

Early Alzheimer's Disease with Frequent Neuritic Plaques Harbors Neocortical Tau Seeds Distinct from Primary Age-Related Tauopathy

Corresponding Author: Dr Allison Kraus

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)

In this study, the authors used postmortem neuropathology tissues from patients with Braak stage II-IV PART and ADNC to compare tau seeding in hippocampus (HP) and middle frontal cortex (MF). A key difference between PART and ADNC is the ultimate development of NFTs in neocortical regions. They use tau RT-qPCR to study changes that occur prior to the development of NFTs in the neocortex. Their primary claim is that there is increased tau seeding in the MF of patients with ADNC but not PART, which correlates with increased tau PTMs, prior to the development of NFTs in the region. The authors posit that tau seeding may be predictive of the future development of NFTs in this region.

While understanding what differentiates ADNC and PART is both interesting and highly relevant, this study suffers from several problems that diminish its impact. First, though they do not mention it, the differences they are seeing could be entirely driven by ApoE genotype, which potentially negates their claim as to the importance of PTMs, or at least shifts the causal focus to genetics. In addition, the reliance only on neuropathological tissue renders this more of an observational study, rather than one that can definitively test a hypothesis, and makes this more appropriate for a specialized audience. Finally, the writing and presentation of the data could be improved. Thus, while we offer constructive critiques below, even if addressed, the key concerns about impact would not be overcome.

Constructive critiques:

- 1) ApoE4 allele frequency is very different in the PART and ADNC subjects. This is unsurprising, but this should be commented on in the body of the paper. A post-hoc analysis should attempt to determine if the group differences are due to this.
- 2) Line 396-398, the authors claim that the S1 fraction contains "comparable seeding activities" to BH. However they do not test the sarkosyl soluble fraction, so this claim is not justified. Further, the data is shown on a log 10 scale, and the seeding propensity is 0.5 lower, suggesting only half the seed competent tau is in the sarkosyl insoluble fraction.
- 1) Given their claims about PTMS, the authors should assess far more than three epitopes.
- 2) In the methods, the dot blot does not fully describe the protocol for immunoreactivity. Additionally, if total protein by ponceau S is used for the loading control, it should be shown in the figure. By eye, there are substantive difference between ADNC and PART in both MF and HP, but the quantification does not reflect this.
- 3) There seems to be more total tau in ADNC and high ADNC on supplemental figure 2, particularly HT7. This should be quantified and displayed. HT7 appears to be capturing a dimer that is not present in the PART sample. This is matched visually on the homogenate from S1.
- 4) The authors should clarify the relevance of Thal when 0-2 defines the PART group and 3-5 is ADNC. Figure 4a makes sense but doesn't seem to be novel.
- 5) The manuscript could benefit from major editorial review. This would include for clarity, accuracy and citations. For example, in the introduction (lines 48-50), the statements about prion mechanisms for the development of NFTs require better, more nuanced descriptions and citations. In addition, the statement that 100% of individuals over 85 have NFTs (lines 45-47) is a misinterpretation of the manuscript, as the reference showed 100% tau PET positivity, not the presence of AD-related NFTs. Finally, this study is densely written and challenging to read.

Reviewer #2

(Remarks to the Author)

Browne et al report their study on the levels of post-translational modifications of tau, and tau seeding activity in pathologically-determined primary age-related tauopathy and Alzheimer's disease. In examining two brain regions, hippocampus vs neocortex, they describe a marked difference in seeding and PTM profile in the neocortex between these conditions. The results are consistent with a model wherein beta-amyloid pathology promotes a distinct tau PTM and seeding phenotype specifically in the cortex but not in the hippocampus. This is a very well performed study, that sheds light on the pathophysiological mechanisms defining PART and AD.

Specific comments:

The ultrastructure of the PART and AD tau filaments has been shown to be identical, which makes comparison between the seeding activity of these strains appropriate, and leads the authors to conclude at PTMs differentiate tau species in cortical PART vs ADNC. However, another difference could be fibril length. For a given quantity of tau input, there might be more seed-competent species if the filaments were shorter. Can this alternative explanation be addressed? For instance, by profiling insoluble species using negative stain EM. Alternatively, if sonication were used and a large increase in PART seeding activity were observed, it could be consistent with the break-up of large fibrils.

Line 47-50: there is a distinction between inter-cellular prion-like spreading and the assembly of tau within a cell which is not captured in this description of tau aggregation. Prion-like seeded aggregation as a hypothesis of the apparent spread of tau pathology across the brain should be presented alongside alternatives.

The authors normalise the input sarkosyl insoluble tau to the seeding reactions. While this is valuable for determining differences in intrinsic seeding ability, it masks the total number of seeds present in tissue. A graph showing the quantities of insoluble tau in the regions/diseases, similar to Fig 1c and 1e would be valuable. Does this correspond to the IHC tau pathology or to the RT-QuIC?

It is not always clear whether the seed quantification assays were performed using homogenate or sarkosyl insoluble input (eg Fig 5 and Supp Fig 6).

The ADNC seeding does not always reach endpoint dilution in the graphs displayed. Can the authors confirm that this was determined?

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

This manuscript describes differences in tau seeding between brain tissue homogenates from PART/possible PART and ADNC middle frontal cortex in Braak stage matched cases. In addition, differences in tau PTM levels are identified, and associations with cognition are made. Overall, it is an interesting study and well-written. However, it is missing some crucial analyses, without which the conclusions appear to be overstated.

Major comments:

Page 2, lines 25-26, and page 3, lines 47-48: While there have been studies which suggest tau seeding as a mechanism for tau propagation, this is not universally accepted and remains somewhat controversial. At minimum, the controversy around this topic should be discussed as this concept has not definitively been proven. Page 3, lines 50-52 are true, but contradict lines 47-48, as this mechanism is not fully understood yet.

Page 2, line 26: This does not necessarily suggest that all "tau seeds" are not equally pathogenic; it could simply suggest that tau without amyloid, or tau without other comorbidities could be less pathogenic.

Page 19, lines 400-410: If this is true, that essentially means more p-tau, leads to more seeding, and this contradicts the digital pathology findings that PART and ADNC in this cohort had equal amounts of p-tau.

Page 20, line 439: Are total tau levels between PART and ADNC equal? Supplementary figures 1 and 2 appear to demonstrate that PART has lower total tau. Please see below as well, regarding page 23, lines 492-494.

Page 21, lines 460-465: These analyses could be performed with intermediate ADNC cases with differing CERAD NP

scores. For example, int ADNC cases with Thal phase 3-5 and Braak III-IV can have CERAD score of sparse, moderate or frequent and still be intermediate ADNC (not PART or possible PART).

Do intermediate ADNC cases with differing CERAD NP scores have different tau seeding levels? If yes, then this tau seeding does not necessarily differentiate PART from ADNC, as the title and manuscript claim. Perhaps tau seeding may simply differentiate progressive CERAD NP levels.

In addition, since Thal phase correlates with seeding, it is possible that amyloid burden would correlate. Therefore, digital quantification for amyloid could be performed and amyloid burden levels could be correlated with seeding levels, similar to the authors' work on tau.

The cognitive data correlation with seeding could simply be due to increased NP density, this possibility should be addressed. Multivariate regression models could be constructed to account for NP density (among other variables).

Page 23, lines 492-494: Does this suggest that more total tau leads to more seeding without other factors?

Page 23, lines 497-500: Given the importance of the relationship between NP density and seeding/MF tau, this concept should be made more central to the paper rather than focusing on "the seeding activity differentiates PART from AD". This is particularly true since the authors do not distinguish their cases as "definite PART" or "possible PART", and so have a mixture of cases with and without neuritic plaques. The authors should either organize their conclusions around the presence/absence/severity of tau (PTMs) and neuritic plaques or separate their cases into more distinct PART/ADNC groups rather than the continuum shown in figure 5g.

Nowhere in the manuscript do the authors demonstrate that PART alone is different from ADNC, only that seeding correlates with NP density, and Thal 4-5 as compared to Thal 0 has greater MF tau seeding, but not greater than Thal 3-4 cases, thus PART (Thal 0) is no different from ADNC with Thal 3-4.

Figure 5g: there appears to be a case labeled as "PART" (presumably possible PART) with a Braak score IV and CERAD 2/moderate. This CERAD NP score is incompatible with a diagnosis of PART as it is currently defined (Reference #3 in the current manuscript).

Minor comments:

Page 3, line 62: PART can extend to the neocortex, albeit rarely and in a limited fashion. This has been shown previously in the literature, and in Figure 1b. The sentence "Importantly, the NFT pathology of PART appears limited to subcortical and limbic regions and does not extend to the neocortex" could be left out, as the next sentence directly contradicts that sentence.

Page 15-16, lines 329-332: Analyzing the contralateral hippocampus and middle frontal cortex for p-tau burden does not necessarily mean that the other side will have an equal amount of p-tau. There is often asymmetry, especially in PART and early stages of ADNC.

Page 17, line 370: Please include Braak stage and CERAD NP score in these analyses (not just "PART" or "ADNC"), as these have previously been found to associate with cognitive measures.

Page 28, lines 616-619: Post-mortem and pre-mortem CSF samples are available at many institutions, this could be tested in the present manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised version of the manuscript by Browne et al. addresses many of our concerns. The authors have analyzed the effect of ApoE4 dosage and tau seeding and found no effect of ApoE4. They also have performed a more nuanced analysis of the link between neuritic plaque density and tau seeding. These analyses, as well as extensive revision to the writing and data presentation, are appreciated and have improved the manuscript.

A few items remain that should be addressed to strengthen the paper:

- 1) As the authors decline to test additional tau PTMs, they will need to thoroughly revise their claims about PTMs, inclusive of the abstract, results and discussion. Specifically, any claim about a PTM should not be generalized but refer to the specific PTM tested or describe the "selected" or "subset" of PTMs. The indiscriminate generalization of limited findings historically has been a major problem in the field of neurodegeneration. This study should take care to avoid this pitfall.
- 2) Address in the discussion why there is no impact of ApoE4 on seeding as this is a surprising finding.
- 3) We appreciate the authors providing the full dot blots of PTMs. It appears that the difference is driven by a few individuals

in the ADNC group in Figure 3e-f; it would be interesting to hear more discussion on these patients.

4) Line 131: "billion" needs a citation

Reviewer #2

(Remarks to the Author)

My comments have been satisfactorily addressed.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors have addressed my original comments.

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Reviewer response, NCOMMS-24-26668

We thank the reviewers for their constructive feedback, and include a point-by-point response below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, the authors used postmortem neuropathology tissues from patients with Braak stage II-IV PART and ADNC to compare tau seeding in hippocampus (HP) and middle frontal cortex (MF). A key difference between PART and ADNC is the ultimate development of NFTs in neocortical regions. They use tau RT-quIC to study changes that occur prior to the development of NFTs in the neocortex. Their primary claim is that there is increased tau seeding in the MF of patients with ADNC but not PART, which correlates with increased tau PTMs, prior to the development of NFTs in the region. The authors posit that tau seeding may be predictive of the future development of NFTs in this region.

While understanding what differentiates ADNC and PART is both interesting and highly relevant, this study suffers from several problems that diminish its impact. First, though they do not mention it, the differences they are seeing could be entirely driven by ApoE genotype, which potentially negates their claim as to the importance of PTMs, or at least shifts the causal focus to genetics.

Please see below **Point 1** for detailed analyses responding to this hypothesis.

In addition, the reliance only on neuropathological tissue renders this more of an observational study, rather than one that can definitively test a hypothesis, and makes this more appropriate for a specialized audience.

We respectfully disagree with this reviewer's opinion, which is in clear contrast to multiple recent Nature Communications articles aimed at a broader audience and based on neuropathological tissue data, including some about Alzheimer's and other neurodegenerative diseases (please see. PMC10960791, 2024; PMC8975902, 2022; PMC11180185, 2024 among others). Additionally, models of ADNC do not fully recapitulate aspects noted in human disease; thus, human neuropathologic tissue has a critical role in defining disease pathophysiology.

Finally, the writing and presentation of the data could be improved.

This has been extensively addressed in our revised manuscript; please see the track-change version.

Thus, while we offer constructive critiques below, even if addressed, the key concerns about impact would not be overcome.

Constructive critiques:

1) ApoE4 allele frequency is very different in the PART and ADNC subjects. This is unsurprising, but this should be commented on in the body of the paper. A post-hoc analysis should attempt to determine if the group differences are due to this.

We have now tested this extensively. As the reviewers note, APOE $\epsilon 4$ is less common in PART but more common in AD, and the $\epsilon 4$ allele is associated with increased A β deposition relative to $\epsilon 3$ or $\epsilon 2$ allele. To test the hypothesis that ApoE $\epsilon 4$ genotype is driving tau seeding and PTMs and leading to the differences between our PART and ADNC groups, we performed multiple analyses described below. **We do not find compelling evidence that ApoE genotype drives the group differences observed, as detailed below.**

A) We used linear regression and two-way ANOVA to demonstrate that the APOE $\epsilon 4$ genotype is not driving differences in seeding or PTMs in PART vs ADNC. Specifically, when a model term for the presence of an APOE $\epsilon 4$ allele is added to the models for seeding and PTMs, the same relationships remain significant, while the $\epsilon 4$ term itself never reaches significance (see **New Supplemental Table 5**).

B) Within our ADNC group, the APOE $\epsilon 4$ allele was present in 52% (i.e approximately half) of our participants. The ADNC cohort is therefore useful to directly test the effect of ApoE $\epsilon 4$ on tau seeding and PTMs. We find no difference in MF seeding or MF tau modifications between the ADNC cohort with $\epsilon 4$ and the ADNC cohort without an $\epsilon 4$ allele (see **New Supplemental Figure 5**).

Overall these findings imply that APOE $\epsilon 4$ genotype is a risk factor for the development of A β pathology (and in turn ADNC, both of which are well established) but that APOE $\epsilon 4$ is not in itself the main driver of our tau modification and seeding results.

This point has now been addressed in the manuscript with:

- (1) **Updated** description of full APOE genotypes (rather than # $\epsilon 4$ alleles) in the demographic **Table 1**.
- (2) **New Supplemental Table 5** showing non-significant effect of the addition of APOE genotype to regression models of seeding and tau modifications.
- (3) **New Supplemental Figure 5** showing no effect of APOE genotype on seeding or PTMs within the ADNC group.
- (4) Inclusion of substantial additional text describing the above findings and rationale for these experiments (lines **589-615** in “all markup” track change version)

2) Line 396-398, the authors claim that the SI fraction contains “comparable seeding activities” to BH. However they do not test the sarkosyl soluble fraction, so this claim is not justified. Further, the data is shown on a log 10 scale, and the seeding propensity is 0.5 lower, suggesting only half the seed competent tau is in the sarkosyl insoluble fraction.

This comparison was intended to demonstrate that the SI fraction harbors significant seeding activities. However, we agree that characterizing how relative amounts of tau seeding activity partition to soluble vs. insoluble fractions is valuable. To address this point, we have added new data quantifying seeding activity in the soluble fraction (everything that remains after isolating the sarkosyl-insoluble fraction) versus the sarkosyl insoluble fraction. The soluble fraction does harbor some seeding activity, albeit at lower levels compared to the sarkosyl insoluble fraction. We have added these data and related discussion to the manuscript (See **Supplemental Figure 1**, and lines 513-514) including revision of the text to note “A majority of tau seeding activity recovered from brain homogenate is retained in the SI fractions compared to the soluble fractions, . . .” Additionally, more biological replicates have been added to **Supplemental Figure 1** as new data was generated while addressing other reviewer comments.

3) Given their claims about PTMS, the authors should assess far more than three epitopes.

We identified and focused on key tau PTMs reported in literature to track with AD neuropathology which are also being examined as AD biomarkers (PMC8168922; PMC10979350; PMC10154225). Our goal was to test the hypothesis that seeding activity differences between MF-ADNC and MF-PART are related to these key epitopes, and we now include additional technical immunoblot replicate analysis in the statistical evaluation (**Updated Supplemental Table 3**). While we agree a comprehensive mapping of PART filament PTMs may be interesting as part of another extended project, it requires specialized technologies (PMC8168922; PMC10154225) that are well outside the scope of this study. Regardless, our observations that pT217, pS202/T205, and C-terminal immunoreactivities are different in Braak-matched MF-PART and MF-ADNC adds evidence that these three epitopes may be important in discriminating PART from ADNC at early Braak stages.

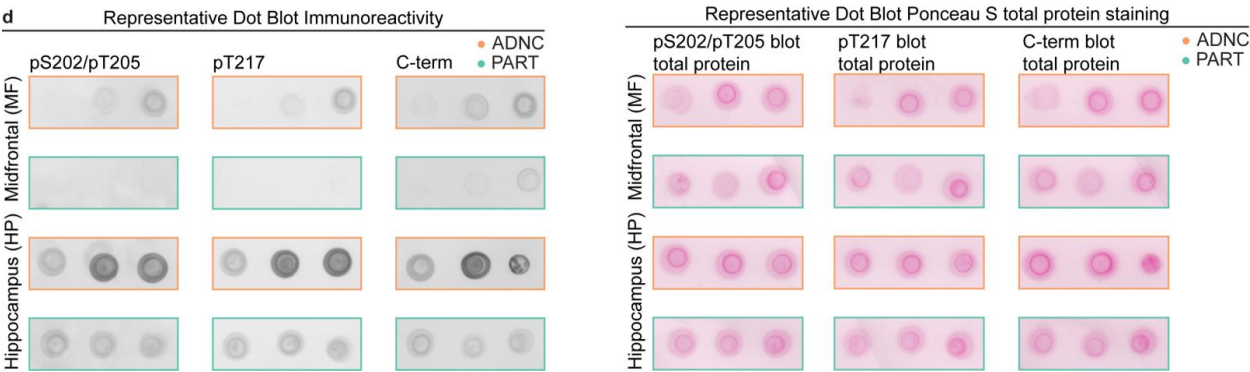
4) In the methods, the dot blot does not fully describe the protocol for immunoreactivity. Additionally, if total protein by ponceau S is used for the loading control, it should be shown in the figure. By eye, there are substantive difference between ADNC and PART in both MF and HP, but the quantification does not reflect this.

In the original methods, we attempted to avoid redundancy by stating that our dot blots were carried out “as described above” and referencing our detailed western immunoblotting protocol. To clarify further, we edited the

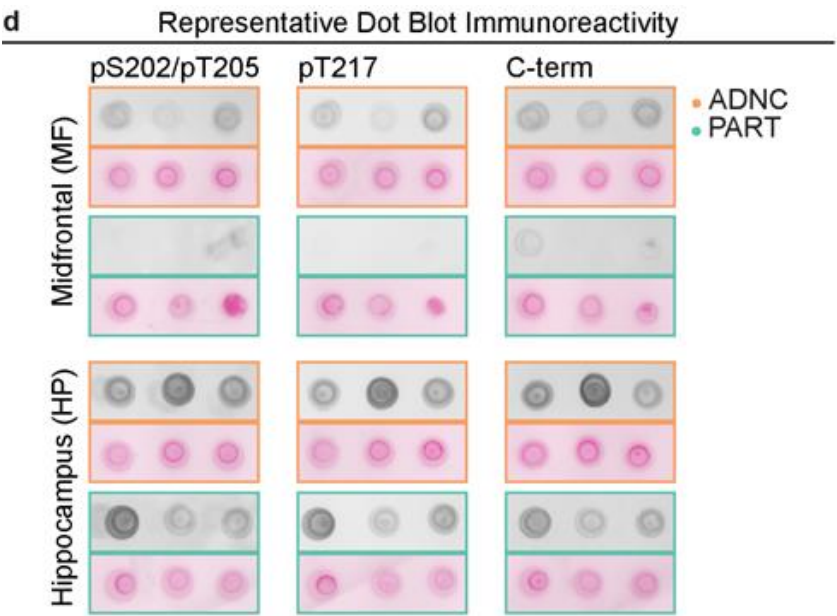
methods for dot blots to state these were carried out “using the methods and antibody incubations described for immunoblotting methods above” (lines 319-320).

We have also incorporated a new representative dot blot example into **Updated Figure 3** that we think better captures the range of immunoreactivities we visualized across ADNC and PART cases. However, for the reviewer, we also include the original with Ponceau S below. All uncropped blots are also included with the manuscript submission.

ORIGINAL WITH ADDED PONCEAU S COMPARISON:



NEW REPLACEMENT:



5) There seems to be more total tau in ADNC and high ADNC on supplemental figure 2, particularly HT7. This should be quantified and displayed. HT7 appears to be capturing a dimer that is not present in the PART sample. This is matched visually on the homogenate from SI.

Original **Supplemental Fig. 2** shows non-identical banding patterns of two mid-domain tau antibodies (HT7 and BT2), even though both HT7 and BT2 are routinely used in the literature as a proxy for all tau isoform species. We

believe this result highlights that measurement of “total tau” is complicated by tau’s existence as multiple isoforms with myriad post-translational modifications, proteolytic cleavage products, and soluble and insoluble species.

We considered these issues carefully when designing our experiments, which is why we present results for two frequently used “total tau” mid-domain antibodies (HT7 and BT2) (as well as the C-term antibody which is also marketed as a “total tau” antibody), and measure total protein levels by Ponceau S. Normalizing our seeding results to each of these (**Supplemental Figure 8**) demonstrates comparable results with all 3 measures.

To directly address this reviewer’s question, we now include measurements of the amounts of HT7-reactive tau and BT2-reactive tau normalized to total protein in both MF and HP in **New Supplemental Figure 4a & b**. Comparing these measures between our ADNC and PART groups suggests a decreased amount of tau marked by these two mid-domain antibodies in PART vs. ADNC in **both** MF and HP that does not explain the region-specific differences in seeding activity or PTMs (see **Supplemental Figure 8**).

We agree that HT7 appears to capture additional high molecular weight species when compared to BT2. The reviewers’ observation that HT7 may capture additional higher molecular weight species present in ADNC but not PART in the single replicates shown here reiterates that a single total tau antibody is not sufficient to identify all tau species. This observation may warrant further follow up to examine prevalence of tau proteoforms in PART and ADNC, but is outside the scope of this paper.

6) The authors should clarify the relevance of Thal when 0-2 defines the PART group and 3-5 is ADNC. Figure 4a makes sense but doesn’t seem to be novel.

Separating the patients by Thal phase allows comparison between Definite PART (Thal 0) and Possible PART (Thal 1-2), which is a critical neuropathologic distinction. Figure 4a is also presented as part of the panel of the three staging systems used in the neuropathologic diagnosis of ADNC, allowing direct comparison of the effects of Thal phase (A β neuroanatomic distribution on IHC) vs. CERAD (density of neuritic plaques) vs Braak (tau neuroanatomic distribution) on seeding. We have updated **Figure 4a** to include color-coding each point to reflect “Definite PART”, “Possible PART”, and “ADNC” cases. We have clarified this as follows:

“An examination of this breakdown allowed comparison of patients that met criteria for “Definite” PART (defined using the preferred criteria as those with Thal phase 0, or CERAD 0 by the alternative criteria) to those with “Probable” PART (Thal 1-2, or alternatively CERAD 1), and demonstrated no significant differences between the two sub-categories of PART (Figure 4a). Further, this analysis revealed that within ADNC, those with relatively more dense neuritic plaques (CERAD 3) had elevated seeding compared to those with relatively fewer plaques (CERAD 2)”. (lines 641-647)

7) The manuscript could benefit from major editorial review. This would include for clarity, accuracy and citations. For example, in the introduction (lines 48-50), the statements about prion mechanisms for the development of NFTs require better, more nuanced descriptions and citations.

We have revised the paper extensively as shown in our track-change comments. We wholly agree with the point that mechanisms of spread and protein-based propagation was insufficiently delineated in our previous statement. We have revised this to provide a more accurate, detailed description of the state of the field (lines 63-73):

“Tau pathology in progressive Alzheimer’s Disease (AD) is characterized by stereotyped regional accumulation of NFTs visible by histology ^{2,3}, leading to the prevailing hypothesis that tau pathology can spread intercellularly throughout the brain. This pathological spread has been posited to occur directly through the intercellular transfer and protein-based propagation of misfolded tau itself (whereby tau ‘seeds’ can recruit natively folded monomers to self-propagate filamentous structures), or through anatomically connected cellular spread of pathological processes that leads to tau post-translational modification, misfolding, and resultant toxicity ^{4,5}. Understanding the mechanisms

that enhance tau misfolding and NFT accumulation is critical as it underlies the characteristic neuropathology and clinical presentation of tauopathies including AD.”

8) In addition, the statement that 100% of individuals over 85 have NFTs (lines 45-47) is a misinterpretation of the manuscript, as the reference showed 100% tau PET positivity, not the presence of AD-related NFTs. Finally, this study is densely written and challenging to read.

We apologize for the mischaracterization. We have now clarified the text to indicate that 100% of individuals over age 85 demonstrate tau-PET positivity. With regard to NFTs specifically, we now cite Braak and Braak (PMID: 22002422), who demonstrated, in a series of 2,332 non-selected autopsy cases ranging from age 1 to 100, that by age 40 all cases had at least pre-tangle pathology and by age 81+ all 559 patients in those groups had at least Braak stage I pathology. The specific changes in the manuscript are as follows (lines 60-63):

“Tau neurofibrillary tangles (NFTs) are a highly prevalent neuropathology in aged individuals, with pre-tangle pathology nearly universal in individuals over age 40, tau-PET positivity in 100% of individuals over age 85, and frank histologic NFTs observed in all individuals by age 81+.”

Reviewer #2 (Remarks to the Author):

Browne et al report their study on the levels of post-translational modifications of tau, and tau seeding activity in pathologically-determined primary age-related tauopathy and Alzheimer’s disease. In examining two brain regions, hippocampus vs neocortex, they describe a marked difference in seeding and PTM profile in the neocortex between these conditions. The results are consistent with a model wherein beta-amyloid pathology promotes a distinct tau PTM and seeding phenotype specifically in the cortex but not in the hippocampus. This is a very well performed study that sheds light on the pathophysiological mechanisms defining PART and AD.

Specific comments:

The ultrastructure of the PART and AD tau filaments has been shown to be identical, which makes comparison between the seeding activity of these strains appropriate, and leads the authors to conclude that PTMs differentiate tau species in cortical PART vs ADNC. However, another difference could be fibril length. For a given quantity of tau input, there might be more seed-competent species if the filaments were shorter. Can this alternative explanation be addressed? For instance, by profiling insoluble species using negative stain EM.

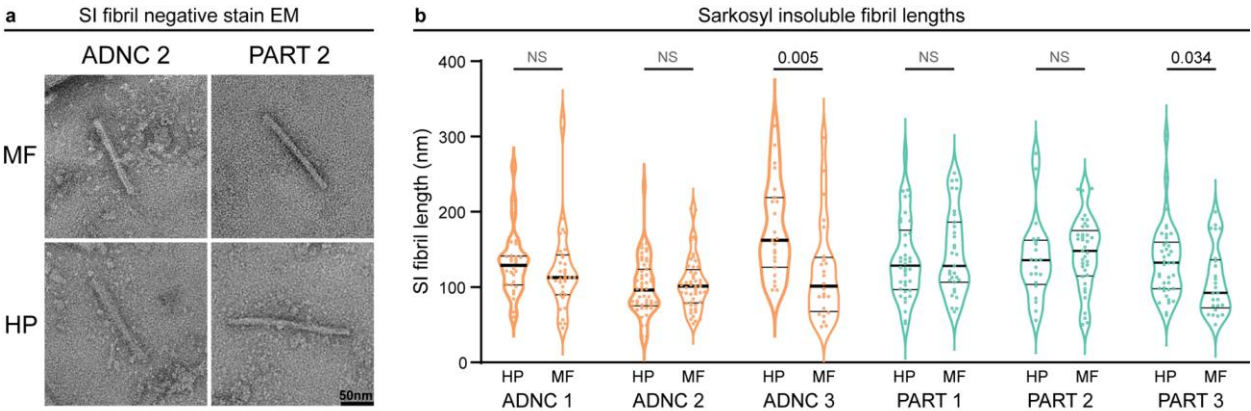
The reviewer makes an astute point that increased fibril length should be inversely related to available seeding ends per amount of misfolded tau. Per the reviewer’s point, we have begun to assess if obvious fibril length differences in representative cases of PART and ADNC using negative stain EM could further explain our findings. We analyzed a small sample of HP and MF derived SI preparations (3 ADNC and 3 PART cases) using negative stain electron microscopy to evaluate the bulk ultrastructural characteristics.

We made the following observations:

1. Fibril lengths vary, within and across both PART and ADNC cases; see Panel B below. We include representative EM images of fibrils in panel A, with our measurements across regions and PART and ADNC tabulated in panel B.
2. Fibril lengths did not differ consistently between HP and MF regions. This includes in PART cases where we noted substantially less seeding activity in MF. This suggests that fibril length alone is not sufficient to explain differences in seeding activities.

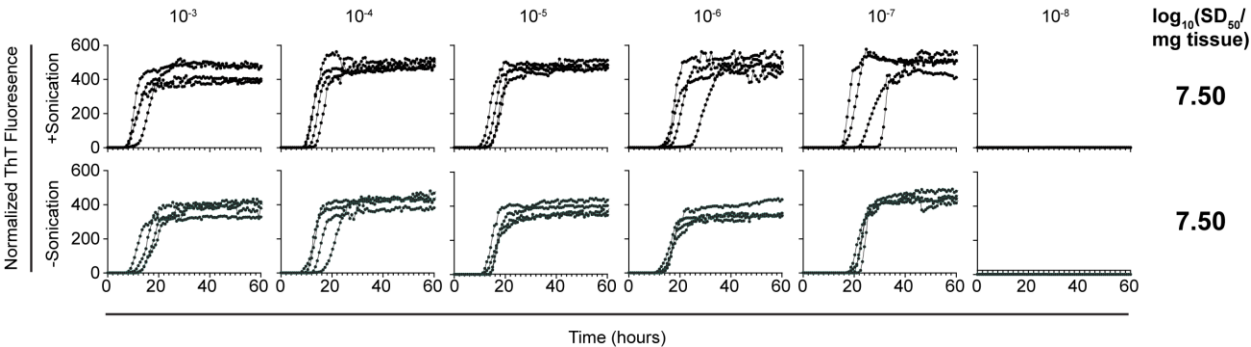
We note this evidence is preliminary. We have opted to not include it in the revised manuscript figures as it is our perspective that such analyses in isolation are unlikely to capture the complexities of structural variation in these preparations (e.g. mixed compositions of polymorphs [paired helical and straight filaments of ADNC]; mixed lengths; mixed maturity in stage of fibril formation at any given pathological stage; etc). While outside the scope of this current paper, we now note in discussion (lines 836-840 in “all markup” track-change version) “Regarding our

observed differences in MF-PART versus MF-ADNC seeding activities; we cannot rule out that collective fibril ultrastructure differences may also contribute (e.g. mixed composition of paired helical and straight filaments [both incorporating the shared amyloid core structure], mixed lengths, mixed fibril maturity at a given pathological stage, etc).”



Alternatively, if sonication were used and a large increase in PART seeding activity were observed, it could be consistent with the break-up of large fibrils.

The technical steps used for this study were equivalently implemented between PART and ADNC. Specifically, while there was a brief sonication step directly after resuspension of the pellet to ensure homogenous distribution of product in solution (i.e. no clumps), this was done under the exact same conditions for PART and ADNC. We have also further tested if our brief sonication protocols alter our seeding readouts and have not noted any difference with sonication of the insoluble material (see Figure below).



Line 47-50: there is a distinction between inter-cellular prion-like spreading and the assembly of tau within a cell which is not captured in this description of tau aggregation. Prion-like seeded aggregation as a hypothesis of the apparent spread of tau pathology across the brain should be presented alongside alternatives.

Per our response to Reviewer 1, we see the reviewer’s point that we were overly brief in our statement. We agree that mechanisms of accumulation, spread, and protein-based propagation are insufficiently delineated. We have revised lines 63-73: Tau pathology in progressive Alzheimer’s Disease (AD) is characterized by stereotyped regional accumulation of NFTs visible by histology ^{2,3}, leading to the prevailing hypothesis that tau pathology can spread intercellularly throughout the brain. This pathological spread has been posited to occur directly through the intercellular transfer and protein-based propagation of misfolded tau itself (whereby tau ‘seeds’ can recruit natively folded monomers to self-propagate filamentous structures), or through anatomically connected cellular spread of

pathological processes that leads to tau post-translational modification, misfolding, and resultant toxicity^{4,5}. Understanding the mechanisms that enhance tau misfolding and NFT accumulation is critical as it underlies the characteristic neuropathology and clinical presentation of tauopathies including AD”.

The authors normalize the input sarkosyl insoluble tau to the seeding reactions. While this is valuable for determining differences in intrinsic seeding ability, it masks the total number of seeds present in tissue. A graph showing the quantities of insoluble tau in the regions/diseases, similar to Fig 1c and 1e would be valuable. Does this correspond to the IHC tau pathology or to the RT-QuIC?

We agree with the reviewer that it is valuable to understand if there is increased SI material per unit of brain tissue in cases with higher seeding activity. Thus, we have now included new data showing there is no difference in the mass of SI material derived per milligram of brain tissue across both regions and diagnoses (new **Supplemental Figure 2**). Additionally, there is no correlation between the amount of SI material derived and BH seeding activity or quantitative tau digital IHC.

It is not always clear whether the seed quantification assays were performed using homogenate or sarkosyl insoluble input (eg Fig 5 and Supp Fig 6).

We have revised to ensure this is specified in each Figure legend for added clarity.

The ADNC seeding does not always reach endpoint dilution in the graphs displayed. Can the authors confirm that this was determined?

Representative curves for RT-QuIC fluorescent readouts were only intended to show examples of primary data for a dilution series, and for ADNC and PART cases (including examples of **Figures 1d** and **3a**). We confirm that endpoint was always reached, and have added this statement to the manuscript (lines 279-280). To better reflect this in the Figures, we have selected another case for **Figures 1d** and **3a**, to clearly show what an ADNC case that reaches endpoint within the dilution series looks like.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for co-reviewing our manuscript, please see our detailed responses.

Reviewer #4 (Remarks to the Author):

This manuscript describes differences in tau seeding between brain tissue homogenates from PART/possible PART and ADNC middle frontal cortex in Braak stage matched cases. In addition, differences in tau PTM levels are identified, and associations with cognition are made. Overall, it is an interesting study and well-written. However, it is missing some crucial analyses, without which the conclusions appear to be overstated.

Major comments:

1) Page 2, lines 25-26, and page 3, lines 47-48: While there have been studies which suggest tau seeding as a mechanism for tau propagation, this is not universally accepted and remains somewhat controversial. At minimum, the controversy around this topic should be discussed as this concept has not definitively been proven. Page 3, lines 50-52 are true, but contradict lines 47-48, as this mechanism is not fully understood yet.

As noted in our response to Reviewer 1, we thank the reviewer for pointing out the nuance lost in our overly brief original statement. We have added additional verbiage (lines 63-73) to more clearly delineate the most prevalent hypotheses for mechanisms of tau propagation and spread.

2) Page 2, line 26: This does not necessarily suggest that all “tau seeds” are not equally pathogenic; it could simply suggest that tau without amyloid, or tau without other comorbidities could be less pathogenic.

We agree that this sentence is overly simplistic and that pathogenic outcomes are very likely to be driven by multiple factors, including comorbid pathologies, cellular milieu, and yet-to-be characterized inflammatory responses. We are actively investigating these questions for future publications. To better capture such nuances in the short space of the abstract, we have revised our statement as follows (additions to text in bold below, lines 30-32):

“Tau neurofibrillary tangles (NFTs) are required for the diagnosis of Alzheimer’s Disease (AD) yet cognitively normal aged individuals frequently exhibit NFTs arising from tau seed accumulation. This **may** suggest that not all tau species are equally pathogenic and raises the question of whether unidentified tau modifications augment tau seeding activity and neurodegeneration in AD.”

Ultimately, more study is required to fully define the main drivers of tau-related pathogenicity; however, our data support a model in which qualitative features of tau seeds such as PTMs may augment tau seeding activity.

3) Page 19, lines 400-410: If this is true, that essentially means more p-tau, leads to more seeding, and this contradicts the digital pathology findings that PART and ADNC in this cohort had equal amounts of p-tau.

Differences in sensitivity of digital quantification of IHC vs. western blot likely account for this apparently discrepant observation (**Figure 1c** and Original **Supplemental Table 2 (now New Supplemental Table 3)** vs. **Figure 3**). For **Fig 1c/Table S3**, we used digital quantification of IHC. Our cohort is by definition Braak IV or lower, so there is very little phospho-tau stain in the midfrontal cortex. For **Fig 1c/Table S3**, measurements are shown on a log scale (ln % area occupancy). Converting from the log scale shows the area positive for AT8 signal in MF is <0.5% in both PART and ADNC cases. In contrast, it is known that immunoblotting methods are more sensitive and are capable of detecting as little as 0.1 ng of protein (e.g. PMID 36513922). This allows western blot to discriminate levels of phospho-tau in MF of ADNC vs. MF of PART even though IHC cannot.

4) Page 20, line 439: Are total tau levels between PART and ADNC equal? Supplementary figures 1 and 2 appear to demonstrate that PART has lower total tau. Please see below as well, regarding page 23, lines 492-494.

Measurement of “total tau” is quite complicated since tau exists as multiple isoforms with a myriad of post-translational modifications, proteolytic cleavage products, and soluble and insoluble species. Measurements of total tau will also capture normally folded endogenous tau in addition to variable misfolded forms of tau. We considered these issues carefully when designing our experiments, which is why we present results for two frequently used “total tau” mid-domain antibodies (HT7 and BT2) as well as measuring total protein levels by Ponceau S and normalizing our seeding results to each of these (**Supplemental Figure 8**). Importantly, comparable results were demonstrated with all 3 measures.

To directly address this reviewer’s question, we now include measurements of the amounts of HT7-reactive tau and BT2-reactive tau normalized to total protein in both MF and HP in new **Supplemental Figure 4a&b**, and evaluated additional technical immunoblot replicates for these and other PTM antibodies in the statistical analyses per Updated **Supplemental Table 3**. Comparing these measures between our ADNC and PART groups suggests a decreased amount of tau marked by these two mid-domain antibodies in PART vs. ADNC in **both** MF and HP that cannot explain the region-specific differences in seeding activity or PTMs (see **Supplemental Figure 8**).

5) Page 21, lines 460-465: These analyses could be performed with intermediate ADNC cases with differing CERAD NP scores. For example, int ADNC cases with Thal phase 3-5 and Braak III-IV can have CERAD score of sparse, moderate or frequent and still be intermediate ADNC (not PART or possible PART).

This is a good idea. We analyzed seeding within the group of intermediate ADNC patients (Thal 3-5, Braak III-IV) across CERAD levels. This subgroup of patients contains 12 individuals (C1:1, C2:5, C3:6) and does show an effect of CERAD on seeding within the ADNC group. We have added the analysis to the results sections as follows:

Lines 647-671: “To establish if this effect was due to an association of higher CERAD scores with more advanced tau pathology, we repeated this analysis in exclusively intermediate ADNC patients (defined by Thal phase 3-5 and Braak stage III-IV), where there was a range of CERAD scores (CERAD1: n = 1, CERAD 2: n = 5, and CERAD 3: n = 6). Within this group of patients, MF seeding remained associated with increasing CERAD score ($F = 8.4$, $p = 0.02$), with a post-hoc difference between those with CERAD 3 versus 2 ($p = 0.03$)”

A summary of comprehensive revisions addressing the relationship of neuritic plaque density to seeding, PTMs, and cognition (points 5-6, 8, 11), including above analysis, follow point 11 below.

6) Do intermediate ADNC cases with differing CERAD NP scores have different tau seeding levels? If yes, then this tau seeding does not necessarily differentiate PART from ADNC, as the title and manuscript claim. Perhaps tau seeding may simply differentiate progressive CERAD NP levels.

As above, in the cohort of intermediate ADNC patients, increased CERAD NP score correlates with increased MF tau seeding and PTMs. This does not negate the ability of seeding to distinguish between ADNC and PART, since differences in CERAD or Thal define PART vs ADNC as per ref. 3, PMID: 25348064. In other words, since cortical seeding correlates with CERAD or Thal, and since PART vs. ADNC is defined by CERAD or Thal, it logically follows that cortical seeding should differentiate between ADNC and PART, which it does.

However, we agree that the relationship to neuritic plaque density was not highlighted sufficiently. **We have revised the title to: “Early Alzheimer's Disease with frequent neuritic plaques harbors neocortical tau seeds distinct from primary age-related tauopathy”** and incorporated extensive additions and revisions listed after **Point 11** to reflect the importance of neuritic plaque density.

We also note that while either Thal or CERAD may be used to diagnose PART, PMID: 25348064 primarily recommends Thal. However, our findings suggest that MF seeding more closely relates to CERAD (cortical neuritic plaque density), than to Thal (neuroanatomic distribution of A β) or overall A β IHC immunoreactivity (see point 7 below). Because neuritic plaques have a tau component, it is tempting to speculate that this neuritic tau component could contribute to seeding, something we plan to pursue in future work. MF tau deposition (i.e. Braak stage >IV) underpins the clinical differences of late-stage ADNC and PART. If increased MF tau seeding foreshadows future MF tau NFTs and if MF tau seeding most strongly relates to neuritic plaques, CERAD rather than Thal-based criteria for the diagnosis of PART may capture a more biologically meaningful group.

-We include **new text to address this idea in our discussion lines 864-868:** “This could suggest that categorizing PART by CERAD neuritic plaque density and not Thal phase may capture a more biologically meaningful patient population; however, this possibility is admittedly speculative and based only on effects on tau modification/seeding. Extensive additional studies would be needed to evaluate the utility of these two possible criteria.”

7) In addition, since Thal phase correlates with seeding, it is possible that amyloid burden would correlate. Therefore, digital quantification for amyloid could be performed and amyloid burden levels could be correlated with seeding levels, similar to the authors' work on tau.

We have now used digital histology to analyze the density of A β across HP and MF in the whole cohort and included it in our results. Across the cohort, there are separate significant correlations of both tau and A β pathology burden and seeding activity in HP. However, when degree of A β pathology is controlled for using multivariable models, ANOVA testing demonstrates that tau burden drives the association with SD50 in HP.

In MF, there are trend level associations of A β pathology with SD50 (also with tau pathology, noting as above that there are low levels of tau on IHC in MF). In a multivariable model, these associations remain trend level (i.e., when

the amount of tau pathology in MF is controlled for, A β pathology is only related to MF SD50 doses at the trend level of $p=0.07$). We note that digital histologic methods capture all immunoreactive A β , rather than just neuritic plaques as is measured by CERAD. This supports our speculation that neuritic plaques may be an especially important player in tau seeding.

-We have added the following text describing our analyses (lines 672-687): “Given that the ordinal CERAD neuritic plaque density and Thal A β phase appeared to demonstrate a consistent effect on cortical tau seeding, we sought to employ a more granular measure of A β density using digital quantification of %AO by A β IHC (as we had done previously with tau). The results indicate that within the entire cohort, there are significant correlations of HP seeding activity separately with both HP tau and HP A β histologic digital area occupied, but when controlling for both in a two-way ANOVA, tau burden drove the seeding association while the A β digital area occupied term was not significant (**Supplemental Table 6**). In the MF, there were only trend-level associations of MF seeding activity separately with both A β and tau histologic frequency (acknowledging that this is scant in Braak II-IV cases). In multivariable models, these analyses remained trend level for both tau and A β (**Supplemental Table 6**). We note that this digital histologic assessment captures the area occupied by any A β immunoreactivity, rather than density only of neuritic plaques as is measured by CERAD. This could suggest that neuritic plaques specifically play a distinct role in this relationship, in line with the stronger association of MF seeding with CERAD compared to Thal phase.”

8) The cognitive data correlation with seeding could simply be due to increased NP density, this possibility should be addressed. Multivariate regression models could be constructed to account for NP density (among other variables).

Page 17, line 370: Please include Braak stage and CERAD NP score in these analyses (not just “PART” or “ADNC”), as these have previously been found to associate with cognitive measures.

Please note that the cognitive models did not include a group term (PART vs ADNC) but instead tested the influence of seeding in models adjusted for age at death and education level. However, your point is well-taken, and we tested if the relationship of cognition with seeding is a proxy for another variable (e.g., CERAD or Braak). As suggested, we constructed multivariable models by adding either Thal, CERAD, or Braak alongside the original seeding model terms. The results of ANOVA testing indicates that MF seeding remains predictive of Executive function, and HP seeding remains predictive of Memory, while Thal, CERAD, or Braak did not reach significance in any of the models analyzed. This suggests that seeding is not simply a proxy for neuropathologic stage in its relationship to cognitive decline.

-We have included this analysis and full model results in **new Supplemental Table 4**.

-We expand on this point in the manuscript in new text (lines 490-503): “The question arises if these cognitive associations are more related to group differences between ADNC and PART (or to the A β pathology which defines them), rather than to seeding activity itself. Mirroring the greater MF seeding activity seen in ADNC, there is a trend towards more impaired executive function ADNC patients compared to PART ($\beta \pm SE = -1.12 \pm 0.57$, $p = 0.06$), which may foreshadow further cognitive decline as ADNC patients accumulate more MF pathology at later disease stages (i.e., Braak V-VI). However, when either CERAD or Thal were included as covariates in the cognitive models of seeding used above, neither demonstrated an association with either memory or executive function (**Supplemental Table 4**). Rather, HP seeding remained a significant predictor of memory, while MF seeding remained a significant (or trend-level alongside CERAD) predictor of executive function (**Supplemental Table 4**). Overall this suggests that tau seeding activity is a more direct correlate of these cognitive measures than CERAD or Thal, rather than a proxy for them.”

9) Page 23, lines 492-494: Does this suggest that more total tau leads to more seeding without other factors?

See response to reviewer 4 point 4, where we addressed this.

10) Figure 5g: there appears to be a case labeled as “PART” (presumably possible PART) with a Braak score IV and CERAD 2/moderate. This CERAD NP score is incompatible with a diagnosis of PART as it is currently defined (Reference #3 in the current manuscript).

Our PART and ADNC groups were defined using the preferred criteria which utilize a combination of Thal phase and Braak stage (See Table 2 of Ref. 7, PMID: 25348064). As noted, one participant from the PART group did have a CERAD score of 2, but had a Thal stage of 2 as well, placing them in the PART group by this criterion. We have now updated **Figure 4a** to provide more detail by coloring the points by their respective groups.

This point highlights some of the open questions regarding diagnosis of PART, which we now expand upon in our response to Point 6 **in discussion lines 864-868 (see above)**.

11) Page 23, lines 497-500: Given the importance of the relationship between NP density and seeding/MF tau, this concept should be made more central to the paper rather than focusing on “the seeding activity differentiates PART from AD”. This is particularly true since the authors do not distinguish their cases as “definite PART” or “possible PART”, and so have a mixture of cases with and without neuritic plaques. The authors should either organize their conclusions around the presence/absence/severity of tau (PTMs) and neuritic plaques or separate their cases into more distinct PART/ADNC groups rather than the continuum shown in figure 5g.

Nowhere in the manuscript do the authors demonstrate that PART alone is different from ADNC, only that seeding correlates with NP density, and Thal 4-5 as compared to Thal 0 has greater MF tau seeding, but not greater than Thal 3-4 cases, thus PART (Thal 0) is no different from ADNC with Thal 3-4.

We demonstrate that in aggregate, PART (Thal 0-2) differs from ADNC (Thal 3-5) in seeding and PTMs, as it is currently defined by the current neuropathologic diagnostic criteria. When this is further subdivided, we see that it is the extremes of each group that differ. However, the lack of identified difference between the intermediate groups is tempered by the reduced statistical power of the smaller subgroups. However, your points on NP density (**points 5-6, 8, 11**) are well-taken, and we have reoriented the framing of the paper, including updating the title, to focus more on neuritic plaque density (i.e., CERAD score) as an important factor as below:

- 1) Revised title to “**Early Alzheimer's Disease with frequent neuritic plaques harbors neocortical tau seeds distinct from primary age-related tauopathy**” better reflecting the importance of neuritic plaque density in the effects we observe (point 6)
- 2) **New analysis of seeding vs. CERAD in intermediate ADNC** with results in **lines 647-671 & 864-868** (points 5,6)
- 3) **New analysis of CERAD, Thal, and Braak in cognitive models** (point 8) with results in **new Supplemental Table 4** and lines **490-503**
- 4) **New analysis of A β digital pathology density** demonstrating a stronger associate with CERAD stage than with Thal Phase or A β digital quantification
- 5) We have **updated Figure 4a** to provide more detail by coloring the points to show PART vs ADNC.
- 6) New text in discussion **lines 864-868**

Minor comments:

Page 3, line 62: PART can extend to the neocortex, albeit rarely and in a limited fashion. This has been shown previously in the literature, and in Figure 1b. The sentence “Importantly, the NFT pathology of PART appears

limited to subcortical and limbic regions and does not extend to the neocortex” could be left out, as the next sentence directly contradicts that sentence.

We have removed the sentence in question, as we agree that the following sentence presents a more nuanced explanation.

Page 15-16, lines 329-332: Analyzing the contralateral hippocampus and middle frontal cortex for p-tau burden does not necessarily mean that the other side will have an equal amount of p-tau. There is often asymmetry, especially in PART and early stages of ADNC.

This is absolutely a limitation of this study, as it is a limitation in much biobanking research. We have now highlighted this particular limitation directly in the Methods, as follows (**lines 256-260**):

“It should be noted that both PART and ADNC exhibit asymmetry in the degree of neuropathology, especially in the early stages evaluated for this project. This is a known limitation of studies using tissue from brain banks that have chosen to divide the brain in half for fixation and freezing, and may attenuate the strength of associations identified.”

Page 28, lines 616-619: Post-mortem and pre-mortem CSF samples are available at many institutions, this could be tested in the present manuscript.

There is no published protocol to detect 3R/4R tau seeding in CSF, though this is an open area of investigation by our group and others. Thus, this cohort was chosen with regard to availability of clinical/cognitive data, paraffin blocks, and frozen tissue, but not for availability of CSF. As such, less than a third of these participants have CSF for analysis. With regard to pTau fluid biomarkers, several published papers shed light on these questions (see doi:10.1001/jamaneurol.2023.2560 and doi: 10.1007/s00401-023-02570-4), and we hope to explore this further in future studies from our laboratories.

We appreciate the feedback from Reviewer 1 and note our responses below.

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript by Browne et al. addresses many of our concerns. The authors have analyzed the effect of ApoE4 dosage and tau seeding and found no effect of ApoE4. They also have performed a more nuanced analysis of the link between neuritic plaque density and tau seeding. These analyses, as well as extensive revision to the writing and data presentation, are appreciated and have improved the manuscript.

A few items remain that should be addressed to strengthen the paper:

1) As the authors decline to test additional tau PTMs, they will need to thoroughly revise their claims about PTMs, inclusive of the abstract, results and discussion. Specifically, any claim about a PTM should not be generalized but refer to the specific PTM tested or describe the “selected” or “subset” of PTMs. The indiscriminate generalization of limited findings historically has been a major problem in the field of neurodegeneration. This study should take care to avoid this pitfall.

We agree with the reviewer’s point about overly broad generalizations and have made certain to incorporate the modifier “select” PTMs comprehensively throughout the manuscript.

2) Address in the discussion why there is no impact of ApoE4 on seeding as this is a surprising finding.

We agree with the reviewer that the effect of ApoE4 is interesting. The above comment may miss the nuance of the effect of Abeta. There is an effect of ApoE4 on tau seeding, however, this effect of ApoE4 is mediated by Abeta. I.e. ApoE4 certainly increases Abeta amount and Abeta amount also increases seeding. However, high Abeta in the absence of ApoE4 has the same effect as Abeta driven by ApoE4. This is discussed in the following sentence:

“Overall these findings imply that *APOE* ϵ 4 genotype is a risk factor for the development of A β pathology (and in turn ADNC, both of which are well established) but that *APOE* ϵ 4 is not in itself the main driver of tau PTMs and seeding results.”

3) We appreciate the authors providing the full dot blots of PTMs. It appears that the difference is driven by a few individuals in the ADNC group in Figure 3e-f; it would be interesting to hear more discussion on these patients.

We agree this is interesting; to this point, the individuals in the ADNC group that show high levels of pT217 and pS202/pT205 in Figure 3e-f are parsed by CERAD score in Figure 4d-e. This demonstrates a clear relationship between CERAD “frequent” and increased pT217 and pS202/pT205.

4) Line 131: “billion” needs a citation
We have added citations.