Novel tau biomarkers phosphorylated at T181, T217 or T231 rise in the initial stages of the preclinical Alzheimer's continuum when only subtle changes in Aβ pathology are detected

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Editor: Jingyi Hou

Transaction Report:

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7th Jul 2020

Dear Dr. Molinuevo,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees acknowledge the potential interest of the study. However, they also raise substantial concerns about your work, which should be convincingly addressed in a major revision of the present manuscript.

While the referee #2 and #3 are supportive, referee#1 is concerned about the overall novelty presented in the study in comparison to the existing studies. In particular, during our pre-decision cross-commenting process (in which the referees are given the chance to make additional comments, including on each other's reports), referee #2 added "My understanding is that the novelty of this study is the use of different, more sensitive N-terminal tau antibodies in the biomarker assays and the combination of 5 different tau biomarkers in all study participants, most of whom were cognitively unimpaired but had some early changes in amyloid beta protein suggesting initiation of AD pathology. I agree with Referee #1 that a comparison of the CSF and plasma levels of N-p-tau181 would be helpful. The authors state that a longitudinal study is needed -- which I am guessing is probably underway. In my opinion, the strengths outweigh the limitations/weaknesses of the paper and add to a growing literature about early tau changes in AD pathogenesis."

In light of referee #1's comment on the novelty, attention should be given to placing the findings in the context of previous literature and to highlighting the novelty of the current study. All other issues raised by the referees need to be satisfactorily addressed as well. We would welcome the submission of a revised version within three months for further consideration. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

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I look forward to receiving your revised manuscript.

Yours sincerely,
Jingyi Hou

Jingyi Hou
Editor
EMBO Molecular Medicine

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***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Two recent studies have investigated the relationship of these p-tau fragments in AD, it is unclear what the present study adds to that literature, other than the focus on cognitively unimpaired individuals.

Referee #1 (Remarks for Author):

Summary:
This paper investigates the relationship of p-tau markers for different phosphorylation sites, and mid or N term fragments in CSF, and N-ptau181 in plasma with amyloid changes in cognitively unimpaired older individuals. CSF ptau-181 and ptau-217 have previously been investigated in relation to clinical status, and tau PET across clinical stages in AD. A strength of this study is the large sample of cognitively unimpaired individuals, of whom a large percentage also has amyloid PET. Still, several issues remain unclear:

-The rationale of the study could be more clearly introduced. Now it presents a lot of literature, but the focus and precise rationale for all analyses performed remains unclear. In particular a more clear presentation of recent studies on these CSF ptau markers (e.g., Janelidze 2020 & Barthelemy 2020 studies), what they found and what new aspects will be researched here, and why it is important to study those questions with both CSF and PET amyloid measures. It is explicitly stated in the introduction section: 'However, within the Alzheimer's continuum [undefined], there is a high correlation between CSF p-tau and t-tau and it is difficult to disentangle to what extent these biomarkers reflect tau pathophysiology, AD-triggered neurodegeneration or both' This would be interesting to investigate indeed, but it is not clear how this study will resolve this question, and to what extent the different tau phosphorylation sites can help answer this question, and most importantly, no measures for tau pathology included other than CSF and plasma are included.
-Also, the rationale for comparing plasma p-tau in with plasma NFL to predict amyloid status is not clear. Correlating and comparing between plasma p-tau and CSF p-tau would be more

straightforward in terms of validation, also given the notion that NFL is increased in many other neurodegenerative disorders than AD?

-Although previous research has shown that CSF abeta can be abnormal before amyloid PET, the discordance between CSF and PET amyloid status in the present study is very large: only 39% of individuals with abnormal CSF have an abnormal PET, compared to e.g., 63% in ADNI and 76% in the Biofinder cohorts as reported in Palmqvist et al.,, (2017) in participants with normal cognition. This suggest that the ab42/40 cutoff used in the present paper may not reflect amyloid pathology. This is further supported by the notion that p-tau predictive values are systematically higher for prediction of amyloid status based PET versus CSF. How can these differences be explained? (also note that the text at p12 implies that predictive accuracy for ptau markers is best for CSF and PET CL12, but this does not match the results presented in table 3, which shows highest absolute number for PET visual read).

The A- group showed a negative relationship with most p-tau measures and CSF ab42/40, while this was positive for the PET CL scale. To dissociate sub-threshold changes from physiological changes it would be of interest to show how CSF ab42/40 and PET CL correlate with each other in this group, and for A- classified based on PET only to see how CSF A+ may influence those results.

-PET amyloid subthreshold effects are explored by comparing subgroups on ptau measures. Most subjects, however, have a CL value < 12: Do the relationships between ptau measures and CL in this group still hold? That would imply that 'subthreshold' may span a larger range of values. Also rerunning analyses with interaction term based on amyloid PET normal/abnormal for the 12 CL threshold would further formally test differences in these relationships between normal and abnormal groups.

Where do the individuals with >12CL fall in the CSF ab42/40 measures? And conversely, where do the CSF ab42/40 abnormal individuals fall in the PET CL plots? Could these be color coded in the regression scatterplots of figures 2 and 3?

-From p13 onward p-tau changes across the preclinical AD stages are modelled cross-sectionally, by taking ab42/ab40 as a proxy for disease progression. However, there is no clear argumentation provided whether this is a valid approach, and this seems to conflict with statements in the introduction section that p-tau measures are more closely related to cognitive decline. It is not clear what these analyses add over the previous more straightforward analyses.

Other points:
-The introduction section is long and unclear in terms of the rationale for the specific analyses that are performed. There are already CSF papers out on the different phosphor-tau's, it should be better explained what the added value of the present analyses is.
-In Table 3 the A+ and A- numbers seem to be swapped.

Referee #2 (Comments on Novelty/Model System for Author):

Examination of novel, highly sensitive tau biomarkers in CSF and plasma of individuals in the preclinical stage of AD is timely and relevant for early diagnosis, tracking disease progression, monitoring drug engagement and therapeutic outcomes.

Referee #2 (Remarks for Author):

Suarez-Calvet and colleagues from the ALFA study program report new and exciting data indicating that several novel p-tau biomarkers including CSF N-p-tau181, CSF N-p-tau217, CSF Mid-p-tau231 and to a lesser degree, plasma N-p-tau181 increase at very early stages of the AD continuum, when only subtle changes in Aβ are detected (between 12 and 30 CL) and individuals

are cognitively unimpaired. The 381 study participants were part of a well-characterized cohort of CU individuals and included a large number of middle-aged (45-75 yo), preclinical AD determined by CSF Aβ ratio (of which 76% were A+ T-). The novel CSF biomarkers increased early in preclinical AD, increased with Aβ pathology and were capable of distinguishing A+ from A- individuals. The novel biomarkers were shown to be more sensitive than the commonly used CSF Mid-p-tau181 or total tau biomarkers. In addition, CSF N-p-tau217 was increased in A+ T- early stage preclinical AD but not in A- T+ non-AD participants, suggesting that it may be useful to distinguish AD from non-AD tauopathies at early stages of pathogenesis. CSF Mid-p-tau231 had the biggest increase and has been detected in pre-neurofibrillary tangles (NFT) before filaments suggesting that it is an early change that occurs before NFT formation. The plasma N-p-tau181 biomarker increase was less robust than the novel CSF tau biomarkers but was higher than the increase in plasma neurofilament light chain (NfL). The authors were careful to note the limitations of this cross-sectional study, including the absence of tau PET data, and acknowledged that longitudinal studies are needed. The paper is extremely well-written, clear and straightforward. The use of such novel, sensitive CSF biomarkers will help inform future clinical studies in the preclinical AD population and may be useful for measuring target engagement in tau therapeutic trials and monitoring tau in anti-amyloid trials.

Minor Comments:

1. Results - Section "Novel p-tau biomarkers detect amyloid-β pathology more accurately in cognitively unimpaired individuals": the last sentence appears to have an error. It reads "For plasma N-p-tau181, however, the AUC to discriminate between Aβ+ and Aβ- in CU individuals was lower (Table 3; Fig 4B, D, F) and similar to that of plasma N-p-tau181, plotted as a comparison."
2. Do the plasma N-p-tau181 levels correlate with the CSF N-p-tau181 levels?
3. The authors mention in the Discussion that "CSF N-p-tau217 was slightly higher in women than men". Should this be in the Results section? Is this the only novel biomarker that showed sex-specific effects (even if only subtle)?
4. Did the presence of an APOE ε4 allele correlate with higher CSF and/or plasma tau biomarker levels?
5. Do the authors know if any of these novel tau biomarkers can be detected in urine?

Referee #3 (Remarks for Author):

Suarez-Calvert, Karikari and coworkers investigate a set of novel p-tau assays as potential biomarkers for AD. The authors find that in N-terminal-inclusive pT181 and pT217 assays, evidence of p-Tau increases in CSF and plasma are evident in early stages of preclinical AD. The authors note the limitations that this is a cross-sectional study and a mix of a analytical platforms may to some degree confound some of the conclusions. Nonetheless, this study can stand alone as an interesting set of observations that could add important insight into how core biomarkers fit into the AD continuum. I have only one comment in regard to additional analysis. Did the authors look at APOE4 carriers in the Ab- group to see if p-tau levels were significantly different? It is somewhat predictable that APOE4 carriers might have very subtle levels of Ab deposition in this group that might lead to increases in p-tau levels for N-term 217 or 181 in CSF.

Please find enclosed the revised version of our manuscript (EMM-2020-12921), entitled "***Novel tau biomarkers phosphorylated at T181, T217 or T231 rise in the initial stages of the preclinical Alzheimer's continuum when only subtle changes in Aβ pathology are detected***". We would like to thank you for giving us the opportunity to submit a revised version of the manuscript. As you kindly suggested, we have emphasized the novelty of our study and we have placed our findings in the context of recently published literature. We also performed the comparison between CSF and plasma N-p-tau181 (see new Table 3 and Figure 4), as you indicated.

We would like to take this opportunity to thank the reviewers for their support and interesting suggestions, which has certainly improved the quality of this article. A point-by-point response follows. The reviewer comments are quoted and in bold, and our responses are in plain text.

Reviewer #1:

1. **"The rationale of the study could be more clearly introduced. Now it presents a lot of literature, but the focus and precise rationale for all analyses performed remains unclear. In particular a more clear presentation of recent studies on these CSF ptau markers (e.g., Janelidze 2020 & Barthelemy 2020 studies), what they found and what new aspects will be researched here, and why it is important to study those questions with both CSF and PET amyloid measures. It is explicitly stated in the introduction section: 'However, within the Alzheimer's continuum [undefined], there is a high correlation between CSF p-tau and t-tau and it is difficult to disentangle to what extent these biomarkers reflect tau pathophysiology, AD-triggered neurodegeneration or both' This would be interesting to investigate indeed, but it is not clear how this study will resolve this question, and to what extent the different tau phosphorylation sites can help answer this question, and most importantly, no measures for tau pathology included other than CSF and plasma are included."**

We agree with the reviewer that clarification of the rationale of this study is required more explicitly in the main text and put it in the context of recent literature. In response, we have done major changes in the introduction section.

We have clarified the rationale of the study and highlighted its novelty:

Introduction, page 5-6: *"Despite the recent breakthrough developments in understanding p-tau as both a CSF and blood biomarker, it is less known whether specific p-tau biomarkers change early in the continuum of sporadic Alzheimer, when only subtle, incipient changes in Aβ pathology are present. Studying these early stages of the disease is particularly relevant for the p-tau biomarkers since their changes probably precedes NFT pathology (Barthélemy et al, 2020c). Amid the new p-tau assays being developed (targeting different phosphorylation sites, targeting N-terminal vs mid region tau or using different platforms), it is important to perform a head-to-head comparison of these assays. Moreover, there are promising tau phosphorylations, such p-tau231, that have not been yet investigated in asymptomatic stages."*

We have stressed the importance of the more recent studies on p-tau, especially those related to p-tau217:

Introduction, page 5: *"Besides p-tau181, recent strong data indicates that p-tau217, measured both in CSF and in plasma, accurately predicts Aβ pathology in both symptomatic and asymptomatic stages and is an excellent biomarker to discriminate AD from healthy controls and other neurodegenerative diseases (Barthelemy et al., 2015; Barthélemy, Bateman, et al., 2020; Barthélemy et al., 2017; Barthélemy, Li, et al., 2020; Janelidze et al., 2020). In autosomal-dominant AD, CSF p-tau217 even increases two decades before tau PET (Barthélemy, Li, et al., 2020)."*

In the discussion, we have also discussed the new plasma p-tau217 studies and we have acknowledged one of the limitations of our study, that is not including plasma p-tau217:

Discussion, page 17: *"This probable higher specificity is in line with recent studies demonstrating that plasma p-tau217 detects AD pathology with a very high accuracy (Barthélemy, Horie, Sato, & Bateman, 2020; Palmqvist et al., 2020)."*

Discussion, page 22: *"Finally, our study did not include plasma p-tau217. While our manuscript was being reviewed, two studies were published showing the high value of this biomarker to identify AD (Barthélemy, Horie, et al., 2020; Palmqvist et al., 2020)."*

We highlighted the importance of including Aβ CSF and PET in the same study:

Introduction, page 6: *"In order to ensure robustness, we used both CSF and PET as biomarkers of Aβ pathology, which reflect different aspects of Aβ pathology (i.e., soluble Aβ and fibrillar Aβ aggregates, respectively)."*

Finally, as suggested by the reviewer, we have shortened the introduction and we have deleted an entire paragraph that do not clearly relate to the aims of the study.

2. **"Also, the rationale for comparing plasma p-tau in with plasma NFL to predict amyloid status is not clear. Correlating and comparing between plasma p-tau and CSF p-tau would be more straightforward in terms of validation, also given the notion that NFL is increased in many other neurodegenerative disorders than AD?"**

We understand the reviewers' point and we concur. In the revised manuscript, we directly compare CSF and plasma N-p-tau181 and we included the following new sentence in the main text:

Results, page 12: *"For plasma N-p-tau181, however, the AUCs to discriminate between A+ and A- in CU individuals were significantly lower (Table 3; Fig 4B) than its CSF counterpart, CSF N-p-tau181. Yet, plasma N-p-tau181 had and similar AUCs to those of plasma NfL (except for Aβ PET CL12 where plasma N-p-tau181 had a significantly higher AUC), plotted as a comparison."*

Moreover, we have merged the CSF and plasma ROC figures (see new Figure 4 below) so that all the ROC curves can be compared in the same graph. Also, we have conducted a comparison of all the biomarkers AUCs with a DeLong test, without separating between CSF and plasma biomarkers (see new Table 3).
The correlation between CSF and plasma N-p-tau181 is shown in Fig EV3 and in the following sentence in the main text:

Results, page 12: *"the correlation between CSF and plasma N-p-tau181 was weaker but still significant (Fig EV3). Of note, the correlation of CSF and plasma N-p-tau181 was higher in the A+ (r_s = +0.353, P < 0.0001) than in the A- group (r_s = +0.191, P = 0.003)"*

New Figure 4. Discrimination of cognitively unimpaired Aβ-positive from Aβ negative individuals by p-tau biomarkers.

A

B

C
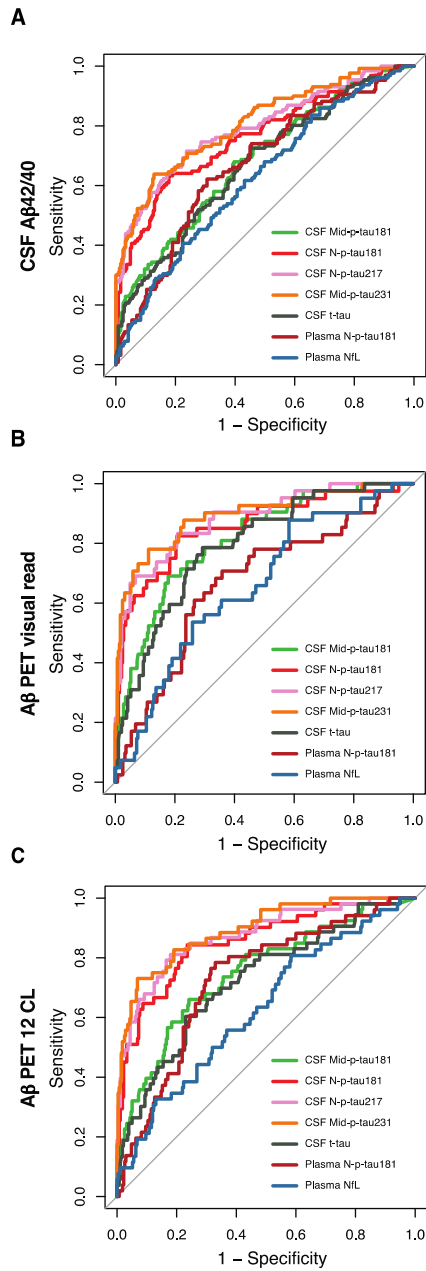
ROC analysis was performed to test the accuracy to discriminate between Aβ-positive from Aβ-individuals. Aβ positivity was defined as CSF Aβ42/40 < 0.071 (A), Aβ PET visual read (B) or Aβ PET Centiloid (CL) > 12 (C).

Abbreviations: CSF, cerebrospinal fluid; Mid, mid-region; NfL, neurofilament light; N, N-terminal; p-tau, phosphorylated tau; t-tau, total tau.

3. **"Although previous research has shown that CSF abeta can be abnormal before amyloid PET, the discordance between CSF and PET amyloid status in the present study is very large: only 39% of individuals with abnormal CSF have an abnormal PET, compared to e.g., 63% in ADNI and 76% in the Biofinder cohorts as reported in Palmqvist et al.,, (2017) in participants with normal cognition. This suggest that the ab42/40 cutoff used in the present paper may not reflect amyloid pathology. This is further supported by the notion that p-tau predictive values are systematically higher for prediction of amyloid status based PET versus CSF. How can these differences be explained? (also note that the text at p12 implies that predictive accuracy for ptau markers is best for CSF and PET CL12, but this does not match the results presented in table 3, which shows highest absolute number for PET visual read)."**

The reviewer raises a very important issue. The reviewer is correct in pointing out that the cohort studied (ALFA+) has a higher number of Aβ CSF and PET mismatches compared to other studies. Regarding this, we would like to emphasise the following points:

a. The ALFA+ cohort, which is enriched by individuals at risk of AD, has a high percentage of individuals that are cognitively unimpaired and are Aβ-positive but Tau-negative (A+T-), using as biomarkers CSF Aβ42/40 and CSF M-p-tau181. In other words, there are a number of individuals in the earliest stage of the Alzheimer's *continuum*, that is in the Alzheimer's pathological change stage (as defined by the 2018 NIA-AA Research Framework). Therefore, it is not surprising we found a higher number of CSF/PET mismatches, because there may be individuals that already have soluble Aβ changes (as shown by CSF) but not Aβ fibrillar changes (as shown by PET). This is in fact one of the main strengths of this cohort.

b. The CSF Aβ42/40 ratio cutoff derived herein is not intended to be used for diagnostic purposes and we acknowledge that it might not be generalizable to clinical cohorts. However, our goal is to derive sensitive cutoffs that capture the very early stages of the disease *continuum*, when Aβ pathophysiological changes are starting. The typical cutoffs derived in symptomatic AD (e.g. based on a comparison against Aβ PET or seeking an optimal discrimination between a control and a pathological group) are very useful in clinical population but they might not detect very early preclinical Alzheimer's. This is why we applied a data-driven approach, namely a Gaussian Mixture Modelling (GMM), which allows to describe a normal range of CSF Aβ42/40 ratios in the non-pathological group and derive a cutoff defined as the mean minus 2 standard deviations of this Gaussian distribution. In fact, GMM has already been used to derive cohort-specific CSF Aβ cutoffs, showing a robust ability to discriminate normal and pathologic distributions of Aβ42 (Bertens, Tijms, Scheltens, Teunissen, & Visser, 2017; De Meyer et al., 2010; Willemse et al., 2018). The cutoff used in the ALFA+ cohort has already been published (Milà‐Alomà et al., 2020) Milà-Alomà et al, JPAD, in press).

Considering these points, it is reasonable that the p-tau biomarkers predictive values are better for prediction of PET than CSF Aβ status. CSF-based Aβ status captures an early stage of the disease than PET-based Aβ status.

We would also like to draw the reviewer's attention to new Figure 3 (see below), where we coloured the CSF-based Aβ-status in the graph with Aβ PET Centiloids. Please note that most CSF-based Aβ-positive (A+) individuals (coloured in Turquoise) are just below the 12CL threshold, which agrees with the idea that CSF cutoffs detects even more subtle Aβ changes than Aβ PET.

Finally, the reviewer is right that that the sentence is page 12 about the predictive accuracy of p-tau biomarkers in not correct and misleading and we have removed it from the revised manuscript.

New Figure 3. Effect of Aβ pathology (Aβ PET) on p-tau biomarkers
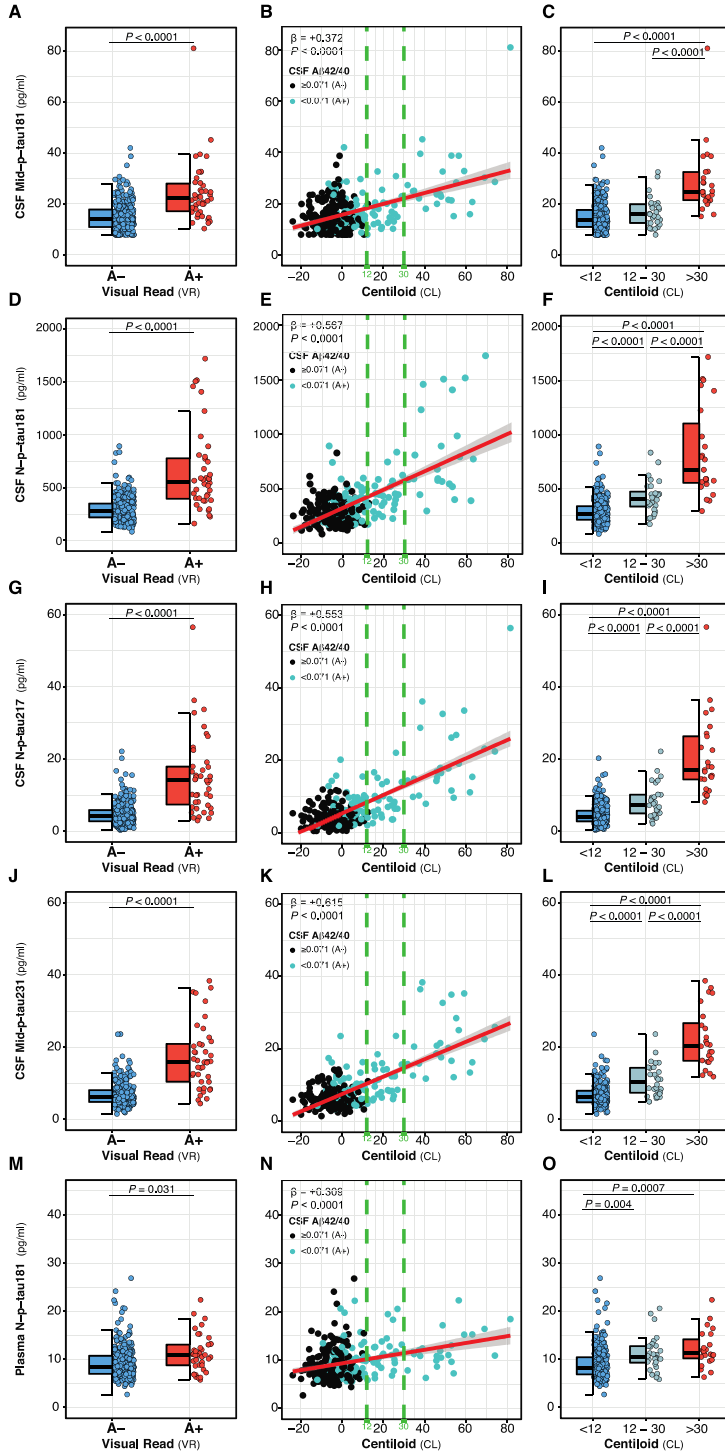
(A, D, G, J, M) Dot and box-plot showing comparison between each of the p-tau biomarker between the Aβ negative (A-; blue) and the Aβ positive (A+; red) groups. Aβ positivity was defined with Aβ PET visual read. *P*-values were assessed by a one-way ANCOVA adjusted by age and sex.

(B, E, H, K, N) Scatter plots depicting the changes between each p-tau biomarker as a function of Aβ PET Centiloids (CL). The standardized regression coefficients (β) and the P-values were computed using a linear model adjusting for age and sex. The solid lines indicate the regression line and the 95% confidence intervals. The dashed green lines indicate the CL12 and CL30 cutoffs. Participants were also colour-coded based on the CSF Aβ42/40 ratio (A-, black; A+, turquoise).

(C, F, I, L, O) Dot and box-plot depicting comparison between each of the p-tau biomarker between Centiloid scale groups. The group between 12 and 30 CL (grey) indicates the subtle Aβ pathology group. P-values were assessed by a one-way ANCOVA adjusted by age and sex, followed by a Bonferroni-corrected *post hoc* pairwise comparison. Abbreviations: CSF, cerebrospinal fluid; Mid, mid-region; N, N-terminal; p-tau, phosphorylated tau.

4. **"The A- group showed a negative relationship with most p-tau measures and CSF ab42/40, while this was positive for the PET CL scale. To dissociate sub-threshold changes from physiological changes it would be of interest to show how CSF ab42/40 and PET CL correlate with each other in this group, and for A- classified based on PET only to see how CSF A+ may influence those results."**

Following reviewer suggestion, we have tested the correlation between CSF Aβ42/40 and Aβ PET CL in the A- group (Figure for the reviewers 1). Of note, CSF Aβ42/40 and Aβ PET CL did not correlate in the A- group (Spearman's correlation coefficient, r_s = -0.049, P = 0.472), while there was a significant correlation in the A+ group (r_s = -0.489, $P < 0.0001$).

Figure for the reviewers 1. Correlation between Aβ PET and CSF Aβ42/40 stratified by Aβ-status (as defined by the Aβ42/04 ratio).
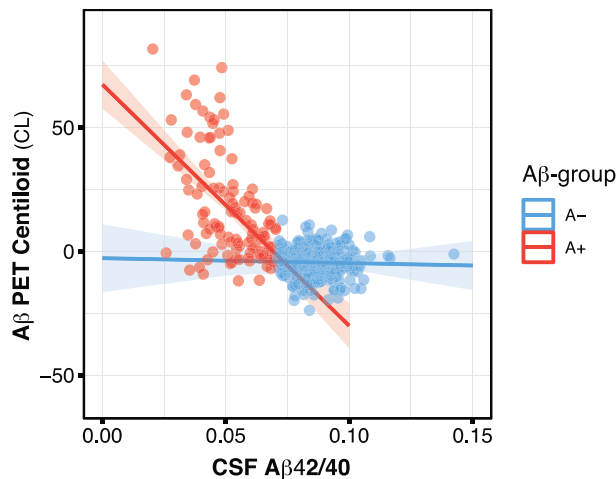
Regarding the second question, there were 73 (25.4%) A+ individuals (based on the CSF Aβ42/40 criteria) among the 287 individuals that were classified as A- based on the Aβ PET visual read. If we remove these CSF A+ individuals from the analyses, the associations between the p-tau biomarkers and Aβ PET CL in the PET A- group are not significant anymore (Table for the reviewer 1).

6

Table for the reviewers 1. Association of p-tau biomarkers and Aβ PET CL scale in A- individuals (as defined by Aβ PET visual read). We show the results removing the 73 individuals that are A+ based on the CSF Aβ42/40 ratio and including them (as in the main text; show here in gray for comparison).

	A- (Aβ PET visual read)					
	Removing CSF Aβ42/40 A+ (n = 214)			Not removing CSF Aβ42/40 A+ (n = 287)		
	β (SE)	*P*	eta^2	β (SE)	*P*	eta^2
CSF Mid-p-tau181	+0.002 (0.069)	0.981	0.000	+0.074 (0.059)	0.210	0.006
CSF N-p-tau181	+0.015 (0.068)	0.828	0.0003	+0.231 (0.058)	<0.0001*	0.054
CSF N-p-tau217	+0.029 (0.069)	0.676	0.0009	+0.266 (0.058)	<0.0001*	0.072
CSF Mid-p-tau231	+0.046 (0.069)	0.502	0.002	+0.271 (0.057)	<0.0001*	0.073
Plasma N-p-tau181	+0.103 (0.069)	0.137	0.011	+0.217 (0.058)	0.0002*	0.048

This later result suggests that the significant associations between p-tau biomarkers and Aβ PET Centiloids observed in the A- (as defined by Aβ PET visual read) are probably driven by those that already have a decreased CSF Aβ42/40 ratio. In figure 3, we have colour-code the individuals based on the CSF Aβ42/40 ratio.

We thank the reviewer for the interesting observation. Although there maybe physiological changes in p-tau biomarkers in healthy individuals, these results suggest that they change as soon as there are changes in Aβ metabolism, even with low Aβ centiloids in Aβ PET.

5. **"PET amyloid subthreshold effects are explored by comparing subgroups on ptau measures. Most subjects, however, have a CL value < 12: Do the relationships between ptau measures and CL in this group still hold? That would imply that 'subthreshold' may span a larger range of values. Also rerunning analyses with interaction term based on amyloid PET normal/abnormal for the 12 CL threshold would further formally test differences in these relationships between normal and abnormal groups. Where do the individuals with >12CL fall in the CSF ab42/40 measures? And conversely, where do the CSF ab42/40 abnormal individuals fall in the PET CL plots? Could these be color coded in the regression scatterplots of figures 2 and 3?"**

The reviewer is right that there is a higher number of individuals that are Aβ PET CL<12. Following reviewer suggestion, we have tested the associations between the p-tau biomarkers and Aβ PET CL in the CL <12 group and we also run interaction analyses including the term 'Aβ PET CL x Aβ status (CL12)'. These analyses have been included in the new expanded view Table EV2 and it is also shown below.

The results using CL12 to classify A+ and A- are similar to those using the Aβ PET visual read (in Table 2 in the main text). In both analyses, there is a significant increase of all p-tau biomarkers (except for Mid-p-tau181) as a function of Aβ PET CL in the A- group. The interaction analyses also render a similar result, except for the fact that the interaction 'Aβ PET CL x Aβ status (CL12)' for Mid-p-tau231 becomes significant. As the reviewer points out, these may imply that there may be Aβ subtle changes even below Aβ PET CL12.

Table EV2. Association of p-tau biomarkers and Aβ PET CL scale stratifying by Aβ status (as defined by Aβ PET CL12 cutoff).

	Aβ PET CL12 (n = 331)						
	A- (n = 278)			A+ (n = 53)			'Aβ PET x Aβ status' interaction
	β (SE)	*P*	eta^2	β (SE)	*P*	eta^2	*P*
CSF Mid-p-tau181	+0.058 (0.060)	0.339	0.003	+0.483 (0.118)	0.0002*	0.254	0.009*
CSF N-p-tau181	+0.115 (0.060)	0.054*	0.014	+0.628 (0.132)	<0.0001*	0.324	0.004*
CSF N-p-tau217	+0.176 (0.060)	0.003*	0.031	+0.610 (0.110)	<0.0001*	0.391	0.246
CSF Mid-p-tau231	+0.165 (0.059)	0.006*	0.027	+0.606 (0.119)	<0.001*	0.350	0.040*
Plasma N-p-tau181	+0.130 (0.060)	0.032*	0.017	+0.253 (0.156)	0.112	0.053	0.859

For each p-tau biomarker we computed the linear regression standardized coefficients (β) and standard errors (SE) as a function of Aβ PET Centiloid scale. The analyses were performed after stratifying for Aβ-negative (A-) and Aβ-positive (A+) groups, as defined by Aβ PET CL12. We report the eta-squared (eta^2) as a measure of the effect size. We also computed the *P*-value for the interaction term 'Aβ biomarker x Aβ status'. All analyses were adjusted by age and sex. *Significant differences.

Abbreviations: CL, Centiloid; CSF, cerebrospinal fluid; Mid, mid-region; N, N-terminal; PET, positron emission tomography; p-tau, phosphorylated tau.

In the revised manuscript, we have included Table EV2 and the following sentence in the main text:

Results, page 10: "We repeated the same analyses after defining the A+ group as having a CL > 12 in Aβ PET, instead of visual read, and the results were similar (Table EV2)."

In the Table for reviewer 2, we answer the next reviewer's question. Among the individuals that were Aβ PET CL>12, only 1 individual (1.9%) was A- and 52 (98%) were A+ using the CSF Aβ42/40 ratio. Among the individuals that were A+ with the CSF Aβ42/40 ratio, 62 (54%) were Aβ PET CL<12 and 52 (46%) are Aβ PET CL≤12. Following reviewer suggestion, we have colour code these individuals in the new Figure 2 (see below) and Figure 3 (see above). Together, these results are consistent with the fact that the CSF Aβ42/40 ratio changes earlier in the disease *continuum* than the Aβ PET.

Table for the reviewers 2. Table with the Aβ-classification agreement between CSF Aβ42/40 and Aβ PET (as defined by CL12).

		Aβ PET CL12		
		A-	A+	Total
CSF Aβ42/40	**A-**	216	1	217
	A+	62	52	114
	Total	278	53	331

New Figure 2. Effect of Aβ pathology (CSF Aβ42/40) on p-tau biomarkers
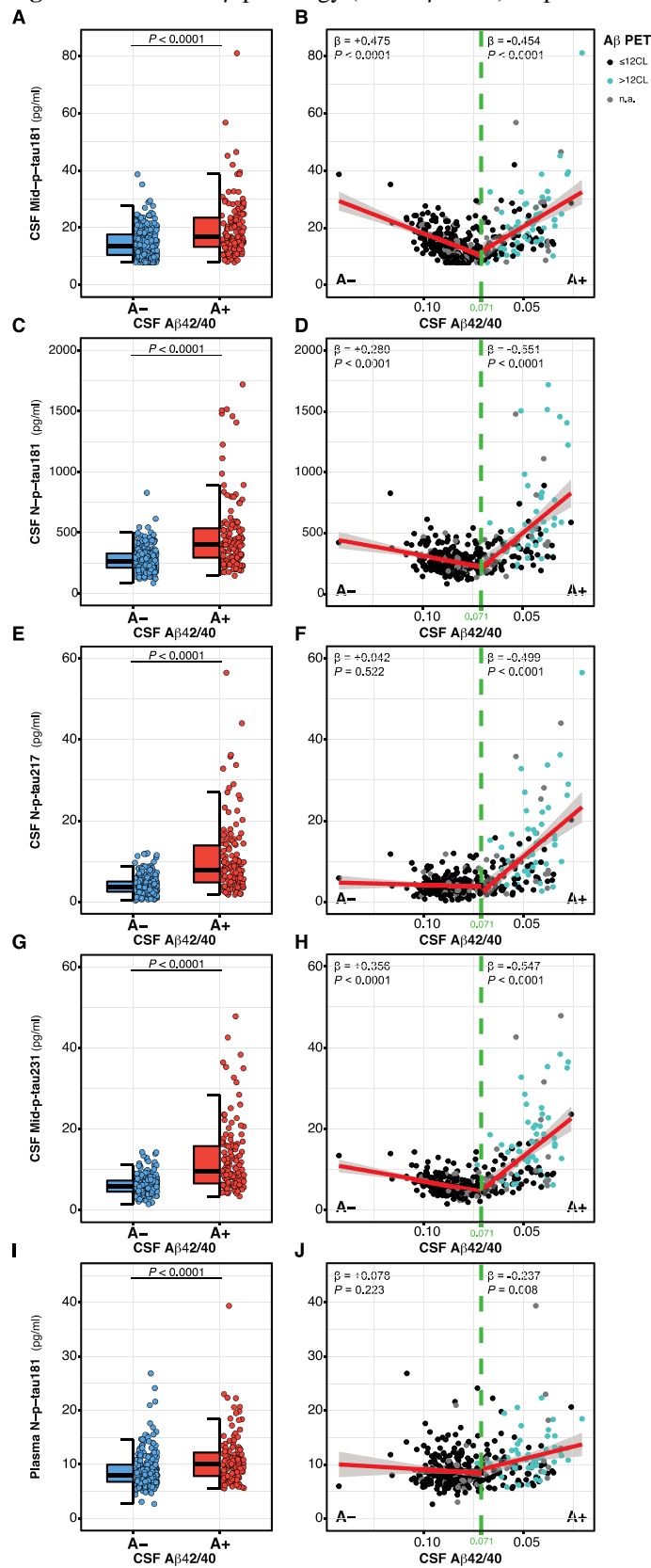
(A, C, E, G, I) Dot and box-plot comparing each of the p-tau biomarker between the Aβ-negative (A-; blue) and the Aβ-positive (A+; red) groups. Aβ positivity was defined as CSF Aβ42/40 ratio < 0.071. The box-

plot depict the median (horizontal bar), interquartile range (IQR, hinges) and 1.5 x IQR (whiskers). *P*-values were assessed by a one-way ANCOVA adjusted for age and sex.

(B, D, F, H, J) Scatter plots depicting the changes between each p-tau biomarker as a function of CSF Aβ42/40. The horizontal-axes directions were inverted; lower CSF Aβ42/40 ratio reflects higher Aβ pathology. For each Aβ status group, we computed the standardized regression coefficients (β) and the *P*-values, adjusted for age and sex. The solid lines indicate the regression line and the 95% confidence intervals for each of the Aβ status groups. The dashed green lines indicate the CSF Aβ42/40 cutoff. Participants were also colour-coded based on the Aβ PET CL scale (≤12CL, black; >A+, turquoise; Aβ PET non-available, grey).

Abbreviations: CSF, cerebrospinal fluid; Mid, mid-region; N, N-terminal; n.a. non-available; p-tau, phosphorylated tau.

6. **"From p13 onward p-tau changes across the preclinical AD stages are modelled cross-sectionally, by taking ab42/ab40 as a proxy for disease progression. However, there is no clear argumentation provided whether this is a valid approach, and this seems to conflict with statements in the introduction section that p-tau measures are more closely related to cognitive decline. It is not clear what these analyses add over the previous more straightforward analyses."**

We understand the reviewer point and we have better explained why we used this approach:

Methods, page 30-31: *"This approach allowed us to define more precisely the biomarker changes in the Alzheimer's continuum and therefore overcome the limitation of simplifying the preclinical stage in discrete stages."*

Studying the long preclinical stage of sporadic AD is difficult because we do not have the concept of "expected years to symptom onset (EYO)" as in autosomal-dominant AD. Also, we cannot use cognitive decline as we are in presymptomatic stages. Our results are consistent using different approaches, which we believe reinforce their robustness.

7. **"Other points:**
-The introduction section is long and unclear in terms of the rationale for the specific analyses that are performed. There are already CSF papers out on the different phosphor- tau's, it should be better explained what the added value of the present analyses is."

We agree with the reviewer and we have rewritten the introduction section accordingly.

"-In Table 3 the A+ and A- numbers seem to be swapped."

Thanks for catching the error, we corrected the Table.

Reviewer #2:

"Examination of novel, highly sensitive tau biomarkers in CSF and plasma of individuals in the preclinical stage of AD is timely and relevant for early diagnosis, tracking disease progression, monitoring drug engagement and therapeutic outcomes."

We appreciate the strong support of the reviewer. They also share some minor comments:

1. **"Results - Section "Novel p-tau biomarkers detect amyloid-β pathology more accurately in cognitively unimpaired individuals": the last sentence appears to have an error. It reads "For plasma N-p-tau181, however, the AUC to discriminate between Aβ+ and Aβ- in CU individuals was lower (Table 3; Fig 4B, D, F) and similar to that of plasma N-p-tau181, plotted as a comparison"**

We thank the reviewer for catching the errors. We corrected them in the revised version as follows:

Results, page 11: *"plasma N-p-tau181 had and similar AUCs to those of plasma NfL (except for Aβ PET CL12 where plasma N-p-tau181 had a significantly higher AUC), plotted as a comparison."*

2. "Do the plasma N-p-tau181 levels correlate with the CSF N-p-tau181 levels?"

Yes, they do, as shown in Fig EV3. In the revised manuscript we have included the statistics in the main text:

Results, page 12: *"the correlation between CSF and plasma N-p-tau181 was weaker but still significant (Fig EV3). Of note, the correlation of CSF and plasma N-p-tau181 was higher in the A+ (r_s = +0.353, P < 0.0001) than in the A- group (r_s = +0.191, P = 0.003)"*

3. "The authors mention in the Discussion that "CSF N-p-tau217 was slightly higher in women than men". Should this be in the Results section? Is this the only novel biomarker that showed sex-specific effects (even if only subtle)?"

We thank the reviewer to highlight these results. We only found sex differences in CSF N-p-tau217, but not in the other p-tau biomarkers. We do believe that sex and gender differences in biomarkers should be a priority in our research field. This result is explained in the results section as follows:

Results, page 9: *"Interestingly, after adjusting for age, levels of CSF N-p-tau217 were slightly, but significantly, higher in women (M = 6.71 pg/ml, SD = 7.01) than men (M = 5.63, SD = 5.31; P = 0.016). This difference remained significant after additionally adjusting for Aβ status (P = 0.005). The rest of the CSF or plasma p-tau biomarkers were not affected by sex."*

4. "Did the presence of an APOE ε4 allele correlate with higher CSF and/or plasma tau biomarker levels?"

This is an interesting point. We conducted a linear regression with each p-tau biomarker as a dependent variable and *APOE-ε4* status, age and sex as independent variables. *APOE-ε4* status had a significant effect on CSF N-p-tau181 (*P* = 0.0001), CSF N-p-tau217 (*P* = 0.00008), CSF Mid-p-tau231 (*P* = 0.019), but not CSF M-p-tau181 (*P* = 0.624) or plasma N-p-tau181 (P = 0.224). However, when we adjusted by amyloid-β status, all the significant results disappeared. This suggests that the effect of *APOE-ε4* on p-tau biomarkers is probably related to Aβ pathology.

5. "Do the authors know if any of these novel tau biomarkers can be detected in urine?"

We are not aware that these p-tau biomarkers can also be measured in urine. We concur with the reviewer that testing biomarkers in matrix different from blood and CSF, such as urine or saliva, would be highly interesting and we will conduct these measurements in future studies.

Reviewer #3:

1. **"Suarez-Calvert, Karikari and coworkers investigate a set of novel p-tau assays as potential biomarkers for AD. The authors find that in N-terminal-inclusive pT181 and pT217 assays, evidence of p-Tau increases in CSF and plasma are evident in early stages of preclinical AD. The authors note the limitations that this is a cross-sectional study and a mix of a analytical platforms may to some degree confound some of the conclusions. Nonetheless, this study can stand alone as an interesting set of observations that could add important insight into how core biomarkers fit into the AD continuum. I have only one comment in regard to additional analysis. Did the authors look at APOE4 carriers in the Ab- group to see if p-tau levels were significantly different? It is somewhat predictable that APOE4 carriers might have very subtle levels of Ab deposition in this group that might lead to increases in p- tau levels for N-term 217 or 181 in CSF."**

We thank the reviewer for their support and for the interesting question. We compared the levels of the p-tau biomarkers between the A- (stratifying between *APOE*-ε4 non-carriers and carriers) and the A+ (including both *APOE*-ε4 carriers and non-carriers). The levels of the p-tau biomarkers in the A- and *APOE*-ε4 carriers' group did not differ from those of the A- and *APOE*-ε4 non-carriers group (see figure for the reviewer 2).

Figure for the reviewers 2. Effect of Aβ pathology (CSF Aβ42/40) and *APOE* on p-tau biomarkers. We conducted an ANCOVA adjusted by age and sex followed by a Bonferroni-corrected *post hoc* pairwise comparison.
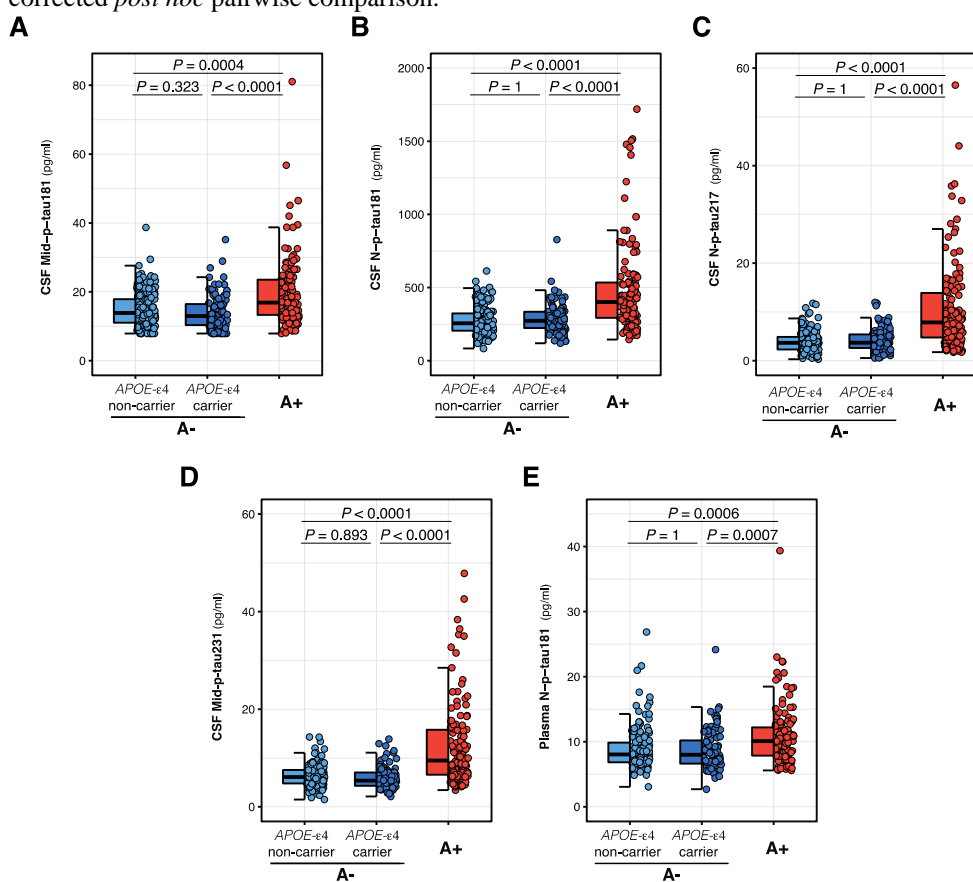
Taken together, we believe we have addressed all the reviewers' comments and hope that you find our manuscript now acceptable for publication in EMBO Molecular Medicine. Many thanks for considering our manuscript.

Sincerely,

José Luis Molinuevo
Kaj Blennow

24th Aug 2020

Dear Dr. Molinuevo,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to re-assess it. As you will see the referee is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following amendments:

1. In the main manuscript file, please do the following:
- Reduce the number of keywords to 5
- Add callouts for Fig 1 A-E (currently only Figure 1 in general is called for). Same for Fig. 4 A & C, Fig. 5A,B,C,D,E.
- remove the yellow color font.
- in legends, provide exact n= and exact p= values, not a range, along with the statistical test used. Some people found that to keep the figures clear, providing an Appendix supplemental table with all exact p-values was preferable. You are welcome to do this if you want to.
- in Material & Methods (and in checklist), include a statement that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
- in Material & Methods, provide the antibody dilutions that were used for each antibody.

2. Table EV1- EV3 need to be uploaded as separate files (.txt, .xls., etc.) using the file type Expanded View File.

3. Please add a "Data availability" section (placed after the Materials & Methods section).
Since there is no large-scale dataset generated in this study, please include the following sentence in this section- "This study includes no data deposited in external repositories".

4. For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

5. We would encourage you to include the source data for figure panels that show essential quantitative information. Additional information on source data and instruction on how to label the files are available at < https://www.embopress.org/page/journal/17574684/authorguide#sourcedata >.

6. Our data editor has made a couple of suggestions on your manuscript (see attached), please address.

7. As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
a. In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent

correspondence relating to the manuscript. Let us know if you do not agree with this.

b. Please note that the Authors checklist will be published at the end of the RPF.

8. Please provide a visual abstract (550px width and max 400px height, jpeg format) to illustrate your article on our homepage.

I look forward to seeing a revised version of your manuscript as soon as possible.

Sincerely,

Jingyi

Jingyi Hou
Editor
EMBO Molecular Medicine

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2) Separate figure files*

3) supplemental information as Expanded View and/or Appendix. Please carefully check the authors guidelines for formatting Expanded view and Appendix figures and tables at https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word
file).

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- the medical issue you are addressing,
- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

6) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

7) Author contributions: the contribution of every author must be detailed in a separate section.

8) EMBO Molecular Medicine now requires a complete author checklist (https://www.embopress.org/page/journal/17574684/authorguide) to be submitted with all revised manuscripts. Please use the checklist as guideline for the sort of information we need WITHIN the manuscript. The checklist should only be filled with page numbers were the information can be found. This is particularly important for animal reporting, antibody dilutions (missing) and exact values and n that should be indicted instead of a range.

9) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one sentence bullet points that summarise the paper. Please write the bullet points to summarise the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

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***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The authors have done a extremely thorough job at addressing each Reviewers' comments and questions. They have included new analyses and the revised the manuscript is greatly improved. This paper will be of high interest to the field.

The authors performed the requested editorial changes.

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: José Luis Molinuevo, Kaj Blennow
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2020-12921

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:
➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

➔ a specification of the experimental system investigated (eg cell line, species name).
➔ the assay(s) and method(s) used to carry out the reported observations and measurements
➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
➔ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
  • exact statistical test results, e.g., P values = x but not P values < x;
  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We studied all the participants of the ALFA+ cohort that were available to ensure the highest power possible.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The ALFA+ study has its inclusion and exclusion crietia (Molinuevo et al, 2016)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The measurements of biomarkers were performed blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	All figueres include a description of the statistics performed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We tested for normality of the distribution for each biomarker using the Kolmogorov-Smirnov test and visual inspection of histograms.
Is there an estimate of variation within each group of data?	We test for the assumption of homoscedasticity.

Is the variance similar between the groups that are being statistically compared?	All assumptions of the tests were met.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All reagents used in the study are described in the methods section and the catalg number included.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The ALFA+ study (ALFA-FPM-0311) was approved by the Independent Ethics Committee "Parc de Salut Mar", Barcelona, and registered at Clinicaltrials.gov (Identifier: NCT02485730).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All participants signed the study's informed consent form that had also been approved by the Independent Ethics Committee "Parc de Salut Mar", Barcelona. The experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	There were not any restrictions
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Measurements of biomarkers can be provided upon request. However, the confidentiality of the participants must be protected and hence the demographic and clinical data that could identify a participant can not be provided.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA