

Supplementary data

Antibody-free measurement of cerebrospinal fluid tau phosphorylation across the Alzheimer's disease continuum

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Supplementary Figure S6. Fold changes (AD versus CU-) for phospho-epitope concentrations compared with phosphorylation ratios

Supplementary Table S1. Precursor and product ions used for quantification.

| | | Product m/z | Product Charge | Fragment Ion |
|-------------------------------------|---------------------|------------------------|---------------------------|-------------------------|
| Phosphorylation Site | Thr-181 | 341.2183 | 1 | y3 |
| Peptide Sequence | TPPAPK[pT]PPSSGEPPK | 367.1976 | 1 | b4 |
| Amino Acids | 175-190 | 490.5727 | 3 | y14 |
| Precursor m/z | 556.6062 | 522.9236 | 3 | y15 |
| Precursor Charge | 3 | 592.3453 | 1 | b6 |
| HCD Collision Energy (%) | 26 | 602.3220 | 2 | y12 -98 |
| Spike-in conc. (fmol/ml CSF) | 120 | 651.3105 | 2 | y12 |
| | | 675.3824 | 1 | b7 |
| | | 686.8290 | 2 | y13 |
| | | 686.3760 | 2 | y14-98 |
| | | 701.3464 | 1 | y7 |
| | | 735.3554 | 2 | y14 |
| | | 798.3992 | 1 | y8 |
| Phosphorylation Site | Ser-199 | 456.7303 | 2 | y10 |
| Peptide Sequence | SGYS[pS]PGSPGTPGSR | 464.1776 | 1 | b5 -98 |
| Amino Acids | 195-209 | 618.2518 | 1 | b7 -98 |
| Precursor m/z | 737.3039 | 758.3791 | 1 | y8 |
| Precursor Charge | 2 | 815.4006 | 1 | y9 |
| HCD Collision Energy (%) | 35 | 912.4534 | 1 | y10 |
| Spike-in conc. (fmol/ml CSF) | 8 | | | |
| Phosphorylation Site | Ser-202 | 370.6879 | 2 | y8 -98 |
| Peptide Sequence | SGYSSPG[pS]PGTPGSR | 399.1987 | 2 | y9 -98 |
| Amino Acids | 195-209 | 447.725 | 2 | y10 -98 |
| Precursor m/z | 737.3039 | 482.1882 | 1 | b5 |
| Precursor Charge | 2 | 496.7135 | 2 | y10 |
| HCD Collision Energy (%) | 35 | 740.3686 | 1 | y8 -98 |
| Spike-in conc. (fmol/ml CSF) | 8 | 797.39 | 1 | y9 -98 |
| | | 894.4428 | 1 | y10 -98 |
| | | 992.4197 | 1 | y10 |
| Phosphorylation Site | Thr-205 | 376.1604 | 2 | y7 |
| Peptide Sequence | SGYSSPGSPG[pT]PGSR | 416.2252 | 1 | y4 |
| Amino Acids | 195-209 | 482.1882 | 1 | b5 |
| Precursor m/z | 737.3039 | 496.7135 | 2 | y10 |
| Precursor Charge | 2 | 751.3134 | 1 | y7 |
| HCD Collision Energy (%) | 35 | 838.3455 | 1 | y8 |
| Spike-in conc. (fmol/ml CSF) | 8 | 877.3686 | 1 | b10 |
| | | 895.3669 | 1 | y9 |
| | | 992.4197 | 1 | y10 |
| Phosphorylation Site | Thr-217 | 374.6731 | 2 | y6 |
| Peptide Sequence | TPSLP[pT]PPTR | 399.2238 | 1 | b4 |

| | | | | |
|-------------------------------------|--------------------------|-----------|---|---------|
| Amino Acids | 212-221 | 470.2722 | 1 | y4 |
| Precursor m/z | 573.7814 | 523.2575 | 2 | y9 |
| Precursor Charge | 2 | 748.3389 | 1 | y6 |
| HCD Collision Energy (%) | 25 | 948.4550 | 1 | y8 |
| Spike-in conc. (fmol/ml CSF) | 8 | | | |
| Phosphorylation Site | Thr-231 | 368.7450 | 2 | b7 -98 |
| Peptide Sequence | KVAVVR[pT]PPKSPSSAK | 417.7335 | 2 | b7 |
| Amino Acids | 225-240 | 449.7533 | 2 | y9 |
| Precursor m/z | 577.9887 | 489.2667 | 1 | y5 |
| Precursor Charge | 3 | 618.8566 | 2 | y12 -98 |
| HCD Collision Energy (%) | 32 | 668.3908 | 2 | y13 -98 |
| Spike-in conc. (fmol/ml CSF) | 4 | 703.9093 | 2 | y14-98 |
| | | 717.3792 | 2 | y13 |
| | | 736.4828 | 1 | b7 -98 |
| | | 752.8978 | 2 | y14 |
| | | 753.4436 | 2 | y15 -98 |
| | | 801.4465 | 1 | y8 |
| | | 802.4320 | 2 | y15 |
| | | 898.4993 | 1 | y9 |
| Phosphorylation Site | Thr-396 | 460.2514 | 1 | y4 |
| Peptide Sequence | TDHGAEIVYK[pS]PVVSGDTSPR | 482.1994 | 1 | b5 |
| Amino Acids | 386-406 | 507.7644 | 2 | y10 |
| Precursor m/z | 765.6933 | 542.2751 | 2 | y11-98 |
| Precursor Charge | 3 | 611.2420 | 1 | b6 |
| HCD Collision Energy (%) | 30 | 632.2998 | 1 | y6 |
| Spike-in conc. (fmol/ml CSF) | 4 | 693.6684 | 3 | y19 |
| | | 818.4003 | 1 | y8 |
| | | 823.3945 | 1 | b8 |
| | | 1014.5214 | 1 | y10 |
| | | 1083.5429 | 1 | y11-98 |

Supplementary Table S2. Method reproducibility assessment. Aliquots of a CSF pool was prepared separately and analyzed at 8 occasions over two days.

| Analyte | Mean conc. [fmol/ml] | CV [%] |
|----------|-------------------------|--------|
| pTau-181 | 17.75 | 7.35 |
| pTau-199 | 2.68 | 3.80 |
| pTau-202 | 1.04 | 3.10 |
| pTau-205 | 1.32 | 12.78 |
| pTau-217 | 1.39 | 19.01 |
| pTau-231 | 1.79 | 10.50 |
| pTau-396 | 0.41 | 14.89 |

Supplementary Table S3. Performance of the pTau biomarkers in biochemically characterized AD patients and controls. 'C' = Controls; AUC = Area under the ROC curve.

| Analyte | Mean Conc. AD [fmol/ml] | Mean Conc. C [fmol/ml] | mean fold change | AUC | Cohen's d |
|----------------|--------------------------------|-------------------------------|-------------------------|------------|------------------|
| pTau-181 | 26.25 | 13.52 | 1.942 | 1.000 | 2.414 |
| pTau-199 | 04.01 | 2.22 | 1.807 | 0.953 | 2.395 |
| pTau-202 | 01.70 | 0.85 | 2.000 | 0.956 | 2.145 |
| pTau-205 | 2.19 | 0.62 | 3.537 | 0.994 | 2.508 |
| pTau-217 | 4.01 | 0.52 | 7.745 | 1.000 | 3.192 |
| pTau-231 | 4.01 | 0.60 | 6.700 | 1.000 | 2.319 |
| pTau-396 | 0.64 | 0.41 | 1.536 | 0.831 | 1.303 |

Abbreviations: AUC, area under the ROC curve.

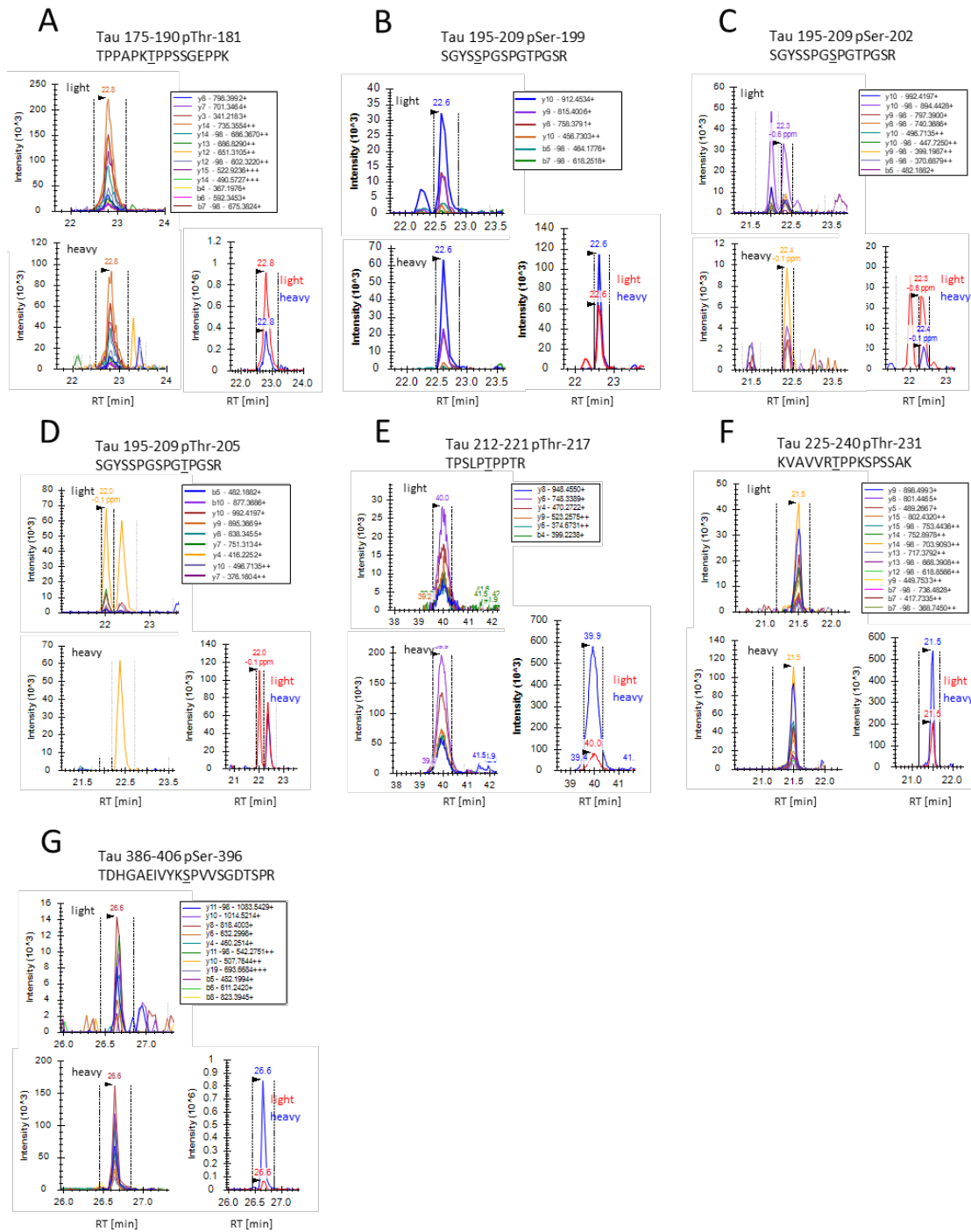
Supplementary Table S4. ROC curve analysis to discriminate groups in the TRIAD cohort.

| | Aβ-PET positive vs CU- | | AD vs CU- | | MCI+ vs CU- | | CU+ vs CU- | |
|----------|--|--------------------|---------------------|--------------------|---------------------|--------------------|---------------------|--------------------|
| | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change |
| pTau-181 | 75.41 (66.68-84.13) | 1.41 | 79.63 (68.28-90.98) | 1.52 | 80.99 (69.57-92.42) | 1.56 | 68.23 (55.58-80.89) | 1.23 |
| pTau-199 | 77.75 (69.4-86.1) | 1.44 | 88.17 (77.31-99.02) | 1.76 | 81.09 (70.11-92.08) | 1.44 | 68.09 (54.87-81.31) | 1.21 |
| pTau-202 | 73.78 (64.7-82.86) | 1.39 | 83.85 (73.51-94.19) | 1.63 | 74.17 (61.29-87.05) | 1.43 | 66.52 (53.41-79.63) | 1.18 |
| pTau-205 | 85.07 (77.79-92.34) | 3.35 | 99.07 (97.21-100) | 5.34 | 90.25 (80.23-100) | 3.96 | 71.58 (58.29-84.87) | 1.51 |
| pTau-217 | 92.8 (87.78-97.81) | 4.26 | 99.71 (99.06-100) | 6.33 | 98.93 (97.36-100) | 4.73 | 83.26 (72.15-94.37) | 2.39 |
| pTau-231 | 93.61 (89.61-97.6) | 5.72 | 98.73 (96.99-100) | 7.64 | 98.54 (96.63-100) | 6.44 | 86.25 (78.15-94.35) | 3.80 |
| pTau-396 | 69.14 (59.58-78.69) | 1.37 | 63.99 (48.72-79.26) | 1.28 | 79.04 (66.62-91.47) | 1.52 | 65.46 (52.09-78.82) | 1.33 |

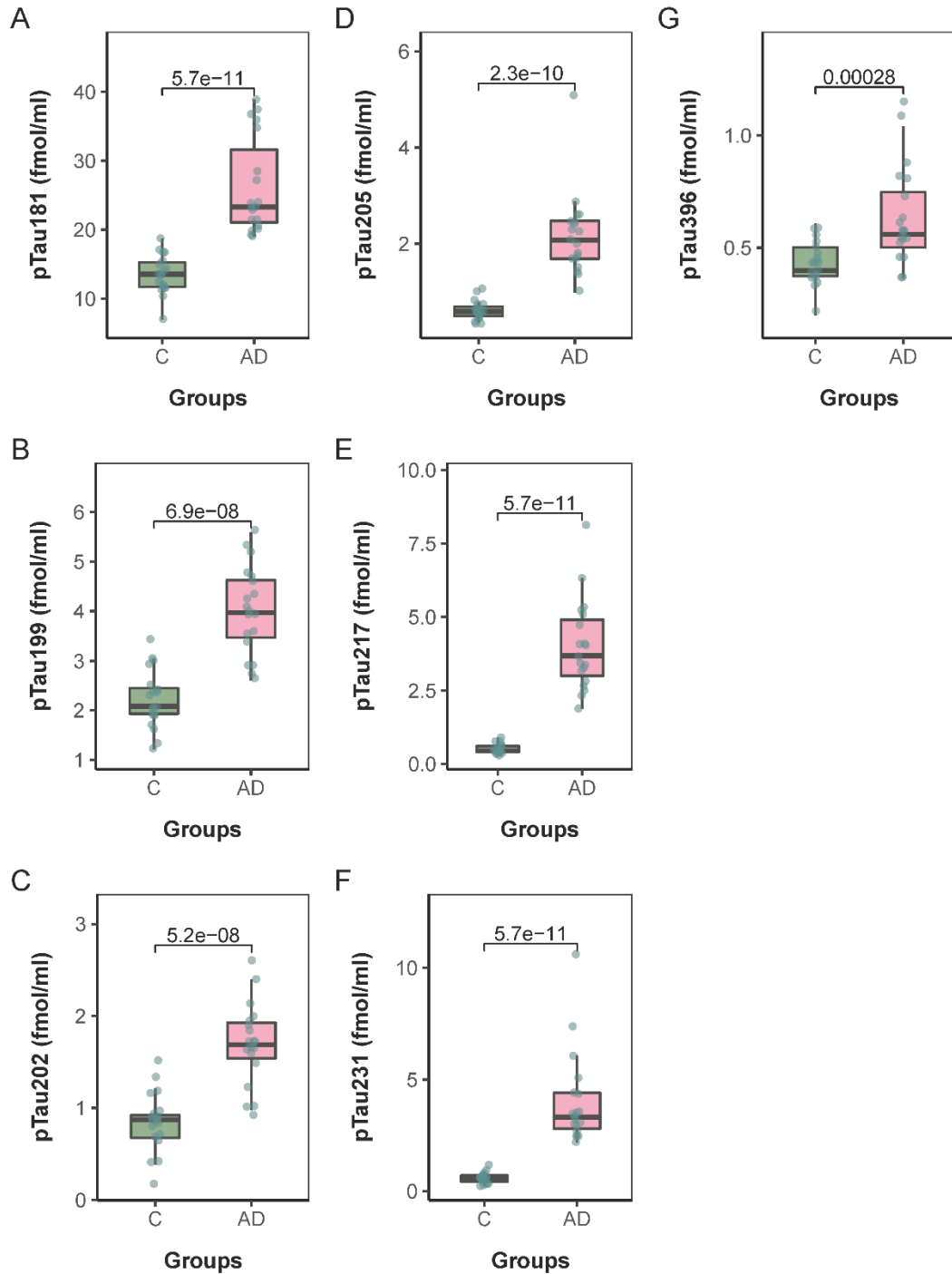
Supplementary Table S5. ROC curve analysis to discriminate groups in the BioFINDER-2 cohort.

| | Aβ+* vs CU- | | AD vs CU- | | MCI+ vs CU- | | CU+ vs CU- | |
|----------|-------------------------------------|--------------------|----------------------|--------------------|---------------------|--------------------|---------------------|--------------------|
| | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change | AUC (95% CI) | Fold-change |
| pTau-181 | 77.87 (73.29-82.45) | 1.58 | 85.37 (79.68-91.05) | 1.87 | 76.31 (68.7-83.90) | 1.54 | 76.43 (69-75-83.12) | 1.41 |
| pTau-199 | 76.18 (71.50-80.87) | 1.58 | 87.12 (82.27-91.96) | 1.99 | 76.03 (68.79-83.27) | 1.51 | 66.54 (59.04-74.04) | 1.31 |
| pTau-202 | 70.63 (65.52-75.74) | 1.52 | 81.38 (75.38-87.38) | 1.86 | 69.05 (61.21-76.90) | 1.41 | 62.86 (54.92-70.80) | 1.35 |
| pTau-205 | 81.99 (77.77-86.20) | 2.50 | 93.51 (90.16-96.85) | 3.92 | 82.55 (76.45-88.66) | 2.11 | 71.11 (63.86-78.36) | 1.70 |
| pTau-217 | 94.50 (92.40-96.61) | 5.45 | 98.12 (95.70-100.00) | 8.83 | 94.79 (91.18-98.41) | 4.86 | 91.05 (86.82-95.27) | 3.29 |
| pTau-231 | 84.44 (80.57-88.31) | 6.40 | 91.89 (88.37-95.41) | 10.65 | 85.45 (80.36-90.54) | 5.62 | 78.69 (72.27-85.12) | 3.65 |
| pTau-396 | 69.97 (64.78-75.16) | 1.30 | 72.02 (64.63-79.42) | 1.33 | 74.31 (66.52-82.10) | 1.59 | 69.30 (61.95-76.65) | 1.21 |

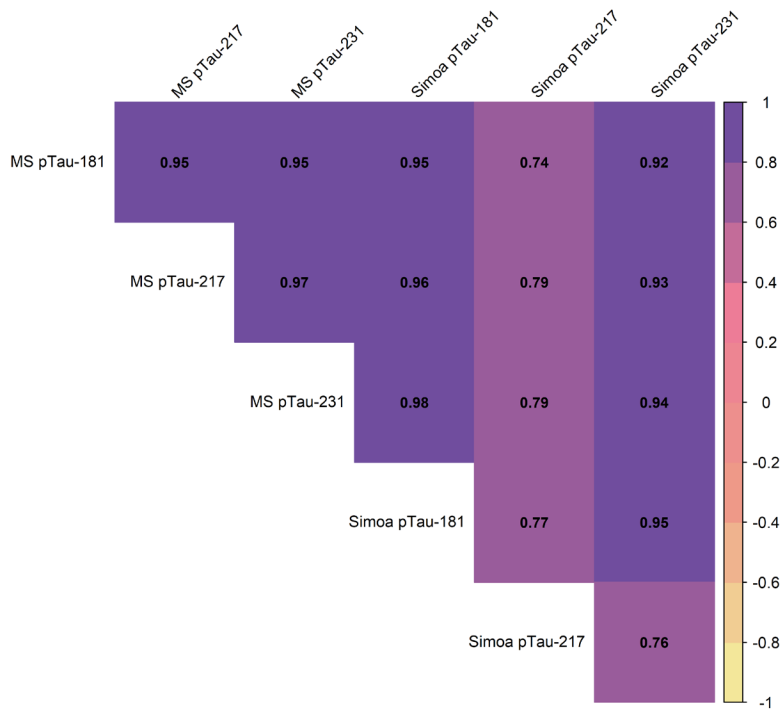
* CSF Ab42/40 ratio



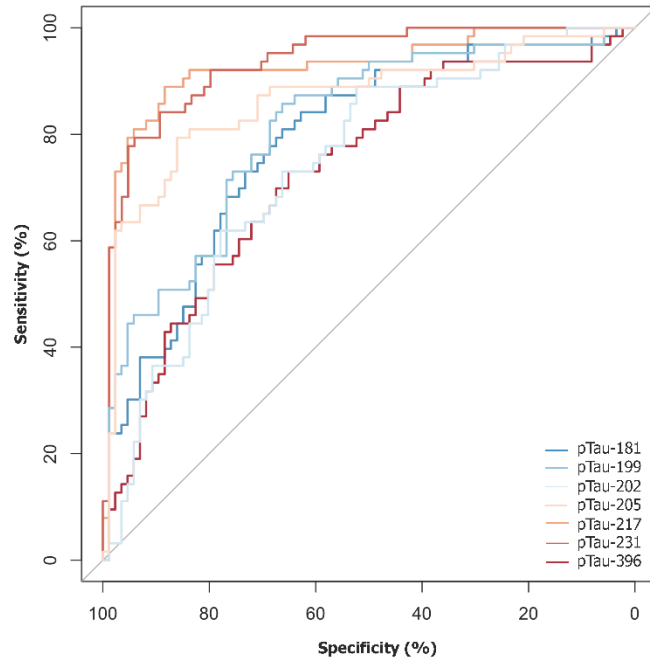
Supplementary Figure S1. Representative PRM chromatograms of the assayed tau phosphopeptides, Tau 175-190 p181 (A), Tau 195-209 p199 (B), Tau 195-209 p202 (C), Tau 195-209 p205 (D), Tau 212-224 p217 (E), Tau 225-240 p231 (F), Tau 386-406 p396 (G). For each phosphopeptide, the chromatographic traces of the included b- and y- fragment ions used for quantification are indicated in the two left panels, for the light (top) and heavy (bottom) form. The summed up peak areas of these traces for the light and heavy forms, used to calculate the light-to-heavy ratio, is shown in the bottom right panel.



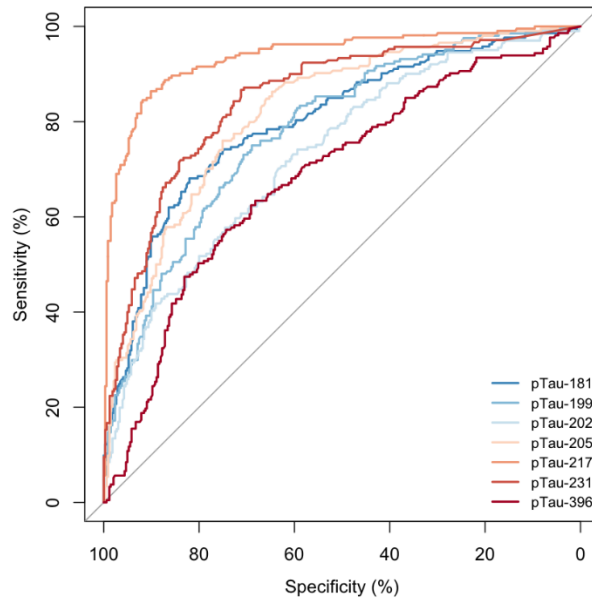
Supplementary Figure S2. Scatter plots of phosphorylated peptide abundances in biochemically characterized AD patients and controls. The phosphorylated epitopes measured were Thr-181 (A), Ser-199 (B), Ser-202 (C), Thr-205 (D), Thr-217 (E), Thr-231 (F) and Thr-396 (G). Concentrations are given as femtomoles of the respective measured tryptic phosphopeptide per mL CSF. The boxplots depict the median (horizontal bar), interquartile range (IQR, hinges) and 1.5 x IQR (whiskers). Abbreviations: AD, Alzheimer's disease; C, control.



Supplementary Figure S3. Correlations (Spearman) between the pTau PRM assay and Simoa immunoassay for pTau-181, pTau-217 and pTau-231.



Supplementary Figure S4. Discriminative accuracy of pTau biomarkers in the TRIAD cohort. ROC curve analysis of the performance of pTau-181, pTau-119, pTau-199, pTau-202, pTau-205, pTau-217, pTau-231, and pTau-396 for distinguishing between A β positive and negative individuals (as indicated by A β PET).



Supplementary Figure S5. Discriminative accuracy of pTau biomarkers in the BioFINDER cohort. ROC curve analysis of the performance of pTau-181, pTau-119, pTau-199, pTau-202, pTau-205, pTau-217, pTau-231, and pTau-396 for distinguishing between A β positive and negative individuals (as indicated by A β PET).

Supplementary Figure S6. Fold changes in AD versus CU- cases for the phospho-epitopes pS199, pS202, pT205 and pT217, and for the phosphorylation ratios pS199/S199, pS202/S202, pT205/T205 and pT217/T217, measured relative to the corresponding non-phosphorylated peptides.

