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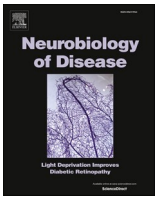

Identification of diagnostic and prognostic biomarkers of PD using a multiplex proteomics approach

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ABSTRACT

Given the complexity of Parkinson's disease (PD), achieving acceptable diagnostic and prognostic accuracy will require the support of a panel of diverse biomarkers. We used Proximity extension assays to measure a panel of 92 proteins in CSF of 120 newly diagnosed PD patients and 45 control subjects without neurological disease. From 75 proteins detectable in the CSF of >90% of the subjects, regularized regression analysis identified four proteins (β-NGF, CD38, tau and NCAN) as downregulated in newly diagnosed PD patients (age at diagnosis 67.2 ± 9.4 years) compared to controls (age 65.4 ± 10.9 years). Higher tau (β −0.82 transformed MMSE points/year, 95% CI −1.37 to −0.27, $P = 0.005$) was also linked to faster cognitive decline over the first ten years after PD diagnosis. These findings provide insights into multiple aspects of PD pathophysiology and may serve as the foundation for identifying new biomarkers and therapeutic targets.

1. Introduction

Parkinson's disease (PD) is a common, progressive, and disabling neurodegenerative movement disorder characterized by cardinal motor symptoms and a combination of secondary motor symptoms and non-motor symptoms (de Lau and Breteler, 2006; Jankovic, 2008). Pathologically, the disorder is defined by degeneration of the substantia nigra and the presence of Lewy bodies, which contain abundant amounts of α-synuclein.

The diagnosis of PD is mainly based on the clinical findings of resting tremors, bradykinesia and rigidity, and the exclusion of other disorders. Accurate diagnosis is challenging, especially early in the disease course when the clinical picture has not yet developed fully. Accordingly, although clinical diagnosis after several years of follow-up correlates highly with neuropathological diagnosis upon post-mortem examination, the accuracy of the initial diagnosis can vary greatly (Adler et al., 2014; Rizzo et al., 2016). Correct, early diagnosis is crucial for patient care and for meaningful research studies and clinical trials, and the current diagnostic precision emphasizes the need for new biomarkers to improve early diagnosis.

Given the complexity of PD, it is unlikely that a single biomarker can provide sufficient accuracy to diagnose PD and a combination of biomarkers will be required to improve diagnostic and prognostic accuracy. In this study, we aimed to identify novel diagnostic protein biomarkers of PD using multiplex proximity extension assay (PEA) technology to analyse a panel of 92 candidate neurological biomarkers. PEA multiplex immunoassays are based on a dual-recognition approach using matched pairs of antibodies labeled with complementary DNA oligonucleotide tags for quantitative real-time PCR-based measurement. Further, we explored the value of the candidate biomarkers as prognostic markers of PD progression.

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2. Methods and data availability

2.1. Study participants and clinical assessments

Patients were from the Norwegian ParkWest study that recruited participants from 2004 to 2006 to investigate the incidence, neurobiology and prognosis of PD (Alves et al., 2009). The PD group consisted of 121 newly diagnosed, mostly drug-naïve patients ($n = 120$). PD was diagnosed according to the UK brain bank criteria (Daniel and Lees, 1993), omitting the family history criterion. Study data up to the 9-year visit was available. The mean follow-up period was 7.9 ± 2.3 years (Supplementary Fig. 1). Before the last clinical visit, 28 (23.3%) participants died and 3 (2.5%) were lost to follow-up. The control group was a set of 45 participants without known brain disease who underwent elective neurological examination or orthopaedic surgery at Stavanger University Hospital.

General medical and neurological examinations and semi-structured interviews were performed at baseline to obtain medical, drug and family history. For this study, we included the Hoehn and Yahr (H&Y) scale for disease staging (Goetz et al., 2004), the Unified PD Rating Scale (UPDRS) for assessment of ADL (part II) and motor function (part III) (Fahn and Elton, 1987), and the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) for assessment of global cognition. MMSE was also available for the control subjects. H&Y and UPDRS were assessed at baseline (BL) and every year thereafter; MMSE was assessed at BL and the visits at year 1, 3, 5, 7 and 9.

The Western Norway Regional Committee for Medical and Health Research Ethics approved the studies. All participants signed written informed consent.

2.2. CSF sampling and processing

Lumbar puncture and CSF collection was conducted according to standardized procedures after overnight fasting. Briefly, the freshly drawn CSF samples were centrifuged at 2000*g* for 10 min at 4 °C, and frozen in polypropylene tubes at −80 °*C. prior* to analysis, the samples were subjected to two freeze–thaw events for aliquotation purposes. The median time from clinical diagnosis to lumbar puncture was 38 days (IQR 20.5–57.5).

2.3. Neurology-related protein biomarker measurement

The CSF samples were analysed blindly as one project at Olink Bioscience (Uppsala, Sweden) using Multiplex Proximity extension assay (PEA) technology (Assarsson et al., 2014; Lundberg et al., 2011). The Proseek Multiplex Neurology panel targets 92 biomarkers (Supplementary Table 1). Validation data are available online (www.olink.com/data-you-can-trust/validation). The data were pre-processed by Olink using the NPX Manager software and are expressed as normalized protein expression (NPX) values. NPX values are an arbitrary unit on a Log2 scale that can be used for relative quantification of the same protein.

One sample failed quality control, leaving 120 PD samples and 45 control samples. Assays with >10% values below the lower limit of detection (LLOD) were excluded from the analysis ($n = 17$ out of 92 proteins; See Supplementary Table 1). For the remaining assays, all values including those below the LLOD were included. Also, a threshold was set for exclusion of individual participants with >10% of assays below the LLOD; applying this criterion, no subjects were excluded.

2.4. Statistical analyses

Group comparisons were performed using two-tailed independent samples *t*-tests (continuous, normally distributed data), Mann-Whitney *U* tests (continuous, not normally distributed data), and χ^2-tests (categorical data). Normality of the data was established using the Kolmogorov-Smirnov test. Regularized logistic regression with elastic net (EN) penalization was used to identify baseline PEA biomarkers associated with each disease group (Lange et al., 2014; Wei et al., 2013). EN analyses were performed in R version 4.2.1, using the glmnet-package version 4.1–6 (Friedman et al., 2010). The level of regularization parameter λ was chosen as the minimal λ that yielded prediction error estimated by leave-one-out cross-validation within one standard error from its minimal value. In the glmnet, the parameter α decides the balance between L1 and L2 regularizations, of which the former is the regularization used in Lasso regression and the latter is used in Ridge regression. The EN was repeated for all α from 0 to 1, with 0.01 increments. Non-zero estimated coefficients (β-values) throughout the entire range of α support the associations between relevant PEA biomarkers and disease status; the markers whose estimated β-values were non-zero across all α range were selected into the panel of candidates.

Further analyses were performed using logistic regression models with AUC (area under the receiver operating characteristic (ROC) curve) and optimism-adjusted AUC measuring model's diagnostic accuracy. First, we evaluated the strength of each candidate biomarker using logistic regression with the disease diagnosis as the outcome and the biomarker expression level, age, and sex as predictors. Second, we assessed the multicollinearity of the data in the selected panel using the variance inflation factor (VIF) and excluded the candidate with VIF > 5. A multiple logistic regression model was then evaluated with the disease diagnosis as outcome and all the remaining candidates as predictors, controlling for age and sex. ROC curves were constructed using R package *pROC* version 1.18.0 (Robin et al., 2011). Optimism-adjusted AUCs were calculated using the bootstrapping method with 2000 replications to compensate overfitting of the models using the R package *boot* version 1.3–28 (Davison, 1997). The 95% confidence interval for optimism-adjusted AUC were obtained using Harrell's bias correction method with 2000 bootstrap samples (Harrell Jr et al., 1996; Noma et al., 2021).

Linear mixed effects analyses performed in R package *lme4* version 1.1–31 (Bates et al., 2015) were used to study the relationships between the two outcome variables (UPDRS part III and MMSE score) and each of the candidate biomarkers, one at a time. The MMSE scores were transformed as described (Philipps et al., 2014). All models included the NPX value for each biomarker, time in years, the interaction between the NPX value and time, age, and sex as fixed effects. Models including MMSE also had education in years as a fixed effect. The random effects were the patient-specific intercepts and time slopes. The NPX value for each biomarker is in Log2 scale, meaning that a 1 NPX difference corresponds to a doubling of protein concentration. Analyses were corrected for multiple comparisons ($n = 4$ biomarkers) using the Benjamini–Hochberg false discovery rate (FDR) method at FDR < 0.05. For visualisation of the predicted slopes, patients were stratified into high and low biomarker level groups based on a cut off using the mean NPX value.

Table 1
Study characteristics.

	Controls	PD	P
N	45	120	
Male, n (%)	22 (48.9)	77 (64.2)	0.074[a]
Age at sampling, mean (SD)	65.4 (10.9)	67.2 (9.4)	0.317[b]
Education, years, mean (SD)	11.0 (3.6)	11.2 (3.1)	0.743[b]
H&Y score, median (IQR)		2.0 (0.5)	
UPDRS II, median (IQR)		8.0 (6.0)	
UPDRS III, median (IQR)		20.0 (14.0)	
MMSE, median (IQR)	29.0 (2.0)	28.5 (2.0)	0.011[c]

Abbreviations: UPDRS III: Unified Parkinson's Disease Rating Scale part III; MMSE: Minimal-Mental State Examinations.
[a] χ^2-test
[b] independent samples *t*-test.
[c] Mann-Whitney *U* test.

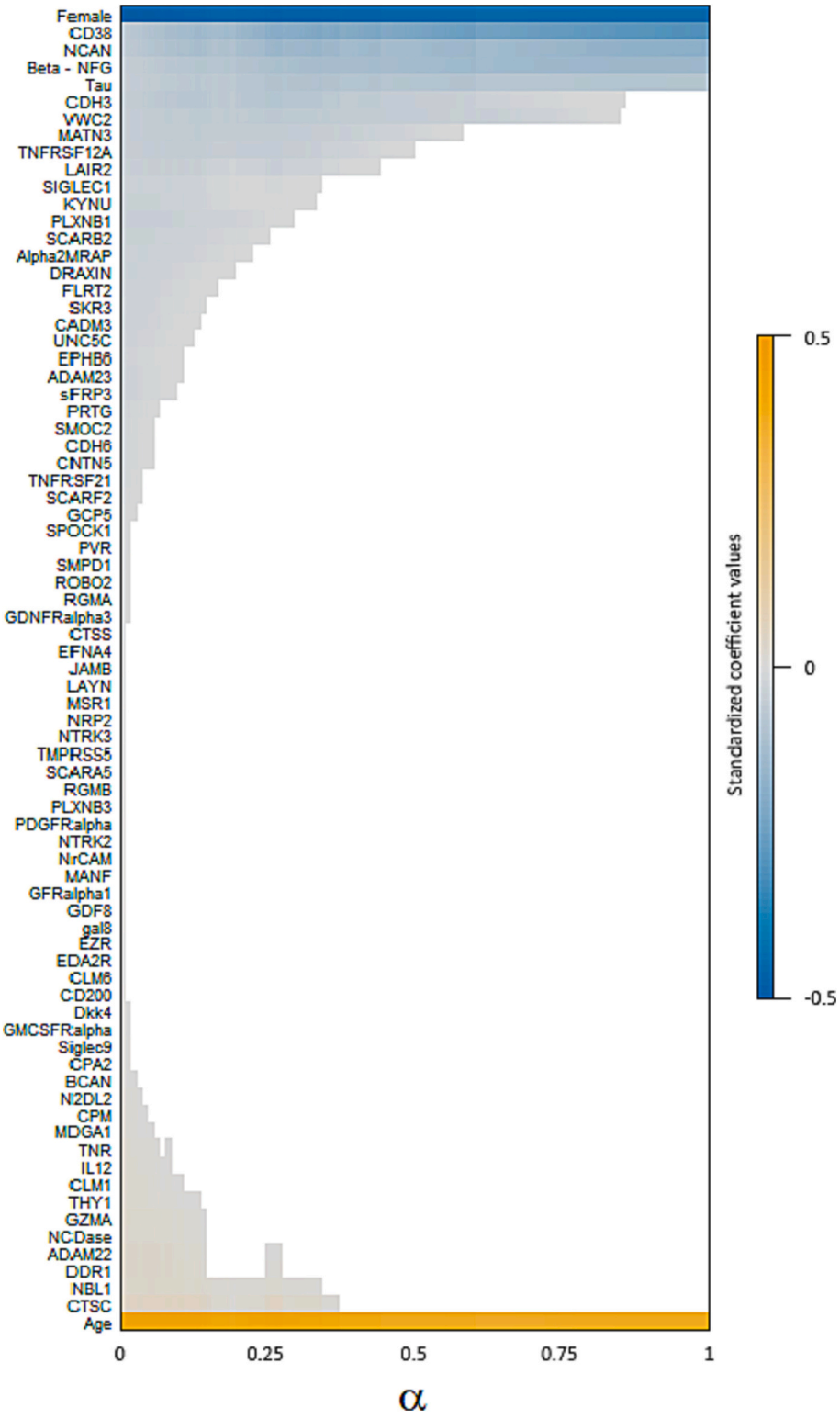

Fig. 1. Results of regularized regression with elastic net penalization for α-values between 0 and 1. Variables are ordered by strength of association from those negatively associated with PD (i.e., decreases risk) highlighted in blue to those positively associated with PD (i.e., increases risk) highlighted with yellow. The intensity of colour corresponds to the standardized coefficient value and reflects the strength of association. PEA biomarkers not associated with PD are white. The selected PEA biomarkers demonstrate a significant association across all levels of α.

3. Results

3.1. Identification of PEA biomarkers associated with PD

A total of 120 PD cases and 45 controls were included in the primary analysis (Table 1). The average age at diagnosis for the PD patients was 67.2 ± 9.4 (s.d.) years. There were no significant intergroup differences regarding age at sampling, sex, or years of education between the two groups. Total MMSE score was as expected slightly lower in PD compared to the control group ($P = 0.011$).

CSF samples from all participants were analysed using PEA for 92 proteins from the predesigned Neurology panel. Seventy-five proteins that were detectable in the CSF of >90% of the subjects (Supplementary Table S1) were included in the analysis, and four of these were shown to be associated with PD across all levels of α in the EN analysis (Fig. 1). Specifically, lower levels of β-NGF, CD38, tau, and NCAN were each

Table 2
Association between PD diagnosis and a clinical/biomarker model.

			Full model,[a] PD vs NC	
Predictors	OR (95% CI)	*P**	AUC (95% CI)	Optimism-adjusted AUC (95% CI)
Age	1.07 (1.02; 1.12)	0.009		
Female	0.32 (0.13; 0.74)	0.008		
CD38	0.59 (0.40; 0.82)	0.003	0.81 (0.74 to 0.88)	0.78 (0.72 to 0.87)
tau	0.59 (0.34; 0.99)	0.049		
NCAN	0.01 (0.00; 0.25)	0.013		

Abbreviations. PD: Parkinson's disease; NC: Normal control; OR: Odds ratio; CI: Confidence interval; AUC: Area under the curve.
[a] ROC curves were constructed using the covariates (age and sex) and the biomarkers.

associated with PD (Supplementary Fig. 2). The biological functions of the significant PEA markers are summarised in Supplementary Table 2. β-NGF was excluded from the full model for VIF >5 with tau, and the remaining 3-biomarker panel found to yield an optimism-adjusted AUC of 0.78 (Table 2).

3.2. Association of PEA biomarkers with disease progression and cognitive decline in PD

The association of the four candidate biomarkers for PD with disease progression and cognitive decline was analysed using linear mixed models to predict the impact of a 1 unit increase in biomarker level on annual changes in scores of motor and cognitive impairments over the first ten years of PD. Tau was associated with faster annual decline in cognitive function (β −0.82 transformed MMSE points/year, 95% CI -1.37 to −0.27, P = 0.005). NCAN was initially associated with faster annual decline in motor function (β 2.60 UPDRS III points/year, 95% CI 0.31 to 4.89, $P = 0.028$), although this association was not significant after adjustment for multiple testing. No other significant associations were observed between repeated measures of clinical scores and the candidate biomarkers (Table 3).

To further explore the changes in clinical scores linked to tau, patients were stratified into two groups based on low or else high biomarker level using the mean NPX value as the cut-off value. The group with higher levels of tau (≥2.58 NPX) were predicted to experience a larger annual decrease in MMSE scores (β −1.4 transformed MMSE points/year, 95% CI -2.50 to −0.32, $P = 0.013$; Fig. 2) compared to patients in the low level group. The estimated drop in MMSE score over 10 years from diagnosis was from around 29 to 28 points for patients in the low tau group and from 29 to 25 points for those in the high level group.

We further explored the association of the CSF tau biomarker with selected variables that have been linked to an increased risk of a cognitive dominant subtype of PD (Sauerbier et al., 2016; Williams-Gray et al., 2009). Higher levels of tau were associated with older age at diagnosis (≥72 years; $p < 0.001$), UPDRS part III score ≥ 25 ($P = 0.046$), and poor performance on the semantic fluency test (<20 words in 90 s; $P = 0.006$), but not with a lower pentagon copying score ($p > 0.05$) or with the non-tremor dominant (non-TD) motor phenotype, which was conversely associated with a lower level of tau compared to the TD group ($P = 0.007$; Supplementary Fig. 3).

4. Discussion

In this PEA study, we analysed a large set of prospectively followed, newly diagnosed PD patients and controls and identified a panel of four neurology-related proteins as candidate biomarkers of PD. Further, we identified tau (microtubule-associated protein tau) as a candidate prognostic marker of cognitive decline in PD. Together, these findings provide insights into biological processes implicated in PD and may serve as the foundation for the identification of diagnostic and prognostic biomarkers to improve patient care.

Few studies have utilized PEA for PD (Jabbari et al., 2019; Santaella et al., 2020; Whelan et al., 2019). Earlier studies have included CSF but otherwise had notable differences in study design, including inclusion of PD patients from a specialized clinical setting (Santaella et al., 2020) or

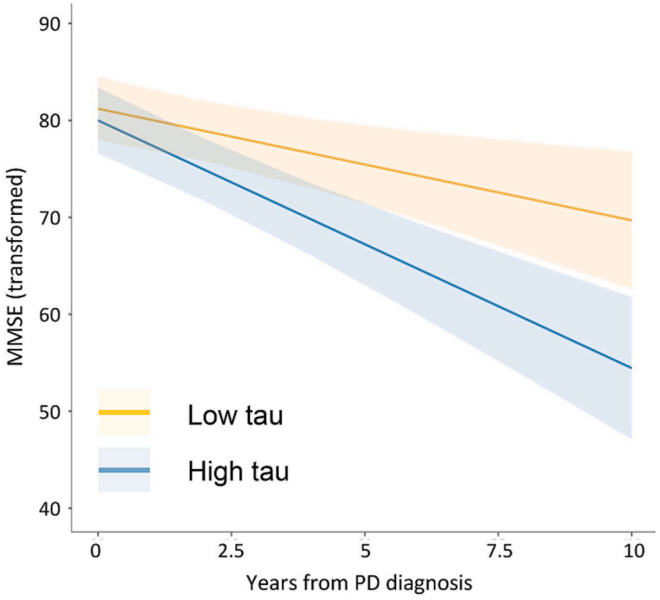

Fig. 2. Prediction of changes in Mini–Mental State Examination (MMSE) scores over time.
Patients were grouped by biomarker level (low tau <2.58 NPX; else high). MMSE scores were transformed before plotting as described in the Methods.

Table 3
Relationship between candidate biomarkers and predicted annual change in scores of motor and cognitive scales.

	UPDRS part III [a]				*MMSE* [a]			
Scores	Main effect[a] β (95% CI)	*P*	Interaction with time[a] β (95% CI)	*P* [b]	Main effect[a] β (95% CI)	*P*	Interaction with time[a] β (95% CI)	*P* [b]
Beta-NGF	0.22 (−5.07 to 5.5)	0.934	−0.04 (−1.39 to 1.3)	0.950	0.49 (−7.36 to 8.33)	0.904	−1.65 (−3.5 to 0.25)	0.091
CD38	0.63 (−0.57 to 1.83)	0.306	−0.10 (−0.40 to 0.20)	0.527	1.08 (−0.71 to 2.88)	0.239	−0.26 (−0.68 to 0.17)	0.241
NCAN	−8.95 (−18.26 to 0.36)	0.062	2.60 (0.31 to 4.89)	0.028	−6.94 (−20.75 to 6.86)	0.326	−2.97 (−6.2 to 0.29)	0.077
tau	−0.71 (−2.340 to 0.92)	0.394	0.35 (−0.05 to 0.75)	0.088	−0.46 (−2.88 to 1.96)	0.710	−0.82 (−1.37–0.27)	**0.005**

Abbreviation: CI, confidence interval; MMSE, Minimal-Mental State Examinations; UPDRS III, Unified Parkinson's Disease Rating Scale part III.
[a] Models adjusted for sex and age (UPDRS) and sex, age and years of education at baseline (MMSE). The main effect indicates the effect of a 1 unit increase in biomarker level on the intercept and the interaction with time indicates the effect of a 1 unit increase in biomarker level on the slope (change in value per year) of the model.
[b] significant *p* values at $p < 0.05$ after BH correction are highlighted in bold.

only amyloid-negative PD patients (Whelan et al., 2019), markedly smaller cohorts (<50 PD) (Jabbari et al., 2019; Santaella et al., 2020), and the choice of different PEA panels. Finally, all previous studies recruited cross-sectional patients from different disease stages, making comparison of the results with our newly diagnosed cohort difficult. Our analysis identified four proteins that were differentially expressed between newly diagnosed PD cases and controls. Of these, β-NGF has also been observed to be lower in PD in a study of 34 amyloid negative healthy controls compared to 119 amyloid negative PD patients (Whelan et al., 2019), and in a study comparing 44 PD and 25 controls (Santaella et al., 2020), although in the latter case the difference was not significant.

We found that higher levels of CSF tau at the time of PD diagnosis were linked to a faster annual decline in global cognitive function. Several studies have explored the association between the levels of different CSF tau species and cognitive impairments in PD: Meta-analysis of cross-sectional studies has shown that CSF total tau (T-tau) and phosphorylated tau (P-tau) are elevated in PD patients with cognitive impairment compared with those without (Hu et al., 2017). Further, higher P-tau was associated with faster decline in some cognitive tasks in patients after levodopa treatment was initiated (Liu et al., 2015), whilst an increase in P-tau (Hall et al., 2016) or both T-tau and P-tau (Baek et al., 2021) has been linked to worsening or worse cognitive impairment, respectively. We further found that higher tau levels were linked to clinical and demographic factors associated with a cognitive dominant subtype of PD (Sauerbier et al., 2016; Williams-Gray et al., 2009), indicating that CSF tau may be a molecular biomarker to support subtyping in PD.

The tau protein is more commonly linked to other neurodegenerative diseases, most notably Alzheimer's disease (AD), in which increased CSF T-tau and P-tau along with decreased amyloid-β are well-established as biomarkers to support AD diagnosis (Jack Jr et al., 2018). Additionally, CSF T-tau levels rise in conditions marked by rapid neurodegeneration without tau or amyloid pathology, like Creutzfeldt-Jakob disease. They also increase in acute conditions such as stroke and brain trauma, correlating with the injury's severity and potentially serving as an indicator of neuronal damage. (Blennow and Zetterberg, 2018). Notably, as we observed in this study but distinctly different from AD, CSF tau is reported to be lower in patients with PD compared to healthy controls (Baek et al., 2021; Kang et al., 2013; Shi et al., 2011), suggesting different roles in the context of disease diagnosis and cognitive decline. One proposal is that an interaction between tau proteins and α-synuclein may limit the release of tau proteins into CSF (Baek et al., 2021; Parnetti et al., 2014). The complexity of the relationship between concomitant pathologies in PD is further illustrated by observations from the PPMI study: at baseline, PD patients had lower CSF tau levels compared to controls, however, tau levels were projected to increase at a faster rate in the PD patient group, particularly among those with cognitive impairment or low amyloid β levels (Baek et al., 2021).

The new proteins identified in this study reflect broader biology than do those biomarkers classically studied in PD, such as α-synuclein, and can give new insights into the processes and pathways underlying neurodegeneration in early PD. The biological processes identified in this study were diverse (Supplementary Table 2), including neuronal adhesion and migration, insulin regulation and signal transduction. Importantly, some of these same pathways have been shown to be affected in PD and PD models in studies of genetic and gene expression data (Edwards et al., 2011; Jia et al., 2020; Kia et al., 2021; Lesnick et al., 2007; Yao et al., 2021), and three of these studies include *NCAN* and *CD38* among their candidate genes (Jia et al., 2020; Kia et al., 2021; Yao et al., 2021), supporting the significance of our findings using PEA.

The study has notable strengths. First, we included a well-defined prospective cohort of PD, diagnosed using accepted clinical criteria, and continued follow up ensured that patients did not subsequently develop signs suggestive of an alternative diagnosis. Further, the samples were analysed in a blinded fashion and the PEA technology is very sensitive, permitting the measurement of small changes in proteins in biofluids. Given that our study was conducted in newly diagnosed patients, this opens the possibility to detect small changes at the earliest clinical stages and the candidate biomarkers identified here may be promising prodromal biomarkers. Still, the study has some weaknesses. First, we did not have access to a validation cohort. Further, the control subjects were not followed longitudinally and so we were unable to analyse the association of the candidate biomarkers with cognitive function over time in this group. Finally, the MoCA is an alternative measure for assessing cognitive function with higher sensitivity to detect subtle cognitive deficits in PD patients than MMSE. However, when the ParkWest study began, the MoCA had not yet been validated (Dalrymple-Alford et al., 2010; Nasreddine et al., 2005). Consequently, the MMSE was initially included and later retained for consistency in follow-up visits.

5. Conclusion

In this work we identified a panel of PEA biomarkers that were different between patients with newly-diagnosed PD and controls. A combination of multiple CSF biomarkers reflecting multiple aspects of PD pathophysiology might enable earlier diagnosis and more accurate prognostic assessment in PD and have the potential to expand our understanding of PD heterogeneity and staging.

Author contribution statement

JMG: Conceptualization, Funding acquisition, Formal analysis, Writing original draft.

AU: Methodology, Formal analysis, Writing - review.

KFP, G.A, and OBT: Data curation, Funding acquisition, Writing - review.

JL: Conceptualization, Data curation, Writing - review.

All authors read and approved the final manuscript.

Declaration of Competing Interest

All authors declare no financial or non-financial competing interests.

Data availability

The datasets used during the current study are not publicly available due to the condition of the study's ethical approvals, but are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106281.

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