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An alpha-synuclein MRM assay with diagnostic potential for Parkinson's disease and monitoring disease progression

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Abstract

Aim—The alpha-synuclein (α -syn) level in human cerebrospinal fluid (CSF), as measured by immunoassays, is promising as a Parkinson's disease (PD) biomarker. However, the levels of total α -syn are inconsistent among studies with large cohorts and different measurement platforms. Total α -syn level also does not correlate with disease severity or progression. Here, we developed a highly sensitive Multiple Reaction Monitoring (MRM) method to measure absolute CSF α -syn peptide concentrations without prior enrichment or fractionation, aiming to discover new candidate biomarkers.

Results—Six peptides covering 73% of protein sequence were reliably identified, and two were consistently quantified in cross-sectional and longitudinal cohorts. Absolute concentration of α -syn in human CSF was determined to be 2.1ng/mL. A unique α -syn peptide, TVEGAGSIAAATGFVK (81-96), displayed excellent correlation with previous immunoassay results in two independent PD cohorts ($p < 0.001$), correlated with disease severity, and its changes significantly tracked the disease progression longitudinally.

Conclusions—An MRM assay to quantify human CSF α -syn was developed and optimized. Sixty clinical samples from cross-sectional and longitudinal PD cohorts were analyzed with this approach. Although further larger-scale validation is needed, the results suggest that α -syn peptide could serve as a promising biomarker in PD diagnosis and progression.

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The authors have declared no conflict of interest.

Keywords

α -syn; diagnosis; MRM; progression; Parkinson's disease

Introduction

Alpha synuclein (α -syn) is essential in the pathogenesis of Parkinson's disease (PD). Extracellular α -syn, particularly in cerebrospinal fluid (CSF), has also been studied extensively as a potential PD biomarker. We and others have used antibody-based assays, such as ELISA or Luminex, to examine CSF α -syn, with the majority finding that its levels were lower in PD patients than controls [1–6]. However, several technical challenges make adaptation of immunoassay-based measurements to a clinical setting difficult. First, the absolute concentrations of CSF α -syn differed significantly between different antibody-based studies, with the mean values of controls ranging from 0.5 to 40 ng/mL [1–6]. Even when an identical sample was used, different assays showed up to a 10-fold-difference in concentrations [7]. One possible explanation was that different capture and/or detection antibodies used in each immunoassay detected different subsets of α -syn molecules. A similar phenomenon was observed in other studies, such as of Alzheimer's disease [8]. Moreover, a number of isoforms of α -syn, including various post-translational modifications (PTMs) are known to exist, further complicating the picture of which species were identified, and therefore included in the reported CSF α -syn levels. Another question that remains unclear is whether or how α -syn correlates with the severity of PD [9–11]. The earlier symptoms of PD are subtle and in some cases, the disease can progress quickly. Therefore, a biomarker for early diagnosis and/or disease severity will be highly applicable in the field.

Mass spectrometry and proteomics, especially targeted proteomics methods have been used widely as alternative approaches for biomarker development. However, few proteomics studies have reported on α -syn and/or its post-translational modifications [12–14] because of several challenges: the concentration of α -syn in human CSF is extremely low [15] (estimated in the ng/mL (~ 10 pM) range based on immunoassays, though these may underestimate the true value), thus it could be masked by more abundant proteins; and the α -syn protein contains a highly acidic C-terminus lacking in sites for digestion by trypsin. The latter complication requires that sequencing of the C-terminus of α -syn be carried out using less efficient digestion strategies featuring chemical methods or other enzymes.

In this study, we used a strategy of targeted mass spectrometry - Multiple Reaction Monitoring (MRM) to develop a sensitive and quantitative α -syn assay. In MRM, specific peptide sequences are isolated and fragmented, and then selected fragments are filtered and measured. MRM methods provide highly selective and sensitive measurements of distinct proteins [16], and also demonstrate good precision and excellent reproducibility [17, 18]. Current MRM sensitivity can reach sub picomolar levels with excellent performance of LC-MS, and sometimes extensive fractionation or enrichment steps before MS analysis [13, 19, 20]. In addition, a two-step digestion strategy using trypsin and AspN enzymes was optimized to enable an efficient digestion. AspN cuts at the N-terminal of aspartic acid

residues, and can therefore generate peptides from the C-terminal region of α -syn. To quantify the absolute concentrations, recombinant unlabeled (light; quantified using amino acid analysis) and ^{15}N -labeled (heavy) α -syn were used to build calibration curves for every quantifiable α -syn peptide. To account for variations due to processes, including protein precipitation, digestion and LC-MS/MS, CSF samples were spiked with a constant amount of heavy α -syn prior to sample preparation. The resultant Light/Heavy (L/H) ratios in CSF samples were used to calculate the absolute concentration according to the calibration curves.

Materials and methods

A scheme of the experiment is shown in Figure 1. Details of materials and experiments are described in the Supplemental Information. Briefly, the Institutional Review Boards of all participating sites including 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 approved this study. All CSF samples were obtained after informed consent from patients. The two PD clinical cohorts UDALL and DATATOP were described in our earlier studies [3, 13, 21–23]. Briefly, the inclusion and exclusion criteria included: all PD subjects met the UK PD Society Brain Bank clinical diagnostic criteria for PD. Healthy control subjects were the patients' spouses or community volunteers in good health. They had no signs or symptoms suggesting cognitive impairment or neurological disease.

“Reference CSF” samples were used to develop the assay, and consisted of pooled samples with low blood contamination following clinical assessment at Harborview Medical Center. All clinical CSF samples were obtained by lumbar puncture in the morning, and followed similar sample collection protocols and quality control procedures at all participating centers, in particular, use of polypropylene collection and storage tubes, rapid separation into single use aliquots, and freezing of samples, to minimize potential site variability. All clinical samples received at the University of Washington were thawed, treated with protease inhibitor cocktail (PIC), aliquoted and kept frozen at -80°C until proteomics analysis. CSF concentrations of α -syn and hemoglobin (Hgb), an index of blood contamination, were measured with immunoassays in our previous studies [3, 13, 22, 23].

Details of the proteomics sample preparation are included in the Supplementary Information. Briefly, 200 μL of CSF samples were spiked with equal amount (4pmol) of heavy α -syn. Proteins were precipitated with TCA, washed and re-constituted in 8M urea in 25mM ammonium bicarbonate (ABC), where the protein concentrations were measured using BCA assay. The proteins were reduced and alkylated by dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Samples were diluted 10-fold in 25 mM ABC, to reduce urea concentration, and digested with Trypsin followed by AspN. The resulted peptides were cleaned up with MicroSpin C18 chromatography prior to LC-MS/MS analysis.

Then samples were fractionated with a nanoACQUITY UPLC (Waters) and analyzed with a TSQ Vantage Triple Quadrupole MS (Thermo Fisher). A 30-cm long C18 column with a laser pulled ESI tip was made in house by packing a fused silica capillary (360 μm o.d. $\times$ 75

µm i.d.) with MAGIC C18AQ 100A 5U beads (Michrom Bioresources). A 2-cm long trap column was prepared similarly by packing a 25cm long frit fused silica capillary with MAGIC C18AQ 200A 5U beads. Mass spectra were collected using the scheduled MRM mode, in which 5–7 transitions were targeted for each peptide during an LC retention time window of 8 min (calibration peak retention time $\pm$ 4 min). Collision energies for each transition were calculated using Skyline software. Both of the Q1 and Q3 peak widths (FWHMs) were set to be 0.70.

The collected MRM data were processed using software Skyline (64-bit) 3.5.0.9319 [24]. Typical settings applied include 0.055Th match tolerance and an m/z range between 50 – 1500, default peak integration and no application of smoothing or transformation algorithm. Manual inspection of datasets was done to ensure correct peak detection and integration. Peptides with S/N of at least 3 were considered detectable. Quantification was performed using peak areas of at least 3–4 best performing transitions for both unlabeled (light) and labeled (heavy) peptides. The lower limits of detection (LLOD) and quantification (LLOQ) were determined by analyzing at least two replications. The LLOD of the peptides was defined as the average of the lowest standard concentration at which 3–4 transitions were confidently identified. Likewise, the LLOQ was defined as the average of the lowest standard concentration at which the CV was less than 20%.

Then, statistical analyses were performed using GraphPad Prism version 6.00 for Windows. In the UDALL cohort, two-tailed unpaired t test was used to compare group differences between PD and controls for both Luminex and MRM assays. For DATATOP, paired t test was used to compare final and baseline samples from the same subject. Pearson Linear regression was performed to detect correlations between Luminex and MRM results, as well as the UPDRS or cognitive scores' relationships with MRM results. Values with $p < 0.05$ were considered significant. However, in several cases, potentially dependent data sets were analyzed together; therefore corrections of multiple comparisons were performed using Benjamini Hochberg method to report a q^* value as the corrected significance level by controlling the false discover rate of 0.05 (worksheet author: Manuel Weinkauff, ver 1.1) [25, 26].

Results and Discussion

The calibration curves of six well characterized peptides in α -syn are shown in Supplemental Figure 1. The LLODs are between 0.04–2.89 ng/mL, and the LLOQs are between 0.17 – 22.56 ng/mL (Supplemental Table 1). The six peptides together covered 73% of the α -syn total sequence (position 13–114 out of 140 amino acids). Two reference CSF samples were used to test the reproducibility of the analysis and the CVs were generally below 10% except for one peptide (Supplemental Table 2).

With this strategy, we selected CSF samples from two well-characterized cohorts, where α -syn concentrations were measured previously using Luminex immunoassays [3, 22, 27]. These included a cross-sectional cohort (UDALL) of 15 patients with PD and 15 age- and gender- matched healthy controls; and a longitudinal cohort (DATATOP) of 15 recently diagnosed, drug-naïve PD subjects. DATATOP subjects underwent two CSF collections, the

first at the baseline and the second at a final endpoint (~2 years later in average) when their disease had progressed sufficiently to require L-DOPA therapy. Because blood contamination of CSF samples could affect the α -syn concentration, only samples with low hemoglobin concentrations, as an index of red blood cell contamination, were included in this study. Table 1 summarizes the demographic information, the Unified Parkinson's Disease Rating Scale (UPDRS) motor scores and previously immunoassay measurements. More detailed individual data can be found in Supplemental Table 3.

In the UDALL cohort, a few peptides could be consistently quantified among all the samples, contrasting substantially with the reference samples, in which six peptides were well-characterized. It likely resulted from the significant differences between the clinical and reference samples, the latter of which was collected from non-PD patients, tested for blood contamination and then pooled. Therefore, individual samples with unusually high or low α -syn concentrations may greatly alter the overall pooled sample's concentration. In addition, the reference CSF samples were generally collected from younger people than PD patients. Nonetheless, at least two peptides, TVEGAGSIAAATGFVK and EGVVAAAEK, were reliably and consistently quantified. The best result was obtained from peptide TVEGAGSIAAATGFVK, which was unique to α -syn and quantified consistently over all thirty samples (Supplemental Figure 2, Supplemental Table 4). To our knowledge, this may be the first report using the MRM-MS approach without efforts prior to mass spectrometry analysis to remove highly abundant proteins (e.g. depletion) or enrichment of proteins/peptides of interest (e.g. immunoprecipitation, mass filtering, extra fractionation etc.) to quantify the absolute concentration of human α -syn. The concentration reported here will be helpful in development of quantitation methods with different platforms (e.g. immunoassays etc.) by providing an estimate of their efficiencies and accuracies, and will enable the comparison of multi-site and/or multi-platform results. The average absolute concentration of α -syn in CSF is 1.75 ± 0.29 ng/mL, comparing with our Luminex immunoassay results of 0.36 ± 0.14 ng/mL. However, the two are significantly correlated ($p = 0.0003$) as shown in Figure 2a. Further, as shown in Figure 2b, the MRM quantitation result can be used to distinguish the PD from control groups ($p = 0.0355$), with a statistical power similar to our Luminex assay [3, 27] (Figure 2c, $p = 0.0063$), and is consistent with the observations of several other large independent studies [5, 6, 28, 29]. This value is slightly higher than another publication[12] using fractionation and MRM, where the α -syn concentration was determined to be 0.1–0.4 ng/mL with MRM and 0.5–2.5 ng/mL with ELISA; and the MRM results showed no alteration in Synucleinopathies.

As discussed previously, the relationship between total CSF α -syn level and PD disease severity remains unclear [22], likely because immunoassays rely on antibodies that capture certain forms or subgroups of α -syn, e.g., monomeric, oligomeric, truncated forms or those with various PTMs. However, using the strategy of non-selective digestion and mass spectrometry, we could target the same peptide generated from all forms of α -syn. Therefore, the most important observation from the cross-sectional (UDALL) cohort may be that the peptide TVEGAGSIAAATGFVK showed a significant correlation with the PD patients' UPDRS motor scores ($p = 0.0007$, $R^2 = 0.6023$), which measure motor functions from patients and can be used as indicators of disease severity/progression over time (Figure 2d). This peptide covers the protein sequence between sites 81 and 96, where a previously

reported phosphorylation site pS87 resides [30]. It is not clear whether this correlation may be caused by differential phosphorylation levels on S87. This might be answered by investigating the corresponding phosphorylated peptide(s) directly, and we will conduct this more challenging MRM-MS analysis with further method development in future studies.

In addition to TVEGAGSIAAATGFVK, peptide EGVVAAAEK was detected in 24 samples, which resides on the N-terminal of the protein (13–22). However it was only quantified in 21 samples with a minimum graphic S/N of 3. This region of α -syn is shared with β -syn, resulting in a higher concentration (12.43ng/mL) without correlation with the Luminex assay ($p > 0.01$). Yet the peptide modestly correlates with the UPDRS score with a negative slope ($p = 0.0185$) (Supplemental Figure 3). The biological or clinical meaning of this correlation is not clear; however, the opposite slopes of the two α -syn peptides may help explain some of the earlier controversy as to whether or how the α -syn levels in CSF correlate with PD severity. It is possible that the concentrations of various isoforms of α -syn were altered in different directions during the course of the disease, leading to conflicting results when using immunoassays where one form might be preferentially detected over others.

In addition, we further tested 30 samples of 15 early stage PD subjects from the longitudinal DATATOP cohort. As mentioned earlier, CSF samples from each PD patient were collected twice (“baseline” and “final” time points), and the patients’ disease statuses were measured with a battery of tests assessing motor and cognitive functions. The patients were not treated with anti-parkinsonism drugs between the baseline and final visits. For peptide TVEGAGSIAAATGFVK, although it was detected in all 30 samples, only 24 were considered quantifiable after filtering with the graphic S/N (Supplemental Table 4, Supplemental Figure 4). In addition, two other samples were excluded due to abnormal observations during the sample preparation (e.g. white cloudiness that cannot be re-dissolved after protein precipitation). So in total 22 samples were further analyzed with statistical tools. As shown in Figure 3a, the MRM results correlate with our earlier Luminex measurements in all 22 samples with $p < 0.0001$ and $R^2 = 0.5938$ (2.48 ± 0.31 vs. 0.46 ± 0.15 ng/mL). When we tested the correlation between the MRM results and the UPDRS motor scores, the baseline samples showed a positive trend ($p = 0.0344$, $q^* = 0.025$, Figure 3b).

Furthermore, the cognitive statuses of the patients were also monitored and tested against MRM results. As shown in Figure 3c, negative trends were observed between MRM and the Symbol Digit Modalities Test (SDMT, a test of visuospatial working memory/processing speed) using either baseline or final data (12 and 10 samples, $p = 0.0062$ and 0.0253 , respectively). Baseline MRM results also showed correlating trends with three other cognitive tests (Supplemental Figure 5, $p = 0.0478$, 0.0478 and 0.0399): Odd-man Out Reaction Time Tests (OMO RT) with Trial 4 Correct, OMO RT with Trial 4 Errors (testing the speed to interpret the relationships and differences among stimuli), and Purdue Pegboard (testing the manual dexterity and bimanual coordination). These results enhanced our earlier observations using the same cohort and immunoassays, which only detected significant cross-sectional associations with a single memory test (SRT-Delayed at baseline; SRT-Total at final time point) [22]. Of particular note, if all of the cognitive tests were related, the p

values needed to be further corrected for multiple comparisons, and the significance cut-off was dropped to 0.002, in which case, the current results were not considered significant.

Furthermore, we evaluated the longitudinal changes using paired t-test and found a slight decrease of α -syn concentrations from baseline to the final samples ($p = 0.4411$), likely due to the small sample size (8 subjects with both quantifiable baseline and final MRM data). Our previous study using ~400 DATATOP subjects demonstrated that CSF levels of α -syn, measured with the Luminex immunoassay, significantly decreased over the study period [22]. These suggest that we might need to test more subjects with the MRM assay to replicate the immunoassay results.

To illustrate the ability of MRM to distinguish faster or more aggressive disease progression, we tested the relationship between the change rate in UPDRS motor score (delta UPDRS motor divided by years passed from baseline to final) and the change in MRM results. Remarkably, despite the small sample size, there was a significant correlation between the concentration decrease of peptide TVEGAGSIAAATGFVK and the increase rate of UPDRS motor score ($p = 0.0167$, $R^2 = 0.6427$) (Figure 3d). In other words, patients with greater decreases in the α -syn concentrations experienced a faster progression of motor symptoms. Interestingly, our previous Luminex data for the same cohort showed that total α -syn levels cannot predict or track the decline of motor functions. This suggests that the peptide, instead of the whole protein, might better track the pathological changes during the disease progression. Alternatively, if we simply compared the baseline MRM results (12 samples) vs. the UPDRS motor scores change rates, a positive trend was observed showing that higher baseline α -syn concentration modestly indicated faster disease progression, yet the result was not statistically significant ($p = 0.1362$, $R^2 = 0.2080$). This observation suggested that the baseline peptide level alone was not predicting, but the small sample size should also be considered.

As mentioned, peptide TVEGAGSIAAATGFVK contains a possible phosphorylation site at the residue Ser87. The relationship between TVEGAGSIAAATGFVK and the PD disease progression may result from changes of the phosphorylation levels. In our previous study, the levels of a phosphorylated α -syn (pS129) negatively correlate with the PD disease severity for early onset patients, while the trend was opposite for later time points [23]. Similarly, the MRM results here may reflect differential PTMs, such as phosphorylation levels on S87. Therefore, a mass spectrometry method that can quantify the pS87 levels is in high demand. However, our current set of experiments did not observe pS87 in either control or PD samples. This is likely due to the low abundance of the modified form. Therefore, a further separation or enrichment method will be necessary to target the pS87 peptide. For example, a two-dimensional HPLC separation coupled with MRM mass spectrometry strategy has been shown to quantify species with sub pg/mL level [20]. Another possible strategy is to develop antibodies to target the specific modified peptide, and perform an immuno-enrichment prior to the MRM analysis [31]. Further, the peptide TVEGAGSIAAATGFVK showed different trends in early onset patients vs. patients with longer disease duration, a finding worthy of dedicated future studies.

Other than the peptide TVEGAGSIAAATGFVK, the N-terminal peptide EGVVAAAEK was also successfully measured from the majority of DATATOP samples (21 out of 30 samples). As discussed, the peptide is shared with β -syn, thus further statistical analyses showed ambiguous information for clinical uses (Supplemental Table 4).

Concluding Remarks

A sensitive MRM assay without target enrichment or CSF fractionation was developed to quantify α -syn levels. Recombinant light and heavy isotopically labeled pure α -syn standard proteins were used to build calibration curves and for normalization, permitting measurement of the absolute concentrations of CSF α -syn. The assay was applied to two small cohorts of PD patients, including cross-sectional and longitudinally collected samples. From the 60 CSF samples, the absolute concentration of α -syn was shown to be about 2.1ng/mL, which is slightly higher than our previous immunoassay reading (about 0.5ng/mL). However, peptide TVEGAGSIAAATGFVK showed excellent correlations with Luminex measurements; it can be used to distinguish control from PD samples; and it showed significant correlations with motor tests in PD subjects. Additionally, changes of the peptide tracked disease progression (motor score changes) in the longitudinal cohorts. Thus this peptide is likely a promising PD disease diagnostic and prognostic biomarker. Our future plan is to continue method development for phosphorylated α -syn in addition to validate our results in larger and independent cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

α-syn	alpha synuclein
CSF	cerebrospinal fluid
PD	Parkinson's disease
PIC	protease inhibitor cocktail
TCA	trichloroacetic acid
ABC	ammonium bicarbonate
FWHM	full width at half maximum
LLOD	lower limit of detection

LLOQ	lower limit of quantitation
UPDRS	Unified Parkinson's Disease Rating Scale
SDMT	Symbol Digit Modalities Test
OM-ORT	Odd-man Out Reaction Time Tests

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Statement of Importance

In the present study, we made several major advances with regard to measuring CSF α -syn levels. First, we developed a highly sensitive Multiple Reaction Monitoring (MRM) method to measure CSF α -syn peptides without prior enrichment or fractionation. Next, we employed this method and quantified α -syn levels in cross-sectional and longitudinal cohorts. We demonstrated the absolute concentration of α -syn in human CSF is about 2.1 ng/mL. Moreover, a unique α -syn peptide displayed excellent correlation with our previous immunoassay results in both cohorts ($p < 0.001$). Despite of the relatively small sample size, we have, for the first time to our knowledge, found a robust elevation of CSF α -syn levels in controls as compared to PD patients, with similar distinguishing power as has been achieved using immunoassays, which is the best performed biomarker quantitation method for PD described to date. More excitingly, this peptide correlated with disease severity, and its changes even tracked the disease progression longitudinally, which were measured with the UPDRS motor scores and the cognitive test scores. Considering the previous total α -syn assays did not show such correlations, the specific peptide, but not the total α -syn, has the potential to help in monitoring or predicting disease progression.

Figure 1.

Scheme of the LC-MS-MRM experiment.

a. Human CSF samples preparation and analysis scheme. Equal amount of heavy isotope (¹⁵N) labeled recombinant α-syn was spiked into 200uL of human CSF samples. The CSF samples were then precipitated with TCA, digested with trypsin and AspN sequentially, and purified with C18 spin columns. The digested samples were analyzed with MRM LC-MS/MS.

b. Calibration samples preparation and analysis scheme. Heavy isotope labeled and non-labeled light recombinant α-syn proteins were digested separately in two Eppendorf tubes, together with a matrix background of BSA. The digestion and purification procedures are the same as the CSF samples. Then the light α-syn peptides were serially diluted to form a calibration curve, while the heavy α-syn peptides were spiked into the light samples with a constant concentration and served as a normalization factor.

Figure 2.

MRM results of peptide TVEGAGSIAAATGFVK in the UDALL cohort.

- MRM results correlated significantly with total α -syn levels measured with Luminex immunoassay ($p = 0.0003$).
- MRM results showed a significant decrease of α -syn levels between PD and control samples ($p = 0.0355$).
- Previous Luminex results showed a significant decrease of α -syn levels between PD and control samples ($p = 0.0063$).
- MRM results showed a significant correlation with UPDRS motor scores ($p = 0.0007$), which indicated PD severity.

Figure 3.

MRM results of peptide TVEGAGSIAAATGFVK in the DATATOP cohort.

- MRM results significantly correlated with the total α -syn levels measured with Luminex immunoassay ($p < 0.0001$).
- MRM results showed a strong correlation with the UPDRS motor scores of the baseline samples ($p = 0.0344$).
- Trends between the patients' MRM results and their cognitive statuses were observed with the Symbol Digit Modalities Test ($p = 0.0062$ for baseline samples and $p = 0.0253$ for final).
- Faster decrease of the peptide concentration of TVEGAGSIAAATGFVK indicated a more aggressive PD progression ($p = 0.0167$).

Table 1

Demographic information and immunoassay measurements of CSF samples. Note: the total protein concentrations were measured with BCA assay after TCA precipitation. The Hemoglobin levels were measured with ELISA and α -syn levels were measured with Luminex assay.

a. Cross-sectional cohort UDALL

	Control	Parkinson's disease (PD)
Number of cases	15	15
Sex (F/M)	7/8	7/8
Age (years)		
Mean $\pm$ SD	56.7 $\pm$ 5.9	57.9 $\pm$ 7.6
Range	42 – 65	41 – 70
CSF total protein (mg/dL)		
Mean $\pm$ SD	22.41 $\pm$ 4.68	24.73 $\pm$ 6.50
Range	15.26 – 27.96	18.00 – 45.29
CSF Hemoglobin (ng/mL)		
Mean $\pm$ SD	32.2 $\pm$ 54.5	55.0 $\pm$ 84.7
Range	0.0 – 199.0	0.0 – 336.01
CSF α -synuclein (ng/mL)		
Mean $\pm$ SD	0.43 $\pm$ 0.14	0.29 $\pm$ 0.11
Range	0.22 – 0.72	0.054 – 0.41
UPDRS score		
Score 15		5
15 < Score 30		5
Score > 30		5

b. Longitudinal cohort DATATOP

	Baseline	Final
Number of cases	15	15
Sex (F/M)	6/9	6/9
Age (years)		
Mean $\pm$ SD	59.1 $\pm$ 10.1	61.1 $\pm$ 10.0
Range	44 – 76	46 – 77
Disease duration (years)		
Mean $\pm$ SD	1.3 $\pm$ 0.5	3.3 $\pm$ 1.0
Range	1 – 2	2 – 5
UPDRS motor score		
Mean $\pm$ SD	14.7 $\pm$ 9.0	27.5 $\pm$ 10.4
Range	0 – 30.5	5.0 – 43.5
CSF total protein (mg/dL)		
Mean $\pm$ SD	30.4 $\pm$ 8.6	28.9 $\pm$ 4.0
Range	21.2 – 49.5	21.0 – 35.1

a. Cross-sectional cohort UDALL

	Control	Parkinson's disease (PD)
CSF Hemoglobin (ng/mL)		
Mean $\pm$ SD	44.8 $\pm$ 36.6	29.4 $\pm$ 27.1
Range	0.0 – 152.6	0.0 – 97.8
CSF α -synuclein (ng/mL)		
Mean $\pm$ SD	0.50 $\pm$ 0.16	0.41 $\pm$ 0.13
Range	0.32 – 0.81	0.25 – 0.66

Note: all patients were not treated with drugs.