Mass spectrometric analysis of Lewy body-enriched α-synuclein in Parkinson's disease

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List of abbreviations:

ACN, acetonitrile; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; DTT, dithiothreitol; FA, formic acid; IP, immunoprecipitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDS, lauryl dodecyl sulfate; LN, Lewy neurites; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; MSA, multiple system atrophy; PBST, phosphate-buffered saline (pH 7.4) with Tween-20; PD, Parkinson's disease; PDD, Parkinson's disease with dementia; PRM, parallel reaction monitoring; PTMs, post-translational modifications; TBS, Tris buffered saline (pH 7.6); TFA, trifluoroacetic acid; WB, western blotting.

Keywords: α-synuclein, Lewy bodies, mass spectrometry, Parkinson's disease, parallel reaction monitoring.

Abstract

Parkinson's disease (PD) is characterized by intra-neuronal inclusions of aggregated α-synuclein protein (so called Lewy bodies) in distinct brain regions. Multiple posttranslational modifications may affect the structure and function of α-synuclein. Mass spectrometry-based analysis may be useful for the characterization and quantitation of α-synuclein forms, but has proven challenging, mainly due to the insolubility of Lewy bodies in aqueous buffer. In the present study, we developed a novel method by combining differential solubilization with immunoprecipitation and targeted proteomics using liquid chromatography and tandem mass spectrometry. Brain tissue homogenization and sample preparation were modified to facilitate analysis of soluble, detergent soluble and detergent insoluble protein fractions (Lewy body-enriched). The method was used to compare α-synuclein forms from cingulate cortex (affected) and occipital cortex (unaffected) in two study sets of PD patients, and controls. We identified ~20 modified α-synuclein variants, including species with N-terminal acetylation and C-terminal truncations at amino acids 103 and 119. The levels of α-synuclein forms Ac-α-syn$_{1-6}$, α-syn$_{13-21}$, α-syn$_{35-43}$, α-syn$_{46-58}$, α-syn$_{61-80}$ and α-syn$_{81-96}$ except Ac-α-syn$_{103-119}$ were significantly increased in PD cingulate region compared to controls in the Lewy body-enriched α-synuclein fraction. In the soluble fraction, only Ac-α-syn$_{1-6}$ was significantly increased in PD compared to controls. None of the detected α-synuclein variants was Lewy body-specific, but acetylated forms should be examined further as potential biomarkers for abnormal α-synuclein accumulation.

Introduction:

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disease affecting approximately 10 million people worldwide (1). Although it typically develops after the age of 65, about 4% of people develop "young-onset" PD before the age of 50 (1). Pathological aggregation of the intrinsically disordered protein α-synuclein into Lewy bodies is a hallmark of PD and other synucleinopathies (2). Neuropathologically, PD is defined by loss of dopaminergic neurons in the substantia nigra, the presence of Lewy bodies and Lewy neurites made of unbranched α-synuclein filaments (3). α-Synuclein pathology predominantly affects the neocortex, hippocampus, substantia nigra (SN), and thalamus (4). The α-synuclein inclusions are also distributed in the peripheral and

the enteric nervous system (5). The typical symptoms of PD include motor dysfunction, bradykinesia, rigidity and tremor in the early stage and at advanced stage cognitive dysfunctions (4). Inclusions of α-synuclein are found in PD, but also in PD dementia (PDD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), collectively known as synucleinopathies (3). In PD, PDD and DLB, the Lewy body-containing insoluble α-synuclein deposits are found primarily in neurons, whilst cytoplasmic inclusions in oligodendrocytes are common in MSA (4, 6). The distinction between PD, PDD and DLB is based on the time of onset of motor symptoms and dementia. However, the regional distribution and progression patterns of α-synuclein pathology may be modified by several factors, including genetic variation (*e.g. SNCA, LRRK2, VPS35, PARK2, PINK1*) and other co-existing pathologies resulting in significant overlap between the synucleinopathies (7-9).

There has been a focus on establishing biomarkers reflecting Lewy body pathology in PD and other synucleinopathies in biofluids, especially in cerebrospinal fluid (CSF). Most available assays measure total amount of α-synuclein, but there are also assays targeting oligomeric and phosphorylated α-synuclein or other modified forms (10-16), as well as novel assays that qualitatively detect the amplified biochemical signal of α-synuclein seeds that may be related to Lewy body pathology (16, 17). Despite considerable variation in assay specificity and performance among the quantitative assays, the general pattern that has emerged is a modest decrease in total α-synuclein and an increase in phosphorylated and oligomeric α-synuclein in PD (18, 19). Even though, the general trend is a decrease in total α-synuclein level in CSF, the large overlap of individual values decreases clinical usefulness (20). Additionally, in Alzheimer´s disease (AD) and Creutzfeldt-Jakob's disease (CJD), CSF α-synuclein concentration is increased and correlates with CSF tau concentration, indicating that α-synuclein might also be a non-specific marker of neurodegeneration (11, 21-23). Increased CSF concentration of α-synuclein has also been reported in DLB, where a competition might exist between the aggregation of α-synuclein into Lewy bodies and its release from degenerating synapses (where it is abundantly expressed), complicating the data interpretation (24). In agreement with this hypothesis, a recently published, multiple reaction monitoring mass spectrometry assay revealed significantly increased CSF concentrations of α-synuclein in AD and CJD, but not in the 'classical' synucleinopathies like PD (15). Yet another problem with α-synuclein as a CSF biomarker is that the measurements may be confounded by α-synuclein release from erythrocytes, where is it highly expressed (25), in cases of blood contamination of the sample.

Full-length α-synuclein comprises 140 amino acids containing three distinct regions: residues 1–60 containing four 11-amino acid repeats (KTKEGV); residues 61–95 containing the hydrophobic and highly amyloidogenic non-Aβ component (NAC) region and residues 96–140, the acidic C-terminal region (26, 27). The physiological function of α-synuclein is largely unknown, but the protein is highly expressed in synapses and may play a role in neuronal plasticity and protect from apoptosis and oxidative damage (28-30).

In vitro studies have suggested that α-synuclein may undergo a conformational transition from helical structure to anti-parallel β-sheet structures exposing the NAC domain that can participate in hydrophobic interactions and initiate the process of aggregation (31, 32).

The highly organized fibrils of aggregated α-synuclein are the major constituent of Lewy bodies (33). α-Synuclein can be modified by multiple covalent posttranslational modifications (PTMs) like phosphorylation, nitration, methionine oxidation, glycosylation, ubiquitination, sumoylation, N-terminal acetylation and truncations that may affect both structure and function of α-synuclein. Phosphorylated α-synuclein (at S87, S129, or Y125) and truncated forms (α-syn1-110, α-syn1-120, α-syn1-121) increase the aggregation propensity of α-synuclein (21, 34-37). One study suggested that aggregation-prone C-terminally truncated α-synuclein forms oligomers and then facilitates the aggregation of full-length α-synuclein leading to formation of fibrils (38). Analysis of PTMs of α-synuclein is challenging due to the insolubility of Lewy bodies in aqueous buffer for tissue homogenization, loss of α-synuclein during the biochemical fractionation of brain tissue procedure, sample preparation steps for immunochemical analysis, efficiency of digestion of α-synuclein and finally, solubility and ionization of digested peptides for mass spectrometry-based analysis (39).

We have previously used immunoprecipitation combined with nanoflow liquid chromatography (LC) coupled to high resolution electrospray ionization Fourier transform ion cyclotron resonance tandem mass spectrometry (ESI-FTICR-MS/MS) to determine known and novel isoforms of α-synuclein (Ac-α-syn$_{1-140}$, Ac-α-syn$_{1-139}$, and Ac-α-syn$_{1-103}$) in brain tissue homogenates (40). In this study, we have expanded the investigation into the detergent insoluble (Lewy body-enriched) fraction of α-synuclein. We have also developed a novel method where we combined immunoprecipitation with targeted proteomics using Parallel Reaction Monitoring Liquid Chromatography-tandem/Mass Spectrometry (PRM LC-MS/MS)

to achieve better quantification of the identified α-synuclein forms. Brain tissue homogenization and sample preparation were modified to facilitate the analysis of soluble, detergent-soluble and detergent-insoluble protein fractions. The developed method was subsequently used to compare soluble, detergent-soluble and detergent-insoluble α-synuclein forms from cingulate cortex and occipital cortex in two cohorts of PD patients and controls.

1. Materials and Methods

1.1. α-Synuclein proteins and heavy isotope labeled peptide standards

Recombinant full length α-synuclein (Mw 14.460 kDa; rPeptide) and uniformly (15)N-labeled α-synuclein (Mw 14.624 kDa; rPeptide) were dissolved in ultrapure water at a concentration of 1 g/L. The heavy stable isotope (SI)-labeled peptide standards of α-synuclein (Ac-α-syn$_{1\text{-}6[K]}$, α-syn$_{13\text{-}21[K]}$, α-syn$_{35\text{-}43[K]}$, α-syn$_{46\text{-}58[K]}$, α-syn$_{61\text{-}80[K]}$, α-syn$_{71\text{-}80[K]}$, α-syn$_{81\text{-}96[K]}$, and α-syn$_{103\text{-}119[L]}$) were custom synthesized with (13)C- and (15)N-labeled lysine [K] or (13)C- and (15)N-labeled leucine [L] at ThermoFisher Scientific, USA (Heavy Peptide FasTrack 1 standards). The purity of the peptides was >95%. IS peptides were dissolved in 1 mL water, aliquoted and stored at −70 °C.

2.2 α-Synuclein antibodies

The following antibodies were used: the mouse monoclonal anti-α-synuclein antibody Clone 42 (BD Transduction Laboratories, USA), which was raised against amino acids 15–123 of rat α-synuclein that recognizes amino acids 85-98 of α-synuclein (41), and the rabbit monoclonal anti-α-synuclein antibody MJFR1 (Abcam, UK), which recognizes amino acids 118-123 of human α-synuclein.

2.3. Human brain tissue samples

Frozen post-mortem brain tissue was obtained from Queen Square Brain Bank, London, UK. Two brain regions, cingulate cortex as disease affected and occipital cortex as disease unaffected, was collected from clinically and pathologically confirmed patients with PD (n = 10) and controls (n = 10) in two sets for two studies (denoted study 1 and 2). The presence of Lewy bodies and α-synuclein was confirmed by immunohistochemistry. The stages of PD were categorized according to the Braak and Braak criteria, which are based on the abundance of α-synuclein in different brain regions. All the PD and control cases belonged to stage 5 or 6 and stage 0, respectively. The control cases were significantly older than the PD cases. Post-mortem

delays were comparable between PD and control cases. The age at onset and duration of the disease in PD cases were not significantly different between study 1 and 2. The clinical and demographic characteristics of the brain samples (study 1 and 2) are presented in table 1.

2.4. Homogenization of brain tissue

The sequential homogenization of brain tissue was performed following the method as described (40) with modifications specified below. During the entire process, the samples were kept on ice. Brain tissue (~100 mg) was homogenized in 1 mL Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.6 containing 137 mM NaCl; Sigma–Aldrich) containing complete protease inhibitor. Centrifugation of the homogenate was performed at 31,000 *g* for 1 h at +4°C and the supernatant was collected (soluble fraction). The pellet was washed two times with TBS containing protease inhibitor. After each wash, the samples were centrifuged at 31,000 g for 10 min at +4°C and supernatants discarded. After adding 0.8 mL 2% Triton-X-100 containing protease inhibitor (Sigma–Aldrich), the pellet was homogenized and sonicated for 30 seconds and then centrifuged as above. The supernatant (detergent-soluble fraction) was collected and the pellet was washed two times with 2% Triton-X-100 containing protease inhibitor (see above). Then, 0.8 mL 90% formic acid (FA; Sigma–Aldrich) was added to the pellet, which was homogenized, sonicated and centrifuged as above and the supernatant (detergent insoluble fraction) was collected. The pellet was stored at -80°C for further analysis. The detergent insoluble fraction was immediately diluted with ultrapure water (1:20, v/v) in presence of 72.5 mM NaCl (Sigma–Aldrich) and lyophilized followed by reconstitution of the dried sample in 0.3% n-octyl β-D-glucopyranoside (n-OGP; Sigma–Aldrich) in 100 mM Na-phosphate (pH 7.4; Sigma–Aldrich). In study 2, (15)N-labeled α-synuclein protein (5 µg) was added in each biochemical fractionation step in order to monitor if any α-synuclein modification were created by the sample preparation. Pooled samples were prepared by mixing equal amount of individual brain tissue extracts from each fraction from individual groups to use it as a quality control sample. The total protein amount in each sample was determined using Protein DC assay reagent (Bio-Rad Laboratories).

2.5. Western blotting (WB)

Brain tissue extracts were mixed (2:1, v/v) with NuPAGE sample buffer (3X) (Thermo Fisher Scientific) containing 12% lithium dodecyl sulphate (LDS, Sigma–Aldrich) and 150 mM dithiothreitol (DTT, Sigma–Aldrich). Pellets were dissolved in NuPAGE sample buffer (1X).

Then, 5 µg (soluble and detergent soluble) and 0.5 µg (detergent insoluble) of total protein were electrophoresed on NuPAGE 12% Bis–Tris gel using the NuPAGE mini-gel system (Thermo Fisher Scientific) at 120 V for 1 h. Before transfer, the membrane and the blotting papers (4X thick filter paper; BioRad) were soaked in transfer buffer (Thermo Fisher Scientific) containing 10% methanol (Thermo Fisher Scientific) for 1 h at room temperature shaking. The gel was soaked in the transfer buffer containing 10% methanol for 15 min at room temperature. The proteins were transferred onto nitrocellulose membranes (0.22 µM; GE Healthcare Life Sciences) using semi-dry blotting technique. The transfer was performed at 0.05 A per gel with 60 cm^2 area for 1h 20 min. Blocking was performed for 3 h at room temperature using 1% bovine serum albumin (BSA, Sigma-Aldrich) in phosphate-buffered saline containing 0.05% Tween-20 (PBST, Sigma-Aldrich). Incubations with the monoclonal anti-α-synuclein antibodies, BD Clone 42 (250 mg/L, dilution 1:1000) or MJFR1 (1 g/L, dilution 1:1000), and without primary antibody (negative control) were performed overnight at +4°C. The membranes were washed three times with PBST and then incubated for 1 h at room temperature with biotinylated anti-mouse (1.5 mg/ml, diluted 1:5000; Vector Laboratories) or biotinylated anti-rabbit (3mg/ml, dilution 1:5000; Sigma-Aldrich) IgG antibody in 1% BSA in PBST. A second PBST wash was performed after which the membranes were incubated for 1 h at room temperature with streptavidin-biotinylated horseradish peroxidase complex (diluted 1:20000 in 1% BSA in PBST; Kem-En-Tec Diagnostics). Following three times washes, the membranes were developed for 2 min with ECL Advanced solution (GE Healthcare Life Sciences) according to the manufacturer's instructions. The emitted signal was detected by a Fujifilm LAS-3000 System (FUJIFILM Corporation) and evaluated using the MultiGaugev2.2software (FUJIFILM Corporation). The amount of in brain tissue extracts to be loaded in gel to get a clear and quantifiable band of α-synuclein were optimized using a standard curve (R^2= 0.9453) prepared by densitometric quantification of bands of different amount (0.0025-0.01µg) of recombinant α-synuclein standard in WB. Band intensities α-synuclein was based on the optical density of each band subtracting the background of the blot. All samples were run and analyzed in duplicate on two separate gels. The coefficient of variation (CV) was less than 10% between two duplicate samples in each gel. The semi-quantitative estimation of the most prominent band of α-synuclein (17 kDa) in all the samples was done by normalizing the band intensities of α-synuclein from individual brain samples against the band intensities of the pooled PD sample from each fraction from cingulate cortex region followed by calculating the mean value of individual samples from two separate gels.

2.6. Immunoprecipitation

Immunoprecipitation (IP) of brain tissue homogenates was performed as described earlier (40, 42). In brief, an aliquot (1 µg) of the mouse monoclonal anti-α-synuclein antibody Clone 42 (250 mg/L) or IgG from murine serum (1 g/L, a negative control) was separately added to 50 µL magnetic DynabeadsM-280 Sheep anti-mouse IgG (Thermo Fisher Scientific) per sample and incubated 1 h on a rocking platform at room temperature. The beads were washed three times with 1 mL of phosphate buffered saline (PBS, pH 7.4). The antibodies were cross-linked using 20 mM dimethylpimelimidate dihydrochloride (DMP; Sigma–Aldrich) and 0.2 M triethanolamine (pH 8.2; Sigma–Aldrich) and the reaction was stopped by addition of 50 mM Tris-HCl, pH 7.5. The cross-linked beads were washed 3 times in PBS and the blocking of unbound sites was performed for 1 h at room temperature using 1 mL Roti®-block (1x) (Carl Roth, GmbH). The samples described below were incubated with antibody coupled magnetic beads in presence of PBS containing 0.025% Tween-20 (soluble fractions) and PBS (detergent soluble and detergent insoluble fractions) overnight on a rocking platform at +4°C. The magnetic beads/sample solution was transferred to tube 1 in the KingFisher magnetic particle processor (Thermo Fisher Scientific). The following three wash steps (tubes 2–4) were conducted for 10 s in 1 mL of each washing buffer: (tube 2) 0.025% Tween-20 in PBS, (tube 3) PBS and (tube 4) 50 mM ammonium bicarbonate (NH_4HCO_3, pH 8.0; Sigma-Aldrich). Then, the washed beads were resuspended in 100 µL 50 mM NH_4HCO_3, pH 8.0 in tube 5 and transferred to polypropylene tubes.

Samples study 1: Soluble (25 µg total protein) brain fractions together with uniformly labeled recombinant (15)N-labeled α-synuclein (500 fmol/sample) were incubated with antibody coupled magnetic beads in presence of PBS containing 0.025% Tween-20 overnight on a rocking platform at +4°C. The detergent soluble (25 µg total protein) and detergent insoluble (10 µg total protein) brain fractions together with (15)N-labeled α-synuclein (500 fmol/sample) were incubated in presence of PBS overnight on a rocking platform at +4°C.

Samples study 2: In study 2, the (15)N-labeled α-synuclein was added at the homogenization step (see section 2.4). The soluble (25 µg total protein) brain fractions were incubated with antibody coupled magnetic beads in presence of PBS containing 0.025% Tween-20 and the detergent soluble (25 µg total protein) and detergent insoluble (10 µg total protein) brain fractions were incubated in presence of PBS overnight on a rocking platform at +4°C.

2.7. Recovery and digestion efficiency

The monitoring of recovery of α-synuclein after IP and vacuum centrifugation using MALDI-TOF-MS analysis and method optimization of digestion of α-synuclein with different enzymes have been described in detail in the supporting information.

2.7. On-bead digestion with trypsin

Disulphide bonds were reduced by adding 10 µL 10 mM dithiothreitol (DTT) in 50mM NH_4HCO_3 to each sample followed by 30 min incubation at +60°C at 400 rpm. After cooling down to +25°C, alkylation was performed by adding 10 µL 10 mM iodoacetamide (IAM) in 50mM NH_4HCO_3, and incubating for 30 min at +25°C at 400 rpm in in dark. Sequencing grade modified trypsin (Promega, 20µg dissolved in 0.01% aqueous hydrochloric acid and diluted to 5 mg/L in 50 mM NH_4HCO_3, pH 8) was added to each sample (10 µL) and incubated overnight at +37°C at 400 rpm. The reaction was stopped by addition of 10 µL of 10% aqueous FA followed by centrifugation 16910 *g* for 10 min. The supernatant was collected and divided into two polypropylene tubes. The samples were dried using vacuum centrifugation.

2.8. Data dependent nanoflow LC–MS/MS analysis

On-bead-digested dried samples were dissolved in 10 µl 20% ACN containing 0.1% TFA, mixed thoroughly and transferred to LC vials (SUN-Sri). The nanoflow LC-ESI-MS/MS spectra were acquired on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer coupled to Dionex Ultimate 3000 RSLC nano system (Thermo Fisher Scientific). A C_{18} column Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. x 15 cm, nanoViper (Thermo Fisher Scientific) was used for high-resolution separation and Acclaim PepMap 100 C18, length 500 mm; i.d. 75µm, particle size 2 µm (Thermo Fisher Scientific) was used for desalting. Mobile phases were 0.1% aqueous FA (v/v) (A) and 0.1% FA in 84% ACN in water (v/v) (B). Loading buffer was 0.05% FA in 2% ACN in water (v/v). The sample (5 µL) was loaded on the trapping column with 5µL/min loading buffer. After 2 minutes of washing the peptides were loaded on the separation column with 3% buffer B at 150 nL/min and subsequently eluted with a 50 min linear gradient of 5%–40% buffer B. The mass spectrometer was set to acquire positive ions in the data-dependent mode, with a scan cycle consisting of single microscans of one full scan mass spectrum followed by up to ten MS2 scans. Settings were as follows: spray voltage (+1.7 kV); capillary temperature (+250°C); resolution (70000 MS, 17500 dd-MS2); AGC target (1e6 MS, 5e4 dd-MS2); maximum injection time (250ms MS, 60ms dd-MS2), m/z isolation window

(m/z 300-1200 MS, m/z 2.0 dd-MS2). MS/MS scans were acquired with a normalized collision energy (NCE) setting of 28.0 for the ten most intense precursor ions with an intensity greater than 1.7e4, charge state 2-4, and a dynamic exclusion for 50 sec. Mascot Distiller v. 2.3.2.0 was used for database search and protein identification. Specified search parameters were database (Swissprot), taxonomy (*Homo sapiens*), enzyme (semi-trypsin), variable modifications (acetyl [N-term] and oxidation [M]), static modification (carbamidomethyl [C]), mass values (monoisotopic), peptide mass tolerance (± 15 ppm), fragment mass tolerance (± 20 mmu), and maximum 2 missed cleavages. The target false discovery rate (FDR) for peptide spectrum matches (PSMs) was 0.01. On average, individual ions scores > 40 indicate identity or extensive homology ($p< 0.05$) was considered for identification. Ions score is −10log(P), where P is the probability that the observed match is a random event.

2.9. Targeted microflow LC-MS/MS analysis

High-resolution parallel reaction monitoring (PRM) analyses were performed on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 chromatography system (Thermo Fisher Scientific). Mobile phases were 0.1% aqueous FA(v/v) (A) and 0.1% FA in 84% ACN in water (v/v) (B). The IS peptides standard stock solutions were thawed, mixed and further diluted in 20% ACN containing 0.1% FA to a final concentration of 10 fmol/µL. The LC gradient, MS scheduling and quantitated transitions were set to avoid interference of co-eluting peptides of similar mass-to-charge ratios belonging to other brain proteins. The IonMax electrospray ion source settings were: spray voltage, +4100 V; capillary temperature, +320 °C; sheath gas pressure setting, 25 arbitrary units; auxiliary gas pressure setting, 10 arbitrary units; and probe heater temperature, +300 °C. The instrument was set to acquire scheduled pairs or triplets of PRM scans in profile mode, allowing parallel detection of fragments from corresponding brain derived α-synuclein peptide, IS peptide and (15)N-labeled peptide derived from the (15)N-labeled recombinant α-synuclein. The settings were as follows: resolution setting, 70,000; AGC target, 3e6; maximum injection time, 250 ms; isolation window 3.0 m/z. The NCE settings were optimized for each peptide by injecting respective IS peptide solution.

Study 1: The dried samples after IP and on-bead digestion were dissolved in 55 µL of a mixture of IS peptides (Ac-α-syn$_{1\text{-}6}$, α-syn$_{13\text{-}21}$, α-syn$_{35\text{-}43}$, α-syn$_{46\text{-}58}$, α-syn$_{61\text{-}80}$n, α-syn$_{81\text{-}96}$, and α-syn$_{103\text{-}119}$) mixed thoroughly for 1 h and transferred to LC vials (SUN-Sri). Samples (50 µL) were loaded directly onto a HypersilGold-C18 column, (length 100 mm, inner diameter 2mm,

particle size 1.9µm, Thermo Fischer Scientific) with 0.1% aqueous FA at 300 µL/min. After 2 min of loading, the peptides were eluted off the column using the following linear gradient steps: 0 min 0%B; 4 min 17%B; 16min 35%B; 17.5 min 100%B; 20 min 0%B.

Study 2: The dried samples after IP and on-bead digestion were dissolved in 55 µL of a mixture of IS peptides (Ac-α-syn$_{1-6}$, α-syn$_{13-21}$, α-syn$_{35-43}$, α-syn$_{61-80}$, α-syn$_{71-80}$, and α-syn$_{103-119}$) mixed thoroughly for 1 h and transferred to LC vials (SUN-Sri). Samples (50 µL) were loaded directly onto a HypersilGold-C18 column, (length 100 mm, inner diameter 2mm, particle size 1.9µm, Thermo Fischer Scientific) with 0.1% aqueous FA at 300 µL/min. After 2 min of loading, the peptides were eluted off the column using the following linear gradient steps: 0 min 0%B; 4 min 5%B; 16min 35%B; 16.5 min 100%B; 20 min 0%B.

2.9.1. Data analysis

Data acquisition was done with Xcalibar software version 2.2 SP1.48 (Thermo Fisher Scientific) and the data analysis for determining selected fragment ion peak areas was performed with Pinpoint 1.3.0 (Thermo Fisher Scientific). The MS accuracy was ±10 ppm centered at 0, a MS/MS accuracy of ±15 ppm and the isolation mode was set to MS/MS with an isolation width of 3.0 u. The peaks were detected using a chromatographic peak with a window size of ± 2.0 min. The complete peak area was determined after using four points of smoothing. The scheduling window size for identified transitions was ± 0.5 min. The detected fragment ion peaks were manually inspected for accuracy and absence of interferences from other peptides than the peptide of interest, including fragments originating from other product ions in the same pair/triplet. The relative amount of each brain derived or (15)N-labeled α-synuclein peptide was calculated by normalizing the measured peak area with the peak area of the corresponding IS peptide.

2.10. Statistical analysis

The statistical (non-parametric) analysis was performed using GraphPad Prism (version 6.07). Data are given as the median (interquartile range) unless otherwise stated. Differences between more than two groups were determined with Kruskal-Wallis test. If there were significant ($P < 0.05$) differences among groups, then the differences between two groups were determined by Mann-Whitney U-test.

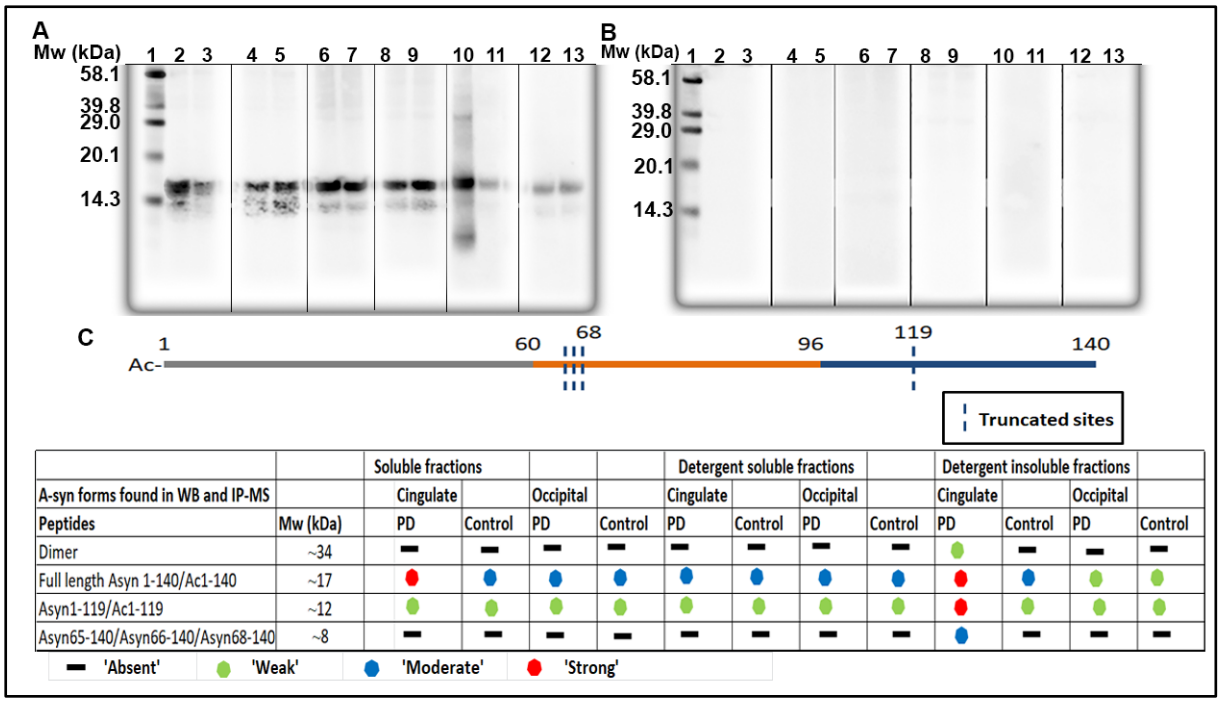
2.11. Ethical approval

The study was approved by the regional ethics committees at the University of Gothenburg and UCL.

2. Results:

2.1. Analysis of brain α-synuclein by WB

The brain homogenate fractions were analyzed by WB to monitor the presence of full length α-synuclein as well as truncated and oligomeric forms. All brain homogenate fractions (soluble, detergent soluble and detergent insoluble) of both brain regions; cingulate cortex and occipital cortex from PD and control brains showed presence of full length α-synuclein corresponding to molecular weight of around 17 kDa in WB (probably full length) (Figure 1). The band intensities of 17 kDa full length α-synuclein in the detergent insoluble fraction from both the gyrus cingulate and occipital cortices were significantly lower in controls than PD brains. In the detergent insoluble fraction of cingulate cortex, but not in occipital cortex, weak additional α-synuclein bands of 34 kDa (possibly an α-synuclein dimer), 12 kDa (probably truncated forms α-syn_{1-119} or Ac-α-syn_{1-119}) and 8 kDa (probably truncated forms α-syn_{65-140}, α-syn_{66-140}, α-syn_{68-140}) were observed. These forms were also identified by IP-MS analysis, which is discussed in details in section 3.3. The 34 kDa band was not present in the soluble and detergent soluble fractions. Instead, the soluble and detergent soluble fractions from both controls and PD brains showed presence of 12 kDa band of α-synuclein (Figure 1). Semi-quantification of the full length α-synuclein (17 kDa) band revealed significant differences between the PD and control samples from detergent insoluble fractions from both regions (Figure 2). The band intensities for the control detergent insoluble fractions from cingulate cortex were significantly lower than the corresponding fractions from PD cingulate cortex (Figure 2). The WB results of three fractions from two regions from PD and control cases (*n* =10) are presented in supplementary figure S1.



		Soluble fractions				Detergent soluble fractions				Detergent insoluble fractions			
A-syn forms found in WB and IP-MS		Cingulate		Occipital		Cingulate		Occipital		Cingulate		Occipital	
Peptides	Mw (kDa)	PD	Control	PD	Control	PD	Control	PD	Control	PD	Control	PD	Control
Dimer	~34	—	—	—	—	—	—	—	—	●	—	—	—
Full length Asyn 1-140/Ac1-140	~17	●	●	●	●	●	●	●	●	●	●	●	●
Asyn1-119/Ac1-119	~12	●	●	●	●	●	●	●	●	●	●	●	●
Asyn65-140/Asyn66-140/Asyn68-140	~8	—	—	—	—	—	—	—	—	●	—	—	—

— 'Absent' ● 'Weak' ● 'Moderate' ● 'Strong'

Figure 1: WB analysis of biochemically fractionated samples from PD and control postmortem brains. WB using mouse monoclonal anti- α-synuclein antibody (BD Clone 42) (A) and without primary antibody (negative control) (B). Molecular markers (lane 1); soluble fraction of cingulate cortex (lane 2 and 3); soluble fraction of occipital cortex (lane 4 and 5); detergent soluble fraction of cingulate cortex (lane 6 and 7); detergent soluble fraction of occipital cortex (lane 8 and 9); detergent insoluble fraction of cingulate cortex (lane 10 and 11) and detergent insoluble fraction of occipital cortex (lane 12 and 13) from PD and control brain respectively. The samples are representatives of 10 samples from each fraction and all the samples were run in duplicates in two separate gels. (C) Schematic diagram of α-synuclein showing N-terminal acetylation and truncations (blue dotted line) at different sites that might be detected by WB and IP-MS analysis of brain tissue samples. The table below shows the abundance (denoted by black line, green circle, blue circle and red circle for absent, weak, moderate and strong band respectively) of the α-synuclein bands of 34, 17, 12, and 8 kDa in different fractions from PD and control brains that correspond to different forms of α-synuclein (α-syn$_{1-140}$, Ac-α-syn$_{1-140}$, α-syn$_{1-119}$, Ac-α-syn$_{1-119}$, α-syn$_{65-140}$, α-syn$_{66-140}$, α-syn$_{68-140}$).

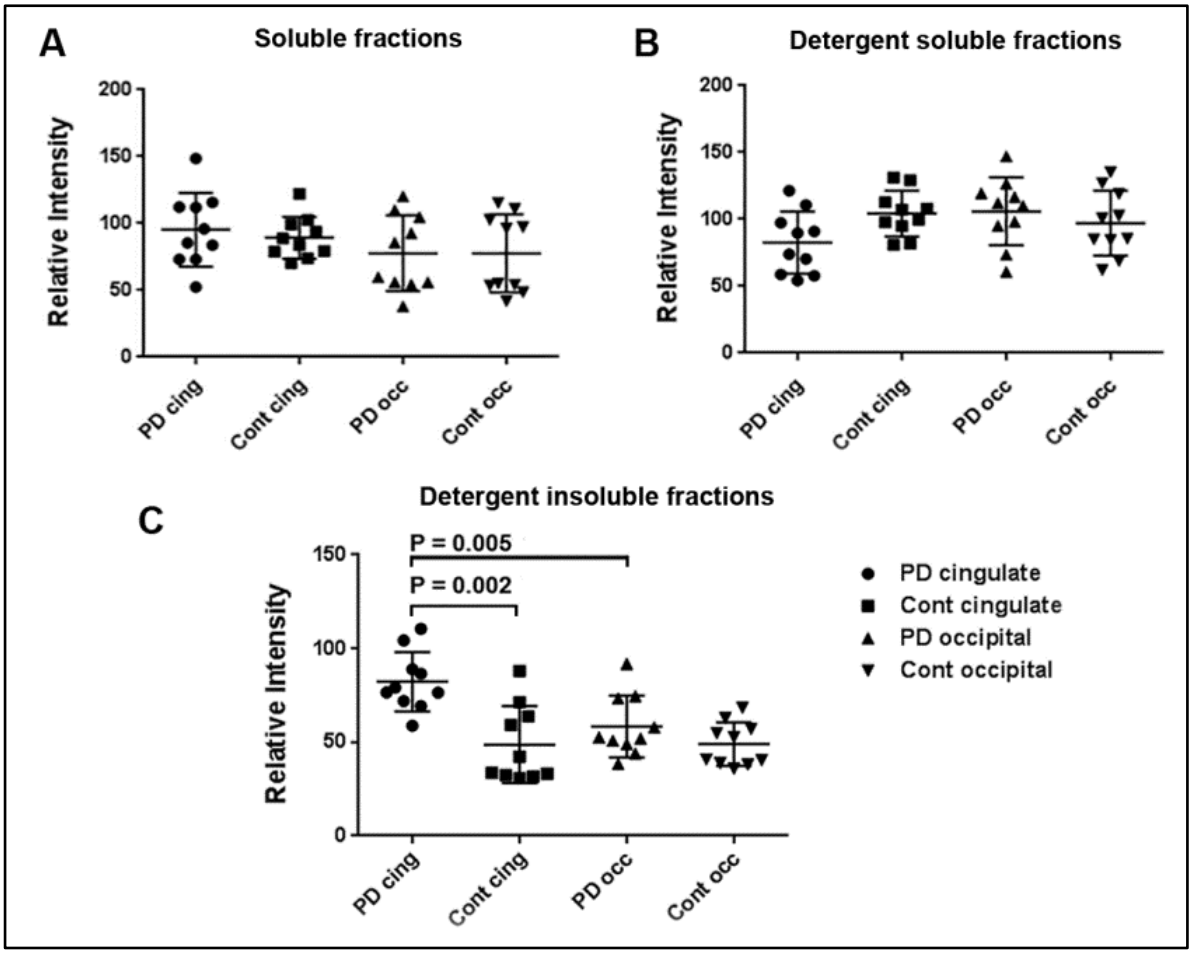


Figure 2: Densitometric quantification of the α-synuclein 17 kDa band recognized by the mouse monoclonal BD clone 42 antibody in WB of PD and control brain samples. Relative intensities of α-synuclein bands of individual samples with mean of corresponding PD pooled samples in soluble (A), detergent soluble (B) and detergent insoluble (C) fractions of cingulate and occipital cortex of PD and control brains. The scatter dot plot shows median with interquartile range. $p < 0.05$ was considered significant.

2.2. Method optimization for recovery of immuno precipitated α-synuclein

In our original method, the immuno-enriched α-synuclein was eluted from the magnetic beads in 0.5% FA followed by drying by vacuum centrifugation (52). However, in this study we found that drying of recombinant α-synuclein in 0.5% FA by vacuum centrifugation resulted in almost complete loss (~90%) of α-synuclein signal in MALDI-TOF-MS compared to a freshly prepared sample (supplementary figure S2). Furthermore, the storage of α-synuclein at

-20°C overnight caused an almost 30% signal loss in MALDI-TOF-MS (supplementary figure S1A-B). To minimize the loss of α-synuclein by vacuum-drying, a method for on-bead digestion of α-synuclein was developed (see supplementary information).

Briefly, on-bead digestion of recombinant α-synuclein was performed with trypsin and Glu-C separately after IP followed by reconstitution in 20% ACN containing 0.1% TFA or 1M GuHCl containing 0.1% TFA. Since the sequence coverage was similar and some of the potentially truncated α-synuclein forms could not be detected after Glu-C digestion we decided to continue with on-bead trypsin digestion and reconstitution in in 20% ACN containing 0.1% TFA. However, the sensitivity of the MS analysis appeared to be significantly lower for the most N-terminal and C-terminal parts of α-synuclein.

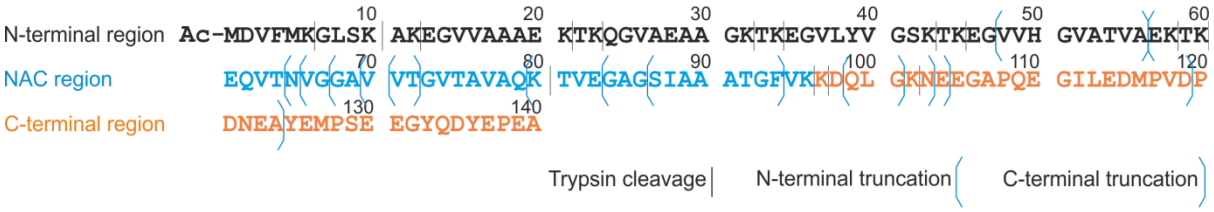
Brain tissue extracts were also immunoprecipitated with either mouse monoclonal BD Clone 42 or rabbit monoclonal MJFR1 anti-α-synuclein antibody that did not show significant difference in sequence coverage.

2.3.Analysis of brain α-synuclein forms by data dependent nanoflow LC–MS/MS

In study 1, data-dependent nanoflow LC-MS/MS analysis was performed on all brain tissue homogenates (soluble, detergent soluble and detergent insoluble) after IP and on-bead digestion to determine which forms of α-synuclein that could be detected (Figure 4A). Here the presence of semi-tryptic α-synuclein peptides indicate possible truncated α-synuclein forms. The LC-MS/MS analysis of soluble fractions yielded very high sequence coverage (85-100%) of α-synuclein along with detection of several semi-tryptic peptides (Figure 4B). Some of these peptides (α-syn$_{81\text{-}103}$, and α-syn$_{97\text{-}119}$, α-syn$_{98\text{-}119}$, α-syn$_{103\text{-}119}$) probably corresponds to two of the endogenous C-terminally truncated forms of α-synuclein, Ac-α-syn$_{1\text{–}103}$ and Ac-α-syn$_{1\text{–}119}$ we identified in our previous study and which have been also been detected by other researchers (53). Four other peptides ending with the lysine at amino acid 80 may well correspond to N-terminally truncated forms earlier found by Kellie *et al.* in the SDS-soluble (α-syn$_{71\text{-}140}$, α-syn$_{68\text{-}140}$, α-syn$_{66\text{-}140}$, α-syn$_{65\text{-}140}$) or SDS-insoluble (α-syn$_{68\text{-}140}$) fractions of human frontal gyrus tissue samples (43). In general, we found that the α-synuclein sequence coverage for the detergent soluble and insoluble fractions were lower, around 50-75%. This could be due to lower amounts of brain derived α-synuclein in these biochemical fractions. Fewer semi-tryptic peptides were also found in the detergent soluble and insoluble fractions (Figure 4B). A potentially interesting exception being the peptide α-syn$_{66\text{-}80}$ that probably

corresponds to N-terminally truncated forms α-$syn_{66\text{-}140,}$ which only was detected in the detergent insoluble fraction. Note also that only the acetylated N-terminal was detected and not the unmodified form. This strengthens our earlier findings that brain-derived α-synuclein is mainly N-terminally acetylated. However, the LC-MS/MS sensitivity for most N-terminal part of α-synuclein is reduced since the Ac-α-syn_{1-6} and Ac-α-syn_{1-10} peptides are relatively hydrophobic and difficult to ionize with a predominantly charge state of one. The expectation values of the tryptic and semi-tryptic peptides of α-synuclein in three fractions of all samples in study 1 have been presented in supplementary table S1-S3.

In study 2, a few samples from each fraction were analyzed by nanoflow LC-MS/MS to check the reproducibility of the method and trypsin digestion efficiency. The result obtained from nanoflow-LC-MS/MS analysis of study 2 was comparable to study 1 in terms of sequence coverage, score and detection of tryptic and semi-tryptic peptides (Figure 4B).



	Soluble fractions				Detergent soluble fractions				Detergent insoluble fractions			
	cingulate		occipital		cingulate		occipital		cingulate		occipital	
	PD	Control	PD	Control	PD	Control	PD	Control	PD	Control	PD	Control
Ac1-6												
Ac1-10	1	1										
Ac1-12		1										
11-23*	10	11	11	11	4	4	4		5	5	6	4
13-23*	1	4	2	8			1			1		
24-43		1	1									
33-43*	11	11	11	11	11	11	11	11	11	11	11	11
35-43*	8	10	5	4	7	9	9	7	10	9	7	5
33-45	3	1										1
44-58*	11	11	11	11	11	11	11	11	11	11	11	11
44-56	1	2										
46-58*	11	11	11	11	11	11	11	11	11	11	11	11
46-60	5	8	5	7		1	2	3	1		1	
46-80	2	2	2	3	1		1					
48-58*	7	10	7	9	2	2						
57-80	1											
59-72	6	3	2	1								
59-80*	11	11	11	11	11	11	11	11	11	11	11	11
61-80*	11	11	11	11	11	11	11	11	11	11	11	11
65-80*	1	1	1	1	1		1	1			1	
66-80*									7	6	10	9
68-80*	11	11	11	10	4	8	5	7				
70-80*	1	2	1	4								
71-80*	5	9	7	9								
80-96	10	11	9	11					3			
81-94		2							2			
81-96*	11	11	11	11	11	11	11	11	11	11	11	11
81-97*	11	11	11	11	11	11	11	11	11	11	11	11
81-101*	7	9	3	2				1				
81-102*	11	10	11	11	10	11	11	11	11	11	11	11
81-103*	1	1							4			
81-104	3	1							3			
84-97*	6	4	3	1								
87-97*	3	2	3									
97-119*	9	5	3	4								
98-119	5	2		1								
98-124	1											
103-119	4	2		1								
103-140*												

Figure 4: Summary of results obtained from nanoflow-LC-MS/MS analysis. A. Sequence of α-synuclein (UniProtKB acc # P37840; SYUA_HUMAN) showing cleavage site of trypsin (black lines) and semi-tryptic endogenous peptides (blue lines). B. Heat map representing how many samples in each fraction/region/group that had a significant data base hit for each peptide (Red 1-4, Yellow 5-7, Green 8-11).

2.4. Quantitation of brain α-synuclein by targeted microflow LC-MS/MS

Study 1

To get a more accurate quantification of α-synuclein in different brain samples, the immunoprecipitated and trypsin-digested samples from PD and control brains were quantified using targeted microflow LC-MS/MS (LC-PRM-MS) after reconstituting the samples in 20% ACN containing 0.1% FA in presence of isotopically (13)C-labeled standard peptides (tryptic Ac-α-syn$_{1-6}$, α-syn$_{13-21}$, α-syn$_{35-43}$, α-syn$_{46-58}$, α-syn$_{61-80}$n, α-syn$_{81-96}$ and semi-tryptic α-syn$_{103-119}$).

In the soluble fractions, only the most N-terminal α-synuclein peptide (Ac-α-syn$_{1-6}$) showed significant differences between PD and control (Figure 5A-G) (supplementary table S4). The levels were significantly increased in the PD cingulate region compared to the control cingulate and PD occipital region. No statistically significant differences were found in the detergent soluble fractions (Figure 5H-N) (Table S5). In the detergent insoluble fractions the levels of all α-synuclein peptides, except Ac-α-syn$_{1-6,}$ and α-syn$_{103-119}$ were significantly increased in cingulate region from PD compared to the controls (Figure 5O-U) (Table S6). Interestingly, the levels of all investigated peptides were significantly increased in occipital region from PD compared to the controls (Figure 5O-U) (Table S6).

Note also that neither the (15)N-labeled Ac-α-syn$_{1-6}$, nor the (15)N-labeled α-syn$_{103-119}$ were detected in quantifiable amounts (Table S4-6), which indicates that these modifications could be mainly brain-derived.

The CVs for individual (13)C-labeled and (15)N-labeled peptides respectively added in different samples including pooled samples during IP and PRM analysis were typically less than 10% and 20%, respectively (supplementary table S4-S6). Low CV indicates that the IP, digestion and LC-PRM-MS methods were relatively robust.

Study 2

In study 2, the following α-synuclein peptides were quantified: tryptic Ac-α-syn$_{1-6}$, α-syn$_{13-21}$, α-syn$_{35-43}$, α-syn$_{61-80}$ and semi-tryptic α-syn$_{71-80}$ and α-syn$_{103-119}$ (Figure 4B). In the soluble fractions most peptides levels were significantly increased in the cingulate region compared to the occipital region both in PD (not α-syn$_{61-80}$ and α-syn$_{71-80}$) and controls (not α-syn$_{71-80}$) (Figure 6A-F) (Table S7). In the detergent-soluble fraction all peptides except α-syn$_{71-80}$ had

significantly increased levels in PD cingulate region compared both to controls cingulate region and PD occipital region (Figure 6G-L) (Table S8). This was also the case in the detergent-insoluble fractions. The exceptions were for Ac-α-syn$_{1-6}$, where the difference between PD cingulate region and PD occipital region was not significant, and α-syn$_{103-119}$, where there was no difference between PD and control in the cingulate region (Figure 6M-R) (Table S9).

A potential problem of using formic acid during protein extraction is the risk of cleavage at sites containing aspartic acid-proline (Asp-Pro) peptide bonds (44-46). However, during the brain extraction in this study the temperature was maintained around +4°C throughout during extraction, homogenization and centrifugation. After that the detergent insoluble fraction was quickly diluted (1:20 v/v) and lyophilized. At these conditions, the possibility of formic acid cleavage should theoretically be extremely low (47). In spite of this, to evaluate the effect of sample preparation on modification of α-synuclein, uniformly (15)N-labeled α-synuclein protein (5 µg) was added to the brain tissues before each step of homogenization in study 2, which was in contrast to study 1, where the uniformly (15)N-labeled α-synuclein protein was added to the samples immediately before the immunoprecipitation step. We found that the relative amounts of (15)N-labeled and brain-derived α-synuclein peptides were the same for all unmodified peptides; ~130% in the soluble fractions, ~500% in the detergent-soluble fractions, and ~3500% in the detergent-insoluble fraction (Table S7-9). This ratio increase is mainly due to the levels of brain-derived α-synuclein decreasing in each extraction step. However, for the acetylated N-terminal (Ac-α-syn$_{1-6}$), the (15)N-labeled levels were considerably lower; 0.4% in soluble fractions, 2% in the detergent soluble fractions, and 16% in the detergent insoluble fraction. This indicates that even if N-terminal acetylation of α-synuclein is probably possible to create in the sample preparation, the risk is in the order of 0.5% or less. The risk of creating a cleavage at α-synuclein amino acid 119 seems to be higher, at least for the detergent insoluble fraction. Due to the very low α-syn$_{103-119}$ levels, it is difficult to make any reliable estimation, but the risk of FA-induced fragmentation could be in the range from roughly 1% in the soluble fraction to 50% in the detergent-insoluble fraction. We found no reliable signal from (15)N-labeled α-syn$_{71-80}$.

The CVs for the individual isotopically (13)C-labeled standard peptides added to the samples at reconstitution were very similar to study 1, indicating that the LC-PRM-MS method was relatively robust (Table S7-9). The CVs for the (15)N-labeled peptides originating from the (15)N-labeled α-synuclein added to the brain tissues at the homogenization were higher than

the corresponding peptides in study 1, typically 10-30% (supplementary table S7-S9). This most likely reflects the variability in the extraction steps.

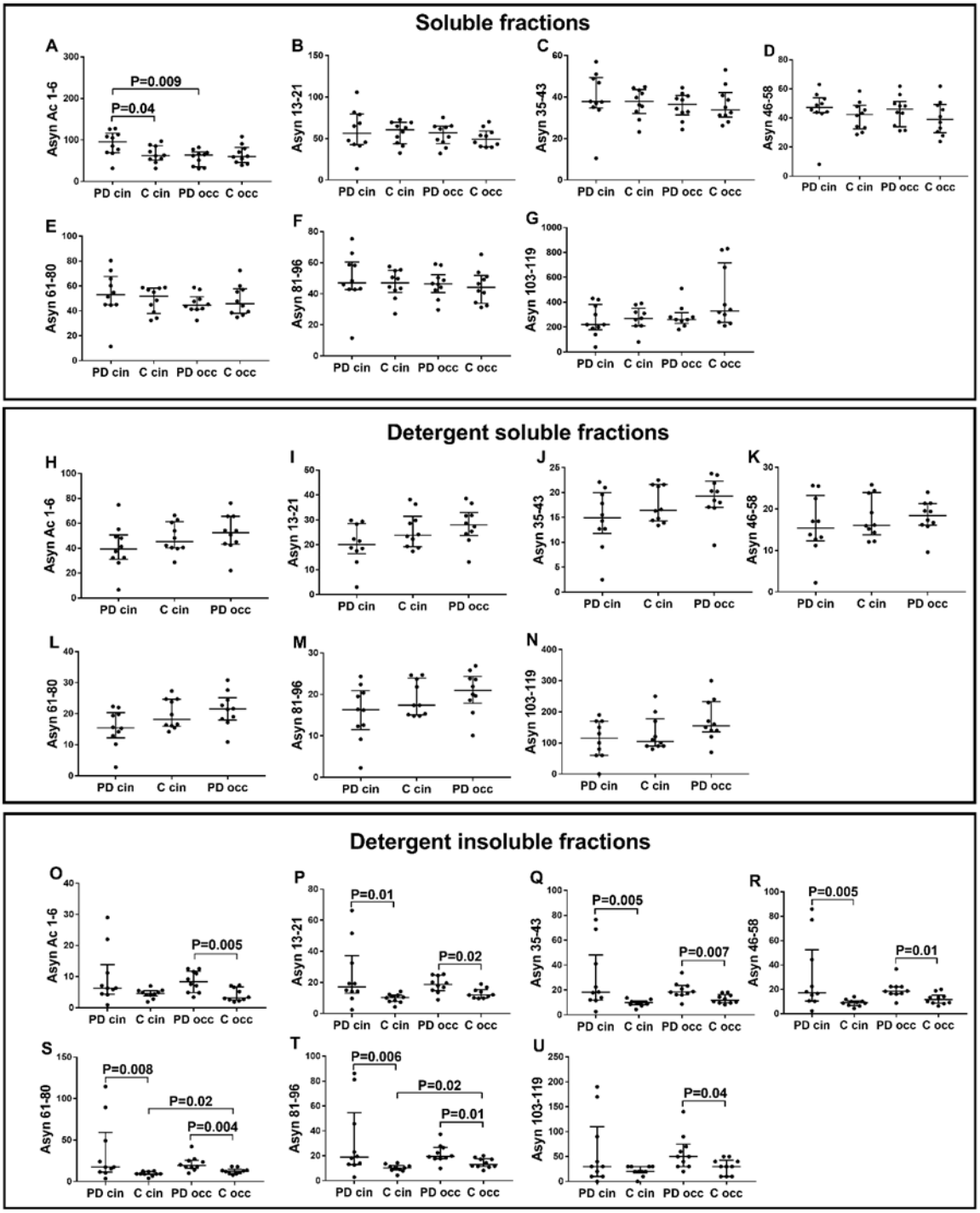


Figure 5: Targeted PRM analyses of α-synuclein in human brain homogenates in study 1. The values of different peptides of α-synuclein were reported as the peak area ratio between the endogenous peptide and the (15)N-labeled corresponding peptide (endogenous peptide/(15)N-labeled peptide). The peak area ratio for tryptic and semi-tryptic (103-119) peptides were

multiplied by 10 and 10000, respectively, to plot in a suitable scale. The scatter dot plot shows the measured levels of six N-terminal tryptic peptides, Ac-1-6 (A, H, O), 13-21 (B, I, P), 35-43 (C, J, Q), 46-58 (D, K, R),61-80 (E, L, S), 81-96 (F, M, T) and one semi-tryptic peptide 103-119 (G, N, U) of α-synuclein from soluble (A-G), detergent-soluble (H-N) and detergent-insoluble (O-U) fractions of PD and control brains. The plot shows median with interquartile range. A probability (*p*) value < 0.05 was considered significant.

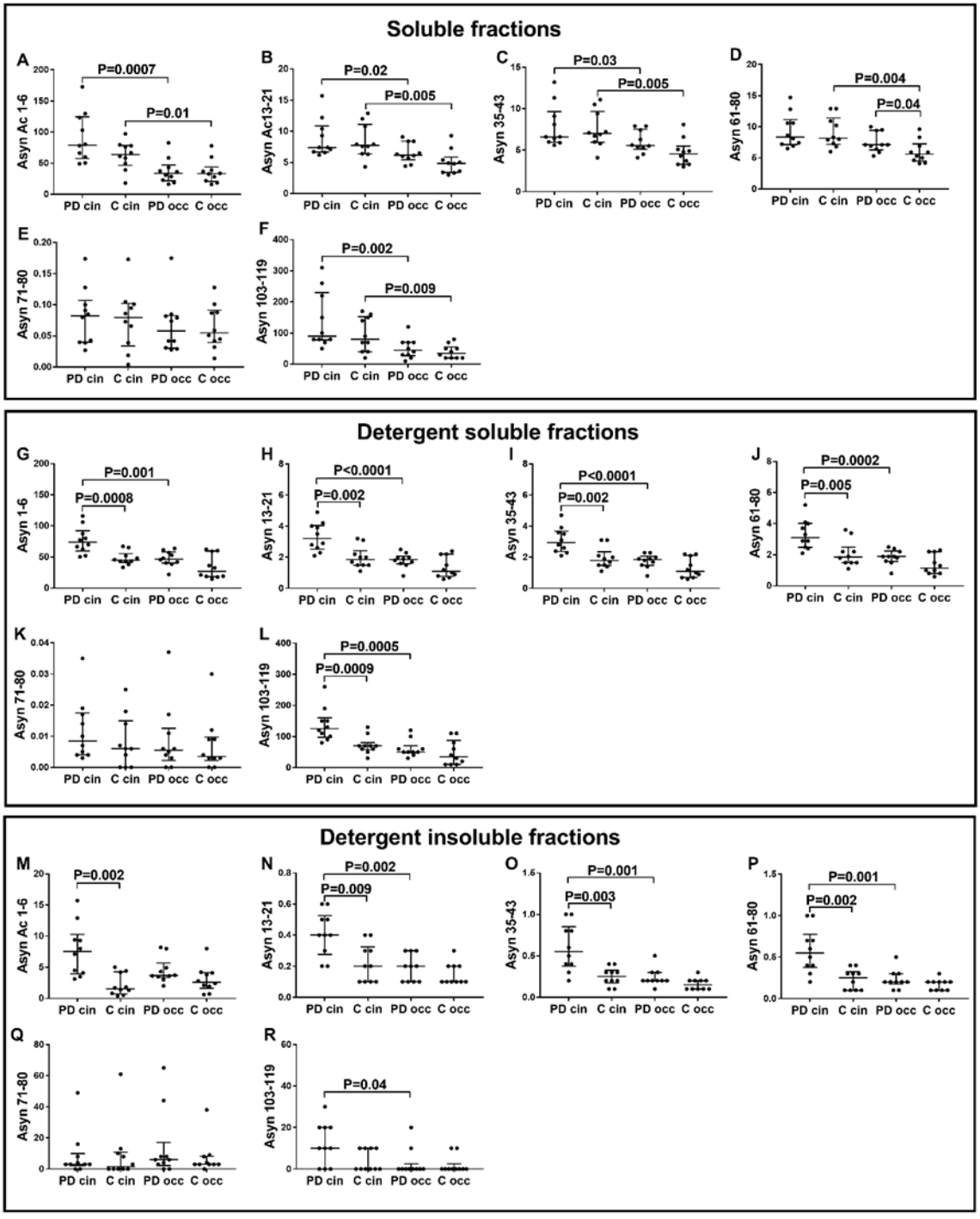


Figure 6: Targeted PRM analyses of α-synuclein in human brain homogenates in study 2. The values of different peptides of α-synuclein were reported as the peak area ratio between the endogenous peptide and the (15)N-labeled corresponding peptide (endogenous peptide/(15)N-labeled peptide). The peak area ratio for tryptic and semi-tryptic (103-119 (F, L, R), and 71-80 (Q)) peptides were multiplied by 10 and 10000 respectively to plot in a suitable scale. The scatter dot plot shows the measured levels of four N-terminal tryptic peptides, Ac-1-6 (A, G, M), 13-21 (B, H, N), 35-43 (C, I, O), and 61-80 (D, J, P), two semi-tryptic peptide 71-80 (E, K, Q) and 103-119 (F, L, R) of α-synuclein from soluble (A-F), detergent-soluble (G-L) and detergent-insoluble (M-R) fractions of PD and control brains. The plot shows median with interquartile range. A probability (p) value < 0.05 was considered significant.

3. Discussion:

In the present study, we evaluated α-synuclein levels in brain tissue by western blotting, and developed an IP-MS-based method for characterization and quantification of different α-synuclein forms in post-mortem brain tissue, which was used in two independent studies to quantify soluble, detergent-soluble and detergent-insoluble α-synuclein in brain tissue from two different brain regions in PD patients and non-demented controls.

HZ: Här tycker jag att det borde komma ett stycke om de viktigaste metodutvecklingsframstegen (on-bead digestion, m.m.) och en konklusion om vad det var för metod vi till slut bestämde oss för att använda. Sedan kan resten komma.

The main finding of our study was that in the detergent-insoluble (Lewy body-enriched) fraction, the PD cingulate cortex had the highest levels of α-synuclein. This was found both in the WB and LC-MS analyses. In our WB analysis, the dimer (34 kDa) and low molecular weight species (12 and 8 kDa) of α-synuclein were only present in the detergent-insoluble fractions of PD brains. These results were consistent with the immunohistochemical findings of Lewy bodies and α-synuclein in the cingulate region of these PD patients and indicate that results obtained from the α-synuclein extraction and sample preparation procedure reflects the pathology (10, 58). In study 1, there was also a significantly increased level of α-synuclein in PD compared to control in the occipital region. For PD patients in latest stage of the disease (Braak stage 6), it is not unusual that Lewy body pathology is present in the occipital regions (59).

In the initial characterization of brain α-synuclein, we found evidence of ~20 modified forms, including N-terminal acetylation and C-terminal truncations at amino acids 103 and 119. Several of these are consistent with modified α-synuclein forms reported in earlier studies (40, 43, 48-51). We did not detect any non-acetylated N-terminal α-synuclein, which is in agreement with earlier studies (40, 43, 49). N-terminal acetylation of cytosolic proteins, especially in proteins initiated with a Met residue often occurs and has been shown to be indispensable for the proper plasma membrane targeting of α-synuclein in yeast (52, 53). Interestingly, in study 1, the most N-terminal α-synuclein peptide (Ac-α-syn$_{1-6}$) was the only part of α-synuclein that had significantly increased levels in PD cingulate cortex compared to control cingulate cortex. Also, in study 2, the levels of soluble Ac-α-syn$_{1-6}$ were more markedly increased than the rest of the protein in PD cingulate compared to PD occipital region. All PD-causing point mutations in α-synuclein occur within the N-terminal membrane-binding domain which suggests a possible association between membrane interaction and PD pathology (54). In the detergent soluble fractions, which mostly contain membrane proteins we did not detect any significant differences between PD and controls in study 1 (67). However, in study 2 there were increased levels in PD cingulate region compared to both control cingulate region and PD occipital. In general the differences between PD patients and controls as well cingulate and occipital brain regions were more pronounced in study 2.

It has earlier been shown that C-terminally truncated forms of α-synuclein increase the aggregation propensity and facilitate the formation of fibrils (39, 48). Murray *et al*. (2003) have performed seeding experiments under conditions where full-length α-synuclein did not readily aggregate, whilst α-synuclein that was C-terminally truncated at 102 or 110 was efficient in seeding full-length α-synuclein aggregation over a range of concentrations (55). According to Murray *et al*. (2003), Ac-α-syn$_{1-103}$ should be more aggregation-prone than Ac-α-syn$_{1-119}$ and longer α-synuclein forms (49). This could explain our result from the detergent-insoluble fraction suggesting that the levels of the longer form of α-synuclein (α-syn$_{103-119}$) were unaltered in PD cingulate cortex compared to control cingulate cortex. Kellie *et al*. (2014) identified and quantified a few truncated forms using an intact protein MS approach and normalized the values to the Ac-α-syn$_{1-140}$ form to show the protein abundance (53). In agreement with our results, they did not find any statistically significant differences in endogenous α-syn$_{1-119}$ levels between disease and control subjects in the SDS-insoluble fractions (43).

Most of the truncated forms found in the current study were N-terminal truncations in the NAC region, *i.e.*, α-syn$_{65\text{-}X}$, α-syn$_{66\text{-}X}$, α-syn$_{68\text{-}X}$, α-syn$_{71\text{-}X}$. We measured the levels of α-syn$_{71\text{-}80}$ in study 2 and found a no significant differences. This is consistent with Kellie *et al*. (2014) who detected the endogenous forms of α-syn$_{71\text{-}140}$, α-syn$_{68\text{-}140}$, α-syn$_{66\text{-}140}$, α-syn$_{65\text{-}140}$ but did not find any statistically significant differences in their abundance between disease and control subjects in either the SDS-soluble or -insoluble fraction (43).

There are reports on differences in the levels of C-terminally truncated forms between PD and control samples (43, 48). Different results between different studies might result from variable methods of brain extraction. Analysis of PTMs of aggregated α-synuclein is limited by insolubility of Lewy bodies and loss of α-synuclein in different extraction steps. A major challenge in studying membrane-bound or LB associated proteins using MS is the use of strong ionic and non-ionic detergents like SDS and Triton X-100; chaotropic agents like urea, thiourea or guanidine and high concentration of organic acids like FA. These reagents are essential for solubilizing these types of proteins; however, they interfere with the IP, enzymatic digestion and MS analysis by suppressing ionization and affecting chromatographic separation (56, 57). In this study, 2% Triton-X-100 and 90% FA were used for solubilization of membrane-bound and Lewy body-enriched detergent-insoluble α-synuclein, respectively. We found that by removing the organic acid by lyophilization followed by reconstitution of the protein in presence of n-OGP in phosphate buffer and diluting the samples in PBS during the IP we achieved a relatively robust method with high recovery and low variability. Another general problem when characterization/quantifying protein forms from brain tissue is that the protein extraction or any other preparation step could create cleavages or other modifications of the targeted protein. In this study we added (15)N-labeled α-synuclein either at the IP or to the brain tissue at the different extraction steps. This made it possible to monitor the risk for cleavages/modifications being created by the sample preparation. Overall, the risk for such a confounding effect was found to be negligible.

4. Conclusions:

We have developed a novel method where we combine differential solubilization with immunoprecipitation and proteomics using PRM LC-MS/MS for quantification of brain-derived α-synuclein. This method was used to analyze soluble, detergent soluble and detergent insoluble α-synuclein in brain tissue fractions from PD patients and controls. Interestingly, in the soluble fraction only the acetylated N-terminal α-synuclein (Ac-α-syn$_{1\text{-}6}$) was significantly

increased in PD compared to controls. In the detergent insoluble fraction, significantly increased levels of most investigated α-synuclein forms were found in PD occipital region compared to controls. In conclusion, we did not identify any Lewy body-specific forms of α-synuclein, but acetylated forms should be investigated further.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author's contributions

PB, AB and AÖ designed experiments, PB and AB performed the experiments, PB, AB, AÖ and HZ analyzed data and interpreted results. PB performed statistical analysis. TL provided the postmortem brain samples and the demographic data. PB, AB, and AÖ wrote the manuscript. HZ, TL and KB critically revised the manuscript and gave conceptual advice; final approval of the version to be published was obtained from all authors.

Supporting information

Experimental procedures: MALDI-TOF-MS analysis, Method optimization for digestion of α-synuclein, Database search parameters

Supplementary figures and tables

Supplementary figure S1: Western blotting (WB) of biochemical fractions of PD and control brains.

Supplementary figure S2: Recovery of α-synuclein after speedvac drying.

Supplementary figure S3: Schematic diagram of trypsin and Glu-C peptides of α-synuclein detected in nano-LC-MS/MS analysis soluble brain fractions.

Supplementary figure S4: Immunoblot of α-synuclein to test the efficiency of immunoprecipitation with mouse monoclonal anti- α-synuclein antibody (BD Clone 42).

Supplementary figure S5: Calibration curve for (15)N- α-synuclein protein using IP-PRM-MS method.

Supplementary table S1: Nanoflow LC-MS/MS analysis of soluble fractions in study 1

Supplementary table S2: Nanoflow LC-MS/MS analysis of detergent soluble fractions in study 1

Supplementary table S3: Nanoflow LC-MS/MS analysis of detergent insoluble fractions in study 1

Supplementary table S4: PRM analysis of soluble fractions in study 1

Supplementary table S5: PRM analysis of detergent soluble fractions in study 1

Supplementary table S6: PRM analysis of detergent insoluble fractions in study 1

Supplementary table S7: PRM analysis of soluble fractions in study 2

Supplementary table S8: PRM analysis of detergent soluble fractions in study 2

Supplementary table S9: PRM analysis of detergent insoluble fractions in study 2

Table 1. Clinical and demographic characteristics of the brain samples

Subjectdetails	Study set 1			Study set 2		
	PD (*n* = 10)	Control (*n* = 10)	*p* value [(b)]	PD (*n* = 10)	Control (*n* = 10)	*p* value
Female	*n* = 4		-	*n* = 4	*n* = 6	-
Male	*n* = 6		-	*n* = 6	*n* = 4	-
Age at death (years)[(a)]	73.1 ± 9.2	86.8 ± 6.1	<0.001	80.7 ± 6.2	85.7 ± 5.0	< 0.05
Age at onset (years)	54.7 ± 8.8	n.a.	-	61.4 ± 10.2	n.a.	-
Duration of the disease (years)	18.4 ± 8.7	n.a.	-	19.9 ± 6.6	n.a.	-
Post-mortem delay (hours)	50.2 ± 31.8	57.0 ± 21.8	> 0.05	62.8 ± 20.9	61.5 ± 34.4	> 0.05

[(a)] The age at death, age at onset, duration of the disease and post-mortem delays presented in the table is the mean ± S.D. of samples (*n* = 10).

[(b)] Mann–Whitney U test was performed for evaluating statistical significant differences (*p* values of < 0.05) between Parkinson's disease (PD) and control cases.

References

1. Truong DD, Bhidayasiri R. Parkinson's Disease. In: RP L, DD T, WM C, R B, editors. International Neurology. 2 ed: Wiley-Blackwell; 2016.
2. McCann H, Stevens CH, Cartwright H, Halliday GM. alpha-Synucleinopathy phenotypes. Parkinsonism Relat Disord. 2014;20 Suppl 1:S62-7.
3. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839-40.
4. Goedert M, Spillantini MG, Del Tredici K, Braak H. 100 years of Lewy pathology. Nat Rev Neurol. 2013;9(1):13-24.
5. Braak H, Del Tredici K. Neuroanatomy and pathology of sporadic Parkinson's disease. Adv Anat Embryol Cell Biol. 2009;201:1-119.
6. Puschmann A, Bhidayasiri R, Weiner WJ. Synucleinopathies from bench to bedside. Parkinsonism Relat Disord. 2012;18 Suppl 1:S24-7.
7. Duda JE, Giasson BI, Mabon ME, Miller DC, Golbe LI, Lee VM, et al. Concurrence of alpha-synuclein and tau brain pathology in the Contursi kindred. Acta Neuropathol. 2002;104(1):7-11.
8. Lashley T, Holton JL, Gray E, Kirkham K, O'Sullivan SS, Hilbig A, et al. Cortical alpha-synuclein load is associated with amyloid-beta plaque burden in a subset of Parkinson's disease patients. Acta Neuropathol. 2008;115(4):417-25.
9. Jellinger KA. A critical evaluation of current staging of alpha-synuclein pathology in Lewy body disorders. Biochim Biophys Acta. 2009;1792(7):730-40.
10. Tokuda T, Qureshi MM, Ardah MT, Varghese S, Shehab SA, Kasai T, et al. Detection of elevated levels of alpha-synuclein oligomers in CSF from patients with Parkinson disease. Neurology. 2010;75(20):1766-72.

11. Mollenhauer B, Locascio JJ, Schulz-Schaeffer W, Sixel-Doring F, Trenkwalder C, Schlossmacher MG. alpha-Synuclein and tau concentrations in cerebrospinal fluid of patients presenting with parkinsonism: a cohort study. Lancet Neurol. 2011;10(3):230-40.
12. Schmid AW, Fauvet B, Moniatte M, Lashuel HA. Alpha-synuclein post-translational modifications as potential biomarkers for Parkinson disease and other synucleinopathies. Mol Cell Proteomics. 2013;12(12):3543-58.
13. Hansson O, Hall S, Ohrfelt A, Zetterberg H, Blennow K, Minthon L, et al. Levels of cerebrospinal fluid alpha-synuclein oligomers are increased in Parkinson's disease with dementia and dementia with Lewy bodies compared to Alzheimer's disease. Alzheimers Res Ther. 2014;6(3):25.
14. Hall S, Ohrfelt A, Constantinescu R, Andreasson U, Surova Y, Bostrom F, et al. Accuracy of a panel of 5 cerebrospinal fluid biomarkers in the differential diagnosis of patients with dementia and/or parkinsonian disorders. Arch Neurol. 2012;69(11):1445-52.
15. Oeckl P, Metzger F, Nagl M, von Arnim CA, Halbgebauer S, Steinacker P, et al. Alpha-, Beta-, and Gamma-synuclein Quantification in Cerebrospinal Fluid by Multiple Reaction Monitoring Reveals Increased Concentrations in Alzheimer's and Creutzfeldt-Jakob Disease but No Alteration in Synucleinopathies. Mol Cell Proteomics. 2016;15(10):3126-38.
16. Shahnawaz M, Tokuda T, Waragai M, Mendez N, Ishii R, Trenkwalder C, et al. Development of a Biochemical Diagnosis of Parkinson Disease by Detection of alpha-Synuclein Misfolded Aggregates in Cerebrospinal Fluid. JAMA Neurol. 2017;74(2):163-72.
17. Fairfoul G, McGuire LI, Pal S, Ironside JW, Neumann J, Christie S, et al. Alpha-synuclein RT-QuIC in the CSF of patients with alpha-synucleinopathies. Ann Clin Transl Neurol. 2016;3(10):812-8.
18. Kang W, Chen W, Yang Q, Zhang L, Zhang L, Wang X, et al. Salivary total alpha-synuclein, oligomeric alpha-synuclein and SNCA variants in Parkinson's disease patients. Sci Rep. 2016;6:28143.
19. Parnetti L, Cicognola C, Eusebi P, Chiasserini D. Value of cerebrospinal fluid alpha-synuclein species as biomarker in Parkinson's diagnosis and prognosis. Biomark Med. 2016;10(1):35-49.
20. Lewczuk P, Riederer P, O'Bryant SE, Verbeek MM, Dubois B, Visser PJ, et al. Cerebrospinal fluid and blood biomarkers for neurodegenerative dementias: An update of the Consensus of the Task Force on Biological Markers in Psychiatry of the World Federation of Societies of Biological Psychiatry. World J Biol Psychiatry. 2018;19(4):244-328.
21. Slaets S, Vanmechelen E, Le Bastard N, Decraemer H, Vandijck M, Martin JJ, et al. Increased CSF alpha-synuclein levels in Alzheimer's disease: correlation with tau levels. Alzheimers Dement. 2014;10(5 Suppl):S290-8.
22. Tateno F, Sakakibara R, Kawai T, Kishi M, Murano T. Alpha-synuclein in the cerebrospinal fluid differentiates synucleinopathies (Parkinson Disease, dementia with Lewy bodies, multiple system atrophy) from Alzheimer disease. Alzheimer Dis Assoc Disord. 2012;26(3):213-6.
23. Wennstrom M, Surova Y, Hall S, Nilsson C, Minthon L, Bostrom F, et al. Low CSF levels of both alpha-synuclein and the alpha-synuclein cleaving enzyme neurosin in patients with synucleinopathy. PLoS One. 2013;8(1):e53250.
24. Kapaki E, Paraskevas GP, Emmanouilidou E, Vekrellis K. The diagnostic value of CSF alpha-synuclein in the differential diagnosis of dementia with Lewy bodies vs. normal subjects and patients with Alzheimer's disease. PLoS One. 2013;8(11):e81654.
25. Barbour R, Kling K, Anderson JP, Banducci K, Cole T, Diep L, et al. Red blood cells are the major source of alpha-synuclein in blood. Neurodegener Dis. 2008;5(2):55-9.
26. Alderson TR, Markley JL. Biophysical characterization of alpha-synuclein and its controversial structure. Intrinsically Disord Proteins. 2013;1(1):18-39.
27. Barrett PJ, Timothy Greenamyre J. Post-translational modification of alpha-synuclein in Parkinson's disease. Brain Res. 2015;1628(Pt B):247-53.
28. Zhu M, Qin ZJ, Hu D, Munishkina LA, Fink AL. Alpha-synuclein can function as an antioxidant preventing oxidation of unsaturated lipid in vesicles. Biochemistry. 2006;45(26):8135-42.
29. Jin H, Kanthasamy A, Ghosh A, Yang Y, Anantharam V, Kanthasamy AG. alpha-Synuclein negatively regulates protein kinase Cdelta expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. J Neurosci. 2011;31(6):2035-51.
30. Burre J, Sharma M, Sudhof TC. alpha-Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation. Proc Natl Acad Sci U S A. 2014;111(40):E4274-83.

31. Emamzadeh FN. Alpha-synuclein structure, functions, and interactions. J Res Med Sci. 2016;21:29.
32. Roeters SJ, Iyer A, Pletikapic G, Kogan V, Subramaniam V, Woutersen S. Evidence for Intramolecular Antiparallel Beta-Sheet Structure in Alpha-Synuclein Fibrils from a Combination of Two-Dimensional Infrared Spectroscopy and Atomic Force Microscopy. Sci Rep. 2017;7:41051.
33. Giraldez-Perez R, Antolin-Vallespin M, Munoz M, Sanchez-Capelo A. Models of alpha-synuclein aggregation in Parkinson's disease. Acta Neuropathol Commun. 2014;2:176.
34. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, et al. alpha-synuclein promotes mitochondrial deficit and oxidative stress. Am J Pathol. 2000;157(2):401-10.
35. Alim MA, Ma QL, Takeda K, Aizawa T, Matsubara M, Nakamura M, et al. Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. J Alzheimers Dis. 2004;6(4):435-42; discussion 43-9.
36. Hashimoto M, Kawahara K, Bar-On P, Rockenstein E, Crews L, Masliah E. The Role of alpha-synuclein assembly and metabolism in the pathogenesis of Lewy body disease. J Mol Neurosci. 2004;24(3):343-52.
37. Danzer KM, Haasen D, Karow AR, Moussaud S, Habeck M, Giese A, et al. Different species of alpha-synuclein oligomers induce calcium influx and seeding. J Neurosci. 2007;27(34):9220-32.
38. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of alpha-synuclein: from structure and toxicity to therapeutic target. Nat Rev Neurosci. 2013;14(1):38-48.
39. Liu CW, Giasson BI, Lewis KA, Lee VM, Demartino GN, Thomas PJ. A precipitating role for truncated alpha-synuclein and the proteasome in alpha-synuclein aggregation: implications for pathogenesis of Parkinson disease. J Biol Chem. 2005;280(24):22670-8.
40. Ohrfelt A, Zetterberg H, Andersson K, Persson R, Secic D, Brinkmalm G, et al. Identification of novel alpha-synuclein isoforms in human brain tissue by using an online nanoLC-ESI-FTICR-MS method. Neurochem Res. 2011;36(11):2029-42.
41. Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, et al. In vivo demonstration that alpha-synuclein oligomers are toxic. Proc Natl Acad Sci U S A. 2011;108(10):4194-9.
42. Portelius E, Tran AJ, Andreasson U, Persson R, Brinkmalm G, Zetterberg H, et al. Characterization of amyloid beta peptides in cerebrospinal fluid by an automated immunoprecipitation procedure followed by mass spectrometry. J Proteome Res. 2007;6(11):4433-9.
43. Kellie JF, Higgs RE, Ryder JW, Major A, Beach TG, Adler CH, et al. Quantitative measurement of intact alpha-synuclein proteoforms from post-mortem control and Parkinson's disease brain tissue by intact protein mass spectrometry. Sci Rep. 2014;4:5797.
44. Landon. Cleavage at aspartyl-prolyl bonds. Methods Enzymol. 1977;47:145-9.
45. Li A, Sowder RC, Henderson LE, Moore SP, Garfinkel DJ, Fisher RJ. Chemical cleavage at aspartyl residues for protein identification. Anal Chem. 2001;73(22):5395-402.
46. Crimmins DL, Mische SM, Denslow ND. Chemical cleavage of proteins in solution. Curr Protoc Protein Sci. 2005;Chapter 11:Unit 11 4.
47. Zhang H, Li M, Shi S, Yin C, Jia S, Wang Z, et al. Design and identification of a high efficient formic acid cleavage site for separation of fusion protein. Protein J. 2015;34(1):9-17.
48. Li W, West N, Colla E, Pletnikova O, Troncoso JC, Marsh L, et al. Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations. Proc Natl Acad Sci U S A. 2005;102(6):2162-7.
49. Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. J Biol Chem. 2006;281(40):29739-52.
50. Beyer K, Domingo-Sabat M, Lao JI, Carrato C, Ferrer I, Ariza A. Identification and characterization of a new alpha-synuclein isoform and its role in Lewy body diseases. Neurogenetics. 2008;9(1):15-23.
51. Lewis KA, Su Y, Jou O, Ritchie C, Foong C, Hynan LS, et al. Abnormal neurites containing C-terminally truncated alpha-synuclein are present in Alzheimer's disease without conventional Lewy body pathology. Am J Pathol. 2010;177(6):3037-50.
52. Bradshaw RA. Protein translocation and turnover in eukaryotic cells. Trends Biochem Sci. 1989;14(7):276-9.

53. Zabrocki P, Bastiaens I, Delay C, Bammens T, Ghillebert R, Pellens K, et al. Phosphorylation, lipid raft interaction and traffic of alpha-synuclein in a yeast model for Parkinson. Biochim Biophys Acta. 2008;1783(10):1767-80.
54. Fortin DL, Nemani VM, Nakamura K, Edwards RH. The behavior of alpha-synuclein in neurons. Mov Disord. 2010;25 Suppl 1:S21-6.
55. Murray IV, Giasson BI, Quinn SM, Koppaka V, Axelsen PH, Ischiropoulos H, et al. Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. Biochemistry. 2003;42(28):8530-40.
56. Mirza SP, Halligan BD, Greene AS, Olivier M. Improved method for the analysis of membrane proteins by mass spectrometry. Physiol Genomics. 2007;30(1):89-94.
57. Vaisar T. Thematic review series: proteomics. Proteomic analysis of lipid-protein complexes. J Lipid Res. 2009;50(5):781-6.