

Plasma eMTBR-tau243 and p-tau205 as T2 Biomarkers for Biological Staging and Prognosis in Alzheimer's Disease

Corresponding Author: Professor Sang Won Seo

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The study "Defining Plasma T2 Tau Biomarkers for Biological Staging and Prognosis in Alzheimer's Disease" by Yim, Sohyun; Lee, Eun Hye et. al. on behalf of the K-ROAD study groups evaluates plasma biomarkers that reflect distinct stages of Alzheimer's disease (AD) that could enable scalable biological staging. The study evaluated phosphorylated tau (p-tau) species across multiple analytical platforms and endogenously cleaved microtubule-binding region containing residue 243 (eMTBR-tau243) using a dedicated LC-MS/MS platform in a well-characterized cohort with amyloid PET and subset with tau PET. They found phosphorylated tau (p-tau181, p-tau217 – referred to as T1 markers) were increased in the preclinical stages of the disease, whereas eMTBR-tau243 and p-tau205 increased later in the disease stage. eMTBR-tau243 showed strongest and stage-dependent associations with regional tau PET and cognition. The authors found that plasma-based staging system integrating p-tau217 and eMTBR-tau243 closely aligned with PET-based tau staging and predicted longitudinal cognitive trajectories. They claim that plasma eMTBR-tau243 as a late phase biomarker (T2 biomarker). This plasma-based staging has the potential for prognosis and therapeutic decision making in AD.

The results presented in this manuscript is timely with significant implications for large scale deployment of blood-based biomarker assessment in older (age above 65) populations that is currently not achievable and possible using imaging (PET) based and other invasive biomarker testing platform (CSF). Inclusion of multiple analytical platforms in this study such as automated single molecule immunoassays and LC-MS/MS assays, enables robust and generalizable assessment of biomarker performance. By doing so this study aims to minimize platform-specific bias and providing cross-validation across diverse assay technologies. While the data presented in this manuscript and supplementary is solid, there are many points that the authors must address in terms of writing and interpretation. The authors don't cite the correct references when describing the literature on validated biomarkers, while some references are missing without which it is hard to assess if the analytical measurements are indeed validated and accurate. Addressing these concerns will not only make this manuscript more robust and reproducible, but it will also help the readers understand the results who are not very much accustomed to the nomenclatures and terminologies of blood-based biomarkers.

Major points

1. The reviewer looked across the manuscript (Introduction, results and discussion section) however could not find detailed definition of T1/T2 biomarker framework. One area that could be strengthened in the Introduction section about the discussion and definition of the T1/T2 biomarker framework. The reference cited don't refer to Jack, Clifford R. et. al. Revised criteria for the diagnosis and staging of Alzheimer's disease, Nature Medicine, 2024, PMID: 38934362; Jack, Clifford R. et.al. Revised criteria for diagnosis and staging of Alzheimer's disease: Alzheimer's Association Workgroup, Alz & Dementia, 2024 (currently cited as reference 5 in the prior paragraph that seems little out of place) PMID: 38934362. While the manuscript refers to certain phosphorylated tau species as "T1 biomarkers" and tau aggregation-associated markers as "T2 biomarkers," however the conceptual basis and nomenclature underlying these categories are not explicitly introduced (Core Biomarkers for defining stages of Alzheimer's disease proteinopathy pathway or pathogenic species). Because this terminology is relatively recent and many researchers across neuroscience as well as neurodegeneration field may not yet be familiar with these, a clearer explanation of what biologically distinguishes T1 from T2 biomarkers would improve accessibility and context for the study rationale.
2. A ~ 23 % reduction of plasma tau phosphorylation p-tau217 in TRAILBLAZER-ALZ trial in anti-amyloid treated

participants (donanemab) have been reported with respect to the placebo participants at 76 weeks compared to their baseline levels (JAMA Neurol. 2022;79(12):1250-1259). Thus plasma biomarker p-tau217 is a candidate reflecting the pharmacodynamic measures of the effect of anti-amyloid therapies. The authors need to include that in their introduction and mention that these early amyloid pathology surrogate markers do have treatment related response.

3. The reference cited for CSF microtubule-binding region derived tau (MTBR-tau243) is not correct. The first paper that describes the CSF MTBR-tau243 assay that should be referred is PMID: 33283854. The reference for the multi-cohort validation of CSF MTBR-tau243 is PMID: 37443334. The authors are recommended to cite the correct references when they discuss the respective relevant biomarkers.

4. The authors cite Horie, K et. al. Nature Medicine 2025 paper (PMID: 33283854) for MTBR-tau243 which is not correct. This Nature Medicine 2025 paper is the first paper to describe the plasma endogenously cleaved MTBR-tau243 fragment (eMTBR-tau243). This Nature Medicine paper clearly states that the tryptic MTBR-tau243 as originally used for the CSF, did not correlate with tau-PET in the plasma samples. They discovered a different non-tryptic peptide (which they call eMTBR-tau243) that has valine residue at the C-terminus (Val256) and preceding amino acid asparagine (Asn255) is deamidated, which was proportional to tau-PET in both the plasma and the CSF samples. The authors are recommended again to cite the corresponding reference along with this description when describing eMTBR-tau243.

5. In relation to the above comment, looking across the method section of this manuscript, it is not clear what is different for the p-tau and eMTBR-tau243 C2N assay. There are no references for the C2N eMTBR-tau243 assay that the authors claim was developed at C2N. If this assay is different from the Nature Medicine 2025 paper, PMID: 33283854, then the authors need to clearly mention that in the method section. Is the target analyte different when the authors state the eMTBR-tau243 was measured using a separate research use only (RUO) assay? This is unclear and no information has been provided to evaluate how reproducible this new assay compared to the original peer reviewed publication. Same for the p-tau multiplex tau mass-assisted assay. If these assays are different to the previously reported and established assays (PMID: 35446396) the authors need to provide either references for the assay validation (limit of quantitation, limit of detection, assay variability) or include them in the method/supporting information. The lack of clarity of what is being monitored in this manuscript is one of the major drawbacks that reduces enthusiasm toward the results presented.

6. Compared to immunoassays, LC-MS/MS was performed only on 20-25 % of the samples. The authors describe aliquot availability, laboratory volume requirements from the participants was the limiting factor for analytical sample size variance for the platform. Thus, it is crucial to describe in the methods the details of each platform, especially if the methods have been modified. Also did the authors analyze using LC-MS/MS assay all the 55 patients with tau-PET. The mean biomarkers values need to be tabulated in Table 1 for each group measured using the different biomarker platforms. Table 1 should also include the cognitive assessments (MMSE and CDR scores) for each group.

7. In the discussion the author claim that eMTBR-tau243 remained relatively stable during the preclinical and prodromal phase and rising sharply only with the onset of dementia. While all other p-tau biomarkers (Supplementary Figure 1) have low quantified levels at the amyloid negative CU stage, eMTBR-tau243 looks like it is very close to zero/or limit of detection (LOD). So, it could be possible that this assay lacks sensitivity to detect this endogenously cleaved peptide in plasma in the early phase of the disease unlike other more abundant phospho-tau species. The authors need to highlight this in the limitations.

8. The authors should include longitudinal CDR-SB trajectories and tau-PET, plasma based staging data, even if in the supporting information, as the number of participants is relatively low n=36 and the analysis may not be powered enough to draw strong conclusions.

Minor Point

1. There is a typo in the abstract last line. It should be "plasma tau-based staging for prognosis..." instead of "plasma tau-base staging...". The authors use multiple different names for this staging. Sometimes it is plasma-based tau staging, plasma tau-based staging etc. They need to be consistent and use one naming nomenclature.

Reviewer #2

(Remarks to the Author)

This is an interesting study discussing biomarkers that associate more strongly with tau pathology than with amyloid. However, several points need to be addressed to improve clarity, and interpretability.

Abstract

The T1 and T2 biomarkers are not defined. Please spell out the full names on first mention.

Results – Study Participants

The participant description is difficult to follow as written. Rather than referring to the measurement platform, please specify the biomarkers directly (platform details belong in the Methods). The current phrasing is confusing and impedes interpretation of the results.

The sentence reporting age and sex proportions — "The median age of participants was 73 [67–78], 72 [66–78], 72 [66–78], and 73 [66–78] years, and the proportion of female was 62.1%, 63.6%, 63.6%, and 63.9% respectively" — does not identify which participant groups the four values correspond to. Please label the groups explicitly. The entire participant description section would benefit from restructuring for clarity.

Because sample sizes differ across biomarkers, this should be clearly stated throughout, particularly given that the paper draws direct comparisons between biomarkers.

In Supplemental Figure 1 the group separation is very difficult to discern.

The meta-temporal ROI is described as representing "early-to-intermediate tau deposition." This characterization, however meta-temporal is not an early tau deposition region. Consider using the medial temporal lobe (MTL) instead, which is consistent stated as an early tau region.

Additional Analyses to Consider

1. Diagnosis-stratified associations. Were associations with tau and amyloid examined separately by diagnostic group? It would be informative to know whether effects differ between cognitively unimpaired individuals and those with cognitive impairment.
2. Longitudinal tau PET. If longitudinal tau PET data are available, analyses examining whether plasma biomarker levels track tau accumulation over time would substantially strengthen the paper.
3. Figure 1 / tau positivity. Why is there no figure showing effect sizes for tau positivity similar to what is shown for amyloid? Additionally, do these biomarkers differentiate Braak stages, or at low/intermediate/high tau burden categories as used in clinical trials? This would be highly relevant to include.
4. Demographic table. Please add the proportion of tau-positive participants to Table 1.
5. Tau staging in the Discussion. The Discussion references tau staging, but the analyses only separate participants into early and late groups, analysis with all stages should be performed to state that.

Limitations

The Limitations section should explicitly acknowledge: (1) lack of replication in diverse populations and clinical settings; (2) unequal sample sizes across biomarkers, which limits direct comparisons; (3) potential influence of comorbidities on biomarker levels.

Reviewer #3

(Remarks to the Author)

The manuscript investigated a comprehensive panel of plasma tau biomarkers measured across multiple platforms and the MTBR-tau243 fragment. I have the following concerns, mainly regarding statistical analyses.

Major Concerns:

1. For PET imaging, how did the authors address heterogeneity across scanners and/or tracers? Did the authors apply any harmonization procedure?
2. For the longitudinal trajectory analysis, it is unclear about the follow-up frequency and % of lost follow-up. Can the authors provide more details? When constructing longitudinal trajectories, how did the authors address the sparsity in the longitudinal observation and how did the authors temporally align the measurements? More details on statistical models used for the trajectory construction should be provided.
3. Statistical analyses Section.
 - (a) Before removing outliers, can the authors provide some diagnosis of why outliers occur? Is it because of imaging quality concerns or potentially some pathological reasons?
 - (b) For group difference analysis in plasma biomarker levels, multiple markers were measured and compared. Did the authors do any multiple testing correction? The same for the association analysis, multiple brain regions, multiple plasma markers, and multiple cognitive outcomes were studied. A correction for multiplicity is needed.
 - (c) In ROC curve analyses, it is unclear what model was used? A logistic regression model?
 - (d) Related to the comment above, LMMs for cognitive trajectories may not be sufficient. How data sparsity and temporal alignment were addressed is unclear. For the association analysis between baseline plasma biomarker and cognitive trajectories, again, the use of LMMs assumes same time lag between visits across individuals. The validity of such an assumption is unclear. Also, multiple testing was not addressed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have now adequately addressed all the concerns about definition of current AD staging criteria, blood based biomarker assay description, proper referencing of eMTBR-tau243 literature and details of the research use biomarker assays with proper limit of detection and quantitations that was highlighted by the reviewer. The manuscript has acknowledged its limitations and highlighted the scope of the findings properly. Staging in Alzheimer's disease using blood based biomarker is becoming vital with the advent of disease modifying therapies and this paper adds to the utility of the two diagnostic biomarkers ptau217 and eMTBR-tau243 that will play major role in future clinical care and therapeutic interventional studies. The paper is ready to be accepted in the current form for publication.

Reviewer #2

(Remarks to the Author)

The manuscript has improved substantially, and the authors have addressed most of the reviewers' comments. I only have a few minor suggestions:

1. Improve the quality and resolution of the figures in the Supplementary Material. Several figures labels are difficult to read.
2. Perform a sensitivity analysis including only participants with measurements for all biomarkers. This direct comparison would help demonstrate that the main findings remain consistent when all biomarkers are evaluated in the same individuals.

Reviewer #3

(Remarks to the Author)

Thanks for taking the effort to address my comments. I have no further concerns.

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RESPONSE TO REVIEWERS' COMMENTS

REVIEWER #1

Major points

1. The reviewer looked across the manuscript (Introduction, results and discussion section) however could not find detailed definition of T1/T2 biomarker framework. One area that could be strengthened in the Introduction section about the discussion and definition of the T1/T2 biomarker framework. The reference cited don't refer to Jack, Clifford R. et. al. Revised criteria for the diagnosis and staging of Alzheimer's disease, Nature Medicine, 2024, PMID: 38934362; Jack, Clifford R. [et.al](#). Revised criteria for diagnosis and staging of Alzheimer's disease: Alzheimer's Association Workgroup, Alz & Dementia, 2024 (currently cited as reference 5 in the prior paragraph that seems little out of place) PMID: 38934362. While the manuscript refers to certain phosphorylated tau species as "T1 biomarkers" and tau aggregation-associated markers as "T2 biomarkers," however the conceptual basis and nomenclature underlying these categories are not explicitly introduced (Core Biomarkers for defining stages of Alzheimer's disease proteinopathy pathway or pathogenic species). Because this terminology is relatively recent and many researchers across neuroscience as well as neurodegeneration field may not yet be familiar with these, a clearer explanation of what biologically distinguishes T1 from T2 biomarkers would improve accessibility and context for the study rationale.

Author's response: We thank the reviewer for this important suggestion. We respectfully note that we had cited the revised AD staging criteria (Jack et al. 2024, Alzheimers Dement — corresponding to PMID 38934362, Jack et al. 2024, Nature Med — corresponding to PMID 38942991) as reference 5 and 6; however, we agree that the citation was placed in a paragraph where the connection to the T1/T2 framework was not made explicit. To address this, we have:

1. Repositioned the Jack et al. 2024 citation to the sentence introducing the T1/T2 framework itself.
2. Added an explicit conceptual definition of T1 and T2 biomarkers in the Introduction.
3. The T2 biomarker descriptions that follow (eMTBR-tau243, p-tau205) have been edited to make the rationale of the present study explicit within this framework.

[Change: introduction]

To address this limitation, in the revised criteria for the biological diagnosis and staging of Alzheimer's disease proposed by Jack et al. [5, 6], plasma and CSF tau biomarkers are conceptually divided into two categories reflecting distinct stages of the tau proteinopathy pathway. T1 biomarkers capture early-phase tau abnormalities — predominantly soluble phosphorylated tau species (e.g., p-tau217, p-tau181, p-tau231) that increase in response to amyloid- β pathology and rise during the preclinical stage. T2 biomarkers (e.g., p-tau205, MTBR-tau243), in contrast, reflect later-phase tau aggregation — the transition from soluble phosphorylated tau to insoluble neurofibrillary tangle pathology — and therefore correspond more closely with tau PET uptake, Braak stage progression, and cognitive decline. Distinguishing T1 from T2 biomarkers is therefore essential for biological staging and prognostic application of plasma tau measurements. Consistent with this framework, Cerebrospinal-fluid (CSF) studies have proposed p-tau205 as a transitional marker bridging early phosphorylation and later aggregation [17, 18], and identified microtubule-binding region (MTBR)-derived fragments such as MTBR-tau243 as late-rising markers that increase only after overt tangle formation [19, 20].

2. A ~ 23 % reduction of plasma tau phosphorylation p-tau217 in TRAILBLAZER-ALZ trial in anti-amyloid treated participants (donanemab) have been reported with respect to the placebo participants at 76 weeks compared to their baseline levels (JAMA Neurol. 2022;79(12):1250-1259). Thus plasma biomarker p-tau217 is a candidate reflecting the pharmacodynamic measures of the effect of anti-amyloid therapies. The authors need to include that in their introduction and mention that these early amyloid pathology surrogate markers do have treatment related response.

Author's response: We thank the reviewer for this important suggestion. We have updated the Introduction to explicitly acknowledge that plasma p-tau217 can also serve as a pharmacodynamic biomarker reflecting treatment response to anti-amyloid therapy, citing the TRAILBLAZER-ALZ trial result (Pontecorvo et al., JAMA Neurol 2022; PMID 36315099), in which plasma p-tau217 decreased by approximately 23% in donanemab-treated participants compared with placebo at 76 weeks.

A complementary statement has also been added to the Discussion linking T1 and T2 biomarkers along the same continuum (T1 → therapy response; T2 → staging and prognosis).

[Change: Introduction]

As a result, plasma biomarkers have emerged as promising, noninvasive alternatives for biological staging. Among these, phosphorylated tau (p-tau) species such as p-tau217, p-tau181, and p-tau231—often referred to as T1 biomarkers—have shown excellent performance in detecting A β positivity and early tau phosphorylation [10-13]. These markers rise early in the disease course, tightly correlate with A β PET, and to a lesser degree with tau PET, thereby serving as sensitive indicators of amyloid-related tau changes [14, 15]. Beyond their diagnostic and prognostic utility, T1 biomarkers — particularly plasma p-tau217 — have also been shown to serve as pharmacodynamic markers of anti-amyloid therapy, with a ~23% reduction observed in donanemab-treated participants at 76 weeks in the TRAILBLAZER-ALZ trial compared with placebo [16]. This reinforces the value of plasma p-tau217 not only as an early diagnostic indicator but also as a measure of treatment-related biological change. However, because they predominantly reflect early phosphorylation events, their capacity to capture later tau aggregation, disease staging, or prognosis remains limited [17].

[Change: Discussion]

Collectively, these findings support a complementary framework in which T1 biomarkers such as p-tau217 sensitively capture early disease-related changes and response to anti-amyloid therapy, whereas T2 biomarkers such as eMTBR-tau243 more accurately reflect disease severity and prognosis.

3. The reference cited for CSF microtubule-binding region derived tau (MTBR-tau243) is not correct. The first paper that describes the CSF MTBR-tau243 assay that should be referred is PMID: 33283854. The reference for the multi-cohort validation of CSF MTBR-tau243 is PMID: 37443334. The authors are recommended to cite the correct references when they discuss the respective relevant biomarkers.

Author's response: We thank the reviewer for this important correction. We confirm that the multi-cohort validation paper (Horie et al., Nat Med 2023; PMID 37443334) was already cited as reference 19. However, the original first description of the CSF MTBR-tau243 assay (Horie et al., Brain 2021; PMID 33283854) had been inadvertently omitted. We have now added this foundational citation in the Introduction at the relevant sentence on CSF MTBR-derived fragments. The reference list has been updated accordingly.

[Change: Introduction]

Consistent with this framework, Cerebrospinal-fluid (CSF) studies have proposed p-tau205 as a transitional marker bridging early phosphorylation and later aggregation [18, 19], and identified microtubule-binding region (MTBR)-derived fragments such as MTBR-tau243 as late-rising markers that increase only after overt tangle formation, first described in Horie et al. [20] and subsequently validated in multi-cohort studies [21, 22].

4. The authors cite Horie, K et. al. Nature Medicine 2025 paper (PMID: 33283854) for MTBR-tau243 which is not correct. This Nature Medicine 2025 paper is the first paper to describe the plasma endogenously cleaved MTBR-tau243 fragment (eMTBR-243). This Nature Medicine paper clearly states that the tryptic MTBR-tau243 as originally used for the CSF, did not correlate with tau-PET in the plasma samples. They discovered a different non-tryptic peptide (which they call eMTBR-tau243) that has valine residue at the C-terminus (Val256) and preceding amino acid asparagine (Asn255) is deamidated, which was proportional to tau-PET in both the plasma and the CSF samples. The authors are recommended again to cite the corresponding reference along with this description when describing eMTBR-tau243.

Author's response: We thank the reviewer for this important clarification. We agree that plasma eMTBR-tau243 is a non-tryptic, endogenously cleaved peptide—terminating at Val256 with deamidation of the preceding Asn255—that is biochemically distinct from the tryptic CSF MTBR-tau243 originally described in Horie et al. (Brain 2021; PMID 33283854), and that this plasma-detectable species was first reported in Horie et al. (Nat Med 2025; reference 23). Although this Nat Med 2025 paper was already in our reference list (reference 23), the citation was not placed at the first mention of plasma eMTBR-tau243, and the biochemical distinction from the tryptic CSF fragment was not made explicit. We have now revised the Introduction to cite this reference at first mention and to state this distinction explicitly, and we have updated the Methods to make the assay target analyte explicit (see also our response to Reviewer 1 #5).

[Change: Introduction]

Consistent with this framework, Cerebrospinal-fluid (CSF) studies have proposed p-tau205 as a transitional marker bridging early phosphorylation and later aggregation [18, 19], and identified microtubule-binding region (MTBR)-derived fragments such as MTBR-tau243 as late-rising markers that increase only after overt tangle formation, first described in Horie et al. [20] and subsequently validated in multi-cohort studies [21, 22]. In contrast to the tryptic MTBR-tau243 fragment originally described in CSF studies [20, 22], which did not correlate with tau-PET in plasma. Horie et al. subsequently identified a distinct endogenously cleaved, non-tryptic peptide which referred to as eMTBR-tau243 [23]. This eMTBR-tau243 peptide is proportional to tau-PET uptake in both CSF and plasma and was therefore selected as the target analyte in the present study.

In this study, we investigated a comprehensive panel of plasma tau biomarkers, including multiple phosphorylated tau species (p-tau181, p-tau205, p-tau217, and p-tau231) measured across multiple analytical platforms and the eMTBR-tau243 quantified using a single assay developed at C2N Diagnostics (C2N eMTBR-tau243 assay).

[Change: Method]

Plasma eMTBR-tau243 was measured with an RUO LC-MS/MS assay at C2N Diagnostics. The assay targets the same analyte reported by Horie et al.[23], namely the endogenous, non-tryptic tau fragment spanning residues 243–256 (tau243–256 dN) in plasma—terminating at Val256 with deamidation of the preceding Asn255.

5. In relation to the above comment, looking across the method section of this manuscript, it is not clear what is different for the p-tau and eMTBR-tau243 C2N assay. There are no references for the C2N eMTBR-tau243 assay that the authors claim was developed at C2N. If this assay is different from the Nature Medicine 2025 paper, PMID: 33283854, then the authors need to clearly mention that in the method section. Is the target analyte different when the authors state the eMTBR-tau243 was measured using a separate research use only (RUO) assay? This is unclear and no information has been provided to evaluate how reproducible this new assay compared to the original peer reviewed publication. Same for the p-tau multiplex tau mass-assisted assay. If these assays are different to the previously reported and established assays (PMID: 35446396) the authors need to provide either references for the assay validation (limit of quantitation, limit of detection, assay variability) or include them in the method/supporting information. The lack of clarity of what is being monitored in this manuscript is one of the major drawbacks that reduces enthusiasm toward the results presented.

Author's response: We thank the reviewer. We have clarified the analytical target and validation of each C2N assay in the Methods/Supplementary Methods, as summarized below. **(1) eMTBR-tau243 assay.** Both assays target the same analyte, namely the endogenous tau fragment spanning residues 243–256 (tau243-256 dN) in plasma. The primary difference is analytical rather than biological: in the C2N RUO assay, a certain residue within the target peptide is intentionally oxidized prior to LC-MS analysis to improve analyte stability and measurement consistency. As a result, the LC-MS readout differs slightly from the WashU implementation, but both assays quantify the same underlying eMTBR-tau243 analyte — further supported by the strong cross-laboratory correlation observed between the C2N and WashU assays.

Analytical performance was evaluated internally at C2N's R&D laboratory using CLSI guidance documents (135087.2413 – C2N-RUO-P0005 eMTBR-tau243 Analytical Validation Report):

- Total imprecision (within-lab): 9.8%–18.3%; within-day imprecision (repeatability): 8.0%–18.5%
- Accuracy: 90.2%–107.2%
- Sensitivity: LoB = 0.10 pg/mL, LoD = 0.11 pg/mL, LoQ set to the LLMI at 0.2 pg/mL
- Carryover: 0.42%; Specificity: no interference for any tested interferent

(2) Multiplex %p-tau-MAA. While the tryptic peptide sequences for the p181/205/217 and np181/205/217 analytes are identical to Barthélemy et al., Nature Medicine 2020, the

methods to extract tau proteins, process the samples, and detect the targets by LC-MS have been substantially modified to allow for higher throughput and analytical robustness in our commercial labs. Extensive internal validations demonstrate equivalent analytical and clinical performance of the %p-tau-MAA to C2N's PrecivityAD2 %p-tau217 assay.

Analytical performance (135087.2447 – C2N-RUO-P0008 Analytical Validation Summary Report):

- Total imprecision (within-device): p-tau217 4.3–12.6%, p-tau205 5.2–10.6%, p-tau181 5.0–8.0%, np-tau217 2.4–12.8%, np-tau205 1.5–11.5%, np-tau181 1.1–6.0%
- Total imprecision (within-day): p-tau217 3.2–10.9%, p-tau205 4.4–11.6%, p-tau181 3.6–6.7%, np-tau217 3.6–8.3%, np-tau205 2.4–4.3%, np-tau181 1.6–3.3%
- Sensitivity, LoD (pg/mL; LoQ = LoD): p217 0.938, p205 1.214, p181 1.262, np217 6.846, np205 8.375, np181 5.598

(3) As in the original manuscript, the reported %p-tau217 derives from this multiplex tau-MAA RUO assay and is distinct from the clinically validated APS2 (PrecivityAD2) assay. These clarifications and the full analytical validation parameters for both C2N assays have been incorporated into the Methods (subsections "Plasma eMTBR-tau243 measurement" and "Plasma multiplex p-tau measurement") and Supplementary Table 8.

[Change: Methods]

All measurements were performed in a blinded manner with standardized protocols, using a single batch of reagents per platform to minimize variability. Details of the two C2N research-use-only (RUO) assays are provided below, and their analytical validation parameters are summarized in Supplementary Table 8.

Plasma eMTBR-tau243 measurement (C2N RUO assay)

Plasma eMTBR-tau243 was measured with an RUO LC-MS/MS assay at C2N Diagnostics. The assay targets the same analyte reported by Horie et al.²³, namely the endogenous, non-tryptic tau fragment spanning residues 243–256 (tau243–256 dN) in plasma—terminating at Val256 with deamidation of the preceding Asn255. The primary difference from the WashU implementation is analytical rather than biological: in the C2N RUO assay, a certain residue within the target peptide is intentionally oxidized prior to LC-MS analysis to improve analyte stability and measurement consistency. As a result, the LC-MS readout differs slightly from the WashU implementation, but both assays quantify the same underlying eMTBR-tau243 analyte, further supported by the strong cross-laboratory correlation observed between the C2N and WashU assays. Briefly, eMTBR-tau243 was directly immunoprecipitated from human plasma using a proprietary antibody, digested with an endoprotease to produce a peptide contained within the first microtubule-binding repeat region (R1), and quantified by stable-isotope-dilution LC-MS/MS, with concentrations expressed in pg/mL.

Analytical performance of the eMTBR-tau243 assay was evaluated internally at C2N's R&D laboratory using CLSI guidance documents for all analytical study designs (135087.2413 – C2N-RUO-P0005 eMTBR-tau243 Analytical Validation Report). Total imprecision (within-laboratory) ranged 9.8–18.3% and within-day imprecision (repeatability) ranged 8.0–18.5%; accuracy ranged 90.2–107.2%. Sensitivity was defined as a limit of blank (LoB) of 0.10 pg/mL, a limit of detection (LoD) of 0.11 pg/mL, and a limit of quantitation (LoQ) set to the lower limit of the measurable interval at 0.2 pg/mL. Carryover was 0.42%, and no interference was observed for any tested interferent (Supplementary Table 8). Measurements below the LoQ were assigned a floor value of 0.1 pg/mL and retained (not excluded) in all analyses; all such values fell at or below the LoD (Supplementary Table 9).

Plasma multiplex p-tau measurement (C2N %p-tau-MAA)

Plasma p-tau181, p-tau205, and p-tau217 (each expressed as a phosphorylated-to-total ratio) were measured with C2N's multiplex tau mass-assisted assay (%p-tau-MAA). The tryptic peptide sequences for the p181/205/217 and np181/205/217 analytes are identical to those in Barthélemy et al. (Nat Med 2020); however, the methods to extract tau proteins, process the samples, and detect the targets of interest by LC-MS were substantially modified to allow for higher throughput and analytical robustness in commercial laboratories. Extensive internal validations were conducted to demonstrate analytical and clinical performance equivalent to C2N's PrecivityAD2 %p-tau217 assay. Both C2N assays are research-use-only (RUO); accordingly, the %p-tau217 reported here is derived from this multiplex tau-MAA and is distinct from the clinically validated Amyloid Probability Score 2 (APS2) assay. Briefly, tau proteins are extracted from human plasma using an anti-tau antibody immunoprecipitation, digested with trypsin endoprotease, desalted by solid-phase extraction, and analyzed by liquid chromatography–tandem high-resolution mass spectrometry (LC-MS/HRMS) on a nano-Acquity ultra-performance LC system (Waters) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) operating in parallel-reaction-monitoring (PRM) mode. Each peptide was quantified against a uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled internal standard using a light-to-heavy peak-area ratio and an exogenous calibration curve, with concentrations expressed in pg/mL.

Analytical performance of the %p-tau-MAA was evaluated internally at C2N's R&D laboratory using CLSI guidance documents (135087.2447 – C2N-RUO-P0008 Analytical Validation Summary Report). Per-measurand limits of detection ranged 0.938–1.262 pg/mL for phosphopeptides and 5.598–8.375 pg/mL for non-phosphopeptides (LoQ = LoD for each measurand), and total imprecision ranged 1.1–12.8% (within-device) and 1.6–11.6% (within-day) across measurands. Full per-measurand values, together with those of the eMTBR-tau243 assay, are provided in Supplementary Table 8.

6. Compared to immunoassays, LC-MS/MS was performed only on 20-25 % of the samples. The authors describe aliquot availability, laboratory volume requirements from the participants was the limiting factor for analytical sample size variance for the platform. Thus, it is crucial to describe in the methods the details of each platform, especially if the methods have been modified. Also did the authors analyze using LC-MS/MS assay all the 55 patients with tau-PET. The mean biomarkers values need to be tabulated in Table 1 for each group measured using the different biomarker platforms. Table 1 should also include the cognitive assessments (MMSE and CDR scores) for each group.

Author's response: We thank the reviewer. We have addressed each point:

- (1) Table 1 expansion. Baseline MMSE and CDR-SB values have been added to Table 1 for each analytical platform. To present platform $\times$ diagnostic group stratified data without overburdening the main table, we have added Supplementary Tables (1 to 4) showing demographics, MMSE, CDR-SB, $\text{A}\beta$ PET positivity, and mean (SD) baseline biomarker values for every diagnostic subgroup ($\text{A}\beta^-$ CU, $\text{A}\beta^+$ CU, $\text{A}\beta^+$ MCI, $\text{A}\beta^+$ DAT, $\text{A}\beta^-$ others, $\text{A}\beta^+$ others) on each platform.
- (2) LC-MS/MS and tau-PET overlap. Among the 55 participants with available tau PET, all 55 (100%) were also measured on the C2N LC-MS/MS platform.
- (3) Platform-specific method details. Additional information on each analytical platform — including assay characteristics, target peptides, and validation parameters (LOD/LOQ/CV) —

has been added to the Methods and to the new Supplementary Methods section (see also our response to Reviewer 1 #5).

[Change: Result, study participants]

Additional demographic and clinical characteristics, including APOE ϵ 4 carrier frequency, A β PET positivity, tau PET positivity, and baseline cognitive scores are summarized in Table 1. Detailed demographic, clinical, and biomarker characteristics stratified by diagnostic group for each analytical platform are provided in Supplementary Table 1-4.

[Change: Table 1, Supplementary table 1-4]

7. In the discussion the author claim that eMTBR-tau243 remained relatively stable during the preclinical and prodromal phase and rising sharply only with the onset of dementia. While all other p-tau biomarkers (Supplementary Figure 1) have low quantified levels at the amyloid negative CU stage, eMTBR-tau243 looks like it is very close to zero/or limit of detection (LOD). So, it could be possible that this assay lacks sensitivity to detect this endogenously cleaved peptide in plasma in the early phase of the disease unlike other more abundant phospho-tau species. The authors need to highlight this in the limitations.

Author's response: We thank the reviewer. We agree that the apparent stability of plasma eMTBR-tau243 during the preclinical and prodromal phases could, in part, reflect assay sensitivity at the lower end of the dynamic range rather than true biological invariance, since baseline levels in the A β - cognitively unimpaired group approach the limit of detection (LoD = 0.11 pg/mL). Accordingly, we now report the proportion of measurements at or below the LoD in each group (A β - CU 97.7%, A β + CU 95.1%, A β + MCI 63.9%, A β + DAT 30.4%; full six-group breakdown in Supplementary Table 9) and clarify in the Methods that values below the measurable interval (LoQ = 0.2 pg/mL) were assigned a floor value of 0.1 pg/mL and retained in all analyses. We have also added this as a limitation to the Discussion: "A limitation specific to plasma eMTBR-tau243 is that a substantial proportion of measurements in cognitively unimpaired and amyloid-negative participants fell at or below the analytical limit of detection of the C2N RUO assay; its apparent stability during the preclinical and prodromal phases may therefore reflect, in part, reduced assay sensitivity at low concentrations rather than purely biological invariance, and future assays with lower limits of detection will be required to distinguish a biological floor from an analytical floor." These changes have been incorporated into the Methods (plasma eMTBR-tau243 subsection) and the new Supplementary Table 9.

[Change: Discussion]

Second, plasma biomarker measurements at the preclinical stage may be subject to assay sensitivity limits; in particular, a substantial proportion of eMTBR-tau243 measurements in A β -negative cognitively unimpaired individuals fell at or below the limit of detection of the C2N RUO assay. Its apparent stability during the preclinical and prodromal phases may therefore reflect, in part, reduced assay sensitivity at low concentrations rather than purely biological invariance—potentially underestimating true early-phase changes—and assays with lower limits of detection will be required to distinguish a biological floor from an analytical floor.

[Change: Methods; Supplementary Table 9]

Measurements below the LoQ were assigned a floor value of 0.1 pg/mL and retained (not excluded) in all analyses; all such values fell at or below the LoD (Supplementary Table 9).

8. The authors should include longitudinal CDR-SB trajectories and tau-PET, plasma based staging data, even if in the supporting information, as the number of participants is relatively low n=36 and the analysis may not be powered enough to draw strong conclusions.

Author's response: We thank the reviewer for this important comment. We would like to clarify that the sample size of n = 36 applies only to the concordance analysis shown in Fig. 3A, which required the simultaneous availability of tau PET, plasma p-tau217 (Lilly_MSD), and plasma eMTBR-tau243 (C2N_LC-MS/MS) data. In contrast, the longitudinal cognitive trajectory analyses in Fig. 3B were conducted in larger samples: the PET-based staging analysis included 55 participants with available tau PET data, whereas the plasma-based staging analysis included 83 participants with both plasma p-tau217 and eMTBR-tau243 measurements. To avoid confusion, we have revised the figure legend to explicitly report the sample size for each analysis.

[Change: Legend of Figure 3]

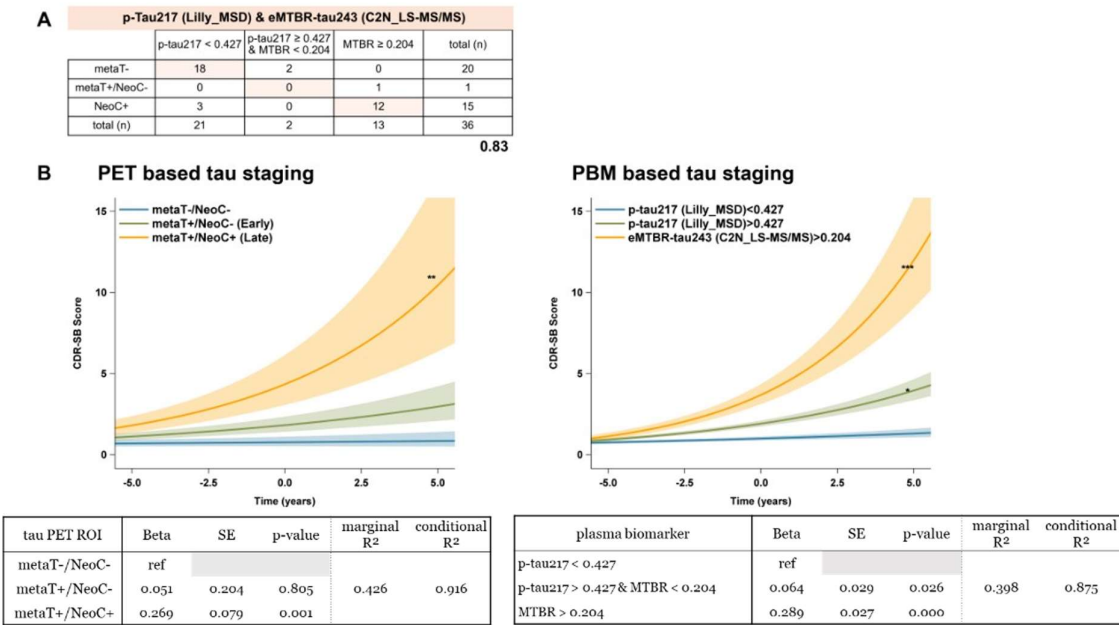


Fig. 3 PET-based and plasma-based regional tau staging and longitudinal cognitive trajectory

A. Comparison of plasma-based and PET-based regional tau staging. Plasma-based tau stages were derived from p-tau217 and eMTBR-tau243 and classified participants into negative, early, and late tau stages. The concordance value (0.83, shown in red) was calculated based on n=36 participants with available tau PET, plasma p-tau217 on the MSD platform, and eMTBR-tau243 data. **B.** Longitudinal CDR-SB trajectories across negative, early, and late tau stages defined by both PET-based and plasma-based tau staging. The PET-based staging analysis included 55 participants with available tau PET data. The plasma-based staging

analysis included 83 participants with both plasma p-tau217 on the MSD platform and plasma eMTBR-tau243 data. Standardized β coefficients and marginal R^2 values from the longitudinal trajectory models are shown below the trajectory. Abbreviations: metaT, meta-temporal; NeoC, neocortical; CDR-SB, Clinical Dementia Rating sum of boxes; C2N_LC-MS/MS, immunoprecipitation-liquid chromatography–tandem mass spectrometry assay at C2N Diagnostics; UGOT_Simoa, immunoassay on the Single Molecule Array platform at the University of Gothenburg; Lilly_MSD, customized immunoassay on the Meso Scale Discovery platform at Eli Lilly; Roche_Elecsys, prototype Elecsys immunoassay developed within the Neuro ToolKit framework at Roche Diagnostics

Minor Point

1. There is a typo in the abstract last line. It should be “plasma tau-based staging for prognosis...” instead of “plasma tau-base staging...”. The authors use multiple different names for this staging. Sometimes it is plasma-based tau staging, plasma tau-based staging etc. They need to be consistent and use one naming nomenclature.

Author's response: We thank the reviewer for identifying this inconsistency. We have corrected the typo in the Abstract and standardized the terminology throughout the manuscript to consistently use “plasma-based tau staging” and “PET-based tau staging.”

[Change: Abstract, Method, Discussion, Figure legends]

REVIEWER #2

1. Abstract

The T1 and T2 biomarkers are not defined. Please spell out the full names on first mention.

Author's response: We thank the reviewer for this suggestion. T1 and T2 do not represent formal expanded acronyms, but rather conceptual biomarker subclassifications introduced in the revised Jack et al. framework to distinguish tau biomarkers based on their temporal relationship to disease progression. In general, T1 corresponds to early-changing/Core 1-associated tau biomarkers, whereas T2 corresponds to later-changing/Core 2-associated tau biomarkers reflecting tau aggregation. To improve clarity, we have expanded the explanation of T1 and T2 biomarkers in the Introduction.

[Change: introduction]

To address this limitation, in the revised criteria for the biological diagnosis and staging of Alzheimer's disease proposed by Jack et al. [5, 6], plasma and CSF tau biomarkers are conceptually divided into two categories reflecting distinct stages of the tau proteinopathy pathway. T1 biomarkers capture early-phase tau abnormalities — predominantly soluble phosphorylated tau species (e.g., p-tau217, p-tau181, p-tau231) that increase in response to amyloid- β pathology and rise during the preclinical stage. T2 biomarkers (e.g., p-tau205, MTBR-tau243), in contrast, reflect later-phase tau aggregation — the transition from soluble phosphorylated tau to insoluble neurofibrillary tangle pathology — and therefore correspond more closely with tau PET uptake, Braak stage progression, and cognitive decline. Distinguishing T1 from T2 biomarkers is therefore essential for biological staging and prognostic application of plasma tau measurements.

2. Results – Study Participants

The participant description is difficult to follow as written. Rather than referring to the measurement platform, please specify the biomarkers directly (platform details belong in the Methods). The current phrasing is confusing and impedes interpretation of the results. The sentence reporting age and sex proportions — "The median age of participants was 73 [67–78], 72 [66–78], 72 [66–78], and 73 [66–78] years, and the proportion of female was 62.1%, 63.6%, 63.6%, and 63.9% respectively" — does not identify which participant groups the four values correspond to. Please label the groups explicitly. The entire participant description section would benefit from restructuring for clarity. Because sample sizes differ across biomarkers, this should be clearly stated throughout, particularly given that the paper draws direct comparisons between biomarkers.

Author's response: We thank the reviewer for this important suggestion. We agree that the original participant description was difficult to follow because it was overly centered on analytical platforms. We have therefore restructured the Results section to emphasize biomarker availability and participant characteristics, while moving detailed platform-specific assay information to the Methods section. We also clarified that sample sizes differed across biomarkers because of differences in sample availability and assay-specific volume requirements, and explicitly labeled all demographic summaries according to each biomarker dataset.

[Change: Result, Study participants]

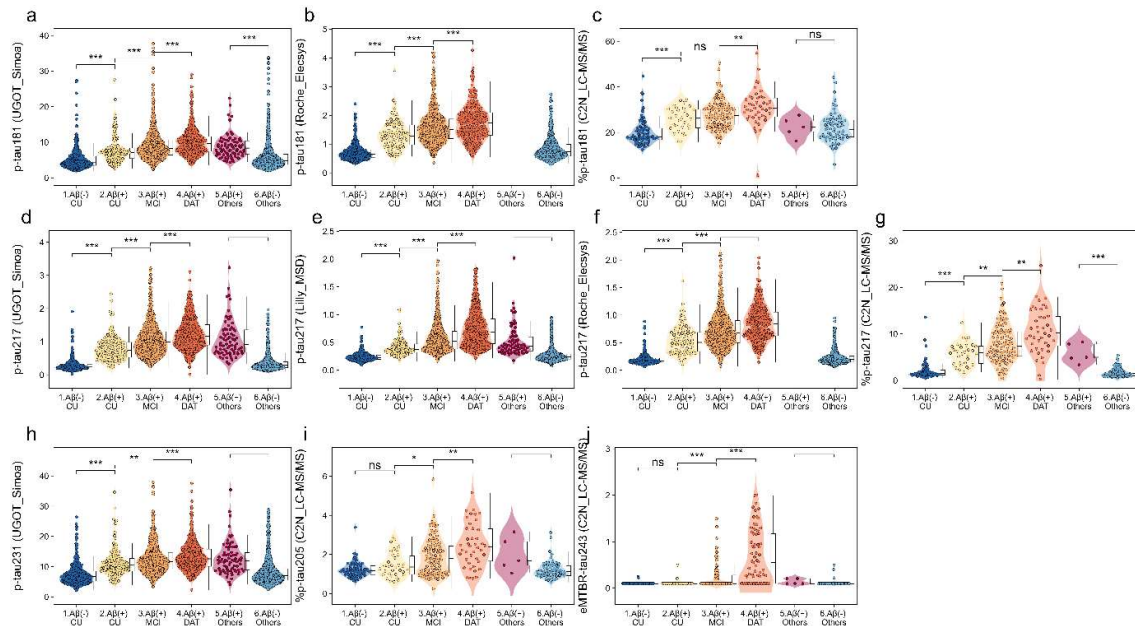
Plasma samples were analyzed using four analytical platforms with differing sample sizes because of differences in sample availability and assay-specific volume requirements. At C2N Diagnostics (C2N_LC-MS/MS; n = 401), %p-tau181, %p-tau205, %p-tau217, and eMTBR-tau243 were quantified. At the University of Gothenburg (UGOT_Simoa; n = 2,893), p-tau181, p-tau217, and p-tau231 were measured. At Eli Lilly (Lilly_MSD; n = 2,873), p-tau217 was measured. At Roche Diagnostics (Roche_Elecsys; n = 1,793), p-tau181 and p-tau217 were measured. Detailed platform-specific assay information is provided in the Methods section. The median age of participants was 73 [67–78] years for the C2N_LC-MS/MS dataset, 72 [66–78] years for UGOT_Simoa, 72 [66–78] years for Lilly_MSD, and 73 [66–78] years for Roche_Elecsys, while the proportion of female participants was 62.1%, 63.6%, 63.6%, and 63.9%, respectively. The distribution of clinical diagnoses (cognitively unimpaired [CU], mild cognitive impairment [MCI], dementia of the Alzheimer type [DAT], subcortical vascular cognitive impairment [SVCI], and frontotemporal dementia [FTD]) was 126 (31.4%)/182 (44.4%)/77 (19.2%)/12 (3.0%)/4 (1.0%) for C2N_LC-MS/MS, 641 (22.2%)/1,372 (47.4%)/607 (21.0%)/184 (6.4%)/86 (3.0%) for UGOT_Simoa, 640 (22.3%)/1,366 (47.6%)/597 (20.8%)/184 (6.4%)/86 (3.0%) for Lilly_MSD, and 448 (25.0%)/1,010 (56.3%)/335 (18.7%)/0 (0%)/0 (0%) for Roche_Elecsys. APOE ε4 carrier frequency was 40.7%, 37.8%, 37.9%, and 37.0%, and Aβ PET positivity was 58.9%, 48.1%, 48.2%, and 45.8% for the C2N_LC-MS/MS, UGOT_Simoa, Lilly_MSD, and Roche_Elecsys datasets, respectively (Table 1).

[Change: Method, Plasma collection and processing for plasma biomarker analysis]

Plasma biomarker measurements were performed using four independent analytical platforms: an immunoprecipitation–liquid chromatography–tandem mass spectrometry (IP-LC-MS/MS) assay at C2N Diagnostics (C2N_LC-MS/MS), the ALZpath immunoassay on the Single Molecule Array platform at the University of Gothenburg (UGOT_Simoa), a customized immunoassay on the Meso Scale Discovery platform at Eli Lilly (Lilly_MSD), and the prototype Elecsys immunoassay developed within the NeuroToolKit framework on the Elecsys platform at Roche Diagnostics (Roche_Elecsys). Plasma p-tau181 was quantified using the UGOT, Roche, and C2N platforms; p-tau217 using all four platforms; p-tau231 using the UGOT platform; and p-tau205 and eMTBR-tau243 using the C2N LC-MS/MS platform. In addition, the C2N platform quantified non-phosphorylated tau (np-tau) species to derive the ratios of p-tau species (%p-tau181, %p-tau205, and %p-tau217).

3. In Supplemental Figure 1 the group separation is very difficult to discern.

Author's response: We thank the reviewer. We have redrawn Supplementary Figure 1 to improve visual discrimination across the six diagnostic groups: (i) distinct color coding by Aβ status and clinical stage, (ii) larger violin elements with overlaid individual data points (jittered), (iii) enlarged group labels in horizontal orientation, and (iv) clearer significance annotations with explicit pairwise post-hoc comparisons. The revised figure replaces the original Supplementary Figure 1.



4. The meta-temporal ROI is described as representing "early-to-intermediate tau deposition." This characterization, however meta-temporal is not an early tau deposition region. Consider using the medial temporal lobe (MTL) instead, which is consistent stated as an early tau region.

Author's response: We thank the reviewer. Our meta-temporal ROI follows the standard Jack et al. (2017) definition and includes the entorhinal cortex, amygdala, and parahippocampal gyrus (canonical early-Braak regions) together with the fusiform, inferior temporal, and lingual gyri (intermediate-Braak regions); it therefore captures both early and intermediate tau deposition, consistent with our original description. To address the reviewer's point, we have added complementary analyses using a narrower medial temporal lobe (MTL) sub-ROI (entorhinal cortex, hippocampus, amygdala, and parahippocampal gyrus) for the association analyses in Supplementary Figure 4 and tau-positivity prediction in Supplementary Table 6 and Supplementary Figure 6. The pattern of associations was consistent with the meta-temporal results. The PET-based topographic staging in Fig. 3 was retained as originally defined (meta-temporal = early; neocortical = late), as this two-tier framework is central to the staging hypothesis being tested and corresponds to the recently proposed AD staging system (Jack et al. 2024).

[Change: Result, Associations of plasma biomarkers with PET uptakes and cognition]

Comparable results were obtained using an alternative tau PET framework based on MTL and neocortical ROIs (Supplementary Fig. 4).

[Change: Result, Plasma-based regional tau staging]

To evaluate whether plasma biomarkers reflect regional tau deposition, receiver operating characteristic (ROC) curve analyses were performed to predict tau PET positivity in the meta-temporal and neocortical ROIs, representing early-to-intermediate and advanced tau stages, respectively (Table 2, Supplementary Fig. 6). Complementary analyses using a medial temporal ROI are provided in Supplementary Table 6 and Supplementary Fig. 6.

5. Additional Analyses to Consider

1) Diagnosis-stratified associations. Were associations with tau and amyloid examined separately by diagnostic group? It would be informative to know whether effects differ between cognitively unimpaired individuals and those with cognitive impairment.

Author's response: We thank the reviewer. We note that our original analyses already include subgroup analyses by A β status (A β -positive subgroup and A β -positive CI subgroup; Fig. 2C, Fig. 4 and Supplementary Table 7). To directly address the reviewer's point, we have now added diagnosis-stratified analyses restricted to the CI group for both the cross-sectional associations with regional tau PET and cognition (Supplementary Figure 5) and the longitudinal cognitive trajectory analyses (Supplementary Figure 10). The pattern of associations was consistent with the main findings, with eMTBR-tau243 showing the strongest signals in the CI group. We did not perform stand-alone analyses in the CU group because eMTBR-tau243 was close to the limit of detection and tau PET was largely negative in this stage, resulting in insufficient variance and statistical power for meaningful stratified inference. This limitation has been added to the Limitations section (also addressing Reviewer 1, point #7).

[Change: Result]

These trends became more pronounced in the A β -positive and A β -positive cognitively impaired (CI) subgroups. Similar associations were observed in analyses restricted to cognitively impaired participants irrespective of A β status (Supplementary Fig. 5).

[Change: Result]

Analyses using MMSE trajectories yielded similar patterns (Fig. 4, Supplementary Fig. 7B, 8B, 9B, and Supplementary Table 7). The overall pattern of associations was also preserved in analyses restricted to the cognitively impaired group irrespective of A β status (Supplementary Fig. 10).

2) Longitudinal tau PET. If longitudinal tau PET data are available, analyses examining whether plasma biomarker levels track tau accumulation over time would substantially strengthen the paper.

Author's response: We thank the reviewer for this valuable suggestion. We performed exploratory analyses to examine whether baseline plasma biomarker levels were associated with subsequent regional tau accumulation among participants with longitudinal tau PET data. Individual annualized meta-temporal and neocortical tau PET slopes were estimated using subject-specific linear regression models among participants with repeated tau PET measurements. Associations between baseline plasma biomarkers and annualized tau PET slopes were assessed using both crude and adjusted linear regression models, with adjustment for age, sex, education, and APOE ϵ 4 status. Because plasma biomarkers were measured on different analytical platforms with differing sample availability and assay-specific requirements, the number of participants with both longitudinal tau PET and corresponding plasma biomarker measurements varied across biomarkers (n = 20–74). Although a nominal association was observed between baseline eMTBR-tau243 and meta-temporal tau PET slopes in the crude analysis, no plasma biomarker remained significantly associated with

longitudinal tau accumulation after adjustment for covariates. Given the limited number of participants with longitudinal tau PET data (e.g., 24 of 55 participants with available tau PET data in the C2N LC-MS/MS platform had longitudinal tau PET), these findings should be interpreted cautiously.

[Change: Result; Supplementary Table 5]

Exploratory analyses examining associations between baseline plasma biomarkers and longitudinal regional tau PET accumulation are provided in Supplementary Table 5.

3) Figure 1 / tau positivity. Why is there no figure showing effect sizes for tau positivity similar to what is shown for amyloid? Additionally, do these biomarkers differentiate Braak stages, or at low/intermediate/high tau burden categories as used in clinical trials? This would be highly relevant to include.

Author's response: We thank the reviewer for this helpful suggestion. To address this point, we performed complementary effect-size analyses based on tau PET positivity and PET-based tau staging and added the results as Supplementary Figure 2. Because tau-positive cognitively unimpaired participants were rare in our cohort, effect sizes were evaluated for the transitions from Tau- CU to Tau+ MCI and from Tau+ MCI to Tau+ DAT using both meta-temporal and neocortical tau PET positivity definitions. We additionally assessed effect sizes across PET-based tau stages (negative, early, and late AD). These analyses demonstrate that plasma biomarkers differentiate increasing tau burden across both clinical and PET-defined tau stages.

In addition, following statistical review, we replaced Cohen's d with Cliff's Delta as the effect-size metric throughout the manuscript, including Fig. 1 and Supplementary Fig. 2. Cliff's Delta is a nonparametric effect-size measure that is more robust to non-normal distributions and unequal group sizes, which are common in biomarker studies across disease stages. The overall pattern of results remained unchanged, supporting the robustness of our conclusions.

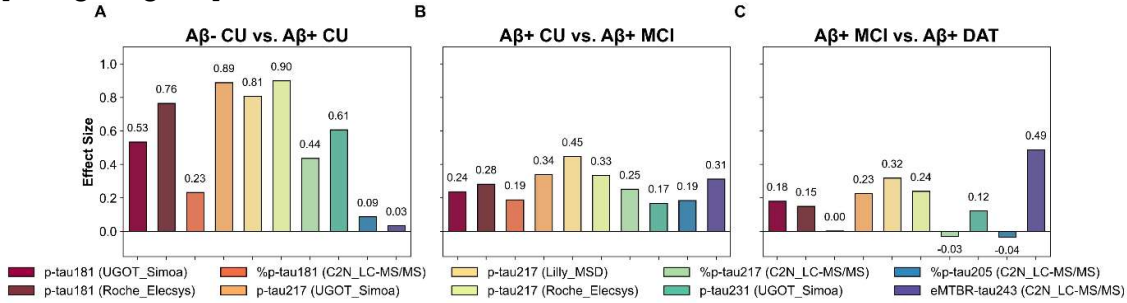
[Change: Result; Figure 1; Supplementary figure 2]

Effect size analyses revealed that for T1 biomarkers, the strongest discrimination occurred between A β - CU and A β + CU (Cliff's Delta = 0.23-0.90), followed by progressively smaller effects during later transitions (A β + CU $\rightarrow$ A β + MCI, 0.17-0.45; A β + MCI $\rightarrow$ A β + DAT, -0.03-0.32) (Fig. 1). By contrast, eMTBR-tau243 showed larger effect size during later transition, with the largest effect observed between A β + MCI and A β + DAT (Cliff's Delta = 0.49), exceeding all p-tau species across platforms. Similar findings were observed in complementary analyses based on tau PET positivity and PET-defined tau stages (Supplementary Fig. 2).

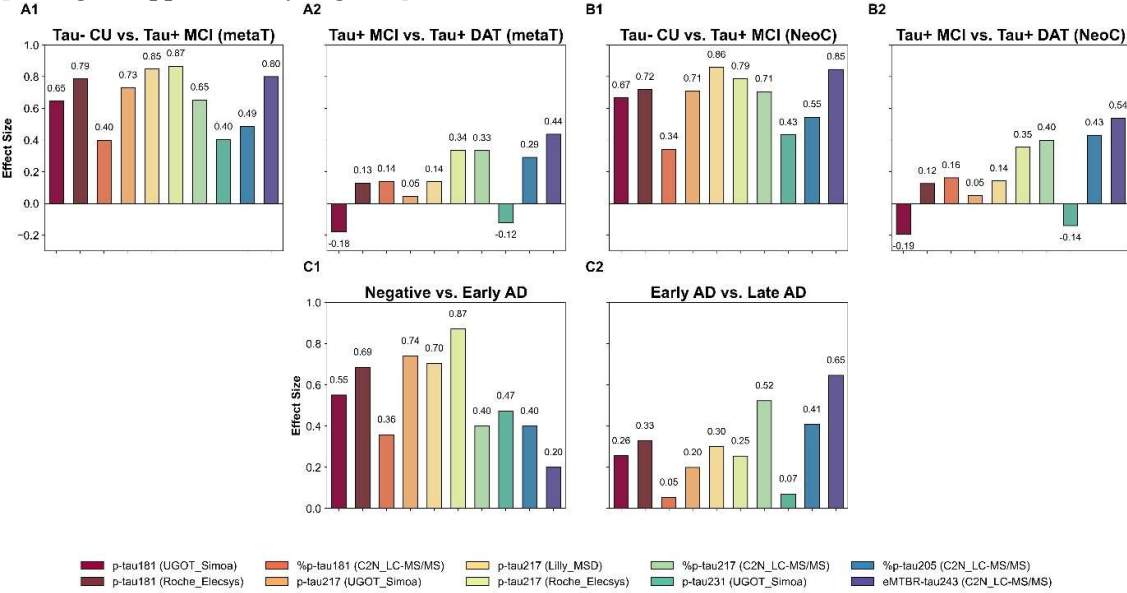
[Change: Method]

Group differences in plasma biomarker levels were analyzed across six diagnostic groups using analysis of covariance (ANCOVA), followed by Tukey-corrected post-hoc pairwise comparisons. Biomarker values were log-transformed when appropriate to improve distributional normality. Cliff's Delta was calculated to quantify the magnitude of group differences between adjacent disease stages. Cliff's Delta is a nonparametric effect size measure ranging from -1 to +1, with absolute values of 0.147, 0.330, and 0.474 interpreted as small, medium, and large effects, respectively.

[Change: Figure1]



[Change: Supplementary Figure2]



4) Demographic table. Please add the proportion of tau-positive participants to Table 1. (meta-temporal ROI)

Author's response: We thank the reviewer for this helpful suggestion. We have revised Table 1 to include the proportion of tau-positive participants based on the meta-temporal ROI definition used in the study.

Table 1. Demographics and clinical characteristics of participants across analytical platforms

	C2N LC-MS/MS (N=401)	UGOT Simoa (N=2,893)	Lilly MSD (N=2,873)	Roche Elecsys (N=1,793)
Age, years	72 [67, 78]	72 [66, 78]	72 [66, 78]	73 [66, 78]
Female, n (%)	249 (62.09%)	1,841 (63.64%)	1,826 (63.56%)	1,146 (63.92%)
Education, years	12 [9, 16]	12 [6, 16]	12 [6, 16]	12 [6, 14]
APOE ε4, n (%)	163 (40.65%)	1,094 (37.82%)	1,090 (37.94%)	663 (36.98%)
Clinical diagnosis, n (%)	126/182/77/12/4	641/1,372/607/184/89	640/1,366/597/184/86	448/1,010/335/0/0
CU/MCI/DAT/SVCI/FTD	(31.42/45.39/19.20/2.99/1.00)	(22.16/47.42/20.98/6.36/3.08)	(22.28/47.55/20.78/6.40/2.99)	24.99/56.33/18.68/0/0)
Aβ PET positivity, n (%)	236 (58.85%)	1,390 (48.05%)	1,384 (48.17%)	821 (45.79%)
Tau PET positivity, n (%)	31 (56.4%)	98 (51.0%)	93 (49.7%)	48 (60.8%)
MMSE	26 [23, 28]	25 [21, 28]	25 [21, 28]	26 [22, 28]
CDR-SB	1.0 [0.5, 3.0]	1.5 [0.5, 3.5]	1.5 [0.5, 3.5]	1.0 [0.5, 3.0]
Biomarker analyzed	%p-tau181 %p-tau205 %p-tau217 eMTBR-tau243	p-tau181 p-tau217 p-tau231	p-tau217	p-tau181 p-tau217

Demographic and clinical characteristics of participants analyzed across the four distinct analytical platforms: C2N LC-MS/MS (%p-tau181, %p-tau205, %p-tau217, and eMTBR-tau243), UGOT Simoa (p-tau181, p-tau217, and p-tau231), Lilly MSD (p-tau217), and Roche Elecsys (p-tau181 and p-tau217). Aβ PET positivity was defined using a cutoff value of 25 Klunk CL. **Tau PET positivity was defined as an SUVR greater than 1.31 in the meta-temporal ROI.** Values are presented as median [IQR] or mean (SD) for continuous variables and number (%) for categorical variables, unless otherwise specified. Abbreviations: CU, cognitively unimpaired; MCI, mild cognitive impairment; DAT, dementia of the Alzheimer type; SVCI, subcortical vascular cognitive impairment; FTD, frontotemporal dementia; Aβ, amyloid-β; APOE, apolipoprotein E; PET, positron emission tomography; **MMSE, Mini-Mental State Examination; CDR-SB, Clinical Dementia Rating sum of boxes;** C2N LC-MS/MS, immunoprecipitation-liquid chromatography–tandem mass spectrometry assay at C2N Diagnostics; UGOT Simoa, immunoassay on the Single Molecule Array platform at the University of Gothenburg; Lilly MSD, customized immunoassay on the Meso Scale Discovery platform at Eli Lilly; Roche Elecsys, prototype Elecsys immunoassay developed within the Neuro Toolkit framework at Roche Diagnostics

5) Tau staging in the Discussion. The Discussion references tau staging, but the analyses only separate participants into early and late groups, analysis with all stages should be performed to state that.

Author's response: We thank the reviewer and would like to respectfully clarify. Our staging analysis was not restricted to early and late groups: it explicitly includes a tau-negative stage as well, yielding a three-stage framework (negative → early [meta-temporal+] → late [neocortical+]). All staging analyses presented in Fig. 3A (concordance between PET-based and plasma-based staging), Fig. 3B (longitudinal CDR-SB trajectories), and Table 2 (cutoff-based performance) were performed across all three stages, in line with the recently proposed simplified AD staging framework (Jack et al. 2024). To make this clearer in the Discussion, we have revised the relevant passages to explicitly refer to the three-stage staging (negative / early / late) throughout.

[Change: Discussion]

Notably, individuals classified into plasma-defined tau-negative, early and late tau stages exhibited stepwise differences in longitudinal cognitive decline, mirroring patterns observed with PET-based tau staging and supporting its biological validity

6. Limitations

The Limitations section should explicitly acknowledge: (1) lack of replication in diverse populations and clinical settings; (2) unequal sample sizes across biomarkers, which limits direct comparisons; (3) potential influence of comorbidities on biomarker levels.

Author's response: We thank the reviewer. We have expanded the Limitations section to explicitly acknowledge the three points raised, in addition to the limitations already noted:

[Change: Discussion]

However, several limitations should be noted. First, only a subset of participants (n=55) underwent tau PET imaging, limiting both regional calibration of plasma biomarkers and direct assessment of whether plasma levels track within-individual tau accumulation over time. Second, plasma biomarker measurements at the preclinical stage may be subject to assay sensitivity limits; in particular, a substantial proportion of eMTBR-tau243 measurements in A β -negative cognitively unimpaired individuals fell at or below the limit of detection of the C2N RUO assay. Its apparent stability during the preclinical and prodromal phases may therefore reflect, in part, reduced assay sensitivity at low concentrations rather than purely biological invariance—potentially underestimating true early-phase changes—and assays with lower limits of detection will be required to distinguish a biological floor from an analytical floor. Third, sample sizes differed across analytical platforms because plasma aliquot availability, species coverage, and laboratory volume requirements varied; this limits the direct quantitative comparison of biomarker performance across platforms, and inter-platform comparisons should therefore be interpreted with appropriate caution. Fourth, although the K-ROAD cohort represents a nationwide memory-clinic–community hybrid population, independent replication in ethnically diverse populations and across different clinical settings will be required to confirm generalizability of the proposed plasma-based staging. Fifth, we did not systematically assess the influence of common comorbidities (e.g., chronic kidney disease, body mass index, diabetes) on plasma biomarker levels, which has been reported to affect plasma p-tau quantification. Nevertheless, despite these limitations,

this study provides novel and robust evidence supporting plasma-based tau staging of tau pathology across the AD continuum.

REVIEWER #3

Major Concerns:

1. For PET imaging, how did the authors address heterogeneity across scanners and/or tracers? Did the authors apply any harmonization procedure?

Author's response:

We thank the reviewer. Heterogeneity across amyloid tracers is a well-recognized issue, which is precisely why the Klunk Centiloid (CL) framework was developed and has since become the field standard for cross-tracer/cross-site harmonization. Following this convention, all A β PET data acquired at multiple K-ROAD sites with two tracers ([^{18} F]florbetaben and [^{18} F]flutemetamol) were transformed to the Centiloid scale, and a single tracer-independent cutoff of 25 CL was applied. Tau PET was acquired at a single center using a single tracer ([^{18} F]flortaucipir) under a uniform acquisition/reconstruction protocol with a consistent reference region (inferior cerebellar gray matter), so no additional harmonization was required.

2. For the longitudinal trajectory analysis, it is unclear about the follow-up frequency and % of lost follow-up. Can the authors provide more details? When constructing longitudinal trajectories, how did the authors address the sparsity in the longitudinal observation and how did the authors temporally align the measurements? More details on statistical models used for the trajectory construction should be provided.

Author's response: We thank the reviewer. The longitudinal cognitive analyses in Figure 4 were performed in AD-continuum participants with available plasma biomarkers shown in Figure 4 measured and at least two MMSE or CDR-SB assessments (n = 2,267). The median follow-up duration was 2.9 years with a median of 4 MMSE assessments, and 2.5 years with a median of 3 CDR-SB assessments (because MMSE and CDR-SB were not always obtained at the same visit, follow-up duration and number of assessments differed between the two measures). We did not observe systematic dropout: participants were retained in the analyses as long as at least two assessments were available, and linear mixed-effects models used all available observations under a missing-at-random assumption without imputation, thereby accommodating participants with incomplete or unequally spaced follow-up rather than excluding them. Time was modeled as years from the baseline plasma sampling date. To address the reviewer's concerns regarding sparsity, unequal visit spacing, and temporal alignment, we used linear mixed-effects models (LMMs), the standard approach for such longitudinal data precisely because they accommodate unbalanced observations, varying numbers of visits, and irregular intervals without requiring imputation. Models included subject-level random intercept and random slope on Time, with fixed effects for biomarker, Time, and biomarker $\times$ Time, adjusted for baseline age, sex, APOE ϵ 4 status, and education; parameters were estimated by REML. These specifications have been added to the Methods.

[Change: Method]

Retrospective and prospective MMSE and CDR-SB scores were collected at the time of blood sampling and during follow-up. Only participants within AD continuum were included in the longitudinal cognitive analyses, given the potential confounding effect of neurodegenerative co-pathologies on clinical progression. 2,267 participants underwent at least two MMSE or CDR-SB. The median follow-up duration was 2.9 years with a median of four MMSE assessments, and 2.5 years with a median of three CDR-SB assessments.

Because MMSE and CDR-SB were not always obtained at the same visit, follow-up duration and the number of assessments differed between the two measures. Because linear mixed-effects models use all available observations under a missing-at-random assumption without imputation, participants with at least two assessments were retained regardless of incomplete or unequally spaced follow-up.

3. Statistical analyses Section.

(a) Before removing outliers, can the authors provide some diagnosis of why outliers occur? Is it because of imaging quality concerns or potentially some pathological reasons?

Author's response: We thank the reviewer for this important comment. Outlier exclusion was applied to plasma biomarker values and was performed separately within A β -positive and A β -negative groups. To better understand the origin of these extreme values, we reviewed the corresponding participants for potential imaging quality concerns and clinical characteristics. We found no consistent evidence of imaging artifacts or quality issues. Interestingly, most outlier values occurred in A β -negative individuals. This observation is notable because previous studies of MTBR-tau243 also reported two extreme outliers, both of whom were A β -negative participants and were subsequently found to be MAPT mutation carriers. Although genetic testing was not systematically performed in our cohort and we therefore cannot determine the underlying etiology, these findings raise the possibility that a subset of outliers may reflect non-amyloid primary tauopathies. We additionally explored whether these participants showed evidence of unusually high cognitive reserve by examining educational attainment; however, education levels were not consistently higher than those of the remaining cohort. We also considered the possibility that some individuals were experiencing rapidly progressive disease and may have been biologically more advanced than suggested by their clinical diagnosis at the time of sampling. Finally, we reviewed available clinical information for several eMTBR-tau243 outliers and found no consistent pattern in body weight or other readily identifiable biological factors.

(b) For group difference analysis in plasma biomarker levels, multiple markers were measured and compared. Did the authors do any multiple testing correction? The same for the association analysis, multiple brain regions, multiple plasma markers, and multiple cognitive outcomes were studied. A correction for multiplicity is needed.

Author's response: We thank the reviewer for this important comment. Post hoc pairwise comparisons following ANCOVA were already adjusted using Tukey's method. To further address the issue of multiplicity, we additionally applied Benjamini–Hochberg false discovery rate (BH-FDR) correction to the longitudinal cognitive trajectory analyses based on linear mixed-effects models. The overall pattern of results and the main conclusions remained unchanged after correction.

(c) In ROC curve analyses, it is under what model was used? A logistic regression model?

Author's response: The ROC analyses were based on univariate logistic regression models, where each plasma biomarker was entered separately as the predictor and tau PET-defined positivity as the binary outcome. Predicted probabilities derived from these models were used to construct the ROC curves and calculate AUC values.

(d) Related to the comment above, LMMs for cognitive trajectories may not be sufficient. How data sparsity and temporal alignment were addressed is unclear. For the association analysis between baseline plasma biomarker and cognitive trajectories, again, the use of LMMs assumes same time lag between visits across individuals. The validity of such an assumption is unclear. Also, multiple testing was not addressed.

Author's response: We thank the reviewer for this important comment. We respectfully clarify that linear mixed-effects models (LMMs) do not assume identical time intervals between visits across individuals. Rather, LMMs are specifically designed to accommodate unbalanced longitudinal data with varying numbers of visits and irregular follow-up intervals. To clarify this point, we have revised the Methods section to provide additional details regarding the model specification, including subject-level random intercepts and random slopes for time, fixed effects for plasma biomarker, time, and the biomarker $\times$ time interaction, adjustment for baseline age, sex, APOE ϵ 4 status, and education, and estimation using restricted maximum likelihood (REML). Time was defined as years from baseline plasma sampling.

Regarding multiple testing, we additionally applied Benjamini–Hochberg false discovery rate (BH-FDR) correction to the LMM analyses. FDR correction was performed separately for each cognitive outcome across all biomarkers and subgroups. The overall pattern of results remained unchanged after correction, including the strong prognostic association of eMTBR-tau243 with longitudinal cognitive decline.

[Change: Method]

Each model included subject-level random intercepts and random slopes for time, with fixed effects for the plasma biomarker, time, and their interaction (biomarker $\times$ time). Time was modeled as years from the date of baseline plasma sampling, allowing unbalanced numbers of visits and irregular intervals across participants without imputation. Parameters were estimated by restricted maximum likelihood (REML).

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1

The authors have now adequately addressed all the concerns about definition of current AD staging criteria, blood based biomarker assay description, proper referencing of eMTBR-tau243 literature and details of the research use biomarker assays with proper limit of detection and quantitations that was highlighted by the reviewer. The manuscript has acknowledged its limitations and highlighted the scope of the findings properly. Staging in Alzheimer's disease using blood based biomarker is becoming vital with the advent of disease modifying therapies and this paper adds to the utility of the two diagnostic biomarkers ptau217 and eMTBR-tau243 that will play major role in future clinical care and therapeutic interventional studies. The paper is ready to be accepted in the current form for publication.

Reviewer #2

The manuscript has improved substantially, and the authors have addressed most of the reviewers' comments. I only have a few minor suggestions:

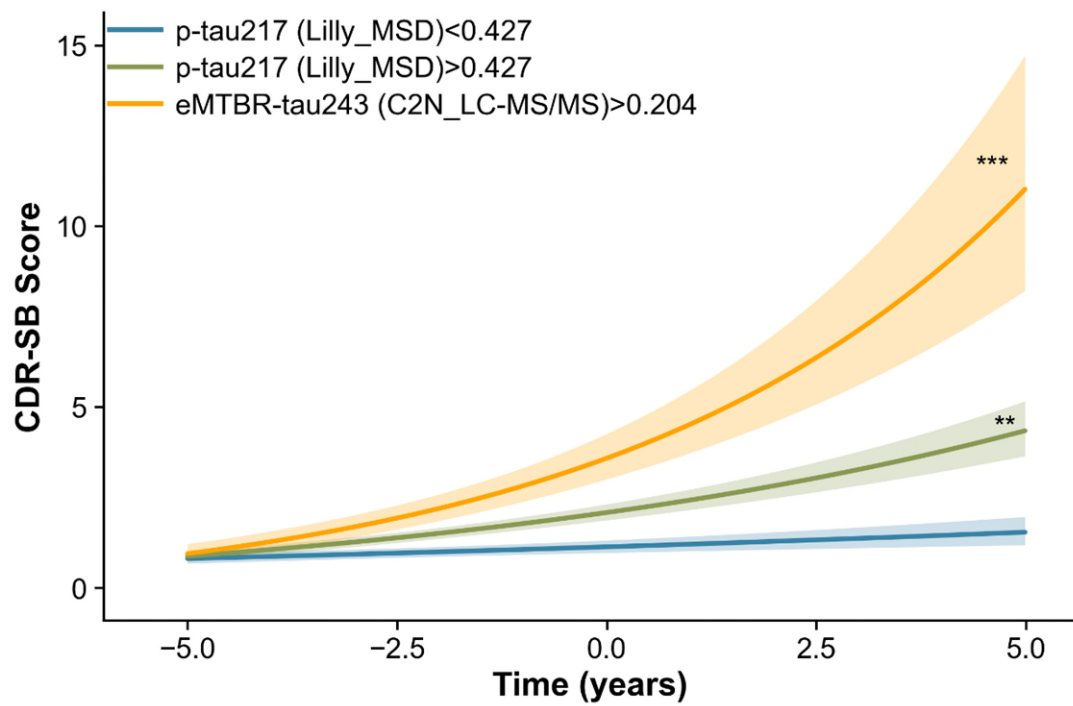
1. Improve the quality and resolution of the figures in the Supplementary Material. Several figures labels are difficult to read.

Author's response: We have revised supplementary material by improving the resolution and readability of the figures and enlarging figure labels where necessary. The updated high-resolution figures have been incorporated into the revised Supplementary Material

2. Perform a sensitivity analysis including only participants with measurements for all biomarkers. This direct comparison would help demonstrate that the main findings remain consistent when all biomarkers are evaluated in the same individuals.

Author's response: We thank the reviewer for this important suggestion. In response to the suggestion, we performed an additional sensitivity analysis restricted to 198 participants with complete measurements for all plasma biomarkers. Using the same biomarker cutoffs as in the primary analysis, we repeated the plasma-based tau staging analysis and evaluated longitudinal CDR-SB trajectories using linear mixed-effects models. The results were consistent with those of the primary analysis. Compared with the negative stage, significant

differences were observed in the intermediate stage ($\beta = 0.102$, $p = 0.010$) and the late stage ($\beta = 0.280$, $p < 0.001$). These results have been added as Supplementary Fig. 7 and described in the revised result section.



tau PET ROI	Beta	SE	p-value	marginal R ²	conditional R ²
p-tau217 < 0.427	ref				
p-tau217 > 0.427 & eMTBR < 0.204	0.102	0.039	0.010	0.353	0.757
eMTBR > 0.204	0.280	0.039	0.000		

Reviewer #3

Thanks for taking the effort to address my comments. I have no further concerns.

The study “Defining Plasma T2 Tau Biomarkers for Biological Staging and Prognosis in Alzheimer’s Disease” by Yim, Sohyun; Lee, Eun Hye et. al. on behalf of the K-ROAD study groups evaluates plasma biomarkers that reflect distinct stages of Alzheimer’s disease (AD) that could enable scalable biological staging. The study evaluated phosphorylated tau (p-tau) species across multiple analytical platforms and endogenously cleaved microtubule-binding region containing residue 243 (eMTBR-tau243) using a dedicated LC-MS/MS platform in a well-characterized cohort with amyloid PET and subset with tau PET. They found phosphorylated tau (p-tau181, p-tau217 – referred to as T1 markers) were increased in the preclinical stages of the disease, whereas eMTBR-tau243 and p-tau205 increased later in the disease stage. eMTBR-tau243 showed strongest and stage-dependent associations with regional tau PET and cognition. The authors found that plasma-based staging system integrating p-tau217 and eMTBR-tau243 closely aligned with PET-based tau staging and predicted longitudinal cognitive trajectories. They claim that plasma eMTBR-tau243 as a late phase biomarker (T2 biomarker). This plasma-based staging has the potential for prognosis and therapeutic decision making in AD.

The results presented in this manuscript is timely with significant implications for large scale deployment of blood-based biomarker assessment in older (age above 65) populations that is currently not achievable and possible using imaging (PET) based and other invasive biomarker testing platform (CSF). Inclusion of multiple analytical platforms in this study such as automated single molecule immunoassays and LC-MS/MS assays, enables robust and generalizable assessment of biomarker performance. By doing so this study aims to minimize platform-specific bias and providing cross-validation across diverse assay technologies. While the data presented in this manuscript and supplementary is solid, there are many points that the authors must address in terms of writing and interpretation. The authors don’t cite the correct references when describing the literature on validated biomarkers, while some references are missing without which it is hard to assess if the analytical measurements are indeed validated and accurate. Addressing these concerns will not only make this manuscript more robust and reproducible, but it will also help the readers understand the results who are not very much accustomed to the nomenclatures and terminologies of blood-based biomarkers.

Major points

1. The reviewer looked across the manuscript (Introduction, results and discussion section) however could not find detailed definition of T1/T2 biomarker framework. One area that could be strengthened in the Introduction section about the discussion and definition of the T1/T2 biomarker framework. The reference cited don’t refer to Jack, Clifford R. et. al. *Revised criteria for the diagnosis and staging of Alzheimer’s disease*, Nature Medicine, 2024, PMID: 38934362; Jack, Clifford R. et.al. *Revised criteria for diagnosis and staging of Alzheimer’s disease: Alzheimer’s Association Workgroup*, Alz & Dementia, 2024 (currently cited as reference 5 in the prior paragraph that seems little out of place) PMID: 38934362. While the manuscript refers to certain phosphorylated tau species as “T1 biomarkers” and tau aggregation-associated markers as “T2 biomarkers,” however the conceptual basis and nomenclature underlying these categories are not explicitly introduced (Core Biomarkers for defining stages of Alzheimer’s disease proteinopathy pathway or pathogenic species). Because this terminology is relatively recent and many researchers across neuroscience as well as neurodegeneration field may not yet be familiar with these, a clearer explanation of what biologically distinguishes T1 from T2 biomarkers would improve accessibility and context for the study rationale.
2. A ~ 23 % reduction of plasma tau phosphorylation p-tau217 in TRAILBLAZER-ALZ trial in anti-amyloid treated participants (donanemab) have been reported with respect to the placebo participants at 76 weeks compared to their baseline levels (JAMA Neurol. 2022;79(12):1250-1259). Thus plasma biomarker p-tau217 is a candidate reflecting the pharmacodynamic measures of the effect of anti-amyloid therapies. The authors need to include that in their introduction and mention that these early amyloid pathology surrogate markers do have treatment related response.
3. The reference cited for CSF microtubule-binding region derived tau (MTBR-tau243) is not correct. The first paper that describes the CSF MTBR-tau243 assay that should be referred is PMID: 33283854. The reference for the multi-cohort validation of CSF MTBR-tau243 is PMID: 37443334. The authors are recommended to cite the correct references when they discuss the respective relevant biomarkers.
4. The authors cite Horie, K et. al. Nature Medicine 2025 paper (PMID: 33283854) for MTBR-tau243 which is not correct. This Nature Medicine 2025 paper is the first paper to describe the plasma endogenously cleaved MTBR-tau243 fragment (eMTBR-243). This Nature Medicine paper clearly states that the tryptic MTBR-tau243 as originally used for the CSF, did not correlate with tau-PET in the

plasma samples. They discovered a different non-tryptic peptide (which they call eMTBR-tau243) that has valine residue at the C-terminus (Val256) and preceding amino acid asparagine (Asn255) is deamidated, which was proportional to tau-PET in both the plasma and the CSF samples. The authors are recommended again to cite the corresponding reference along with this description when describing eMTBR-tau243.

5. In relation to the above comment, looking across the method section of this manuscript, it is not clear what is different for the p-tau and eMTBR-tau243 C2N assay. There are no references for the C2N eMTBR-tau243 assay that the authors claim was developed at C2N. If this assay is different from the Nature Medicine 2025 paper, PMID: 33283854, then the authors need to clearly mention that in the method section. Is the target analyte different when the authors state the eMTBR-tau243 was measured using a separate research use only (RUO) assay? This is unclear and no information has been provided to evaluate how reproducible this new assay compared to the original peer reviewed publication. Same for the p-tau multiplex tau mass-assisted assay. If these assays are different to the previously reported and established assays (PMID: 35446396) the authors need to provide either references for the assay validation (limit of quantitation, limit of detection, assay variability) or include them in the method/supporting information. The lack of clarity of what is being monitored in this manuscript is one of the major drawbacks that reduces enthusiasm toward the results presented.
6. Compared to immunoassays, LC-MS/MS was performed only on 20-25 % of the samples. The authors describe aliquot availability, laboratory volume requirements from the participants was the limiting factor for analytical sample size variance for the platform. Thus, it is crucial to describe in the methods the details of each platform, especially if the methods have been modified. Also did the authors analyze using LC-MS/MS assay all the 55 patients with tau-PET. The mean biomarkers values need to be tabulated in Table 1 for each group measured using the different biomarker platforms. Table 1 should also include the cognitive assessments (MMSE and CDR scores) for each group.
7. In the discussion the author claim that eMTBR-tau243 remained relatively stable during the preclinical and prodromal phase and rising sharply only with the onset of dementia. While all other p-tau biomarkers (Supplementary Figure 1) have low quantified levels at the amyloid negative CU stage, eMTBR-tau243 looks like it is very close to zero/or limit of detection (LOD). So, it could be possible that this assay lacks sensitivity to detect this endogenously cleaved peptide in plasma in the early phase of the disease unlike other more abundant phospho-tau species. The authors need to highlight this in the limitations.
8. The authors should include longitudinal CDR-SB trajectories and tau-PET, plasma based staging data, even if in the supporting information, as the number of participants is relatively low n=36 and the analysis may not be powered enough to draw strong conclusions.

Minor point

1. There is a typo in the abstract last line. It should be “plasma tau-based staging for prognosis...” instead of “plasma tau-base staging...”. The authors use multiple different names for this staging. Sometimes it is plasma-based tau staging, plasma tau-based staging etc. They need to be consistent and use one naming nomenclature.