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Cerebrospinal fluid biomarkers of neuropathologically diagnosed Parkinson's disease subjects

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Abstract

1. Objectives—Parkinson's disease (PD) afflicts approximately 1-2% of the population over 50 years of age. No cures or effective modifying treatments exist and clinical diagnosis is currently confounded by a lack of definitive biomarkers. We sought to discover potential biomarkers in the cerebrospinal fluid (CSF) of neuropathologically confirmed PD cases.

2. Methods—We compared postmortem ventricular CSF (V-CSF) from PD and normal control (NC) subjects using two-dimensional difference gel electrophoresis (2D-DIGE). Spots exhibiting a 1.5-fold or greater difference in volume between PD patients and controls were excised from the 2D gels, subjected to tryptic digestion and identification of peptides assigned using mass spectrometric/data bank correlation methods.

3. Results—Employing this strategy six molecules: fibrinogen, transthyretin, apolipoprotein E, clusterin, apolipoprotein A-I and glutathione-S-transferase-Pi were found to be different between PD and NC populations.

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4: Discussion—These molecules have been implicated in PD pathogenesis. Combining biomarker data from multiple laboratories may create a consensus panel of proteins that may serve as a diagnostic tool for this neurodegenerative disorder.

Keywords

2D-DIGE; cerebrospinal fluid; Parkinson's disease; proteomics

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder resulting from poorly elucidated genetic and/or environmental factors. Approximately 1-2% of the population over 50 years of age is afflicted with PD¹. Clinical signs of PD include motor (resting tremor, bradykinesia, postural instability, rigidity), cognitive (dementia), neuropsychiatric (depression and anxiety) and autonomic (hypotension and constipation) dysfunctions^{1;2}. A prominent neuropathological manifestation of PD is the degeneration of neurons containing neuromelanin in the substantia nigra that results in a loss of dopamine^{1;2}. Another important neuropathological observation is the presence of intraneuronal Lewy bodies, composed mainly of α -synuclein filaments. While medications alleviate PD symptoms, there is no cure nor are there clearly effective disease modifying treatments.

There is no definitive biomarker or imaging test for PD and the diagnosis is determined from clinical observations and confirmed only by postmortem neuropathological analysis. Parkinson's disease symptoms resemble other neurodegenerative disorders leading to an estimated clinical diagnostic accuracy ranging between 75 and 90%³⁻⁵. Great efforts have been invested in the identification of biomarkers that can diagnose PD in the early stages of clinical development so that pharmacological interventions can be more effectively provided. Unfortunately, by the time PD symptoms are clinically evident, 50-60% of neurons in the substantia nigra have been lost and dopamine levels in the striatum are 80-85% depleted⁶⁻⁸. Even during the asymptomatic phase of Lewy body disease, striatal dopaminergic markers are depleted by approximately 50%⁹⁻¹².

In an effort to identify possible cerebrospinal fluid (CSF) biomarkers in PD, we compared postmortem ventricular CSF (V-CSF) from neuropathologically confirmed PD and normal control (NC) subjects by using two-dimensional difference gel electrophoresis (2D-DIGE). We identified six molecules in PD patients that deviated from control specimens. Ventricular CSF has higher concentrations of molecules generated from the brain which may not correspond directly to the levels observed in lumbar CSF from early stage PD. We are aware of the fact that the use of postmortem V-CSF has the disadvantage of time dependant degradation of proteins. We try to minimize this potential artifact by using V-CSF with the shortest possible postmortem intervals. However, postmortem CSF has the enormous advantage of originating from neuropathologically diagnosed cases.

2. Materials and Methods

2.1 Clinical Assessments and Patient Selection

2.1.1 Subjects—Subjects were derived from those enrolled in the Banner Sun Health Research Institute Brain and Body Donation Program, Sun City, Arizona and were clinically examined longitudinally¹³⁻¹⁵. This cohort is made up of volunteers from the community as well as PD patients from the clinical practices of the physicians who work in the program. All subjects or their legal representatives signed the written informed consent approved by the Banner Health Institutional Review Board. Annual standardized assessments were performed on most subjects, including movement¹⁶ and cognitive batteries¹³. The diagnostic

criteria for PD included: 2 of 3 cardinal signs (rest tremor, bradykinesia, cogwheel rigidity). DSM IV and Emre et al. criteria for PD-dementia were used¹⁷.

2.1.2 Neuropathological Examination—Subjects were chosen by searching the Brain and Body Donation Program database for cases that had received a neuropathological diagnosis of PD without a concurrent neuropathological diagnosis of Alzheimer's disease, progressive supranuclear palsy or other major neurodegenerative conditions. The NC group was composed of subjects without evidence of dementia (normal elderly subjects) or parkinsonism (**Table 1 and Supplementary Table 1**).

Subjects received standardized neuropathological examinations. Specific clinicopathologic diagnostic criteria were used for PD¹⁸ including a clinical diagnosis as well as Lewy bodies and pigmented neuronal loss in the substantia nigra. Gross and microscopic neuropathologic assessments were made by a single observer (TGB) without the knowledge of the clinical history or clinical diagnosis; subsequently the clinical history was reviewed in order to make an appropriate clinicopathologic diagnosis. Diagnostic histologic methods were performed on standard blocks of tissue that were fixed in 4% neutral-buffered formaldehyde and then either dehydrated and embedded in paraffin or cryoprotected and cut on a freezing, sliding microtome. Each case was first staged according to the Unified Staging System for Lewy Body Disorders (**Table 1 and Supplementary Table 1**) with a standard set of brain sections as previously described¹⁹.

2.2 Cerebrospinal fluid

Polypropylene tubes and syringes were used for collection, storage and 2D-DIGE experiments. Postmortem CSF was collected by syringe aspiration from the lateral ventricles in the immediate postmortem period (median postmortem interval = 3.3 hours), centrifuged at $1600 \times g$ for 10 min and the supernatant fractionated into 1 ml samples and stored at -80°C until analysis. All CSF samples collected for this study were collected between 1992-2008. The total protein concentration of each individual's CSF was measured with the Micro BCA protein assay kit (Pierce, Rockford, IL). In the PD group, 5 cases were eliminated because of high total protein levels ($> 1.5 \text{ mg/ml}$), while one case was eliminated for low levels ($< 0.3 \text{ mg/ml}$). In the NC group, one case was eliminated for high total protein levels and one case was eliminated for low total protein levels. Equal amounts of total protein from each subject were pooled in PD (final $n = 43$) or NC (final $n = 49$) groups (**Table 1**).

2.3 2D-DIGE Analysis

Albumin and IgG were removed from the V-CSF using a kit from GE Healthcare (Piscataway, NJ). Briefly, the V-CSF pools were incubated with an affinity resin for 90 min at 4°C on a rocking platform with unbound V-CSF proteins separated from the resin by centrifugation at $6500 \times g$ for 5 min in microspin columns.

In order to prepare the V-CSF pools for 2D-DIGE analysis, the remaining proteins were precipitated with acetone/trichloroacetic acid (TCA) by mixing with 3 volumes of cold 13.3% TCA in acetone (stored at -20°C) and incubating at -20°C overnight. The samples were centrifuged at $15,000 \times g$ for 30 min and the supernatant discarded. The pelleted proteins were washed with the same volume of cold acetone, vortexed and incubated at -20°C for 30 min. Samples were centrifuged at $15,000 \times g$ for 15 min and the supernatant discarded. The V-CSF protein pellets were air dried for 5 min, and then suspended in 7 M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 2% 3-[N,N-Dimethyl(3-myristoylamino)propyl]ammonio]propanesulfonate (ASB-14), 15 mM Tris, pH 8.3. A pooled

reference sample, loaded on every analytical gel, was created by combining 30 µg from each sample group and all samples were diluted to 2 mg/ml using the above buffer. Thirty µg of each individual sample group was labeled with 200 pmol minimal Cy3 and Cy5 NHS ester (GE Healthcare), and the pooled reference sample labeled in bulk with minimal Cy2 NHS ester (at a ratio of 200 pmol dye:30 µg sample) at 4°C for 30 min. The labeling reaction was quenched with 10 nmol of lysine. Labeled protein extracts were combined and proteins separated on 24 cm pH 4-7 IPG strips for 60,000 Vhr using the IPGphor (GE Healthcare) in 7 M urea, 2 M thiourea, 2% CHAPS, 2% ASB-14, 0.5% IPG buffer, 18.2 mM dithiothreitol (DTT) and 0.002% bromophenol blue using active rehydration at 30V. After isoelectric focusing, IPG strips were equilibrated in 6 M urea, 12% SDS, 65 mM DTT, 30% glycerol and 10 mM Tris, pH 6.8 for 15 min at room temperature (RT). The IPG strips were equilibrated in the above buffer, replacing DTT with 135 mM iodoacetamide, for 15 min at RT. Proteins were then separated on 20 × 26 cm 8-15% SDS polyacrylamide gels using a Dalt II (GE Healthcare) electrophoresis apparatus. The gels were scanned at 3 wavelengths on a GE Healthcare Typhoon 9410 Imager. These images were analyzed with the differential in-gel analysis module using the GE Healthcare DeCyder software package (version 6.5). The gels were stained with Sypro Ruby, in order to visualize and excise the spots of interest from the gels. The Ettan Spot Picker (GE Healthcare) was used to robotically excise the spots from the gel and transfer them into a 96-well microtiter plate. Spots were subjected to robotic tryptic (Promega) digestion on a GE Healthcare Ettan TA Digester. A portion of the digest (1-1000 fmols) was desalted using a C18 ZipTip. The C18 ZipTip (P10 size) was washed with 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) and then equilibrated with 0.1% TFA (3 × 20 µl). The sample was aspirated and then expelled back into the original sample well eight times and the ZipTip was then washed 5X with 20 µl 0.1% TFA. Peptides/proteins were eluted from the ZipTip with 3 µl of 60% acetonitrile, 0.1% TFA containing 3.5 mg/ml α-cyano-4-hydroxy cinnamic acid matrix. A volume of 0.8 µl was loaded onto the MALDI target plate. Internal calibrants, 1 fmol of bradykinin (protonated monoisotopic mass = 1060.569) and 2 fmol ACTH clip 18-39 (protonated, monoisotopic mass = 2465.199) were added with the matrix. MALDI-ToF/ToF of protein identification was performed on an Applied Biosystems Model 4800 MALDI-ToF/ToF mass spectrometer. Reflectron mass spectrometry (MS) analysis sums 1250 laser shots to generate the peptide fingerprint map, and the spectra were internally calibrated using the bradykinin internal standard. Masses were chosen by the Applied Biosystems 4000 Series Explorer software (version 3.0) for MS/MS acquisition. MS/MS analysis was performed first on the masses with the highest intensity, ensuring that several MS/MS spectra of good quality were obtained before the MALDI spot was depleted. Up to 10 MS/MS spectra were acquired and 10,000 laser shots were combined for each MS/MS spectra. Mascot analysis utilized the NCBI nr database and a combined peptide mass fingerprint/MS/MS database search was performed. Only data with significant Mascot scores of 80 or greater were utilized.

3. Results and Discussion

The demographic characteristics of the PD and NC groups used in this study as well as the mean postmortem intervals, mean brain weight, apolipoprotein E (ApoE) allelic frequencies and the Unified Lewy body stage are shown in **Table 1**. There were no statistically significant differences between PD and NC postmortem intervals, which had an average of 3.3 h, and there was a trend towards larger brain weights in the PD group, although this also did not reach statistical significance ($p = 0.064$). The mean age of death was significantly higher in the NC group (PD = 79 years; NC = 83 years; $p = 0.002$). Polymorphic ApoE allelic frequencies did not demonstrate significant differences between the two populations.

Figure 1A shows the 2D-DIGE protein spots with 1.5-fold or greater differences in spot volume. Blue represents an increase in the PD over NC volume and red denotes an increase

in the NC over PD. An enlarged caption of the area that contains the spots of interest is provided in **Supplementary Figure 1. Figure 1B** illustrates the spot distribution (NC versus PD) with the left Y-axis corresponding to spot frequency and the right Y-axis representing the maximum spot volume of a given pair. The red curve demonstrates the frequency distribution of log volume. The blue curve corresponds to the normalized model frequency fitted to the spots proportions so that the modal peak is zero. The black vertical lines are set at 1.5-fold difference in the PD/NC spot volume.

The spots which demonstrated more than 1.5-fold changes in intensity units, were robotically excised, submitted to tryptic digestion and analyzed by MS/database reference comparisons. The PD/NC differences, accession numbers, protein identifications, molecular weights, isoelectric points, database search scores, total ion scores, percentage of coverage and number of tryptic peptides identified are given in **Table 2. Supplementary Table 2** provides the amino acid sequences of the tryptic peptides of the proteins identified in **Table 2**. The identified molecules were: fibrinogen (FIB, 2 spots), transthyretin (TTR, 2 spots), ApoE, clusterin (apolipoprotein J), apolipoprotein A-1 (ApoA-1) and glutathione-S-transferase-Pi (GST-Pi).

One of the major limitations in biomarker discovery is that 2D gel methodologies yield numerous and convoluted sets of spots and hence it is very difficult to establish whether a representative protein in a fluid or tissue homogenate is elevated or decreased from the heterogeneous number of isoforms that, in most cases, are generated by a single protein. In many instances, there is only one particular isoform that deviates from a molecule with different pIs, or there are real or spurious aggregated forms which makes difficult to deduce the biological meaning of the observation. ELISA techniques are also limited to the detection of those peptides with antigenic determinants restricted to a specific antibody(s) that may or may not correspond to the same peptide detected by 2D-DIGE. Therefore, direct comparisons are challenging. Western blots suffer the same limitations. In spite of these limitations, a comparative examination of results against other analytical technologies such as 2D-DIGE or ELISA or Western blots is justified because multiple and correlated qualitative and quantitative studies that identify the same protein have some potential biological meaning.

FIB is a coagulation cascade glycoprotein that is synthesized by the liver, and is converted to fibrin by thrombin which is then crosslinked by to factor XIII to form a clot. Both isoforms of the β -chain of FIB discovered by 2D-DIGE analysis were decreased in the PD group (4.34 and 3.94 times) as compared to the NC group (**Table 2**). A previous study using lumbar CSF and two-dimensional gel electrophoresis (2DE) also found the β -chain of FIB to be decreased in PD patients relative to NC subjects²⁰, while another study using isobaric Tagging for Relative and Absolute protein Quantification (iTRAQ), chromatography and MS/MS identification found no change²¹. Dysfunctions in blood coagulation have been observed in PD patients taking anti-parkinsonian drugs²² and high blood FIB levels have been linked to a greater risk of PD in older men²³. FIB can generate a large number of different molecules due to alternative splicing and post-translational modifications, thus, individual fluctuations are great and complicate quantification (reviewed in Hofmann M, 2008²⁴).

TTR, a transporter of thyroxine and retinol, is synthesized in the liver and choroid plexus and makes up one-quarter of all CSF protein²⁵. In PD individuals, TTR (also known as prealbumin) was elevated in two spots of the 2D-DIGE analysis (2.17 and 2.01 times) relative to the NC pool (**Table 2**). In a similar proteomic study of human lumbar CSF, TTR levels were decreased in the PD group²⁰. The levels of TTR are altered in many neurodegenerative disorders including PD, Alzheimer's disease, familial amyloid

polyneuropathy and psychiatric disorders (reviewed in Fleming CE, et al., 2009²⁶). In animal models with PD-like lesions, TTR is increased in the CSF relative to normal controls as assessed by 2DE^{27,28}. We and others have observed that in CSF proteomics, TTR can be dimeric²⁹⁻³², therefore a simple explanation for the molecular mass observed at ~28 kDa (**Figure 1A**) is that TTR is dimerized.

Clusterin (or apolipoprotein J) is widely expressed in human tissues and has many functions in health and disease (reviewed in Bettuzzi S, 2009³³; Klock G, et al., 2009³⁴; Falgarone G, et al., 2009³⁵). The 2D-DIGE analysis revealed a 2 -fold increase in PD clusterin compared to the NC cohort (**Table 2**). In another proteomic study, four isoforms of clusterin were also elevated in PD 2DE gels compared to NC gels²⁰. Additionally, individuals with less than 2 years with PD showed increased clusterin levels relative to NC cases, however this phenomenon was not observed in individuals with more than 2 years with this neurodegenerative disorder³⁶. Immunohistochemical analysis has shown that clusterin co-localizes with α -synuclein in Lewy bodies. In these lesions, a strong clusterin staining correlated with a weaker α -synuclein staining suggesting that clusterin may protect against α -synuclein aggregation³⁷.

ApoE is a multifunctional protein of paramount importance in the brain since it is the main carrier of cholesterol and fatty acids. In Alzheimer's disease, ApoE ϵ 4 is the best known risk factor for this dementia. In our 2D-DIGE analysis ApoE was increased in the PD CSF (2.01 fold) relative to NC (**Table 2**). Other studies have demonstrated contradictory results. An immunobead-based multiplex assay of lumbar CSF revealed that the NC individuals had significantly more ApoE than the PD group³⁸. In contrast, a comparable study utilizing 2DE and 1D Western blotting showed that the NC group had less ApoE than the PD group³⁹. Another proteomics study of lumbar CSF found no changes in ApoE levels between PD and NC²¹. ApoE was decreased in the CSF of a PD animal model relative to normal animals as assessed by 2DE²⁷. Intriguingly, in a transgenic mouse model of α -synuclein overexpression, neurodegeneration caused a large increase in ApoE levels in the spinal cord, sciatic nerve, and to a lesser degree in the brain. When ApoE expression was deleted in these mice, soluble α -synuclein increased and insoluble α -synuclein decreased and although neurodegeneration still occurred, it was delayed and diminished⁴⁰.

ApoA-1 is the major structural protein of the HDL and can activate lecithin cholesterol acyltransferase. In our study, the levels of ApoA-1 were decreased (-1.6 times) in PD relative to controls (**Table 2**). This trend was also previously reported in lumbar CSF by Zhang J, et al., 2008³⁸. Other proteomic studies of lumbar CSF also found ApoA-1 to be lower in PD cases in comparison to NC cases^{20,41}. Lumbar CSF investigated by 2D-DIGE revealed that one isoform of ApoA-1 was increased while another was decreased when PD and NC individuals were compared. Subsequent Western blot examination of these specimens showed ApoA-1 to be significantly elevated in the PD cases. These discrepancies could be explained by the total levels of the holo ApoA-1 being metabolized differently than its isoforms⁴².

GST-Pi is a molecule that belongs to a family of cytosolic neuroprotective transferases that are involved in detoxification by conjugation of reduced glutathione to several hydrophobic and electrophilic substrates. Glutathione-S-transferases are involved in the inactivation of oxidative stress metabolites, in the synthesis of leukotrienes, prostaglandins, testosterone and progesterone and in the degradation of tyrosine (reviewed in Hayes JD, et al., 2005⁴³; Dourado DF, et al., 2008⁴⁴). In the current study, GST-Pi was decreased (1.6 times) in the PD 2D-DIGE analysis in comparison to the NC pool (**Table 2**). Glutathione levels have been observed to be deficient in the substantia nigra of PD cases^{45,46}. *O*-quinones, a byproduct of dopamine, conjugated to glutathione by way of glutathione-S-transferases may

have a protective role against dopaminergic system degeneration (reviewed in Hayes JD, et al., 2005⁴³). In a proteomic study of frontal cortex synaptosomes, PD cases contained increased levels of GST-Pi relative to NC cases⁴⁷. This increase in GST-Pi has been interpreted as an adaptive mechanism as the disease progresses.

We are familiar with the standardization procedures proposed for the lumbar CSF collection and banking in living patients⁴⁸, and recognize concerns regarding the unavoidable postmortem interval (PMI) in relation to protein degradation. Therefore, the use of postmortem V-CSF as a medium for identifying diagnostic markers must be approached with caution, as recent proteomic studies have shown that many proteins show postmortem concentration changes⁴⁹⁻⁵¹. However, this potential confound can be substantially minimized by a shorter PMI. In addition, postmortem V-CSF focuses on proteomic changes in the later stages of PD, which may differ from those in the early stages. However, the suggestion that such limitations entirely negate the clinical value of postmortem V-CSF for the investigation of human neurological disease must be firmly rejected. The investigation of postmortem brain and CSF chemistry has a long and productive tradition within the biomedical sciences, and has yielded what might arguably be regarded as the most relevant and useful discoveries within the field of neurodegenerative disease. The cholinergic and dopaminergic neurotransmitter deficits found within the postmortem brains of subjects with AD and PD, respectively, remain the basis of the major current pharmacologic therapies for these diseases. The data presented in the current study would appear to represent genuine disease-related changes, as the diagnostic groups did not differ in terms of their mean PMI. In addition, we have conducted several studies on postmortem V-CSF in Alzheimer's disease^{29;52;53} using proteomic methodologies and ELISA and have obtained results similar to those published by many other groups, who have all used lumbar CSF from living subjects. The benefits obtained by having precise neuropathological diagnoses for subjects outweighs the disadvantages conferred by the presence of any minor postmortem changes¹⁴. Other parameters to be considered are that the concentration of CSF proteins, that originate from neurons and glial cells, is higher in ventricular than lumbar CSF⁵⁴. Therefore, the chances of finding good biomarkers of AD might be better in V-CSF. Because postmortem degradation may produce artifacts in the V-CSF, any potential diagnostic panel discovered in postmortem V-CSF needs to be confirmed in living individuals to determine its utility, a standard and essential precaution for any such efforts.

4. Conclusions

The search for PD biomarkers has been enhanced by the use of sophisticated systems such as 2D-DIGE coupled with MS protein identification. These technologies permit the precise separation of multiple isoforms resulting from differential splicing and post-translational modifications which culminate in a constellation of molecules with different isoelectric points and molecular masses. We sought potential PD biomarkers in pools of postmortem V-CSF samples from definitively-diagnosed cases. The quest for biomarkers of disease is confounded by the fact that individuals exhibit considerable levels of variability due to individual genotypes, complicated pleiotropic interactions, general health status, diet, lifestyle differences and medications. Our 2D-DIGE analyses revealed detectable isoforms of FIB, ApoA-1 and GST-Pi were decreased in PD versus NC subjects while TTR, clusterin and ApoE were elevated in PD when compared to the NC group. Interestingly, ApoE, ApoA1 and clusterin have been implicated in lipid metabolism and transport as well as in membrane repair and remodeling. These molecules, as well as other potential biomarkers, may be indicative of generalized neurodegeneration rather than a specific disease marker. In PD patients there is an elevation of GST-Pi suggesting a neuroprotective redox compensatory mechanism and may explain the reduced levels of this protein observed in CSF. The lower levels of FIB in the V-CSF of our PD cases, which have also been observed

in lumbar CSF, stand in contrast with the higher levels of this coagulation factor and acute phase reactant observed in the plasma of patients with PD. The elevation of TTR in PD V-CSF is intriguing and may be functionally equivalent to the putative sequestration of A β by TTR in AD, but in this case, α -synuclein. However, this molecular interaction has not yet been investigated. Overall, the differences in these molecules show clear quantitative trends for specific isoforms in 2D-DIGE in CSF. In addition, their specific identification as well as their expression level dynamics has been confirmed in multiple previous studies performed using CSF from living individuals. Our analyses were performed in neuropathologically confirmed PD cases with short postmortem intervals. The observed correspondence in results suggests that, despite the different experimental conditions and status of the studied subjects (living or deceased), combining data from multiple studies may ultimately enable the creation of an expanded sensitive and specific roster of accurate PD biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

2D-DIGE	two-dimensional difference gel electrophoresis
2DE	two-dimensional gel electrophoresis
ABS-14	3-[N,N-Dimethyl(3-myristoylamino)propyl]ammonio]propanesulfonate
ApoA-1	apolipoprotein A-1
ApoE	apolipoprotein E
ApoJ	apolipoprotein J
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CSF	cerebrospinal fluid
DTT	dithiothreitol
FIB	fibrinogen
GST-Pi	glutathione-S-transferase-Pi
LB	Lewy body
MS	mass spectrometry
NC	normal control
PD	Parkinson's disease
PMI	postmortem interval

RT	room temperature
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TTR	transthyretin
V-CSF	ventricular cerebrospinal fluid

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Figure 1.

A) 2D-DIGE master gel showing spots with 1.5x or greater differences in spot volume. Spots encircled in blue are increased in the PD group and spots encircled in red are decreased in the PD group, relative to the NC cohort. The two spots on the acidic side were likely artifacts and not included in the study. Note that pI and kDa are approximate. **B)** The spot distribution in PD and NC 2D-DIGE gels. The left Y-axis represents spot frequency and the right Y-axis represents the maximum spot volume of a given pair of spots. The red curve signifies the frequency of distribution of the log volume ratios while the blue curve is the normalized model frequency fitted to the spot ratios so that the modal peak is zero. The black vertical lines are set at a 1.5-fold difference in PD/NC spot volume ratio (red spots = decrease in PD; blue spots = increase in PD).

Table 1

Personal characteristics of the Parkinson's disease and normal control populations under study

	PD = 43	NC = 49	^a <i>p</i> =
Age, years *	79 (64-90)	83 (68-97)	0.002
Gender, % male	70%	63%	
PMI, hours *	3.3 (1.2-13.8)	3.3 (1.5-24.0)	0.982
Brain weight, g *	1256 (1085-1480)	1214 (980-1560)	0.064
ApoE allelic frequency	$\epsilon 2=0.07$ $\epsilon 3=0.83$ $\epsilon 4=0.10$	$\epsilon 2=0.09$ $\epsilon 3=0.76$ $\epsilon 4=0.15$	
Unified Lewy body stage ^b	8 brainstem-predominant 2 limbic-predominant 18 brainstem and limbic 7 neocortical		

PD = Parkinson's disease; NC = normal control; PMI = postmortem interval; ApoE = apolipoprotein E.

* Mean values given with ranges are in parentheses.

^a *p* = unpaired, two-tailed t-test^b Eight cases in the PD group did not have enough data to allow Lewy body staging

Table 2

Identification of spots of interest by mass spectrometry

Spot #	PD/NC fold change	Accession #	Protein Identified	MW (Da)	pI	Database Search Score	Total ion score	% Coverage	# of Peptides
1262	-4.34	IP100298497	Fibrinogen, β -chain	55892	8.5	68	51	22	9
1282	-3.94	IP100298497	Fibrinogen, β -chain	55892	8.5	111	81	28	12
1484	2.17	IP100022432	Transferrin	15877	5.5	613	564	80	7
1521	2.01	IP100022432	Transferrin	15877	5.5	563	513	80	7
1521	2.01	IP100021842	ApoE	36132	5.7	430	378	46	15
1521	2.01	IP100291262	Clusterin (ApoJ)	52461	5.9	114	92	24	8
1919	-1.60	IP100021841	ApoA-I	30759	5.6	598	477	78	19
1919	-1.60	IP100219757	GST-Pi	23341	5.4	279	233	58	9

PD = Parkinson's disease; NC = normal control; MW = molecular weight; ApoE = apolipoprotein E; ApoJ = apolipoprotein J; ApoA-I = apolipoprotein A-I; GST-Pi = glutathione-S-transferase-Pi.

* Theoretical molecular weight and pI are reported.