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## Quantitative Appraisal of Ventricular Cerebrospinal Fluid Biomarkers in Neuropathologically Diagnosed Parkinson's Disease Cases Lacking Alzheimer's Disease Pathology

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**Abstract:** Identifying biomarkers that distinguish Parkinson's disease (PD) from normal control (NC) individuals has the potential to increase diagnostic sensitivity for the detection of early-stage PD. A previous proteomic study identified potential biomarkers in post-mortem ventricular cerebrospinal fluid (V-CSF) from neuropathologically diagnosed PD subjects lacking Alzheimer's disease (AD) neuropathology. In the present study, we assessed these biomarkers as well as p-tau<sup>181</sup>, A $\beta$ 42, and S100B by ELISA in PD (n = 43) and NC (n = 49) cases. The p-tau<sup>181</sup>/A $\beta$ 42 ratio and ApoA-1 showed statistically significant differences between groups. Multiple regression analysis demonstrated that p-tau<sup>181</sup>/A $\beta$ 42 had a significant odds ratio: OR = 1.42 (95% confidence interval [CI], 1.12–1.84), *P* = 0.006. Among the molecules investigated, intriguing correlations were observed that require further investigation. Our results suggest co-existent AD CSF biomarkers within the PD group notwithstanding that it was selected to minimize AD neuropathological lesions.

**Keywords:** Parkinson's disease, biomarkers, ventricular cerebrospinal fluid, apolipoprotein A-1, p-tau<sup>181</sup>/A $\beta$ 42 ratio

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## Introduction

Parkinson's disease (PD) is a neurodegenerative disorder clinically characterized by motor and nonmotor abnormalities. Neuropathological analyses reveal loss of dopaminergic neurons and the presence of Lewy bodies, composed of aggregated  $\alpha$ -synuclein and other proteins. Although Lewy bodies are a prominent feature of PD, there is debate about whether they play a primary role in the pathogenesis of the disease or are a secondary epiphenomenon.

Discovering biomarkers that distinguish PD from normal control (NC) individuals would greatly enhance the understanding of the etiology of the disease and, more importantly, lead to earlier, more effective treatment strategies. Cerebrospinal fluid (CSF) has been extensively studied for potential biomarkers due to its close proximity to and intercommunication with the brain. Although alterations in total tau, phosphorylated-tau (p-tau) and amyloid- $\beta$  (A $\beta$ ) peptides have become accepted biomarkers for Alzheimer's disease (AD), there has been a large effort to investigate these molecules in the CSF of PD subjects, as recently reviewed.<sup>1</sup> While there is some interlaboratory variability in results, the consensus is total tau or p-tau levels are increased and A $\beta$ 42 is decreased in PD patients when compared with normal control (NC) subjects.<sup>1</sup> Interestingly, these findings are similar to those observed in Alzheimer's disease (AD) CSF biomarker studies.<sup>2,3</sup>

The application of proteomic techniques such as two-dimensional difference gel electrophoresis (2D-DIGE) to neurodegenerative disorders has greatly enhanced the discovery of specific disease biomarkers. In the present study, we attempted to individually validate proteins that were differentially altered in pools of freshly obtained postmortem ventricular CSF (V-CSF) from neuropathologically diagnosed PD and NC individuals.<sup>4</sup> Combining powerful, proteomic separations<sup>4</sup> with sensitive enzyme-linked immunosorbent assay (ELISA) methods, we compared V-CSF samples from 43 PD cases and 49 NC subjects. All candidate biomarker molecules, fibrinogen (FIB), transthyretin (TTR), clusterin (CLU), apolipoprotein E (ApoE), apolipoprotein A-1 (ApoA-1), and glutathione S-transferase Pi (GST-Pi) have been previously linked to PD.<sup>4</sup> In addition, due to the recently reported findings mentioned above, A $\beta$ 42 and tau phosphorylated at amino acid 181 (p-tau<sup>181</sup>) were also quantified. An increased presence of S100B in

the CSF is suggestive of astrocytic damage;<sup>5-7</sup> therefore, the levels of S100B were also investigated.

## Materials and Methods

### Patient selection and study cases

#### Cases

All individuals in this study were enrolled in the Banner Sun Health Research Institute Brain and Body Donation Program, Sun City, Arizona. All individuals or their legal representatives signed the written informed consent approved by the Banner Health Institutional Review Board. Subjects are community volunteers from the metropolitan Phoenix, Arizona, communities as well as patients from clinical private practices (CHA, HS, MNS) and were clinically examined longitudinally<sup>8-10</sup> using standardized assessments that included movement<sup>11</sup> and cognitive batteries.<sup>8</sup> An antemortem PD diagnosis required 2 of 3 cardinal signs (rest tremor, bradykinesia, cogwheel rigidity) and responsiveness to dopaminergic medication.<sup>12</sup> Private medical records were also reviewed and abstracted for all subjects.

### Neuropathological examination

The Brain and Body Donation Program databases were searched to find cases with an uncomplicated diagnosis of PD free from other concurrent neuropathological conditions, such as AD, dementia with Lewy bodies, frontotemporal lobar degeneration, progressive supranuclear palsy, vascular dementia, or hippocampal sclerosis. The NC group included subjects without evidence of dementia or parkinsonism. Standardized neuropathological examinations were performed on all cases.<sup>9</sup> Neuropathological evaluations, at the gross and microscopic levels, were performed by a single observer (TGB) blinded to the clinical information. For the diagnosis of PD, specific clinicopathologic criteria were used<sup>13</sup> that included the clinical diagnosis of PD and the presence of Lewy bodies and pigmented neuronal loss in the substantia nigra. At a later stage, the clinical history was appraised in order to adjudicate a definitive clinicopathologic diagnosis. Blocks of tissue were fixed in 4% neutral-buffered formaldehyde and then either dehydrated and embedded in paraffin or cryoprotected and sectioned on a freezing sliding microtome. Paraffin-embedded sections were stained immunohistochemically for phosphorylated  $\alpha$ -synuclein. Individual cases were



staged following the Unified Staging System for Lewy Body Disorders with a standard set of brain sections as previously published.<sup>14</sup> Detailed descriptions of the neuropathological grading for plaque score (maximum 15), neurofibrillary tangle (NFT) score (maximum 15), cerebral amyloid angiopathy (CAA) score (maximum 12) and white matter rarefaction (WMR) score (maximum 12) are given elsewhere.<sup>9,15,16</sup> A summary of demographic and relevant neuropathological data is provided in Table 1.

### Ventricular cerebrospinal fluid

Ventricular-CSF was removed in the immediate post-mortem from the lateral ventricles by syringe aspiration and centrifuged at 1600 × rpm for 10 minutes. The supernatants were divided into 1 mL samples and stored at -80 °C. The Micro BCA protein assay kit (Pierce, Rockford, IL) was used to quantify the total protein concentration. We initially selected 49 PD and 51 NC cases for study. In the NC group, 2 cases were eliminated from the study: 1 for high total protein levels (>1.5 mg/mL) and 1 for low total protein levels (<0.3 mg/mL). In the PD group, 5 cases were eliminated because of high total protein levels while one case was eliminated for low levels. The final numbers of analyzed specimens compared in this study were 43 PD cases and 49 NC cases.

**Table 1.** Group comparisons for gender distribution, age at death, PMI, brain weight and neuropathological assessments.

	NC	PD	P-value
Gender (M/F)	31/18	30/13	0.51*
Mean age at death (yrs)	83.5 (7.5)	78.7 (6.6)	0.02†
Mean PMI (h)	3.3 (3.2)	3.3 (2.1)	0.98†
Mean brain weight (g)	1,214 (124)	1,256 (84)	0.06†
Mean total plaque score	3.7 (4.12)	3.1 (3.97)	0.66††
Mean NFT score	3.7 (2.23)	3.4 (1.67)	0.68††
Mean CAA score	1.8 (2.90)	1.0 (1.84)	0.13††
Mean WMR score	1.0 (3.16)	1.3 (2.02)	0.13††
Median Braak stage	IV	III	0.72††
Median Lewy body stage	0	III	<0.001††

**Notes:** \*Chi-square; †two sample *t*-test; ††Kruskall–Wallis test. For mean values, standard deviations are given in parentheses.

**Abbreviations:** NC, normal control; PD, Parkinson's disease; M, male; F, female; yrs, years; PMI, postmortem interval; h, hours; NFT, neurofibrillary tangles; CAA, cerebral amyloid angiopathy; WMR, white matter rarefaction.

### ELISA analysis

Ten potential biomarkers were measured with the following ELISA kits: S100B (BioVendor R&D, Candler, NC, catalog # RD192090100R); Aβ42 (Innogenetics, Gent, Belgium, catalog # 80177), tau phosphorylated at amino acid 181 (p-tau<sup>181</sup>, Invitrogen, Carlsbad, CA, catalog # KHO0631), FIB (Immunology Consultants Laboratory, Inc., Newberg, OR, catalog # E-80FIB); TTR (or prealbumin, Immunology Consultants Laboratory, Inc., catalog # E-80PRE); ApoE (MBL Int., Woburn, MA, catalog # 7635); CLU (or apolipoprotein J; BioVendor R&D, Candler, NC, catalog # RD194034200R); ApoA-1 (GenWay, San Diego, CA, catalog # 40-288-20069), and GST-Pi (Alpco, Salem, NH, catalog # K7960). All assays were performed according to the manufacturers' instructions. The technical parameters of each ELISA kit such as intraassay and interassay CV% and sensitivity are provided by each manufacturer in the data sheets. Samples were run in duplicate. Samples that did not fall within the range of the standard curve or showed greater than 20% variability between duplicates were reassayed.

### Statistical analysis

A two-sample *t*-test was carried out to discern group differences (PD vs. NC) on age at death, postmortem interval (PMI) and brain weight. Chi-square analysis was employed to determine if the proportion of males and females between the groups was significantly different. The Kruskal–Wallis test was used to compare total plaque score, total NFT score, total CAA score, total WMR score, and the median Braak and Lewy body stages for each group. Analysis of covariance (ANCOVA) was performed with the biomarker variables set as dependent variables and group status (PD vs. NC) as the independent variable. Age at death and gender were entered as covariates in order to account for their effect. Similar ANCOVAs were also carried out for the PD and NC groups separately using ApoE ε4 carrier status as the independent variable while also adjusting for age at death and gender. Cohen's *d* was used to assess the effect size of the group differences for each of the biomarker variables for all analyses.

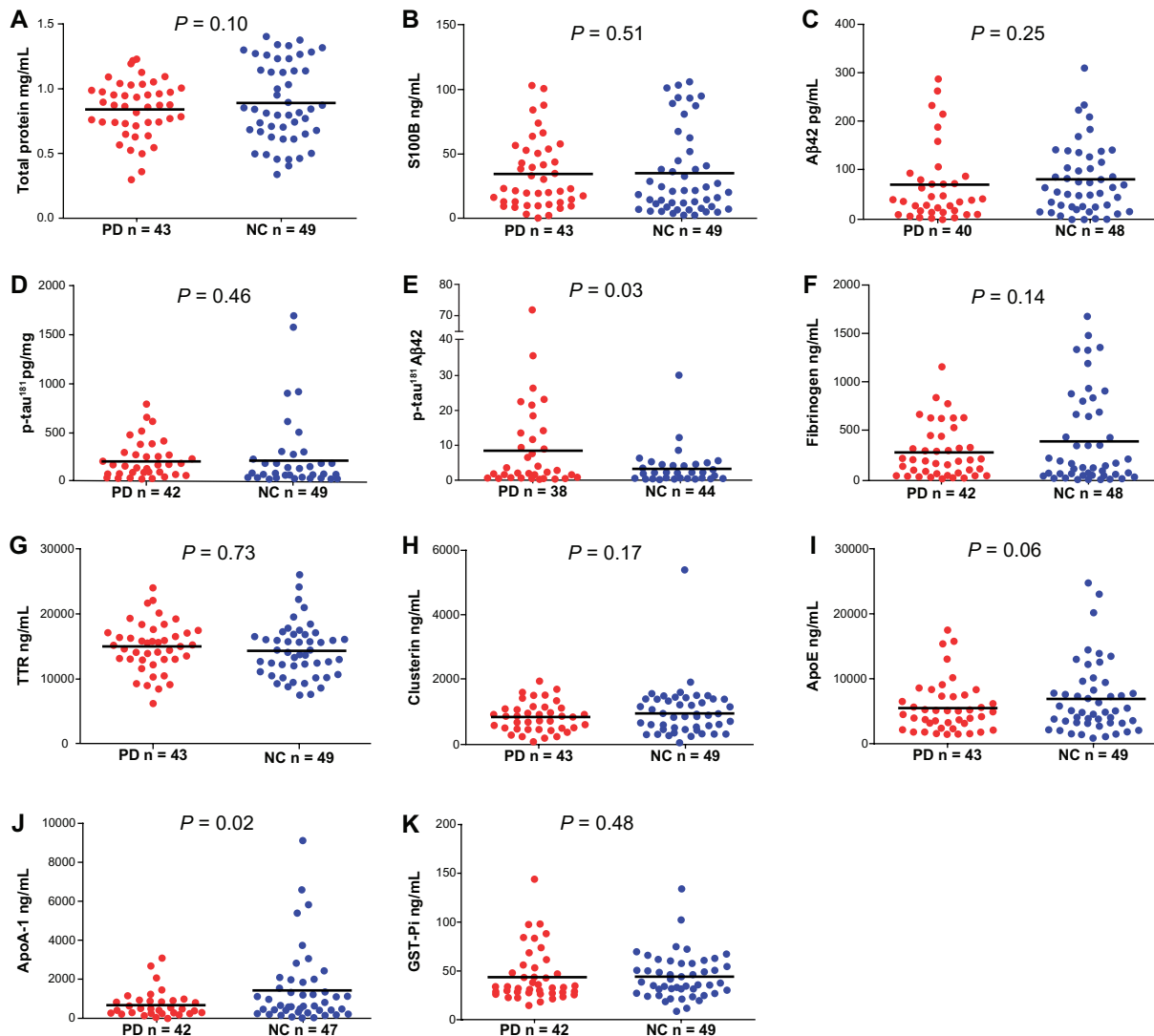
To assess the predictive value of the biomarkers, a multiple logistic regression model was carried out with group status as the dependent variable and the individual biomarkers as independent variables while adjusting for age at death and gender.

## Results

The mean age of death was 78.7 (standard deviation [SD] = 7.5) years for the PD group and 83.5 (SD = 6.6) years for the NC group ( $P = 0.02$ ) (Table 1). There was no difference in gender distribution, PMI, or brain weight between the groups (Table 1). The ApoE allelic frequency did not differ between groups, NC being  $\epsilon 2 = 0.09$ ,  $\epsilon 3 = 0.76$ , and  $\epsilon 4 = 0.15$ ; and PD being  $\epsilon 2 = 0.07$ ,  $\epsilon 3 = 0.83$ , and  $\epsilon 4 = 0.10$ . The PD and NC groups did not significantly differ in NFT or total plaque scores nor CERAD or Braak stage, the neuropathological hallmarks of AD (Table 1). In addition,

there was no difference in WMR or CAA scores between the groups (Table 1). The NC group did not have any subjects with Lewy bodies while the median Unified Lewy body stage of the PD cohort was III (Table 1).

Figure 1 displays the group differences (PD vs. NC) for the 10 biomarker measures as well as total protein levels, and Table 2 shows the results of the statistical analyses. The p-tau<sup>181</sup>/A $\beta$ 42 ratio and ApoA-1 were the only biomarkers that differed statistically between the groups (Table 2). The PD group demonstrated higher p-tau<sup>181</sup>/A $\beta$ 42 ratios (Fig. 1E,  $P = 0.03$ ) and



**Figure 1.** Scatter plots of total protein concentration and ELISA measurements in CSF. (A) Total protein concentration in mg/mL. (B) S100B concentration in ng/mL. (C) A $\beta$ 42 concentration in pg/mL. (D) p-tau<sup>181</sup> concentration in pg/mL. (E) p-tau<sup>181</sup>/A $\beta$ 42 ratio. (F) Fibrinogen concentration in ng/mL. (G) TTR concentration in ng/mL. (H) Clusterin concentration in ng/mL. (I) ApoE concentration in ng/mL. (J) ApoA-1 concentration in ng/mL. (K) GST-Pi concentration in ng/mL.

**Notes:** Mean values are indicated by the horizontal line. Results of the statistical analyses are given in Table 2.

**Abbreviations:** PD, Parkinson's disease; NC, normal control; A $\beta$ 42, amyloid-beta 42; p-tau<sup>181</sup>, tau phosphorylated at amino acid 181; TTR, transthyretin; ApoE, apolipoprotein E; ApoA-1, apolipoprotein A-1; GST-Pi, glutathione S-transferase Pi.

**Table 2.** Group differences for biomarker measures.

	NC	PD	F-value	P-value	Effect size
Total protein (mg/mL)	0.89 (0.31)	0.84 (0.21)	2.84	0.10	0.19
S100B (ng/mL)	35 (32)	35 (27)	0.43	0.51	0.00
A $\beta$ 42 (pg/mL)	81 (70)	68 (72)	1.35	0.25	0.19
p-tau <sup>181</sup> (pg/mL)	205 (359)	194 (190)	0.55	0.46	0.04
p-tau <sup>181</sup> /A $\beta$ 42	2.87 (4.55)	8.15 (12.98)	5.24	0.03	0.54
FIB (ng/mL)	417 (469)	302 (267)	2.24	0.14	0.30
TTR (ng/mL)	14,342 (4,099)	14,981 (3,774)	0.12	0.73	0.16
CLU (ng/mL)	986 (784)	848 (434)	1.88	0.17	0.22
ApoE (ng/mL)	6,920 (5,507)	5,665 (3,946)	3.51	0.06	0.26
ApoA-1 (ng/mL)	1,454 (1,845)	718 (630)	6.04	0.02	0.53
GST-Pi (ng/mL)	44 (23)	44 (27)	0.50	0.48	0.00

**Notes:** Mean (SD); df = (1, 88); effect size—Cohen's *d*.

**Abbreviations:** NC, normal control; PD, Parkinson's disease; A $\beta$ 42, amyloid-beta 42; p-tau<sup>181</sup>, tau phosphorylated at amino acid 181; FIB, fibrinogen; TTR, transthyretin; CLU, clusterin; ApoE, apolipoprotein E; ApoA-1, apolipoprotein A-1; GST-Pi, glutathione S-transferase Pi.

lower ApoA-1 (Fig. 1J,  $P = 0.02$ ) levels relative to the NC group. The effect sizes for p-tau<sup>181</sup>/A $\beta$ 42 ratio and ApoA-1 were medium, indicating these differences are of moderate importance (Table 2).

Multiple logistic regression analysis (Table 3) was used to assess the predictive value of the individual biomarkers, and only the p-tau<sup>181</sup>/A $\beta$ 42 ratio yielded a significant effect (odds ratio [OR] = 1.42 (95% confidence interval [CI], 1.12, 1.84),  $P = 0.006$ ).

Within-group analyses of the NC and PD cohorts were conducted to determine the effect of ApoE  $\epsilon$ 4 carrier status on the individual biomarkers. For the NC subjects, ApoE  $\epsilon$ 4 carriers had significantly higher ApoE levels than  $\epsilon$ 4 noncarriers ( $P = 0.003$ ). Within the NC group,  $\epsilon$ 4 carriers also had significantly higher

TTR levels ( $P = 0.04$ ). For the PD group, there were no significant differences between  $\epsilon$ 4 carriers and noncarriers on any of the biomarkers.

Correlations among the biomarkers are displayed in Table 4. The p-tau<sup>181</sup>/A $\beta$ 42 ratio correlated moderately with S100B ( $r = 0.42$ ). However, this correlation is probably due to the effect of p-tau<sup>181</sup> ( $r = 0.65$ ) because the correlation of A $\beta$ 42 and S100B is  $-0.01$ . On the other hand, p-tau<sup>181</sup> and A $\beta$ 42 did not show significant effects as individual variables. ApoA-1 demonstrated a strong correlation with FIB ( $r = 0.62$ ) and moderate correlations with CLU ( $r = 0.57$ ) and ApoE ( $r = 0.45$ ). ApoE correlated strongly with CLU ( $r = 0.74$ ) and moderately with A $\beta$ 42 ( $r = 0.45$ ). In addition, FIB showed a moderate correlation with S100B ( $r = 0.59$ ) (Table 4).

In our recent proteomic analysis of the same PD and NC subjects, FIB, ApoA-1, and GST-Pi had lower density values in the PD pool compared to the NC pool, while TTR, ApoE, and CLU values were higher in the PD group relative to the NC.<sup>4</sup> In the current ELISA study, the average quantities of FIB, TTR, and ApoA-1 followed the same trends as in the proteomic study. Both ApoE and ApoA-1 had lower average ELISA values in the PD group, the opposite of the 2D-DIGE results. GST-pi had the same average quantities in the ELISA study. Due to the wide individual variability in the ELISA quantifications (Fig. 1), the average fold change in the proteins ranged from 1.04 to 1.4 with the exception of ApoA-1, which was 2X lower in the PD subjects (Table 2). The 2D-DIGE analysis revealed stronger changes between PD and ND pools (1.6–4.3X).<sup>4</sup>

**Table 3.** Multiple logistic regression analysis for biomarkers.

Biomarker	Odds ratio	95% CI	P-value
Total protein	0.38	0.03, 5.12	0.47
S100B	0.99	0.96, 1.02	0.53
A $\beta$ 42	1.01	1.00, 1.02	0.17
p-tau <sup>181</sup>	1.00	0.99, 1.00	0.26
p-tau <sup>181</sup> /A $\beta$ 42	1.42	1.12, 1.84	0.006
FIB	1.00	1.00, 1.00	0.56
TTR	1.00	1.00, 1.00	0.41
CLU	1.00	1.00, 1.00	0.10
ApoE	1.00	1.00, 1.00	0.27
ApoA-1	1.00	1.00, 1.00	0.24
GST-Pi	1.00	0.98, 1.02	0.95

**Abbreviations:** A $\beta$ 42, amyloid-beta 42; p-tau<sup>181</sup>, tau phosphorylated at amino acid 181; FIB, fibrinogen; TTR, transthyretin; CLU, clusterin; ApoE, apolipoprotein E; ApoA-1, apolipoprotein A-1; GST-Pi, glutathione S-transferase Pi.

**Table 4.** Correlation analysis of biomarkers.

	S100B	Aβ42	p-tau <sup>181</sup>	p-tau <sup>181</sup> /Aβ42	FIB	TTR	CLU	ApoE	ApoA-1	GST-Pi
S100B	–	–0.01	0.65*	0.42*	0.59*	0.38*	0.36*	0.19	0.38*	0.34*
Aβ42	–0.01	–	0.07	–0.30	0.10	0.08	0.38*	0.45*	0.19	0.06
p-tau <sup>181</sup>	0.65*	0.07	–	0.51	0.54	0.19	0.31	0.25*	0.38	0.27
p-tau <sup>181</sup> /Aβ42	0.42*	–0.30	0.51	–	0.34*	0.14	0.04	–0.05	0.05	0.02
FIB	0.59*	0.10	0.54	0.34*	–	0.15	0.40*	0.26	0.62*	0.11
TTR	0.38*	0.08	0.19	0.14	0.15	–	0.30	0.09	0.13	0.19
CLU	0.36*	0.38*	0.31	0.04	0.40*	0.30	–	0.74*	0.57*	0.20
ApoE	0.19	0.45*	0.25*	–0.05	0.26	0.09	0.74*	–	0.45*	0.14
ApoA-1	0.38*	0.19	0.38	0.05	0.62*	0.13	0.57*	0.45*	–	0.14
GST-Pi	0.34*	0.06	0.27	0.02	0.11	0.19	0.20	0.14	0.14	–

Note: \* $P < 0.05$ .

Abbreviations: Aβ42, amyloid-beta 42; p-tau<sup>181</sup>, tau phosphorylated at amino acid 181; FIB, fibrinogen; TTR, transthyretin; CLU, clusterin; ApoE, apolipoprotein E; ApoA-1, apolipoprotein A-1; GST-Pi, glutathione S-transferase Pi.

## Discussion

Proteomic analysis combining 2D separation of V-CSF pools of neuropathologically diagnosed PD and NC, revealed the presence of 6 biomarker molecules that differed between these groups.<sup>4</sup> These 6 proteins were individually measured on the same group of PD and NC individuals by ELISA. Due to the large body of information regarding alterations in the levels of Aβ42, p-tau<sup>181</sup> and, to a lesser degree, S100B in lumbar-derived CSF in PD, we also quantified these molecules in our study. The p-tau<sup>181</sup>/Aβ42 ratio was significantly higher in the PD group, while ApoA-1 level was significantly lower in the PD group relative to the NC group. The effect size for these differences was medium, indicating they are of moderate importance.

The p-tau<sup>181</sup>/Aβ42 ratio yielded a small, but statistically significant OR. This finding was seen despite specifically excluding pathologically confirmed PD subjects with concomitant AD. Other studies, using lumbar-CSF, have reported significantly higher p-tau levels in PD with dementia (PDD) than in PD and NC<sup>17</sup> and a trend or significantly lower values for Aβ42 in PDD and/or PD compared with NC.<sup>17–21</sup> However, a review of 7 independent studies in which Aβ42 levels were appraised in lumbar-CSF found no change in levels of this peptide between PD and NC in 6 studies, and only one group observed lower levels of Aβ42 in PD, but the results were not statistically significant.<sup>22</sup> Additionally, total tau and p-tau have also been reported to have no significant changes between PD without dementia and/or PDD compared with NC cases.<sup>19,20</sup> In a longitudinal study of CSF, lower

baseline Aβ42 levels predicted cognitive decline in PD patients but without significant alterations in total tau and p-tau.<sup>23</sup> It is known that α-synuclein interacts with tau, inducing the aggregation of both of these molecules,<sup>24</sup> and that the binding of α-synuclein to tau also causes tau phosphorylation.<sup>25</sup> Similarly, overexpression of APP/Aβ has been shown to induce the accumulation of neuronal α-synuclein.<sup>26</sup> The diversity of observations reveals the challenge posed by attempting to compare results obtained with living patients with evolving and uncertain pathology and postmortem studies of end-stage disease. Postmortem studies have the advantage of clearly delineated pathology. Many subjects with PD also have a relatively high level of AD histopathology, and many, in fact, meet neuropathological criteria for AD.<sup>14</sup> Although the PD subjects in this study were selected for relatively low levels of AD histopathology, it is possible that the biochemical changes of AD may still be more advanced in these PD subjects as compared with NC individuals. It is also possible that some proteins such as Aβ42 and p-tau may simply be generally associated with neurodegeneration and cellular failure, as they have been linked to other neurological disorders including AD,<sup>27,28</sup> progressive supranuclear palsy and corticobasal degeneration,<sup>29</sup> Creutzfeldt-Jakob disease,<sup>30–33</sup> and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).<sup>34</sup> Therefore, despite its statistical significance, the predictive value of the p-tau<sup>181</sup>/Aβ42 ratio in PD may be somewhat limited.

While some investigators have reported a decrease in Aβ42 levels in ApoE ε4 carriers compared with



ApoE  $\epsilon 4$  noncarriers with PD,<sup>23,35</sup> we did not find any correlations of this type, nor did we observe correlations between ApoE  $\epsilon 4$  carriers and the remainder of the CSF biomarkers. However, a moderately significant and positive correlation was apparent between A $\beta$ 42 and ApoE carrier status when the PD and NC cohorts were combined. Intriguingly, within the NC group only, ApoE  $\epsilon 4$ -positive subjects had significantly higher ApoE levels and TTR levels.

S100B is a multifunctional Ca<sup>2+</sup> binding protein that can act as an intracellular regulator or extracellular signal molecule that interacts with a wide variety of proteins including the receptor for advanced glycation-end products (RAGE).<sup>36</sup> S100B can be neuroprotective at low/physiological levels or toxic at higher concentrations.<sup>7,36,37</sup> Although not connected to a PD diagnosis, increased levels of S100B in the serum of PD patients positively correlated with the progression of disease.<sup>38</sup> Although controversial, this suggests S100B may also serve as a general marker of brain injury or neurodegeneration due to its abundant presence in oligodendrocytes and astrocytes.<sup>7,37</sup> In this context, it has been reported that increased levels of S100B result in glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and hyperphosphorylation of tau.<sup>39</sup> Our analyses revealed that S100B was significantly and positively correlated with p-tau<sup>181</sup>.

CLU and ApoE as well as FIB and ApoA-1 were strongly correlated. However, the significance of these statistical associations is not clear. It was recently observed that the CLU genetic locus is apparently associated with PD and that this association is independent of ApoE  $\epsilon 4$  status.<sup>40</sup> The amounts of CLU in the CSF in PD patients are variable according to the duration of the disease, being more elevated during the first 2 years.<sup>41</sup> Increased levels of plasma FIB have been linked to an elevated risk for PD.<sup>42</sup> There seems to be a positive association between elevated high density lipoprotein cholesterol and, hence, ApoA-1, with enhanced fibrin clot permeability and lysis<sup>43</sup> that may have some protective effects. ApoA-1 has also been found to be decreased in individuals with other neurodegenerative diseases.<sup>44,45</sup> Oxidation of this molecule can lead to elevations of tumor necrosis factor- $\alpha$  that can contribute to neuronal death.<sup>46</sup> As decreased levels of blood lipoproteins, including ApoA-1, are known to occur during fasting, and as PD and other chronic diseases are associated with weight loss,

it is possible that the decreased ApoA-1 concentrations in PD CSF are secondary to fasting, undernourishment, and weight loss. We had previously reported decreased ApoA-1 in AD CSF,<sup>44</sup> another chronic disease that is also associated with weight loss.

Our initial strategy was to investigate, by 2D-DIGE/mass spectrometry, if there were specific molecular differences in pooled CSF specimens originating from neuropathologically diagnosed PD, without other neurodegenerative comorbidities, when compared with an age-matched pool of NC CSF. Those molecules that were substantially increased or decreased in the pooled pair-wise comparisons were subsequently investigated at the individual level by ELISA. However, it should be recognized that ELISA and 2D-DIGE technically differ. ELISAs identify the total level of a given protein based on specific antibody-antigen binding, while 2D-DIGE analyses detect all molecular forms of a given molecule including those that differ by size and charge. Therefore, initial screening by 2D-DIGE may identify a specific isoform of a protein as a potential biomarker that may not be detected by the more broad specificity antibody-antigen binding of ELISAs.

In summary, the search for adequate biomarkers for complex neurodegenerative disorders such as AD and PD has been fraught with frustrations. This reflects the tremendous amount of human variability as well as the heterogeneous presentations of disease illustrated by the different pathological expression and clinical course of a particular neurodegenerative disorder that, not infrequently, has a parallel course with other concurrent morbidities. In addition, there is a large amount of genetic diversity among humans, which is compounded by complicated genetic pleiotropic interactions, epigenetic alterations, and transcriptomic interactions as well as posttranslational modifications and specific protein/ligand events that, at the phenotypic level, dictate the final qualitative and quantitative molecular expression in a given subject. Moreover, the different disease stages at which the fluids are collected and the diverse methodologies utilized in their processing and storage further complicate comparison and validation of biomarker appraisals. The wide range of human variability is reflected in a proteomics evaluation of V-CSF that revealed that out of 249 proteins identified in 10 elderly subjects, 38% were unique to 1 case and not found in the other 9.<sup>47</sup>



Even more surprising was that only 6% of the identified proteins were present in all 10 subjects.<sup>47</sup>

The actual utility of biomarkers identified at the end of a long duration, chronic disease process to serve as an early PD indicator is uncertain and must be assessed in additional studies since postmortem studies in neuropathologically confirmed PD may vary substantially from values obtained in living subjects. Our decision to use postmortem CSF was due to fact that physiological specimens obtained from living patients are at diverse stages of disease while postmortem subjects are all at the end of the disease course. Furthermore, studies of living patients do not have the definite diagnosis of PD that comes with postmortem studies. The use of samples from living subjects is additionally complicated because some PD patients have other concurrent neurodegenerative conditions that will generate confounding factors that are difficult to interpret. However, setting these situations in the context of previous investigations does reveal the extraordinary complexity of PD and its evolution. Efforts to define PD biomarkers in both postmortem and living subjects have demonstrated an exceptional diversity of pathological sequences and reactions that unfold as the disease appears and progresses. These findings suggest that molecular diagnosis may require a constellation of markers and assessments.

### Author Contributions

Conceived and designed the experiments: CLM, TGB, CHA, MM-A, TAK, AER. Analyzed the data: CLM, MM-A, BND, DGW, AER. Wrote the first draft of the manuscript: CLM, TAK, AER. Contributed to the writing of the manuscript: CHA, TGB, BND, HAS, SAJ, MNS. Agree with manuscript results and conclusions: CLM, TGB, CHA, MM-A, TAK, BND, DGW, HAS, SAJ, MNS, AER. Jointly developed the structure and arguments for the paper: CHA, TGB, MNS, AER. Made critical revisions and approved final version: CLM, TGB, CHA, MM-A, TAK, BND, DGW, HAS, SAJ, MNS, AER. All authors reviewed and approved of the final manuscript.

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### Competing Interests

CLM, MM-A, TAK, BND, DGW, SAJ and AER have no conflicts of interest to declare. TGB receives funding from AVID-Radiopharmaceuticals, Bayer Healthcare and GE Healthcare. CHA has received consulting payments from Eli Lilly, Merck Serono, Informa Healthcare, Impax, Ipsen, Merz, and Teva. CHA has grants/grants pending from the Michael J. Fox Foundation and has received royalties from Springer. HAS is a consultant for Ipsen and Merz pharmaceuticals, as well as Medtronic. HAS receives research support from Chelsea Therapeutics, Teva Neuroscience, Schering-Plough, Kalaco Scientific and Avid Radiopharmaceuticals. MNS receives grant/contract support from Avid, Bayer, Baxter, BMS, Lilly, GE, Janssen, Celgene, Ceregene and Pfizer. MNS is a consultant/advisor for EASAI, Bayer, Lilly, Avid, Takeda, Amerisciences and BMS. MNS receives royalties from Amerisciences and Wiley.

### Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclo-





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