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Species fractionation in a case-control study concerning Parkinson´s disease: Cu-amino acids discriminate CSF of PD from controls

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ABSTRACT

Background: Parkinsoń´s disease is affecting about 1% of the population above 65 years. Improvements in medicine support prolonged lifetime which increases the total concentration of humans affected by the disease. It is suggested that occupational and environmental exposure to metals like iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) can influence the risk for Parkinson´s disease. These metals play a key role as cofactors in many enzymes and proteins.

Methods: In this case-control study, we investigated the Mn-, Fe-, Cu- and Zn-species in cerebrospinal fluid (CSF) by size-exclusion chromatography hyphenated to inductively coupled plasma mass spectrometry (SEC-ICP-MS) and the total concentration of these metals by inductively coupled plasma sector field mass spectrometry (ICP-sf-MS).

Results: The investigation of total metal concentration and speciation provided only minor changes, but it produced strong significance for a number of ratios. The analysis revealed a strong change in the ratio between total concentration of Fe and the amino acid-fraction of Cu. This could be observed when analyzing both the respective element concentrations of the fraction (which also depends on individual variation of the total element concentration) as well as when being expressed as percentage of total concentration (normalization) which more clearly shows changes of distribution pattern independent of individual variation of total element concentrations.

Conclusion: Speciation analysis, therefore, is a powerful technique to investigate changes in a case-control study where ratios of different species play an important role.

1. Introduction

Parkinsońs disease (PD) is the second most common neurodegenerative disease und was first described in 1817 by James Parkinson [1]. PD is age-dependent and affects about 0.6% of people between 65–69 years, but 3,5% between 85 and 89 years [2]. PD is a slow progressive movement disorder, with four main symptoms: resting tremor, bradykinesia, rigidity and postural instability [3]. As cause for PD a multifactorial etiology is accepted, although the underlying mechanism is incompletely understood. In literature environmental and occupational factors, i.e. exposure to metals [4–7], pesticides and fungicides [8,9], ge-

Abbreviations: AA, amino acid; DRC, dynamic reaction cell; Cit, citrate; GLM, general linear model; HMM, high molecular mass; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; ID, inner diameter; IOS, inorganic species; LMM, low molecular mass; PD, Parkinsońs disease; PEEK, polyether ether ketone; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, retention time; SCX, strong cation exchange chromatography; SEC, size exclusion chromatography; sf, sector field; SOD, superoxide dismutase; UV, ultraviolet.

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netic factors, i.e. mutations of PARK-genes [10,11], aging, i.e. depletion of antioxidants [12,13] and biological factors, i.e. aggregation and misfolding of proteins [14,15] are discussed as factors involved in PD-progression.

Most likely PD-progression relates to genetic susceptibility combined with other factors since about 90–95% of PD-cases are sporadic and 5–10% have a familial background [16]. All factors mentioned above show connections to the metals in brain. Notably, metals play an ambivalent role in pathogenetic concepts: on the one hand, organic metal-compounds can have substantial toxic effects. On the other hand, metal-chelates contribute to detoxification and antioxidant protective actions, e.g. the Cu-Zn-superoxide dismutase (SOD) [17,18].

Especially the transition metals Fe, Cu, Mn and Zn are in focus. There is substantial evidence that these redox-active metals are mediators of oxidative stress in neurodegeneration [19]. Notably, copper- and iron-containing metallo-enzymes contribute to altered redox-balance. Sayre et al. outline the important role of trace redox-active transition metals in the neuropathology of disorders such as PD [19].

Genetic mutations and exposure to metals accelerate α-Synuclein formation and significantly high levels of Fe(III) have been found in Lewy bodies [20]. Abnormal copper homeostasis is considered as one risk factor for developing PD [12,15].

On a biochemical level, the toxicity of the redox active metals Fe, Cu and Mn is due to their ability to form reactive oxygen species (ROS) via Fenton reaction [21]. The resulting hydroxyl radical is highly reactive and can lead to lipid peroxidation, being connected to increased oxidative stress. Oxidative stress plays a key role in the pathogenesis of PD. Additionally Fe can form complexes with neuromelanin which again induces oxidative stress and a depletion of dopaminergic neurons [22,23]. The ability to form ROS is dependent on the oxidation state of the metal. The oxidation states Fe(II) and Fe(III) are bioavailable in humans. While Fe(III) has nearly no toxicity, Fe(II) can be highly toxic by inducing oxidative stress. A differentiation of these oxidation states is possible by using a strong cation exchange chromatography (SCX) and ICP-MS for detection [24]. Several studies are dealing with redox-speciation in neurodegeneration [25–27]

For these reasons a strict homeostasis of metals is mandatory for proper brain function. Any misbalance regarding the metals or metal ratios can finally lead to oxidative stress, cell death and PD.

The quantification of the metal content in brain of living individuals is impossible. Cerebrospinal fluid (CSF) is much easier available and in close contact to the brain. CSF is well-established in human based neurobiological studies related to the metal exposure [28]. CSF directly contacts extracellular space of brain parenchyma [29], so depletion of elements or change of element-species in the brain is likely to be reflected in this media [30]. Investigations regarding metal content in CSF are present in the literature, but show partly contrary results. In other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), speciation analysis helped to explain controversial findings between molecular biology and epidemiologic studies [31–33]. Such additional information provides deeper insights and shows changes in disease. A comprehensive literature search gained no previous speciation studies in CSF of Parkinson-patients.

This paper first will present the results of a species characterization in a PD-case-control study. The species of Fe, Cu, Mn and Zn were measured by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry (SEC-ICP-MS). Additionally, the total metal-concentration was determined by inductively coupled plasma sector field mass spectrometry (ICP-sf-MS). Finally, element-species-ratios were calculated and compared between the two investigated groups. Several species-ratios were shifted and could have the potential to significantly distinguish PD-cases from controls.

2. Methods

2.1. Study participants

A total of 134 CSF samples taken by standardized lumbar puncture at Cologne University Hospital have been analyzed. Out of these samples, 33 were taken from PD-patients (age ± SD: 65.1 ± 12.9 years; sex: 10 female/23 male; disease-duration ± SD: 1.39 ± 3.7 years) and 101 samples were neurological healthy controls (age ± SD: 44.8 ± 17.3 years; sex: 63 female/38 male). After lumbar puncture samples remained at room temperature up to 6 h for routine diagnostics. Subsequently, samples were stored at −20 °C temporary and later at −80 °C until measurement. The erythrocytes number was semi-quantitatively determined in a counting chamber (negative = no erythrocytes, isolated < 5erythrocytes/μL, + < 90erythrocytes/μL, ++ > 90erythrocytes/μL, +++ > 350erythrocytes/μL, plentiful = overlying erythrocyte layers). One sample had high erythrocyte contamination (+++), but was not excluded, because measured values were within the range of all samples. The study was approved by the Ethics Committee of the University Cologne (09.12.2014, no. 14–364). All patients consented to the scientific use of their CSF samples.

2.2. Chemicals

Tris (Carl Roth, Karlsruhe, Germany), MeOH (Merck, Darmstadt, Germany), elemental standards (Perkin Elmer, Rodgau-Jügesheim, Germany), NH_4Ac, human serum albumin (99%), bovine-γ-globulin, bovine-apo-transferrin (98%), dichloromanganese tetrahydrat (99,99%), citric acid, reduced and oxidized glutathione, arginase, ferritin, β-lactoglobulin (each from Sigma Aldrich, Steinheim, Germany).

2.3. Species characterization by SEC-ICP-MS

The species characterization of Fe, Mn, Cu and Zn was realized by coupling a High Performance Liquid Chromatography (HPLC)-system (Knauer 1100 Smartline inert Series, Berlin, Germany) to an ICP-MS-system (NexIon 300D, Perkin Elmer, Rodgau-Jügesheim, Germany). The HPLC-system was equipped with two columns to ensure the separation of high and low molecular mass (HMM/LMM) compounds. The first column (600 × 10 mm ID) was packed with Toyopearl TSK HW 55S (TosoHaas, Stuttgart, Germany) with a separation range from 1 to 700 kDa and a second PEEK column (250 × 8 mm ID) was packed with Toyopearl TSK HW 40S (TosoHaas, Stuttgart, Germany) for the separation below 2 kDa. The eluent was chosen according to Neth et al. [34]. The effluent of the columns passed a UV-detector (254 nm, 220 nm) and was introduced through a Meinhard nebulizer and a cyclon spray-chamber to ICP-MS. Dynamic Reaction Cell (DRC) mode with NH_3 as reaction gas eliminated isobaric interferences. ICP-MS parameters were RF power 1300 W, plasma gas flow 17 L Ar/min, nebulizer gas flow 0.92 L Ar/min, cell gas flow 0.52 mL NH_3/min, dwell time 1000 ms, monitored isotopes ^{55}Mn, ^{57}Fe, ^{63}Cu, and ^{66}Zn.

Prior to sample measurement, a mass calibration was carried out for both SEC columns. The retention times (RT) of the standards ferritin (440 kDa), γ-globulin (190 kDa), arginase (107 kDa), transferrin (78 kDa), HSA (66 kDa), β-lactoglobulin (37 kDa), oxidized and reduced glutathione (612 Da, 307 Da), citrate (192 Da), inorganic Mn and Fe (55 Da, 56 Da) were recorded and RTs were correlated to respective molecular masses. For HMM-compounds the calibration equation $\log(MW) = -0.0009RT^3 + 0.0977RT^2 - 3.5058RT + 46.998$

($R^2 = 0.9942$) and for LMM-species $\log(MW) = -0.014RT + 3.0747$ ($R^2 = 0.9592$).

The SEC-separation has a low resolution but only limited non-desired interactions between the separated species and the column-matrix. Therefore, it is suitable for the separation of labile Mn-species, e.g. Mn-transferrin. The hyphenation to ICP-MS, operating with NH_3 in DRC mode, allows for the on-line measurement of metals in the separated size fractions with spectral interferences on measures isotopes minimized. The measurements were done in the frame of our continuously running Mn-speciation approaches including successful recovery and accuracy measurements, as reported already in [34,35].

2.4. Determination of total concentration by ICP-sf-MS

The total concentration of the metals Fe, Mn, Cu and Zn was determined by ICP-sf-MS (Element2, Thermo Fisher Scientific, Germany). The CSF-samples were diluted 1:10 or 1:20 in Milli-Q water (18.2 mΩ cm; Milli-Q Purification System). A 4-point-calibration was carried out with a multi-elemental standard to quantify concentrations. The standard composed of 0, 250, 500 and 1000 ng/L of each metal. Rhodium (Rh) was continuously introduced as internal standard at 1 µg/L (final concentration). The instrumental settings are RF power 1170 W, plasma gas flow 16 L Ar/min, nebulizer gas flow 0.99 L Ar/min auxiliary gas 0.65 L Ar/min, monitored isotopes ^{55}Mn, ^{56} Fe, ^{63} Cu, ^{66}Zn, and ^{103}Rh in medium resolution.

2.5. Statistical analysis

Previous to the statistical analysis the variables "total metal concentration" and "concentration of fractions" were $\lg(x + 1)$ transformed in order to reach the normality of the errors and to reduce the skewed distributions. A general linear model (GLM) was applied to the transformed dataset. In the GLM the least square means were calculated to find differences between controls and Parkinson-patients. A significance level $p < .05$ was considered to be statistically significant (* $p < .05$, ** $p < .01$, *** $p < .001$). The evaluation was done in SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). The visualization of the box plot was done in RStudio (Version 1.0.136 – © 2009–2016 RStudio, Inc.), package ggplot2 [36].

3. Results and discussion

3.1. Total concentration of metals does not differentiate between case/control

Metals are suggested to play a role in the development of PD. Hence, the total concentrations of metals in CSF are reported in literature. Table 1 lists our results compared to literature data. Absolute concentrations varied considerably on an individual base but no significant changes were detected. Differences to previously reported concentrations were detected; however, trends were contradictory between different references. Bocca et al. have found a significantly decreased Fe-concentration (control, 45 µg/L; PD, 28 µg/L) [37] and Hozumi et al. significantly increased Cu- (control, 10.2 µg/L; PD, 18.8 µg/L), Mn- (control, 1.9 µg/L; PD, 3.3 µg/L) and Zn-concentrations (control, 5.3 µg/L; PD, 14.5 µg/L) [38].

Table 1 shows that the total concentrations from this study are in agreement with literature values but tend to be at the lower end of published ranges, especially for Mn. The difference can be explained by individual variation depending on e.g. origin or diet. The Fe-concentration, being insignificantly higher in cases, opposes to the results obtained by Bocca et al. but is in agreement with the results of Hozumi et al. We also found elevated levels in cases for Cu and Zn corresponding again with Hozumi et al. Notably, the differences in metal concentrations were insignificant between controls and PD cases from our study as demonstrated by respective *p*-values. The high inter-individual variation of total element concentrations in both groups – being also reported in literature – may be a reason for the missed differentiation. Therefore, total element concentration is unsuitable to differentiate between case and control.

Age was insufficiently matched within the sample groups. Therefore, we checked for age-dependence by means of linear regression, but neither for all participants nor in a single group a linear relationship between age and concentration existed (exemplarily for Fe in Supporting Information). Our result agrees with Hozumi et al. where no correlation with increasing age was detected, although there was an age-difference of about 20 years between cases and controls [38].

3.2. Species characterization in CSF gives no clear differentiation between case/control

Previous investigations on Mn-dependent PD showed changes in speciation [34]. Therefore a speciation approach seemed to be promising, too, since differentiation based on total element concentrations appeared to be unsuccessful. A comprehensive literature search revealed no results for speciation or species characterization of Fe, Mn, Cu and Zn in CSF in PD. But the speciation analysis turned out to be helpful in the analysis of neurodegenerative diseases like Manganism [35] or ALS [31]. Four different mass-characterized species-fractions were separated as shown in Fig. 1 for Mn (characteristic chromatograms of Cu, Fe and Zn in Supporting Information): 350 – 30 kDa ("HMM",), 0.3 – 0.2 kDa ("Citrate", (Cit)), 0.2 – 0.1 kDa ("Amino acids", (AA)) and < 0.1 kDa ("Inorganic species", (IOS)). It should be noted that these fractions consist of several, unspecified molecules and compounds having similar masses.

The evaluation of the chromatograms was carried out by PeakFit™ for identification of species-fractions and calculation of peak-areas. Cu is mainly bound to HMM-species (60–70 %) and is not available as inorganic species. This is in accordance with Nischwitz et al. [39] who found Cu to be mainly bound to HMM in CSF of healthy controls. About 35% of Cu in CSF is bound to ceruloplasmin according to the study of Capo et al. [40]. Ceruloplasmin is an antioxidant which is able to bind up to 6 Cu-ions. It was determined in CSF of control- and Parkinson-samples with concentrations of 1566 ± 157 µg/L for controls

Table 1
Comparison of literature-values for determination of Fe, Cu, Mn and Zn in CSF and measured concentrations of the elements in CSF of Parkinson-patients and controls [29,30,51–55].

element	Concentration controls ± SEM [this paper]	Concentration PD-patients ± SEM [this paper]	p-values [this paper]	Concentration controls-literature [29,30,51–55]	Concentration PD-patients-literature [29,30,51]
Fe	18.84 ± 0.05 µg/L	24.93 ± 0.01 µg/L	0.4340	16–275.9 µg/L	28–263.9 µg/L
Mn	0.50 ± 0.05 µg/L	0.45 ± 0.01 µg/L	1.0000	0.8–5.7 µg/L	0.69–5.4 µg/L
Cu	38.57 ± 0.05 µg/L	43.18 ± 0.01 µg/L	0.9913	10–87.5 µg/L	18.8–67.7 µg/L
Zn	15.80 ± 0.05 µg/L	18.98 ± 0.01 µg/L	0.8914	5–120 µg/L	14.5–30 µg/L

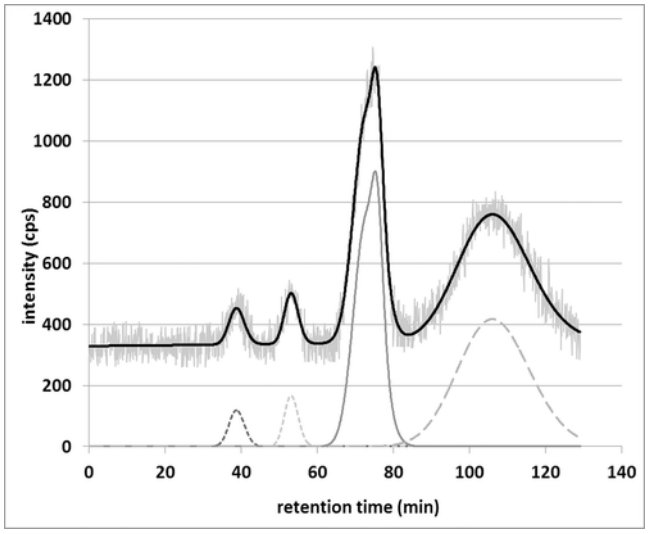

Fig. 1. Observed SEC-ICP-MS-chromatogram (, with graphical off-set) of Mn in CSF with fractions aligned (, aligned chromatogram, with graphical off-set) by use of PeakFit™ software. Species detected are „HMM" (·····), „Citrate" (·····), „Amino acids" (——) and „Inorganic species" (– – ·).

and 1915 ± 223 µg/L in Parkinson-patients [41]. In contrast to Cu, the elements Mn, Fe and Zn mainly exist as inorganic species, especially Fe with more than 90%. These three metals are bound to HMM-compounds only in a minor part. They are mainly bound to HMM-compounds in serum and have a controlled transport into CSF. The scrutiny for LMM-species is less distinct and therefore transport into CSF is almost without control [42]. Ways of transportation for the mentioned metals are reviewed by Yokel [43]. The essential metal Mn was mainly bound to LMM-compounds, especially to citrate [39,44,45], and can cross neural barriers with slight control [44,45]. Our results showed the inorganic fraction rather than the citrate-fraction to be the predominantly species available in CSF. With NMR-investigations of CSF standing at room temperature (RT) Levine et al. showed degradation or transformation of citrate up to 50% after 72 h [46]. Since the samples from this study were standing at room temperature during clinical investigation up to six hours before being stored at −20 °C, in analogy to Levine et al., partial degradation of Mn-citrate to inorganic Mn was not completely excluded. This might explain our finding.

The inter-individual variation of total element concentrations could hide intra-individual species shifts or ratios, which might have been caused by PD-progression. Therefore, we additionally calculated the species percentages-distribution by normalizing to total element concentration (= 100%) of each individual sample, so changes in the distribution of species pattern will be reflected more clearly.

A comparison between case and control can shed light on changes in chemical species-distribution of the elements in CSF and therefore on mechanisms affected through disease. The partial least square means of fractions were calculated after log(x + 1)-transformation. Differences were calculated after Scheffe adjustment. The statistical analysis showed no significant differentiation for any fraction between case and control, however *trends* were actually observed. Such insignificant changes (PD compared to controls) were: 1.) Shift from LMM-Cu-species – mainly Cu-amino acid fraction- toward HMM-Cu-species ($p = 0.12$), 2.) Increase of IOS-Zn ($p = 0.18$) and 3.) Increase of IOS-Fe ($p = 0.16$). No trends were monitored for Mn species fractions.

It is known from the literature that element-ratios, and even more specifically species-ratios, have to be regulated and balanced for an intact brain function and lowering risk for oxidative stress [27,47]. Small, though insignificant changes in species fractions, however, could result in significant changes of species ratios when one species concentration in the enumerator tends to increase, while another species concentration in denominator tends to decrease. In spite of being not significant changes as such, these changes of different element fractions tended into opposite directions, could change the important element species-ratios considerably. Therefore, such ratios were calculated and checked whether significant changes occurred between controls and PD-cases.

The age-dependence was again checked by linear regression and in parallel to the total concentration of metals, the species characterization showed no linear relationship.

3.3. Ratios of different fractions give hints about changes in the brain status

Several ratios of different element fractions or of fractions vs. the total concentration provide highly significant differentiation between controls and PD. Ten significant ratios were found which are listed in Table 2.

Notably, each ratio from Table 2 includes small Cu-fractions in the denominator, either general LMM-Cu-fraction, but mostly specific Cu-AA-fraction. These changes show all the same trend, i.e. increasing in PD. When applying this calculation to the actual metal concentrations (as µg/L) in fractions also 10 ratios appear to be significant (presented in Supporting Information). Here, too, the AA-fraction is involved in half of the shifted ratios but aside from Cu the AA-fraction is also associated with Fe and Mn, influencing the balance between different Fe- and Mn-fractions.

The shifted ratios show misbalances between different elements and element-fractions mainly from the redox-active elements Cu, Fe and Mn. The expansive involvement of AA-fraction could play a crucial role in development and progress of PD. A change in AA-metabolism was also found by Neth et al. in a rat model study with induced manganism [34]. However, for deeper insights the exact amino acids have to be determined, the differences among case and control and the mechanisms which are influenced.

The significant ratios were again analyzed with linear regression for an age-dependence among all patients and the control-/case-group. Fig. 2 shows the three most significant ratios, the distribution of age-dots shows no correlation with the determined values.

Our data confirmed earlier findings that total element concentrations of transition metals in CSF have only limited suitability or are even unsuitable for differentiation. To our best knowledge to date there are no publications available comparing element species/fractions in CSF of PD-patients vs. controls. Nischwitz et al. [39] investigated tran-

Table 2
Significant ratios between different fractions or from fractions vs. total concentration of elements, calculated from percentage values of fraction. Arrows show the direction of alteration for PD, p and F values indicate significance ($p < 0.05$ or $F > 2$: significant; total c = total concentration; AA = amino acid fraction; LMM = low molecular mass; IOS = inorganic species fraction; Cit = citrate fraction).

ratio	Alteration (control to PD)	p-value	F-value
total $c_{(Fe)}$ vs. $AA_{(Cu)}$	↑	< 0.001	20.46
total $c_{(Zn)}$ vs. $LMM_{(Cu)}$	↑	0.0018	10.14
$IOS_{(Mn)}$ vs. $AA_{(Cu)}$	↑	0.0048	8.23
$LMM_{(Mn)}$ vs. $AA_{(Cu)}$	↑	0.0088	7.06
total $c_{(Fe)}$ vs. $LMM_{(Cu)}$	↑	0.0139	6.22
$AA_{(Mn)}$ vs. $LMM_{(Cu)}$	↑	0.0176	5.78
$LMM_{(Mn)}$ vs. $LMM_{(Cu)}$	↑	0.0211	5.45
$Cit_{(Zn)}$ vs. $AA_{(Cu)}$	↑	0.0327	4.66
$IOS_{(Zn)}$ vs. $AA_{(Cu)}$	↑	0.0361	4.48
$LMM + IOS_{(Zn)}$ vs. $AA_{(Cu)}$	↑	0.0455	4.08

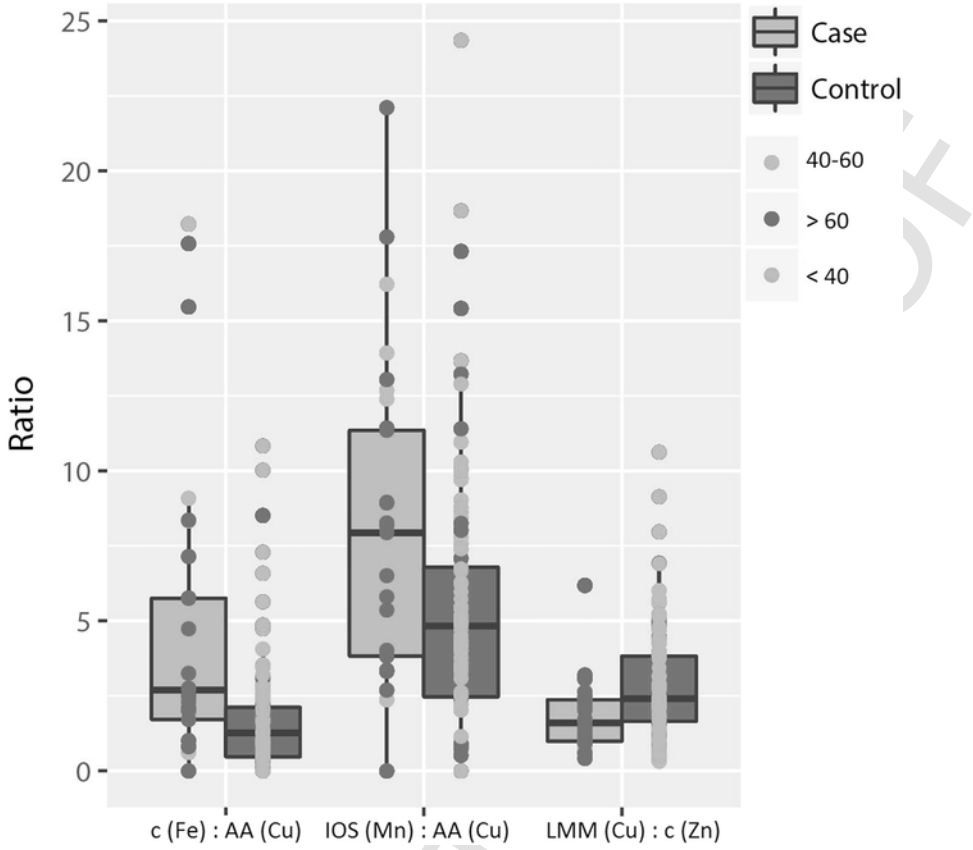

Fig. 2. Box-Whisker-Plot of the three most significant ratios in comparison between case and control and with all gained values colored according to age.

sition metal fractions in CSF from neurological healthy donors but they had no PD–CSF-samples for comparison. Such an extended approach was the first aim of this work. However, the species characterization did not significantly improve differentiation between cases and controls. Discrimination between both groups was even not achieved when inter-individual variations were eliminated by normalization for each sample combined with a subsequent comparison of possible changes in species pattern. However, it appears worth to note, that in brain aside from strict control of species concentration patterns within a single element, a strict regulation of metal-ratios from different elements (e.g. Mn/Fe, Cu/Mn or Zn/Cu) is well established, too [48,49]. Such "inter-metal-ratios", are known to be important for maintaining proper brain function. Any dysregulation could lead to neural damage. In this context, Gaeta and Hider [12] reported that PD is characterized by abnormal protein components, i.e. misfolded α-Synuclein and Lewy bodies, both well known as hallmarks of PD. They state that metals such as Fe and Cu play an important role in protein aggregation and therefore are likely to link protein aggregation and oxidative damage. This is even more likely as both elements (together with Mn) are redox-active metals. Such literature data gave reason in our study to investigate metal-ratios however, exceeding former studies being based only on total metal-ratios, we analyzed species-fraction-ratios for deeper insight into neurodegenerative mechanisms. Importantly, we found twenty different metal-ratios to significantly differentiate between PD and controls. For normalized fractions in each of the significant ratios LMM Cu compounds are involved whereas for ratios based on total concentrations also Mn LMM-compounds are involved. The importance of copper species is supported by Rowinska-Zyrek et al. [50]. They report that the redox activity of copper is an important issue for understanding the molecular basis of PD. Both, Cu^{+}- and Cu^{2+}-α-Synuclein complexes are capable to cause oxidative cell damage via ROS production. Cu^{2+}-α-Synuclein complexes are usually found outside the cell, in plasma, and CSF [50]. Coincidently to our result of Cu-LMM-species tending to lower concentrations in PD, Sayre et al. found decreased copper in CSF, too [19]. Cu^{2+}/Cu^{+}-redox chemistry has been assessed as one of the most important events in the pathophysiology of PD, caused by oxidation of protein backbone, protein fragmentation or by oxidation of amino acid residue side chains. Copper in redox-active form can catalyze hydroxyl radicals in a Fenton-like reaction [19,50,51]. Disturbances in copper homeostasis in brain were described e.g. in Menkes disease and Wilson´s disease [51]. On the other hand, Cu deficiency led to decreased Cu-Zn-SOD, reducing oxidative stress defense [52]. The partial breakdown of the metal-homeostatic mechanisms in PD impact on Cu-dependent cellular signaling [53], reduces protective (Cu-Zn-SOD) mechanisms [52] and/or increases ROS [19,50,51] and finally can lead to copper yielding biphasic changes in neurotransmission [53].

Apart from copper, Mn was involved in metal-fraction-ratios. Mn is described to promote PD-prevalence and can lead to Mn-dependent, PD-like conditions [7,54]. Mn-speciation has been performed by our group in CSF and brains of Mn-exposed rats [34,39,55]. Mn toxicity has been linked to impaired iron homeostasis, specifically shifts in Fe(II)/Fe(III)-ratio [27], changes in neurotransmitter concentrations and formation of ROS/RNS [27,56,57]. From these previous findings the involvement of Mn-LMM species fractions was likely. The well-established competition between manganese and iron can explain that iron species fractions act as antipode to Mn-species fractions. Expectantly, Fe-fraction concentrations tend to increase whereas Mn-fraction concentrations behave oppositely. As copper, Fe(II) can generate reactive hydroxyl radicals via Fenton reaction and modulate oxidative stress [27,53]. Several Zn/Cu-species fractions significantly increased

under PD, too. This might be interrelated with the competition with Cu for the peptide binding sites [53].

The excess influx of zinc into neurons has been found to result in neurotoxicity and damage to postsynaptic neurons [58]. Zinc appears to have an effect on oxidative stress: high and extremely low zinc concentrations are associated with increased oxidative and nitrosative stress, whereas intermediate concentrations were found to be neuroprotective [59].

4. Conclusion

A determination of total concentration of elements and species characterization by SEC-ICP-MS was applied for new insights into ongoing mechanisms in PD and to understand the influence of the metals Cu, Fe, Mn and Zn. The determination of the total concentration gained no significant differences between case and control and hence is unsuitable as marker of the disease due to high inter-individual differences. With the species-characterization by SEC-ICP-MS we found small differences among cases and controls. In PD CSF-samples Fe and Zn showed an increase in the IOS-fraction although the inter-individual variation of element concentration prevented further insight. Therefore, normalization per samples of species fractions as percent of total was calculated to detect changes in species pattern. With normalization, an increase of HMM-species together with a decrease in LMM-species, especially in the AA-fraction, was observed. However, while the observed changes were below significance, a couple of ratios from different element species fractions gained even high significance. Cu is involved in many ratios. If Copper is in the denominator those ratios are increasing significantly in PD. Aside from Cu also Fe and Mn play an important role in misbalanced ratios. The AA-fraction is involved in many ratios and an altered AA-metabolism seems to play a crucial role in the etiology of PD. This study gives deeper insights into the distribution of the mentioned metals to different size-fractions, misbalances and changes ongoing in PD. Further studies are needed to pinpoint specific compounds involved in altered neuro-mechanisms to enlarge knowledge and find possible markers of the disease.

Conflict of interest

Michael Schroeter got personal compensation for talks and advisory boards by Biogen, Sanofi/Genzyme, Grifols, Merck, Miltenyi Biotec, Novartis, Roche.

The other authors declare no conflict of interest.

Author´ś contribution

Conceived and designed experiments: DW, BM, PSK. Performed experiments: DW, BM. Analyzed data: DW, ML, BM, PSK. Contributed to samples and medical data: MG, MS, AS, BM. Wrote paper: DW, ML, PSK, MG, MS, AS, BM.

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[60–64].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jtemb.2018.01.005.

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