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Original Article

Phosphorylated Ubiquitin as a Clinical Biomarker for Mitochondrial Damage in Neurodegenerative Diseases

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ABSTRACT: Phosphorylated ubiquitin (pS65-Ub) is generated by the kinase-ligase pair PINK1-Parkin to selectively label damaged mitochondria for degradation via the autophagy-lysosome system (mitophagy). Consistent with increasing mitochondrial and lysosomal dysfunctions, pS65-Ub accumulates with aging in human autopsy brain and in mice. pS65-Ub levels are strongly and independently elevated in brains from subjects with Alzheimer's or Parkinson's disease compared to age-matched, neurologically normal controls. Furthermore, pS65-Ub levels have been used to identify disease risk and potential resilience factors in cells and in human brain. However, it remains unknown whether pS65-Ub measured in biofluids may also be suitable as a clinical biomarker. Here, we used a validated sandwich ELISA based on the Mesoscale discovery platform to assess pS65-Ub levels in over 1500 plasma samples from different cohorts across a spectrum of mild cognitive impairment, Alzheimer's disease, or Parkinson's disease. We further analyzed almost 150 CSF samples from two independent case-control series with Parkinson's disease to determine whether pS65-Ub levels are associated with disease status and other clinical parameters. While pS65-Ub levels are significantly changed with disease compared to controls in certain samples, current measurements in plasma are not sufficiently discriminatory to serve as a robust diagnostic marker. However, in CSF, pS65-Ub levels were decreased in patients with Parkinson's disease compared to controls, and there was better discrimination between these groups. Our data indicate that pS65-Ub shows promise as a biomarker in CSF but will require further replication in larger cohorts and possibly in combination with additional other measures.

Keywords: Alzheimer's disease, autophagy, mitophagy, Parkin/PRKN, Parkinson's disease, PINK1

INTRODUCTION

Mitochondrial dysfunction and impairments in lysosomal degradation are both hallmarks of aging and are also shared, early features of neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) [1]. The enzymatic pair, PINK1-Parkin, identifies and jointly labels selectively damaged

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mitochondria to direct their elimination via the autophagy-lysosome system (mitophagy) [2]. While biallelic loss-of-function mutations in *PINK1* and *PRKN* are the most common causes of familial, early-onset PD [3], the collective data suggest a broader role of the pathway during aging and across a spectrum of neurological diseases [4, 5]. Given the neuroprotective functions of both genes, impairments in this mitochondrial quality control pathway may also contribute to idiopathic, late-onset forms of AD and PD [6-10]. Thus, determining PINK1 and Parkin protein levels or their joint enzymatic activity may provide opportunities to biologically subtype and stratify patients. Upon mitochondrial damage, PINK1 locally accumulates on the outer mitochondrial membrane and phosphorylates ubiquitin (Ub) at Serine-65 to generate pS65-Ub which acts as a receptor and allosteric activator of Parkin [2]. Once fully activated, PINK1 and Parkin engage in a positive feedback loop to amplify the pS65-Ub signal, which also serves as the mitophagy label, facilitating the removal of damaged mitochondria. Inability to recognize mitochondrial damage, inadequate labeling, or incomplete degradation all lead to failure of the PINK1-Parkin pathway, albeit at different steps of a dynamic process [2].

Although pS65-Ub is thought to be only transient, we have demonstrated an increase of pS65-Ub during aging in mouse and human tissue and independently in human brains affected by AD, PD, and related disorders [11-16]. Despite a seemingly general increase of pS65-Ub, the underlying causes may differ as both activation of PINK1-Parkin signaling (elevated induction) or block in autophagic clearance (impaired flux) can elevate levels of this mitophagy marker. Regardless of the exact mechanism(s) leading to its accumulation, pS65-Ub can be exploited as a quantitative marker of mitochondrial damage or ineffective mitochondrial quality control. We and others have already used pS65-Ub levels to screen for genetic modifiers in experimental cell models and in human autopsy brain and thereby identified known disease risk and novel, potential resilience factors [17-21].

Beyond imaging-based approaches and to measure the very low basal levels of activity that are not detectable with other methods, we have recently developed an ultrasensitive sandwich enzyme-linked immunosorbent assay (ELISA) on the Mesoscale Discovery (MSD) platform. This assay discriminates between basal levels in WT and hetero- and homozygous PINK1 KO animals. We also demonstrated that pS65-Ub is also present and detectable in plasma and validated the detection using plasma from a carrier with PINK1 loss-of-function mutation [22, 23]. In addition to its suitability to monitor mitochondrial health and potential as a disease biomarker, pS65-Ub may also be useful as a treatment biomarker to show target engagement and efficacy. As both PINK1 and Parkin cooperate to build the pS65-Ub signal that marks damaged mitochondria for degradation, small molecule mitophagy activators may show a dynamic response with an initial increase of the mitophagy marker followed by turnover of the cargo and a decline in pS65-Ub levels.

In preparation for future clinical trials, we herein set out to evaluate whether plasma or CSF pS65-Ub can serve as a biofluid-based biomarker and to assess its association with age and neurodegenerative disease status across different patient-control cohorts.

MATERIALS AND METHODS

Study design

The goal of this study was to assess biofluid pS65-Ub as a potential diagnostic or prognostic biomarker for neurodegenerative diseases. pS65-Ub was measured using MSD-ELISA in a blinded fashion in different plasma and cerebrospinal fluid (CSF) cohorts. Our primary aim was to determine whether pS65-Ub levels were associated with disease, age at sample collection, and clinical features such as disease severity.

We measured pS65-Ub in plasma samples from four different cohorts. The first cohort included plasma from a Mayo Clinic cohort of age/sex-matched trios consisting of neurological normal controls, individuals with mild cognitive impairment (MCI), or AD (N=135 in each of the three groups, N=405 total), where age was matched by ± 3 years. Probable AD diagnosis was based on clinical criteria [24]. Information was collected regarding age, sex, mini-mental state examination (MMSE) score, and AD dementia clinical severity (AD patients only) as determined by the treating physician. Data regarding MMSE scores were missing for a substantial number of subjects (N=192), while information regarding AD severity was missing for 5 AD patients; characteristics of this cohort are displayed in Supplementary Table 1.

To assess plasma pS65-Ub as a potential biomarker for PD, we use three different cohorts. The first of these three additional cohorts consisted of 111 PD patients and 110 controls collected as part of the Harvard Biomarkers Study (HBS; https://amp-pd.org/unified-cohorts/hbs) [25-28]. Information was collected regarding age, sex, race, ethnicity, age of disease onset, disease duration, MMSE score, Unified Parkinson's disease rating scale (UPDRS) total score, and Hoehn & Yahr (HY) stage. There was a small amount of missing data for MMSE score (N=13 missing), UPDRS total score (N=9 missing), and HY stage (N=7 missing); subject characteristics are shown in Supplementary Table 2.

In the second of the three additional cohorts, to study the behavior of pS65-Ub levels over time within a given

individual, we used samples that were longitudinally collected through the Morris K. Udall Center of Excellence for Parkinson's disease and the American Parkinson Disease Association Center for Advanced Research at Mayo Clinic Jacksonville. This cohort consisted of samples from 69 individuals with PD with at least two and up to five visits for a total of 196 samples; subject characteristics are shown in Supplementary Table 3.

Finally, in the third of the three additional cohorts, we measured plasma pS65-Ub in a large case-control series collected by the LRRK2 Cohort Consortium (LCC; www.michaeljfox.org/lcc). The plasma cohort consisted of 273 PD patients and 440 controls. Information was collected regarding age, sex, age of disease onset, disease duration, presence of a *LRRK2* mutation, Montreal Cognitive Assessment (MoCA) score, UPDRS part III score, and HY stage. There was a small to moderate amount of missing data for age of disease onset (N=76 missing), disease duration (N=76 missing), presence of LRRK2 mutation (N=25 missing), MoCA score (N=202 missing), UPDRS part III score (N=262 missing), and H&Y stage (N=190 missing); characteristics of the LCC cohort are provided in Supplementary Table 4A and also in Supplementary Table 4B when stratifying by *LRRK2* mutation status.

pS65-Ub was also measured in CSF samples of a subset of the LCC cohort (N=45 PD patients and N=49 controls). Three controls who had pS65-Ub measured in CSF did not have pS65-Ub measured in plasma. The second case-control CSF cohort (N=25 PD patients and N=30 controls) was obtained from the HBS. Subject characteristics for these two CSF cohorts are shown in Supplementary Tables 5 and 6, respectively.

pS65-Ub MSD-ELISA

The pS65-Ub MSD-ELISA was developed and validated previously using PINK1 and PRKN knockout cell lines as well as mouse brain [22]. Spike-in experiments with recombinant pS65-Ub chains in plasma revealed matrix effects with a lower limit of quantitation of 2.9 fM and good linearity of the signal [22]. Here, we carried out the assay as described previously. In brief, 1 µg/ml pS65-Ub antibody (Cell Signaling Technology #62802 lot#1 for control/MCI/AD cohort, Cell Signaling Technology #37642 lot#1 for HBS CSF, Cell Signaling Technology #62802 lot#5 was used for all other cohorts) in sodium carbonate buffer (200 mM Na_2CO_3, pH: 9.7) was used for coating 96-well ELISA plates (#L15XA lot 21J42A for control/MCI/AD trios and HBS CSF, lot 21LOIA for all other cohorts) overnight. While we used different batches of reagents for individual cohorts, all samples of one cohort were run with the exact same reagents. Samples were visually checked for hemolysis and vials were spun at 20,000 g for 15 min at 4 °C before neat plasma or CSF samples were loaded onto plates in duplicates. Wells with pooled samples were run on each plate per cohort and used later to normalize results across cohort plates. Raw values of each cohort were adjusted to the average of pooled samples calibrators across all plates. Total ubiquitin detecting antibody clone P4D1 (Santa Cruz, sc-8017, lot #K0119 for control/MCI/AD cohort, and Enzo Life Science BML-PW0930, lot 11032026 for all others) was used at 1 µg/ml, followed by incubation with sulfo-tag coupled anti-mouse secondary antibody (#R32AC-1 lot W0018427S control/MCI/AD cohort and HBS CSF, lot W0020699S for HBS plasma and longitudinal analysis and lot W0021078S for LCC plasma and CSF). Signal was read in MSD Read Gold buffer on a MESO QuickPlex SQ120 (Meso Scale Diagnostics, Rockville, MD, USA). Samples with a coefficient of variation of higher than 20% between duplicate sample measurements were excluded from the analysis.

Data Analysis

Continuous variables were summarized with the sample median and range. Categorical variables were summarized with number and percentage of subjects. For the control, MCI, and AD trio series, comparisons of demographic and disease characteristics between the three groups were made using a Kruskal-Wallis rank sum test (continuous and ordinal variables) or Fisher's exact test (categorical variables). For the HBS and LCC series, comparisons of demographic and disease characteristics between PD patients and controls were made using a Wilcoxon rank sum test (continuous and ordinal variables) or Fisher's exact test (categorical variables). Due to the skewed distribution of pS65-Ub and presence of outliers, we used a rank transformation [29] for pS65-Ub in all of the subsequently described regression analyses involving pS65-Ub in plasma for the control/MCI/AD trio series, the HBS series, and the LCC series; a higher rank corresponds to a higher pS65-Ub level. No outliers were excluded in any analyses.

Because of observed matrix effects in plasma and because pS65-Ub can be present in biological samples in various forms ranging from monomers to chains of different lengths and linkages [13, 30, 31], we have refrained from generating absolute concentrations using recombinant, un-/conjugated pS65-Ub protein as a standard as this likely does not reflect the actual complexity of the pS65-Ub signal. Instead, we use plate-corrected MSD-ECL raw values of each cohort separately to perform comparisons of pS65-Ub signal between controls and disease groups (PD, MCI, and AD) using unadjusted and multivariable linear regression models.

Multivariable models were adjusted for age and sex for all series; race and ethnicity were also added when examining pS65-Ub in plasma in the HBS series. Regression coefficients (denoted as β) and 95% confidence intervals were estimated and are interpreted as the difference in mean pS65-Ub value (on the rank scale for the previously described measures) compared to controls. Additionally, to evaluate the ability of pS65-Ub to discriminate between different disease groups, we estimated the area under the curve (AUC) of the receiver operating characteristics (ROC) curve, where an AUC equal to 0.5 represents no discriminatory ability and an AUC equal to 1.0 represents perfect discrimination.

In separate disease groups, associations of pS65-Ub level with demographic and disease characteristics were examined using unadjusted and multivariable linear regression models. Multivariable models were adjusted for age and sex. β coefficients and 95% CIs were estimated and are interpreted as the change in mean pS65-Ub level (on the rank scale for the previously described measures) corresponding to a specified increase (continuous and ordinal variables) or presence of the given characteristic (categorical variables). To evaluate whether pS65-Ub levels change over time within a given individual in the longitudinal cohort, we first estimated the rate of change in pS65-Ub levels over time in subjects with at least two pS65-Ub measurements that were at least one year apart. Specifically, separately for each subject, a linear regression model was utilized where pS65-Ub was the outcome and time since the first pS65-Ub measurement was the predictor variable, and the beta coefficient corresponding to the time variable was the rate of change in pS65-Ub over time for the given subject. A one-sample t-test was then used to assess whether pS65-Ub changes over time. *p* values less than 0.05 were considered statistically significant. All statistical tests were two-sided. Statistical analyses were performed using R Statistical Software (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism software was used to visualize data (Prism 10 for Mac version 10.4.1).

Ethics approval

This study was approved by the Mayo Clinic Institutional Review Board (IRB#20-011781).

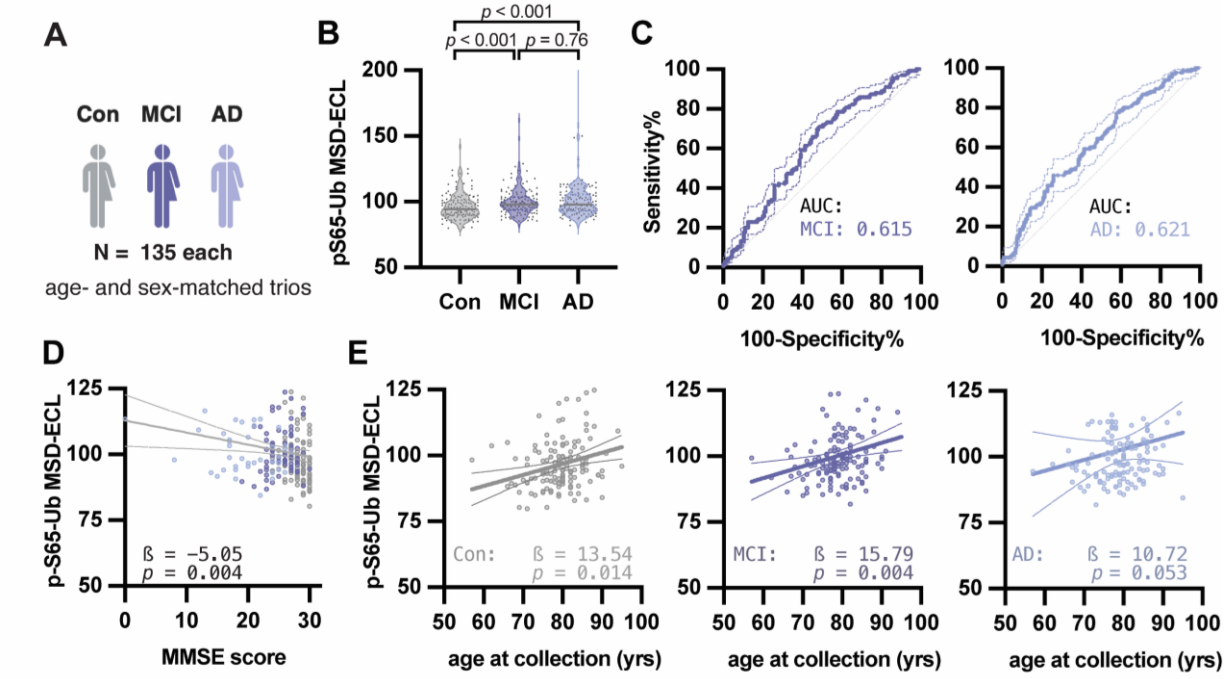

Figure 1. Elevated pS65-Ub plasma levels in cognitively impaired individuals. (**A**) Age- and sex-matched plasma samples from cognitively normal control (Con), individuals with MCI or probable AD (N=135 each) were used for pS65-Ub MSD-ELISA. Details on the study cohort can be found in Supplementary Table 1. (**B**) Shown are violin plots with averaged, plate normalized pS65-Ub MSD-ECL values for each sample. Median and interquartile range are indicated by lines. Plasma samples from subjects with MCI or AD had significantly increased pS65-Ub levels compared to controls. (**C**) Receiver operating characteristic curves indicate sensitivity and specificity of pS65-Ub plasma levels. The 95% CI are indicated as dashed lines and the AUC is given. (**D**) Scatterplot of pS65-Ub plasma levels vs. MMSE scores. (**E**) Scatterplot of pS65-Ub plasma levels in controls, subjects with MCI or AD vs. age of plasma extraction. B, D, E: Shown is the *p* value and ß coefficient (D, E) from multivariable linear regression models that were adjusted for age and sex. $p < .05$ was considered significant.

RESULTS

pS65-Ub plasma levels are increased in cognitively impaired individuals

To test whether pS65-Ub can be used as a clinical biomarker for dementia, we determined levels by MSD-ELISA in a cohort of 405 plasma samples from Mayo Clinic (Fig. 1A). These included 135 trios of cognitively normal individuals, subjects that fulfilled the criteria of MCI, and individuals with dementia that were diagnosed as probable AD by clinical criteria. All samples were age- and sex-matched, and as expected, significant differences were observed in cognitive measures such as MMSE while AD dementia clinical severity was skewed towards mild to moderate disease (Supplementary Table 1).

Interestingly, in analysis that was adjusted for age and sex, pS65-Ub plasma levels were significantly higher in MCI (β: 46.4, $p<0.001$) and AD (β: 50.09, $p<0.001$) or both combined (β: 48.26, $p<0.001$) , compared to controls, with no difference seen between MCI and AD (β: 2.85, $p=0.76$) (Fig. 1B, Table 1). However, AUC values of 0.615 (MCI vs. controls), 0.621 (AD vs. controls), and 0.618 (MCI/AD vs. controls) indicate that pS65-Ub plasma levels alone offered inadequate diagnostic accuracy to distinguish between all groups (Fig. 1C). Regardless, there was a significant association of pS65-Ub plasma levels with cognitive impairment in the combined cohort (β: -5.05, $p=0.004$) (Fig. 1D). Yet, this association was lost when individual groups were analyzed, and there was no statistically significant association of pS65-Ub with AD severity (Supplementary Table 6). pS65-Ub MSD levels were strongly associated with age (β: 38.45, $p<0.001$) when combining all three groups together and consistent with previous findings in human and mouse brain [11, 23]. This association remained mostly present for each separate group and upon adjustment for age and sex (controls: β: 13.54, $p=0.014$, MCI: β: 15.79, $p=0.004$), although for AD the p value was just above the significance threshold (AD: β: 10.72, $p=0.053$) (Fig. 1E, Supplementary Table 7).

In summary, pS65-Ub plasma levels were significantly higher in MCI and AD compared to neurologically normal controls and were associated with the degree of cognitive impairment and with age, but there was poor diagnostic discrimination between groups.

Table 1. Comparison of pS65-Ub level in plasma between control, individuals with MCI or AD in the Control/MCI/AD cohort.

			Unadjusted analysis		Adjusting for age and sex	
Group	**N**	**Median (minimum, maximum) pS65-Ub level**	**β (95% CI)**	***p* value**	**β (95% CI)**	***p* value**
Controls	135	94.46 (79.86, 141.94)	0.00 (reference)	N/A	0.00 (reference)	N/A
MCI	135	97.95 (81.75, 161.97)	45.89 (18.34, 73.44)	**0.001**	46.43 (19.45, 73.41)	**<0.001**
AD	135	97.90 (81.85, 361.09)	49.49 (21.94, 77.04)	**<0.001**	50.09 (23.11, 77.07)	**<0.001**
Controls	135	94.46 (79.86, 141.94)	0.00 (reference)	N/A	0.00 (reference)	N/A
MCI/AD	270	97.92 (81.75, 361.09)	47.69 (23.86, 71.52)	**<0.001**	48.26 (24.92, 71.60)	**<0.001**
MCI	135	97.95 (81.75, 161.97)	0.00 (reference)	N/A	0.00 (reference)	N/A
AD	135	97.90 (81.85, 361.09)	2.82 (-15.93, 21.56)	0.77	2.85 (-15.49, 21.20)	0.76

β=regression coefficient; CI=confidence interval. β coefficients, 95% CIs, and p values result from linear regression models. β coefficients are interpreted as the difference in mean pS65-Ub level (on the rank scale) in comparison to the reference group. The area under the ROC curve of pS65-Ub is 0.615 in distinguishing between MCI patients and controls, 0.621 in distinguishing between AD patients and controls, 0.618 in distinguishing between controls and the combined group of MCI and AD patients and is 0.510 in distinguishing between MCI and AD patients. Significant differences are highlighted in bold.

pS65-Ub plasma levels are increased in subjects with sporadic PD

We next measured pS65-Ub levels by MSD-ELISA in plasma from a cohort of 221 unaffected controls and PD patients (Fig. 2A) with similar age and sex that were obtained from the Harvard Biomarkers Study (Supplementary Table 2). pS65-Ub levels were significantly increased in PD plasma compared to controls after adjusting for age, sex, race, and ethnicity (β: 17.86, $p=0.041$) (Fig. 2B, Table 2). Examination of AUC values showed pS65-Ub plasma levels were inadequate to clearly distinguish PD patients from controls in both sexes (AUC=0.578) (Fig. 2C). In multivariable analysis assessing associations with pS65-Ub, there was no significant association of pS65-Ub with MMSE scores (β: -1.24, P=0.67), consistent with mostly normal cognition in this cohort (MMSE>=24) (Fig. 2D, Supplementary Table 8). Moreover, pS65-Ub plasma levels were also not associated with the age at collection in either the control (β: 0.23, P=0.94) or the PD group (β: -2.99, P=0.38) (Fig. 2E) There was no association of pS65-Ub plasma levels

with the age of onset (β: -4.37, P=0.19) (Fig. 2G) or with clinical scores to assess the severity and progression of PD, such as UPDRS part III (β: -0.13, P=0.95), which mostly assesses motor function, and the HY stage (β: -2.87, P=0.71), which assesses the functional disability associated with PD (Fig. 2G, H). Although there was no association of pS65-Ub with disease duration in the HBS cohort (Supplementary Table 8), we further analyzed pS65-Ub levels in a longitudinal cohort from Mayo Clinic (Supplementary Fig. 1A). However, there was no significant change in pS65-Ub levels over time on the individual level (Supplementary Fig. 1B, Supplementary Table 9).

In summary, while pS65-Ub plasma levels were significantly higher in PD patients compared to controls, pS65-Ub levels were not associated with clinical severity or disease duration, and there was poor discrimination between groups.

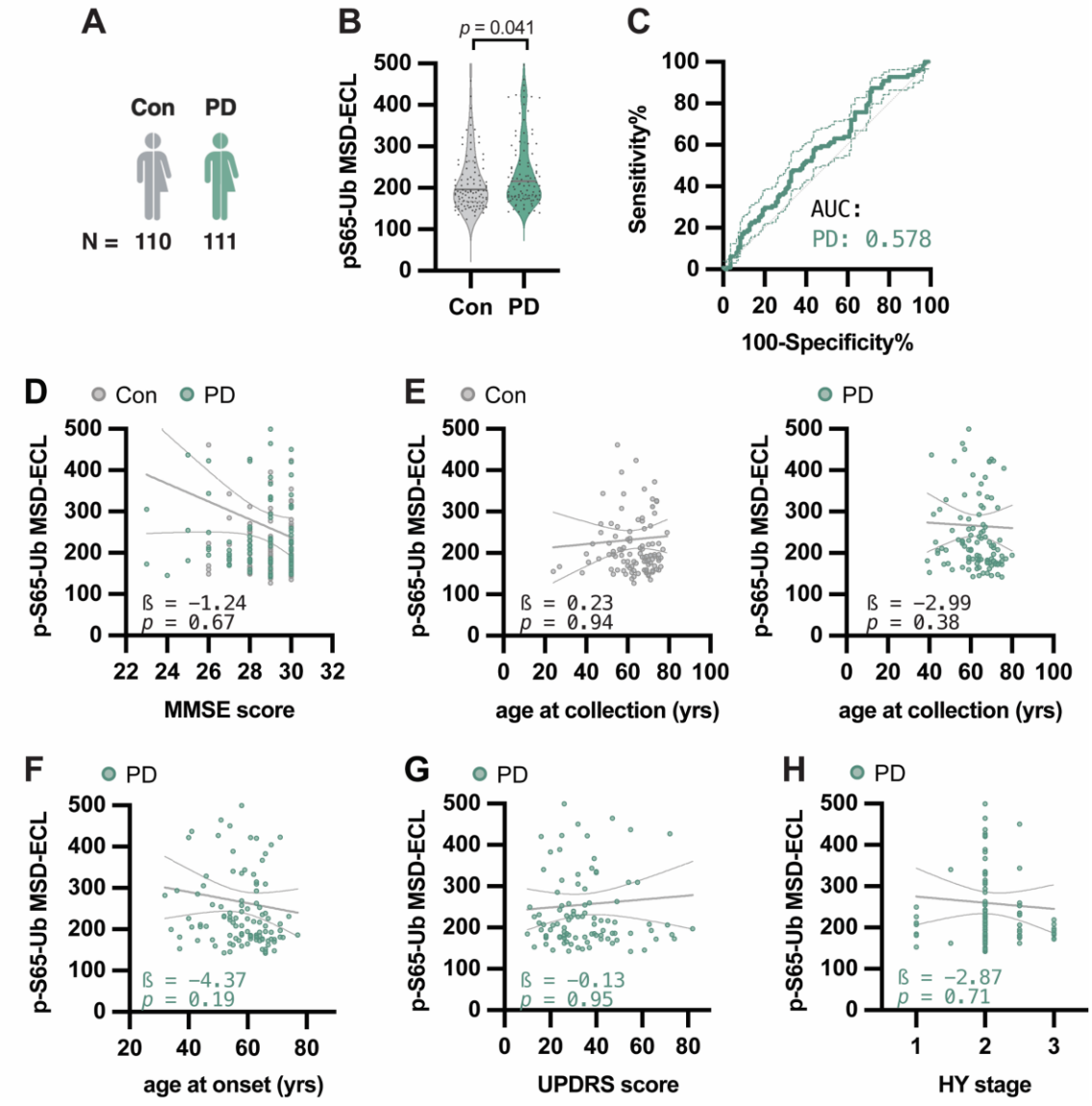

Figure 2. Elevated pS65-Ub plasma levels in individuals with PD. (**A**) Plasma samples from individuals with PD (N=111) and controls (N=110) were subjected to pS65-Ub MSD-ELISA. Details on the study cohort can be found in Supplementary Table 2. (**B**) Shown are volcano plots with averaged, plate normalized pS65-Ub MSD-ECL values for each sample. Median and interquartile range are indicated by lines and the adjusted *p* value is indicated. pS65-Ub MSD-ECL values were significantly elevated in plasma from PD patients compared to controls. (**C**) Receiver operating characteristic curves indicate sensitivity and specificity of pS65-Ub plasma levels. The 95% CI are indicated as dashed lines and the AUC is given. (**D-I**) Scatterplot of pS65-Ub plasma levels vs. (D) MMSE score, (E) age at collection of controls and PD patients and (F-H) age at onset, UPDRS3 score, and HY stage. B, D-H: Shown is the *p* value and ß coefficient (B-H) from multivariable linear regression models that were adjusted for age and sex. $p < .05$ was considered significant.

Table 2. Comparison of pS65-Ub level in plasma between controls and PD patients in the HBS cohort.

			Unadjusted analysis		Adjusting for age and sex, race and ethnicity	
Group	**N**	**Median (minimum, maximum) pS65-Ub level**	**β (95% CI)**	***p* value**	**β (95% CI)**	***p* value**
Controls	110	195.97 (127.47, 2953.03)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD patients	111	216.54 (142.08, 945.52)	17.18 (0.34, 34.02)	**0.046**	17.86 (0.73, 34.98)	**0.041**

β=regression coefficient; CI=confidence interval. β coefficients, 95% CIs, and *p* values result from linear regression models. β coefficients are interpreted as the difference in mean pS65-Ub level (on the rank scale) for PD patients in comparison to the reference group of controls. The area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.578. Significant differences are indicated in bold.

Plasma pS65-Ub levels are inconspicuous in a large PD case-control study, independent of *LRRK2* mutation status

To validate our findings, we obtained blinded samples from another large multi-center case-control study of the LRRK2 cohort consortium consisting of a total of 440 controls and 273 PD patients with or without pathogenic variants in *LRRK2* (Supplementary Tables 4A and 4B, Fig. 3A). In contrast to the HBS cohort, there was no difference in pS65-Ub levels when comparing controls versus PD (β: -3.48, P=0.84) (Fig. 3B), and correspondingly the AUC was 0.510 (Table 3). Separating the non-*LRRK2* mutation and *LRRK2* mutation carriers, there was also no significant difference in pS65-Ub between individuals without or with PD (Non-*LRRK2*, β: 12.83, P=0.24, AUC=0.545; *LRRK2*, β: -18.17, P=0.20, AUC=0.511) (Fig. 3C, Table 3). Linear regression models that were adjusted for age and sex showed pS65-Ub plasma levels were significantly associated with male sex in controls (β: 28.46.11, P=0.021) but not in PD patients (β: 13.65, P=0.16) (Supplementary Table 10). There was no significant association of pS65-Ub with the age of collection (β: 3.58, P=0.54) (Fig. 3D). In PD patients, there were no significant associations of pS65-Ub MSD levels with age at onset, UPDRS part III score, or HY stage (Fig. 3E-G, Supplementary Table 10).

In summary, there was no discriminatory ability of pS65-Ub in the LCC case-control plasma cohort.

Table 3. Comparison of pS65-Ub level in plasma between controls and PD in the LCC series.

			Unadjusted analysis		Adjusting for age and sex	
Group	**N**	**Median (minimum, maximum) pS65-Ub level**	**β (95% CI)**	***p* value**	**β (95% CI)**	***p* value**
Overall series						
Controls	440	193.00 (104.40, 11633.47)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	273	190.16 (96.34, 1206.66)	6.74 (-24.43, 37.91)	0.67	-3.48 (-38.03, 31.07)	0.84
No *LRRK2* mutation						
Controls	180	186.44 (104.40, 11633.47)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	69	194.43 (127.77, 725.50)	11.16 (-8.92, 31.24)	0.28	12.83 (-8.50, 34.15)	0.24
***LRRK2* mutation**						
Controls	249	196.51 (112.09, 1400.86)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	190	183.30 (96.34, 1206.66)	-4.93 (-28.97, 19.12)	0.69	-18.17 (-45.85, 9.51)	0.20

β=regression coefficient; CI=confidence interval. β coefficients, 95% CIs, and *p* values result from linear regression models. β coefficients are interpreted as the difference in mean pS65-Ub level on the rank scale for PD patients in comparison to the reference group of controls. For the overall series, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.510. For *LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.511. For non-*LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.545.

Sporadic PD patients have significantly lower pS65-Ub CSF levels

Uncertainty remains regarding the ideal biofluid in the assessment of pS65-Ub as it correlates to CNS neuropathology. Thus, we next analyzed pS65-Ub in available CSF samples. The first set was obtained from the LCC and contained samples from 49 controls and 45 PD patients (Fig. 4A) with or without *LRRK2* mutations (Supplementary Table 5). In general, in CSF, the measured pS65-Ub levels showed a lower spread compared to data obtained in plasma (Fig. 4B). In contrast to plasma, pS65-Ub MSD levels were significantly decreased in PD patients compared to controls in CSF in age/sex-adjusted analysis (β: -24.51, P=0.022); the estimated AUC was 0.628, and this was similar for *LRRK2* mutation carriers (AUC=0.610) and non-carriers (AUC=0.652) (Table 4). There was no significant association of pS65-Ub with demographic or clinical

variables in controls or PD patients (Supplementary Table 11).

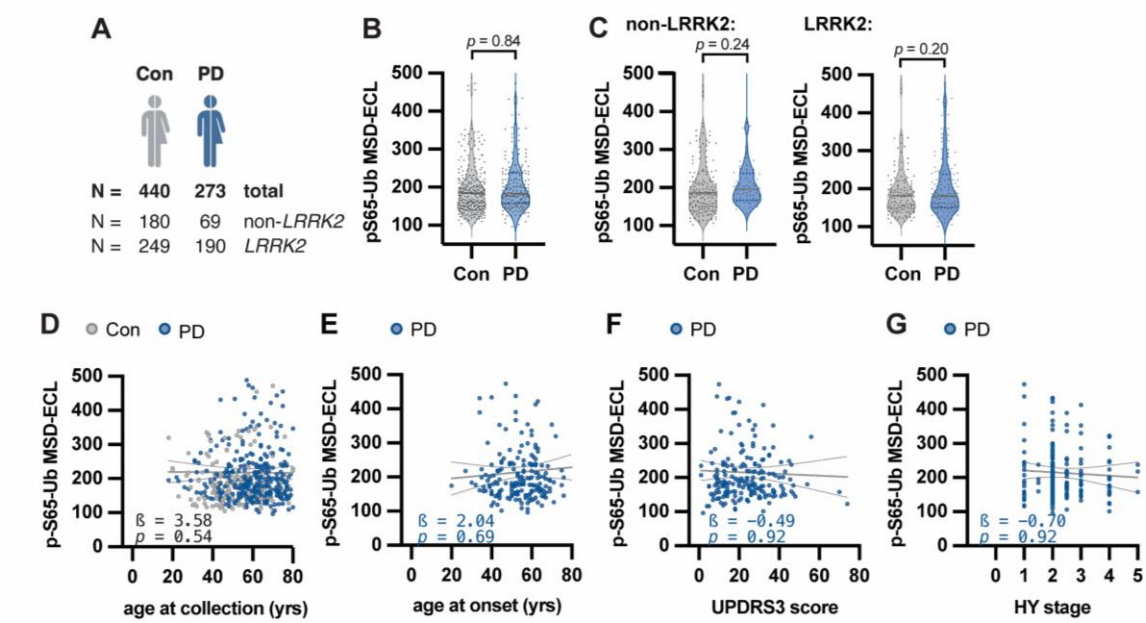

Figure 3. pS65-Ub plasma levels are unchanged between PD and control in the LCC series. (**A**) pS65-Ub levels were measured in 713 plasma samples in the case-control series from the LCC. (**B**) Shown are volcano plots with averaged, plate normalized pS65-Ub MSD-ECL values for each sample. Median and interquartile range are indicated by lines and adjusted *p* value is indicated. pS65-Ub plasma levels from subjects with PD were undistinguishable from controls. (**C**) Samples were separated into two groups for *LRRK2* mutation presence and analyzed separately using adjusted Wilcoxon rank sum tests. (**D-G**) Shown are scatterplots of pS65-Ub plasma levels vs. age at collection (D) and (in PD patients only) with age at onset (E), UPDRS3 score (F) and Hoehn and Yahr stage (G). B-G: Shown is the *p* value and ß coefficient (D-G) from multivariable regression models adjusted for age and sex. $p < .05$ was considered significant.

Case-control CSF samples from the HBS (Fig. 4D, Supplementary Table 6) were used as a replication cohort. As was the case in the LCC CSF cohort, pS65-Ub MSD CSF levels were significantly lower in PD compared to control samples in multivariable analysis (β: -9.12, P=0.005) (Fig. 4E, Table 4); the AUC of 0.715 indicates a moderate ability to discriminate between cases and controls. While there was limited association of pS65-Ub with most clinical parameters, there was a significant association of pS65-Ub CSF levels with disease duration in multivariable linear regression models (β: 6.01, P=0.041) (Supplementary Table 12), suggesting that pS65-Ub MSD CSF levels change over time in PD patients.

Table 3. Comparison of pS65-Ub level in plasma between controls and PD in the LCC series.

			Unadjusted analysis		Adjusting for age and sex	
Group	**N**	**Median (minimum, maximum) pS65-Ub level**	**β (95% CI)**	***p* value**	**β (95% CI)**	***p* value**
Overall series						
Controls	440	193.00 (104.40, 11633.47)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	273	190.16 (96.34, 1206.66)	6.74 (-24.43, 37.91)	0.67	-3.48 (-38.03, 31.07)	0.84
No *LRRK2* mutation						
Controls	180	186.44 (104.40, 11633.47)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	69	194.43 (127.77, 725.50)	11.16 (-8.92, 31.24)	0.28	12.83 (-8.50, 34.15)	0.24
***LRRK2* mutation**						
Controls	249	196.51 (112.09, 1400.86)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	190	183.30 (96.34, 1206.66)	-4.93 (-28.97, 19.12)	0.69	-18.17 (-45.85, 9.51)	0.20

β=regression coefficient; CI=confidence interval. β coefficients, 95% CIs, and *p* values result from linear regression models. β coefficients are interpreted as the difference in mean pS65-Ub level on the rank scale for PD patients in comparison to the reference group of controls. For the overall series, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.510. For *LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.511. For non-*LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.545.

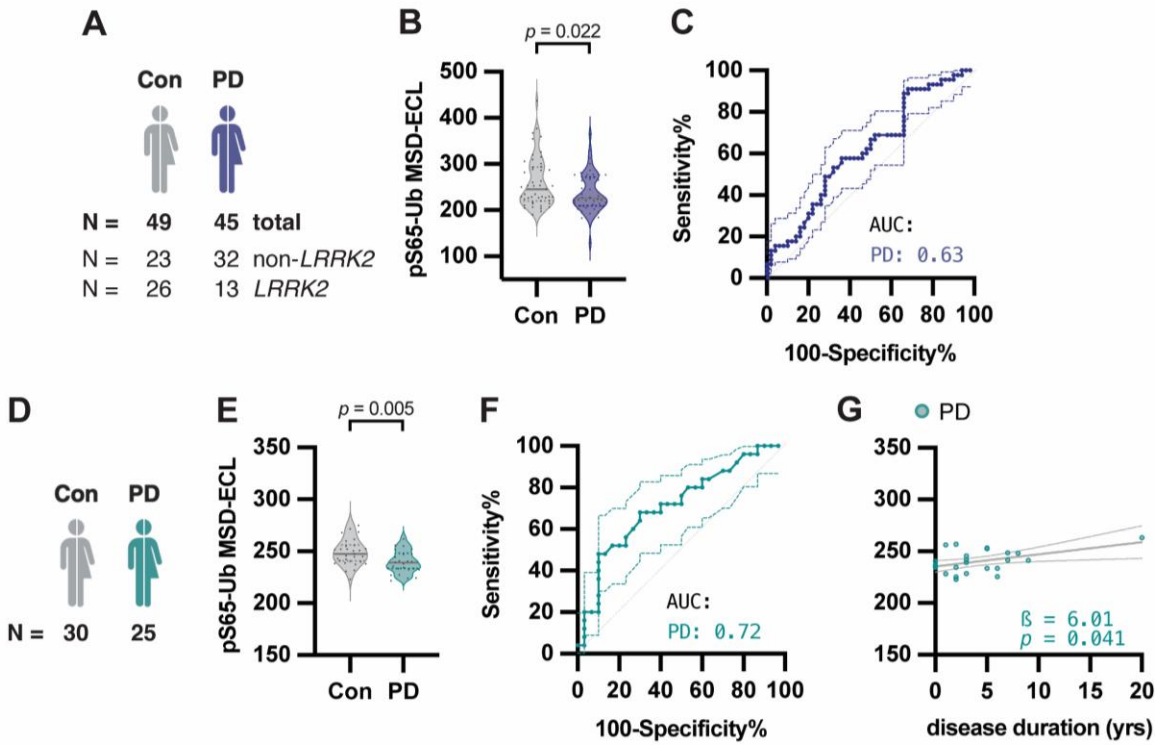

Figure 4. PD patients have significantly lower pS65-Ub CSF levels. (**A**) pS65-Ub levels were measured in 94 CSF samples in the case-control series from the LCC. (**B**) Shown are volcano plots with averaged, plate normalized pS65-Ub MSD-ECL values for each sample. Median and interquartile range are indicated by lines. CSF samples from subjects with PD have significantly lower pS65-Ub levels compared to controls. Shown is the age- and sex-adjusted *p* value. (**C**) Receiver operating characteristic curves indicate sensitivity and specificity of pS65-Ub plasma levels. The 95% CI are indicated as dashed lines and the AUC is given. (**D**) pS65-Ub levels were measured in 55 CSF samples in the case-control series from the HBS. (**E**) Shown are volcano plots with averaged, plate normalized pS65-Ub MSD-ECL values for each sample. Median and interquartile range are indicated by lines. CSF samples from subjects with PD have significantly lower pS65-Ub levels compared to controls. Shown is the age- and sex-adjusted *p* value. (**F**) Receiver operating characteristic curves indicate sensitivity and specificity of pS65-Ub plasma levels. The 95% CI are indicated as dashed lines and the AUC is given. (**G**) Scatterplot of pS65-Ub CSF levels in PD patients with disease duration. B, E, G: Shown is the *p* value and ß coefficient (G) from a multivariable linear regression model that was adjusted for age and sex. $p < .05$ was considered significant.

In summary, pS65-Ub MSD measurements in CSF revealed a higher degree of discrimination between controls and PD with a possible association with disease duration.

DISCUSSION

pS65-Ub is generated in response to mitochondrial damage and has been successfully used to identify disease risk and potential resilience factors in cultured cells and in human autopsy brain [17-21]. Herein, we evaluated the suitability of this mitophagy label as a potential disease biomarker in clinical samples, capitalizing on a sensitive and thoroughly validated sandwich assay that allows detection of basal (i.e., non-stress) PINK1-Parkin signaling in experimental models that currently cannot be captured otherwise [11, 22, 23]. We measured pS65-Ub levels in over 1,500 plasma and almost 150 CSF samples that were obtained from independent single or multi-center studies and applied stringent statistical methods, adjusting for age and sex.

In plasma from two different case-control cohorts, we found pS65-Ub to be significantly increased in diseased individuals compared to healthy subjects. These included age- and sex matched individuals with MCI or AD as well as patients with PD each compared to their respective normal controls (MCI/AD: N=405 and PD: N=221, respectively). There was an association of plasma pS65-Ub levels with age in some but not all groups, and this was potentially dependent on different age ranges of the cohorts. There was also an association with male sex in some but not all controls cohorts. It is possible that sex difference in pS65-Ub could be driven by higher oxidative stress levels in males [32].

Table 4. Comparison of pS65-Ub level in CSF between controls and PD in the LCC and HBS series.

			Unadjusted analysis		Adjusting for age and sex	
Group	**N**	**Median (minimum, maximum) pS65-Ub level**	**β (95% CI)**	***p* value**	**β (95% CI)**	***p* value**
Overall LCC series						
Controls	49	245.64 (189.62, 436.74)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	45	225.96 (129.26, 364.72)	-23.29 (-43.55, -3.03)	**0.025**	-24.51 (-45.47, -3.56)	**0.022**
***No LRRK2* mutation**						
Controls	23	249.63 (197.44, 353.93)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	32	222.97 (182.36, 364.72)	-19.34 (-42.40, 3.71)	0.098	-29.46 (-53.01, -5.90)	**0.015**
***LRRK2* mutation**						
Controls	26	231.35 (189.62, 436.74)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	13	227.16 (129.26, 330.20)	-28.37 (-69.23, 12.50)	0.17	-31.30 (-73.49, 10.89)	0.14
HBS series						
Controls	30	247.65 (224.10, 276.15)	0.00 (reference)	N/A	0.00 (reference)	N/A
PD	25	239.03 (222.89, 263.26)	-9.52 (-15.86, -3.17)	**0.004**	-9.12 (15.35, -2.89)	**0.005**

β=regression coefficient; CI=confidence interval. β coefficients, 95% CIs, and *p* values result from linear regression models. β coefficients are interpreted as the difference in mean pS65-Ub level (on the rank scale for pS65-Ub in plasma) for PD patients in comparison to the reference group of controls. For the overall LCC series, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.628. For *LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.610. For non-*LRRK2* mutation carriers, the area under the ROC curve of pS65-Ub in distinguishing between PD patients and controls is 0.652 for pS65-Ub in CSF. The area under the ROC curve of pS65-Ub in CSF of the HBS cohort is 0.715.

Environmental, dietary or lifestyle factors (e.g., exercise) could override such pattern in some cohorts and an association with male sex might also be dependent on the hormonal status of females included in the study. Given that male sex was also associated with pS65-Ub in some of PD cohorts in the unadjusted analysis, we recommend continuing of sex-stratified analyses in future studies. We further noted an inverse relationship with cognitive function in the MCI and AD groups as measured by the MMSE, but not for motor symptoms or disease duration in PD. The reason for a limited correlation with clinical progression markers remains unclear. It is possible that pS65-Ub correlates with the underlying pathophysiology but in a manner that is not proportional to the degree of clinical impairment. Given that disease progression in neurodegenerative disorders might not be caused by the worsening primary mechanism(s) but instead might be mostly driven by the loss of compensatory strategies, it is plausible that pS65-Ub as a marker of the underlying pathophysiology would not accumulate further. While we were unable to further confirm an association of plasma pS65-Ub levels with disease status in a multi-center PD case-control series with or without pathogenic variants in *LRRK2* (MJFF/LCC: N=713), an independent group described the significant association of pS65-Ub in a large overlapping cohort, but using another immunoassay platform (SMCxPro) with reported several-fold higher sensitivity compared to MSD-ELISA [33]. It is important to mention that plasma pS65-Ub levels seem generally low with considerable matrix interference approaching the lower limit of quantitation especially with the MSD-ELISA. As such, despite significant differences between cases and controls in most cohorts, the discrimination in plasma was inadequate (AUC ~0.6) to serve as a diagnostic biomarker. We question whether advances in pS65-Ub detection technology are needed to improve the signal-to-noise ratio and matrix interference and to reevaluate diagnostic fitness of pS65-Ub in plasma. Alternatively, it is possible that plasma pS65-Ub is a poor marker of initial diagnosis but be useful as pharmacodynamics marker upon treatment of individuals with modulators of mitophagy that are currently being developed for the clinic [5]. Samples from pharmacodynamics experiments in animal models will be crucial to determine whether plasma pS65-Ub is reflective of CNS changes, or results from other tissues [34].

In contrast to plasma, pS65-Ub levels were found to be significantly decreased in CSF from PD patients compared to normal controls, and this was seen in samples from two independent cohorts (LCC: N=94 and HBS: N=55). Overall, and compared to plasma, pS65-Ub levels appeared higher in CSF with less matrix effects resulting in more precise measurements, especially in the HBS cohort. This was also reflected in the better diagnostic accuracy (AUC up to 0.72) seen in both cohorts. While the limited sample size did not allow studying detailed associations with clinical features beyond disease status, there was a positive association with disease duration and CSF pS65-Ub. However, future analysis of longitudinal samples is required to corroborate this as the used cohorts contained only few samples from individuals with longer disease duration. It will also be worth investigating at-risk individuals in future prognostic cohorts and to follow them over time to assess their phenoconversion and how this relates to baseline or altered pS65-Ub levels.

It was unexpected that pS65-Ub levels were lower in PD CSF given that the mitophagy label significantly accumulates in brain with age and independently with different neurodegenerative disease [12-16]. We have previously shown that pS65-Ub deposits are mostly found in neurons [14], and it is plausible that the neuronal accumulation of pS65-Ub with disease causes lower clearance into the CSF similar to the lower CSF levels of Aβ seen in AD [35]. Additional research would be needed to answer this question including paired assessments of pS65-Ub central nervous system parenchymal burden and CSF/plasma levels.

Our study has several limitations. The diverse sources of biologic specimens resulted in substantial differences in sample collection and storage techniques, which likely impact the performance of our assay. Furthermore, the AD samples were collected prior to routine biomarker-based diagnostic confirmation and the diagnosis relied on clinical criteria alone, increasing the likelihood of alternative pathologies beyond AD were included in the samples. Multiple neuropathologic correlation studies demonstrate that approximately 12-23% of individuals clinically diagnosed with probable AD lack sufficient AD pathology at autopsy [36-38]. Additionally, some of our cohorts had distributions skewed toward more mild or early disease, which limited a fair assessment of the spread over the full natural history. Also, the majority of *LRRK2* PD patients in the LCC series are thought to be carriers of the G2019S mutation, but the exact genetic status was not known for most of them. While MSD-ELISA pS65-Ub measures in CSF seem quite robust, sensitivity might be further improved by translating the assay to other single molecule counting technologies that will allow automated and ultrasensitive quantification of pS65-Ub in biofluids which may further improve precision and increase accessibility. Yet, several factors might also affect assay performance. Because remeasuring the same samples upon freeze-thaw led to a drop in ECL values, we suspect that temperature fluctuations during storage and transport of samples might affect the outcome. While many of the outcomes presented herein could principally be confirmed using different reagents and we have confirmed the successful execution of each plate using positive and negative controls, it is possible that lot-to-lot variability and the use of different reagents that we did not normalize or control for in this setting contributed to non-identical results between cohorts. However, the generation of newly available recombinant monoclonal pS65-Ub antibodies [39] should further enhance reproducibility and allow standardization of assay conditions. Finally, although the sample size was not small overall, the possibility of a type II error (i.e., a false-negative finding) is still important to consider, especially in analyses of smaller subgroups (such as CSF cohorts) and in plasma where the signal over background ratio was very low. We cannot conclude that a true difference or association does not exist simply due to the occurrence of a non-significant *p*-value in this study, and emphasis is best placed on 95% confidence limits when interpreting our results.

A few recent studies have already reported a general increase in levels of PINK1, Parkin, and other mitophagy associated proteins both in plasma/serum and in CSF from patients with PD or Parkinsonian disorders or MCI and AD [40-42]. However, it is unclear whether these commercially available ELISAs have been validated in experimental knockout models and tested in relevant patients' matrices. In addition to the pS65-Ub assay used herein [22], we have very recently developed such MSD-ELISA also for PINK1 [43] and Parkin (in preparation) that could be deployed and potentially multiplexed in the future. It will be crucial to reach consensus and ensure all assays are specific, reproducible, and widely accessible in order to compare data between laboratories. Ultimately, a combination of these assays will likely help shed light on those who have significant mitochondrial dysfunction as part of their underlying pathophysiology.

The suitability of pS65-Ub as a physiological biomarker certainly needs to be further explored. An important next step would be the analysis of longitudinal CSF samples to validate whether pS65-Ub levels in CSF are indeed associated with disease duration. For most conclusive results large, well-defined sample cohorts are needed. These ideally would contain samples from biomarkers or autopsy-confirmed individuals, with comprehensive clinical records as well as longitudinal collection of plasma and CSF. Disease heterogeneity of neurodegenerative diseases provides significant challenges for biomarker discovery that are based on physiological as opposed to pathological changes. An integrative approach combining data from different sample types and multiple analytes however may allow biological subtyping of cases which will also facilitate stratification as aberrant pS65-Ub levels in biofluids may reflect compromised mitochondrial quality control or altered neuronal homeostasis, triggered by the accumulation of specific neuropathological aggregates. The autophagy/mitophagy space has been recognized for its enormous potential to furnish diagnostic or prognostic biomarkers [44, 45]. Companion markers such as LC3-II and p62, key proteins involved in autophagosome formation and clearance, could provide valuable complementary information [46, 47]. As these novel biological markers mature, they will likely be combined with other emerging readouts such as quantitative alpha-synuclein seed amplification assays that are currently being developed and standardized [48, 49]. A panel combining mitochondrial, autophagy, and pathological

markers would help to clarify the underlying dysfunction driving altered pS65-Ub levels in the context of neuropathology, improving both disease characterization and the development of targeted therapeutic strategies. While the PINK1-Parkin pathway can be considered as a measure of mitochondrial health, given the recent development of small-molecule mitophagy activators, pS65-Ub and other mitophagy readouts should also be investigated as pharmacodynamic markers to probe target engagement and treatment efficacy in upcoming clinical trials [5, 50].

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Potential conflicts of interests

This research was conducted in compliance with Mayo Clinic conflict of interest policies. All authors declare they have no relevant competing interests. Mayo Clinic, F.C.F., and W.S. hold a patent related to PRKN activators (Small Molecule Activators of Parkin Enzyme Function, US patent,11401255B2; August 02, 2022). Additional funding sources to disclose but not pertinent to the current study include a grant from Amazentis SA (to W.S.). C.K. serves as a medical advisor to Centogene and has received consulting fees or speakers' honoraria from Takeda, Bial, and Biogen, as well as royalties from Oxford University Press and Springer Nature). Z.K.W. serves as PI or Co-PI on Biohaven Pharmaceuticals, Inc. (BHV4157-206), Vigil Neuroscience, Inc. (VGL101-01.002, VGL101-01.201, PET tracer development protocol, Csf1r biomarker and repository project, and ultra-high field MRI in the diagnosis and management of CSF1R-related adult-onset leukoencephalopathy with axonal spheroids and pigmented glia), and ONO-2808-03 projects/grants. He serves as Co-PI of the Mayo Clinic APDA Center for Advanced Research and as an external advisory board member for the Vigil Neuroscience, Inc., and as a consultant for Eli Lilly & Company and for NovoGlia, Inc.

Author contributions

F.C.F., K.L., C.K., D.P.N., C.R.S., N.E.-T., N.R.G.-R., Z.K.W., O.A.R., and W.S. contributed to the conception and design of the study, F.C.F., J.O.W., M.G.H., S.B., M.J.R., and M.K. contributed to the acquisition and analysis of data, F.C.F., M.G.H., M.J.R., M.K., and W.S. contributed to drafting the text or preparing the figures.

Data availability statement

Anonymized datasets used and/or analyzed during this study are available from the corresponding author on reasonable request

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.1220.

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