Multiplexed Mass Spectrometry Assay Identifies Neurodegeneration Biomarkers in CSF

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Abstract

Early detection markers are critical for neurodegenerative diseases since substantial, irreversible damage to the brain occurs by the time of clinical diagnosis. Markers of disease progression are also important, as established clinical assessment tools can require up to a year or more to detect changes. Current efforts in our laboratory include developing such markers in Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases. Candidate biomarkers were identified in the literature for each disease and targeted multiple reaction monitoring mass spectrometry assays (LC-MRM/MS) were assembled. In the case of HD, very few candidate markers were known in the literature, so an unbiased mass spectrometry discovery study (LC-MS/MS) was performed in CSF to identify novel markers for inclusion in the targeted assay. The targeted LC-MRM/MS assays were used to compare disease versus healthy control (HC) CSF samples for each disease, thus enabling subsets of candidate biomarkers to be verified. The results from each study are described, including the affected proteins and pathways, and the similarities and differences in CSF proteins across the different diseases.

A single, unified assay is now established which enables the multiplexed measurement of 205 neurodegenerative disease-related proteins in 50µL CSF which can be deployed in support of clinical biomarker and drug development efforts.

Targeted MRM to Identify CSF Biomarkers in Alzheimer's Disease

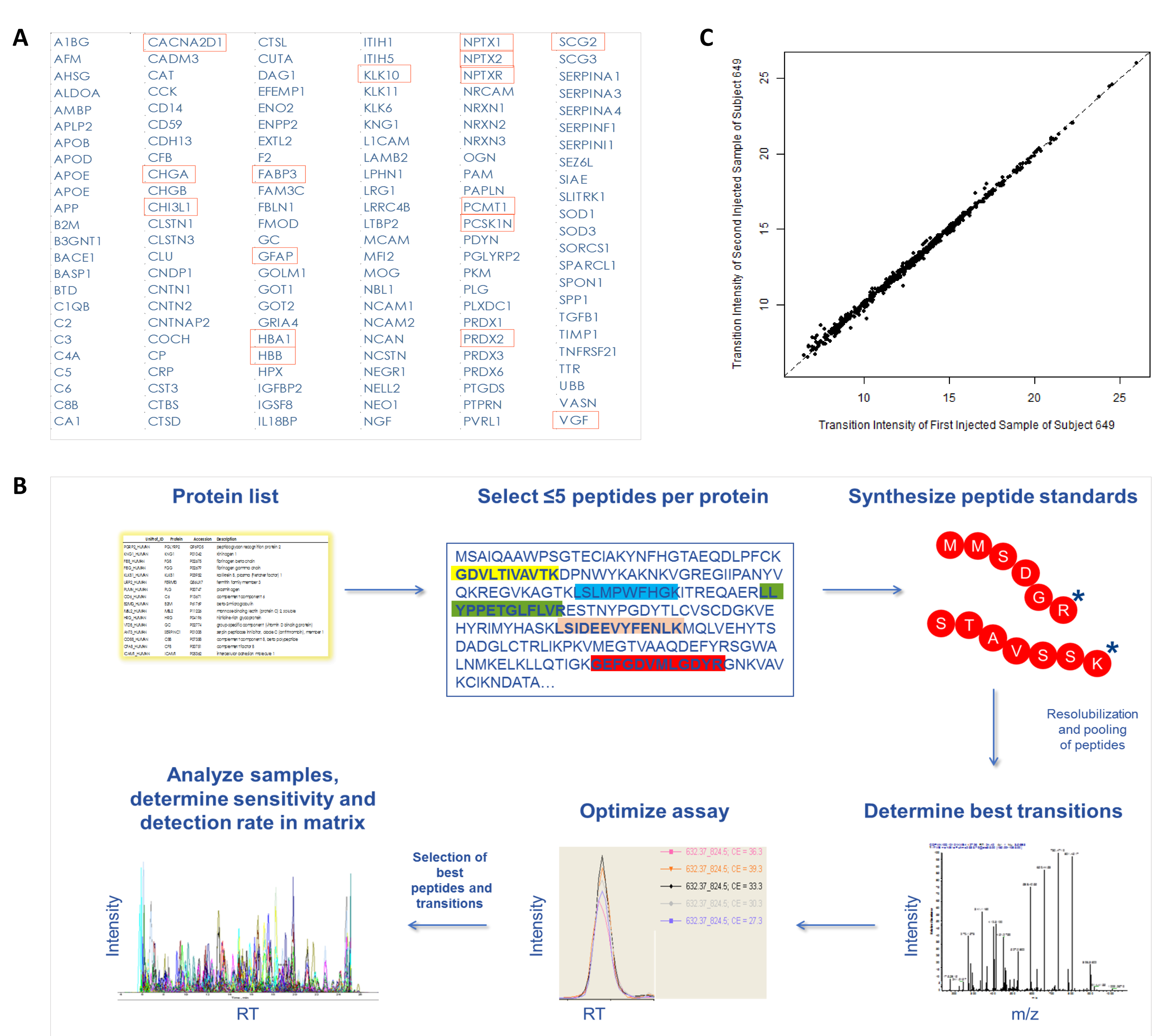
Background and Goals

- Collaborative public-private partnership with the FNIH/Biomarkers Consortium.
- Overall study goal to use a proteomics-based approach for the identification of novel CSF AD markers: Results of this study have been published in Spellman et al., 2015 (1).

Study Design and Overview

A large list of candidate biomarkers (**Figure 1A**) related to AD were assembled into a single multiplexed LC-MRM/MS assay (**Figure 1B**). The proteins were then quantified in a cross-sectional study of AD (n=66) and mild cognitive impairment (MCI) (n=134) patients as well as HC (n=85), with samples provided by the Alzheimer's Disease Neuroimaging Initiative (ADNI). Another 45 identical replicates of a pooled CSF sample were interleaved with the study samples as quality controls. Duplicates of 16 study samples were also distributed across the study as another way to monitor reproducibility (**Figure 1C**).

Figure 1 (counter-clockwise from top left). **(A) List of 142 proteins in the initial MRM assay:** Proteins in red boxes achieved significance in comparison of AD/MCI/HC. **(B) LC-MRM/MS assay development:** Five tryptic peptides selected to represent each protein in the MRM assay were synthesized as stable isotope-labeled (SIL) standards. The AD study used unlabeled peptides, but for improved quantification and confident peptide ID, spiked SIL peptides were used for all subsequent studies. **(C) Very high analytical reproducibility of the LC-MRM/MS platform:** Relative quantitation of all transitions in 2 replicates of the same study sample, thawed, processed and analyzed in different batches.



Sixteen (16) proteins were differentially expressed in disease (see red boxes in **Figure 1A**), including proteins previously shown to correlate with disease (FABPH, **Figure 2**). Although the ApoE e4 allele is associated with a high risk of AD, protein concentration levels did not change in MCI or AD (**Figure 3**). These data provide insights into the biology of CSF in AD and MCI progression and a novel tool for AD researchers and clinicians working to improve diagnostic accuracy, evaluation of treatment efficacy, and early diagnosis. Study results are currently being verified in another set of ADNI subjects, with a focus on longitudinal performance of the markers.

Figure 2. FABPH was verified as differentially expressed in MCI and AD in this study. Consistent with previous report by Chiasserini et al., 2010 (2). For other differentially expressed proteins verified in this study, see red boxes in **Figure 1A**.

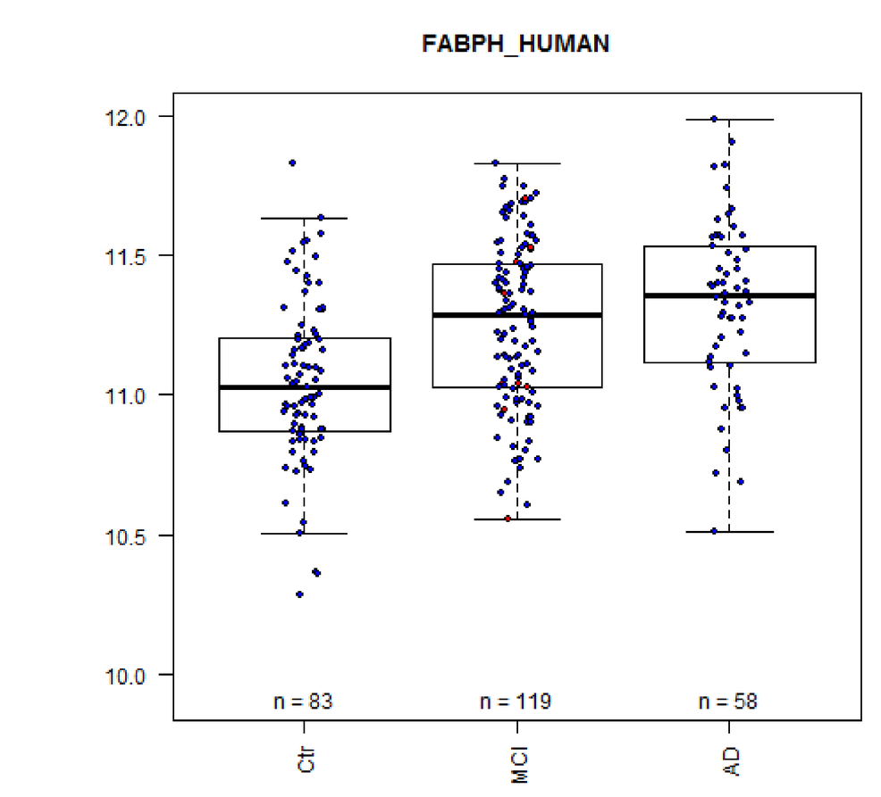
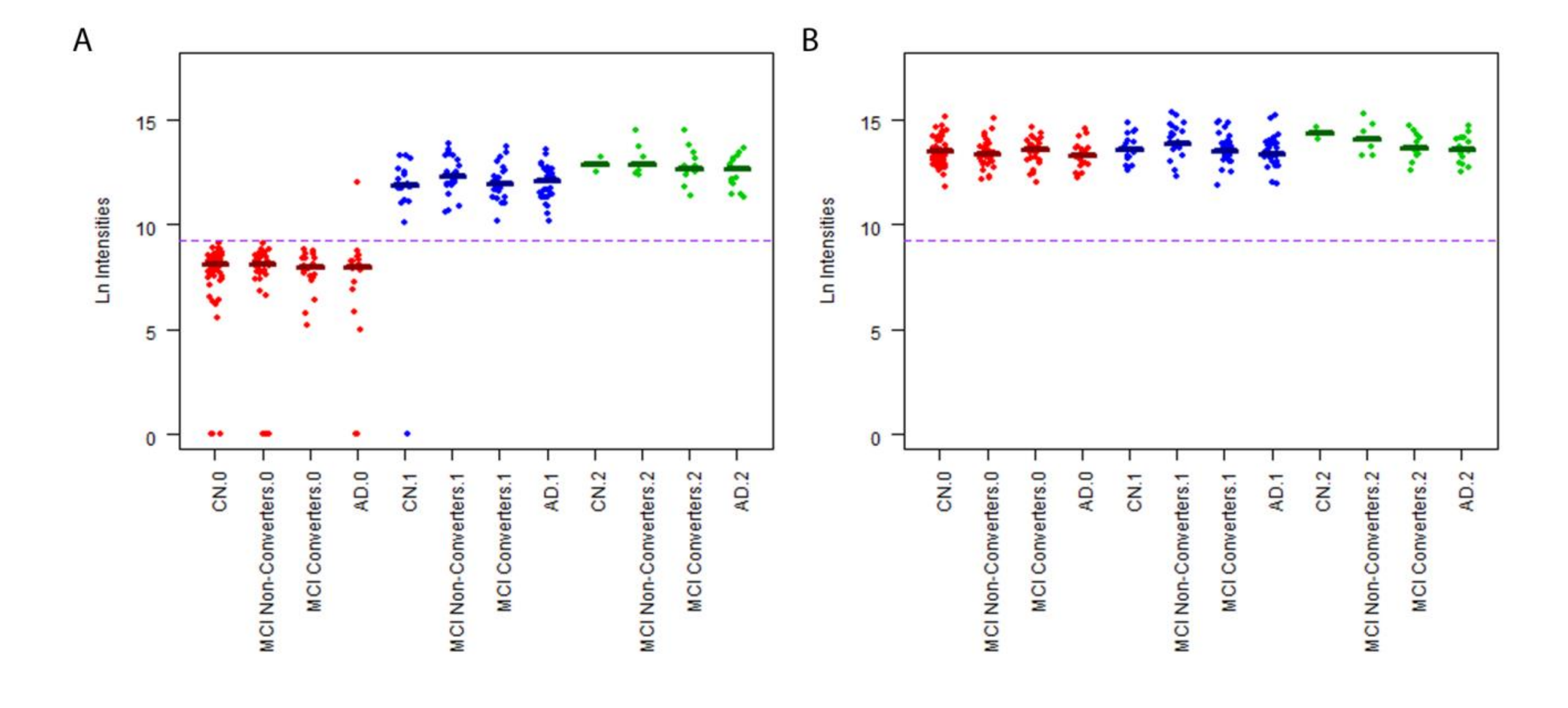


Figure 3. ApoE peptide levels do not differ by disease state in CSF. (A) Levels of ApoE e4 allele-specific peptide (LGADMEDVR) are below the limit of quantitation (dashed line) in non-carriers but detectable in all carriers with twice the levels in homozygous individuals. **(B)** Total levels of common ApoE peptide (LGPLVEQGR) are the same independent of genotype (color by ApoE4 allele count: red, 0; blue, 1; green, 2; Group by disease state: CN, normal; MCI, mild cognitive impairment; AD, Alzheimer's disease; MCI converters progressed to AD within 3 years of sample collection.



Biomarker Discovery in Parkinson's Disease

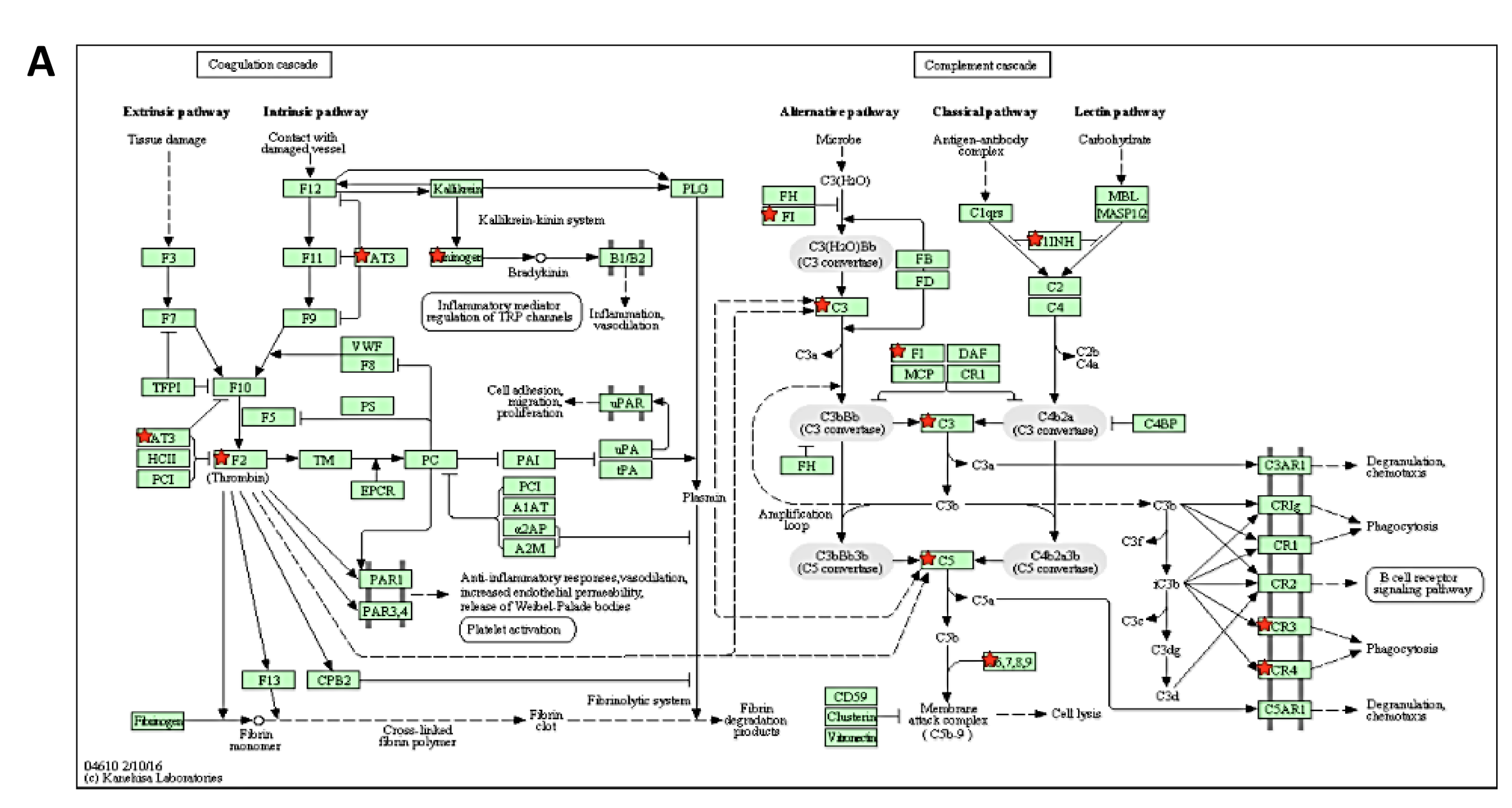
Background and Goals

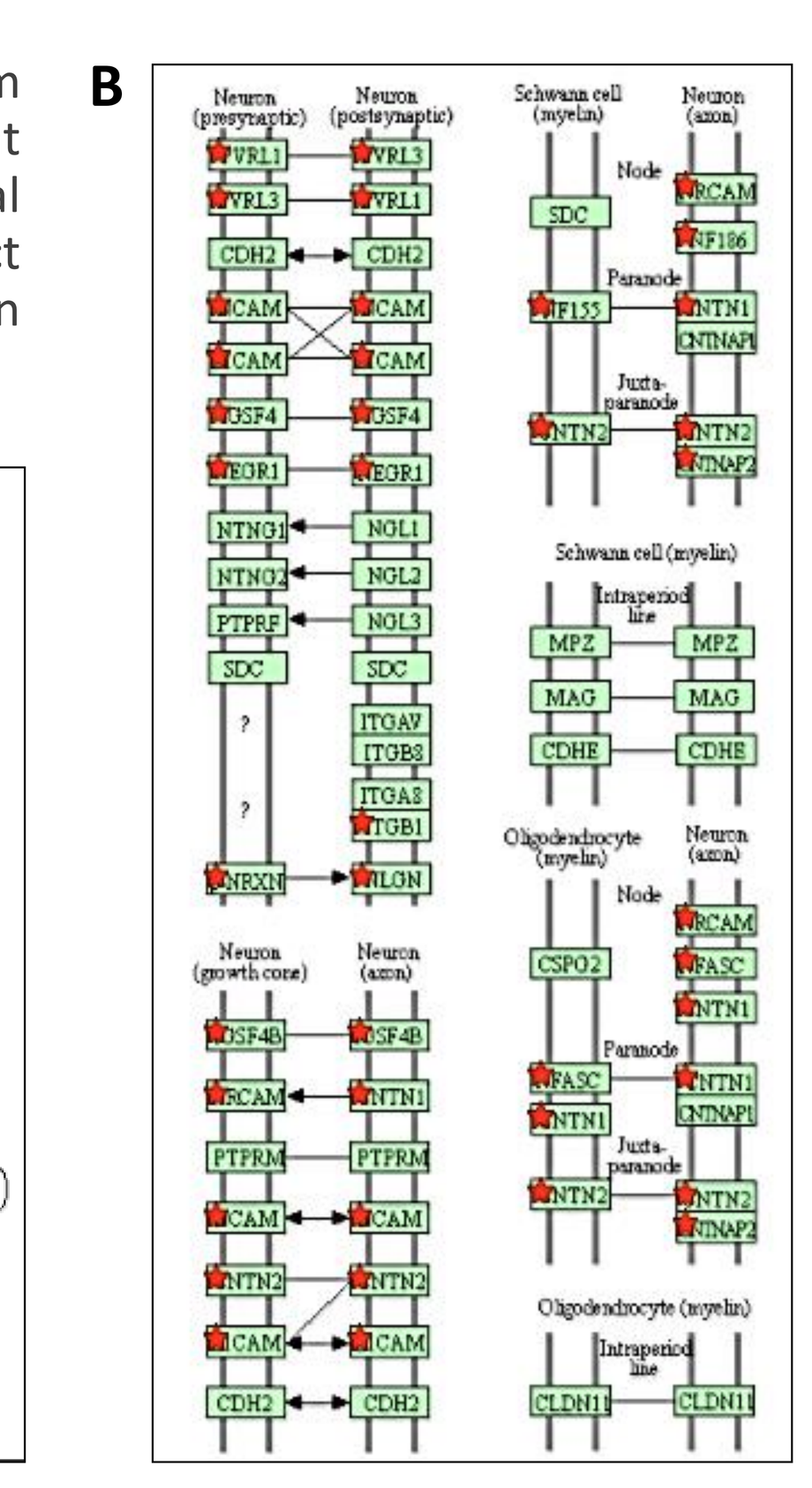
- Supported by the Michael J Fox Foundation.
- Study goal to identify cross-sectional biomarkers that may serve as an early diagnostic, a means of measuring disease progression, and/or a measure of drug efficacy in clinical trials.

Study Design and Overview

The LC-MRM/MS assay developed for AD was supplemented with 50 proteins previously associated with PD. An effort was made to include proteins associated with the degeneration of dopamine neurons in the substantia nigra, protein misfolding and aggregation, dysregulation of the autophagy-lysosome system, calcium homeostasis, mitochondria and endoplasmic reticulum. Following MRM assay development, PD samples (n=109) were compared to HC (n=84) from the BioFIND study, across 8 clinical sites. Of the 185 protein targets, 154 were well detected and quantified (83%). In a comparison of the PD and HC groups, 39 proteins were differentially expressed after correcting for multiple testing ($p \leq 0.05$, $q \leq 0.05$). Eleven (11) up-regulated proteins were associated with inflammation, complement, immune response and coagulation (**Figure 4A**), while 28 proteins were down-regulated with disease, including proteins involved in neural cell adhesion (**Figure 4B**). Efforts are underway to verify these results in an independent set of CSF samples.

Figure 4. Pathway analysis of differentially expressed proteins (Figures exported from KEGG). **(A)** (below) Up-regulated proteins involved in the coagulation and complement cascades are highlighted by ★. **(B)** (right) Down-regulated proteins belonging to the neural cell adhesion family found at the synapse are highlighted by ★. Cell-cell adhesions connect neurons with supporting Schwann cells and oligodendrocytes, and are also involved in axon-axon contacts.





Biomarkers in Huntington's Disease

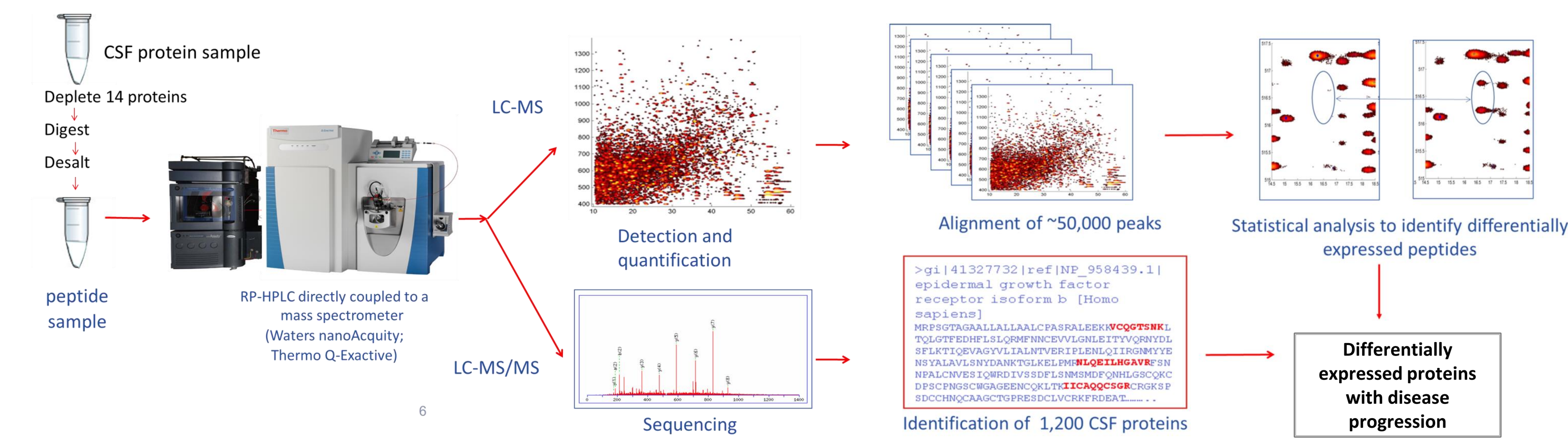
Background and Goals

- Funded by NIH/NINDS 5 U01 NS082089-03.
- Primary study goal to identify biomarkers of HD that could serve to detect early signs of disease as well as to quantify disease progression, particularly in a clinical drug trial.
- Since very few candidate biomarkers of HD exist in the literature, an unbiased mass spectrometry discovery study was performed to identify new biomarker candidates.

Study Design and Overview

An unbiased mass spectrometry discovery study (LC-MS/MS) was performed in CSF (120 subjects) from the PREDICT_HD study with the identification and relative quantification of 1,200 CSF proteins (**Figure 5**). Proteins that were differentially expressed in one of several comparisons between healthy gene-negative, pre-symptomatic gene-positive and symptomatic individuals, were incorporated into the targeted LC-MRM/MS assay, originally designed for AD markers. The discovery samples were then reanalyzed by the MRM assay to qualify the initial results. In this study, the pre-symptomatic group was stratified into three sub-groups, based on the product of their age and number of CAG repeats (CAP score). The "low" group was generally younger and with fewer repeats while the medium and high groups were progressively older with higher numbers of repeats. The hypothesis is that the "low" group is farthest from expressing clinical symptoms of disease while the "high" group is closest. In addition, two time points, approximately one year apart, were analyzed for a subset of the subjects (38 out of 111 subjects).

Figure 5. LC-MS/MS workflow used for discovery analysis of CSF samples. CSF was processed as for MRM but analyzed with a different mass spectrometer that alternates between peptide ion detection and quantification (LC-MS) and fragmentation (LC-MS/MS). The LC-MS spectra, consisting of approximately 50,000 ion peaks, were aligned across all samples to determine the relative abundance of each peak in each sample. Meanwhile, the LC-MS/MS fragmentation spectra were matched to idealized spectra, based on the human protein sequence database, in order to find a match. Peptides with a good match were clustered by homology into their parent proteins and linked to the peptide abundance data. In this way, the relative expression of each protein was determined across all samples and between groups.



A total of 1,200 proteins were identified and quantified in CSF. After correcting for multiple testing 18 proteins were determined to be significant. Among these, only 2 proteins were found to be downregulated, pro-enkephalin (PENK) and the related pro-dynorphin (PDYN) (**Figure 6**), whereas multiple proteins were found to be upregulated. PENK and PDYN proteins are processed into neuroactive peptides that serve various functions including pain perception, response to stress and neuroprotection.

Coagulation proteins increased in abundance with progression from controls and the low CAP group to the diagnosed group, as shown for the MRM study (**Figure 7**). Generally, most of the change occurs in the clinically diagnosed group, rather than in the pre-manifest carriers. Increases in these proteins are commonly linked with inflammation and immune response in most diseases.

Figure 6. MRM analysis of PENK and PDYN show down-regulation prior to clinical signs of disease.

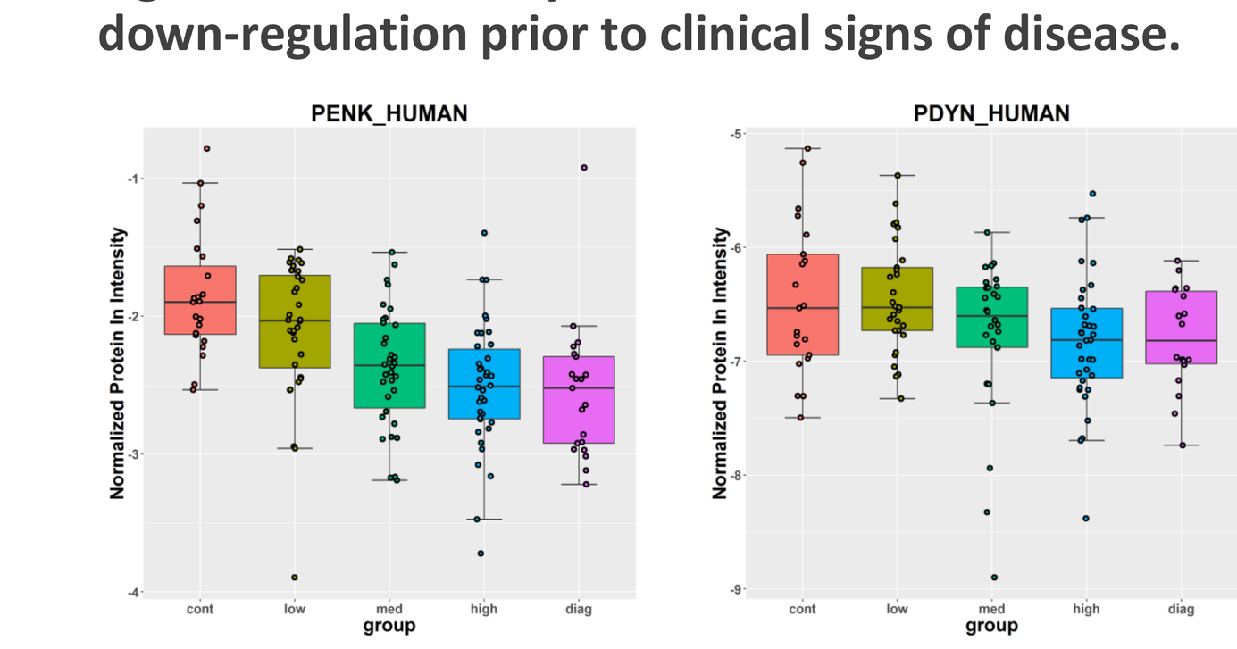
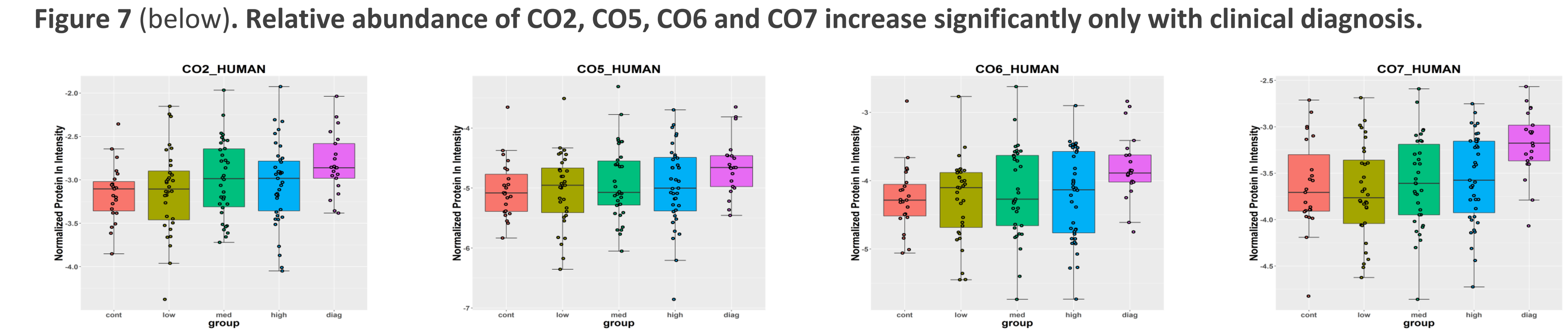


Figure 7 (below). **Relative abundance of CO2, CO5, CO6 and CO7 increase significantly only with clinical diagnosis.**



Similarities and Differences Across CNS Diseases

A subset of proteins have been analyzed by MRM in AD, PD and HD and found to be up- or down-regulated in more than one disease. Shared down-regulated proteins in AD and PD are dominated by neuropeptide hormones (VGF, CCKN, SCG3) or axonal proteins (NPTXR, NRCAM, PTPRN). These proteins, however, are not differentially expressed in HD, consistent with a different underlying biology.

The story is different for up-regulated proteins with the observation that significant proteins in PD are similarly up-regulated in HD. They serve more general functions in inflammation, immune response, and coagulation, proteins expected to increase in response to multiple diseases. While other similar proteins were up-regulated in AD, these proteins were not. Thus, in spite of overlaps, these three neurodegenerative diseases have distinctly unique proteomic profiles (**Table 1**).

Table 1 (right). **Overlapping, yet distinct proteomic profiles in AD, PD and HD.**

	Alzheimer's Disease			Parkinson's Disease			Huntington's Disease		
Protein	FC	p-Value	q-Value	FC	p-Value	q-Value	FC	p-Value	q-Value
VGF	0.77	**0.001**	**0.013**	0.73	**0.000**	**0.002**	1.03	0.843	0.872
CCKN	0.84	**0.006**	**0.057**	0.73	**0.000**	**0.001**	1.07	0.619	0.714
CA2D1	0.83	**0.003**	**0.042**	0.81	**0.001**	**0.011**	1.11	0.218	0.385
NPTXR	0.78	**0.000**	**0.000**	0.81	**0.001**	**0.010**	0.95	0.612	0.713
NRCAM	0.88	**0.020**	**0.094**	0.82	**0.001**	**0.013**	1.06	0.480	0.613
PTPRN	0.84	**0.008**	**0.061**	0.82	**0.001**	**0.011**	0.92	0.451	0.602
FAM3C	0.87	**0.014**	**0.094**	0.84	**0.002**	**0.018**	1.07	0.392	0.560
SCG3	0.87	**0.011**	**0.079**	0.87	**0.011**	**0.047**	0.94	0.521	0.648
PGRP2	1.08	0.324	0.475	1.21	**0.011**	**0.047**	1.34	**0.031**	0.131
AMBP	1.06	0.485	0.577	1.21	**0.008**	**0.043**	1.52	**0.002**	**0.025**
AACT	1.10	0.210	0.360	1.23	**0.001**	**0.013**	1.40	**0.001**	**0.016**
CO5	1.00	0.977	0.741	1.24	**0.006**	**0.037**	1.34	**0.021**	0.112
CO9				1.25	**0.007**	**0.038**	1.46	**0.008**	**0.054**

Conclusions

Targeted MRM mass spectrometry provides the means to rapidly and confidently quantify over 200 proteins in a single assay, requiring only 50µL of CSF. Reproducibility of the assays is very high, particularly with the use of spiked stable isotope-labeled peptide standards for every protein being monitored. Differentially expressed proteins were identified in each of three CNS diseases, providing insight into the underlying biology as well as candidate biomarkers of disease and disease progression. Proteins associated with inflammation, coagulation and complement were typically up-regulated in all diseases, while proteins involved in neuronal signalling, axon guidance and cellular adhesion were often down-regulated. The proteomics profiles of AD, PD and HD were distinct overall but commonalities between pairs of diseases were also found. The current MRM assay has been compiled from all studies and includes proteins found to be significant in each disease. Verification studies are currently underway in AD, PD and HD, all using the same unified assay.

REFERENCES: (1). Spellman DS, Wildsmith KR, Honigberg LA, Tuefferd M, Baker D, Raghavan N, et al. Development and evaluation of a multiplexed mass spectrometry based assay for measuring candidate peptide biomarkers in Alzheimer's Disease Neuroimaging Initiative (ADNI) CSF. Proteomics Clin Appl. 2015;9(7-8):715-31. I: 10.1002/prca.201400178 (2) Chiasserini D, Parnetti L, Andreasson U, Zetterberg H, Giannandrea D, Calabresi P, et al. CSF levels of heart fatty acid binding protein are altered during early phases of Alzheimer's disease. J Alzheimers Dis. 2010;22(4):1281-8. DOI: 10.3233/JAD-2010-101293