RESEARCH ARTICLE

CSF Protein Level of Neurotransmitter Secretion, Synaptic Plasticity, and Autophagy in PD and DLB

Stefanie Lerche, PhD,[1,2] (iD) Simon Sjödin, PhD,[3,4] Ann Brinkmalm,[3,4] Kaj Blennow, MD,[3,4] Isabel Wurster, MD,[1,2]
Benjamin Roeben, MD,[1,2] Milan Zimmermann, MD,[1,2] Ann-Kathrin Hauser,[1,2] Inga Liepelt-Scarfone, PhD,[1,2]
Katharina Waniek, PhD,[5] Ingolf Lachmann, PhD,[5] Thomas Gasser, MD,[1,2] Henrik Zetterberg, MD,[3,4,6,7] and
Kathrin Brockmann, MD[1,2]* (iD)

[1]Center of Neurology, Department of Neurodegeneration and Hertie-Institute for Clinical Brain Research,
University of Tuebingen, Tübingen, Germany
[2]German Center for Neurodegenerative Diseases, University of Tuebingen, Tuebingen, Germany
[3]Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the
University of Gothenburg, Mölndal, Sweden
[4]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden
[5]Roboscreen GmbH, Leipzig, Germany
[6]Department of Neurodegenerative Disease, UCL Institute of Neurology, London, United Kingdom
[7]UK Dementia Research Institute at UCL, London, United Kingdom

ABSTRACT: Background: Molecular pathways associated with α-synuclein proteostasis have been detected in genetic studies and in cell models and include autophagy, ubiquitin-proteasome system, mitochondrial homeostasis, and synaptic plasticity. However, we lack biomarkers that are representative for these pathways in human biofluids.
Objective: The objective of this study was to evaluate CSF protein profiles of pathways related to α-synuclein proteostasis.
Methods: We assessed CSF protein profiles associated with neurotransmitter secretion, synapse plasticity, and autophagy in 2 monocentric cohorts with α-synucleinopathy (385 PD patients and 67 DLB patients). We included 80 PD patients and 17 DLB patients with variants in the glucocerebrosidase gene to serve as proxy for accelerated α-synuclein pathology with pronounced clinical trajectories.
Results: (1) Proteins associated with neurotransmitter secretion, synaptic plasticity, and endolysosomal autophagy were lower in PD and DLB patients compared with healthy controls. (2) These patterns were more pronounced in DLB than in PD patients, accentuated by *GBA* variant status in both entities. (3) CSF levels of these proteins were positively associated with CSF levels of total α-synuclein, with lower levels of proteostasis proteins related to lower levels of total α-synuclein. (4) These findings could be confirmed longitudinally. PD patients with low CSF profiles of proteostasis proteins showed lower CSF levels of α-synuclein longitudinally compared with PD patients with a normal proteostasis profile.
Conclusion: CSF proteins associated with neurotransmitter secretion, synaptic plasticity, and endolysosomal autophagy might serve as biomarkers related to α-synuclein proteostasis in PD and DLB. © 2021 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: lysosome; secretion; synaptic plasticity; CSF; PD; GBA

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*Correspondence to: Dr. Kathrin Brockmann, Department of Neurodegeneration, Hertie Institute for Clinical Brain Research, German Center for Neurodegenerative Diseases, University of Tuebingen, Hoppe Seyler-Strasse 3, 72076 Tüebingen, Germany; E-mail: kathrin.brockmann@uni-tuebingen.de

In the large majority of patients with α-synucleinopathies such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB), the common pathologic feature is the aggregation of abnormal conformations of the protein α-synuclein.[1] Subsequently, several molecular pathways associated with α-synuclein proteostasis have been detected in genetic studies as well as in cell and animal models. These include impairment in autophagy with, for example, endolysosomal dysfunction, ubiquitin-proteasome system, mitochondrial homeostasis, and synaptic plasticity.[2] Despite this clear evidence for pathways involved, we lack validated biomarkers that are clearly disease- and/or pathway-specific in human biofluids. A recent study performed a targeted approach and explored cerebrospinal fluid (CSF) peptide/protein profiles associated with autophagic endolysosomal and ubiquitin-proteasome function using solid-phase extraction combined with parallel reaction monitoring mass spectrometry in 3 cohorts of PD patients, Alzheimer's disease (AD) patients, and healthy controls. Interestingly, CSF protein levels of lysosomal-associated membrane glycoprotein 1 and 2 (LAMP1 and LAMP2), cathepsin F (CTSF), ganglioside GM2 activator (GM2A), AP-2-complex subunit beta (AP2B1), and ubiquitin were lower in PD patients than in AD patients, prodromal AD patients, and healthy controls.[3] With advancing techniques, a recent unbiased quantitative proteomics approach using liquid chromatography–tandem mass spectrometry in data-independent acquisition mode reported decreased CSF levels of proteins associated with neurotransmitter secretion (granin family) as well as with adherens junctions in sporadic PD patients compared with healthy controls.[4] Specifically, CSF protein levels of neurosecretory protein VGF, secretogranin 2 (SCG2), secretogranin 3, and secretogranin 5, chromogranin-A (CHGA) and chromogranin-B, and cadherins were lower in PD. Following this line of evidence, we aimed to evaluate CSF protein profiles associated with pathways of α-synuclein proteostasis in 2 large monocentric cohorts with α-synucleinopathy including a total of 385 PD patients and 67 DLB patients compared with healthy controls. As impaired lysosomal chaperone-mediated autophagy represents a key mechanism in PD, we specifically included PD patients (80 of 385) and DLB patients (17 of 67) with variants in the glucocerebrosidase (GBA) gene. Given the accelerated α-synuclein pathology and pronounced clinical trajectories in DLB compared with PD and in patients with GBA variants compared with the wild-type condition, our primary hypothesis was that profiles in those patient groups would be more distinct.

Participants and Methods

Participants

Between 2005 and 2018, PD patients and DLB patients were recruited from the outpatient clinic and/or ward for Parkinson's disease at the University Hospital of Tuebingen for scientific lumbar puncture. In total, CSF samples from 305 sporadic PD patients without the GBA variant (PD$_{GBA-wildtype}$), 80 PD patients with the GBA variant (PD$_{GBA}$), 50 DLB patients without the GBA variant (DLB$_{GBA-wildtype}$), and 17 DLB patients with the GBA variant (DLB$_{GBA}$) were available. Clinical data and CSF levels of total α-synuclein of 210 PD patients with a median follow-up time of 4 years were available for longitudinal analysis. Spouses of patients and volunteers were clinically assessed to have no neurodegenerative disease and served as control participants (HCs, n = 31).

Genetic Analysis

Genetic screening of all exons of the GBA gene was performed by Sanger sequencing.

We performed GBA subgroup classification for variant severity according to established variant risks reported for PD. Of note, some variants that have been reported as nonrelevant for Gaucher disease have been proven to increase the risk for PD and therefore have been included in our analysis, for example, p.E326K and p.T369M. GBA variants were categorized as: GBA$_{low\_risk}$, E326K, T297S, T369M; GBA$_{mild}$, N370S; GBA$_{severe}$, c1265_1319del55bp, D409H, Ex3 + 4 recombination, IVS2 + 1 g > a, L444P, P182L, R120Q, R359X, W184R.

Clinical Investigations

All participants were examined by a neurologist who specialized in movement disorders. Diagnosis of PD was defined according to the UK Brain Bank Society criteria.[5] Diagnosis of DLB was made according to the revised consensus criteria for DLB developed by the third report of the DLB consortium.[6] PD and DLB patients were assessed in the dopaminergic ON state, and levodopa-equivalent daily dose (LEDD) was calculated. We assessed severity of motor symptoms using part III of the Unified Parkinson's disease Rating Scale (UPDRS-III), from 2006 to 2008 the old version and from 2009 on the MDS-UPDRS.[7] Disease stage was categorized by the modified Hoehn and Yahr Scale (H&Y).[8] Cognitive function was tested using the Montreal Cognitive Assessment (MoCA)[9] and/or the Mini–Mental Status Examination (MMSE).[10] Because the MoCA was available only from 2009 on, all previously obtained MMSE scores were converted to MoCA-equivalent scores.[11] Mood disturbances were assessed using Beck's Depression Inventory II (BDI-II).[12]

Collection of CSF

Spinal tap was performed between 9:00 AM and 1:00 PM. Samples were directly taken from the bedside and centrifuged within 60 minutes and frozen at −80°C

within 90 minutes. Samples with abnormal routine CSF diagnostics (white blood cell count > 6 cells/μL, immunoglobulin subtype G index > 0.7, erythrocytes > 1/μL) were excluded.

CSF Measurement of Total α-Synuclein, Aβ$_{1-42}$, Total-Tau, Phospho-Tau

CSF levels of total α-synuclein were assessed using hSYN total enzyme-linked immunosorgent assay (ELISA) from AJ Roboscreen (Germany). The assay uses a monoclonal capture antibody recognizing amino acids 119–126 and a detection antibody to the C-terminus of α-synuclein. Intra-assay imprecision was 4.4%, and interassay imprecision was below 10%.

CSF levels of Aβ$_{1-42}$, total-tau (t-tau), and phospho-tau (p-tau; phosphorylated at T181) were measured using commercially available ELISA kits (INNOTEST; Fujirebio GmbH, Germany). The intra-assay coefficients of variation for each CSF parameter were below 15%. Concomitant AD CSF profile was defined if Aβ$_{1-42}$ < 650 pg/mL and either h-tau > 450 pg/mL or p-tau > 61 pg/mL.

CSF Measurements of Proteins Related to α-Synuclein Proteostasis

1. *Neurotransmitter secretion*: CHGA, SCG2, VGF.
2. *Synapse plasticity*: NPTX1.
3. *Autophagy, including endocytosis, lysosomal function, ubiquitin-proteasome system*: AP2B1, CTSF, GM2A, LAMP2, ubiquitin.

Sample Preparation

Measurement of protein concentrations was performed as previously described.[3,13] Briefly, 50 μL of CSF was mixed with 50 μL of an internal standard mixture containing stable isotope-labeled peptides (JPT Peptide Technologies GmbH, Germany; Thermo Fisher Scientific Inc., Waltham, MA), 13C-labeled ubiquitin (Silantes, GmbH, Germany), and bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO) diluted in 50 mM NH$_4$HCO$_3$. Reduction and alkylation were performed by the addition of 50 μL of 15 mM 1,4-dithiothreitol in 50 mM NH$_4$HCO$_3$, shaking for 30 minutes at 60°C, cooling down at room temperature for 30 minutes, and the addition of 25 μL of 70 mM iodoacetamide in 50 mM NH$_4$HCO$_3$, followed by shaking at room temperature in the dark for 30 minutes. Samples were digested by the addition of 25 μL of 0.08 μg/μL sequencing-grade modified trypsin (Promega Co., Madison, WI) diluted in 50 mM NH$_4$HCO$_3$ and incubated at 37°C shaking for 18 hours. Digestion was ended by the addition of 25 μL of 10% trifluoroacetic acid. Solid-phase extraction was performed using Oasis HLB 96-well μElution Plates (2 mg sorbent and 30-μm particle size; Waters Co., Milford, MA) by conditioning

(2 × 300 μL methanol), equilibration (2 × 300 μL H$_2$O), loading of samples, washing (2 × 300 μL H$_2$O), and elution (2 × 100 μL methanol). Samples were dried by vacuum centrifugation and stored at −80°C.

Parallel Reaction Monitoring Mass Spectrometry

Samples were dissolved by the addition of 50 μL of 50 mM NH$_4$HCO$_3$ and shaken at room temperature for 1 hour. A total of 40 μL of sample was injected and separated using a Dionex UltiMate 3000 standard-LC system (Thermo Fisher Scientific Inc., Waltham, MA) and a Kinetex EVO C18 column (length, 150 mm; inner diameter, 2.1 mm; particle size, 1.7 μm; Phenomenex Inc., Torrance, CA) with a SecurityGuard ULTRA cartridge prefilter (Phenomenex Inc.). With a 60-minute method, with solvents A (0.1% formic acid in H$_2$O [v/v]) and B (84% acetonitrile and 0.1% formic acid in H$_2$O [v/v]), using a flow rate of 300 μL/min, the gradient went from 3% to 5% B over 1 minute, followed by 5% to 26% B over 48 minutes. The column temperature was set to 50°C. Separation by high-performance liquid chromatography was performed in online mode coupled to a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.). Using a HESI-II ionization probe (Thermo Fisher Scientific Inc.), electrospray ionization was performed in positive ion mode with the following settings: spray voltage, +4.1 kV; heater temperature, 400°C; capillary transfer tube temperature, 380 °C; sheath gas flow rate, 25; auxiliary gas flow rate, 10; and S-Lens RF level, 60. Acquisition of data was performed using single microscans in parallel reaction monitoring (PRM) mode with an isolation window of m/z 2 centerd on the second isotope of the precursor ion. The resolution setting was 70 k, with an AGC target of 1 × 106 and a 256-millisecond injection time. Fragmentation was performed using higher-energy collision-induced dissociation.

Data Extraction

Skyline v.19.1 was used to calculate and export fragment ion peak areas and to monitor fragment ion traces and ratios. The ratio between tryptic peptide and isotope-labeled peptide peak area was used for quantification.

Statistics

Statistical analysis was performed using IBM SPSS 26.0 software. Dichotomous data were analyzed using the Fisher's exact test or the chi-square test. Homogeneity of variance of the variables was evaluated using Levene's test, which showed $P > 0.05$. Intergroup comparisons of demographics, clinical markers, and CSF protein levels were calculated using analysis of variance/analysis of covariance (ANOVA/ANCOVA) including sex, age, and disease duration as covariates as

appropriate. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were calculated to evaluate discriminant capabilities of CSF proteins between patient groups and controls. Pearson's correlation was used to evaluate associations between CSF protein levels.

In PD patients, for each CSF protein associated with pathways related to α-synuclein proteostasis, we assessed the optimal cutoff for pathological levels by calculating Youden's index (Table S3). Values greater than the optimal diagnostic cutoff were considered normal ($PD_{normal\_proteostasis\_profile}$), whereas those below indicated pathologically reduced protein ($PD_{pathological\_proteostasis\_profile}$).

Longitudinal analysis of CSF levels of total α-synuclein and clinical trajectories between $PD_{pathological\_proteostasis\_profile}$ versus $PD_{normal\_proteostasis\_profile}$ was done using a linear mixed model with the fixed factors *group* ($PD_{pathological\_proteostasis\_profile}$, $PD_{normal\_proteostasis\_profile}$) and *time* (time of follow-up in years), their *interaction*, and the random variable *subject*, modeled by random intercepts. The addition of random slopes did not improve model fit. CSF levels of total α-synuclein, MoCA, UPDRS-III, H&Y stage, BDI-II were defined as dependent variables. We analyzed the fixed effect of *group*, *time*, and the interaction of *group and time* on the dependent variable.

Standard Protocol Approval, Registrations, and Patient Consents

The study was approved by the Ethics Committee of the University of Tuebingen (26/2007BO1, 404/2010BO1, 199/2011BO1, 702/2013BO1). All participants gave written informed consent.

Results

Demographics and Clinical Characteristics

PD_{GBA} was younger in mean age (64 vs 66 years; $P = 0.034$) and in mean age at onset (56 vs 60 years; $P = 0.002$) and had a longer mean disease duration (8 vs 7 years; $P = 0.018$) compared with $PD_{GBA\_wildtype}$. UPDRS-III, H&Y staging, MoCA, BDI-II score, and LEDD were not significantly different between PD_{GBA} and $PD_{GBA\_wildtype}$. Demographics and clinical variables did not differ significantly between $DLB_{GBA\_wildtype}$ and DLB_{GBA}. All PD and DLB groups were older than healthy control participants ($P \leq 0.001$; Table 1).

CSF Total α-Synuclein, Aβ$_{1-42}$, Total-Tau, and Phospho-Tau

PD_{GBA} showed lower mean CSF α-synuclein compared with $PD_{GBA\_wildtype}$ (549 vs 623 pg/mL; $P = 0.022$). The same pattern was seen in DLB_{GBA} compared with $DLB_{GBA\_wildtype}$ (457 vs 518 pg/mL;

$P = 0.032$). Mean CSF levels of Aβ$_{1-42}$, total-tau, and phospho-tau did not differ between PD and DLB patients with *GBA* variants compared with those without *GBA* variant. DLB patients more often presented with CSF profiles indicative of AD pathology compared with PD patients and with HCs (21%, 7%, 3%; $P \leq 0.001$). In DLB patients, those with $DLB_{GBA\_wildtype}$ more often showed CSF AD profiles compared with DLB_{GBA} and HCs (26%, 6%, 3%; $P = 0.011$). No differences in CSF AD profiles were found when comparing PD patients with versus those without the *GBA* variant (8%, 5%; $P = 0.447$).

CSF Proteins Related to α-Synuclein Proteostasis

Neurotransmitter Secretion

CSF levels of CHGA, SCG2, and VGF were lower in $PD_{GBA\_wildtype}$, PD_{GBA}, $DLB_{GBA\_wildtype}$, and DLB_{GBA} compared with healthy controls ($P \leq 0.001$; Table 1).

Within the group of PD patients, PD_{GBA_severe} patients had lower CSF levels of SCG2 compared with $PD_{GBA\_wildtype}$ patinets (0.80 vs 1.02) and lower CSF levels of VGF compared with $PD_{GBA\_low\_risk}$ and $PD_{GBA\_wildtype}$ patients (0.70 vs 1.05 vs 1.04, respectively; $P < 0.05$; Table S1).

Within the group of DLB patients, $DLB_{GBA\_mild\_severe}$ had lower CSF levels of CHGA and SCG2 compared with $DLB_{GBA\_wildtype}$ (CHGA, 0.38 vs 0.83, respectively; SCG2, 0.50 vs 0.83, respectively; $P < 0.05$; Table S2).

Synaptic Plasticity

CSF levels of NPTX1 showed a trend toward being lower in PD_{GBA} ($P = 0.087$) and were significantly lower in $DLB_{GBA\_wildtype}$ and DLB_{GBA} compared with healthy controls ($P \leq 0.001$; Table 1).

Stratification according to *GBA* variant severity revealed no significant differences between the PD and DLB groups (Tables S1 and S2).

Autophagy Including Endocytosis, Lysosomal Function, Ubiquitin-Proteasome System

CSF levels of AP2B1 were lower in $PD_{GBA\_wildtype}$ (1.01), PD_{GBA} (0.94), $DLB_{GBA\_wildtype}$ (0.96), and DLB_{GBA} (0.84) compared with healthy controls (1.13); $P \leq 0.01$. CSF levels of GM2A and LAMP2 were lower in $DLB_{GBA\_wildtype}$ (0.98 and 1.04) and DLB_{GBA} (0.87 and 0.87) compared with healthy controls (1.07 and 1.14); $P < 0.05$ and $P < 0.01$, respectively. CSF levels of ubiquitin were lower in $PD_{GBA\_wildtype}$ (1.00) and PD_{GBA} (0.94) compared with healthy controls (1.14); $P \leq 0.001$. CSF levels of CTSF showed no significant differences between groups.

TABLE 1 *CSF levels of pathway-associated proteins in PD patients, DLB patients, and controls*

	Controls, n = 31	PD$_{GBA\_wildtype}$, n = 305	PD$_{GBA}$, n = 80	*P* overall	DLB$_{GBA\_wildtype}$, n = 50	DLB$_{GBA}$, n = 17	*P* overall
Sex (male), n (%)	16 (52)	200 (65)	55 (69)	0.569	34 (68)	14 (82)	0.257
Age (years)	57 ± 13	66 ± 9[c]	64 ± 10[c]	0.034	73 ± 6	71 ± 5	0.131
Age at onset (years)		60 ± 10	56 ± 10	0.002	70 ± 7	67 ± 6	0.199
Disease duration (years)		7 ± 4	8 ± 5	0.018	3 ± 2	3 ± 1	0.888
Hoehn & Yahr		2.1 ± 0.6	2.3 ± 0.7	0.202	2.5 ± 0.6	2.3 ± 0.4	0.354
UPDRS-III		26 ± 12	28 ± 12	0.303	n.a.	n.a.	
MoCA		25 ± 4	24 ± 5	0.161	14 ± 4	15 ± 5	0.761
BDI-II	6 ± 5	9 ± 7	10 ± 7	0.188	n.a.	n.a.	
LEDD		553 ± 379	609 ± 385	0.247	306 ± 265	272 ± 244	0.646
Chromogranin A	1.29 ± 0.65	1.04 ± 0.66[b]	0.92 ± 0.58[c]	0.005	0.83 ± 0.59[c]	0.57 ± 0.21[c]	≤0.001
Secretogranin 2	1.36 ± 0.55	1.02 ± 0.46[c]	0.93 ± 0.46[c]	≤0.001	0.83 ± 0.42[c]	0.66 ± 0.24[c]	≤0.001
Neurosecretory protein VGF	1.45 ± 0.69	1.04 ± 0.60[c]	0.93 ± 0.63[c]	≤0.001	0.70 ± 0.48[c]	0.50 ± 0.24[c]	≤0.001
Neuronal pentraxin 1	1.15 ± 0.37	1.02 ± 0.41	0.96 ± 0.39	0.087	0.85 ± 0.46[c]	0.73 ± 0.24[c]	≤0.001
AP2 complex subunit beta	1.13 ± 0.33	1.01 ± 0.40[b]	0.94 ± 0.35[c]	0.006	0.96 ± 0.49[b]	0.84 ± 0.29[c]	0.004
Cathepsin F	1.02 ± 0.20	1.01 ± 0.27	0.97 ± 0.23	0.400	0.99 ± 0.31	0.99 ± 0.21	0.098
Ganglioside GM2 activator	1.07 ± 0.28	1.01 ± 0.35	0.98 ± 0.35	0.072	0.98 ± 0.48[a]	0.87 ± 0.24[b]	0.020
LAMP2 area	1.14 ± 0.49	1.00 ± 0.46	1.00 ± 0.49	0.117	1.04 ± 0.49[b,d]	0.87 ± 0.24[c]	0.001
LAMP2 ratio	1.13 ± 0.48	1.00 ± 0.45	0.99 ± 0.51	0.095	1.07 ± 0.52[a,d]	0.73 ± 0.30[c]	0.001
Ubiquitin	1.14 ± 0.47	1.00 ± 0.34[c]	0.94 ± 0.35[b]	0.001	1.09 ± 0.51	0.89 ± 0.20[a]	0.033
α-Synuclein (pg/mL)	584 ± 169	623 ± 311[d]	549 ± 260	0.309	518 ± 289[d]	457 ± 281[a]	0.060
Abeta42 (pg/mL)	850 ± 289	678 ± 255[c]	704 ± 253[b]	0.003	494 ± 256[c]	607 ± 212[b]	≤0.001
Total-tau (pg/mL)	207 ± 103	240 ± 125	241 ± 141	0.463	362 ± 231	229 ± 122	0.067
Phospho-tau (pg/mL)	42 ± 17	42 ± 17	40 ± 15	0.221	51 ± 27	38 ± 18[a]	0.089
AD-positive profile, n (%)	1 (3)	21 (7)	4 (5)	0.647	11 (22)[a]	1 (6)	0.033

Data are presented as mean ± standard deviation. Statistical analysis was done by ANCOVA with sex, age, and disease duration (if appropriate) as covariables.
[a]$P < 0.05$ compared with controls;
[b]$P < 0.01$ compared with controls;
[c]$P \leq 0.001$ compared with controls;
[d]$P < 0.05$ compared with GBA.

Stratification according to *GBA* variant severity revealed no significant differences between the PD groups (Table S1).

Within the group of DLB patients, DLB$_{GBA\_mild\_severe}$ (0.42) had lower CSF levels of LAMP2 than DLB$_{GBA\_low\_risk}$ (0.91) and DLB$_{GBA\_wildtype}$ (1.04); $P < 0.05$ (Supplemental Table S2).

ROC Analysis and AUC

Five proteins consistently separated all disease groups (PD$_{GBA\_wildtype}$, PD$_{GBA}$, DLB$_{GBA\_wildtype}$, DLB$_{GBA}$) from healthy controls: CHGA, SCG2, VGF, NPTX1, and AP2B1 and thereby representing all 3 analyzed pathways, AUC > 0.6–0.9.

In addition to the abovementioned proteins, the lysosomal protein ubiquitin discriminated between PD$_{GBA}$ patients and healthy controls (AUC, 0.657), whereas the lysosomal protein LAMP2 discriminated between DLB$_{GBA}$ patients and healthy controls (AUC, 0.735). LAMP2 also discriminated between DLB$_{GBA}$ and DLB$_{GBA\_wildtype}$ (AUC, 0.695); see Figure 1.

Adding α-synuclein as a covariable to the model did not improve the AUC of the proteins in any of the models.

Associations of CSF Proteostasis Protein Levels with CSF Levels of Total α-Synuclein

In PD$_{GBA\_wildtype}$, PD$_{GBA}$, DLB$_{GBA\_wildtype}$, and DLB$_{GBA}$, lower CSF levels of α-synuclein were highly
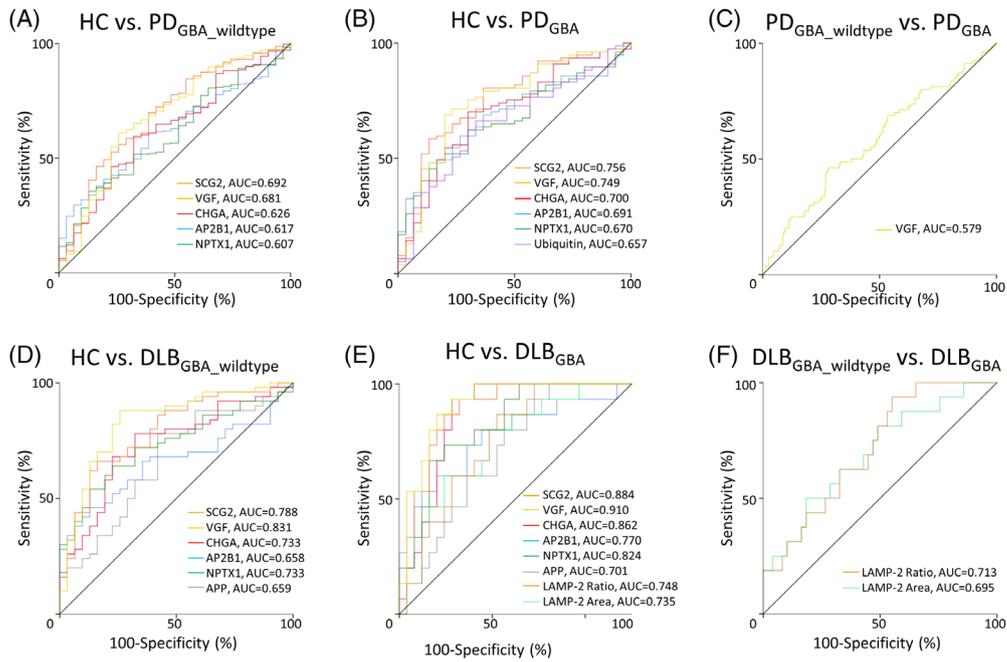
FIG. 1. ROC curves and analysis of proteostasis-related CSF proteins stratified by diagnosis and *GBA* variant status. (**A**) Controls and PD$_{GBA\_wildtype}$. (**B**) Controls and PD$_{GBA}$. (**C**) PD$_{GBA\_wildtype}$ and PD$_{GBA}$. (**D**) Controls and DLB$_{GBA\_wildtype}$. (**E**) Controls and DLB$_{GBA}$. (**F**) DLB$_{GBA\_wildtype}$ and DLB$_{GBA}$. AP2B1, AP-2 complex subunit beta; CHGA, chromogranin-A; LAMP2, lysosome-associated membrane glycoprotein 2; NPTX1, neuronal pentraxin-1; SCG2, secretogranin-2; VGF, neurosecretory protein VGF. [Color figure can be viewed at wileyonlinelibrary.com]

associated with lower CSF levels of all proteins identified in the ROC analysis (AP2B1, CHGA, SCG2, VGF, NPTX1, LAMP2, and ubiquitin), $P \leq 0.01$ (Fig. 2).

Associations of CSF Proteostasis Protein Levels with Demographics and Clinical Scales

Male sex and higher age were weakly associated with lower CSF levels of AP2B1, CHGA, SCG2, VGF, and NPTX1 ($P < 0.05$). Higher H&Y staging and lower MoCA were associated very weakly with lower CSF levels of SCG2, VGF, and NPTX1 ($P < 0.05$). LEDD was not associated with any of the proteins.

PD Patients with a Pathological CSF Protein Profile Related to α-Synuclein Proteostasis Versus Those with a Normal Profile

To discriminate between PD patients with a pathologically low CSF proteostasis profile (PD$_{pathological\_proteostasis\_profile}$) from those patients with a profile similar to healthy controls (PD$_{normal\_proteostasis\_profile}$), Youden's Index was calculated for each CSF protein related to α-synuclein proteostasis (Table S3). We detected 101 PD patients with a pathological CSF protein level below the cutoff in all 6 proteins, whereas 284 showed a normal profile. PD$_{pathological\_proteostasis\_profile}$ patients were younger in mean age (62 vs 67 years; $P \leq 0.001$) and in mean age at onset (56 vs 60 years; $P \leq 0.001$) compared with PD$_{normal\_proteostasis\_profile}$ patients. Also, they were more often male (78% vs 62%; $P = 0.002$), had higher

mean H&Y scores (2.2 vs. 2.1; $P = 0.012$), and lower mean CSF level of total α-synuclein (380 vs 690 pg/mL; $P \leq 0.001$). Mean UPDRS-III, MoCA, and BDI-II scores were not significantly different between PD$_{pathological\_proteostasis\_profile}$ and PD$_{normal\_proteostasis\_profile}$ (Table 2).

Longitudinal Analysis in PD Patients with a Pathological CSF Protein Profile Related to α-Synuclein Proteostasis Versus Those with a Normal Profile

Linear mixed-model analysis revealed that CSF levels of total α-synuclein were influenced by the fixed factor *group* (PD$_{pathological\_proteostasis\_profile}$ vs PD$_{normal\_proteostasis\_profile}$) but not by the factor *time* and the interaction *group/time*. The predicted mean was 397 pg/mL in PD$_{pathological\_proteostasis\_profile}$ vs. 662 pg/mL in PD$_{normal\_proteostasis\_profile}$; $P \leq 0.001$.

H&Y stage and MoCA score were influenced by the factor *time*. For both groups the yearly increase in H&Y was 0.05 points and the yearly decrease in MoCA score was 0.2 points. No effects of group, time, or group/time interaction were seen for UPDRS-III and BDI-II.

Discussion

By assessing CSF protein levels representative for major pathways associated with α-synuclein proteostasis, we show: (1) Proteins associated with
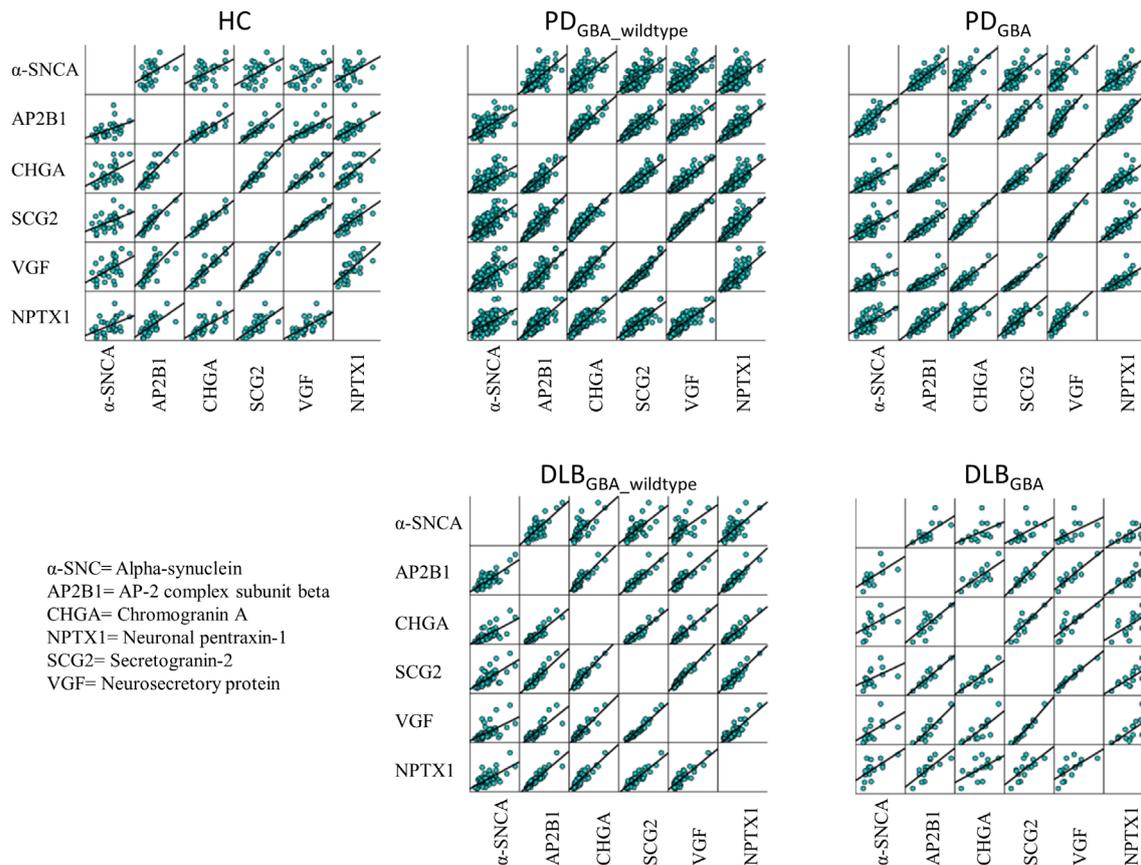
FIG. 2. Correlations of CSF level of pathway-associated proteins with CSF level of total α-synuclein. [Color figure can be viewed at wileyonlinelibrary.com]

neurotransmitter secretion (CHGA, SCG2, VGF), synaptic plasticity (NPTX1), and endolysosomal autophagy (AP2B1, GM2A, LAMP2, ubiquitin) were lower in PD patients and/or DLB patients compared with healthy controls. (2) These patterns were more pronounced in DLB than in PD and were accentuated by *GBA* variant status in both entities, PD and DLB. (3) In all analyzed cohorts, CSF levels of these proteostasis-related proteins were positively associated with CSF levels of total α-synuclein, meaning that lower levels of these proteins were associated with lower levels of total α-synuclein. (4) These findings could be confirmed longitudinally. PD patients with low CSF profiles of proteins related to proteostasis showed lower CSF levels of α-synuclein longitudinally compared with PD patients with a normal proteostasis profile. (5) Lower CSF levels of these proteostasis-related proteins were associated with lower MoCA scores and with higher H&Y staging linking biochemical profiles with clinical outcome parameters.

Decreased CSF levels of the same proteins associated with neurotransmitter secretion (CHGA, SCG2, VGF) as found in our study have recently been identified in an unbiased quantitative proteomics approach in PD patients compared with controls.[4] Results were consistent across 3 US cohorts, one of them longitudinally. These proteins play an essential role in the regulated secretory pathway, which controls the delivery of neurotransmitters and biologically active peptides. They are co-stored in dense-core vesicles and regulate levels of catecholamines such as dopamine. Secretoneurin, a SCG2-derived peptide stimulates the release of dopamine from nigrostriatal neurons.[14,15]

A small study reported CSF levels of CHGA to be reduced in drug-naive PD patients compared with healthy controls. As we did not find an association between LEDD and CSF level of CHGA, these findings suggest that this pattern is already present at diagnosis and not driven by dopaminergic treatment.[16] Interestingly, postmortem findings show that CHGA accumulates in cortical Lewy bodies in a developmental stage-related manner.[17,18]

Using an unbiased proteomics approach in 2 cohorts of patients with DLB, it was recently shown that CSF levels of SCG2 and VGF are also decreased in DLB compared with age-matched control participants.[19] We can confirm this finding. As VGF is associated with learning and memory, the same group investigated CSF levels of VGF not only in DLB but also in AD. They found lower CSF levels of VGF in patients with DLB

TABLE 2   *Demographics and clinical variables in PD patients stratified by proteostasis profile*

	PD$_{normal\_proteostasis\_profile}$, n = 284	PD$_{pathological\_proteostasis\_profile}$, n = 101	*P*
Sex (male), n (%)	175 (62)	79 (78)	0.002
Age (years)	67 ± 9	62 ± 10	≤ 0.001
Age at onset (years)	60 ± 10	56 ± 10	≤ 0.001
Disease duration (years)	7 ± 5	6 ± 5	0.231
Hoehn & Yahr	2.1 ± 0.7	2.2 ± 0.6	0.012[a]
UPDRS-III	26 ± 12	27 ± 11	0.453[a]
MoCA	25 ± 5	25 ± 4	0.338[a]
BDI-II	9 ± 7	9 ± 6	0.969[a]
α-Synuclein (pg/mL)	690 ± 300	380 ± 158	≤ 0.001[a]
Prevalence *GBA* mutation, n (%)	55 (19%)	25 (25%)	0.256

Data are presented as mean ± standard deviation. Statistical analysis was done by ANOVA.

[a]ANCOVA with sex and age as covariables.

compared with either AD patients or controls. Although CSF levels of VGF were positively associated with CSF levels of α-synuclein, no association was found with CSF levels of Abeta$_{1-42}$. In DLB patients, lower levels of VGF were related to more advanced cognitive impairment.[20] VGF is a secreted neuronal precursor protein, and its expression is induced by brain-derived neurotrophic factor, a protein that has been repeatedly shown to be decreased in α-synucleinopathies and has been linked to dopaminergic neuronal survival.[21] VGF is processed into several bioactive peptides that are implicated in energy homeostasis, synaptic biogenesis and synaptic plasticity.[14]

Impaired synapse plasticity is emerging as an important early event in the development of α-synucleinopathies and found already in the period of brain development, as shown in a *LRRK2* p.G2019S mouse model.[22] Neuronal pentraxin-1 (NPTX1) belongs to a family of proteins that are involved in synaptic plasticity. Using whole-genome expression profiling, it was shown that NPTX2 is colocalized with α-synuclein aggregates in the substantia nigra and cerebral cortex in PD.[23] Decreased CSF levels of NPTX1 and NPTX2 have also been shown in patients with frontotemporal dementia and even in presymptomatic mutation carriers (*GRN*, *C9orf72*, or *MAPT*).[24]

With the identification of variants in the *GBA* gene as major genetic risk factor for PD and DLB, it became clear that impairment in lysosomal-mediated autophagy represents a key event in the pathogenesis of α-synucleinopathies.[25,26] We think that the ROC-based differentiation between DLB$_{GBA}$ and DLB$_{wildtype}$ by CSF LAMP2 protein profiles further supports the importance of lysosomal dysfunction based on GBA mutations. We now seem to make progress in the field of pathway-specific biofluid markers in patient cohorts.

This was further underpinned by the finding that genetic variants in other genes associated with lysosomal storage disorders also increase the risk of developing PD.[27] A recent study also found AP2B1, GM2A, and LAMP1 and LAMP2 to be decreased in PD compared with prodromal AD, AD, and healthy controls.[3]

Importantly, lower CSF levels of these proteostasis-related proteins (neurotransmitter secretion, synaptic plasticity, and endolysosomal autophagy) were associated with lower CSF levels of total α-synuclein cross-sectionally and longitudinally. Previous analyses in PD and DLB demonstrated CSF levels of total α-synuclein to be decreased in both diseases compared with healthy controls with even more pronounced decrease in patients with *GBA* variants.[28-31] This mechanistic link between proteostasis-associated proteins and α-synuclein as pathologic hallmark was previously shown in postmortem studies and cell models[32-37] but seems now also detectable in vivo in patient-derived CSF. It is of importance that results and hypothesis coming from cell and animal models can be translated into patient-derived biofluid-marker studies. This field is just beginning to emerge, and we often failed to translate findings/hypothesis into patient cohort. One might imagine that composite profiles of several biofluid markers that are representative of different pathways upstream or downstream of α-synuclein might help to stratify patients for future pathway-specific trials. Of note, the pattern of reduced CSF protein profiles was more pronounced in DLB than in PD and accentuated by *GBA* variant status in both entities. This highlights the currently widely discussed pathomechanistic continuum between PD and DLB[38] with additional acceleration because of *GBA* variants. It will be interesting to evaluate these protein profiles in patients with biallelic variants in recessively inherited PD genes such

as *parkin* and *PINK1*, as these patients often show nigral degeneration without the typical α-synuclein aggregation.

The association of these proteostasis-related proteins with main clinical outcomes such as disease staging by H&Y and cognitive function measured by MoCA warrants further investigation. It will be valuable to assess these proteins in prospective longitudinal cohorts in which CSF along with more complex clinical data and longitudinal clinical trajectories is available from de novo PD patients with several years of follow-up. Such analysis will help to evaluate whether these proteins might serve as biological progression markers that relate to clinical outcomes.

Strengths of our study are: (1) the large monocentric standardized collection of high-quality CSF samples minimizing variance in sample collection and processing; and (2) the validation of findings in longitudinal PD samples and in another α-synucleinopathy, namely, DLB, which represents a histopathological continuum with even more pronounced profiles.

The study had these limitations. (1) As our cohort comprised early- to mid-stage patients, we cannot conclude whether findings are causation or consequence. Therefore, we suggest to explore these findings in de novo and, ideally, prodromal cohorts. (2) The investigated proteins may not be fully representative of the involved pathways that have been discussed. (3) The number of healthy controls is smaller than the number of PD and DLB patients. (4) We lack a direct validation in brain samples from the same patients from whom these CSF profiles were assessed. (5) It is technically possible to measure at least some of the pathway-specific proteins in blood samples, which would be less invasive. However, we do not know whether blood levels of these proteins reflect the CNS milieu. Therefore, it would be important to analyze CSF/blood pairs in future studies to answer this question.

Taken together, our findings suggest that proteins associated with neurotransmitter secretion, synaptic plasticity, and endolysosomal autophagy might serve as biomarkers related to α-synuclein proteostasis in PD and DLB. ◼

Data Availability Statement

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Author Roles

S.L.: Conception, design of the study; execution of the statistical analysis, writing of the first draft
A.B.: Acquisition of study data, critical review of the manuscript
K.B.: Acquisition of study data, critical review of the manuscript
I.W.: Acquisition of study data, critical review of the manuscript
B.R.: Acquisition of study data, critical review of the manuscript
M.Z.: Acquisition of study data, critical review of the manuscript
A.-K.H.: Acquisition of study data, critical review of the manuscript
I.L.-S.: Acquisition of study data, critical review of the manuscript
K.W.: Acquisition of study data, critical review of the manuscript
I.L.: Acquisition of study data, critical review of the manuscript
S.S.: Acquisition of study data, critical review of the manuscript
T.G.: Acquisition of study data, critical review of the manuscript
H.Z.: Acquisition of study data, critical review of the manuscript
K.B.: Conception, organization and execution of the study, manuscript preparation, critical review of the manuscript

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