N-terminal and mid-region tau fragments as fluid biomarkers in neurological diseases

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1 Abstract

2 Brain-derived tau secreted into CSF and blood consists of different N-terminal and mid-domain

3 fragments, which may have a differential temporal course and thus, biomarker potential across

4 the Alzheimer's disease *continuum* or in other neurological diseases. While current clinically

5 validated total-tau (t-tau) assays target mid-domain epitopes, comparison of these assays with

6 new biomarkers targeting N-terminal epitopes using the same analytical platform may be

7 important to increase the understanding of tau pathophysiology. We developed three t-tau

8 immunoassays targeting specific N-terminal (NTA and NTB t-tau) or mid-region (MR t-tau)

9 epitopes, using single molecule array technology. After analytical validation, the diagnostic

10 performance of these biomarkers was evaluated in CSF and compared with the Innotest t-tau

11 (and as proof of concept, with N-p-tau181 and N-p-tau217) in three clinical cohorts ($n = 342$

12 total). The cohorts included participants across the Alzheimer's disease *continuum* ($n = 276$),

13 other dementia ($n = 22$), Creutzfeldt-Jakob disease ($n = 24$), acute neurological disorders ($n = 18$)

14 and progressive supranuclear palsy ($n = 22$). Furthermore, we evaluated all three new t-tau

15 biomarkers in plasma ($n = 44$) and replicated promising findings with NTA t-tau in another

16 clinical cohort ($n = 50$). In CSF, all t-tau biomarkers were increased in Alzheimer's disease

17 compared with controls ($P < 0.0001$) and correlated with each other ($r_s = 0.53$-0.95). NTA and

18 NTB t-tau, but not other t-tau assays, distinguished amyloid-positive and amyloid-negative mild

19 cognitive impairment with high accuracies (AUCs 84% and 82%, $P < 0.001$) matching N-p-

20 tau217 (AUC 83%; DeLong test $P = 0.93$ and 0.88). All t-tau assays were excellent in

21 differentiating Alzheimer's disease from other dementias ($P < 0.001$, AUCs 89-100%). In

22 Creutzfeldt-Jakob disease and acute neurological disorders, N-terminal t-tau biomarkers had

23 significantly higher fold changes versus controls in CSF (45-133-fold increase) than Innotest or

24 MR t-tau (11-42-fold increase, $P < 0.0001$ for all). In progressive supranuclear palsy, CSF

25 concentrations of all t-tau biomarkers were similar to those in controls. Plasma NTA t-tau

26 concentrations were increased in Alzheimer's disease compared with controls in two

27 independent cohorts ($P = 0.0056$ and 0.0033) while Quanterix t-tau performed poorly ($P = 0.55$

28 and 0.44). Taken together, N-terminal-directed CSF t-tau biomarkers increase ahead of standard

29 t-tau alternatives in the Alzheimer's disease *continuum*, increase to higher degrees in

30 Creutzfeldt-Jakob disease and acute neurological diseases and show better potential than

1 Quanterix t-tau as Alzheimer's disease blood biomarkers. For progressive supranuclear palsy,

2 other tau biomarkers should still be investigated.

3 **Keywords:** Alzheimer's disease; tau; biomarker; cerebrospinal fluid; plasma

4 **Abbreviations:** Aβ, beta-amyloid peptide; AUC, area under the curve; CJD, Creutzfeldt-Jakob

5 disease; MCI, mild cognitive impairment; MR t-tau, t-tau assay targeting mid-region bearing

6 fragments; MMSE, mini-mental state examination; N-p-tau181, p-tau assay targeting N-

7 terminal-directed tau phosphorylated at threonine-181; N-p-tau217, p-tau assay targeting N-

8 terminal-directed tau phosphorylated at threonine-217; NTA, N-terminal-directed t-tau assay A;

9 NTB, N-terminal-directed t-tau assay B; t-tau, total tau.

10

1   Introduction

2   Tauopathy is an umbrella term used to classify neurodegenerative diseases in which misfolded
3   and aggregated tau protein constitutes the key pathology.[1] The molecular mechanisms behind
4   tauopathies such as Alzheimer's disease, progressive supranuclear palsy and frontotemporal
5   dementia are likely distinct and, additionally, they differ in terms of clinical presentation,
6   anatomical distribution and cell types affected by the tau aggregates.[1] Although a definitive
7   diagnosis of a tauopathy requires neuropathological examination post-mortem to confirm the
8   presence and distribution of specific tau accumulations in the brain, differential diagnosis can be
9   aided by CSF biomarkers.[2, 3]

10 CSF total tau (t-tau), referring to methods reacting to both phosphorylated and non-
11 phosphorylated tau isoforms, is an established core Alzheimer's disease biomarker[2] that, together
12 with β-amyloid ($A\beta_{1-42}$ or $A\beta_{1-42}/A\beta_{1-40}$) and phosphorylated tau at threonine 181 (p-tau181), is
13 used for biological definition of Alzheimer's disease, in accordance with the
14 amyloid/tau/neurodegeneration (ATN) classification framework.[4] The first CSF t-tau assay was
15 developed in the 1990s, and increased CSF t-tau has been traditionally suggested to reflect tau
16 release due to neuronal injury and/or axonal degeneration.[5] This interpretation was supported by
17 the rapid increases of CSF t-tau seen also upon acute neuronal injury, such as stroke[6] or brain
18 trauma[7, 8], and in diseases with aggressive neurodegeneration, such as Creutzfeldt–Jakob
19 disease.[9] However, since normal CSF t-tau levels are typically detected in non-Alzheimer's
20 disease dementias and primary tauopathies commonly absent of Aβ pathology,[3, 10] the increase in
21 Alzheimer's disease has been hypothesized to be caused at least partly by a change in tau
22 metabolism in neurons affected by Aβ toxicity,[11] possibly from the dystrophic neurites
23 surrounding the plaques.

24 Immunoassays measuring t-tau in clinical routine, such as the fully automated Elecsys and
25 Lumipulse methods, utilize antibodies targeting epitopes located in mid-region of the protein.[12,
26 13] Since these assays can recognize all six tau isoforms, they are stated to measure "total tau".
27 However, in addition to its various post-translational modifications,[14, 15] soluble tau is known to
28 exist in proteolytic fragments of different lengths[16], and shorter N-terminal fragments lacking the
29 mid-region cannot be detected by the classic t-tau assays. Previous work targeting different N-
30 terminal epitopes of tau in CSF have shown that these fragments are increased in Alzheimer's

disease at mild cognitive impairment (MCI) and dementia stages.[17-19] In addition, p-tau assays targeting N-terminal fragments phosphorylated at threonine-181 (N-p-tau181) and threonine-217 (N-p-tau217) have shown earlier abnormal levels in CSF in comparison to mid-region p-tau181 across the Alzheimer's disease *continuum*.[20, 21]

Recently, tau was shown to consist of N-terminal and mid-region species in CSF and predominantly N-terminal forms in blood.[19, 22, 23] This knowledge has been applied to develop new p-tau immunoassays for use in blood, targeting different epitopes than the validated assays. In blood, N-p-tau181, N-p-tau217 and N-p-tau231 biomarkers (**Fig. 1**) are increased early in the Alzheimer's disease *continuum* starting from preclinical stage, and correlate well with CSF p-tau, t-tau, amyloid PET and tau PET.[21, 24-27] Similarly, a commercial Simoa t-tau assay (the most widely used blood t-tau biomarker) targeting N-terminal-to-mid-region epitopes is widely available. While CSF t-tau (directed at mid-region epitopes; **Fig. 1**) is an established Alzheimer's disease biomarker, the plasma t-tau alternative is only marginally increased in Alzheimer's disease compared with controls, shows large overlap between diagnostic groups, and correlates poorly with CSF t-tau.[28-30] It also does not change in relation to grey matter volume loss, cross-sectionally or longitudinally.[30] Recently, an N-terminal-targeted plasma t-tau alternative (NT1 tau) was seen to be increased in Alzheimer's disease at MCI and dementia stages versus controls,[18] and in individuals who later progressed to dementia.[31] These findings, in agreement with p-tau data, suggest that truncation leading to shorter N-terminal fragments of tau is an early event in Alzheimer's disease pathophysiology and that N-terminal tau forms might provide a superior biomarker performance in plasma. However, it is unclear how different tau fragments compare as neurodegeneration markers in Alzheimer's disease *continuum* and other neurodegenerative disorders.

The aim of this study was to develop and validate novel t-tau immunoassays targeting N-terminal and mid-region epitopes using Simoa technology and to subsequently investigate the levels of these new biomarkers versus clinically validated mid-region t-tau, p-tau and N-p-tau assays in CSF in clinical cohorts across the Alzheimer's disease *continuum*, as well as across other neurological diseases including those known for high (e.g., Creutzfeldt-Jakob disease and a heterogeneous group of other acute neurological disorders) and normal (e.g., progressive supranuclear palsy) t-tau levels. In addition, we performed an exploratory analysis in plasma,

1 evaluating the biomarker potential of the different t-tau immunoassays versus the Quanterix t-tau

2 in two independent cohorts.

Materials and methods

Study design

5 This was a cross-sectional, observational study conducted in collaboration with three centers

6 (The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden; University

7 Medical Centre Ljubljana, Ljubljana, Slovenia; and Université de Paris, Paris, France).

Immunoassay development and validation

9 Based on our recent success developing N-terminal-directed N-p-tau181, N-p-tau217 and N-p-

10 tau231 biomarkers,[20, 21, 24-26, 32] we developed two immunoassays targeting N-terminal-bearing

11 tau fragments: **NTA t-tau** containing the N-terminal epitope mapped between amino acids (aa)

12 6-18 and 159-163 and **NTB t-tau** containing epitopes at aa 6-18 and 194-198 . In addition, we

13 developed an assay targeting the mid-region aa 159-163 and 194-198 epitopes (referred as **MR t-**

14 **tau**) as a Simoa-based replica of the Innotest t-tau assay to enable direct comparison on the same

15 analytical platform. For NTA and MR t-tau, mouse monoclonal antibody targeting aa 159-163

16 (HT7, #MN1000, Thermo Scientific) was used as a capture antibody. Mouse monoclonal

17 antibodies targeting aa 6-18 (Tau12, #806502, BioLegend) and aa 194-198 (BT2, #MN1010,

18 Thermo Scientific) were used as detectors, respectively. For the NTB t-tau assay, mouse

19 monoclonal antibodies targeting aa 6-18 (1-100, #816601, BioLegend) and aa 194-198 (BT2,

20 #MN1010, Thermo Scientific) were used for capture and detection, respectively. Recombinant

21 non-phosphorylated full-length Tau-441 (#T08-54N, SignalChem) was used as a calibrator in all

22 the in-house t-tau assays. After optimizing the assay protocols, quantification limits, dilution

23 linearity, precision, accuracy, and spike recovery were assessed for all three assays. More

24 detailed description of the immunoassay development and validation processes are available in

25 **Supplementary material**.

26

7

1 Studied biomarkers

2 All biomarkers studied in this study are presented in **Figure 1**. Analytical and clinical details of

3 the Innotest® hTau Ag ELISA (t-tau) (Fujirebio, Ghent, Belgium), Innotest® Phosphotau (181P)

4 ELISA (Fujirebio, Ghent, Belgium), in-house N-p-tau181 and in-house N-p-tau217 Simoa

5 measurements from the clinical CSF cohort have been reported previously[20] and were included

6 to enable direct comparison between the classical mid-region (t-tau and p-tau181), N-t-tau and

7 N-p-tau biomarkers. Plasma samples were analyzed with commercial t-tau assays from

8 Quanterix, i.e., the single-analyte Tau 2.0 kit (#101552) for the pilot cohort and the multiplexed

9 Neurology 3-Plex kit (#101995), in-house N-p-tau181 and N-p-tau231 for the clinical cohort

10 using previously published methods.[24, 26] Note that both of the Quanterix t-tau assays target

11 identical epitopes.

12 Participants for CSF t-tau study

13 Pilot cohort

14 The pilot study included samples from CSF biomarker-positive Alzheimer's disease patients (n =

15 22) and biomarker-negative control patients (n = 22) clinically assessed in the Sahlgrenska

16 University Hospital, Gothenburg, Sweden. Patients included in the Alzheimer's disease group

17 were assessed due to suspected Alzheimer's disease, and no evidence of other neurological

18 condition was present. Patients in the neurological control group had consulted the clinic due to

19 minor neurological or psychiatric symptoms, but diagnosis of neurocognitive disorders were

20 excluded, and Alzheimer's disease core CSF biomarkers were within normal range. The groups

21 were defined by their core Alzheimer's disease CSF biomarker profiles (CSF $A\beta_{42}$ < 530 ng/l,

22 CSF p-tau181 > 60 ng/l, and CSF t-tau > 350 ng/l for Alzheimer's disease and levels within

23 normal range for control patients).

24 Clinical cohort 1 (the Alzheimer's *continuum*)

25 To study the profiles of different t-tau biomarkers in Alzheimer's disease *continuum*, a clinic-

26 based prospective memory center cohort from the University Medical Centre, Ljubljana,

27 Slovenia, was used. The cohort included individuals with biologically-defined Alzheimer's

28 disease, having both biomarker positivity and clinical presentation (n = 115; A+/T+/N+, mini-

29 mental state examination (MMSE) score = 21(16-25)), $A\beta$ positive MCI (n = 33, A+/T-/N-,

MMSE = 26(24-27)), Aβ negative MCI (*n* = 58; A-/T-/N-, MMSE = 27(26-28)), non-Alzheimer's disease dementia (*n* = 22; A-/T-/N-, MMSE = 21(19-23)) and Aβ- cognitively unimpaired neurological controls (*n* = 26; A-/T-/N-, MMSE = 29(29-30)). The control group presented with subjective cognitive concerns or sensory disturbances but had normal CSF Alzheimer's disease biomarker profile. The non-Alzheimer's disease dementia group included individuals with a diagnosis of alcohol-related dementia (*n* = 3), vascular dementia (*n* = 4), mixed vascular and non-Alzheimer's disease cortical dementia (*n* = 7) and unspecified dementia (*n* = 8). A/T/N profiles were defined by core Alzheimer's disease CSF biomarkers with local cut-offs (CSF Aβ$_{42}$ < 570 ng/l, CSF Aβ$_{42/40}$ < 0.07, CSF p-tau181 > 60 ng/l, and CSF t-tau > 400 ng/l).

Clinical cohort 2 (other neurodegenerative disorders)

In addition to Alzheimer's disease, we investigated individuals with a diagnosis of either Creutzfeldt-Jakob disease (*n* = 24), acute neurological disorders (*n* = 18, including individuals with status epilepticus (*n* = 9), ischemic stroke (*n* = 7), hepatic encephalopathy (*n* = 1), and limbic encephalitis (*n* = 1)) or progressive supranuclear palsy (*n* = 22), from the University Medical Centre, Ljubljana, Slovenia. Comparison between groups included the Alzheimer's disease and neurological control groups described above.

Participants for plasma t-tau study

Pilot cohort

The plasma pilot cohort included samples from CSF biomarker-positive Alzheimer's disease patients (*n* = 22) and biomarker negative control patients (*n* = 22) assessed in the Sahlgrenska University Hospital, Gothenburg, Sweden. Groups were defined as described above for the pilot CSF cohort.

Clinical cohort

Pilot findings with plasma NTA t-tau were replicated using a clinic-based prospective memory center cohort, the BioCogBank Paris Lariboisiére Cohort (Paris, France). CSF biomarker results, neuropsychological assessment, brain magnetic resonance imaging and respective diagnostic criteria were used to establish reliable diagnosis of Alzheimer's disease and other disorders. The cohort included patients with Alzheimer's disease (*n* = 19, A+T+N+, MMSE =

1 19 (13-24)), Aβ positive MCI (n = 6, A+T+N+, MMSE = 23 (22-26)), Aβ negative MCI (n =

2 13, A-T-N-, MMSE = 25 (24-27)), non-Alzheimer's disease dementia (n = 3, MMSE = 24 (17-

3 28)) and neurological controls (n = 8, A-T-N-, MMSE = 28 (26-30)). The non-Alzheimer's

4 disease dementia group included individuals with a diagnosis of vascular (n = 1, A-T-N-) or

5 frontotemporal dementia (n = 2, A-T-N+ and A-T+N+). A/T/N profiles were defined by core

6 Alzheimer's disease CSF biomarkers with local cut-offs (Lumipulse CSF Aβ$_{42/40}$ < 0.061, CSF

7 p-tau181 > 61 ng/L, CSF t-tau > 469 ng/L).

Informed consents

9 Ethical permission was obtained from the ethics committee at the university of Gothenburg

10 (#EPN140811, pilot CSF/plasma cohorts), the Ministry of Health, Republic of Slovenia (0120-

11 442/2017/3, clinical CSF cohorts 1 and 2) and the Bichat Hospital at the Paris University (n°10-

12 037 18/03/2010, clinical plasma cohort). Informed consent was obtained from all participants

13 according to the Declaration of Helsinki.

Biomarker measurements

15 All MR, NTA and NTB t-tau measurements were performed at the Neurochemistry laboratory at

16 University of Gothenburg (Mölndal, Sweden) using Simoa HD-X or HD-1 instruments

17 (Quanterix) between December 2020 and June 2021. Before measurements, assay beads and

18 helper beads were suspended in bead diluent, biotinylated detector antibodies in Tau 2.0 assay

19 buffer (#101556, Quanterix), and the enzyme streptavidin-conjugated β-galactosidase (SBG)

20 concentrate (#103397, Quanterix) in SBG diluent (#100376, Quanterix). For CSF, randomized

21 samples were thawed, vortexed briefly, plated, and diluted 1:4 in Tau 2.0 assay diluent.

22 Additional measurements with 1:10 dilution were needed for a subset of Creutzfeldt-Jakob

23 disease (7/24 for NTA, 5/24 for NTB, 11/24 for MR t-tau) and acute neurological disorders

24 samples (3/18 for NTA, 1/18 for NTB, 1/18 for MR t-tau) due to extremely high signals. For

25 plasma, randomized samples were thawed, vortexed, centrifuged (4000g, 10 min) and diluted 1:2

26 with Tau 2.0 assay diluent. An eight-point calibrator curve from recombinant non-

27 phosphorylated full-length Tau-441, and two internal quality control (iQC) samples were

28 included on each plate before and after the analyzed samples to control for inter and intra-assay

29 variability. Calibrators and iQC samples were run as duplicates and CSF/plasma samples as

10

singlicates in each plate. For commercial t-tau measurements, reagents and samples were prepared following the manufacturer's instructions. Further methodological details can be found in **Supplementary materials.**

Statistical analysis

Data is presented as median (interquartile range). Statistical analyses were performed with GraphPad Prism v. 9.0.1 (GraphPad, San Diego, California, USA) and MedCalc (Ostend, Belgium). Normality of data was inspected visually and with D'Agostino & Pearson normality test, and because not all biomarker data followed Gaussian distribution even after transformations, non-parametric tests were used. Differences in continuous variables in group demographics and t-tau biomarker concentrations were evaluated using either Mann-Whitney U-test (pilot studies, two groups) or Kruskal-Wallis test with Dunn's multiple comparison test (clinical cohorts, multiple groups). Fisher's exact test was used for comparison of categorical variables between groups (sex). Fold changes for all diagnostic groups were calculated by dividing t-tau concentration by the mean concentration of the Aβ- control group. Diagnostic accuracy of the measured biomarkers was evaluated with area under the curve (AUC) from receiver operating characteristic (ROC) analysis. Statistical differences between AUC values were determined using DeLong test. Spearman's correlation was used to evaluate association of different biomarker concentrations with each other, age and MMSE. Numbers of biomarker values below quantification limits or without a read are found in **Supplementary results** and were not included in the analysis. ROC and correlation analysis included only samples that gave readings with all seven assays to enable reliable comparison. Statistical significance level was set on $P < 0.05$ (two-tailed).

Data availability

Blinded data is available upon reasonable request from the corresponding author.

1 Results

2 Analytical validity of the NTA, NTB and MR t-tau assays in CSF

3 All developed t-tau assays had appropriate analytical performance. Defined quantification limits
4 for all assays are presented in **Supplementary Table 1**. Repeatability and intermediate precision
5 of CSF samples were < 30% (results from the validation experiments are presented in
6 **Supplementary Table 2** and from the clinical CSF cohorts in **Supplementary Table 3**), mean
7 spike recoveries were 79-145% (**Supplementary Table 4**) and recovery % with the used sample
8 dilution (1:4) was 78-79% for all t-tau assays (**Supplementary Fig. 1**).

9 Patient demographics

10 Demographics for the CSF cohorts are presented in **Table 1**. In the pilot CSF cohort ($n = 44$),
11 Alzheimer's disease group was older than the control group ($P = 0.0015$), and included more
12 females (73% vs 32%, $P = 0.015$). No correlation between age and any of the t-tau biomarker
13 levels was present within the diagnostic groups ($r_s = -0.28$-0.26, $P > 0.21$ in all).

14 In clinical CSF cohort 1 ($n = 228$), controls were younger than other diagnostic groups ($P \leq$
15 0.012), and other dementia patients older than Alzheimer's disease patients ($P = 0.019$). There
16 were no sex differences between the groups ($P = 0.50$). No correlation between age and any of
17 the t-tau biomarker levels was present within the diagnostic groups ($r_s = -0.10$-0.31, $P > 0.06$ in
18 all). MMSE scores decreased gradually from controls (29 (29-30)) to Alzheimer's disease
19 participants (21 (16-25)).

20 In clinical cohort 2, progressive supranuclear palsy and acute neurological disorders groups had
21 normal levels of CSF $A\beta_{1-42}$. CSF t-tau and p-tau levels were significantly higher in Creutzfeldt-
22 Jakob disease and acute neurological disorders versus progressive supranuclear palsy ($P <$
23 0.0001).

24 Demographics for the plasma cohorts are presented in **Table 2.** The Alzheimer's disease group
25 was older ($P = 0.0015$) and included more females (73% vs 32%, $P = 0.015$) in the pilot cohort
26 for NTB t-tau and Quanterix t-tau. There were no age or sex differences between other groups. In
27 clinical plasma cohort, there were no age or sex differences between groups ($P = 0.71$ and $P =$

1   0.40, respectively). MMSE scores were significantly higher for control ($P = 0.0005$) and Aβ-

2   MCI groups ($P = 0.024$) compared with Alzheimer's disease.

CSF t-tau biomarkers across Alzheimer's disease *continuum*

4   In the pilot cohort, all CSF t-tau biomarkers were significantly increased in Alzheimer's disease

5   participants versus controls ($P \leq 0.001$, **Fig. 2A**). Median fold changes were similar in

6   Alzheimer's disease (2.3 (1.7-4.2) for NTA, 1.8 (1.1-2.0) for NTB and 1.7 (1.5-2.9) MR t-tau, *P*

7    $= 0.22$) and all t-tau assays discriminated Alzheimer's disease from control patients (AUCs 78%-

8   88%; **Fig. 2B**). ROC analysis showed significantly higher AUCs for MR-t-tau (AUC [95%

9   confidence interval]: 88% [77-100%], DeLong$_{MR-NTB}$ $P = 0.038$) and NTA t-tau (AUC 88% [75-

10   100%], DeLong$_{NTA-NTB}$ $P = 0.043$) compared with NTB t-tau (AUC 78% [64-92%]). All in-

11   house CSF t-tau biomarkers were significantly correlated with each other ($r_s \geq 0.81$, $P < 0.0001$

12   for all) both in the whole cohort (**Fig. 2C**) and with Innotest t-tau within diagnostic groups ($r_s \geq$

13   0.44, $P < 0.040$ for all, **Supplementary Fig. 2).**

14   Results from the clinical CSF cohort 1 agreed with the pilot CSF cohort; All CSF t-tau

15   biomarkers were significantly increased in Alzheimer's disease compared to controls, Aβ- MCI

16   and other dementias (**Fig. 3A,** $P < 0.0001$ for all comparisons). For Innotest or MR t-tau, no

17   significant differences between Aβ- MCI and Aβ+ MCI ($P = 0.58$ and $P > 0.99$, respectively) or

18   Aβ+ MCI and controls ($P = 0.06$ and $P > 0.99$, respectively) were observed. On the contrary,

19   both NTA and NTB t-tau showed significantly higher concentrations already in Aβ+ MCI in

20   comparison with controls ($P = 0.0006$ for NTA; $P = 0.0013$ for NTB) and Aβ- MCI (p<0.0001

21   for both, **Fig. 3A**).

22   ROC curve analysis verified that when differentiating controls from Aβ+ MCI, NTA and NTB t-

23   tau assays performed statistically similar to all p-tau assays and Innotest t-tau (AUCs 74-85%,

24   DeLong $P > 0.53$ for all comparisons), and significantly better than MR t-tau (AUC 63% [46-

25   81%]; DeLong$_{MR-NTA}$ $P = 0.014$; DeLong$_{MR-NTB}$ $P = 0.0088$, **Fig. 3B**). In addition, both NTA and

26   NTB t-tau accurately distinguished Aβ- MCI from Aβ+ MCI (AUC$_{NTA}$ 84% [73-94%], AUC$_{NTB}$

27   82% [72-92%]) and had similar performances to N-p-tau217 (AUC 83% [74-92%]; DeLong$_{Np217-}$

28   $_{NTA}$ $P = 0.93$; DeLong$_{Np217-NTB}$ $P = 0.88$) and N-p-tau181 (AUC 75% [64-86%]; DeLong$_{Np181-NTA}$

29   $P = 0.20$; DeLong$_{Np181-NTB}$ $P = 0.30$). In addition, NTA and NTB t-tau assays performed

1 significantly better than MR t-tau (AUC 59% [46-72%]; DeLong$_{\text{MR-NTA}}$, P = 0.0015; DeLong$_{\text{MR-}}$

2 $_{\text{NTB}}$, P = 0.0012), Innotest t-tau (AUC 70% [58-82%]; DeLong$_{\text{Inno t-tau-NTA}}$, P = 0.046; DeLong$_{\text{Inno}}$

3 $_{\text{t-tau-NTB}}$, P = 0.056) and Innotest p-tau181 (AUC 65% [52-78%]; DeLong$_{\text{Inno p-181-NTA}}$, P = 0.016;

4 DeLong$_{\text{Inno p181-NTB}}$, P = 0.024, **Fig. 3C**) in the same scenario. On the contrary, MR t-tau had

5 nearly perfect cross-diagnostic performance discriminating Alzheimer's disease from other

6 dementias with significantly higher accuracy (AUC 98% [96-100%]) in comparison to NTA and

7 NTB t-tau (AUCs 90-91%, DeLong$_{\text{MR-NTA}}$, P = 0.013; DeLong$_{\text{MR-NTA}}$, P = 0.012, **Fig. 3D**). For

8 this comparison, N-t-tau assays had the same accuracy as N-p-tau assays (DeLong, P > 0.06 for

9 all comparisons). Innotest t-tau and p-tau181 were used to stratify patients into diagnostic groups

10 (hence the expected perfect differentiation marked with dashed line in **Fig. 3D**).

11 In clinical CSF cohort 1, all measured CSF tau-species had strong and positive correlation with

12 each other ($r_s \geq 0.63$, and P <0.0001 for all, **Fig. 3E**). As expected, **strong correlations e**xisted

13 between NTA and NTB t-tau (r_s = 0.95, P < 0.0001) and MR and Innotest t-tau (r_s = 0.90, P <

14 0.0001). Each t-tau biomarker had moderate negative correlation with CSF A$\beta_{1\text{-}42}$ in the whole

15 cohort ($r_s \geq$ -0.46, P < 0.0001 for all correlations), but no correlation was observed within any of

16 the diagnostic groups (**Supplementary Fig. 3**). All new t-tau biomarkers had moderate negative

17 correlation with MMSE score ($r_s \leq$ -0.53, P < 0.0001 for all correlations, **Supplementary Fig.**

18 **4**).

19 We also performed an exploratory analysis within the non-Alzheimer's disease dementia group.

20 Even though our sample sizes were small, NTB t-tau was seen to be significantly lower in

21 vascular dementia (P = 0.042), and MR t-tau and Innotest t-tau in mixed dementia (P_{MR} = 0.014,

22 $P_{\text{Innotest t-tau}}$ = 0.022) compared to unspecified dementia. All results are presented in

23 **Supplementary Table 5**.

CSF t-tau biomarkers in Creutzfeldt-Jakob disease and acute neurological disorders

26 All t-tau biomarkers were significantly higher in Creutzfeldt-Jakob disease and acute

27 neurological disorders when compared with controls or Alzheimer's disease (P <0.0001 for all

28 comparisons, **Fig. 4A**). Median fold changes versus controls were significantly higher for NTA

29 and NTB t-tau compared with MR and Innotest t-tau (**Supplementary table 6**); for Creutzfeldt-

14

1 Jakob disease, the median fold changes were 42 (21-89) for MR t-tau, 57 (32-102) for NTA and

2 133 (56-341) for NTB t-tau. Similar differences were found for acute neurological disorders,

3 where mean fold changes were 11 (7.5-20) for MR t-tau, 45 (25-84) for NTA, and 61 (33-136)

4 for NTB t-tau. Both NTA and NTB t-tau differentiated Creutzfeldt-Jakob disease from

5 Alzheimer's disease with an AUC of 99%, performing similar to the mid-region assays (AUCs

6 90-98%; DeLong $P > 0.089$ for all comparisons) but significantly better that both N-p-tau assays

7 (AUCs 67-81%; DeLong$_{NTA-Np217}$, $P < 0.0001$; DeLong$_{NTA-Np181}$, $P = 0.0006$; DeLong$_{NTB-Np217}$, P

8 < 0.0001; DeLong$_{NTB-Np181}$, $P = 0.0003$, **Fig. 4B**). Interestingly, Innotest t-tau, MR t-tau and N-p-

9 tau assays performed better in distinguishing acute neurological disorders and Creutzfeldt-Jakob

10 disease compared with MR p-tau181, NTA and NTB t-tau (**Fig. 4C**). In both groups,

11 concentrations of all CSF t-tau assays were positively correlated, with the strongest association

12 between NTA and NTB t-tau ($r_s = 0.91$, $P < 0.0001$ in Creutzfeldt-Jakob disease, **Fig. 4D**; $r_s =$

13 0.93, $P < 0.00001$ in acute neurological disorders, **Fig. 4E**).

14 An exploratory analysis within the acute neurological disorders group showed no differences in

15 tau concentrations between ischemic stroke and status epilepticus, whereas all CSF t-tau

16 biomarkers were significantly higher after ischemic stroke compared to Alzheimer's disease ($P <$

17 0.01 for all biomarkers). Hepatic encephalopathy and limbic encephalitis were not included in

18 this analysis ($n = 1$ for both). All results are presented in Supplementary Table 5.

19 ## CSF t-tau biomarkers in progressive supranuclear palsy

20 All CSF t-tau biomarkers were low in progressive supranuclear palsy, concentrations being

21 similar to controls ($P > 0.12$ for all assays, **Fig. 5A**). Median fold changes versus controls were

22 similar and below one for all t-tau assays ($P = 0.076$, **Supplementary table 5**) and none of the t-

23 tau and p-tau assays discriminated progressive supranuclear palsy from controls (AUCs 51-64%

24 for all, **Fig. 5B**). NTA and NTB t-tau displayed very strong positive correlations with each other

25 also in progressive supranuclear palsy ($r_s = 0.83$, $P < 0.0001$), and moderate to strong with

26 Innotest and MR t-tau ($r_s = 0.47$-0.62, $P \leq 0.059$, **Fig. 5C**). There was no association between the

27 N-t-tau and N-p-tau concentrations in progressive supranuclear palsy ($r_s = $ -0.21-0.41, $P \geq 0.10$).

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Plasma t-tau biomarkers in Alzheimer's disease

In pilot plasma cohort, NTA t-tau levels showed significantly higher concentrations in Alzheimer's disease than in control patients ($P = 0.0056$) and clearest differentiation between the two groups with an AUC of 75% [59-91%] (**Fig. 6A**). More overlap but statistically significant difference between the groups was seen also in MR t-tau levels ($P = 0.043$), whereas Quanterix Tau 2.0 and NTB t-tau showed similar concentrations in both groups ($P = 0.55$ and $P = 0.48$, respectively, **Supplementary Fig. 5**). However, in this small cohort, plasma t-tau biomarker concentrations did not correlate with CSF Innotest t-tau ($r_s = 0.29$, $P = 0.069$ for MR t-tau; $r_s = 0.29$, $P = 0.071$ for NTA t-tau; $r_s = 0.15$, $P = 0.35$ for NTB t-tau, **Supplementary Fig. 6**).

Based on the pilot results, we aimed to replicate the promising findings with NTA in another clinical cohort comprising patients across the Alzheimer's disease *continuum*. Again, higher plasma NTA t-tau concentrations were measured in patients with Alzheimer's disease than in controls ($P = 0.0033$) or patients with Aβ- MCI ($P = 0.027$), whereas no statistically significant differences were observed with the Quanterix t-tau ($P = 0.44$ and 0.23, respectively, **Fig. 6B**). Plasma NTA t-tau differentiated Alzheimer's disease patients and controls with an AUC of 94% [85-100%], performing similar to plasma N-p-tau181 (AUC 97% [96-100%]) and N-p-tau231 (AUC 95% [88-100%]) and better than Quanterix plasma t-tau (AUC 76% [56-96%]). Plasma NTA t-tau also showed strong correlation with CSF t-tau ($r_s = 0.63$, $P > 0.0001$), plasma N-p-tau181 ($r_s = 0.68$, p<0.0001) and plasma N-p-tau231 ($r_s = 0.69$, $P < 0.0001$) in the whole cohort, whereas no correlation with Quanterix plasma t-tau was observed ($r_s = 0.22$, $P = 0.13$) (**Supplementary Fig. 7**).

Discussion

Based on current understanding about the complexity of tau protein, it has become obvious that it is a much more challenging biomarker to interpret than previously understood.[11] In brain, tau is mostly present as a full-length protein, whereas many different fragments of diverse lengths are known to exist in CSF, and increase in concentrations during Alzheimer's disease pathological process.[17-19, 33-35] Studies using immunoprecipitation followed by mass spectrometry have shown that tau peptides C-terminal to position 254 are not detectable in CSF or blood, suggesting that tau content in these fluids consists of tau x-254 forms.[19, 22, 24, 26] However, extracting conclusions about the biomarker potential of different fragments is difficult since no direct comparison

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between assays targeting mid-region and N-terminal phosphorylated and non-phosphorylated species has been performed on the same analytical platform or in the same large clinical cohorts. Since N-terminal tau forms are ubiquitous to CSF and blood, targeting this part of the tau molecule could generate biomarkers applicable to both fluid systems. We took advantage of our recent successful development of N-terminal-directed p-tau181, p-tau217 and p-tau231 biomarkers[20, 21, 24-26, 32] and designed two novel ultrasensitive t-tau immunoassays (NTA and NTB t-tau) targeting N-terminal epitopes. To enable cross-biomarker comparisons using an identical analytical technology, we developed a third assay (MR t-tau) using the same antibodies used in the gold-standard Innotest t-tau. Thereafter, we performed diagnostic comparisons between the new versus classical t-tau biomarkers (with N-p-tau assays included for proof of concept) across Alzheimer's disease *continuum* and other neurological disorders using the same Simoa platform.

N-terminal bearing CSF tau biomarkers in Alzheimer's disease

Consistent with previous findings in CSF[17-19, 35], we showed that all measured CSF mid-region and N-terminal t-tau fragments were increased in Alzheimer's disease compared with controls and had high diagnostic accuracies for differentiating the two groups (AUCs 90-98% for all assays). However, in our study, only levels of N-terminal-bearing CSF tau biomarkers measured by NTA (HT7/Tau12) and NTB (1-100/BT2) t-tau increased significantly in early Aβ+ MCI when compared with controls. In addition, only NTA and NTB t-tau were able to distinguish between Aβ+ MCI and Aβ- MCI showing equal performances as N-p-tau217 and N-p-tau181. On the contrary, all t-tau assays measured in CSF distinguished Alzheimer's disease and other dementia cases with high accuracy (AUCs 90-100%). In this context, CSF MR t-tau performed significantly better than NTA or NTB (DeLong $P \leq 0.0012$), but there were no significant differences between CSF N-t-tau and N-p-tau assays (Delong $P \geq 0.06$). Together, our findings agree with previously presented hypothesis that during Alzheimer's disease pathophysiological process, tau fragments including the N-terminus are released early by neurons that are presumably affected by Aβ toxicity but still only at risk for developing tangle pathology.[11, 22] We also showed that this early increase can be detected with N-terminal directed assays targeting both phosphorylated and non-phosphorylated epitopes.

CSF t-tau is an established core Alzheimer's disease biomarker, and previous studies have shown increased concentrations measured with classical mid-region assays already in MCI stage of Alzheimer's disease.[2, 36, 37] However, in our study, mid-region bearing fragments were still not significantly different in MCI (both Aβ- and Aβ+) than in controls. These seemingly contradictory findings could be explained by the fact that earlier Aβ+ MCI (A+/T-/N-) patients were included to our CSF study, and conversely, significant increases only in N-terminal-bearing tau biomarkers could already be observed at this stage (**Fig. 3A**). This is in agreement with the previous findings using p-tau assays in the same cohort, namely that CSF mid-p-tau181 likely reflects more established tau pathology in AD, whereas abnormal levels of N-p-tau181 or N-p-tau217 in early MCI were suggested to have closer association with initial Aβ changes.[20]

The developed NTA and NTB t-tau assays use mid-region targeted antibodies for capture (HT7 and BT2), meaning that the mid-region t-tau assays should be able to also capture the shorter N-terminal species bearing these epitopes. However, in our study, this was not the case. This apparent discrepancy might be explained by differences in quantity of the different fragments. Using the same recombinant tau as the assay calibrator, we observed that the levels of CSF t-tau measured by NTA (0-85 pg/mL) and NTB (0-480 pg/mL) assays were pronouncedly lower than those measured by MR t-tau (0-2200 pg/mL) and Innotest t-tau (0-2600 pg/mL). Furthermore, tau truncation and excretion are regulated processes, and it has been hypothesized that whereas full length tau is passively secreted from the neurons, truncated forms are released through active secretion.[38] Thus, the subtle but meaningful changes in the levels of N-terminal fragments could be diluted by the excess of other, longer tau fragments in CSF, captured by mid-region total tau assays.

CSF tau biomarkers in non-Alzheimer's disease dementia

In addition to exploring different stages within the Alzheimer's continuum, it would be interesting to investigate differences in various CSF t-tau markers between different non-Alzheimer's disease dementias. Here, we reported that all tau biomarkers were significantly higher in Alzheimer's disease in comparison to other dementias and some differences were also observed within the non-Alzheimer's disease dementia group; NTB t-tau was significantly lower in vascular dementia, and all mid-region fragments (MR t-tau, Innotest t-tau, and Innotest p-tau181) in mixed dementia in comparison to unspecified dementia. However, due to our small

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sample size (3-8 per group) and heterogeneous in nature of different dementias, we want to emphasize that these findings should be interpreted with caution and investigated in more detail on a larger non-Alzheimer's disease dementia cohort in the future.

N-terminal bearing CSF tau biomarkers in other neurological diseases

In addition to Alzheimer's disease, CSF t-tau is known to be highly increased in Creutzfeldt-Jakob disease and acute neurological disorders due to rapid and aggressive neurodegeneration in these diseases. As expected, we also observed highly elevated concentrations of all investigated CSF t-tau biomarkers in both groups. Fold changes calculated against controls were higher for the N-t-tau biomarkers in comparison to MR t-tau and measuring the N-terminal fragments improved the diagnostic accuracy between Alzheimer's disease and Creutzfeldt-Jakob disease. However, the groups were well distinguished by all CSF t-tau measured biomarkers, thus this difference is likely not clinically relevant. In our study, we did not see differences between the CSF mid-region or N-terminal assays in their ability to differentiate non-Alzheimer's disease dementia or progressive supranuclear palsy from controls. Previously, abnormally low levels of CSF t-tau were reported in progressive supranuclear palsy, using an in-house ELISAs targeting the same N-terminal epitopes that our in-house NTA and NTB t-tau assays for Simoa.[3] Our results support the present understanding that tau deposition and metabolism in primary tauopathies differ from that of Alzheimer's disease, and other forms of tau with better biomarker potential should still be explored to address these disorders.

Short N-terminal bearing tau fragments as plasma biomarkers in Alzheimer's disease

Due to less invasive sampling and higher cost-effectiveness, blood biomarkers hold enormous potential for screening and diagnosis of Alzheimer's disease when compared with both CSF and imaging biomarkers. Plasma t-tau has also been shown to be increased in Alzheimer's disease when compared to controls and Aβ+ MCI, however, high overlap between the diagnostic groups and lack of correlation with CSF t-tau has hindered its usability.[28, 29] This could be due to interference caused by peripheral expression of tau, and/or rapid metabolism and fragmentation of tau in plasma, resulting in fragments that might not be recognized by the commercial t-tau

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assays. Recently, plasma NT1 targeting shorter, N-terminal-bearing fragment of tau (BT2/Tau12) was able to differentiate Alzheimer's disease from control subjects and predict future cognitive decline, suggesting that N-t-tau fragments could be more suitable blood biomarkers.[18, 31, 39] In this study, we identified a novel plasma N-terminal biomarker (NTA) that also showed higher concentrations in Alzheimer's disease compared with controls both in the pilot and clinical cohorts. Interestingly, plasma NTA concentrations in the clinical cohort also correlated strongly with CSF t-tau, as well as both plasma p-tau181 and p-tau231. These findings agree with previous studies suggesting that short, N-terminal bearing fragments in plasma could be presenting the same early response to Aβ seen in CSF and less prone to degradation than the longer fragments.[18, 31]

In contrast to CSF, where our NTA t-tau showed similar performance with NTB t-tau, the NTA t-tau assay (requiring a short minimum aa sequence of 6-159) performed better in plasma compared with NTB t-tau (requiring longer minimum sequence of 6-198, similar to NT1[18]). Previously, Chen *et al*[18] have also shown that NT1 performed better than another N-terminal biomarker NT2 (ADx202/Tau12), that requires a longer sequence (aa 6-224). When we compared our N-terminal assays with the commercial Quanterix t-tau (that could also be considered to target a N-terminal sequence, since it requires a sequence ranging from aa 16 to aa 222) we saw poor performance of this biomarker in plasma in two different cohorts compared with both NTA and NTB t-tau. Even though the NTA and NTB t-tau still need to be further optimized and validated for blood, our findings (together with the earlier reports with NT1 and NT2) support the view that assays targeting minimal N-terminal sequences (including aa 6 targeted by the Tau12 and 1-100 antibodies) provide a superior performance detecting Alzheimer's disease-relevant tau species in plasma when compared with assays targeting longer N-terminal or mid-region fragments.

Strengths and limitations

A clear strength of this study is the identification of a novel short NTA t-tau biomarker measurable using the Simoa technology. Another strength of the present study was the comparison of our in-house mid-region and N-terminal t-tau biomarkers with both classical CSF tau biomarkers (Innotest t-tau and p-tau181), and previously described in-house N-terminally targeted p-tau biomarkers (N-p-tau181 and N-p-tau217)[20] in the same memory clinic cohort

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translating well into real world clinical setting. Different assays were also evaluated both in CSF and in plasma. In addition, we developed MR t-tau that mimics the gold standard Innotest t-tau in Simoa, thus we can be sure that analytical platform or technical effects do not influence our comparison. However, our study does not go without limitations. Firstly, due to the cohorts being comprised of individuals recruited from a memory clinic setting, our study does not include any samples from an early, preclinical phase of Alzheimer's disease. Thus, we were unable to investigate how early in the Alzheimer's disease continuum the N-terminal t-tau fragments become abnormal. Secondly, due to the cross-sectional nature of this study, we were not able to compare the longitudinal changes of the different biomarkers across the Alzheimer's disease *continuum*. In addition, APOE status was not available for all participants in the clinical cohorts, thus we could not investigate the effect of APOE on the t-tau biomarker levels in this study.

Conclusions

In conclusion, we developed new t-tau immunoassays for the Simoa platform targeting both N-terminal and mid-region epitopes of tau and showed that different t-tau assays have different biomarker potential in Alzheimer's disease *continuum* both in CSF and plasma. NTA and NTB t-tau were able to discriminate MCI with and without underlying Aβ pathology, and therefore detecting early Alzheimer's disease-related abnormalities in CSF, whereas all t-tau assays had excellent performance differentiating Alzheimer's disease from other dementias. In addition, N-terminal-directed CSF t-tau biomarkers were seen to be increased to higher degrees in Creutzfeldt-Jakob disease and acute neurological disorders, both characterized with aggressive neurodegeneration. Most notably, plasma NTA t-tau was able to successfully differentiate Alzheimer's disease from controls and correlated strongly with both plasma N-p-tau181 and N-p-tau231. Based on our findings, N-terminal bearing forms of tau seem to be secreted into CSF in an early phase of Alzheimer's disease pathological process, and like N-p-tau, N-t-tau biomarkers could provide added value in the variety of tau assays available for further research.

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11 Supplementary material

12 Supplementary material is available at *Brain online*.

13

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Figure legends

Figure 1 Schematic presentation of all t-tau and p-tau biomarkers included in the study.
Immunoassays developed during this study are presented with a bolded font.

Figure 2 CSF t-tau biomarker concentrations and their diagnostic performance in Alzheimer's disease. (A) Box plots presenting in-house MR, NTA and NTB t-tau concentrations in the pilot cohort composed of core CSF biomarker positive Alzheimer's disease (AD) and biomarker negative control patients. **(B)** Areas under the curve (AUC, with 95% confidence intervals (CI)) from receiver operating characteristic analysis showing the diagnostic accuracy of the in-house t-tau assays to distinguish the groups. (**C**) Correlation matrix presenting Spearman's correlations for all measured t-tau biomarkers with each other and with CSF β-amyloid$_{1\text{-}42}$ in the whole cohort. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, non-significant.

Figure 3 CSF t-tau biomarker concentrations and their diagnostic performance across Alzheimer's disease *continuum*. (A) Box plots presenting CSF concentrations of Innotest t-tau, in-house MR, NTA and NTB t-tau in clinical cohort 1, including subjects across the Alzheimer's disease *continuum*. (**B**) Areas under the curve (AUC, with 95% confidence intervals (CI)) from receiver operating characteristic analysis showing the diagnostic accuracies of all studied CSF biomarkers to distinguish between neurological control and amyloid positive (Aβ+) mild cognitive impairment (MCI) cases; (**C**) amyloid negative (Aβ-) and Aβ+ MCI cases; and (**D**) Alzheimer's disease (AD) and non-Alzheimer's disease dementia cases (including alcohol-related dementia, vascular dementia, mixed dementia and unspecified dementia). (**E**) Correlation matrix presenting Spearman's correlations for all measured t-tau and p-tau assays with each other and with CSF β-amyloid$_{1\text{-}42}$ in the whole cohort. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, non-significant.

Figure 4 CSF t-tau biomarker concentrations and their diagnostic performance in Creutzfeldt–Jakob disease and acute neurological disorders. (**A**) Box plots presenting CSF concentrations of Innotest t-tau, in-house MR, NTA and NTB t-tau in Creutzfeldt–Jakob disease (CJD) and acute neurological disorders (AND, including individuals with status epilepticus, ischemic stroke, hepatic encephalopathy and limbic encephalitis). (**B**) Areas under the curve

27

1   (AUC, with 95% confidence intervals (CI)) from receiver operating characteristic analysis

2   showing the diagnostic accuracies of the tau biomarkers to distinguish between Creutzfeldt–

3   Jakob disease and Alzheimer's disease (AD) or (**C**) acute neurological disorders. (**D**) Correlation

4   matrix showing Spearman's correlations for all measured t-tau and p-tau concentrations in

5   Creutzfeldt–Jakob disease and (**E**) acute neurological disorders. *, $P < 0.05$; **, $P < 0.01$; ***, P

6   < 0.001; ****, $P < 0.0001$; ns, non-significant.

7   **Figure 5 CSF t-tau biomarker concentrations and their diagnostic performance in**

8   **progressive supranuclear palsy. (A)** Box plots presenting CSF concentrations of Innotest t-tau, in-

9   house MR, NTA and NTB t-tau in progressive supranuclear palsy (PSP). (**B**) Areas under the

10   curve (AUC) from receiver operating characteristic analysis presenting diagnostic accuracies of

11   the tau biomarkers to distinguish between progressive supranuclear palsy and controls. (**C**)

12   Correlation matrix showing Spearman's correlations for all measured t-tau and p-tau

13   concentrations in progressive supranuclear palsy. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$;

14   ****, $P < 0.0001$; ns, non-significant.

15   **Figure 6 Plasma NTA and Quanterix t-tau concentrations and its diagnostic performance**

16   **in Alzheimer's disease.** Box plots presenting plasma Quanterix t-tau and NTA t-tau

17   concentrations and areas under the curve (AUC) from receiver operating characteristic analysis

18   (**A**) in the pilot cohort composed Alzheimer's disease (AD) and control patients, and (**B**) in a

19   clinical cohort including subjects with Alzheimer's disease, amyloid negative (Aβ-) and amyloid

20   positive (Aβ+) mild cognitive impairment (MCI), controls and non-Alzheimer's disease

21   dementia. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, non-significant.

22

1   **Table 1 Demographics and biomarker concentrations of the CSF cohorts**

	Pilot CSF cohort			Clinical CSF cohort 1 (Alzheimer's continuum)						Clinical CSF cohort 2 (other neurological disorders)			
	Control	AD	*P*	Control	Aβ-MCI	Aβ+ MCI	AD	Non-AD dementia	*P*	CJD	AND	PSP	*P*
N	22	22		26	58	33	115	22		24	18	22	
Age (years)	71.5 (64.8–75.0)	79.0 (71.0–83.5)	**0.0015**	66.9 (62.7–72.2)*	73.4 (67.8–78.1)#	73.7 (67.7–78.1)#	73.3 (67.5–78.3)#	77.8 (76.1–82.5)*#	**<0.0001**	67.7 (56.1–78.0)	77.2 (63.9–83.6)	68.0 (65.2–75.0)	0.054
Sex (F/M, N)	7/15	16/6	**0.015**	15/11	30/28	23/10	64/52	11/11	0.50	11/13	11/7	7/15	0.18
MMSE	–	–	–	29 (29.0–30.0)*	27 (25.5–28.0)*	26 (24.0–27.0)*	21 (16.0–25.0)	21 (19–23)	**<0.0001**	3.0 (2.0–8.0)	NA	NA	–
CSF Aβ42 (pg/ml)	909 (793–1063)	545 (479–596)	**<0.0001**	1282 (1141–1424)*	1216 (1090–1377)*	592 (483–801)#	555 (6491–631)#	1096 (919–1198)*	**<0.0001**	851 (742–1219)	894 (640–1303)	947 (756–1138)	0.97
CSF Innotest ® t-tau (pg/ml)	324 (228–382)	576 (460–978)	**<0.0001**	227 (181–267)*	281 (209–335)*	316 (291–380)*	777 (610–1020)#	254 (215–323)*	**<0.0001**	6579 (2545–11596)¤	2169 (1701–2389)¤	208 (177–278)	**<0.0001**
CSF p-tau181 (pg/ml)	53.0 (41.8–60.3)	80.0 (67.8–118)	**<0.0001**	41.0 (30.0–47.3)*	46.0 (36.8–52.3)*	51.0 (47.5–55.5)*	103 (85.0–133)#	38.0 (32.8–44.3)*	**<0.0001**	57.0 (43.8–78.3)¤	58.0 (47.0–68.5)¤	34.5 (28.0–42.8)	**<0.0001**
CSF MR t-tau (pg/ml)	210 (116–234)	310 (268–525)	**<0.0001**	114 (76.0–157)*	118 (87.1–170)*	149 (130–169)*	412 (278–587)#	131 (74.4–153)*	**<0.0001**	5409 (2691–11210)¤	1376 (949–2585)¤	76.1 (54.2–131)	**<0.0001**
CSF NTA t-tau (pg/ml)	6.80 (2.90–7.80)	13.8 (9.96–24.9)	**<0.0001**	1.83 (1.26–2.68)*	2.24 (1.05–3.40)*	8.80 (6.04–10.4)#	9.95 (5.9–16.2)#	1.43 (0.56–3.67)*	**<0.0001**	149 (84.6–268)¤	120 (65.0–222)¤	2.04 (0.82–2.84)	**<0.0001**
CSF NTB t-tau (pg/ml)	83.9 (49.5–97.4)	142 (86.1–222)	**0.001**	10.8 (7.26–17.7)*	11.1 (5.77–19.4)*	34.6 (24.7–44.6)#	47.7 (22.9–72.2)#	8.38 (33.4–)*	**<0.0001**	2076 (880–5309)¤	959 (519–2127)¤	9.59 (5.84–16.4)	**<0.0001**

2   Data are presented median (interquartile range). Differences between groups were tested with Mann-Whitney U-test (discovery
3   cohort) and Kruskall Wallis test with Dunns multiple comparison (Clinical cohorts) for continuous variables. Fisher's exact test was
4   used for categorical variables (sex). P value presents overall difference between groups. Significant differences in pairwise
5   comparisons to Alzheimer's disease (AD, *), controls (#) and progressive supranuclear palsy (PSP, ¤) groups are also presented.
6   Abbreviations: Aβ , beta-amyloid; Aβ -, amyloid negative; Aβ +, amyloid positive; AND, acute neuronal disorders; CJD,
7   Creutzfeldt-Jacob disease; MCI, mild cognitive impairment; MMSE, mini-mental state examination.

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1   **Table 2 Demographics and biomarker concentrations of the plasma cohorts**

	Plasma pilot cohort (MR and NTA t-tau)			Plasma pilot cohort (NTB and Quanterix t-tau)			Plasma clinical cohort					
	Control	AD	*P*	Control	AD	*P*	Control	Aβ-MCI	Aβ+MCI	AD	Non-AD dementia	*P*
N	20	20		22	22		8	13	6	19	3	
Age (years)	69.0 (59.5–77.0)	76.0 (68.3–80.8)	0.077	71.5 (64.8–75.0)	79.0 (71.0–83.5)	**0.0015**	67.0 (54.8–76.5)	66.0 (59.5–77.0)	67.5 (62.8–81.3)	70.0 (65.0–75.0)	71.0 (60.0–76.0)	0.71
Sex (F/M, N)	9/11	10/10	>0.999	7/15	16/6	**0.015**	4/4	7/6	5/1	15/4	2/1	0.4
MMSE	NA	NA	NA	NA	NA	NA	27.5 (26.3–30.0)*	25.0 (24.0–27.0)*	22.5 (21.8–26.3)	19.0 (13.0–24.0)	24.0 (17.0–28.0)	**0.0006**
CSF Aβ42 (pg/ml)	966 (849)	480 (434–498)	**<0.0001**	909 (793–1063)	545 (479–596)	**<0.0001**	955 (788–1418)*	1032 (617–1108)*	468 (349–680)	547 (450–630)	1571 (730–1821)*	**<0.0001**
CSF t-tau (pg/ml)	243 (216–278)	915 (800–978)	**<0.0001**	324 (228–382)	576 (460–978)	**<0.0001**	213 (165–265)*	200 (149–306)*	625 (471–803)	888 (582–1268)	580 (165–1811)	**<0.0001**
CSF p-tau181(pg/ml)	42.5 (37.5–45.0)	98.0 (79.5–114)	**<0.0001**	53.0 (41.8–60.3)	80.0 (67.8–118)	**<0.0001**	30.4 (22.0–39.0)*	27.0 (23.6–39.4)*	94.0 (69.7–127)	139 (99.8–204)	49.6 (21.4–62.9)	**<0.0001**
Plasma MR t-tau (pg/ml)	44.4 (27.8–64.8)	65.0 (52.9–74.0)	**0.043**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Plasma NTA t-tau (pg/ml)	0.034 (0.022–0.053)	0.10 (0.050–0.13)	**0.0056**	NA	NA	NA	0.025 (0.019–0.081)*	0.035 (0.023–0.074)*	0.086 (0.034–0.13)	0.14 (0.095–0.18)	0.041 (0.039–0.074)	**0.0021**
Plasma NTB t-tau (pg/ml)	NA	NA	NA	116 (86.9–149)	128 (292.7–167))	0.477	NA	NA	NA	NA	NA	NA
Plasma Quanterix t-tau (pg/ml)	NA	NA	NA	1.40 (0.83–1.98)	1.10 (0.98–1.50)	0.55	0.30 (0.18–0.32)	0.27 (0.22–0.34)	0.28 (0.19–0.41)	0.38 (10.30–0.43)	0.28 (0.19–0.38)	0.11

2   Data are presented median (interquartile range). Differences between groups were tested with Mann-Whitney U-test (pilot
3   cohort) and Kruskall Wallis test with Dunns multiple comparison (Clinical cohort) for continuous variables. Fisher's exact
4   test was used for categorical variables (sex). *P* value presents overall difference between groups. Significant differences in
5   pairwise comparisons to Alzheimer's disease (AD, *) are also presented. Quanterix t-tau was measured with Tau 2.0 kit
6   (pilot cohort) and Neurology 3-plex kit (clinical cohort), both assays targeting identical epitopes. Abbreviations: Aβ , beta-
7   amyloid; Aβ -, amyloid negative; Aβ +, amyloid positive; MCI, mild cognitive impairment; MMSE, mini-mental state
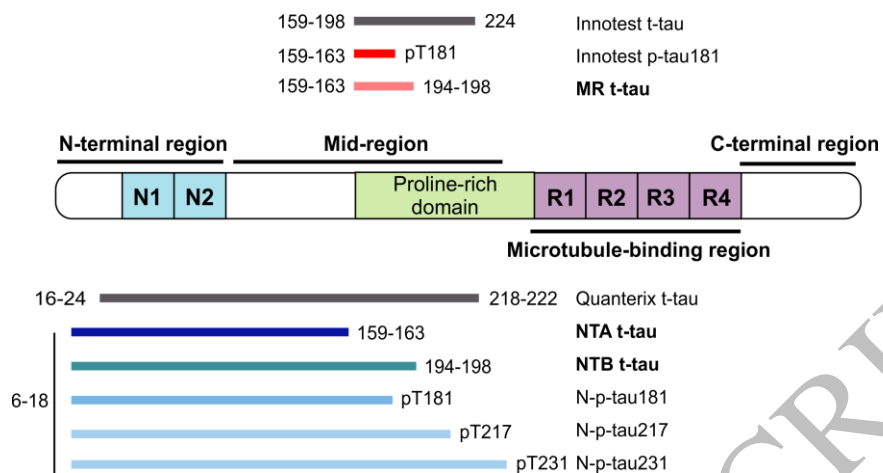8   examination.

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159-198 ━━━━━ 224   Innotest t-tau
159-163 ━━ pT181   Innotest p-tau181
159-163 ━━━━ 194-198   **MR t-tau**

N-terminal region   **Mid-region**   **C-terminal region**

N1 | N2 | Proline-rich domain | R1 | R2 | R3 | R4

Microtubule-binding region

16-24 ━━━━━━━ 218-222   Quanterix t-tau
━━━━━━ 159-163   **NTA t-tau**
━━━━━━ 194-198   **NTB t-tau**
6-18 ━━━━━ pT181   N-p-tau181
━━━━━ pT217   N-p-tau217
━━━━━ pT231   N-p-tau231

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Figure 1
115x61 mm (0.3 x DPI)

4
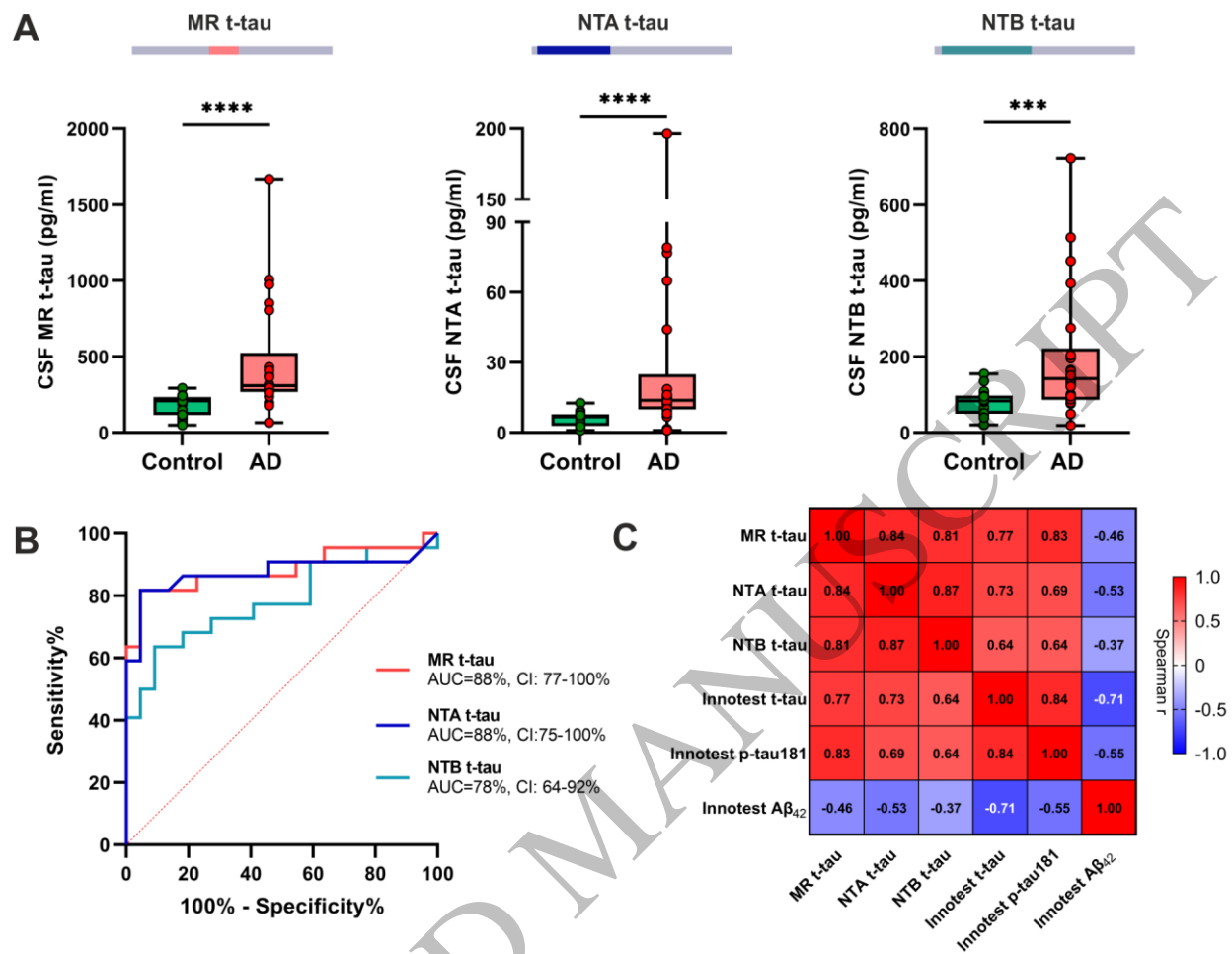
Figure 2
165x127 mm (0.3 x DPI)
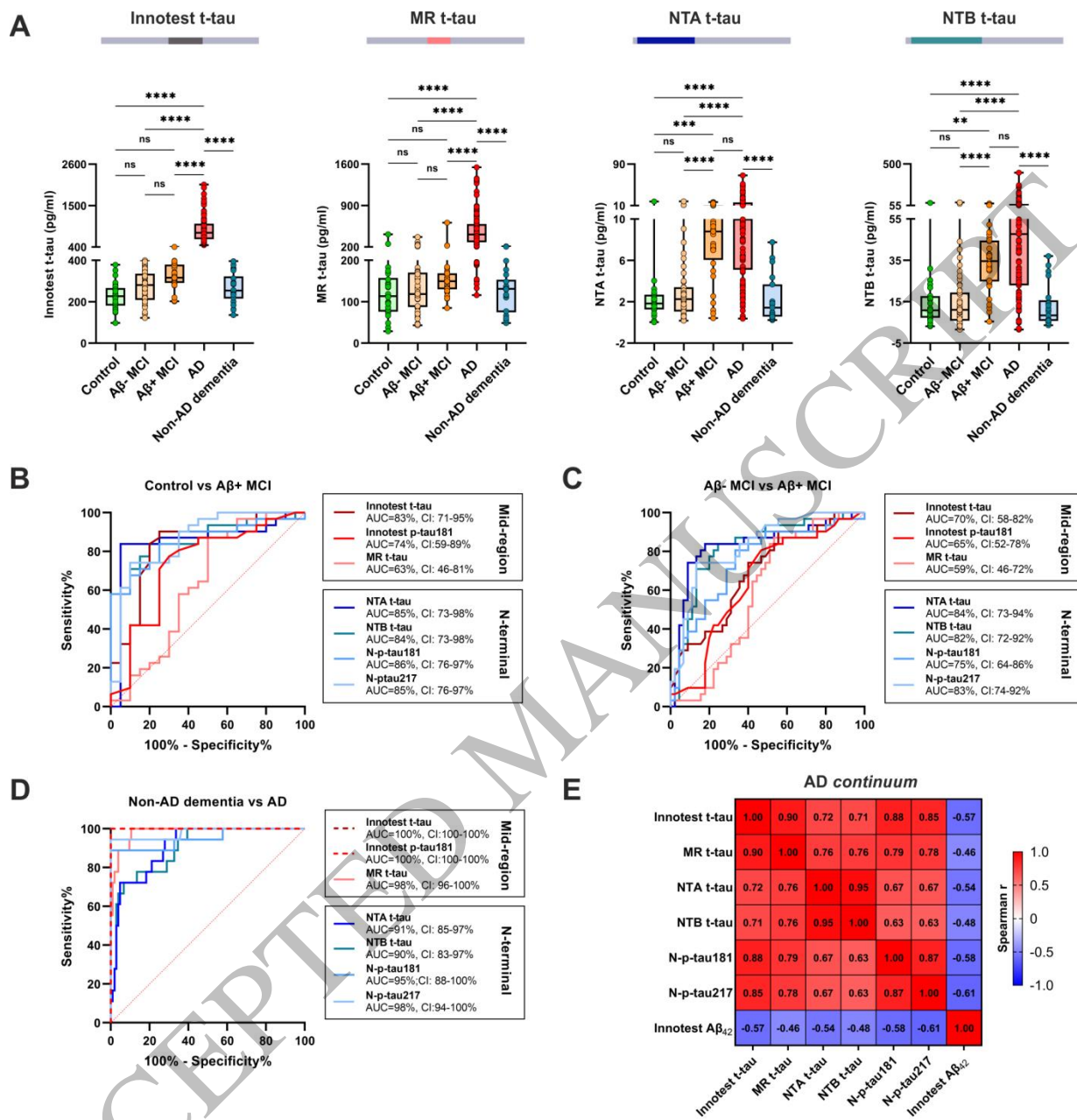
Figure 3
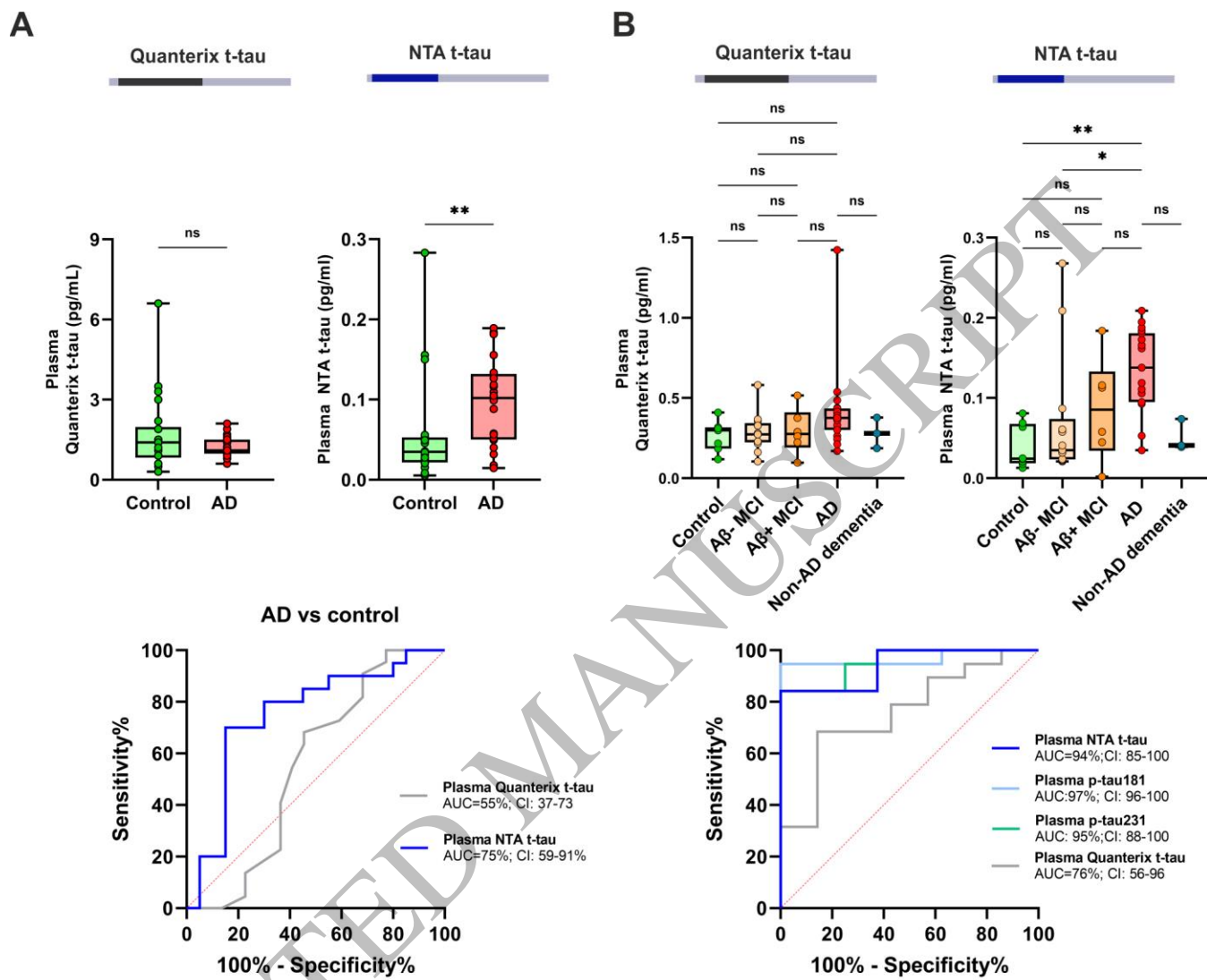**186x190 mm (0.3 x DPI)**

33
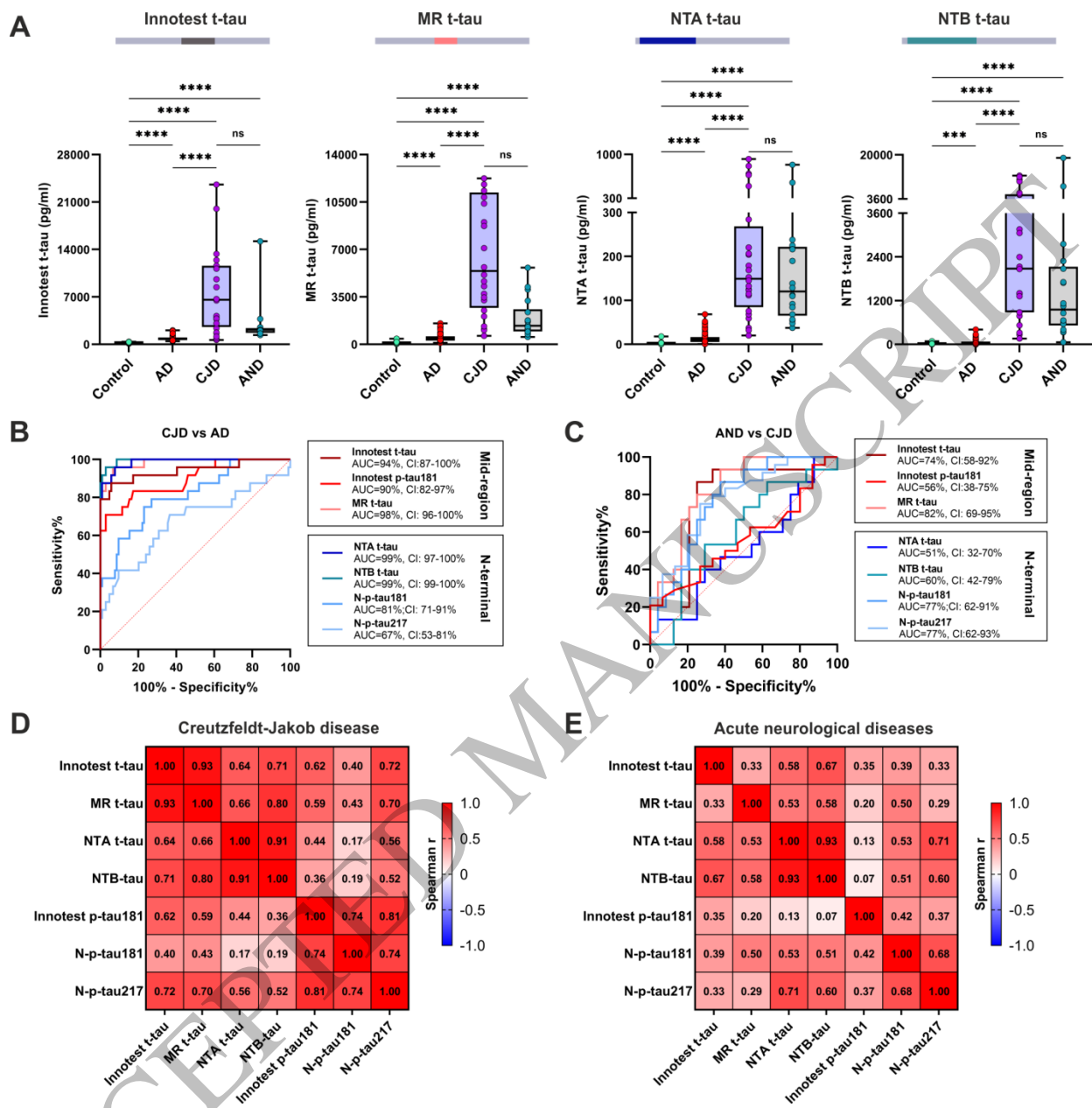
Figure 4
190x192 mm (0.3 x DPI)
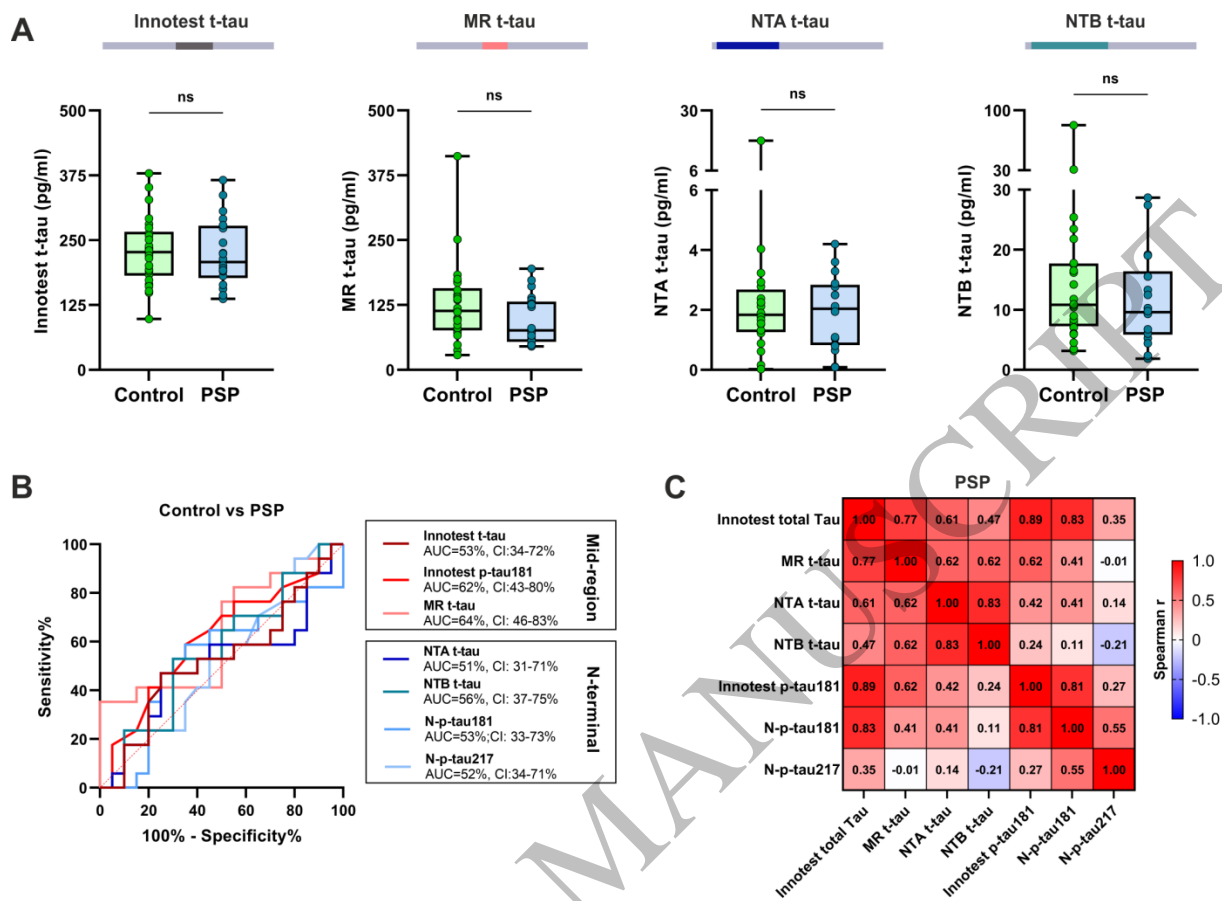
Figure 5
196x140 mm (0.3 x DPI)

Figure 6
184x145 mm (0.3 x DPI)