

RESEARCH ARTICLE

Evaluation of cerebrospinal fluid proteins as potential biomarkers for early stage Parkinson's disease diagnosis

Marcia Cristina T. dos Santos¹, Dieter Scheller², Claudia Schulte³, Irene R. Mesa⁴, Peter Colman⁴, Sarah R. Bujac⁴, Rosie Bell^{1#a}, Caroline Berteau^{1#b}, Luis Tosar Perez⁵, Ingolf Lachmann⁶, Daniela Berg^{3,7}, Walter Maetzler^{3,7}, Andre Nogueira da Costa^{1*}

1 Translational Medicine, UCB Biopharma SPRL, Braine L'Alleud, Belgium, **2** Consultancy Neuropharm, Neuss, Germany, **3** Hertie Institute for Clinical Brain Research, Department of Neurodegeneration, University of Tuebingen and German Center for Neurodegenerative Diseases, Tuebingen, Germany, **4** Exploratory Statistics, Global Exploratory Development, UCB Pharma SA, Slough, United Kingdom, **5** Bioanalytical Sciences, Non Clinical Development, UCB Biopharma SPRL, Braine L'Alleud, Belgium, **6** Research and Development, Analytik Jena, Jena, Germany, **7** Department of Neurology, Christian-Albrechts-University Kiel, Kiel, Germany

^{#a} Current address: Centre for Misfolding Diseases, University of Cambridge, Cambridge, United Kingdom.

^{#b} Current address: Leeds Institute of Biomedical and Clinical Sciences, University of Leeds, Leeds, United Kingdom.

* andre.dacosta@ucb.com



OPEN ACCESS

Citation: T. dos Santos MC, Scheller D, Schulte C, Mesa IR, Colman P, Bujac SR, et al. (2018) Evaluation of cerebrospinal fluid proteins as potential biomarkers for early stage Parkinson's disease diagnosis. PLoS ONE 13(11): e0206536. <https://doi.org/10.1371/journal.pone.0206536>

Editor: Stephen D. Ginsberg, Nathan S Kline Institute, UNITED STATES

Received: March 15, 2018

Accepted: October 15, 2018

Published: November 1, 2018

Copyright: © 2018 T. dos Santos et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from the German Federal Ministry for Education and Research (BMBF) within the BioPharma initiative Neuroallianz, project D13 B and by UCB Pharma GmbH (Monheim, Germany). The D13B study supports the investigation of biomarkers in prodromal and clinical phases of Parkinson's disease, and supports specifically the TREND study

Abstract

Cerebrospinal fluid (CSF) has often been used as the source of choice for biomarker discovery with the goal to support the diagnosis of neurodegenerative diseases. For this study, we selected 15 CSF protein markers which were identified in previously published clinical investigations and proposed as potential biomarkers for PD diagnosis. We aimed at investigating and confirming their suitability for early stage diagnosis of the disease. The current study was performed in a two-fold confirmatory approach. Firstly, the CSF protein markers were analysed in confirmatory cohort I comprising 80 controls and 80 early clinical PD patients. Through univariate analysis we found significant changes of six potential biomarkers (α -syn, DJ-1, A β 42, S100 β , p-Tau and t-Tau). In order to increase robustness of the observations for potential patient differentiation, we developed—based on a machine learning approach—an algorithm which enabled identifying a panel of markers which would improve clinical diagnosis. Based on that model, a panel comprised of α -syn, S100 β and UCHL1 were suggested as promising candidates. Secondly, we aimed at replicating our observations in an independent cohort (confirmatory cohort II) comprising 30 controls and 30 PD patients. The univariate analysis demonstrated A β 42 as the only reproducible potential biomarker. Taking into account both technical and clinical aspects, these observations suggest that the large majority of the investigated CSF proteins currently proposed as potential biomarkers lack robustness and reproducibility in supporting diagnosis in the early clinical stages of PD.

of the Neurology Department of the University of Tübingen, Germany. Samples were obtained from the Neuro-Biobank of the University of Tuebingen, Germany. This biobank is supported by the local University, the Hertie Institute and the DZNE. UCB Biopharma SPRL, Consultancy Neuropharm, UCB Pharma SA and Analytik Jena provided support in the form of salaries for authors MCTDS, RB, CB, ANDC, DS, IRM, PC, SRB, LTP and IL, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Competing interests: We have the following interests. Marcia Cristina T. dos Santos, Rosie Bell, Andre Nogueira da Costa, Luis Tosar Perez and Caroline Berteau are employed by UCB Biopharma SPRL, Dieter Scheller by Consultancy Neuropharm, Irene R. Mesa Peter Colman and Sarah R. Bujac by UCB Pharma SA and Ingolf Lachmann by Analytik Jena. This study was partly funded by UCB Pharma GmbH. IRM and ANdC have share options from UCB Biopharma SPRL and PC has share options from UCB Biopharma SPRL. DB is a full professor at Kiel University and the director of the Department of Neurology at UKSH, Campus Kiel. She has served on scientific advisory boards for Novartis, UCB/ SCHWARZ PHARMA, Lundbeck, and Teva Pharmaceutical Industries Ltd.; has received funding for travel or speaker honoraria from Boehringer Ingelheim, Lundbeck Inc., Novartis GlaxoSmithKline, UCB/ SCHWARZ PHARMA, Merck Serono, Johnson & Johnson, and Teva Pharmaceutical Industries Ltd.; and has received research support from Janssen, Teva Pharmaceutical Industries Ltd., Solvay Pharmaceuticals, Inc./Abbott, Boehringer, UCB, Michael J Fox Foundation, BMBF, dPV (German Parkinson's disease association), Neuroallianz, DZNE and the Center of Integrative Neurosciences. WM is a full professor at Kiel University. He received and receives funding from the European Union, the Michael J. Fox Foundation, Robert Bosch Foundation, Neuroalliance, Lundbeck and Janssen, and holds part of a patent for the assessment of dyskinesias (German patent office, 102015220741.2). He received speaker honoraria from GlaxoSmithKline, Abbvie, UCB, Licher. MT and Rölke Pharma, and was invited to Advisory Boards of Market Access & Pricing Strategy GmbH and Abbvie. There are no further patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder which leads to progressive impairment of motor function caused by prominent loss of dopamine-secreting neurons within the substantia nigra [1, 2]. PD diagnosis so far is based only on clinical criteria by identification of neuro-motor symptoms, the accuracy of which is limited during the first years of the disease [3]. Recent findings in the literature reveal the emergence of non-motor symptoms years before a clinical diagnosis of PD can be made [4]. Currently, it is well known that non-motor symptoms are major contributors to disease morbidity as the disease progresses, frequently receding motor symptoms and involving central and peripheral non-dopaminergic signaling [4, 5]. It is believed that during this initial phase, neurodegeneration is not extensive and severe, thus offering a perfect window for novel therapies and disease modifying treatments. However, diagnosis of early PD would require further parameters in addition to the clinical criteria.

To date, PD is still incurable; treatments available consist of drugs designed to increase dopamine levels and relieve motor symptoms but not to alter disease progression [6]. The development of improved standard of care and novel therapies, that may impede degeneration and delay PD progression, is limited by the already significant degeneration of a large number of dopaminergic neurons at the time clinical diagnosis can be established [7, 8]. The deployment of biomarkers reflecting the onset and progression of the pathology accurately may have a profound impact on diagnosis and detection of the disease. The identification of clinically applicable biomarkers for the early clinical stages of PD, therefore, will not only facilitate clinical diagnosis, but also help monitoring disease progression, enable personalized therapies and open a window for disease modifying treatments.

Currently, biomarkers for PD diagnosis have been extensively investigated, however their clinical applicability still warrants further efforts [9]. Several imaging approaches have been developed to provide in-depth information on brain structure and functioning [10]. However, these techniques are not easily employed in the clinical setting due to the high costs and the demands on infrastructure and therefore are limited when considering routine use for early diagnosis. Molecular biomarkers detectable in body fluids are considered the ideal approach to facilitate clinical diagnosis. Concerning neurodegenerative diseases, CSF is the preferred biofluid for biomarker investigations due to its supposed surrogacy to the brain [11]. Despite the popularity of CSF for biomarker research in PD, validated biomarkers applicable in a clinical setting are still limited. Several studies have highlighted altered levels of α -synuclein (α -syn), DJ-1, amyloid beta (A β) and Tau proteins in late stage, symptomatically identified PD patients compared with controls [12–17]. Nevertheless, these findings are not consistently observed in different studies [18].

The current study was designed to explore the suitability of a panel of 15 CSF protein markers which had been proposed as potential biomarkers based on corresponding discovery studies performed in late stage PD patients, with clinical diagnosis obtained between 74 to 136 months of already existing symptoms. This study aimed at exploring those protein markers in order to confirm their potential as biomarkers applicable to early clinical PD patients that were diagnosed during a symptomatic period of up to 36 months after first signs of the disease.

Our approach closely mimicked suggestions for the performance of a reliable biomarker study in the Alzheimer disease (AD) field. As suggested by a consensus report for biomarkers in AD, a biomarker should make use of a fundamental feature of neuropathology and be investigated in neuropathologically confirmed cases; furthermore, it should have a high diagnostic sensitivity and specificity (of >80%) for distinguishing other dementias. The potential

biomarker should be investigated by at least two independent studies, in our case consisting of at least two separate cohorts [19, 20].

In this study, the confirmation was conducted in a two-step approach: a first confirmatory phase I (employing the confirmatory cohort I) to evaluate previously reported observations and to establish, if possible, a panel of potential biomarkers that would support early PD diagnosis; and a second confirmatory phase II (employing the confirmatory cohort II) to replicate independently our findings. In the first step, we had identified biomarker discovery studies performed in late stage PD patients, which employed appropriate analytical method and were sufficiently powered to select CSF protein markers. To this end, we selected 15 protein markers—Aβ40, Aβ42, α-syn, p-Tau, t-Tau, OPN, HMGB1, NFL, IL-6, DJ-1, UCHL1, FLT3LG, MMP2, S100β and ApoA1—for measurement in the CSF of confirmatory cohort I (comprising 80 early clinical PD patients and 80 controls) and subsequently evaluated their robustness to distinguish early clinical PD patients from controls. All selected protein markers participate in crucial pathways involved in PD pathogenesis and were previously considered as promising protein markers for late stage PD patients [13, 15, 21–44]. Through machine learning and univariate analysis, we aimed to identify protein biomarkers in CSF, which are robust and reproducible enough to utilize in the diagnosis of the early stages of PD.

Material and methods

Sample collection and patients

Early clinical PD patients and controls were recruited from the outpatient clinic at the Neurodegenerative Department of the University of Tübingen, Germany, and clinical data was collated (Table 1). The study was approved by the Ethics Committee of the Medical Faculty of the University of Tübingen (480/2015BO2). All participants provided written informed consent. PD was diagnosed according to the United Kingdom Brain Bank Society Criteria [45]. All patients were investigated by movement disorders specialists, to keep the risk of misdiagnosis at a minimum. Control individuals were assessed as having no neurological disease. Early clinical PD patients were chosen to represent a homogeneous cohort with very early disease state

Table 1. Cohort summary.

Confirmatory Cohort I	Confirmatory Cohort II	
	PD	Controls
Individuals (n)	80	80
gender (male in % (m/f))	51% (54/26)	49% (51/29)
age (in years mean +/- SD)	64.28 ± 9.8	62.74 ± 10.2
Disease duration (in years mean)	2 ± 1.1	NA
LEDD median (IQR)	160 (353)	NA
H&Y median (range 1–4)	2	NA
MMSE median (IQR)	29 (3)	30 (1)
MoCA median (IQR)	27 (4)	28 (3)
UPDRS III median (IQR)	23 (15)	0 (1)
BDI (median IQR)	7 (5)	2 (4)

A total of 220 individuals were included in the this study. Confirmatory cohort I comprised 80 early clinical PD patients and 80 controls. For confirmatory phase II, a total of 60 individuals were included, 30 early clinical PD patients and 30 controls. Although collected at the same institution, the cohorts were independent from each other with regard to recruitment time point and time of analysis. In more detail, samples of the second cohort were analyzed entirely independently from the samples of the first cohort, including a later time point and fit for purpose assays. Gender, age and disease duration were calculated for both groups and are presented below. H&Y = Hoehn and Yahr staging; UPDRS III = Unified Parkinson's Disease Rating Scale. NA = not available. IQR = interquartile range (Q3-Q1).

<https://doi.org/10.1371/journal.pone.0206536.t001>

(mean disease duration = 1.89 years, median Hoehn and Yahr stage (H&Y) = 2, and median Unified Parkinson's disease rating scale III (UPDRS III) = 23) and to have the akinetic-rigid subtype of PD [46, 47]. We included only akinetic-rigid patients as there is increasing evidence that tremor-dominant and akinetic-rigid subtypes are the consequence of different pathophysiologicals, to increase the probability to find (subtype-) specific results [48, 49]. CSF was collected by lumbar puncture in fasting patients according to standardized guidelines previously described in the literature [50]. To prevent blood contamination, CSF samples were tested for hemoglobin. Hemoglobin was determined by photometric Hb concentration measurements at 545 nm, conducted using ADVIA 1800 (Siemens Healthcare, Erlangen, Germany). In all measured samples hemoglobin concentration was <0.01 g/L. CSF samples free of blood were centrifuged (1600 g, 4°C, 15 min), frozen within 30–40 min after the puncture and stored at -80°C according to CSF collection and storage guidelines [51].

Ligand binding assay measurement

Quantitative determination of selected markers was done by ELISA following manufacturer's guidelines (Table 2) and validated fit-for-purpose as proposed by Jani et al [52].

Statistical methods

All analyses were conducted using R-Studio (2016; Version v1.0.136). Demographic and baseline characteristics of the cohorts were assessed using summary statistics. Differences in means between early clinical PD and Controls were assessed using Analysis of Variance (ANOVA); differences in proportions were assessed using chi-squared tests.

Analysis of CSF protein data

Firstly, univariate association analyses comparing early clinical PD and controls were conducted for each biochemical marker using a Wilcoxon Signed-Rank Test. A False Discovery Rate (FDR) adjustment was applied to control the type I error within each sample type and sensitivity analyses were conducted to assess the potential effect of outliers.

Table 2. Analyte information.

Analyte	Dilution	Limit of detection	Intra assay precision	Company
α-syn	1:1	0.37pg/mL	<15% CV	Analytik-Jena, Germany
DJ-1	8-fold	12.0 pg/mL	<10% CV	Meso Scale Discovery, USA
FLT3LG	8-fold	0.49 pg/mL	<10% CV	Meso Scale Discovery, USA
UCHL1	2-fold	0.31 ng/mL	<10% CV	Millipore, USA
MMP2	2-fold	200 pg/mL	<5.4% CV	Millipore, USA
S100β	2-fold	2.7 pg/mL	<4% CV	Millipore, USA
p-Tau	1:100	7.8 pg/mL	<15% CV	Fujirebio, Germany
t-Tau	1:100	7.8 pg/mL	<15% CV	Fujirebio, Germany
Aβ42	1:100	7.8 pg/mL	<15% CV	Fujirebio, Germany
Aβ40	1:100	7.8 pg/mL	<15% CV	Fujirebio, Germany
ApoA1	2-fold	0.7 ng/mL	<10% CV	Millipore, USA
HMGB1	1:1	2.5 ng/mL	<15% CV	IBL International, Germany
OPN	5-fold	5 ng/mL	<8% CV	IBL International, Germany
NFL	5-fold	100 ng/L	<10% CV	IBL International, Germany
IL-6	2-fold	1.56 pg/mL	<10% CV	IBL International, Germany

<https://doi.org/10.1371/journal.pone.0206536.t002>

Multivariate analyses were conducted using the CARET package in R. Data CSF markers were modelled independently. Initial pre-processing of the biomarker data included: centre and scale, removal of near-zero variance predictors, replacement of values below the limit of quantification (BLQ) with a value of half the lower limit of detection, and imputation of other missing values using multivariate K-Nearest Neighbour imputation (KNN). The final step before analysis was to randomise subjects into training and test sets in a ratio of 3:1.

Two multivariate methods were employed to build models predictive of disease status (PD vs. control): Elastic Net (Regularized Regression; GLMNET) and Gradient Boosted (GBM) Regression. Repeated 10-fold cross validation of the training set was used (for both methods) to give an indication of the accuracy of the resulting predictive models. The best Elastic Net and GBM models were chosen based on predictive accuracy. The selected models were then applied to the data in the test set and predictive probabilities were generated for each subject. Confusion matrices were produced and model fit was assessed using the following parameters: sensitivity, specificity and area under the Receiver Operating Characteristics (ROC) curve. For the confirmatory phase II, selected univariate and multivariate hypotheses generated above, were tested on an independent cohort of early clinical PD and controls. Univariate replication of the most promising CSF markers was assessed using a two group Wilcoxon Signed-Rank Test. Only one multivariate model, built by GBM using CSF markers, was tested for replication in the confirmatory cohort II. A selection of the most promising CSF markers was used to fit an example tree from the GBM model to the training set (early clinical PD and controls only) using CARET. Predictive probabilities were generated for each subject and the best cut-off from the training set was used to construct the confusion matrix, and calculate sensitivity, specificity and area under the Receiver Operating Characteristics (ROC) curve.

Results

Selection of potential CSF markers for the early diagnosis of PD

Our study was designed in two steps aligned to patient cohorts: confirmatory cohort I and confirmatory cohort II. Potential biomarkers or panels of biomarkers identified during the first confirmatory step, were taken forward to confirmatory step 2, where markers identified in step 1 should be confirmed in an independent cohort (confirmatory cohort II comprising 30 early clinical PD and 30 controls)(Fig 1)

The demographic characteristics of the cohorts are summarized in [Table 1](#).

Univariate analysis of selected markers

Univariate analyses comparing early clinical PD and controls were conducted for each protein marker. From the 15 protein markers selected, 6 were found significantly dysregulated in early clinical PD. An overview of the results is shown in [Fig 2](#). A β 42, t-Tau, p-Tau, α -syn and DJ-1 were decreased in early clinical PD patients compared to the controls (unadjusted $p = 0.002$, $p = 0.033$, $p = 0.021$, $p = 0.015$, $p = 0.022$, $p = 0.002$, respectively), whereas S100 β levels was increased in early clinical PD patients ($p = 0.025$) ([Fig 2A–2F](#)).

To confirm these findings, we measured S100 β , DJ-1, UCHL1, A β 42, t-Tau, p-Tau and α -syn levels in the CSF of an independent, confirmatory cohort II, comprised of 30 early clinical PD patients and 30 controls. Of the 6 protein markers analysed, only A β 42 levels were found to be significantly changed with levels decreased in early clinical PD patients compared to controls([Fig 3](#)). Receiver Operating Characteristic (ROC) curve analysis was performed to determine the diagnostic accuracy of A β 42, which presented 62% of area under the curve (AUC) ([Fig 2](#) and [S1 Fig](#)).

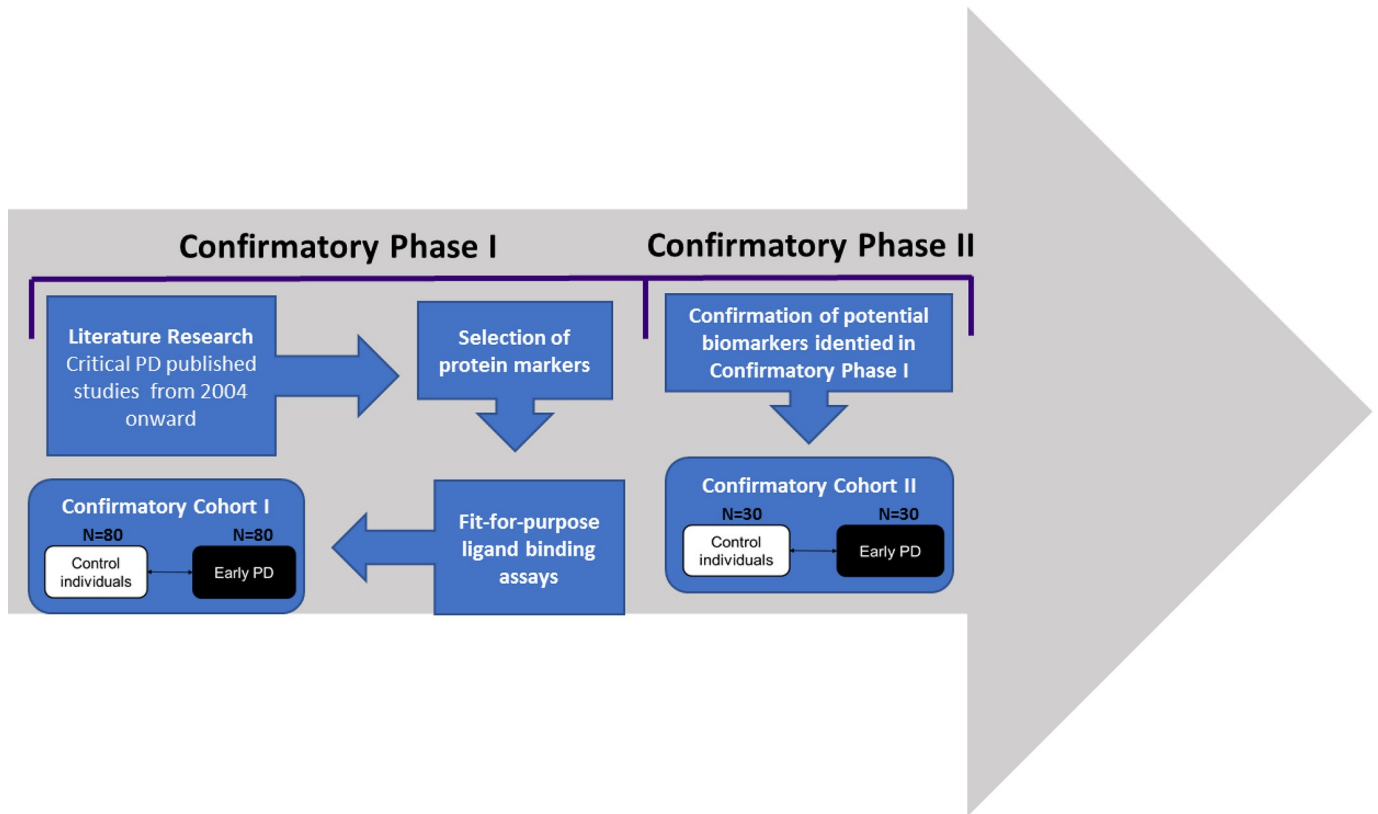


Fig 1. Biomarker study pipeline. Illustrative scheme representing study design. The study was divided in confirmatory phase I and confirmatory phase II. The confirmatory phase I comprised of literature research for selection of biochemical markers and analysis of selected markers in confirmatory cohort I. The confirmatory phase II comprised the validation of markers and models created in an independent cohort (confirmatory cohort II).

<https://doi.org/10.1371/journal.pone.0206536.g001>

Multivariable analysis revealed a potential panel for the early diagnosis of PD

We employed machine learning approaches to investigate if the combination of markers could be used to accurately distinguish early clinical PD from controls. In order to identify robust panels, we employed two different machine learning methods: Elastic Net (Regularized Regression; GLMNET) and Gradient Boosted (GBM) Regression.

Through Elastic Net algorithm, we first defined the ideal threshold to differentiate early clinical PD from controls. From the 15 markers analysed, 14 were taken forward and consequently we found a potential model comprising 16 variables: A β 40, A β 42, α -syn, p-Tau, t-Tau, OPN, NFL, IL-6, DJ-1, UCHL1, FLT3LG, MMP2, S100 β , ApoA1, ratio A β 40/A β 42 and p-Tau/t-Tau. The model was able to distinguish early clinical PD from controls with 90% sensitivity, 50% specificity, 83% and 64% positive and negative predictive values, respectively. ROC analysis revealed AUC = 0.71 (Fig 4).

In order to define a better classifier, we relied on the gradient boosted method. We employed a boosted decision tree algorithm to build an ensemble of 60 decision trees with an optimal interaction depth of four markers with 85% sensitivity, 75% specificity, 77% and 83% of positive and negative predictive values, respectively (Fig 5A). The analysis of diagnostic accuracy showed an AUC = 0.77 (Fig 5B). We used the ensemble of trees to rank the markers most frequent among all the trees and identified S100 β , α -syn, MMP2 and UCHL1 as the most influential markers (Fig 5C). Among all trees presented in the model, the gradient boosted

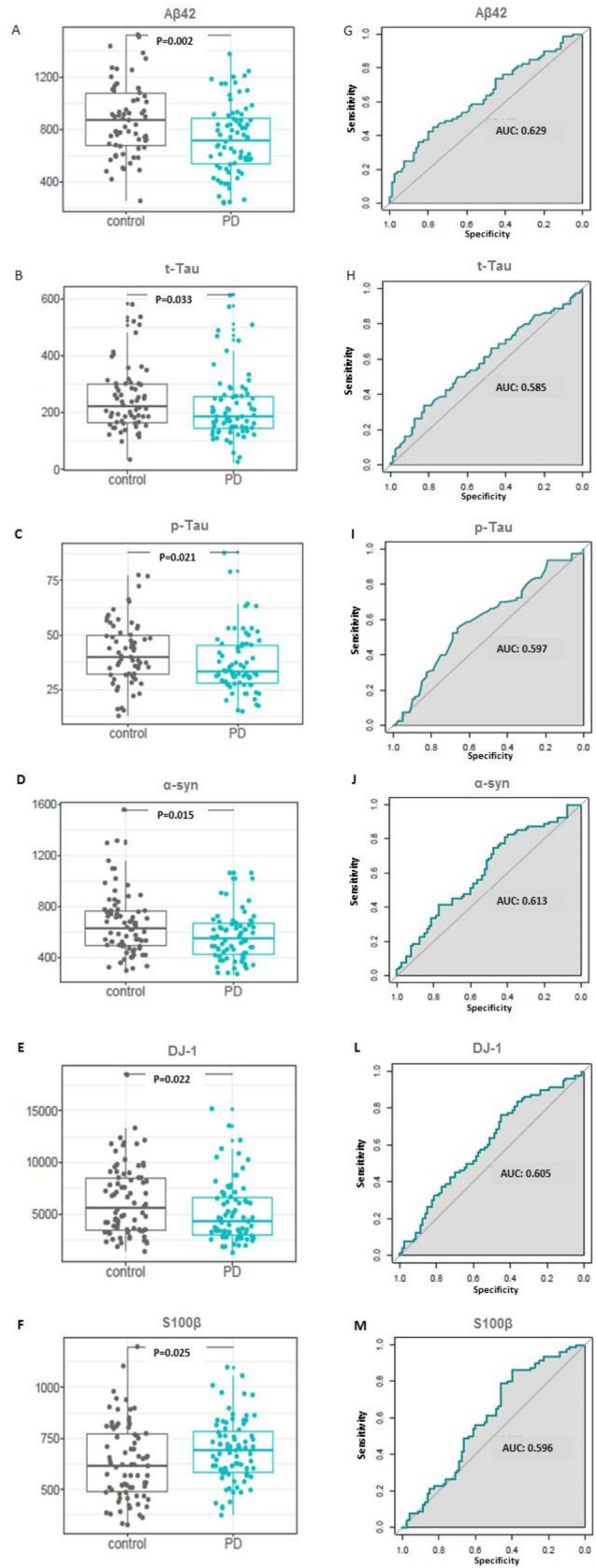


Fig 2. Univariate analysis of selected markers in confirmatory cohort I. (A-F) Boxplots of markers showing statistically significant changes in early clinical PD patients. Grey dots represent controls and blue dots represent early PD. P-values are calculated using the Wilcoxon signed-rank test. (G-M) Corresponding ROC-AUC analysis of significant markers.

<https://doi.org/10.1371/journal.pone.0206536.g002>

method suggests an interaction between S100 β , α -syn and UCHL1, which seemed to be able to distinguish early clinical PD from controls (example tree shown in Fig 5D).

To validate the decision tree and associated protein markers, we applied the same analysis methods and cut-off values obtained with confirmatory cohort I in the confirmatory cohort II. The potential panel of markers were not reproducible in the independent cohort.

Discussion

The discovery, development and validation of sensitive and specific biomarkers to support the early diagnosis of PD is currently an extensively debated topic. Although substantial progress in the discovery and development of low molecular weight and protein markers has been achieved, only a few candidates have been reported as promising biomarkers; however, none of them could be validated so far.

In this study, we aimed at confirming potential protein markers for PD as previously reported and assessing their applicability for early diagnosis during the first years of PD. Here, patients suffered from the disease for up to 36 months after first clinical diagnosis. To this end, we reviewed publicly available literature data from 2004 onward. We identified critical studies where biomarker discovery was conducted to identify PD potential biomarkers using well-characterized cohorts of sufficiently large numbers and in which the proposed biomarkers had a biological contextualization to PD [13, 15, 21, 23–31, 33, 34, 37, 53, 54]. These studies proposed protein markers differently affected mainly in late stage PD patients compared with age-matched controls. Therefore, we sought to confirm these protein markers in two cohorts of early clinical PD and to assess any potential diagnostic value. In total, 16 protein markers were selected, all of which are proposed to be associated with the major pathways of PD pathogenesis, such as protein aggregation (A β 40, A β 42, α -syn, p-Tau, t-Tau and NFL), inflammation (FLT3LG, MMP2, HMGB1, OPN and IL-6), oxidative stress (DJ-1 and S100 β), lipid metabolism (ApoA1) and ubiquitin proteasome proteolysis (UCHL1) [13, 15, 21, 23–31, 33, 34, 37, 53, 54].

Given the importance of bioanalytical validity in the process of implementing biomarkers in the clinical setting, we pursued to the use of ligand binding assays to measure each marker. All commercial assays acquired as research-use-only were fit-for-purpose validated in order to ensure robustness of performance by means of improved analytics (S1 table) [52]. Each of the selected biochemical candidate markers was measured in CSF samples of a first confirmatory cohort comprised of 80 early clinical PD patients and 80 controls.

For statistical evaluation, we first employed an univariate analysis approach to evaluate which markers could be suitable to distinguish early clinical PD patients from controls. Power analysis had been conducted beforehand based on publicly available data in order to determine sample size for confirmatory cohort I. The univariate analysis revealed six protein candidates being significantly dysregulated in early clinical PD patients compared to controls: A β 42, t-Tau, p-Tau, α -syn, DJ-1 and S100 β . However, although these potential protein markers were found to be significantly different when comparing both groups, subsequent ROC-AUC analysis demonstrated limited robustness as diagnostic tools (Fig 2).

As many publications lack the replication of the findings in an independent cohort, we aimed at reinvestigating our observations in the confirmatory cohort II. The size of the cohort

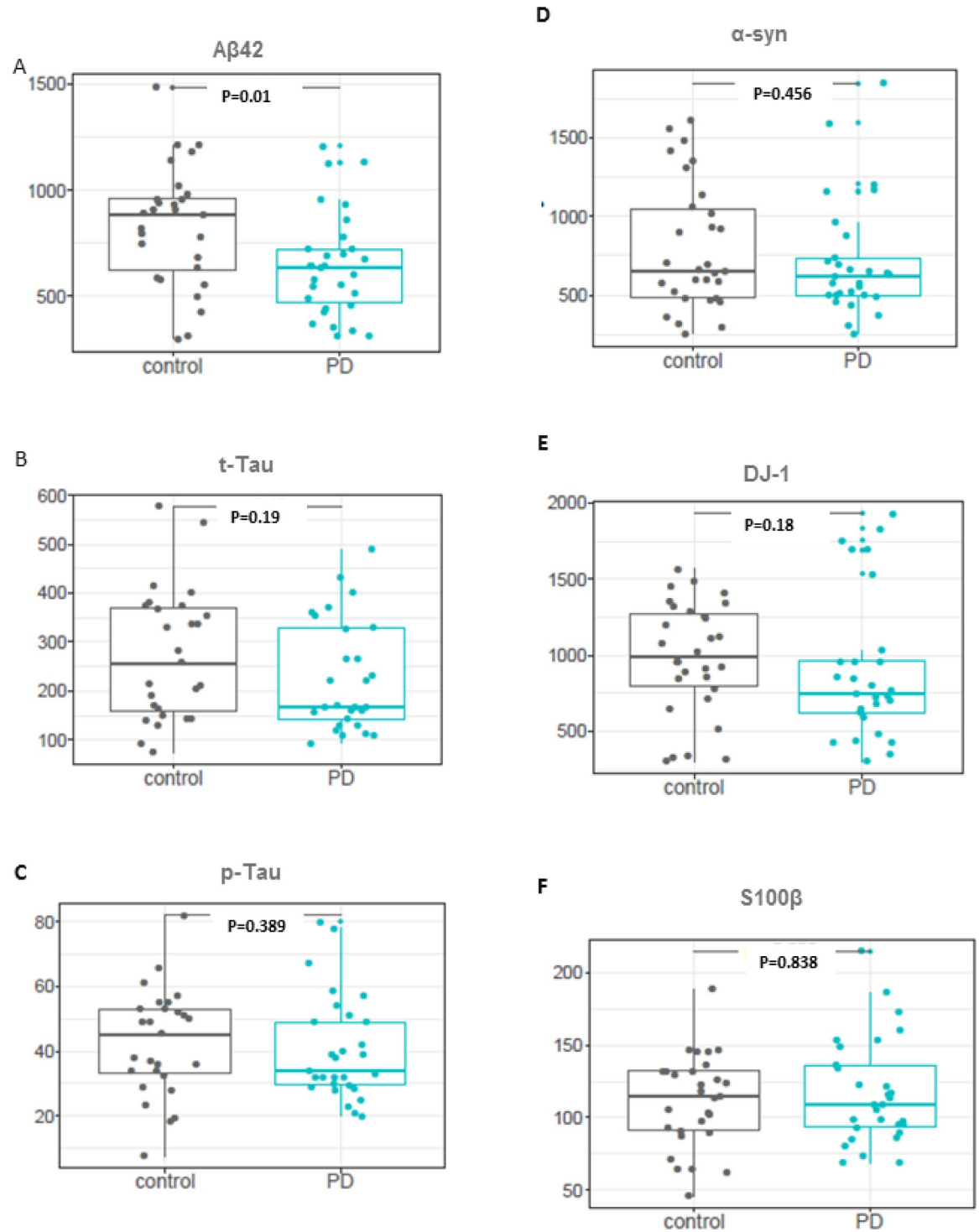


Fig 3. Univariate analysis of selected markers in confirmatory cohort II. (A-F) Boxplots of markers showing statistically significant changes in early clinical PD patients. P-values are calculated using the Wilcoxon signed-rank test. Grey dots represent controls and blue dots represent early PD.

<https://doi.org/10.1371/journal.pone.0206536.g003>

was based on power analysis based on data from confirmatory cohort I. Furthermore, as with confirmatory cohort I, the same randomization procedure was used for sample selection from

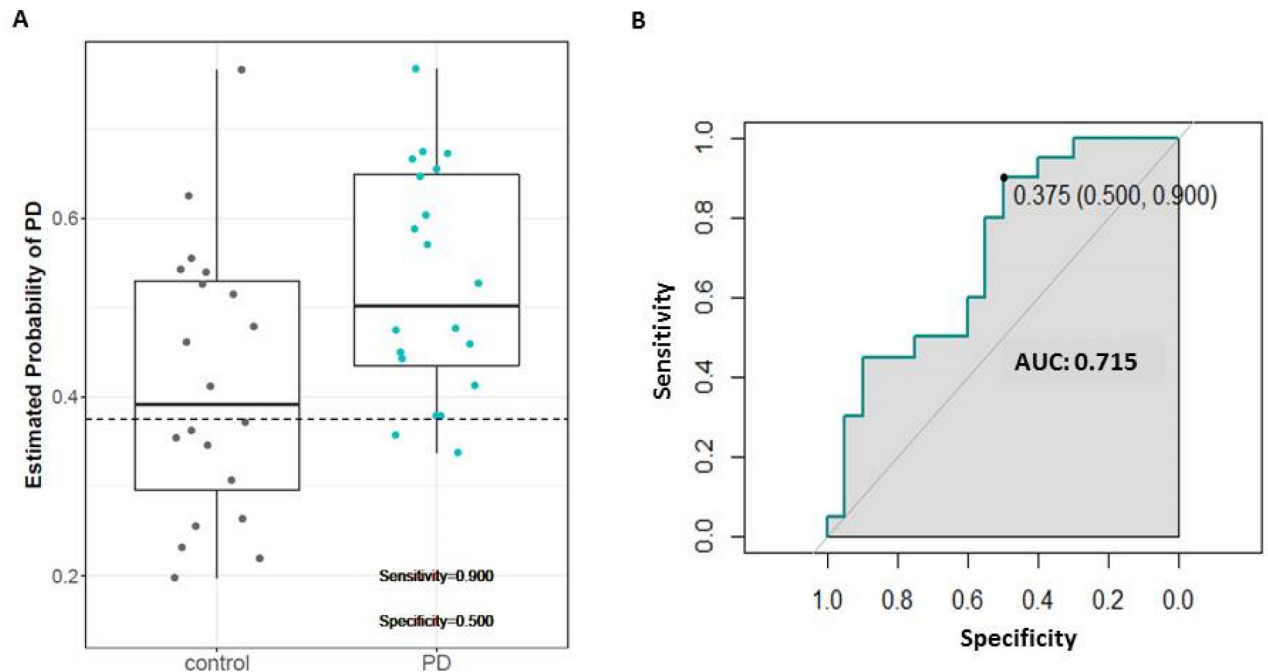


Fig 4. Diagnostic accuracy of potential model from Elastic Net Regression. (A) Predictive probabilities of PD from the most promising Elastic Net Model. The horizontal line corresponds to a predictive probability cut-off of 0.375 to classify PD and control. (B) Corresponding ROC curves, showing the AUC, optimal cut-off, sensitivity and specificity of the test.

<https://doi.org/10.1371/journal.pone.0206536.g004>

the available much larger patient sample base; the bioanalytical and statistical procedures were identical to those applied in step 1.

The analysis of the data showed that of the 6 protein markers significantly dysregulated in confirmatory cohort I, only A β 42 levels were confirmed as being significantly decreased in the confirmatory cohort II when compared to controls using univariate statistics (Fig 3). Decreased A β 42 levels in CSF samples from PD patients had been reported recently, suggesting it may be a reliable candidate [55]. However, in this study, ROC analysis showed that this potential marker was not suitable for diagnostic purposes (Fig 2 and S1 Fig).

Classical statistical methods are powerful tools but recently have been complemented by emerging new tools, such as machine learning approaches, which can manage large numbers of variables and identify potential signatures based on correlated multivariate analysis. Furthermore, machine learning has the advantage of integrating multiple variables and clinical endpoints by using data mining and generating predictive algorithms to provide a meaningful representation of the relationship across the data. In line with that, we reanalyzed our data by using two different machine learning methods. We first used Elastic Net, an algorithm that identifies linear relations within the dataset, to find a model with the best combination of variables and the highest predictive values. Our analysis revealed a model with 16 variables (A β 40, A β 42, α -syn, p-Tau, t-Tau, OPN, NFL, IL-6, DJ-1, UCHL1, FLT3LG, MMP2, S100 β , ApoA1, ratio of A β 40/A β 42 and ratio of p-Tau/t-Tau) able to distinguish early clinical PD from controls with 71% of diagnostic accuracy (AUC-ROC). Although high diagnostic sensitivity (90%) was achieved, diagnostic specificity (50%) was low suggesting lack of predictive value (Fig 4). When considering the biological relevance of the model, we observed that half of variables included in the model are associated with protein aggregation, one of the most important pathways of PD pathogenesis.

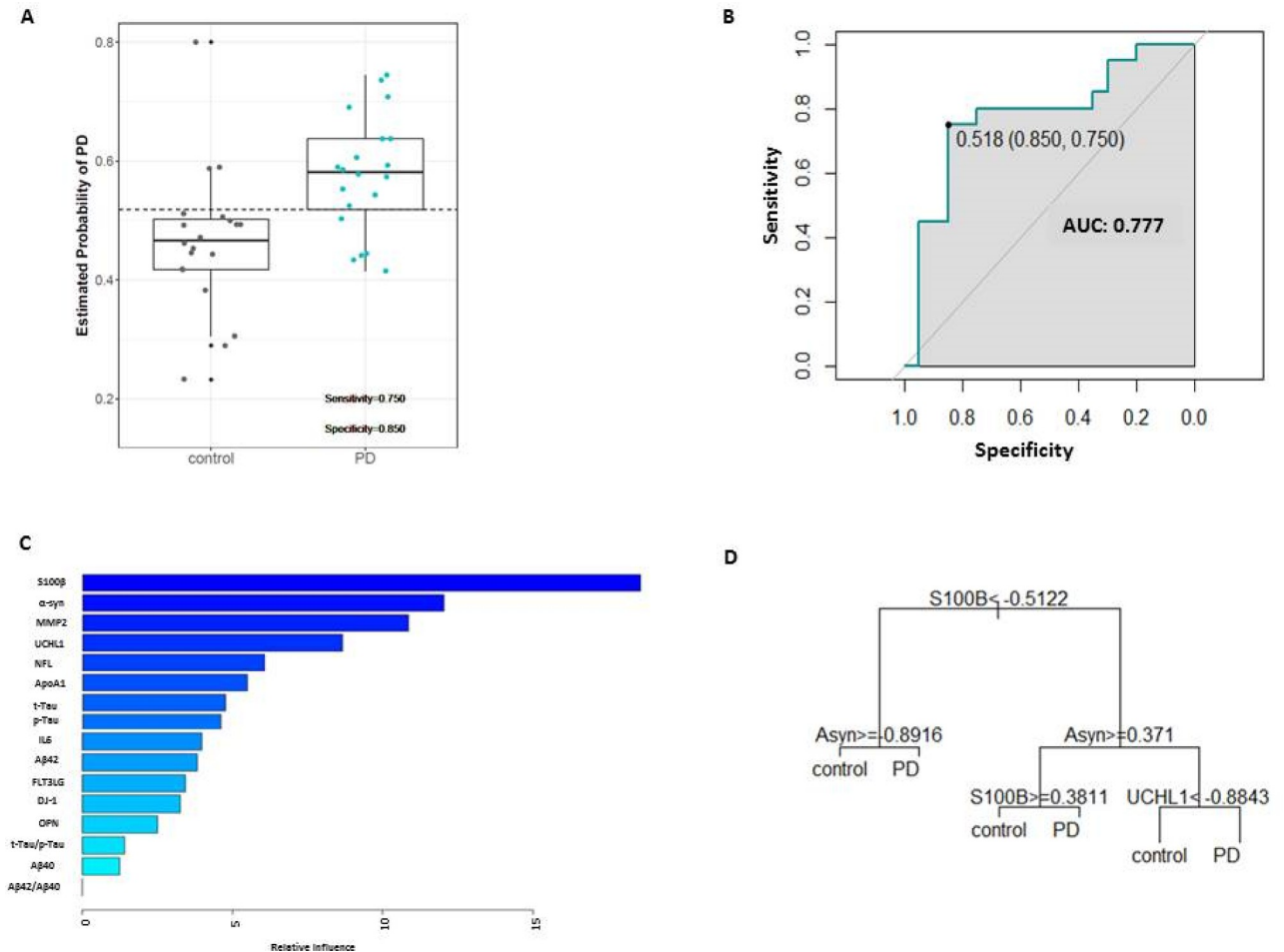


Fig 5. Diagnostic accuracy of gradient boosted model. (A) Predictive probabilities of PD from the most promising GBM. The horizontal line corresponds to a predictive probability cut-off of 0.518 to classify PD and control. (B) Corresponding ROC curves, showing the AUC, optimal cut-off, sensitivity and specificity of the test ROC curves of model created by gradient boosted regression. (C) Graph of most influential frequent variables. (D) Example of a decision tree.

<https://doi.org/10.1371/journal.pone.0206536.g005>

To improve predictivity and to better understand the relationship among the variables measured, we also used Gradient Boosted (GBM) Classification to define a model with higher predictivity and better interpretation. Through this method, we were able to identify a model comprising an ensemble of 60 decision trees with 85% diagnostic sensitivity, 75% diagnostic specificity, and 77% AUC-ROC (Fig 5A and 5B). We examined which markers impacted on the development of the model and found S100β, α-syn, MMP2 and UCHL1 as the most important markers to distinguish early clinical PD from controls (Fig 5C). Interestingly, this model aligned with findings published in the literature, where α-syn is characterized as the hallmark protein of PD, closely involved in the progression of neuronal degeneration and subsequent motor impairments, while S100β has been considered a possible marker for the accompanying neurodegeneration [56, 57]. Due to the novelty of this approach in the PD arena, we also aimed at confirming the model in confirmatory cohort II. When reproducing the model in this cohort, the decision tree could not be confirmed. Among potential reasons for this lack of reproducibility are (1) the less homogenous population included in the confirmatory cohort II and (2) the inherited, and previously documented, diagnostic limitation of the markers used in the model (3) imbalances in baseline characteristic between disease groups may have impacted

the power of the replication study. Whereas the genders in confirmatory cohort I are equally distributed (controls 49% males, patients 51% males), the genders in the control group in cohort II are dominated by females (controls 43% males) whereas the patient group is dominated by males (63% males) (Table 1). This imbalance is also reflected in the distribution of DJ-1, S100 β , t-Tau and p-Tau (Table 1, S2 Fig).

Overall, in contrast to our expectations from recent literature data, we could only identify changes to A β 42 in line with an earlier report [55]. Changes of a-syn, the hallmark of PD, could not be reproducibly detected in our two cohorts even with stringent experimental precautions. In a previous review, Mollenhauer et al. discussed the reliability of assays for the detection of a-syn in CSF in general as well as the desirable study design and CSF sampling conditions (ideally to be standardized between the analytical labs) [20]. Besides the pre-analytical handling of the samples, she also discussed whether post-translational modifications of a-syn (phosphorylation, ubiquitination, truncation, truncation and oxidization) and the various species of a-syn (monomeric, oligomeric, translationally modified) could be at least some of the reasons for the high variability and lack of reproducibility of reported findings between different groups and even—as in our case—even in the same laboratory. To this end, efforts of 18 different laboratories collaborating in the EU-BIOMARKAPD project exploring alternative approaches, essential requirements and standardization procedures are ongoing in the hope of finally finding ways for biomarker identification. Taking these proposals into account, we can state that our investigations have been performed in line with many of the proposed requirements. Thus, our findings rather raise further questions around the suitability of CSF as source of protein markers for PD, the potentially still insufficient clinical differentiation of PD subtypes, the need for further implementation of standardized experimental procedures and also the optimization of statistical evaluations.

To our knowledge, this is the first study which evaluated the performance of literature-based protein markers proposed for late stage PD in early clinical PD patients in line with previously proposed requirements from the neuroscience arena [19]. Our data shows that univariate analysis of CSF protein analysis performed for late stage PD may have limited value in early clinical PD diagnosis. A β 42 levels only could be shown to be reproducible in both of our cohorts. However, whether changes of A β 42 levels may contribute to the differentiation of PD from other neurodegenerative diseases remains a question. Alternative data analysis using a multivariate approaches such as the machine learning methods employed here might be of help in the development of a PD diagnostic algorithm.

Conclusions

In this study, we selected CSF proteins proposed as potential biomarkers for PD and assessed their applicability in two sets of samples from patients in early clinical stages of PD. We optimized and employed appropriate experimental conditions to measure and analyse these markers in the CSF the patients and controls. We demonstrate here that the currently proposed protein CSF markers for PD diagnosis, as identified in late stage PD cohorts, lack robustness and reproducibility when applied in the early clinical stages of akinetic-rigid PD. Thus, further efforts as the EU-BIOMARKAPD project might support the development of potential protein CSF biomarkers for diagnosis or disease monitoring in early clinical PD.

Supporting information

S1 Fig. Diagnostic accuracy of A β 42. (A) Predictive probabilities of PD from a univariate model of A β 42. The horizontal line corresponds to a predictive probability cut-off of 0.559 to classify PD and control. (B) Corresponding ROC curves, showing the AUC, optimal cut-off,

sensitivity and specificity of the test.
(TIF)

S2 Fig. Assessment of potential confounding factors. Figures (A-F) show the relationship between age, gender and protein levels in the two groups.
(TIF)

S1 Table. Summary of the validation criteria.
(XLSX)

Author Contributions

Conceptualization: Marcia Cristina T. dos Santos, Dieter Scheller, Claudia Schulte, Rosie Bell, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Data curation: Marcia Cristina T. dos Santos, Claudia Schulte, Peter Colman, Sarah R. Bujac, Rosie Bell, Andre Nogueira da Costa.

Formal analysis: Marcia Cristina T. dos Santos, Claudia Schulte, Irene R. Mesa, Peter Colman, Sarah R. Bujac, Rosie Bell, Walter Maetzler, Andre Nogueira da Costa.

Funding acquisition: Dieter Scheller, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Investigation: Marcia Cristina T. dos Santos, Dieter Scheller, Claudia Schulte, Rosie Bell, Caroline Berteau, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Methodology: Marcia Cristina T. dos Santos, Dieter Scheller, Claudia Schulte, Rosie Bell, Caroline Berteau, Luis Tosar Perez, Ingolf Lachmann, Daniela Berg, Andre Nogueira da Costa.

Project administration: Marcia Cristina T. dos Santos, Claudia Schulte, Walter Maetzler, Andre Nogueira da Costa.

Resources: Marcia Cristina T. dos Santos, Claudia Schulte, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Software: Marcia Cristina T. dos Santos, Claudia Schulte, Irene R. Mesa, Andre Nogueira da Costa.

Supervision: Marcia Cristina T. dos Santos, Dieter Scheller, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Validation: Marcia Cristina T. dos Santos, Dieter Scheller, Claudia Schulte, Irene R. Mesa, Sarah R. Bujac, Rosie Bell, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

Visualization: Marcia Cristina T. dos Santos, Claudia Schulte, Daniela Berg, Andre Nogueira da Costa.

Writing – original draft: Marcia Cristina T. dos Santos.

Writing – review & editing: Marcia Cristina T. dos Santos, Dieter Scheller, Claudia Schulte, Irene R. Mesa, Sarah R. Bujac, Daniela Berg, Walter Maetzler, Andre Nogueira da Costa.

References

1. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkman J, et al. Parkinson disease. *Nat Rev Dis Primers*. 2017; 3:17013. Epub 2017/03/24. <https://doi.org/10.1038/nrdp.2017.13> PMID: 28332488.

2. Gelpi E, Navarro-Otano J, Tolosa E, Gaig C, Compta Y, Rey MJ, et al. Multiple organ involvement by alpha-synuclein pathology in Lewy body disorders. *Mov Disord*. 2014; 29(8):1010–8. Epub 2014/01/08. <https://doi.org/10.1002/mds.25776> PMID: 24395122.
3. Adler CH, Beach TG, Hentz JG, Shill HA, Caviness JN, Driver-Dunckley E, et al. Low clinical diagnostic accuracy of early vs advanced Parkinson disease: clinicopathologic study. *Neurology*. 2014; 83(5):406–12. Epub 2014/07/01. <https://doi.org/10.1212/WNL.0000000000000641> PMID: 24975862; PubMed Central PMCID: PMC4132570.
4. Wolters E. Non-motor extranigral signs and symptoms in Parkinson's disease. *Parkinsonism Relat Disord*. 2009; 15 Suppl 3:S6–12. [https://doi.org/10.1016/S1353-8020\(09\)70770-9](https://doi.org/10.1016/S1353-8020(09)70770-9) PMID: 20083010.
5. Martinez-Martin P, Rodriguez-Blazquez C, Kurtis MM, Chaudhuri KR, Group NV. The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease. *Mov Disord*. 2011; 26(3):399–406. <https://doi.org/10.1002/mds.23462> PMID: 21264941.
6. Meissner WG, Frasier M, Gasser T, Goetz CG, Lozano A, Piccini P, et al. Priorities in Parkinson's disease research. *Nat Rev Drug Discov*. 2011; 10(5):377–93. <https://doi.org/10.1038/nrd3430> PMID: 21532567.
7. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci*. 1973; 20(4):415–55. Epub 1973/12/01. PMID: 4272516.
8. Kordower JH, Olanow CW, Dodiya HB, Chu Y, Beach TG, Adler CH, et al. Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain*. 2013; 136(Pt 8):2419–31. Epub 2013/07/26. <https://doi.org/10.1093/brain/awt192> PMID: 23884810; PubMed Central PMCID: PMC3722357.
9. Miller DB, O'Callaghan JP. Biomarkers of Parkinson's disease: present and future. *Metabolism*. 2015; 64(3 Suppl 1):S40–6. <https://doi.org/10.1016/j.metabol.2014.10.030> PMID: 25510818; PubMed Central PMCID: PMC4721253.
10. Algarni MA, Stoessl AJ. The role of biomarkers and imaging in Parkinson's disease. *Expert Rev Neurother*. 2016; 16(2):187–203. <https://doi.org/10.1586/14737175.2016.1135056> PMID: 26829357.
11. Olsson B, Lautner R, Andreasson U, Ohrfelt A, Portelius E, Bjerke M, et al. CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *Lancet Neurol*. 2016; 15(7):673–84. Epub 2016/04/14. [https://doi.org/10.1016/S1474-4422\(16\)00070-3](https://doi.org/10.1016/S1474-4422(16)00070-3) PMID: 27068280.
12. Majbour NK, Vaikath NN, van Dijk KD, Ardah MT, Varghese S, Vesterager LB, et al. Oligomeric and phosphorylated alpha-synuclein as potential CSF biomarkers for Parkinson's disease. *Mol Neurodegener*. 2016; 11:7. Epub 2016/01/20. <https://doi.org/10.1186/s13024-016-0072-9> PMID: 26782965; PubMed Central PMCID: PMC4717559.
13. Buddhala C, Campbell MC, Perlmuter JS, Kotzbauer PT. Correlation Between Decreased CSF α -Synuclein and A β (1–42) in Parkinson Disease. *Neurobiology of aging*. 2015; 36(1):476–84. <https://doi.org/10.1016/j.neurobiolaging.2014.07.043> PMC4268043. PMID: 25212463
14. Herbert MK, Eeftens JM, Aerts MB, Esselink RA, Bloem BR, Kuiperij HB, et al. CSF levels of DJ-1 and tau distinguish MSA patients from PD patients and controls. *Parkinsonism Relat Disord*. 2014; 20(1):112–5. Epub 2013/10/01. <https://doi.org/10.1016/j.parkreldis.2013.09.003> PMID: 24075122.
15. Heywood WE, Galimberti D, Bliss E, Sirka E, Paterson RW, Magdalinou NK, et al. Identification of novel CSF biomarkers for neurodegeneration and their validation by a high-throughput multiplexed targeted proteomic assay. *Mol Neurodegener*. 2015; 10:64. Epub 2015/12/03. <https://doi.org/10.1186/s13024-015-0059-y> PMID: 26627638; PubMed Central PMCID: PMC4666172.
16. Parnetti L, Chiasserini D, Persichetti E, Eusebi P, Varghese S, Qureshi MM, et al. Cerebrospinal fluid lysosomal enzymes and alpha-synuclein in Parkinson's disease. *Mov Disord*. 2014b; 29(8):1019–27. Epub 2014/01/18. <https://doi.org/10.1002/mds.25772> PMID: 24436092; PubMed Central PMCID: PMC4282452.
17. Parnetti L, Farotti L, Eusebi P, Chiasserini D, De Carlo C, Giannandrea D, et al. Differential role of CSF alpha-synuclein species, tau, and Abeta42 in Parkinson's Disease. *Front Aging Neurosci*. 2014a; 6:53. Epub 2014/04/20. <https://doi.org/10.3389/fnagi.2014.00053> PMID: 24744728; PubMed Central PMCID: PMC3978246.
18. Teixeira Dos Santos MC, Bell R, da Costa AN. Recent developments in circulating biomarkers in Parkinson's disease: the potential use of miRNAs in a clinical setting. *Bioanalysis*. 2016; 8(23):2497–518. Epub 2016/11/20. <https://doi.org/10.4155/bio-2016-0166> PMID: 27855513.
19. Trojanowski JQ, Growdon JH. A new consensus report on biomarkers for the early antemortem diagnosis of Alzheimer disease: current status, relevance to drug discovery, and recommendations for future research. *J Neuropathol Exp Neurol*. 1998; 57(6):643–4. PMID: 9630242.

20. Mollenhauer B. Quantification of alpha-synuclein in cerebrospinal fluid: how ideal is this biomarker for Parkinson's disease? *Parkinsonism Relat Disord.* 2014; 20 Suppl 1:S76–9. [https://doi.org/10.1016/S1353-8020\(13\)70020-8](https://doi.org/10.1016/S1353-8020(13)70020-8) PMID: 24262194.
21. Aasly JO, Shi M, Sossi V, Stewart T, Johansen KK, Wszolek ZK, et al. Cerebrospinal fluid amyloid beta and tau in LRRK2 mutation carriers. *Neurology.* 2012; 78(1):55–61. <https://doi.org/10.1212/WNL.0b013e31823ed101> PMID: 22170881; PubMed Central PMCID: PMC3466497.
22. Bernhardt D, Muller HP, Ludolph AC, Dupuis L, Kassubek J. Body fat distribution in Parkinson's disease: An MRI-based body fat quantification study. *Parkinsonism Relat Disord.* 2016; 33:84–9. <https://doi.org/10.1016/j.parkreldis.2016.09.016> PMID: 27665038.
23. Carvalho DZ, Schonwald SV, Schumacher-Schuh AF, Braga CW, Souza DO, Oses JP, et al. Overnight S100B in Parkinson's Disease: A glimpse into sleep-related neuroinflammation. *Neurosci Lett.* 2015; 608:57–63. <https://doi.org/10.1016/j.neulet.2015.10.010> PMID: 26453767.
24. Chahine LM, Stern MB, Chen-Plotkin A. Blood-based biomarkers for Parkinson's disease. *Parkinsonism Relat Disord.* 2014; 20 Suppl 1:S99–103. [https://doi.org/10.1016/S1353-8020\(13\)70025-7](https://doi.org/10.1016/S1353-8020(13)70025-7) PMID: 24262199; PubMed Central PMCID: PMC34070332.
25. Chen YC, Wu YR, Mesri M, Chen CM. Associations of Matrix Metalloproteinase-9 and Tissue Inhibitory Factor-1 Polymorphisms With Parkinson Disease in Taiwan. *Medicine (Baltimore).* 2016; 95(5):e2672. <https://doi.org/10.1097/MD.0000000000002672> PMID: 26844501; PubMed Central PMCID: PMC34748918.
26. Grunblatt E, Mandel S, Jacob-Hirsch J, Zeligson S, Amariglio N, Rechavi G, et al. Gene expression profiling of parkinsonian substantia nigra pars compacta: alterations in ubiquitin-proteasome, heat shock protein, iron and oxidative stress regulated proteins, cell adhesion/cellular matrix and vesicle trafficking genes. *J Neural Transm (Vienna).* 2004; 111(12):1543–73. <https://doi.org/10.1007/s00702-004-0212-1> PMID: 15455214.
27. Jimenez-Jimenez FJ, Alonso-Navarro H, Garcia-Martin E, Agundez JA. Cerebrospinal fluid biochemical studies in patients with Parkinson's disease: toward a potential search for biomarkers for this disease. *Front Cell Neurosci.* 2014; 8:369. <https://doi.org/10.3389/fncel.2014.00369> PMID: 25426023; PubMed Central PMCID: PMC34227512.
28. Khandelwal PJ, Herman AM, Moussa CE. Inflammation in the early stages of neurodegenerative pathology. *J Neuroimmunol.* 2011; 238(1–2):1–11. <https://doi.org/10.1016/j.jneuroim.2011.07.002> PMID: 21820744; PubMed Central PMCID: PMC3176984.
29. Lindqvist D, Hall S, Surova Y, Nielsen HM, Janelidze S, Brundin L, et al. Cerebrospinal fluid inflammatory markers in Parkinson's disease—associations with depression, fatigue, and cognitive impairment. *Brain Behav Immun.* 2013; 33:183–9. <https://doi.org/10.1016/j.bbi.2013.07.007> PMID: 23911592.
30. Maarouf CL, Beach TG, Adler CH, Malek-Ahmadi M, Kokjohn TA, Dugger BN, et al. Quantitative appraisal of ventricular cerebrospinal fluid biomarkers in neuropathologically diagnosed Parkinson's disease cases lacking Alzheimer's disease pathology. *Biomark Insights.* 2013; 8:19–28. <https://doi.org/10.4137/BMI.S11422> PMID: 23533154; PubMed Central PMCID: PMC3603385.
31. Maetzler W, Apel A, Langkamp M, Deuschle C, Dilger SS, Stirnkorb JG, et al. Comparable autoantibody serum levels against amyloid- and inflammation-associated proteins in Parkinson's disease patients and controls. *PLoS One.* 2014; 9(2):e88604. <https://doi.org/10.1371/journal.pone.0088604> PMID: 24586351; PubMed Central PMCID: PMC3931625.
32. Maetzler W, Schmid SP, Wurster I, Liepelt I, Gaenslen A, Gasser T, et al. Reduced but not oxidized cerebrospinal fluid glutathione levels are lowered in Lewy body diseases. *Mov Disord.* 2011; 26(1):176–81. Epub 2010/09/16. <https://doi.org/10.1002/mds.23358> PMID: 20842692.
33. Qiang JK, Wong YC, Siderowf A, Hurtig HI, Xie SX, Lee VM, et al. Plasma apolipoprotein A1 as a biomarker for Parkinson disease. *Ann Neurol.* 2013; 74(1):119–27. <https://doi.org/10.1002/ana.23872> PMID: 23447138; PubMed Central PMCID: PMC3773265.
34. Schaf DV, Tort AB, Fricke D, Schestatsky P, Portela LV, Souza DO, et al. S100B and NSE serum levels in patients with Parkinson's disease. *Parkinsonism Relat Disord.* 2005; 11(1):39–43. <https://doi.org/10.1016/j.parkreldis.2004.07.002> PMID: 15619461.
35. Shi M, Movius J, Dator R, Aro P, Zhao Y, Pan C, et al. Cerebrospinal fluid peptides as potential Parkinson disease biomarkers: a staged pipeline for discovery and validation. *Mol Cell Proteomics.* 2015; 14(3):544–55. <https://doi.org/10.1074/mcp.M114.040576> PMID: 25556233; PubMed Central PMCID: PMC349976.
36. Shi M, Furay AR, Sossi V, Aasly JO, Armaly J, Wang Y, et al. DJ-1 and alphaSYN in LRRK2 CSF do not correlate with striatal dopaminergic function. *Neurobiol Aging.* 2012; 33(4):836 e5–7. <https://doi.org/10.1016/j.neurobiolaging.2011.09.015> PMID: 22019052; PubMed Central PMCID: PMC3279603.
37. Yu SY, Zuo LJ, Wang F, Chen ZJ, Hu Y, Wang YJ, et al. Potential biomarkers relating pathological proteins, neuroinflammatory factors and free radicals in PD patients with cognitive impairment: a cross-

- sectional study. *BMC Neurol.* 2014; 14:113. <https://doi.org/10.1186/1471-2377-14-113> PMID: 24884485; PubMed Central PMCID: PMC4035732.
38. Kang JH, Mollenhauer B, Coffey CS, Toledo JB, Weintraub D, Galasko DR, et al. CSF biomarkers associated with disease heterogeneity in early Parkinson's disease: the Parkinson's Progression Markers Initiative study. *Acta Neuropathol.* 2016; 131(6):935–49. <https://doi.org/10.1007/s00401-016-1552-2> PMID: 27021906; PubMed Central PMCID: PMC45031365.
 39. Mollenhauer B, Caspell-Garcia CJ, Coffey CS, Taylor P, Shaw LM, Trojanowski JQ, et al. Longitudinal CSF biomarkers in patients with early Parkinson disease and healthy controls. *Neurology.* 2017; 89(19):1959–69. <https://doi.org/10.1212/WNL.0000000000004609> PMID: 29030452; PubMed Central PMCID: PMC5679418.
 40. Noelker C, Hampel H, Dodel R. Blood-based protein biomarkers for diagnosis and classification of neurodegenerative diseases: current progress and clinical potential. *Mol Diagn Ther.* 2011; 15(2):83–102. <https://doi.org/10.2165/11534870-000000000-00000> PMID: 21623645.
 41. Parkinson Progression Marker I. The Parkinson Progression Marker Initiative (PPMI). *Prog Neurobiol.* 2011; 95(4):629–35. <https://doi.org/10.1016/j.pneurobio.2011.09.005> PMID: 21930184.
 42. Rosenthal LS, Drake D, Alcalay RN, Babcock D, Bowman FD, Chen-Plotkin A, et al. The NINDS Parkinson's disease biomarkers program. *Mov Disord.* 2016; 31(6):915–23. <https://doi.org/10.1002/mds.26438> PMID: 26442452; PubMed Central PMCID: PMC4824671.
 43. Gwinn K, David KK, Swanson-Fischer C, Albin R, Hillaire-Clarke CS, Sieber BA, et al. Parkinson's disease biomarkers: perspective from the NINDS Parkinson's Disease Biomarkers Program. *Biomark Med.* 2017; 11(6):451–73. <https://doi.org/10.2217/bmm-2016-0370> PMID: 28644039; PubMed Central PMCID: PMC5619098.
 44. O'Bryant SE, Xiao G, Zhang F, Edwards M, German DC, Yin X, et al. Validation of a serum screen for Alzheimer's disease across assay platforms, species, and tissues. *J Alzheimers Dis.* 2014; 42(4):1325–35. <https://doi.org/10.3233/JAD-141041> PMID: 25024345; PubMed Central PMCID: PMC4400808.
 45. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry.* 1992; 55(3):181–4. Epub 1992/03/01. PMID: 1564476; PubMed Central PMCID: PMC4014720.
 46. Goetz CG, Poewe W, Rascol O, Sampaio C, Stebbins GT, Counsell C, et al. Movement Disorder Society Task Force report on the Hoehn and Yahr staging scale: status and recommendations. *Mov Disord.* 2004; 19(9):1020–8. Epub 2004/09/17. <https://doi.org/10.1002/mds.20213> PMID: 15372591.
 47. Fahn SE, R. Unified Parkinson's disease rating scale. *Recent Developments in Parkinson's Disease.* 2: Macmillan Health Care Information; 1987. p. 153–63, 293–304.
 48. Wang Z, Chen H, Ma H, Ma L, Wu T, Feng T. Resting-state functional connectivity of subthalamic nucleus in different Parkinson's disease phenotypes. *J Neurol Sci.* 2016; 371:137–47. Epub 2016/11/23. <https://doi.org/10.1016/j.jns.2016.10.035> PMID: 27871435.
 49. Huertas I, Jesus S, Lojo JA, Garcia-Gomez FJ, Caceres-Redondo MT, Oropesa-Ruiz JM, et al. Lower levels of uric acid and striatal dopamine in non-tremor dominant Parkinson's disease subtype. *PLoS One.* 2017; 12(3):e0174644. Epub 2017/03/31. <https://doi.org/10.1371/journal.pone.0174644> PMID: 28358829; PubMed Central PMCID: PMC5373593.
 50. Lewczuk P, Kornhuber J, Wiltfang J. The German Competence Net Dementias: standard operating procedures for the neurochemical dementia diagnostics. *J Neural Transm (Vienna).* 2006; 113(8):1075–80. Epub 2006/07/13. <https://doi.org/10.1007/s00702-006-0511-9> PMID: 16835693.
 51. Teunissen CE, Petzold A, Bennett JL, Berven FS, Brundin L, Comabella M, et al. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. *Neurology.* 2009; 73(22):1914–22. Epub 2009/12/02. <https://doi.org/10.1212/WNL.0b013e3181c47cc2> PMID: 19949037; PubMed Central PMCID: PMC2839806.
 52. Jani D, Allinson J, Berisha F, Cowan KJ, Devanarayan V, Gleason C, et al. Recommendations for Use and Fit-for-Purpose Validation of Biomarker Multiplex Ligand Binding Assays in Drug Development. *Aaps j.* 2016; 18(1):1–14. Epub 2015/09/18. <https://doi.org/10.1208/s12248-015-9820-y> PMID: 26377333; PubMed Central PMCID: PMC4706274.
 53. Bernhard FP, Heinzl S, Binder G, Weber K, Apel A, Roeben B, et al. Insulin-Like Growth Factor 1 (IGF-1) in Parkinson's Disease: Potential as Trait-, Progression- and Prediction Marker and Confounding Factors. *PLoS One.* 2016; 11(3):e0150552. <https://doi.org/10.1371/journal.pone.0150552> PMID: 26967642; PubMed Central PMCID: PMC4788352.
 54. Shi M, Bradner J, Hancock AM, Chung KA, Quinn JF, Peskind ER, et al. Cerebrospinal fluid biomarkers for Parkinson disease diagnosis and progression. *Ann Neurol.* 2011; 69(3):570–80. <https://doi.org/10.1002/ana.22311> PMID: 21400565; PubMed Central PMCID: PMC3117674.

55. Rochester L, Galna B, Lord S, Yarnall AJ, Morris R, Duncan G, et al. Decrease in Abeta42 predicts dopa-resistant gait progression in early Parkinson disease. *Neurology*. 2017; 88(16):1501–11. <https://doi.org/10.1212/WNL.0000000000003840> PMID: 28330963; PubMed Central PMCID: PMC5395075.
56. Steiner J, Bogerts B, Schroeter ML, Bernstein HG. S100B protein in neurodegenerative disorders. *Clin Chem Lab Med*. 2011; 49(3):409–24. <https://doi.org/10.1515/CCLM.2011.083> PMID: 21303299.
57. Yordan T, Erenler AK, Baydin A, Aydin K, Cokluk C. Usefulness of S100B protein in neurological disorders. *J Pak Med Assoc*. 2011; 61(3):276–81. PMID: 21465945.