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Elemental fingerprint: Reassessment of a cerebrospinal fluid biomarker for Parkinson's disease

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ABSTRACT

The aim of the study was to validate a predictive biomarker machine learning model for the classification of Parkinson's disease (PD) and age-matched controls (AMC), based on bioelement abundance in the cerebrospinal fluid (CSF). For this multicentric trial, participants were enrolled from four different centers. CSF was collected according to standardized protocols. For bioelement determination, CSF samples were subjected to inductively coupled plasma mass spectrometry. A predefined Support Vector Machine (SVM) model, trained on a previous discovery cohort was applied for differentiation, based on the levels of six different bioelements. 82 PD patients, 68 age-matched controls and 7 additional Normal Pressure Hydrocephalus (NPH) patients were included to validate a predefined SVM model. Six differentiating elements (As, Fe, Mg, Ni, Se, Sr) were quantified. Based on their levels, SVM was successfully applied to a new local cohort (AUROC 0.76, Sensitivity 0.80, Specificity 0.83), without taking any additional features into account. The same model did not discriminate PD and AMCs / NPH from three external cohorts, likely due to center effects. However, discrimination was possible in cohorts with a full elemental data set, now using center-specific discovery cohorts and a cross validated approach (AUROC 0.78 and 0.88, respectively). Pooled PD CSF iron levels showed a clear correlation with disease duration ($p = .0001$). In summary, bioelemental CSF patterns, obtained by mass spectrometry and integrated into a predictive model yield the potential to facilitate the differentiation of PD and AMC. Center-specific biases interfere with application in external cohorts. This must be carefully addressed using center-defined, local reference values and models.

Abbreviations: AMC, age matched control; AUROC, area under the receiver operating characteristic curve; H&Y, Hoehn and Yahr stage; ICP-OES, inductively coupled plasma optical emission spectrometry; ICP-sf-MS, inductively coupled plasma-sector field mass spectrometry; LED, levodopa equivalent dose; LOD, limit of detection; LOOCV, leave-one-out cross validation; LOQ, limit of quantification; MMSE, Mini–Mental State Examination; MoCA, Montreal Cognitive Assessment Score; NPH, Normal Pressure Hydrocephalus; PCA, principal component analysis; PD, Parkinson's disease; PDNMS, Parkinson's disease non-motor symptoms questionnaire; SVM, Support Vector Machine; UPDRS, Unified Parkinson's Disease Rating Scale

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1. Introduction

The clinical diagnosis of Parkinson's disease (PD) is based on expert diagnosis applying MDS clinical diagnostic criteria (Postuma et al., 2015). Misdiagnosis in early stages is common and a reliable molecular marker is still not available. Chemical bioelements contribute to multiple disease mechanisms in PD and might thus represent a promising alternative biomarker.

Iron, for example, has an outstanding position for PD pathogenesis: next to its well-known accumulation in the substantia nigra and other regions of the Parkinsonian brain (Dexter et al., 1989), its interaction with a-synuclein (Uversky et al., 2001), its involvement in ferroptotic cell death (Dixon et al., 2012) and its role as a drug target in clinical trials involving chelating agents (deferiprone in the FAIRPARK trials (Devos et al., 2014); www.fairpark2.eu) substantiate its importance in PD pathology. Other bioelements like selenium (Se) and the associated selenoproteins are known for their involvement in the oxidative stress response (Ellwanger et al., 2016) and for their dysregulation in the post-mortem Parkinsonian brain (Bellinger et al., 2011, 2012; Boukhzar et al., 2016). Arsenic (As), magnesium (Mg), strontium (Sr) and nickel (Ni) were also implicated in PD pathogenesis based on data from epidemiological studies and different PD models (Cholanians et al., 2016; Slotkin and Seidler, 2011; Sun, 2018). In PD mimics like dementia with Lewy bodies or normal pressure hydrocephalus (NPH), different bioelements and metal-binding protein were also discussed to function as potential biomarkers (Boström et al., 2009; Murakami et al., 2018).

CSF as biofluid reflects many properties of adjacent CNS tissue, including elemental composition and is thus particularly suited as biomarker source. So far, however, most studies on CSF bioelements were conducted with only low patient numbers and with a low level of evidence. All reported trials are exploratory studies, partially showing contradictory results for different elements (Jiménez-Jiménez et al., 2014; Mariani et al., 2013), and a multicentric validation in larger independent cohorts is lacking.

Based on the results of our monocentric exploratory cohort on bioelements in the CSF of patients with PD and age-matched control subjects (AMC) (Maass et al., 2018), we now validated the discriminative power of a predictive machine learning model based on the abundance of six bioelements in the CSF (As, Se, Fe, Mg, Ni, Sr), defined within the exploratory cohort. For the exploratory discovery cohort, we initially quantified 19 different bioelements and used machine learning for prediction of PD and AMC. Thereafter, we reduced the number of elements needed for predictive modeling by applying a stepwise removal of the least informative element, finally presenting a discriminative minimum cluster of the six bioelements, as mentioned above. We hypothesized that using predictive models based on elemental patterns instead of the levels of individual single bioelement, might increase the robustness as a biomarker.

Here, we present the results of the validation of our discovery cohort in four independent cohorts. In addition to an independent validation cohort from our own center (C1), we analyzed the accuracy of prediction in an external clinically matched cohort of similar size (C2), as well as two additional smaller cohorts with different patient characteristics (C3 and C4), also including NPH patients as a frequent differential diagnosis of PD. A Support Vector Machines (SVM) model, based on the bioelemental abundance quantified in the discovery cohort, was used for the discrimination of PD patients and AMC, allowing us to provide class II level of evidence for the potential discriminative power of bioelements in PD.

2. Material and methods

2.1. Subjects

PD patients were diagnosed according to UK Brain Bank criteria and compliant with the MDS criteria (Postuma et al., 2015) and age-matched control patients without signs of neurodegenerative, neuroinflammatory, neurooncological or acute ischemic central nervous diseases (AMC), in most cases having a lumbar puncture for exclusion diagnosis, were recruited from the patient pool of the out- and in-patient clinics of four different neurological centers in Germany, primarily for PD-related prospective research projects. PD patients were included independent of disease duration or disease severity. All PD patients underwent neurological examination and history taken by movement disorder specialists, including the assessment of motor (Unified Parkinson's Disease Rating Scale, part III) and cognitive function (Montreal Cognitive Assessment Score or Mini–Mental State Examination). If only Mini–Mental State Examination was available, a conversion into Montreal Cognitive Assessment Score was applied for further analysis (Lawton et al., 2016). The levodopa equivalent dose (LED) was calculated according to Tomlinson et al. (2010). Most patients were part of cohorts, which have regular scheduled follow-up assessments, allowing for the reassessment of the diagnosis, which increases with time (Adler et al., 2014).

2.1.1. Cohort C1 (Göttingen)

30 PD patients and 25 AMC were selected from the CSF Biobank of the Department of Neurology, University of Göttingen, Germany. Age-matched controls had no clinical signs of neurodegeneration. C1 represents an internal validation cohort, because the initial exploratory discovery cohort was also enrolled at this department (Maass et al., 2018).

2.1.2. Cohort C2 (Tübingen)

31 PD patients and 29 AMC were obtained from the Neuro-Biobank of the University of Tübingen, Germany. This biobank is supported by the local University, the Hertie Institute and the German Center for Neurodegenerative Diseases (DZNE). Age-matched controls were assessed to have no neurological diseases. C2 presents a clinically matched, external validation cohort of similar size.

2.1.3. Cohort C3 (Bochum)

9 PD and 9 AMC patients were obtained from the CSF Biobank of the Department of Neurology, Ruhr-University of Bochum, Germany. Age-matched controls had no clinical signs of neurodegeneration. C3 presents an additional cohort with slightly different characteristics, including PD patients in an earlier disease stage.

2.1.4. Cohort 4 (Kassel/Göttingen)

7 PD samples were obtained from the Kassel cohorts (described e.g. (Mollenhauer et al., 2011)) of the Paracelsus-Elena Klinik, Kassel, Germany (specialized center for movement disorders). In addition, 7 patients with idiopathic normal pressure hydrocephalus (NPH) by MRI and spinal tap without PD or other neurodegenerative disorders were also enrolled. Therefore, C4 presents an additional cohort including a non-neurodegenerative disease control.

Despite the small sample size concerning cohort 3 and cohort 4, patients were not pooled in this analysis to allow for the detection of center specific differences and construction of center specific models.

Permissions of the local ethics committees have been obtained prior to the initiation of the study (Ethics committee of the University Medical Center Göttingen, Nr. 13/11/12, 9/7/04 and 36/7/02; Ethics committee of the Faculty of Medicine at the University of Tübingen, Nr. 199/2011BO1; Local University ethics committee of the Ruhr University Bochum, Nr. 17-6119). Written consent was provided by all patients or care givers. The study conforms with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. CSF sampling procedures

Sample collection was performed according to standard biomarker protocols (Teunissen et al., 2009). In brief, the first 2–4 ml were used

for routine diagnostics and at least 10 ml of CSF were collected for biobanking purposes using polypropylene tubes. Samples were centrifuged, aliquoted and stored at −80 °C within 1–2 h. Samples with relevant blood contamination (according to a red blood cell count > 100/µl or semiquantitative hemoglobin detection) were excluded.

2.3. Sample analysis by ICP-OES and ICP-sf-MS

Element determination (As, Fe, Mg, Ni, Se, Sr) was performed as described before (Maass et al., 2018). In brief, an inductively coupled plasma-optical emission spectrometry (ICP-OES) system was used for element determination and an inductively coupled plasma-sector field-mass spectrometry (ICP-sf-MS) instrument was employed for determination of elements, which were below the limit of quantification from ICP-OES (technical details are given in the supplement).

2.4. Statistical analysis

Quantitative data of two groups was compared using either *t*-test or Mann–Whitney *U* test, as appropriate. Qualitative data was compared using chi-squared test. Multiple groups were compared using either one-way or two-way ANOVA with Tukey's multiple comparisons test. Element levels were log-10 transformed and values below the limit of quantification were imputed using model-based robust expectation-maximization for left-censored data (Helsel, 2005; Palarea-Albaladejo and Martín-Fernández, 2015). Principal component analysis (PCA) of scaled data was used to visualize potential clusters. A scree plot was used for the visualizing of the explained variance of the different components. A radial kernel Support Vector Machine (SVM) algorithm was trained on preprocessed (centered, scaled) CSF levels of As, Fe, Mg, Ni, Se, Sr of a recent discovery cohort (Maass et al., 2018) using 10 times 10-fold cross validation for hyperparameter tuning and after that applied to predict PD and AMC / NPH of the different centers (cohort C1-C4) of the validation cohort. The resulting estimates for the area under the receiver operating characteristic (ROC) curve were visualized. The same SVM algorithm was again trained and tested on each center of the validation cohort separately, using 10 times 10-fold cross validation (C1, C2) or leave-one-out cross validation (LOOCV) (C3, C4), for the definition of center-specific discovery cohorts. The performance was again visualized using ROC curves. Correlation between element levels and clinical parameter was performed using Spearman correlation after adjusting for multiple testing according to Bonferroni. Correlation between Fe and disease duration was controlled for age using multiple linear regression. GraphPad Prism 7.04 and R version 3.4.3 (R-packages given in the supplement) were used for data analysis.

3. Results

3.1. Participants characteristics

A total of 157 patients (82 PD, 68 AMC and 7 NPH) were recruited in four different neurological centers (cohort C1-C4). Within each cohort, there were no significant age or sex differences between the PD and AMC or NPH group, respectively ($p > .05$). Demographical and clinical characteristics of the study population are given in Table 1.

Cohort 1 and cohort 2 represent clinically matched validation cohorts (cohort 1: independent local validation cohort; cohort 2: external validation cohort) without differences in age, H&Y stage, disease duration or UPDRS III score ($p > .05$). Inter-cohort comparison of the clinical characteristics can be found in Supplementary Table S1. Cohort 3 and cohort 4 represent two additional smaller cohorts with different patient characteristics (cohort 3: PD patients with a shorter disease duration; cohort 4: patients with NPH instead of AMC).

3.2. CSF baseline element levels and center effects

To validate a set of six differentiating elements in the CSF (As, Fe, Mg, Ni, Se, Sr; as described in Maass et al. (2018)), we quantified the abundance of the same six elements in the current study. Due to limited CSF volume, only levels of As, Fe, Se and Ni could be quantified for cohort 3. Levels of Sr and Mg were disregarded in this cohort and were not imputed to avoid biases. Values under LOQ were observed only for a small part of all quantifications (total left-censored values combining all cohorts: 11 single values in all PD patients, resulting imputation-rate 2.2%; 19 single values in AMC/NPH, resulting imputation rate 4.2%. Those values were imputed (robust expectation-maximization for left-censored data) based on the LOQ, calculated for each diluted sample.

Comparing the elemental levels between PD and AMC in each cohort (cohort C1-C4) respectively, we found significant differences in individual cohorts, which, however, were never reproduced in the other cohorts (visualized in Fig. 1, summarized in Supplementary Table S2). Inter-cohort comparisons of bioelement levels within the PD and the AMC group respectively, revealed multiple significant differences in the levels of As, Ni, Se and Sr, while levels of Fe and Mg showed a relatively stable abundance (Supplementary Table S3).

As suggested by the inter-cohort comparison, the principal component analysis (PCA) reveals multiple center effects (Supplementary Fig. S1).

Table 1
Demographical and clinical characteristics of the study population.

	Cohort 1		*Cohort 2*		*Cohort 3*		*Cohort 4*	
	Göttingen		*Tübingen*		*Bochum*		*Kassel*	
	PD	AMC	PD	AMC	PD	AMC	PD	NPH
	n = 35	*n* = 30	*n* = 31	*n* = 29	*n* = 9	n = 9	*n* = 7	*n* = 7
Age, years	66.8 (10.3; 58–75)	65.6 (12.6; 57–76)	63.4 (10.7; 54–72)	63.2 (10.3; 56–72)	66.3 (11.7; 54–77)	55.3 (13.7; 44–70)	71.1 (5.5; 66–74)	74.0 (6.2; 68–80)
Male/female (% female)	18/17 (48.6)	17/13 (43.3)	14/17 (54.8)	13/16 (55.2)	6/3 (33.3)	5/4 (44.4)	6/1 (14.3)	5/2 (28.6)
Hoehn & Yahr stage	2.3 (0.8; 2.0–3.0)		2.0 (0.6; 2.0–2.5)		2.0 (0.7; 1.0–2.0)		3.5 (1.4, 1.5–4.0)	
Disease duration, years	6.7 (5.2; 2–9)		7.1 (4.9; 4–9)		2.1 (1.8; 1–2)		3.0 (1.6; 1–4)	
UPDRS III	29.2 (12.4; 19.5–37.5)		26.3 (9.3; 19.5–33.5)		16.5 (2.3; 15.3–19)		30.8 (16.3; 15.5–42.3)	
MoCA	24.4 (5.0; 20–29)		25.2 (4.1; 24–29)		27.4 (2.2; 26–30)		22.5 (5.3; 18.8–27)	

Data is presented as mean (standard deviation; 25-75th quantile), while the median is reported for Hoehn & Yahr stage. PD = Parkinson's disease, AMC = age-matched controls, NPH = idiopathic Normal Pressure Hydrocephalus. UPDRS = Unified Parkinson's Disease Rating Scale. MoCA = Montreal Cognitive Assessment.

3.3. Classification of PD and AMC/NPH patients by support vector machines

In order to classify PD and AMC/NPH patients, a non-linear SVM model was trained on the CSF abundance of the same six elements (As, Se, Fe, Mg, Sr, Ni) that were quantified in our recent discovery cohort (36 PD and 42 AMC, as described in (Maass et al., 2018)). The characteristics of the discovery cohort can be found in the supplement (Supplementary Table S4 and S5). This predefined model was used to classify PD and AMC or NPH patients of a new local cohort (C1, same center as in the discovery cohort) and three external cohorts (C2-C4).

Using the predefined SVM model, we were able to classify PD and AMC patients of the new local validation cohort (cohort 1, Fig. 2A) with an AUROC of 0.76 (Sensitivity 0.80, Specificity 0.83 at Youden index)

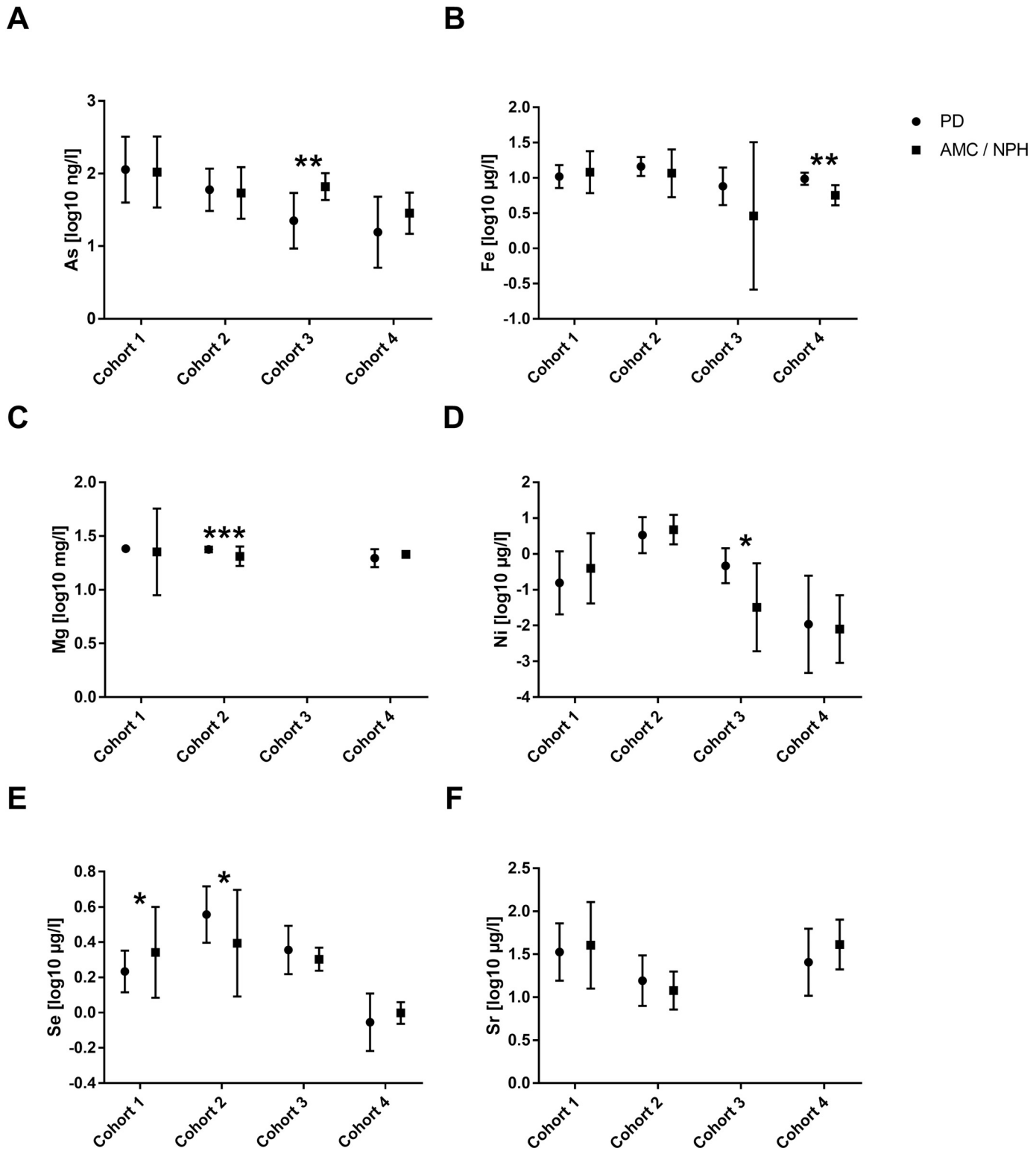

Fig. 1. Bioelement levels presented as log10 transformation ± SD. Two-sided *t*-test was applied for comparison within each cohort. PD = Parkinson's disease, AMC = age-matched controls, NPH = Normal Pressure Hydrocephalus. Mg and Sr levels were not quantified in cohort 3.

purely based on the abundance of those six elements without taking any additional features into account. Adding age and CSF albumin concentration, increased the accuracy further (AUROC 0.79, Sensitivity 0.83, Specificity 0.83 and AUROC 0.82, Sensitivity 0.77, Specificity 0.86 at Youden index, respectively; Fig. 2B and C). This model, however, was not able to discriminate the patients of cohort 2 (clinical matched, external cohort, AUROC 0.57, Fig. 2D) and cohort 3 (external cohort with different characteristics, AUROC 0.52, Fig. 2E). A discrimination of PD and NPH patients in cohort 4 was similarly not feasible using this model (AUROC 0.51, Fig. 2F).

3.4. Evaluation of new center specific models using SVM

The inability of the SVM trained on the discovery cohort to correctly classify patients from cohorts 2 to 4 could be either due to a general lack of information in the elemental data set or derived by a particular center bias. If the six elements were to lack the classifying information, SVM applied to each cohort independently would not be able to separate PD from AMC/NPH patients. On the other hand, there could exist a specific center bias, which would require a center-specific training cohort for each single center. To answer this question, we applied SVM to all four cohorts (C1-C4), which - for this purpose - were each defined as new centre-specific discovery cohorts. Using a 10 times 10-fold cross validation approach (C1, C2) or a leave-one-out cross validation (C3, C4), the SVM algorithm was indeed able to discriminate PD and AMC patients in cohort 1 and 2 (Fig. 3A and B, AUROC 0.76 and 0.78), and PD and NPH patients in cohort 4 (Fig. 3D, AUROC 0.88). The algorithm failed, however, to build a functional model for cohort 3 (Fig. 3C, AUROC 0.31), where only four (Fe, Se, As, Ni) of the initially six determined elements could be quantified due to limited sample volume.

3.5. Correlation with the clinical data

To determine if elemental abundance correlates with clinical parameters (age, H&Y stage, UPDRS part III, disease duration, MoCA, LED), spearman correlation analysis was applied. PD patients of all cohorts were pooled for this analysis. After Bonferroni correction (adjusted alpha ≤ 0.0014), there were significant correlations between Mg and

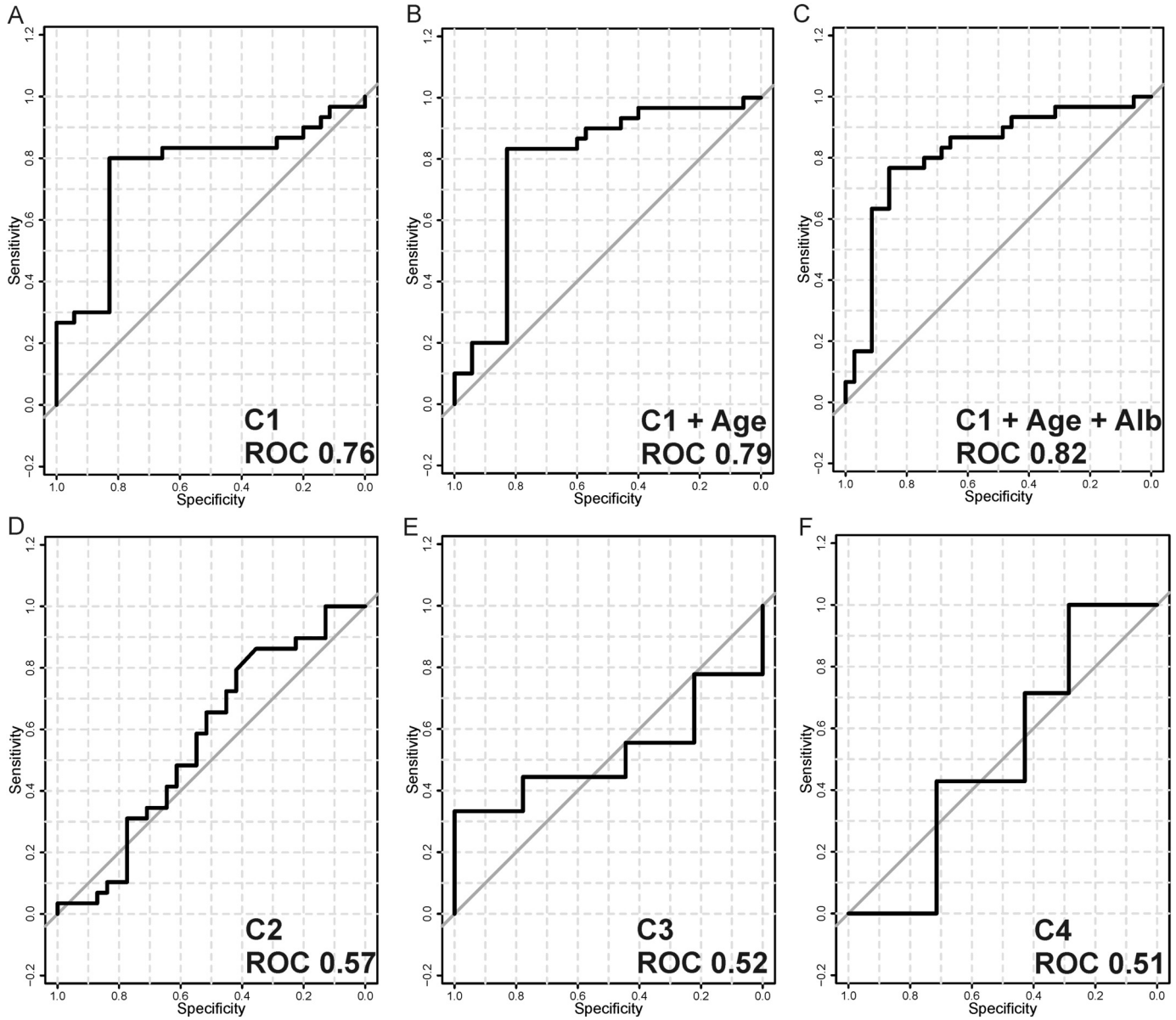

Fig. 2. Classification PD vs. AMC/NPH. SVM was trained on the As, Se, Fe, Mg, Ni, Sr abundance of PD and AMC patients from an exploratory cohort. The model was applied to the validation dataset (C1-C4).

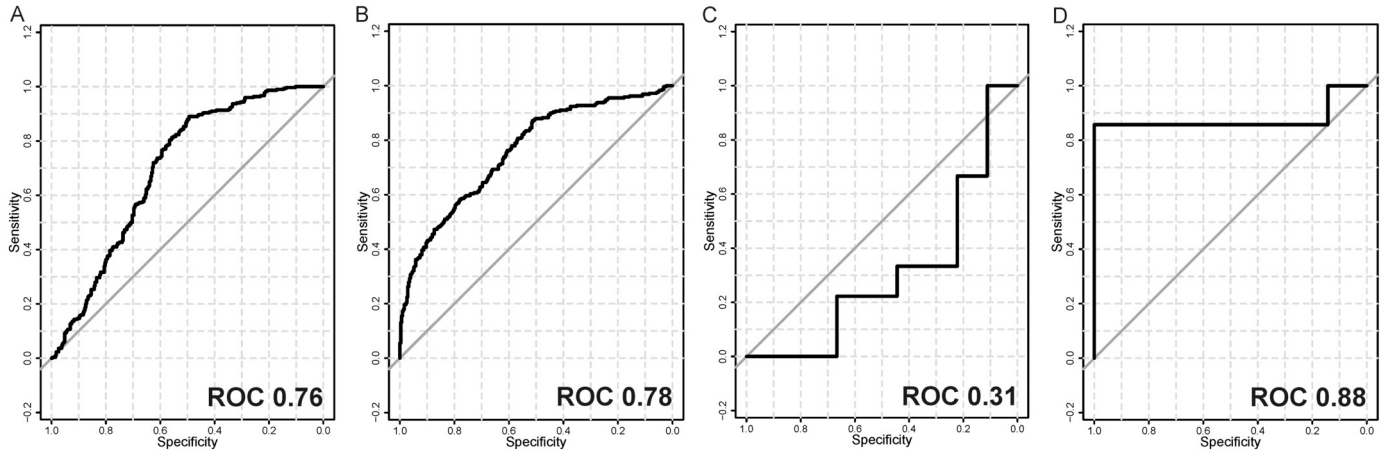

Fig. 3. SVM performance, ROC curves. Using 10 times, 10-fold CV (C1, C2) or leave-one-out CV (C3, C4), SVM was applied on the As, Se, Fe, Mg, Ni, Sr abundance of PD and AMC (C1-C3) or PD and NPH (C4).

the LED (p = .0013, Supplementary Fig. S2) and more importantly between iron and disease duration (p = .0001, Fig. 4). Multiple linear regression was applied to control this relationship for age, still showing a significant relationship (p = .0018).

3.6. Environmental influences on bioelemental CSF concentrations: soil and groundwater concentrations

Bioelements are taken up in the brain and CSF by various mechanisms and partially depend on their environmental supply. Therefore, we investigated whether levels of these six elements in soil or groundwater correlated with the levels detected in our CSF samples.

Environmental concentrations of As, Fe, Mg, Ni, Se, and Sr in top soil and ground water for the geographical regions of the four cohorts (within 50 km range), were provided by the Federal Institute for Geosciences and Natural Resources, Hannover, Germany (Supplementary Fig. S3).

Although the concentrations of some elements showed substantial variations between the different locations, particularly in groundwater (e.g. Fe, Ni, Sr), we could not find any significant correlation between the median soil and groundwater elemental concentrations and the corresponding mean CSF levels (p > .05 in each case; correlation matrix in Supplementary Table S6).

4. Discussion

Here we present the first multicentric trial on the accuracy of an elemental signature in CSF to differentiate patients with PD and control subjects. In contrast to previously published, mainly monocentric exploratory analyses without further validation (e.g. trials on CSF iron levels (Alimonti et al., 2007; Forte et al., 2004; Gazzaniga et al., 1992; Hozumi et al., 2011; Jiménez-Jiménez et al., 1998; Pall et al., 1987; Sanyal et al., 2016; Willkommen et al., 2018), our data relies on CSF samples from four independent cohorts, including patients with different disease durations and NPH as a frequently encountered PD mimic. Employing an SVM model, we were able to validate the elemental profile in the local part of the validation cohort but not in the external cohorts. However, applying independent center specific SVM models for the differentiation of PD and controls demonstrated discriminative value of the elemental profile in two out of three external cohorts.

Bioelements have many advantages as compared to other biomarker molecules, e.g. proteins, RNA or metabolites: chemical elements as such are not affected from degradation and they remain stable at low and moderate temperature (only at critically elevated temperature volatile elements such as iodine, mercury may be affected: this does not apply to herein investigated elements), pH or sampling-analysis delay (Bornhorst et al., 2005; Tevis et al., 2018; Wiberg et al., 2001). Moreover, a number of individual elements have been attributed particular roles in the pathogenesis of PD (e.g. iron, copper, manganese) (Davies et al., 2014; Dexter et al., 1989; Mortimer et al., 2012).

To obtain an elemental fingerprint by a combination of six pre-defined elements, we used the Support Vector Machines (SVM) algorithm: it was previously successfully used for discrimination of patients with PD and Multiple System Atrophy based on proteomics data (Mattison et al., 2012), it was efficient in multicenter settings (Lindemer et al., 2018) and it is able to tolerate high noise levels in training data (Kumar et al., 2011).

Applying SVM to the local validation cohort (C1) demonstrates the plausibility of the proposed model with an AUROC of 0.76 for the discrimination of PD and AMC, purely based on the abundance of As, Fe, Mg, Ni, Se and Sr. Although we observed a significant decrease in Se levels in the PD group, this was not the case in other cohorts (C2-C4). This is consistent with our hypothesis that the abundance of individual elements alone lacks predictive power, because multiple exogenous and endogenous factors influence their abundance and result in fluctuating levels depending on the context (Jiménez-Jiménez et al., 2014; Mariani et al., 2013). The combination of elements, however, may provide a more robust signature considering their interactions and thus is more suited as a biomarker.

Adding additional characteristics to the model (age and CSF albumin) resulted in a moderate increase in discriminative power, resulting in an AUROC of 0.82 for the local cohort. Both parameters were included based on their potential availability in the clinical routine, so they could be easily integrated into the model in a translational approach. Whereas age is a well-known independent risk factor for PD

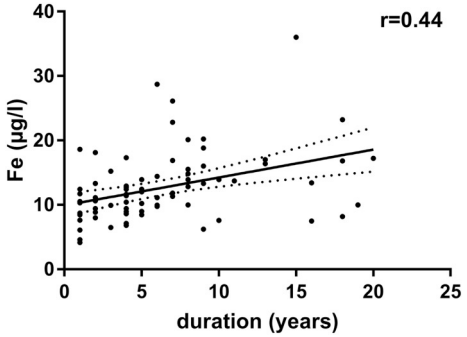

Fig. 4. Correlation of CSF iron levels and PD disease duration. PD patients of cohort 1–4 were pooled for analysis (Spearman correlation, r = 0.44, p = .0001, Bonferroni adjusted alpha level ≤ 0.0014).

(Hindle, 2010), increased CSF albumin levels reflect blood-brain-barrier dysfunction, which has been observed in different neurodegenerative disorders including PD (Sweeney et al., 2018).

When applied to the external validation cohorts (C2-C4), the SVM model trained on the original discovery cohort did not correctly discriminate PD from AMC (C2, C3) or NPH (C4). However, when the validation cohorts C2-C4 were defined as new isolated discovery cohorts and SVM was again trained and tested on each of these single cohorts (10 times, 10-k CV/LOOCV), the algorithm successfully was able to discriminate the different patient groups in C2 and C4 (AUROCs of 0.78 and 0.88, respectively), also showing the ability to discriminate PD and NPH. Because of the small sample size in C4, this finding remains uncertain and has to be evaluated in future studies. The algorithm failed, however, to build a functional model for cohort C3 (AUROC 0.31). In this cohort, only four out of six suggested elements could be determined due to the paucity of CSF volume suggesting that a minimum of six differentiating elements is required for sufficient differentiation, as suggested by the feature selection and step-wise removal of the least informative elements-procedure in the initial discovery cohort (Maass et al., 2018).

The successful establishment of a center specific functional model based on the data from validation cohorts C2 and C4, but not from the original discovery cohort argues in favor of a substantial center bias, which is also suggested by center-dominated clustering in the PCA. Such center biases have been described for different biomarkers and can be attributed to 1) differences in pre-analytical or analytical factors, 2) differences in patient characteristics or medication and 3) differences in environmental factors and diet (Lewczuk et al., 2018; Mattsson et al., 2010; Mollenhauer et al., 2015).

Even though sample collection was done according to standardized biomarker protocols (Teunissen et al., 2009), center differences in sample processing, collected CSF fractions and their corresponding ventricular/lumbar gradient, minor blood contaminations with subsequent release of serum derived elements, different equipment (with unknown potential of element interaction) and in particular the clinical heterogeneity in health status of the control groups might influence elemental levels. Standardized biomarker protocols applying more rigorously standards compared to protein research might be needed to reduce these influential factors. As all mass spectrometry analyses were performed simultaneously at one analytical facility, including internal and external standards, we can largely exclude that the analysis method contributes to the observed variability.

Regarding clinical characteristics, we did not observe significant differences in age, disease duration, Hoehn & Yahr stage, UPDRS III- or MoCA scores between the local validation cohort C1 and the clinical matched cohort C2, which together represent 85% of all analyzed patients in this study. The only significant correlation between the levodopa equivalent dose (LED) and elemental abundance was observed for magnesium, but the clinical relevance of this finding remains elusive (Supplementary Fig. S2). Interestingly, we found a significant correlation between iron levels and disease duration, even if controlled for age. Progressive iron deposition has been described in the substantia nigra of PD patients (Dexter et al., 1989) and our analysis shows for the first time, to the best of our knowledge, correlation of iron CSF levels with disease duration. Non-Parkinsonian factors like concomitant diseases or general drug intake might affect CSF levels by influencing blood-brain-CSF equilibria, but larger cohort sizes would be needed to robustly analyze these diverse factors.

Environmental factors are suspected to account for the main part of center variability and have been previously shown to correlate with clinical parameters. For example, a recent epidemiological study linked Se, Sr and Mg concentrations in top soil to PD mortality rates (Sun, 2018). These and other elements that are present in our environment (e.g. in drinking water, food, soil, dust) can be incorporated and affect element levels in different tissues (also see www.atsdr.cdc.gov). Uptake, storage and release of numerous elements (e.g. iron or manganese) is tightly regulated in the CNS (Bowman et al., 2011; Mills et al., 2010; Nischwitz et al., 2008). Selenium and iron, reflecting two important factors in our model, are rather robust to changes in serum levels e.g. by dietary habits due to the regulation of their intake via the blood-brain-barrier. Selenium shows total CSF levels which are largely independent of its serum levels and this is also true for the most important selenoprotein SEPP1 (Solovyev et al., 2013). Similarly, brain iron levels are not easily altered with diet, particularly in adults (in contrast to the critical time point of weaning (Hare et al., 2013)). Concentrations of other elements, however, largely depend on their environmental abundance and nutritional supply (e.g., magnesium or arsenic) (Morris, 1992; Nischwitz et al., 2008; Yang et al., 2012). For example, arsenic in the form of arsenate resembles nutrient phosphate and is taken up by phosphate transport systems (Yang et al., 2012). Thus, dietary habits might confound with elemental levels but cannot explain group differences on their own. We obtained elemental concentrations from soil and ground water for the geographical regions of the four cohorts (within 50 km range), which did not demonstrate a significant correlation with the respective CSF levels. Most likely, the number of centers and patients in our study did not yield sufficient power to detect environmental influences (Sun, 2018). Furthermore, intake by air and food are also potentially influencing factors, which were not considered. It is also likely that a substantial number of patients do not live in the 50 km-radius of the centers, due to referral from more distant areas. Additionally, individual relocation-, travel and exposure history might confound our data.

In summary, this first multicenter validation trial on CSF bioelements and corresponding signatures in PD adds new evidence to our proposed predictive biomarker model. While this model currently cannot be used as an individual biomarker in clinical routine, its successful validation at the original center and the establishment of individual signatures for another two independent sites supports the overall hypothesis that bioelements have the potential to differentiate disease cohorts. Further analysis is needed to get a deeper understanding of the factors which contribute to the center bias. More stringent preanalytical standards adapted to the analysis of elements could further reduce confounding factors. Prospective inclusion of PD mimics will yield important information on the specificity of our model towards Parkinson's disease.

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Authors' contributions

Design / conceptualization or execution of the study: F.M., B.Mi., D.W., A.L., C.S., L.T., B.Mo, C.T., D.R., P.L., I.Z., M.B., M.Bö. / Design and execution of the biostatistical analysis: F.M., A.L., P.L. / Drafting the manuscript: F.M., P.L. / Revising the manuscript: F.M., B.Mi., D.W., A.L., C.S., L.T., B.Mo, D.R., C.T., M.Bö, M.Bä, I.Z., P.L. All authors read and approved the final manuscript.

Declaration of Competing Interest

None of the authors have anything to disclose in regard to this manuscript.

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Appendix A. Supplementary data

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