Targeted Discovery and Validation of Plasma Biomarkers of Parkinson’s Disease

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Supporting Information

ABSTRACT: Despite extensive research, an unmet need remains for protein biomarkers of Parkinson’s disease (PD) in peripheral body fluids, especially blood, which is easily accessible clinically. The discovery of such biomarkers is challenging, however, due to the enormous complexity and huge dynamic range of human blood proteins, which are derived from nearly all organ systems, with those originating specifically from the central nervous system (CNS) being exceptionally low in abundance. In this investigation of a relatively large cohort (∼300 subjects), selected reaction monitoring (SRM) assays (a targeted approach) were used to probe plasma peptides derived from glycoproteins previously found to be altered in the CNS based on PD diagnosis or severity. Next, the detected peptides were interrogated for their diagnostic sensitivity and specificity as well as the correlation with PD severity, as determined by the Unified Parkinson’s Disease Rating Scale (UPDRS). The results revealed that 12 of the 50 candidate glycopeptides were reliably and consistently identified in plasma samples, with three of them displaying significant differences among diagnostic groups. A combination of four peptides (derived from PRNP, HSPG2, MEGF8, and NCAM1) provided an overall area under curve (AUC) of 0.753 (sensitivity: 90.4%; specificity: 50.0%). Additionally, combining two peptides (derived from MEGF8 and ICAM1) yielded significant correlation with PD severity, that is, UPDRS (r = 0.293, p = 0.004). The significance of these results is at least two-fold: (1) it is possible to use a targeted approach to identify otherwise very difficult to detect CNS related biomarkers in peripheral blood and (2) the novel biomarkers, if validated in independent cohorts, can be employed to assist with clinical diagnosis of PD as well as monitoring disease progression.

KEYWORDS: Parkinson’s disease, targeted mass spectrometry, peripheral biomarkers, selected reaction monitoring

INTRODUCTION

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders, with an estimated prevalence of approximately 1 to 2% among people 65 years of age or older. Clinically, PD patients show motor (e.g., tremor and rigidity) and nonmotor (e.g., dementia and depression)
The other patients are already at relatively advanced stages pathologically. The other difficulty is lack of objective assessment of disease progression, which has led to extensive research on biomarkers that can be employed for early diagnoses of PD or assessing its progression. As of today, the best PD protein biomarkers are those found in cerebrospinal fluid (CSF), the body fluid that is in direct contact with brain and spinal cord. Discovery of peripheral biomarkers reflecting motor components of PD is highly desirable because CSF-based tests depend on the comparatively more invasive process of lumbar puncture that requires more specialized training to collect samples and are therefore not acceptable by some subjects, particularly those without apparent clinical symptoms. However, peripheral biomarker discovery has been largely unsuccessful, due primarily to factors intrinsic to blood; specifically, the plasma or serum proteome is quite complex, proteins are present across a wide dynamic range, and target proteins are often of extraordinarily low abundance. Thus, accurately detecting and quantifying brain-derived or disease-specific proteins in blood is challenging for current technologies. That said, a few blood biomarkers discovered recently, for example, EGF and ApoA1, appear to be related to cognitive impairment or disease onset in PD. Additionally, it has been suggested recently that α-synuclein pathology, a key component of PD pathogenesis, exists in peripheral nerves, leading to a report demonstrating abnormal autonomic nerve staining in skin biopsies in PD. To facilitate peripheral biomarker discovery, in the current investigation, we began with proteins identified in our previous proteomic investigations using human brain tissues obtained at autopsy and CSF obtained from living patients, with a focus on glycosylated proteins, which are highly enriched in body fluids, including plasma. We hypothesized that some of the brain or CSF-derived proteins will reach plasma via mechanisms yet to be defined and that a subset of these proteins or peptides will be detectable in plasma using sufficiently sensitive measurements.

Table 1. Demographic Data of Participating Subjects

| sample | group       | cases | age (mean ± SD) | M/F | UPDRS (mean ± SD) |
|--------|-------------|-------|-----------------|-----|-------------------|
| Initial Validation | | | | | |
| control | 1 | 10 | 65.2 ± 7.5 | 6/4 | | |
| | 2 | 10 | 65.3 ± 7.7 | 6/4 | | |
| | 3 | 10 | 63.8 ± 7.2 | 6/4 | | |
| AD | 1 | 5 | 71.6 ± 13.8 | 2.3 | 12.4 ± 2.1 | |
| | 2 | 5 | 71.3 ± 13.2 | 2.3 | 9.6 ± 4.8 | |
| | 3 | 5 | 71.0 ± 4.7 | 2.3 | 11.2 ± 1.3 | |
| PD (UPDRS < 15) | 1 | 5 | 63.6 ± 9.2 | 2.3 | | |
| | 2 | 5 | 62.6 ± 11.1 | 2.3 | 9.6 ± 4.8 | |
| | 3 | 5 | 63.2 ± 11.5 | 1/4 | 11.2 ± 1.3 | |
| PD (UPDRS 15–30) | 1 | 10 | 63.8 ± 7.1 | 6/4 | 20.5 ± 5.3 | |
| | 2 | 10 | 63.8 ± 4.5 | 6/4 | 22.0 ± 3.2 | |
| | 3 | 10 | 63.2 ± 6.4 | 7/3 | 20.6 ± 3.6 | |
| PD (UPDRS > 30) | 1 | 10 | 69.9 ± 8.6 | 6/4 | 47.6 ± 9.1 | |
| | 2 | 10 | 70.6 ± 8.5 | 6/4 | 45.9 ± 8.7 | |
| | 3 | 10 | 71.5 ± 8.2 | 6/4 | 47.5 ± 7.7 | |
| subtotal | | 120 | | | | |
| Final Validation | | | | | | |
| AD | | | | | | |
| control (age 50+) | | 15 | 67.7 ± 8.8 | 9.6 | | |
| | 34 | 66.4 ± 8.6 | 15:19 | | | |
| control (age ≤ 50) | | 15 | 33.3 ± 9.0 | 9.6 | | |
| PD All | | 98 | 65.1 ± 9.4 | 75:23 | 23.2 ± 12.5 | |
| PD (Age ≥ 50) | | 96 | 65.6 ± 8.8 | 74:22 | 23.3 ± 12.5 | |
| PD (UPDRS < 15) | | 33 | 63.8 ± 8.8 | 20:13 | 10.5 ± 4.6 | |
| PD (UPDRS 15–30) | | 43 | 64.9 ± 10.2 | 35:8 | 22.3 ± 5.7 | |
| PD (UPDRS > 30) | | 22 | 67.3 ± 8.4 | 20:2 | 40.8 ± 9.8 | |
| subtotal | | 162 | | | | |
SRM), dramatically increasing their relative concentration; however, such techniques are limited by the need for highly specific and high-affinity antibodies, which are either not available for all targets or expensive to generate. We chose to use the N-glycocapture technique, which is an antibody-free, hydrazide-based approach to selectively enrich N-glycopeptides.27 Additionally, glycosylation is known to be important in PD,21,28 and glycoproteins, which are prevalent in extracellular surface proteins and secreted proteins, are ideal sources of biomarkers.

Therefore, to test our hypothesis in this study, N-glycoproteins were captured from plasma of a larger cohort of PD patients, along with healthy and diseased controls, by a hydrazide-based solid-phase capturing approach,27 followed by quantification of peptides that are uniquely associated with PD diagnosis or severity based on our previous experiments.21

**MATERIALS AND METHODS**

### Characterization of Subjects and Sample Collection

The Institutional Review Boards of all participating sites approved the current study. A total of 282 subjects, including patients with PD or Alzheimer’s disease (AD) and age-matched controls, recruited at the Veterans Affairs Puget Sound Health Care System/University of Washington School of Medicine, the Oregon Health and Science University, and the University of California at San Diego, were included in the investigation. The subjects consisted of two subcohorts: those collected prior to 2011 (75 PD, 15 AD, and 30 controls) and those collected more recently (98 PD, 15 AD, and 49 controls). All plasma samples were obtained after informed consent from patients, and all patients underwent medical history evaluation, physical, and neurological examinations, laboratory tests, and neuropsychological assessments. All PD subjects met the UK PD Diagnosis or severity based on our previous experimentations.21,28 AD patients and age-matched controls were previously investigated.21 All N-glycopeptides with quantitative alterations in brain or CSF were selected in the initial candidate library. To evaluate the utility of these glycoproteins as diagnostic markers for PD, we tested the 133 SRM assay feasible glycopeptides (derived from 73 N-linked glycoproteins) in the initial pilot study using a few pooled plasma samples from PD and controls. Peptide selection criteria include: (1) length of 8–20 amino acid residues; (2) no chemically unstable residues (e.g., NG, DG, QG, N-terminal N, and N-terminal Q); (3) fully tryptic; (4) avoiding cysteine residue if possible; and (5) sequence specific for the target protein (i.e., proteotypic peptides).

All peptides used in this study were evaluated using BLAT (http://genome.ucsc.edu) and Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches to ensure uniqueness of the target proteins at both proteomic and genomic levels. Finally, an SRM theoretical collision calculator tool (http://proteomicsresource.washington.edu/cgi-bin/srmcalc.cgi) was applied to confirm the uniqueness of every Q1/Q3 pair for the target peptides. Mass tolerance for both Q1 and Q3 was ±0.35 Da. Deaminated (N → D) glycopeptide sequences were applied for SRM.

### Liquid Chromatography–MS Analysis of Target N-Glycopeptides by SRM

All SRM analyses were performed on a TSQ Vantage triple quadrupole (QQQ) mass spectrometer (Thermo Scientific) at the University of Washington Proteomics Resource. The mass spectrometer was coupled to a nanoelectrospray ionization source and a Waters nanoACQUITY UPLC system. Glycopeptides from 1 μL of original plasma (~1 μg peptides) were loaded onto a C18 trap column (20 mm long, 75 μm ID) and separated by a 150 mm C18 column (75 μm ID) over a 60 min 2–35% linear acetonitrile gradient. Spray voltage was set at 1700 V. Scheduled SRM was performed with 5 min retention time windows for most of the peptides and an instrument cycle time of 2000 ± 500 ms. Dwell times were varied depending on the number of concurrent transitions; in all cases they were at least 10 ms.

### Quantification of Target Peptides in Plasma

Both unpurified (Thermo-Fisher Scientific, Germany) and purified (AQUA-grade, Sigma-Aldrich, USA) peptide standards that correspond to natural counterparts (“light” peptides) were synthesized with heavy isotopic lysine (13C6-15N2) or arginine (13C6-15N4) at the C-termini (“heavy” peptides). Collision energies (CEs) were determined using the default formula from Thermo (0.034 × precursor mass /z + 3.3140) and then optimized with four additional CE steps (±5 V, ±10 V). The top four abundant transition (Q1/Q3) pairs, including 4 “light”
and 4 “heavy” transitions, were selected. To build the calibration curve and determine the limits of detection (LOD) and quantification (LOQ) for each target glycopeptide, we titrated heavy peptides at seven concentration points in a reference glyco-captured plasma matrix. Two replicates were performed. A linear regression algorithm was used for fitting the seven serial dilution data points for each curve. The endogenous peptides were also monitored in these assays to help determine the amount of each peptide standard to spike-in. All raw SRM data were processed using the Skyline Targeted Proteomics Environment (v1.3) (McCoss Lab, University of Washington) software developed for SRM data sets.\(^{34}\) Settings including 0.055 Th match tolerance \(m/z\), default peak integration, and Savitzky–Golay smoothing algorithm were applied. All data were manually inspected to ensure correct peak detection and accurate integration. Peptides with signal-to-noise ratio of at least 3 were considered detectable. Information including peak area and area ratio of light/heavy peptide pair were output from Skyline to a text-delimited format worksheet.

It should also be noted that the glycopeptide capturing method used in the current study has been well-optimized and widely employed\(^{27,35,36}\) with CVs typically controlled in 15–20% range.\(^{26}\) To further control this variable, in this study, we included one to two reference plasma samples in every batch of sample preparations as inter- and intrabatch controls. These samples showed an average of 16.5 and 14% for preanalytical (capture) and analytical (SRM) variation (CV), respectively; that is, the variations associated with the capture and SRM stages were reasonable in our investigation.

### Statistical Analysis

The key challenge for the present analysis is a high dimension of the feature vector (large number of potentially predictive proteins) versus size of samples within the data set. Our preprocessing selection included peptides that have no more than 50% missing data. Missing data were handled using \(k\)-nearest neighbor imputation algorithms \((k = 10)\). Repeated (duplicate) measurements for the same protein–peptide-\(m/z\) combination were averaged. All peptide data were then log-10-transformed to achieve more normal distribution. These methods have been previously described.\(^{37}\)

All other analyses were generated in Prism 6.0 (GraphPad software, La Jolla, CA) or SPSS 18.0 (IBM, Chicago, IL). One-way analysis of variance (ANOVA), followed by the Tukey’s HSD post hoc test, was used to compare differences between groups. Receiver operating characteristic (ROC) curves were used to calculate the relationship between sensitivity and specificity for PD versus the healthy control group and hence to evaluate the diagnostic performance of the analytes, either individually or in combinations. Logistic regression was used to determine the best linear combination of peptide analytes for predicting disease status (versus healthy controls), followed by ROC analysis on the linear combination. The “optimum” cutoff value from a ROC curve is determined when the sum of sensitivity and specificity is maximal. Additionally, relationships between the analytes and the unified Parkinson’s disease rating scale (UPDRS) were analyzed with bivariate correlation using Pearson’s correlation coefficients. Stepwise multiple linear regression analysis was used to screen for the best predictors (linear combination of peptide analytes) that correlate the disease severity (UPDRS). Values with \(p < 0.05\) were regarded as significant in this pilot investigation.

### RESULTS

#### Development of SRM Assay

To enrich secreted proteins in body fluids, in the past few years, we profiled \(N\)-linked glycoproteins in the brain and CSF of patients with PD as compared with age-matched healthy and diseased controls (patients with AD). A subset of proteins has been previously published.\(^{21}\) Drawing from our previous work, we identified candidate peptides with quantitative alterations (defined by ±50% over control cases; full list not shown) and selected a total of 133 \(N\)-glycopeptides, representing 73 \(N\)-linked glycoproteins based on peptide characteristics, especially amino acid sequence, suitable for SRM assays, as defined in the Methods section. With the aid of synthetic heavy-isotopic labeled peptides, SRM conditions including \(Q1/Q3\) transitions, collision energy (CE), retention time (RT), and the spiked-in amount of heavy peptide, were first optimized with a set of pooled samples obtained from healthy controls and PD patients. On the basis of the detectability of target peptides in the pooled samples (either control or PD), 50 formerly \(N\)-glycosylated peptides (deamidation happens during glyco-capture, the \(N\)-glycopeptides monitored in SRM are not glycosylated anymore) derived from 40 glycoproteins (Supplemental Table 1 in the Supporting Information, along with reported biological functions) were selected as SRM targets for further analysis, with spiked synthetic corresponding heavy peptides in the initial stage of the validation to be discussed later. A diagram, Figure 1, is provided to illustrate the workflow.

**Figure 1.** Brief workflow outlining the pipeline for screening plasma PD glycoprotein biomarkers by SRM. In the Pilot study, a primary synthetic peptide library containing 133 unpurified, deamidated \(N\)-glycopeptides was used to generate SRM assays and test the detectability of these formerly \(N\)-glycosylated peptides in glyco-captured plasma. Refined SRM assays with optimized settings including retention time (RT) were applied in next stages in the pipeline. In initial validation, 50 \(N\)-glycopeptides that could be detected in the Pilot study were measured in a cohort including 15 PD, AD, and control samples pooled from 75 PD, 15 AD, and 30 control individuals, respectively. The final SRM assay library containing 12 purified, deamidated \(N\)-glycopeptides was then used to detect the candidate biomarkers in an independent validation cohort for ROC calculation and assessment of disease severity association \((n = 162)\).
Figure 2. Heat map of hierarchical clustering analysis of the 50 N-glycopeptides that were detected in the initial validation cohort containing 15 pooled control, AD, and PD plasma samples. The analysis was based on SRM measured peptide relative abundance against average of three pooled controls and conducted using average linkage and Euclidean distance. PD1, PD2, and PD3 represent PD at early, middle, and late stages of the disease, respectively, based on UPDRS. (Also see Table 1.)

Table 2. Information of the 12 N-Glycopetides Monitored in SRM

| UniProt  | gene symbol | peptide sequence | LOD (fmol/μL) | LOQ (fmol/μL) | estimated fmol/μL in plasma | estimated ng/mL in plasma | description | MW (KDa) |
|----------|-------------|------------------|---------------|---------------|-----------------------------|---------------------------|-------------|----------|
| O00533 CHL1 | IGVN^LTQK | 0.008 | 0.008 | 0.104 | 1.4 × 10^1 | neural cell adhesion molecule L1-like protein | 135.071 |
| O00533 CHL1 | IIPSN'NSGTFR | 0.02 | 0.04 | 0.104 | 1.4 × 10^1 | neural cell adhesion molecule L1-like protein | 135.071 |
| Q8NB4 GOLM1 | AVLVNN'ITTGER | 0.04 | 0.04 | 0.203 | 9.2 × 10^0 | golgi membrane protein 1 | 45.333 |
| P98160 HSPG2 | ALVN'FTR | 0.0016 | 0.008 | 0.256 | 1.2 × 10^2 | heparan sulfate proteoglycan 2 | 468.830 |
| Q9Y4L1 HYOU1 | AEPPLN^ASASDQGEK | 0.016 | 0.08 | 0.041 | 4.6 × 10^0 | hypoxia up-regulated 1 | 111.335 |
| P05862 ICAM1 | AN^LTVVLLR | 0.2 | 0.4 | 1.228 | 7.1 × 10^1 | intercellular adhesion molecule 1 | 57.825 |
| Q13449 LSAMP | LGVTN^ASLVLFR | 0.2 | 0.2 | 0.428 | 1.6 × 10^1 | limbic system-associated membrane protein | 37.393 |
| Q7Z7M0 MEGF8 | ALLTN^VSSVALGSR | 0.08 | 0.2 | 5.543 | 1.7 × 10^2 | multiple EGF-like domains 8 | 303.100 |
| P13591 NCAM1 | DQGLPSSN^YSNIK | 0.04 | 0.04 | 0.349 | 3.3 × 10^2 | neural cell adhesion molecule 1 | 94.574 |
| P04156 PRNP | GEN'FTETDVK | 0.008 | 0.008 | 0.293 | 8.1 × 10^0 | major prion protein | 27.661 |
| Q92854 SEMA4D | AAN^YTSSSLNPDK | 0.008 | 0.02 | n/a | n/a | semaphorin-4D | 96.150 |
| P24821 TNC | N^TTSYVLLR | 0.008 | 0.02 | 0.174 | 4.2 × 10^1 | tenasin | 240.853 |

Notes: N^, deamidated asparagine residues. The limit of detection (LOD) of each peptide was obtained from the average of lowest concentration point at which all three or four transitions were confidently detected. The limit of quantification (LOQ) was obtained from the average of lowest concentration point at which the intensity of the most abundant transition is on the linear scale along with the calibration curves. Protein LOD/LOQ estimates assume complete trypsin digestion. Mass spec spectral counts estimated protein concentrations cited from Farrah et al. 2011. According to amino acid sequence. Oligosaccharide chain is not included.
having identified peptides that are reliably detectable in plasma, we then aimed to narrow down the panel of potential biomarker candidates to those showing the largest alterations under the disease conditions. A total of 120 cases from PD, AD, and age-matched controls were included in this study, with the samples pooled based on disease status. To allow approximation of disease stage correlations, we further split plasma samples pooled based on disease status. To allow approximate sensitivity and specificity of these 12 peptides, we used AQUA level purification heavy isotopic-labeled peptides, with SRM running conditions of the 12 glycopeptides (both endogenous and spiked-in standard) are shown in Supplemental Figure 2 in the Supporting Information. With the exception of peptide SEMA4D-AADYTSSLNLPSDK, the remaining 11 peptides were detected in more than 50% of the individuals 

**Diagnostic Sensitivity and Specificity**

Pool samples, even with multiple pools as performed in our initial validation previously discussed, do not provide precise sensitivity and specificity information. Accordingly, in the final validation stage (Figure 1), a total of 162 individual plasma samples from an independent cohort of subjects recruited from the same medical center were investigated. Additionally, to achieve higher-level accuracy, we used AQUA level purified heavy isotopic-labeled peptides, with SRM running conditions further optimized for the new peptide standards (Supplemental Table 3 in the Supporting Information). Peptide sequences, estimated plasma concentrations, and limits of detection and quantification of these 12 peptides are presented in Table 2. Of note, calibration curves were generated in a reference glycoprotein plasma matrix to fully characterize the performances of SRM assays. Calibration curves for each AQUA peptide are plotted on linear scales in Supplemental Figure 1 in the Supporting Information. Representative SRM chromatograms of the 12 glycopeptides (both endogenous and spiked-in standard) are shown in Supplemental Figure 2 in the Supporting Information. With the exception of peptide SEMA4D-AADYTSSLNLPSDK, the remaining 11 peptides were detected in more than 50% of the individuals (Supplemental Table 4 in the Supporting Information). P values and posthoc comparisons between each diagnostic group and age-matched controls were calculated for each of the 12 SRM peptides using one-way ANOVA, followed by Tukey’s HSD post hoc tests (Table 3). Three of the 12 N-glycopeptides, derived from glycoproteins PRNP (prion protein), GOLM1 (Golgi membrane protein 1), and NCAM1

### Table 3. Multiple Comparisons (Tukey’s HSD post hoc tests) of the 12 N-Glycopeptides in Final Validation Cohort

| protein | peptide sequence | CTRL (age ≤ 50) | PD (UPDRS < 15) | PD (UPDRS 15−30) | PD (UPDRS > 30) | PD-All (age 50+) | AD |
|---------|-----------------|----------------|-----------------|-----------------|----------------|-----------------|-----|
| CHL1    | BGVYN’LTQK      | −6.778b        | 1.367           | 3.789           | 0.503          | 3.048           | −1.059        |
| CHL1    | IIPSN’NSGTFR    | −4.399c        | 1.389           | 2.445           | 1.629          | 2.593           | 3.564         |
| GOLM1   | AVLVYN’TTTGER   | −5.191b        | 2.835           | 3.961c          | 2.393          | 4.507c          | 1.908         |
| HSPG2   | ALVNL’FTTR      | −7.967b        | −0.102          | 0.545           | 0.162          | 0.336           | −0.164        |
| HYOU1   | AEEPPLN’ASASDQGEK | −0.962        | −0.144          | 4.076c          | 2.243          | 2.787           | 1.336         |
| ICAM1   | ANLTVVLRR       | −2.847         | 2.474           | 2.458           | 0.785          | 2.737           | 1.585         |
| LSAMP   | LGVTN’ASVLFRL   | 3.13           | 3.109           | 1.472           | 0.21           | 2.13            | 0.542         |
| MEGF8   | ALLTN’VSSVALGRS | −0.537         | −0.288          | 1.485           | −0.016         | 0.67            | 0.228         |
| NCAM1   | DGGQLPSSN’YSNIK | 1.431          | 3.296           | 3.32            | 2.737          | 4.563c          | 4.39c         |
| PRNP    | GEN’FTEDVK      | −1.923         | 2.403           | 4.39c           | 2.298          | 4.42c           | 0.642         |
| SEMA4D  | AA’YTSSSLNLDPDK | −1.414         | 3.002           | 2.61            | 0.099          | 1.621           | 0.721         |
| TNC     | N’TTYSVLRL      | −4.816b        | 0.676           | 0.922           | −0.01          | 0.881           | 0.443         |

**Notes:** N-deamidated asparagine residues. Settings in Tukey’s HSD post hoc tests: alpha = 0.05; n = average sample numbers in the two comparison groups. Proteins and peptides in bold are those with statistical alterations unique to PD (vs age-matched controls and AD). #Significance level: p < 0.01. *Significance level: p < 0.05.

### Table 4. ROC and Correlation Data

| N-glycopeptide marker | missing values | AUC | p value | cut-off value | sensitivity (%) | specificity (%) |
|-----------------------|----------------|-----|---------|---------------|-----------------|-----------------|
| PRNP                  | 1              | 0.648 | 0.011  | −1.7496       | 76.8            | 47.1            |
| GOLM1                 | 0              | 0.632 | 0.023  | −0.5819       | 69.8            | 52.9            |
| NCAM1                 | 0              | 0.677 | 0.002  | −0.5611       | 93.8            | 52.9            |
| PRNP, HSPG2, MEGF8, and NCAM1 | 4 | 0.753 | 0.000 2 | 0.613        | 90.4            | 50              |

Initial Validation

Having identified peptides that are reliably detectable in plasma, we then aimed to narrow down the panel of potential biomarker candidates to those showing the largest alterations under the disease conditions. A total of 120 cases from PD, AD, and age-matched controls were included in this study, with the samples pooled based on disease status. To allow approximation of disease stage correlations, we further split plasma from PD subjects into three subgroups according to UPDRS-defined disease severity. To reduce the within-pool heterogeneity and facilitate statistical analysis, we combined samples in each group into three small pools (control: n = 3, early PD [UPDRS < 15]: n = 3, intermediate PD [UPDRS 15−30]: n = 3, late PD [UPDRS > 30]: n = 3, and AD: n = 3). Each pooled sample consisted of 10 individuals with age and gender evenly distributed, except the AD and early PD groups, where plasma from only five individuals was used because of limited samples in these subcohorts (Table 1). Quantitative assessment of 15 pooled samples revealed clear differences among different diagnostic groups or between different stages of the disease (Supplemental Table 2 in the Supporting Information). Figure 2 shows the hierarchical clustering analysis, demonstrating a clear separation of PD with UPDRS >15 (right side, PD2.* and PD3.* columns) from the three controls (left side columns). Interestingly, this phenomenon is missing in the three AD pools as well, suggesting that these elevations are unique in PD with UPDRS >15. The three UPDRS <15 samples (PD1_1, PD1_2, and PD1_3) did not present obvious changes compared with the controls, indicating that the current set of glycoproteins may lack the ability to distinguish PD in the early stage from controls, at least in these pooled samples. Overall, a total of 12 N-glycopeptides derived from 11 glycoproteins were detectable, with more than 50% difference between PD versus controls or between the stages of disease. Consequently, these 12 N-glycopeptides were selected to be the targets in the next stage of validation experiments.
(neural cell adhesion molecule 1), were significantly altered between PD versus age-matched controls (age \( \geq 50 \); PD, \( n = 96 \); control, \( n = 34 \); \( p < 0.05 \)). Among them, the plasma concentration of peptide NCAM1 was also significantly increased in AD patients compared with age-matched controls (\( p < 0.05 \)), suggesting its change may be related to neurodegeneration but not specific to PD. Furthermore, levels of 5 N-glycopeptides (CHL1_IIP, CHL1_ISG, GOLM1, HSPG2, and TNC) were lower in younger controls (age \(< 50 \)) indicating that the blood level of these glycoproteins was likely associated with aging processes.

On the basis of the performance of each individual peptide on the group difference, a logistic regression-based multivariate analysis was performed to evaluate the sensitivity and specificity of the three PD unique peptides, alone or in combination with the rest of the 12 peptides for discriminating of PD from healthy controls (age \( \geq 50 \)) (Table 4). As shown in Supplemental Figure 3 in the Supporting Information, the best individual performing peptide was PRNP (area under curve (AUC): 0.648; sensitivity: 76.8%; and specificity: 47.1%). The performance of ROC became progressively better when more peptides were added to the panel; a combination of four peptides (PRNP, HSPG2, MEGF8, and NCAM1) improved AUC to 0.753, sensitivity to 90.4%, and specificity to 50.0% (Figure 3A). Notably, however, adding more peptides only enhanced ROC performance slightly; for example, a panel of 10 peptides (PRNP, HSPG2, MEGF8, NCAM1, ICAM1, TNC, GOLM1, CHL1_IIP, CHL1_ISG, and LSAMP) achieved the following values: AUC, 0.840; sensitivity, 71.7%; and specificity, 83.3%. Also, as expected, there were more cases having missing values (i.e., peptides not detectable) when more peptides were included; specifically, the number of missing values for PRNP, 4-peptide combination, and 10-peptide combination were 1, 4, and 53, respectively.

Correlation with PD Severity

Significant differences among PD subgroups at different disease stages (UPDRS < 15, UPDRS 15–30, UPDRS > 30) and age-matched controls were also observed (Table 3). Next, the correlation of PD severity (as determined by UPDRS) with plasma levels of single peptide or a combination of peptides was evaluated by Pearson’s correlation. A stepwise multiple linear regression analysis was used to screen for the best predictors (linear combination of peptide analytes) for disease severity. Although none of the single peptides significantly correlated with UPDRS, when peptides were considered together, a combination of MEGF8 and ICAM1 was identified to correlate significantly with UPDRS (Figure 3B; \( r = 0.293, p = 0.004 \)).

■ DISCUSSION

It has been exceptionally challenging to detect peripheral markers unique to CNS diseases, including PD, largely because human blood is extremely complex, with proteins contributed from many organ systems, and CNS-derived proteins are exceedingly low in concentration. In this study, we took a targeted approach, using previously identified CNS-related proteins with changes specific to PD as the starting point. Several objectives are achieved in this study, including (1) detection of 11 CNS-related glycoproteins (related to PD diagnosis or severity in previous proteomics profiling) in plasma, a much less invasive sample source (than brain, CSF, or skin biopsy), (2) a combination of several peptides, that is, PRNP, HSPG2, MEGF8, and NCAM1, provided good diagnostic sensitivity (90.4%) and specificity (50.0%) between PD and controls, and (3) a combination of two peptides, MEGF8 and ICAM1, significantly correlated with PD severity, as measured by UPDRS.

All 12 of the N-glycopeptides studied in the final validation study are either relatively specific to the CNS or have functions potentially important to PD pathogenesis or CNS diseases in general. However, those that provided good sensitivity and
specificity, alone and in combination, warrant further discussion. PRNP (prion protein or PrP) is most predominantly expressed in the nervous system but occurs in many other tissues throughout the body.\textsuperscript{38,39} It reportedly may cross the blood–brain barrier (BBB)\textsuperscript{40} and, when aggregated, becomes a major contributor to a variety of cognitive deficiencies and neurodegenerative diseases, especially Creutzfeldt–Jakob disease.\textsuperscript{41} PrP has been implicated as a receptor and binding partner for A\textsubscript{β} oligomers in AD,\textsuperscript{42,43} and its discovery as a protein altered in PD suggests interesting potential for a role in PD pathogenesis as well. Neural cell adhesion molecule 1 (NCAM1) plays important roles in the inflammatory mechanisms associated with neurodegeneration and participates in the neuroprotective response in neurodegeneration.\textsuperscript{44} Its up-regulation in PD cases could be a compensatory mechanism during the disease process. Similarly, hepan sulfate proteoglycan 2 (HSPG2) is an extracellular matrix protein that is primarily synthesized by vascular endothelial and smooth muscle cells, with several proposed functions, including maintenance of the integrity of the BBB,\textsuperscript{45} which has been reported to be compromised in PD.\textsuperscript{46} Finally, very little is known about MEGF8 (multiple epidermal growth factor-like domains 8) and its role in either PD or any other neurodegenerative disorder. Interestingly, a recent study has suggested that this protein could potentially be involved in phagocytic function of astrocytes.\textsuperscript{47}

It should be stressed that while the four-peptide panel probably does not reach sufficient (≥80%) sensitivity/specificity to be used as a sole diagnostic criterion, it performed similarly to the best biomarkers discovered thus far. For example, in a previous study of α-synuclein and DJ-1 in CSF of PD patients, sensitivity and specificity for distinguishing control from PD were 94 and 50% for DJ-1 and 93 and 39% for α-synuclein, respectively.\textsuperscript{48} This result is especially notable when considering that the previous results were obtained in CSF, a biological fluid in direct contact with the extracellular space in the brain, while the current study used a peripheral fluid. Thus, while the current test performed similarly, it provides at least a promising lead to use a less invasive sample by sensitive detection of CNS-related proteins in blood samples. That said, even a test without ideal sensitivity or specificity could be useful in clinical and research settings (e.g., selecting patients for clinical trials or screening for preclinical/premotor patients), where they may be useful for screening subjects or patients whose diagnosis would then be confirmed by more expensive or more invasive tests.

Of note is also the observation that another glycoprotein, GOLM1 (Golgi membrane protein, also known as Golgi phosphoprotein 2 or Golgi membrane protein GP73), was significantly changed between PD and controls but did not perform well in logistic regression screening for ROC performance. However, this does not necessarily mean that it is not important to PD pathogenesis or development. Indeed, the Golgi complex, including GP73, plays a key role in the sorting and modification of proteins exported from the endoplasmic reticulum, which has been demonstrated to be dysfunctional in PD or PD models.\textsuperscript{49}

To date, biomarkers that could robustly correlate with PD severity or progression (assessed in longitudinal collected samples) are quite rare, if there are any.\textsuperscript{50} In fact, we are unaware of any such PD candidate markers (or AD for that matter) that have been validated by independent studies. To this end, our candidate markers, although vetted through discovery and validation phases, require yet another round of validation by independent groups, particularly for the identified models/combinations. That said, two candidate peptides, MEGF8 and ICAM1, were associated with PD severity in this study. As previously indicated, there is no clear evidence that MEGF8 is involved in CNS disease at this point, and its potential role should be investigated, particularly if these findings are validated, as it may be a novel PD-related protein. Intercellular adhesion molecule 1 (ICAM1) is a cell-surface glycoprotein that is typically expressed on endothelial cells and cells of the immune system.\textsuperscript{51} One of the proposed CNS functions of ICAM1 is involvement in vasoconstriction associated with subarachnoid hemorrhage,\textsuperscript{52,53} indicating its role in endothelial dysfunction. In addition to regulating endothelial functions, ICAM1 is also a mediator of cellular inflammation and reported to be regulated by DJ-1 and α-synuclein, two proteins intimately involved in PD,\textsuperscript{54,55} during neurodegeneration.\textsuperscript{56} The increase in circulating ICAM1 as a function of increasing PD severity is in line with the argument that there is persistent inflammation during PD development and progression.\textsuperscript{55,56} It remains to be determined, though, whether plasma ICAM1 is originated from the CNS or the other way around.

The two-peptide panel that correlated with disease severity may also be useful in developing an assay to track the progression of PD. However, as in all biomarker studies, the biomarkers studied here must be further validated in future studies. Moreover, because of the unbiased nature of the discovery stage of this study, some of the peptides discovered here (including the pair correlating with UPDRS) have not previously been associated with PD and are therefore also new putative candidates for future studies of PD pathogenesis.

Interestingly, most of these peptides showed a pattern of greatest increase in the midstage PD group, followed by a decrease in the late PD group. The reasons for this pattern are not yet clear, but it should be emphasized that UPDRS does not reflect a linear pattern of degeneration in specific brain regions but rather a combination of the neurodegenerative and compensatory processes occurring throughout the brain. Moreover, PD processes are highly heterogeneous, and individual disease courses vary substantially. Thus, biomarker patterns may similarly be nonlinear and require interpretation depending on disease stage (e.g., screening tests at early stages vs. tests for following progression at more advanced stages).

It is also notable that although several peptides showed significant differences between control and PD subjects no single peptide correlated with PD severity. This result resembles those observed for α-synuclein, which is reduced in the CSF of PD patients but does not correlate with disease severity.\textsuperscript{58} A number of factors could contribute to this outcome. For example, some biomarkers show floor/ceiling effects, in which the maximal change occurs early in the disease and therefore no correlation is observed with further progression. Furthermore, proteins involved in the disease process may undergo nonlinear changes, such as early compensatory up-regulation, followed by decreases accompanying more severe neurodegeneration. Moreover, it must be considered that the current results were obtained in plasma, while the proteins measured may have originated in the CNS. Therefore, alterations in peripheral clearance or mechanisms of transfer across the BBB may change during the course of the disease as well, so plasma levels reflect a complex combination of disease processes.
Several caveats affect the preliminary portions of our study. In the original selection of candidate peptides, we included peptides that changed ≥50% compared with controls. While this criterion is lenient, the low threshold could be justified in the preliminary steps, where the goal was choosing a pool of candidates for further analysis. Because further elimination of candidates would occur in following steps, to narrow a large number of detectable peptides to a feasible number of promising candidates for SRM, failing to identify a candidate was more problematic than including a peptide that may not really change. An additional limitation was the pooling of subjects used for the first test set. In the preliminary validation step, the primary goal was to identify which of the candidate peptides were most altered in the disease conditions. We followed our previous strategy of combining samples into small pools by disease status.57 This represents a compromise, limiting costs by decreasing the samples to be processed and analyzed but also allowing statistical analyses. This strategy does have statistical costs, most notably dramatic reduction of the power to detect subtle changes (due to the reduction from the number of subjects to the number of pools) and masking the true variability of individual subjects. However, this limitation can be justified in the context of a preliminary study, in which the goal was to rank peptide candidates by the magnitude of their changes. That is, while we may not have detected subtle differences between groups in some peptides, these differences may not be the changes of the greatest relevance.

Finally, an important aspect of the current study is the increase in efficiency of the use of large proteomics data sets. In biomarker research, unbiased studies can produce very large pools of candidate biomarkers, but validation of these candidates is hampered by limitations in assay development for individual proteins. Thus, this strategy of prioritizing peptides that change in the CNS under the disease condition, while moving to peripheral fluids and using sensitive, relatively high-throughput assays such as SRM, may allow improved use of these data in the future.

In conclusion, using a relatively large cohort totaling 282 subjects, several CNS-related protein markers, implicating potential novel mechanisms in PD pathogenesis were readily detected in human plasma. Several of them provided good diagnostic sensitivity/specificity for PD or correlated with PD severity. These results, if validated in independent investigations, could potentially help with establishing CNS specific markers in blood for early disease diagnosis, monitoring disease progression or assessment of treatment effects.

ASSOCIATED CONTENT
Supporting Information
Characterizing individual SRM assays by generating calibration curves in a reference glyco-captured plasma matrix. Representative SRM chromatogram of the 12 glycopeptides monitored in the validation cohort. The ROC curve analysis of the ability of three individual N-glycopeptides to discriminate between PD and controls. Biological functions of the 40 N-linked glycoproteins (50 glycopeptides) monitored in this study. 12 N-linked glycopeptides as biomarker candidates in an initial validation cohort (15 pooled samples). SRM running methods applied in initial and final validation cohorts. Twelve N-linked glycopeptides measured by SRM in the final validation cohort including 162 individuals. This material is available free of charge via the Internet at http://pubs.acs.org. All data sets will be submitted to the PeptideAtlas SRM Experiment Library (PASSEL, http://www.peptideatlas.org/passel/).

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ABBREVIATIONS
CE, collision energy; CNS, central nervous system; CSF, cerebrospinal fluid; PD, Parkinson’s disease; RT, retention time; SRM, selected reaction monitoring; UPDRS, unified Parkinson’s disease rating scale

REFERENCES
(1) Lang, A. E.; Lozano, A. M. Parkinson’s disease. First of two parts. N. Engl. J. Med. 1998, 339 (15), 1044–1053.
(2) Thomas, B.; Beal, M. F. Parkinson’s disease. Hum. Mol. Genet. 2007, 16 (Spec No. 2), R183–194.
(3) Shulman, J. M.; De Jager, P. L.; Feany, M. B. Parkinson’s disease: genetics and pathogenesis. Annu. Rev. Pathol. 2011, 6, 193–222.
(4) Jankovic, J. Parkinson’s disease: clinical features and diagnosis. J. Neurol. Neurosurg. Psychiatry 2008, 79 (4), 368–376.
(5) Sollinger, A. B.; Goldstein, F. C.; Lah, J. J.; Levey, A. I.; Factor, S. A. Mild cognitive impairment in Parkinson’s disease: subtypes and motor characteristics. Parkinsonism Relat. Disord. 2010, 16 (3), 177–180.
(6) Reichmann, H.; Schneider, C.; Lohle, M. Non-motor features of Parkinson’s disease: depression and dementia. Parkinsonism Relat. Disord. 2009, 15 (Suppl 3), S87–S92.
(7) Moore, D. J.; West, A. B.; Dawson, V. L.; Dawson, T. M. Molecular pathophysiology of Parkinson’s disease. Annu. Rev. Neurosci. 2005, 28, 57–87.
(8) Schapira, A. H. Recent developments in biomarkers in Parkinson disease. Curr. Opin. Neurol. 2013, 26 (4), 395–400.
(9) Shi, M.; Bradner, J.; Hancock, A. M.; Chung, K. A.; Quinn, J. F.; Pescind, E. R.; Galasko, D.; Jankovic, J.; Zabetian, C. P.; Kim, H. M.; Leverenz, J. B.; Montine, T. J.; Ginghina, C.; Kang, U. J.; Cain, C. K.; Wang, Y.; Aasly, J.; Goldstein, D. S.; Zhang, J. Cerebrospinal Fluid Biomarkers for Parkinson Disease Diagnosis and Progression. Ann. Neurol. 2011, 69 (3), 570–580.
(10) Mollenhauer, B.; Locascio, J. J.; Schulz-Schaeffer, W.; Sixt-Döring, F.; Teenkwalder, C.; Schlossmacher, M. G. alpha-Synuclein and tau concentrations in cerebrospinal fluid of patients presenting with parkinsonism: a cohort study. Lancet Neurol. 2011, 10 (3), 230–240.
(11) Zhang, J.; Goodlett, D. R.; Montine, T. J. Proteomic biomarker discovery in cerebrospinal fluid for neurodegenerative diseases. *J. Alzheimers Dis.* 2005, 8 (4), 377–386.

(12) Farrah, T.; Deutsch, E. W.; Omenn, G. S.; Sun, Z.; Watts, J. D.; Yamamoto, T.; Shemyenberg, D.; Harris, M. S.; Moritz, R. L. State of the Human Proteome in 2013 as Viewed through PeptideAtlas: Comparing the Kidney, Urine, and Plasma Proteomes for the Biology- and Disease-Driven Human Proteome Project. *J. Proteome Res.* 2014, 13 (1), 60–75.

(13) Breen, D. P.; Michell, A. W.; Barker, R. A. Parkinson’s disease—the continuing search for biomarkers. *Clin. Chem. Lab. Med.* 2011, 49 (3), 393–401.

(14) Haab, B. B.; Paulovich, A. G.; Anderson, N. L.; Clark, A. M.; Downing, G. J.; Hermjakob, H.; Labaer, J.; Uhlen, M. A reagent resource to identify proteins and peptides of interest for the cancer community: a workshop report. *Mol. Cell. Proteomics* 2006, 5 (10), 1996–2007.

(15) Parker, C. E.; Pearson, T. W.; Anderson, N. L.; Borchers, C. H. Mass-spectrometry-based clinical proteomics—a review and prospective. *Analyst* 2010, 135 (8), 1830–1838.

(16) Chen-Plotkin, A. S.; Hu, W. T.; Siderowf, A.; Weintraub, D.; Goldmann Gross, R.; Hurtig, H. I.; Xie, S. X.; Arnold, S. E.; Grossman, M.; Clark, C. M.; Shaw, L. M.; McCluskey, L.; Elman, L.; Van Deelen, V. M.; Lee, V. M.; Soares, H.; Trojanowski, J. Q. Plasma epidermal growth factor levels predict cognitive decline in Parkinson disease. *Ann. Neurol.* 2011, 69 (4), 655–663.

(17) Pellecchia, M. T.; Santangelo, G.; Picillo, M.; Pivonello, R.; Longo, K.; Pivonello, C.; Vitale, C.; Amboni, M.; De Rosa, A.; Mocchia, M.; Erro, R.; De Micheile, G.; Santoro, L.; Colao, A.; Barone, P. Serum epidermal growth factor predicts cognitive functions in early, drug-naive Parkinson’s disease patients. *J. Neurool.* 2013, 260 (2), 438–444.

(18) Qiang, J. K.; Wong, Y. C.; siderowf, A.; Hurtig, H. I.; Xie, S. X.; Lee, V. M.; Trojanowski, J. Q.; Yeartow, D.; J. B. L.; Montine, T. J.; Stern, M.; Mendic, S.; Jennings, D.; Zabetian, C.; Marek, K.; Chen-Plotkin, A. S. Plasma apolipoprotein A1 as a biomarker for Parkinson disease. *Ann. Neurol.* 2013, 74 (1), 119–127.

(19) Derkinderen, P.; Rouaud, T.; Lebouvier, T.; Bruley des Varannes, S.; Neunlist, M.; De Giorgio, R. Parkinson disease: the enteric nervous system spits its guts. *Neurology* 2011, 77 (19), 1761–1767.

(20) Wang, N.; Gibbons, C. H.; Laflo, J.; Freeman, R. alpha-Synuclein in cutaneous autonomic nerves. *Neurology* 2013, 81 (18), 1604–1610.

(21) Hwang, H.; Zhang, J.; Chung, K. A.; Zabetian, C. P.; Peskind, E. R.; Galasko, D.; Jankovic, J.; Leverenz, J. B.; Zhang, J. Significance and confounders of peripheral DJ-1 and alpha-synuclein in Parkinson’s disease. *Neurosci. Lett.* 2010, 480 (1), 78–82.

(22) Zhang, H.; Loriaux, P.; Eng, J.; Campbell, D.; Keller, A.; Moss, P.; Bonneau, R.; Zhang, N.; Zhou, Y.; Wollscheid, B.; Cooke, K.; Yi, E. C.; Lee, S.; Peskind, E. R.; Zhang, J.; Smith, R. D.; Aebersold, R.; Unipep—a database for human N-linked glycosites: a resource for biomarker discovery. *Genome Biol.* 2006, 7 (8), R73.

(23) Tian, Y.; Zhou, Y.; Elliott, S.; Aebersold, R.; Zhang, H. Solid-phase extraction of N-linked glycopeptides. *Nat. Protoc.* 2007, 2 (2), 334–339.

(24) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 2010, 26 (7), 966–968.

(25) Lai, Z. W.; Nice, E. C.; Schilling, O. Glycocapture-based proteomics for secretome analysis. *Proteomics* 2013, 13 (3–4), 512–525.

(26) Wollscheid, B.; Bausch-Fluck, D.; Henderson, C.; O’Brien, R.; Bibel, M.; Schiess, R.; Aebersold, R.; Watts, J. D. Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nat. Biotechnol.* 2009, 27 (4), 378–386.

(27) Qin, S.; Zhou, Y.; Lok, A. S.; Tsodikov, A.; Yan, X.; Gray, L.; Yuan, M.; Moritz, R. L.; Galas, D.; Omenn, G. S.; Hood, L. SRM targeted proteomics in search for biomarkers of HCV-induced progression of fibrosis to cirrhosis in HALT-C patients. *Proteomics* 2012, 12 (8), 1244–1252.

(28) Weissmann, C. The state of the prion. *Nat. Rev. Microbiol.* 2004, 2 (11), 861–871.

(29) Zomosa-Signoret, V.; Arnaud, J. D.; Fontes, P.; Alvarez-Martinez, M. T.; Llortaud, J. P. Physiological role of the cellular prion protein. *Vet. Res.* 2008, 39 (4), 9.

(30) Banks, W. A.; Robinson, S. M.; Diaz-Espinoza, R.; Urayama, A.; Soto, C. Transport of prion protein across the blood-brain barrier. *Exp. Neurol.* 2009, 218 (1), 162–167.

(31) Head, M. W.; Ironside, J. W. Review: Creutzfeldt-Jakob disease: prion protein type, disease phenotype and agent strain. *Neuropathol. Appl. Neurobiol.* 2012, 38 (4), 296–310.

(32) Forloni, G.; Balducci, C. beta-amyloid oligomers and prion protein: Fatal attraction? *Prion 2011,* 5 (1), 10–15.
(43) Um, J. W.; Nygaard, H. B.; Heiss, J. K.; Kostylev, M. A.; Stagi, M.; Vortmeyer, A.; Wisniewski, T.; Gunther, E. C.; Strittmatter, S. M. Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nat. Neurosci. 2012, 15 (9), 1227−1235.

(44) Wielgat, P.; Braszko, J. J. Significance of the cell adhesion molecules and sialic acid in neurodegeneration. Adv. Med. Sci. 2012, 57 (1), 23−30.

(45) Deguchi, Y.; Okutsu, H.; Okura, T.; Yamada, S.; Kimura, R.; Yuge, T.; Furukawa, A.; Morimoto, K.; Tachikawa, M.; Ohtsuki, S.; Hosoya, K.; Terasaki, T. Internalization of basic fibroblast growth factor at the mouse blood-brain barrier involves perlecan, a heparan sulfate proteoglycan. J. Neurochem. 2002, 83 (2), 381−389.

(46) Desai, B. S.; Monahan, A. J.; Carvey, P. M.; Hendey, B. Blood-brain barrier pathology in Alzheimer’s and Parkinson’s disease: implications for drug therapy. Cell Transplant. 2007, 16 (3), 285−299.

(47) Loov, C.; Hillered, L.; Ebendal, T.; Erlanson, A. Engulfing astrocytes protect neurons from contact-induced apoptosis following injury. Plas One 2012, 7 (3), e33090.

(48) Hong, Z.; Shi, M.; Chung, K. A.; Quinn, J. F.; Peskind, E. R.; Galasko, D.; Jankovic, J.; Zabetian, C. P.; Leverenz, J. B.; Baird, G.; Montine, T. J.; Hancock, A. M.; Hwang, H.; Pan, C.; Bradner, J.; Kang, U. J.; Jensen, P. H.; Zhang, J. DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson’s disease. Brain 2010, 133 (Pt 3), 713−726.

(49) Fan, J.; Hu, Z.; Zeng, L.; Lu, W.; Tang, X.; Zhang, J.; Li, T. Golgi apparatus and neurodegenerative diseases. Int. J. Dev. Neurosci. 2008, 26 (6), 523−534.

(50) McGhee, D. J.; Royle, P. L.; Thompson, P. A.; Wright, D. E.; Zajicek, J. P.; Counsell, C. E. A systematic review of biomarkers for disease progression in Parkinson’s disease. BMC Neurol. 2013, 13, 35.

(51) Lawson, C.; Wolf, S. ICAM-1 signaling in endothelial cells. Pharmacol. Rep. 2009, 61 (1), 22−32.

(52) Mannam, V. K.; Lewis, R. E.; Cruse, J. M. The fate of renal allografts hinges on responses of the microvascular endothelium. Exp. Mol. Pathol. 2013, 94 (2), 398−411.

(53) McNally, R. S.; Davis, B. K.; Clements, C. M.; Accavitti-Loper, M. A.; Mak, T. W.; Ting, J. P. DJ-1 enhances cell survival through the binding of Cezanne, a negative regulator of NF-kappaB. J. Biol. Chem. 2011, 286 (6), 4098−4106.

(54) Klegeris, A.; Giasson, B. I.; Zhang, H.; Maguire, J.; Pelech, S.; McGeer, P. L. Alpha-synuclein and its disease-causing mutants induce ICAM-1 and IL-6 in human astrocytes and astrocytoma cells. FASEB J. 2006, 20 (12), 2000−2008.

(55) Deleidi, M.; Gasser, T. The role of inflammation in sporadic and familial Parkinson’s disease. Cell. Mol. Life Sci. 2013, 70 (22), 4259−4273.

(56) Taylor, J. M.; Main, B. S.; Crack, P. J. Neuroinflammation and oxidative stress: co-conspirators in the pathology of Parkinson’s disease. Neurochem. Int. 2013, 62 (5), 803−819.

(57) Lin, X.; Cook, T. J.; Zabetian, C. P.; Leverenz, J. B.; Peskind, E. R.; Hu, S. C.; Cain, K. C.; Pan, C.; Edgar, J. S.; Goodlett, D. R.; Racette, B. A.; Checkoway, H.; Montine, T. J.; Shi, M.; Zhang, J. DJ-1 isoforms in whole blood as potential biomarkers of Parkinson disease. Sci. Rep. 2012, 2, 954.

(58) Farrah, T.; Deutsch, E. W.; Omenn, G. S.; Campbell, D. S.; Sun, Z.; Bletz, J. A.; Mallick, P.; Katz, J. E.; Malmstrom, J.; Ossola, R.; Watts, J. D.; Lin, B.; Zhang, H.; Moritz, R. L.; Aebersold, R. A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. Mol. Cell. Proteomics 2011, 10 (9), M110 006353.