

Cerebrospinal fluid peptides as potential Parkinson disease biomarkers: a staged pipeline for discovery and validation – Supplemental Data

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Supplemental Methods

1. Participants

The inclusion and exclusion criteria were previously described (1, 2). Briefly, all Parkinson disease (PD) patients met UK PD Society Brain Bank clinical diagnostic criteria for PD (3) except that having "more than one affected relative" was not considered an exclusion criterion. Alzheimer disease (AD) patients were diagnosed with probable AD according to NINDS-ADRDA criteria (4). Control subjects were the patients' spouses or community volunteers in good health. They had no signs or symptoms suggesting cognitive impairment or neurological disease; all subjects had a Mini Mental Status Examination (MMSE) score between 28 and 30, a Clinical Dementia Rating (CDR) score of 0, and New York University paragraph recall scores (immediate and delayed) of >6.

2. Collection of CSF and quality control

Following written informed consent, individuals were placed in the lateral decubitus position and the L4-5 interspace was infiltrated with 1% lidocaine. Lumbar puncture (LP) was performed with a 24G spinal needle. Individuals remained at bed rest for one hour following LP. The first 2 mL of CSF were sent to a local laboratory for determination of protein, glucose and cell count. Up to 25 ml CSF was then taken from each subject, with every 5 mL pooled in one fraction. These were aliquoted into polypropylene cryotubes in 0.5 mL aliquots (labeled 1st-50th fraction, corresponding to 1st-25th mL), flash frozen, and stored at -80°C. All LPs were performed in the morning to limit potential circadian fluctuation of CSF proteins and metabolites. Before analysis, all CSF samples were only thawed once when 10% protease inhibitor cocktail (Sigma, St Louis, MO, USA) was added and samples were further aliquoted.

Reference CSF refers to the CSF samples obtained from the clinical laboratory at Harborview Medical Center (Seattle, WA), and only the samples from subjects who had been determined neurologically as well as biochemically normal were pooled and used.

Blood contamination in CSF samples was controlled with careful and correct practice during sample collection. Additionally, the hemoglobin levels in CSF samples were chosen as an index of the degree of red blood cell contamination of CSF and were measured as described (1, 2). Samples with high blood contamination (hemoglobin \geq 200 ng/mL) were excluded from analyses when necessary.

3. SCX fractionation for pooled reference CSF samples

For assay development, reference CSF samples were pooled from >5 healthy control samples collected at a UW clinic (see above). For CSF observable peptide selection, tryptic digests of 990 μL reference CSF were desalted using 1cc Sep-Pak[®] Vac C18 cartridges (Waters, Milford, MA, USA). Briefly, the columns were rinsed with 2 mL of methanol and equilibrated with 2 mL of 0.1% trifluoroacetic acid (TFA). The sample was slowly applied to the column, washed with 3 mL of 0.1% TFA, and eluted with 0.8 mL of 80% acetonitrile / 0.1% TFA. Finally, the sample was concentrated by vacuum centrifugation and rehydrated in \sim 1 mL of strong cation-exchange (SCX) equilibration buffer (buffer A: 5 mM KH_2PO_4 , 20% ACN, pH 2.8). SCX fractionation was then carried out using a PolySulfo-ethyl A ($200 \times 2.1 \text{ mm} \times 5\text{-}\mu\text{m}$, 300 \AA) column (PolyLC, Columbia, MD, USA) on a Biologic Duo-Flow LC system (Bio-Rad, Hercules, CA, USA). One (1) mL of sample was loaded onto the column in 100% buffer A and separated with a linear gradient of 0% buffer B (5 mM KH_2PO_4 , 25% ACN, 600 mM KCl, pH 2.8) to 100% B for 125 min at a flow rate of 0.2 mL/min. Fractions were collected and pooled to give approximately equal mass of total peptide per fraction based on a UV trace. Ten SCX pools were made for each CSF sample and desalted using C18 MicroSpin columns (The Nest Group, Southborough, MA, USA) according to the manufacturer's protocol. Samples were dried down and then resuspended in 25-90 μL of 2% acetonitrile/0.1 % formic acid prior to mass spectrometry (MS) analysis.

4. Q-Exactive analysis for CSF observable peptide selection

The +2 charge state for each of the 13,879 candidate peptides was used to generate two inclusion lists for targeted analysis on a Q-Exactive mass spectrometer (Thermo Scientific, Rockford, IL, USA) based on the number of precursors that could be monitored in one run. The splitting was done according to the predicted retention time (SSRCalc (5)) of the desired peptides (peptides ranked by their hydrophobicity were alternatively assigned into two lists to best avoid co-eluting). Liquid chromatography was performed using a Waters NanoAcquity UPLC; peptides were separated online with 75 μm i.d. \times 20 cm home-packed fused silica columns (ReproSil-Pur C18-AQ, 3 μm , Dr Maisch GmbH, Ammerbuch-Entringen, Germany) with a 120 min 2-80% acetonitrile/water gradient containing 0.1 % formic acid. Three to Five μL of tryptic peptides were injected onto the column and separated using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. Binary gradient elution was carried out using 2% to 10% B for 1 min, 10% to 35% B for 89 min, 35% to 80% B for 1 min and held constant for another 9 min. Finally, an 80% to 2% B for 1 min was performed and column was re-equilibrated with 2% B for 19 min. Each SCX pool was run 4 times: 2 data-dependent acquisition (DDA) runs, and 2 coupled with the inclusion lists. Typical instrument settings include capillary temperature of 325 $^\circ\text{C}$, spray voltage of 2000 V, and the top 20 most abundant ions were subjected to high-energy collision-induced dissociation (HCD) with normalized collision energy of 25%. Q-Exactive analysis was performed in positive ionization mode with at least 70,000 mass resolving power.

5. SRM analysis

All SRM analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific) coupled to a nanoAcquity UPLC (Waters). Reversed-phase chromatography was performed on capillary columns (75 $\mu\text{m} \times 20 \text{ cm}$; Polymicro Technologies, Phoenix, AZ, USA) packed with 100 \AA Magic C18 (Bruker) and a flow rate of 0.3 $\mu\text{L}/\text{min}$ using 0.1% formic acid in water as mobile phase A

and 0.1% formic acid in acetonitrile as mobile phase B. Four (4) μL of tryptic digest were injected onto the column and separated using a binary gradient of 2-35% B for 30 min, 35-80% B for 1 min and held constant for 9 min. Finally, an 80% to 2% B for 1 min was carried out and the column was re-equilibrated at 2% B for another 19 min. Mass spectra were recorded in positive ion mode with a capillary temperature of 325 °C and vaporizer temperature of 400 °C. TSQ MS method settings include Q2 collision gas pressure at 1.5 mTorr and Q1 and Q3 were set at unit resolution of 0.70 FWHM. Scheduled SRM were performed with dwell times of 20 ms and retention time windows of 4 min and 5 min for the training set and the validation set, respectively.

Collision energy (CE) optimization was performed by loading 100 fmol each of pooled peptide standards on column. A step-wise variation with 1V increments (-5 to +5) from the default CE was used. A total of 11 CE voltage values for each fragment ion were evaluated. Optimized CE was chosen where the combined signal intensities of the individual transitions yielded the highest peak area among the CE voltage values tested. These optimized CE values were then used for experiments with the samples in the validation set.

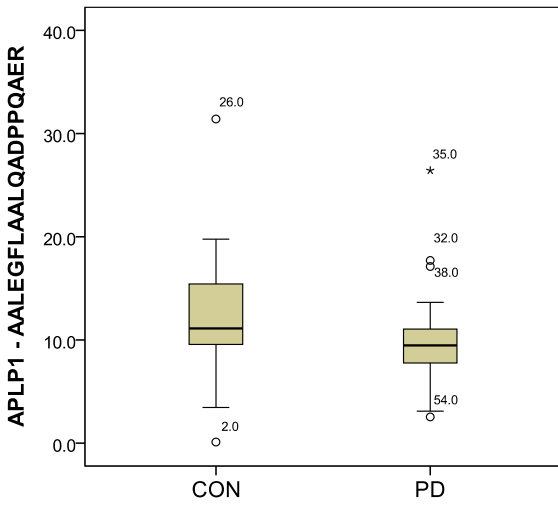
To control for the stability of the measurement, 1-2 reference CSF samples were included during the assay development and the clinical sample measurement. These samples showed an average of $\leq 15\%$ for variation (CV%). The clinical samples were prepared randomly and analyzed alternatively in batches (e.g., control1, PD1, control2, PD2, and so on). After every 8 injections, a peptide standard consisting of angiotensin and neurotensin was run to evaluate the reproducibility of the chromatography and fluctuations on the instrument sensitivity over time during the analysis.

References

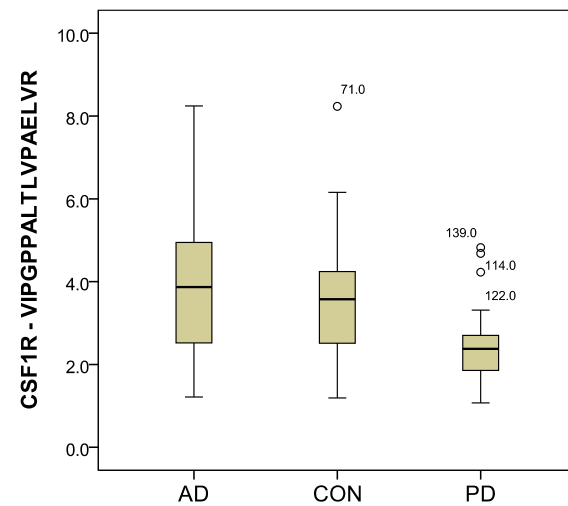
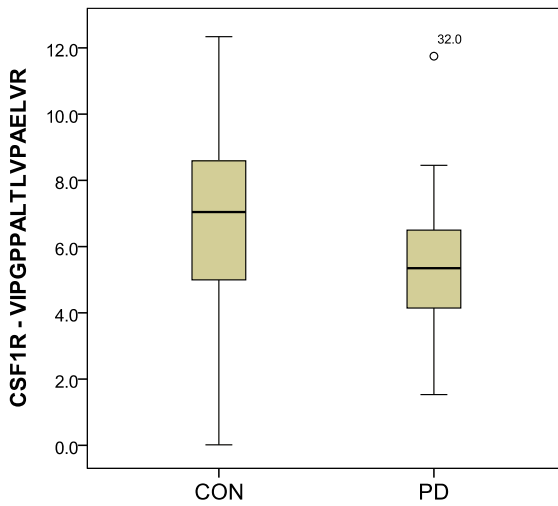
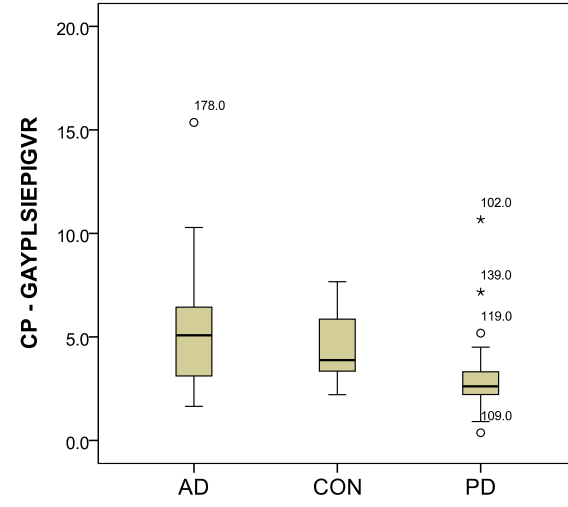
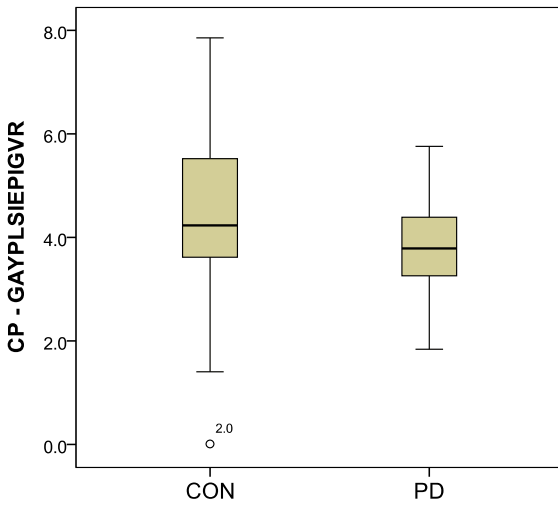
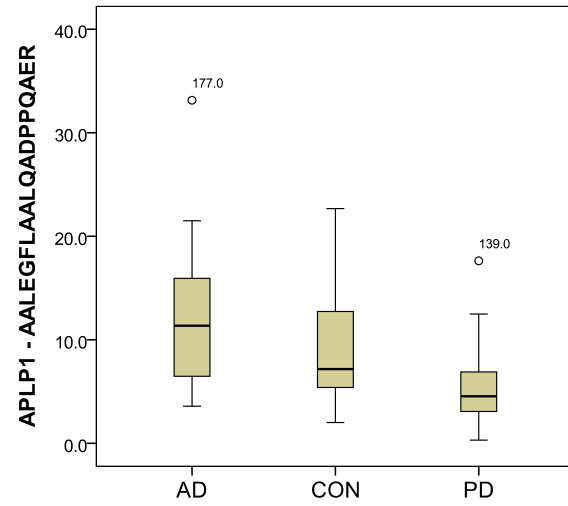
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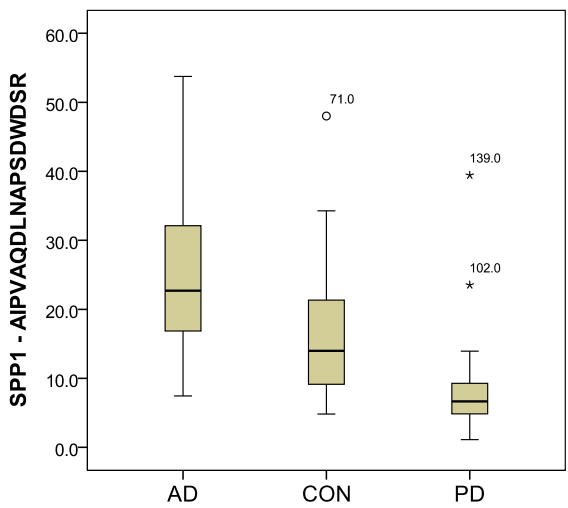
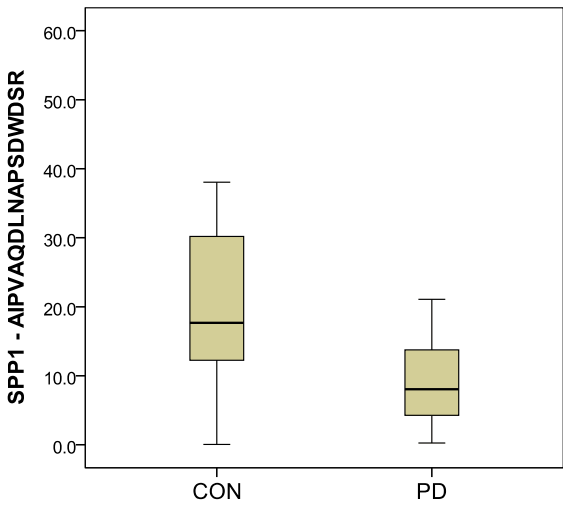
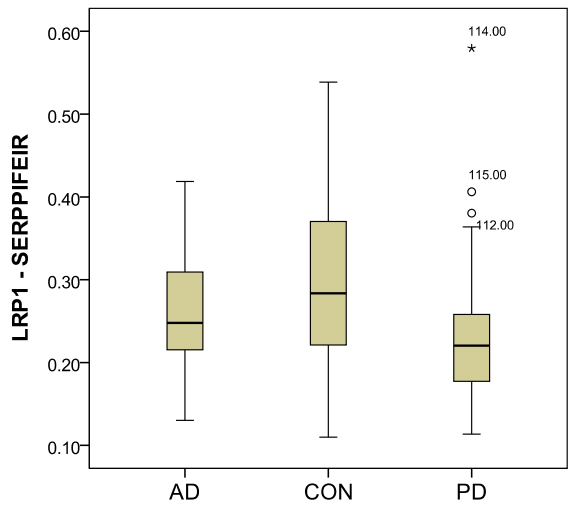
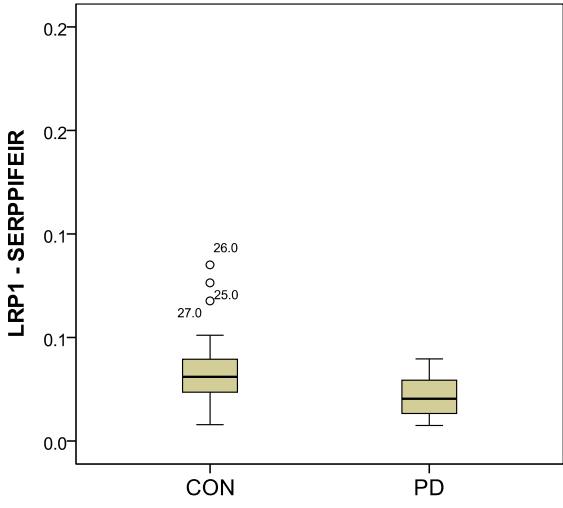
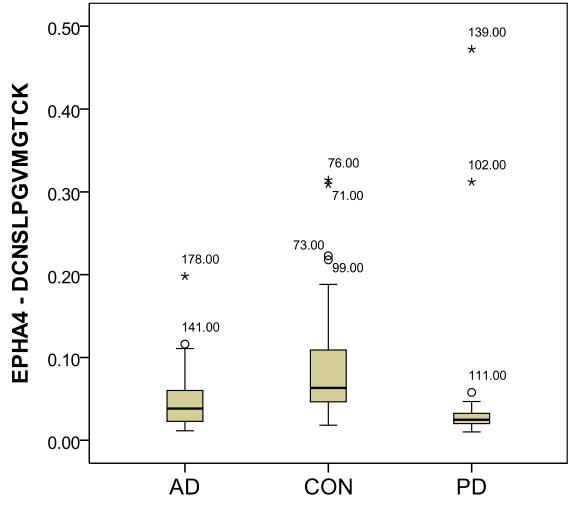
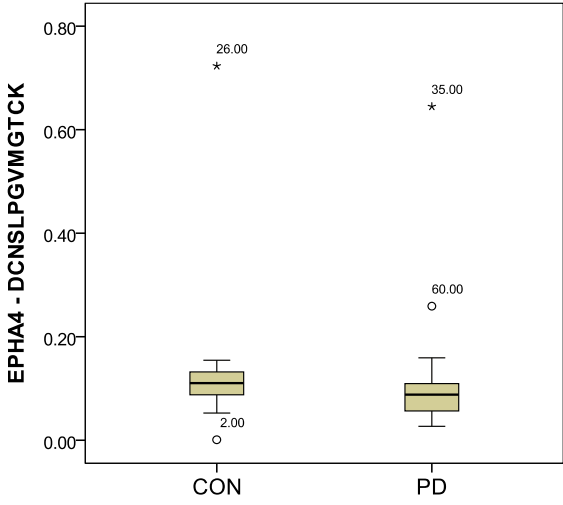
Supplemental Figures

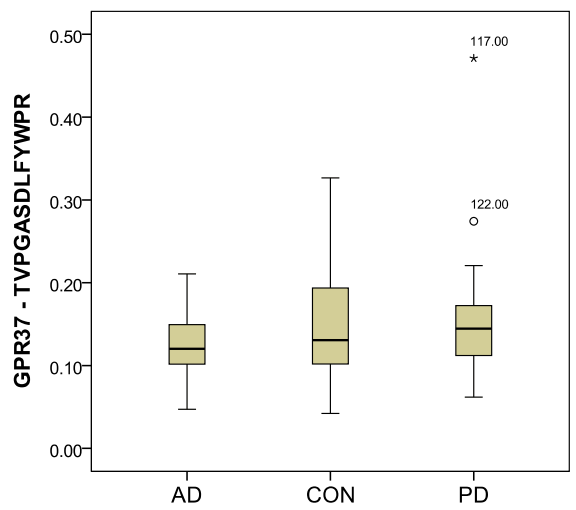
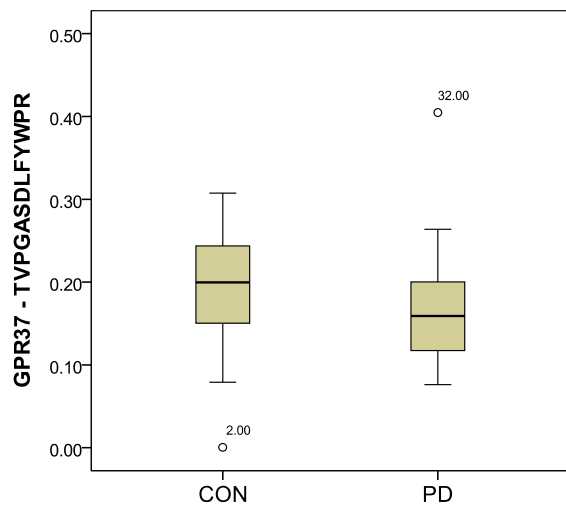
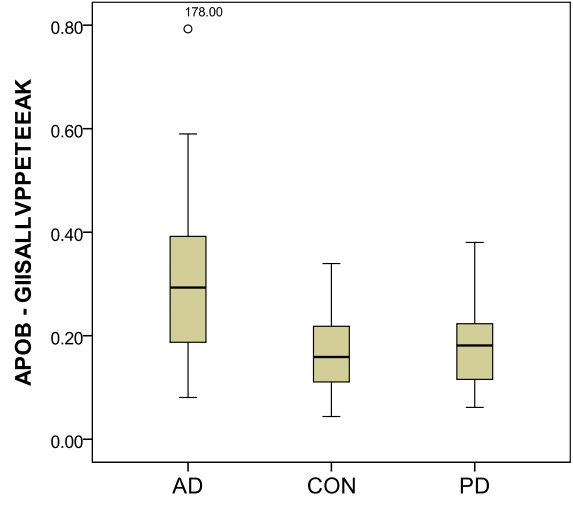
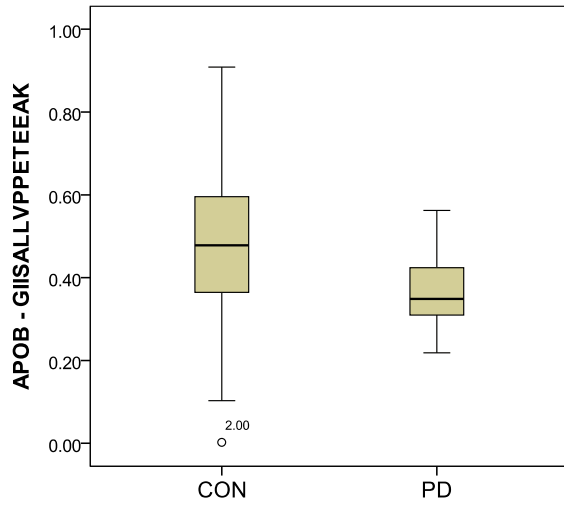
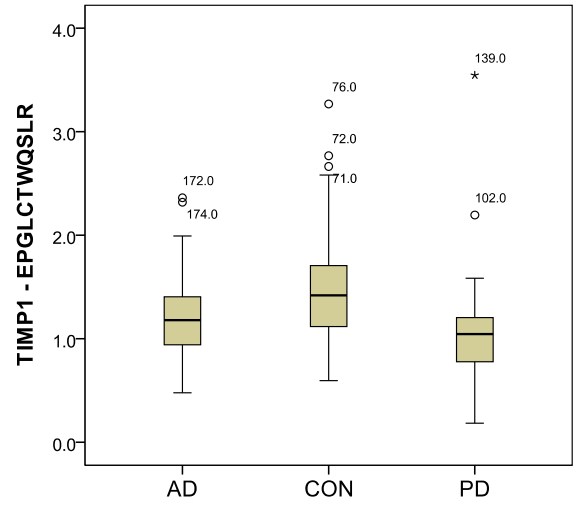
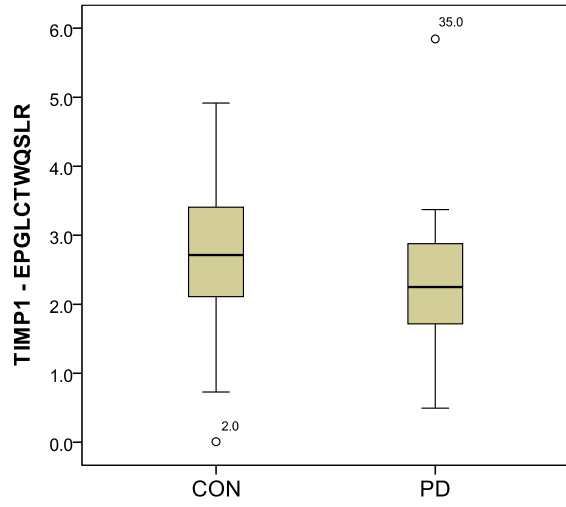
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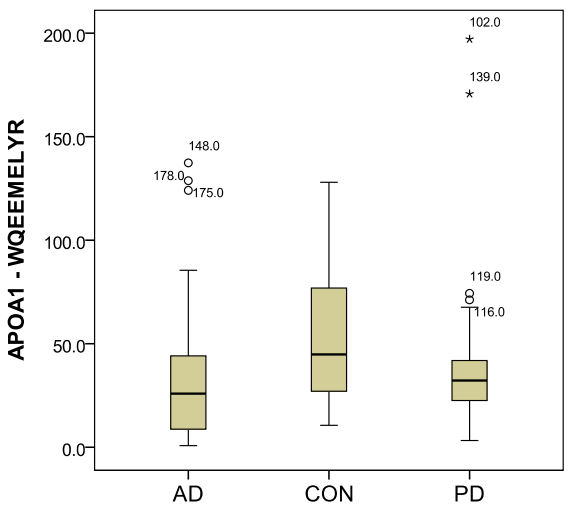
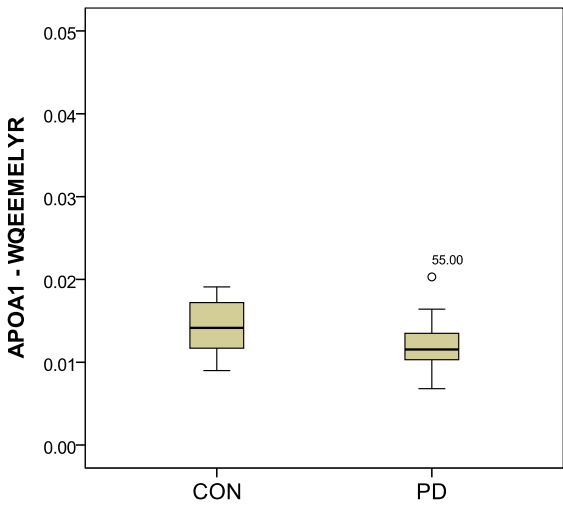
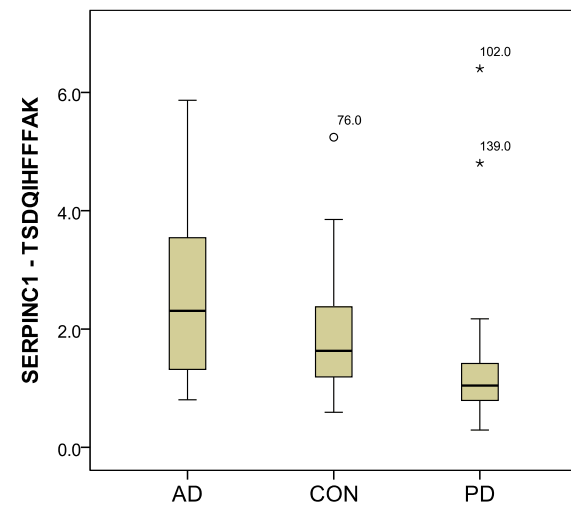
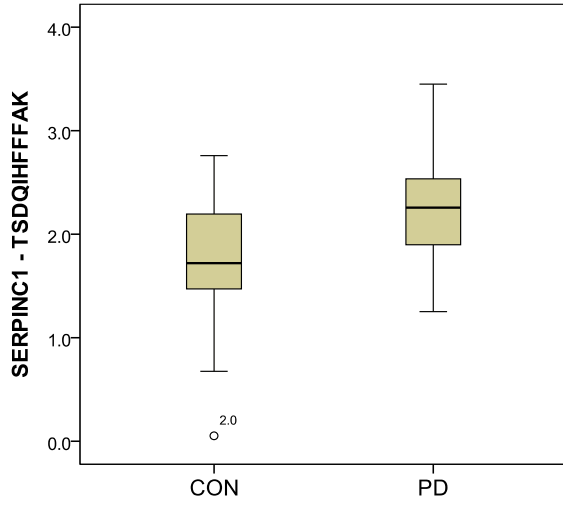
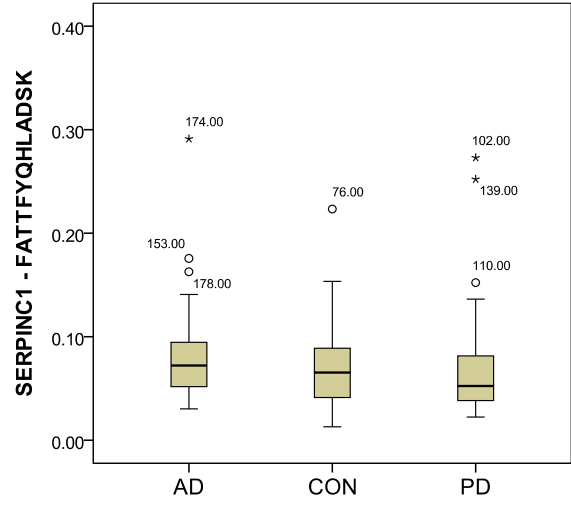
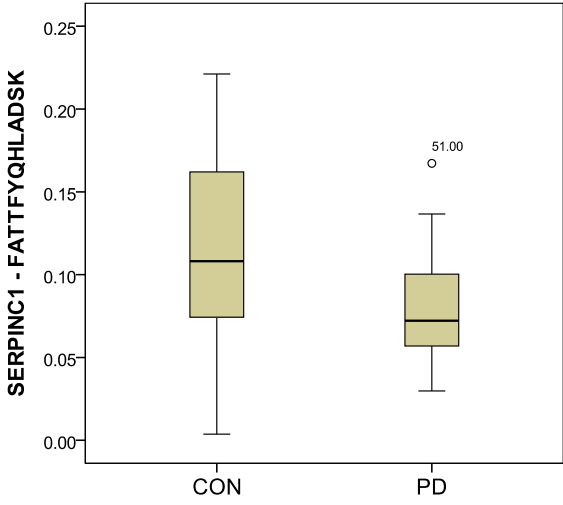


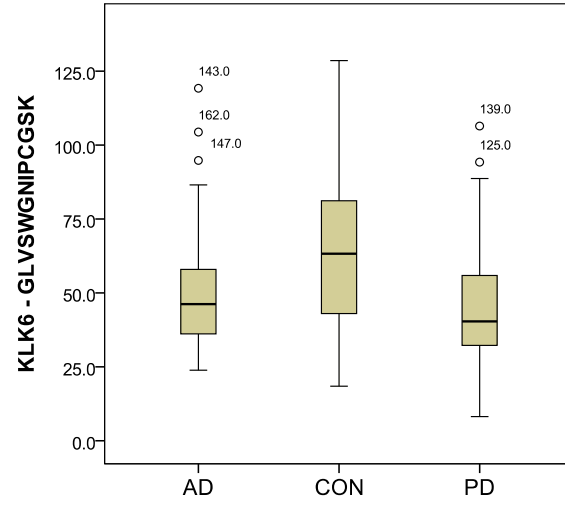
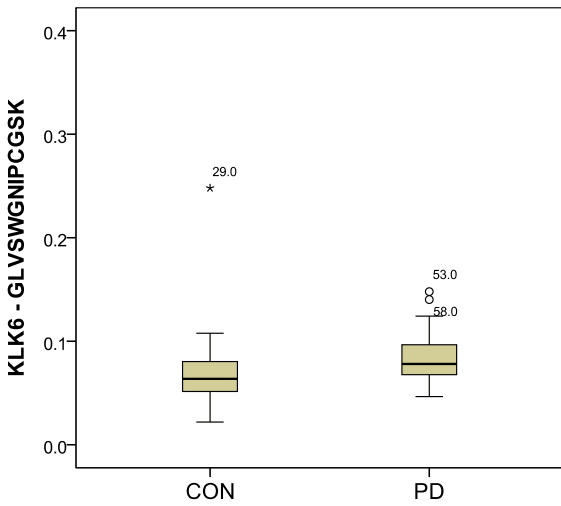
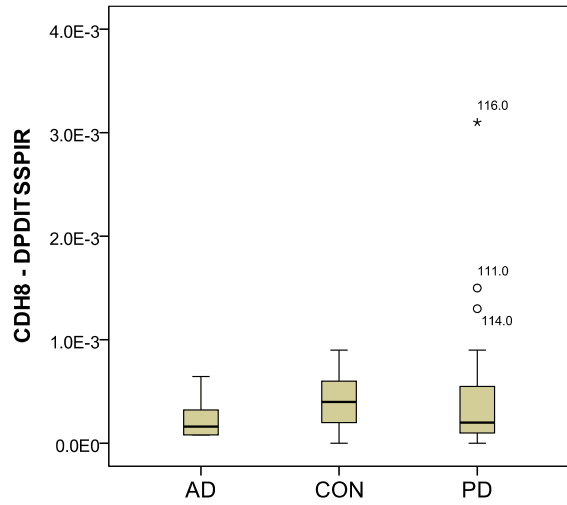
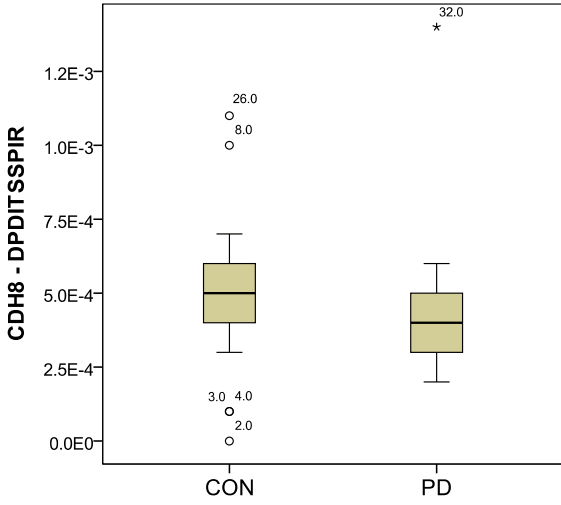
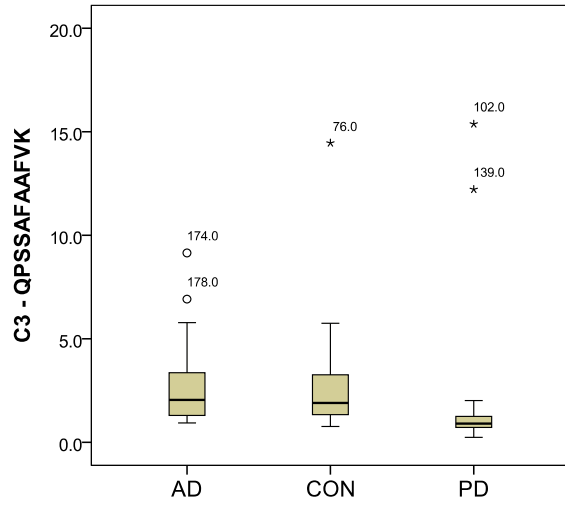
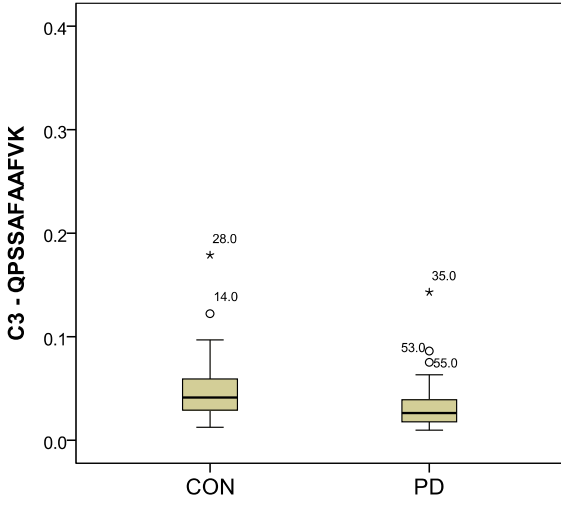
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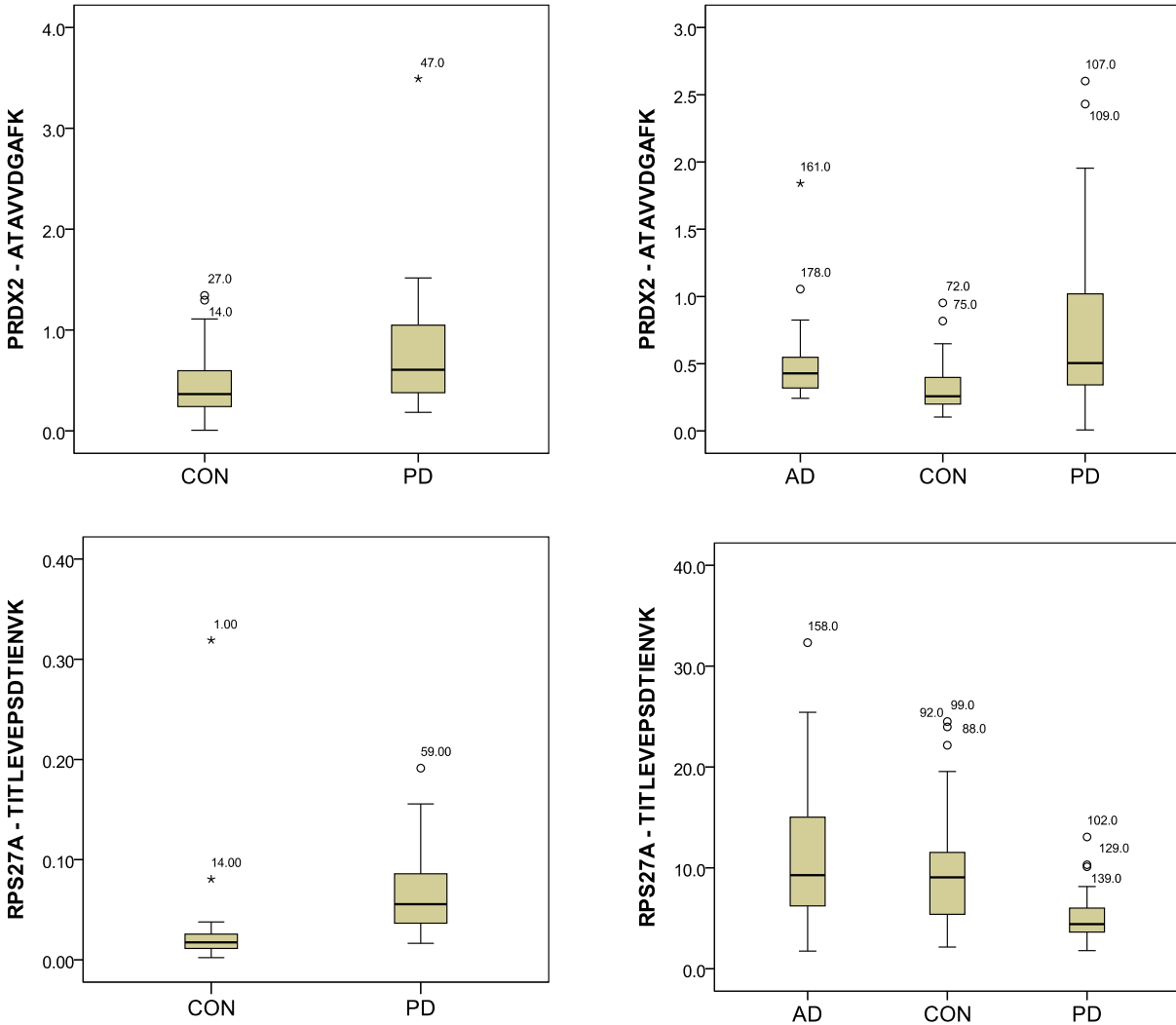




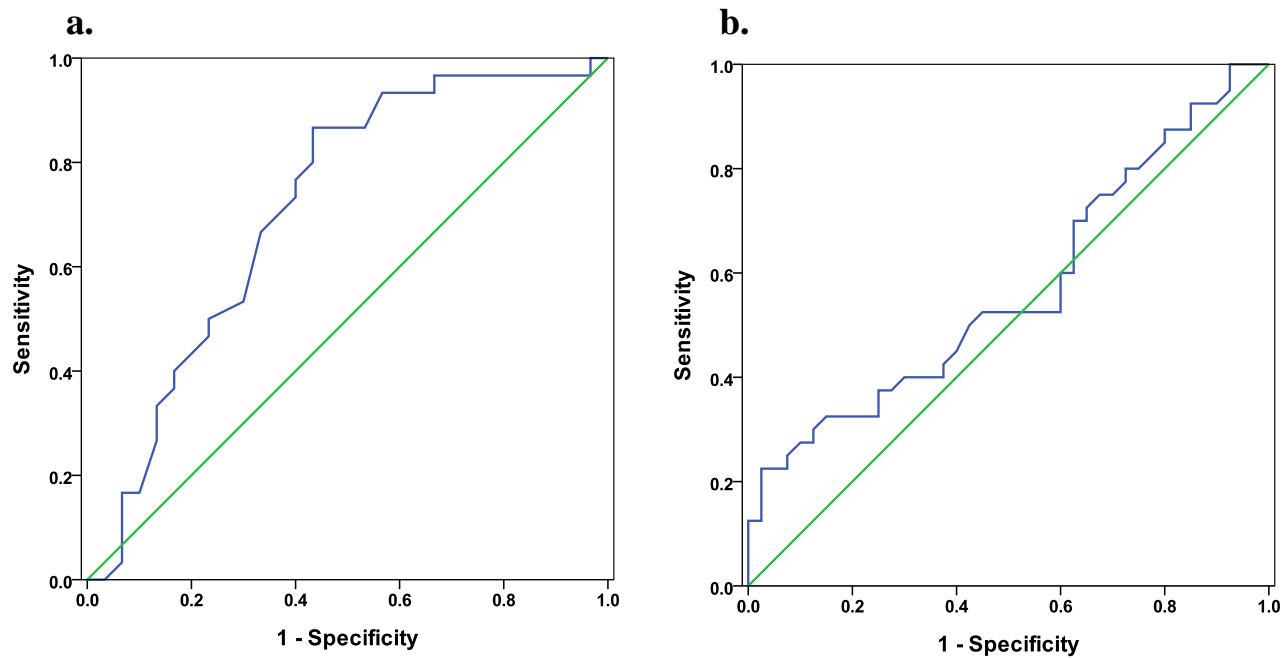




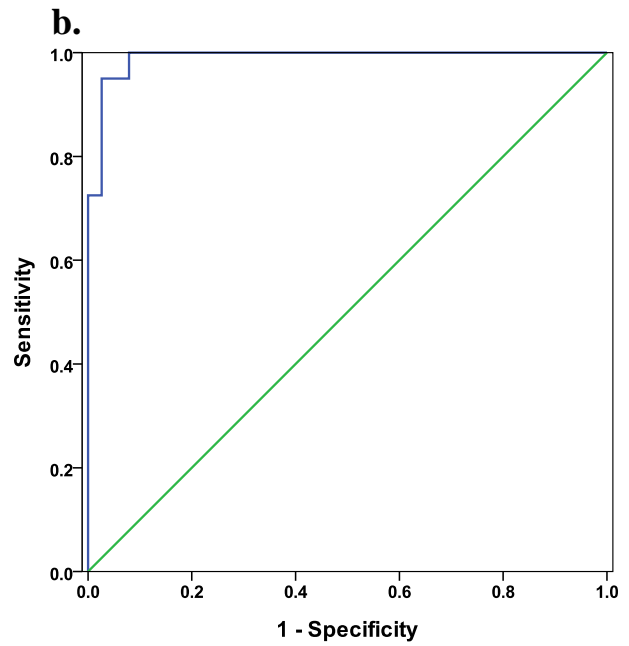
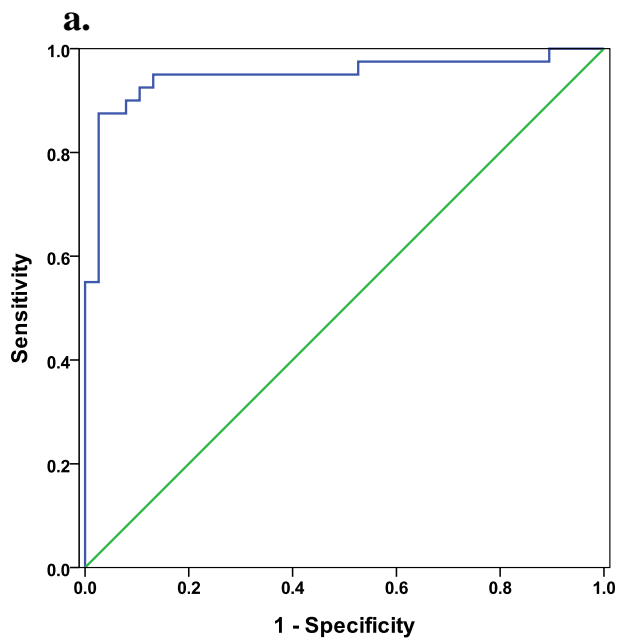




Suppl. Fig. 1 Box plots of levels of 17 CSF peptides in the training and validation sets. Peptide data shown are the raw light/heavy ratios. The dark line in the middle of the boxes represents the median, the bottom and top of the box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), and the ends of the whiskers represent the lowest datum still within 1.5 interquartile range (IQR) of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile, respectively. Note that the data were normalized (Box-Cox transformed) for all other analyses, and there were 6 peptides (APOA1, C3, CDH8, KLK6, PRDX2, and RPS27A) that failed to meet our criteria (see **Experimental Procedures**) for robust and reliable detection and quantification in the training or validation set.



Suppl. Fig. 2 Receiver operating characteristic (ROC) analysis of CSF α -synuclein differentiating patients with PD and healthy controls. CSF α -synuclein protein concentrations were measured using a Luminex immunoassay and evaluated in **a)** the training set and **b)** the validation set.



Suppl. Fig. 3 Receiver operating characteristic (ROC) analysis of CSF peptides differentiating patients with PD and patients with AD in the validation set. a) CSF peptide AIPVAQDLNAPSDWDSR (derived from the protein SPP1), and b) a combination of 5 CSF peptides derived from SPP1, LRP1, CSF1R, EPHA4, and TIMP1.