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# Diverse Serum Manganese Species affect Brain Metabolites depending on Exposure Conditions

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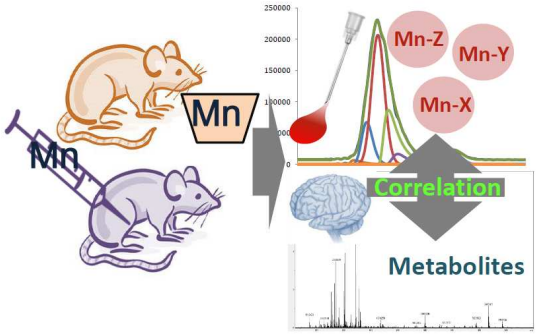

**Abstract**

Occupational and environmental exposure to increased concentrations of Manganese (Mn) can lead to an accumulation of this element in the brain. The consequence is an irreversible damage of dopaminergic neurons leading to a disease called manganism with a clinical presentation similar to the one observed in Parkinson´s Disease. Human as well as animal studies indicate that Mn is mainly bound to low molecular mass (LMM) compounds such as Mn-citrate when crossing neural barriers. The shift towards LMM compounds might already take place in serum due to elevated Mn concentrations in the body. In this study we investigated Mn-species pattern in serum in two different animal models by size exclusion chromatography - inductively coupled plasma mass spectrometry (SEC-ICP-MS). A subchronic feeding of rats with elevated levels of Mn led to an increase in LMM compounds, mainly Mn-citrate and Mn bound to amino acids. In addition, a single *i.v.* injection of Mn showed an increase in Mn-transferrin and Mn bound to amino acids one hour after injection, while species values were more or less rebalanced four days after the injection. Results from Mn-speciation were correlated to the brain metabolome determined by means of electrospray ionization ion cyclotron resonance Fourier transform mass spectrometry (ESI-ICR/FT-MS). The powerful combination of Mn-speciation in serum with metabolomics of the brain underlined the need for Mn-speciation in exposure scenarios instead of determination of whole Mn concentrations in blood. The progress of Mn-induced neuronal inury might therefore be assessed on basis of known serum Mn-species.

## Introduction

Besides the phenotype of idiopathic Parkinson´s Disease (PD) there are several indices for the prevalence of Parkinsonian disturbances induced by the trace element manganese (Mn). Although endogenous Mn concentrations are usually strictly controlled in the body, exposure to elevated levels of Mn can lead to accumulation in the brain, resulting in a disease called manganism. Overall occupationally exposed welders or miners subjected to highly concentrated Mn-dusts were shown to develop Mn-induced Parkinsonism or manganism. Exact numbers of prevalence are not known, while in China a 0.5-2% rate was reported amongst exposed workers.[1] Moreover, also individuals living near such alloy-producing industries are discussed to are at higher risk for developing Mn-induced Parkinsonism.[2] By the use of Mn in methylcyclopentadienyl Mn tricarbonyl as anti-knock agent in gasoline and also in fungicides such as maneb, the general population is likewise subjected to increased environmental concentrations of Mn.[3, 4]

Once there is an onset of manganism, the disease is progressive and irreversible. The early stage is termed "manganese madness" due to emotional lability accompanied by weakness, anorexia, behavioral and psychiatric disturbances as well as attention disorders.[5] Following neurodegenerative mechanisms in manganism share both, physiological and cellular similarities to PD such as the dysfunction of mitochondria.[5, 6] Oxidative stress, dopamine autoxidation as well as impairment of cellular metabolisms count to be the most important Mn-induced mechanisms finally leading to cell death of dopaminergic neurons in the basal ganglia.[7-10] While Levodopa is state of the art treatment in PD, there is commonly no effect in cases of manganism.[11] Chelating therapy was effective in some cases reversing Mn-induced intoxication.[12, 13] However, follow-up studies as well as large controlled clinical trials are still lacking for establishment of efficient treatment strategies.[7] To find such treatment options,

deciphering the versatile mechanisms of Mn-induced neurodegeneration is one major task to prevent onset or progression of this disorder.

A prerequisite for early and reliable detection of the onset of manganism is to have a useful biomarker for the Mn status in exposed humans, which is of high interest to clinical investigators of Mn neurotoxicity and occupational health.[14] Measurement of Mn concentrations in blood (or serum and plasma) is commonly used for determination of the body Mn status. However, there is a relatively large variation in blood Mn among individuals due to diet or environmental influences disqualifying blood Mn for clinical uses.[15] The investigation of a reliable biomarker of Mn exposure is still ongoing and serum would be a preferred matrix due to the easy sampling procedure. Addressing this topic, Michalke and colleagues carried out extensive speciation studies assuming that during or after Mn-exposure mainly low molecular mass (LMM) compounds, presumably Mn-citrate, are formed in the body.[16, 17] As formation of citrate carriers for Mn correlated with Mn in serum from a certain concentration on, the prevalence of LMM compounds in serum accompanied with Mn concentrations above that described point was discussed to be useful for biomonitoring attempts.[15]

These results prompted us to investigate the formation of Mn-species in serum under two different Mn-exposure scenarios in rats (subchronic vs. acute). The results from speciation were then correlated to the brain metabolome determined by means of electrospray ionization ion cyclotron resonance Fourier transform mass spectrometry (ESI-ICR/FT-MS). This powerful combination of metallomics with metabolomics clearly validates the need of considering serum Mn-species more than total Mn values in blood. With the knowledge of Mn-species in serum reliable conclusions about brain inflammatory status might be drawn in clinical diagnosis of the early onset of manganism or Mn-induced Parkinsonism

## Experimental procedure

*Keeping of animals and treatment*

The animal experiments were in accordance with the institutional Animal Welfare Committee as well as the German regulations for experimental animal's treatment, and approved by the Bavarian federal state government under the file number 55.2-1-54-2531-180-12. 12 male Sprague-Dawley rats (RjHan:SD) were purchased from Janvier (Janvier S.A.S., France) directly after weaning at three weeks of age to ensure sustenance by only mother´s milk. The animals were kept in pairs in polycarbonate cages type III under specified pathogen-free conditions at a 12/12 hours light cycle. The cages were embedded by hemp mats to facilitate quantitative collection of feces for analysis, and paper houses were given as environmental enrichment. All animals were supplemented with filtered tap water and fodder ad libitum. For euthanasia, animals were deeply narcotized with 5% isoflurane until total loss of consciousness. After cutting through the *aorta abdominalis* with a ceramic scalpel, blood was collected in Eppendorf tubes and centrifuged subsequently for 10 minutes at 12000xg and 4°C to collect serum, which was stored at -80°C until analysis. Also brains were taken out and immediately shock frozen in liquid nitrogen until sample preparation. Feeding experiment: As explained in [18], the control group (n=6) received standard diet with 23 mg Mn/kg fodder (ssniff EF R/M AIN 93G, Ssniff Spezialdiäten, Soest, Germany) whilst the test group received the same but Mn enriched fodder (500 mg/kg), which is still in the range of recommended feeding levels. Feeding duration was 56 days until sacrifice. Injection experiment: During an adaption time of 2 weeks, animals were fed with the above mentioned standard diet. Then animals were anesthetized with isoflurane for a single i.v. injection in the tail vein of either 100 µl isotonic saline 0.9% (SteriPharm, Berlin, Germany) for control rats or 100 µl of 1.5 mg Mn/kg body weight in isotonic saline for test rats (sterile filtered). One

hour after injection, blood was collected from the *Vena sublingualis* by punctuation under isoflurane anesthesia for analysis. Four days later, animals were sacrificed, as Mn brain concentrations were found to be highest after four days following injection [19].

*Determination of Mn in serum by ICP-sf-MS*

ICP-sf-MS (Element2, Thermo Fisher Scientific, Germany) was carried out to determine Mn concentrations in serum. Serum was thawed at 4°C over night and diluted 1:30 in aqua bidest. Before measurement, a so called peak search was carried out to set the respective mass offset for each isotope for correct evaluation of masses. Calibration and quantification of respective concentrations was carried out by a five point calibration with 0, 100, 250, 500 and 1000 µg/L Mn with a multi elemental standard. Rhodium (Rh) was used as internal standard, which was continuously introduced and mixed with the sample solution before introduction into the spray chamber by the prepFast system (Elemental Scientific, Mainz, Germany), resulting in a final concentration of 1 µg/L in the samples. Plasma conditions were 15 L Ar/min for plasma gas, 1.45 L Ar/min for auxiliary gas and 1.14 L Ar/min for sample gas at a RF power of 1260 W. The measurements were carried out in medium resolution with determination of $^{55}$Mn and $^{103}$Rh isotopes. Accuracy of the instrument was checked by measurement of certified reference material (bovine liver, BCR185, nitric acid digestion, n=5). The measured average value for Mn was 8.8±0.2 mg/kg (certified value: 9.1±0.3 mg/kg), representing a good accuracy.

*ESI-ICR/FT-MS: extraction and measurement parameters*

Brains were roughly homogenized under liquid nitrogen and 50 mg were suspended with 500 µL 50% MeOH. After sonication on ice for 20 minutes, the samples were transferred into a 2 mL glass homogenizer (Fortuna, Neolab, Germany) with 250 µL of 50% MeOH. After

homogenization the extract was transferred back into the eppendorf tube with 250 µL 50% MeOH and again sonicated on ice for 20 minutes. After centrifugation for 30 minutes at 2°C and 18900g, the supernatant was diluted with 70% MeOH (1:10). The extracts were stored at -80°C until measurement with ESI-ICR/FT-MS.

Ultra-high resolution mass spectra of brain extracts were acquired by a Fourier transform ion cyclotron resonance mass spectrometer (ICR/FT-MS, Solarix, Bruker, Bremen, Germany) equipped with a 12-T superconducting magnet (Magnex Scientific, Varian Inc., Oxford, UK) and an electrospray source (ESI, Apollo II; Bruker Daltonics, Bremen, Germany). The applied ICR/FT-MS allows assignment of masses of molecular ions with a mass accuracy of <0.1 ppm, and the respective molecular compositions can be calculated with very high precision.[20] Measurements and external calibration were performed in negative ionization mode with a liquid flow rate of 2 µL/min and a temperature of 180°C. External mass calibration was performed by analyzing an 3 mg/L arginine solution in MeOH with calibration errors below 0.1 ppm (analyzed arginine clusters: [M-H]- m/z 173.10440, 347.21607, 521.32775, 695.43943). The ESI voltage difference between the electrode and the counter electrode was 3500 V. An additional voltage drop of 500 V was maintained between the counter electrode and an inner cone inside the electrospray to further accelerate the ions toward the mass spectrometer. The dry gas flow rate was maintained at 4 L/min and the ESI nebulizer gas flow rate was kept at 2 L/min. The spectra were recorded in a mass-to-charge-ratio (m/z) range of 123-1000 with an ion accumulation time of 300 ms for achieving better sensitivity.

The ICR/FT-MS raw spectra were processed with DataAnalysis Version 4.1 (Bruker Daltonik GmbH, Bremen, Germany). First, they were calibrated internally by using the exact masses of known rat brain metabolites with an error below 0.1 ppm. After the internal calibration,

spectra were exported to peak lists at a signal to noise ratio (S/N) of 4 and an intensity threshold of 0.01%. The spectra were aligned within an error of 1 ppm by an in-house written software. The new data matrix was uploaded to MassTRIX web server in order to perform annotation of masses within an error range of 1 ppm.[21]

*SEC-ICP-MS of serum samples*

For optimum separation of Mn-species in serum a combination of two columns was applied, equipped with a pre-column to retain contaminants (Securityguard™, Phenomenex, USA). The first Peek column body (250x8mm) was packed with Toyopearl HW55SF (TosoHaas, Stuttgart, Germany) for separation of HMM (high molecular mass) compounds, while the second Peek column body (200x8mm) was packed with Toyopearl HW40S (TosoHaas, Stuttgart, Germany) for separation of LMM. This combination provided a satisfactory separation of LMM compounds like Mn-citrate from HMM compounds. The isocratic eluent was composed of 90% Eluent A (50 mM $NH_4Ac$, pH 5.8) mixed with 10% Eluent B (10 mM Tris, 50 mM $NH_4Ac$, 5% (v/v) MeOH, pH 8). Eluent delivery was performed by the metal-free Knauer Smartline HPLC system (Knauer, Berlin, Germany) with an isocratic flow rate of 0.7 ml/min. Before entering the meinhard nebulizer and an in-house-made spray chamber of the ICP-MS, the column effluent passed an UV meter for detection of disulfide bonds, which correspond to proteins. The injection volume was 25 µl. The mass calibration of the columns was carried out by analysis of alpha-2-macroglobulin (725 kDa), ferritin (440 kDa), γ-globulin (190 kDa), arginase (107 kDa), transferrin (78 kDa), human serum albumin (66 kDa), lysozyme (14.4 kDa), oxidized and reduced glutathione (612 Da, 307 Da), cysteine (240 Da), citrate (192 Da), L-glutamic acid (147 Da) and inorganic Mn (55 Da). Due to the use of two columns with two separation ranges, two column mass calibrations (HMM, eluting before 19 minutes and LMM, eluting after 19 minutes) were gained, which are characterized

by the equations (ln MW/RT): HMM: ln(MW)=-0.6209xRT+15.264, $R^2$=0.926; LMM: ln(MW)= -0.1245xRT+1.098, $R^2$=0.963. After each sample, the columns were cleaned by application of 100% eluent B for 10 minutes and then the regular eluent until column equilibration, which was checked by detecting the wash through. Furthermore, columns were cleaned separately overnight by a mixture of 20% 500 mM NaCl and 80% MeOH at 0.1 ml/min in reversed flow to wash off elemental as well as organic or protein contaminants. The ICP-MS (Nexion300D, Perkin Elmer, MA, USA) was run in DRC mode to remove interferences, optimized according to manufacturer´s instrument operation instructions with further parameters: plasma gas 15 L Ar/min; nebulizer gas 0.94 mL Ar/min; cell gas A 0.7 ml $NH_3$/min; RF Power 1250 V; band pass q=0.8; monitored isotope: $^{55}$Mn, $^{103}$Rh. The system was regularly optimized by daily performance checks due to manufacturer´s protocol. Normalization of data by the Rh signal had no advantage. Peaks were integrated and aligned by use of PeakFit™ v4.11 (Systat, Erkrath, Germany). Quality control: Recovery was calculated by standard addition of Mn single standard to serum samples and comparison of peak areas. The average recovery of all samples (serum feeding, serum one hour and serum four days) was 73-127%. LoD for Mn was determined by the 3σ criteria, which was 17 ng/L.

*Manganese standards for SEC calibration*

Stock solutions of 1000 mg/L of HSA, γ-globulin, transferrin, ferritin and citric acid were prepared in eluent A. A stock solution of 5 g/L Mn was prepared of $MnCl_2.4H_2O$ also in eluent A. The final standard solution was a mixture of 4 parts protein/citric acid and 1 part Mn stock solution. It was aliquoted in Eppendorf tubes and kept at room temperature for a week to fulfill complexation. Aliquots were stored at -20°C in the dark and working solutions were freshly prepared before analysis.

*Statistical evaluation*

The data from Mn-speciation was log transformed in order to improve the linear relation between input and output variables and to make the last one approximately normal distributed. The data of the feeding study were then analyzed by a two-way ANOVA with "SEC-fraction" and "diet" as main effects and calculation of least square means to obtain contrasts between the different SEC-fractions. This increased the explained variance to $R^2$=92%. A generalized linear model with repeated measures (GLM) was applied to the data of the injection study (one hour after injection and four days after injection). All the elaboration was done in SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). P-values ≤ 0.05 were considered to be statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). The ICR/FT-MS data were analyzed with different multivariate techniques in order to extrapolate and visualize significant features, related to the different nature of the experimental groups. Orthogonal partial least square discriminant analysis (OPLS-DA) was done on scaled data (Unit Variance) applying a 7-round cross validation. The elaborations were done in SIMCA-P 13.0.3.0 (Umetrics, Umea, Sweden) and the different heatmaps were generated in R version 3.1.1. The OPLS-DA model generated from the metabolome corresponding the acute Mn response revealed $Q^2(cum)=0.6$ and $R^2Y(cum)=0.9$ as values for the goodness for prediction and for the fit, comparing the metabolome of subchronic Mn exposure which had values of $Q^2(cum)=0.9$ and $R^2Y(cum)=0.9$.

## Results and Discussion

*Total Mn concentration in serum does not reflect body Mn status under non-acute situations*

Taking blood samples is the routine method in medical diagnostics to monitor mineral and trace element body levels. Due to the fact that blood is the transport medium for Mn towards

the brain, having a marker in blood, which is indicative of the brain status, would be of great benefit in examination of exposed persons. As the relationship of total Mn concentrations in whole blood to external exposure is poorly understood, investigations have been based on analysis of serum and plasma. In our study, we exposed rats to a subchronic, Mn-elevated feeding, for simulating a low-dose chronic exposure. In contrast, we also applied a single low-dose Mn injection in rats, simulating a single time exposure, as it is for example the case by the use of the Mn-containing contrast agent MnDPDP for magnet resonance imaging.[22] Total Mn concentration in serum of control and Mn-treated rats were measured by ICP-sf-MS. The total concentration of Mn in serum after the subchronic feeding was comparable to the one of control rats (4.24 and 4.40 µg/L, Supplementary Figure S1A). Similar results were found for Mn levels four days after *i.v.* injection of Mn, when rats were sacrificed (6.05 and 6.94 µg/L in control and injected rats, respectively, Supplementary Figure S1C). For control of Mn injection, blood was taken one hour after injection and serum concentrations of Mn were found to be 6.95 µg/L in control rats and 193.25 µg/L in injected rats (Supplementary Figure S1B). Therefore, determination of the total concentration of Mn in serum would only be reliable for Mn body status shortly after direct exposure. These findings are in line with a study by Smith et al., where whole blood as well as plasma and urine Mn concentrations of low, moderate and high exposed workers were measured.[23] After trying to find correlations to air Mn levels, the authors generally summarized that the relationship between Mn exposure and blood Mn levels is highly complex and is depending on exposure properties as well as on blood sampling time. It should be emphasized that all rats in our study were treated in the same way with regard to food and drinking concentrations of Mn. This is not the case for human samples, where individual variations in Mn concentrations in blood exist due to their different nutritional habits, which influences Mn levels in the body. One of these influences is age as Mn concentrations in blood seem to decrease with increasing age.[24] This was also

found to be true herein as the rats of the feeding study were older at the time of sacrifice (aged 74 days) and had lower Mn levels in serum compared to the younger rats in the injection study (aged 38 days). Furthermore, the rapid back-change of Mn in serum as observed in our study was also stated by Zheng et al., who estimated the half-life time of Mn in blood to be 1.8 h after *i.v.* injection of $MnCl_2$.[25] This leads to the fact that determination of total Mn concentration in blood/serum is only indicative of the blood Mn status in a very recent scenario but not in detection of historical exposure to Mn.

*SEC-ICP-MS peak assignment*

SEC-ICP-MS was applied for separation of labile Mn-species in serum. As this chromatographic method is a mild separation technique, no degradation of native Mn-species throughout the analysis was ensured. The comparison of species peaks in serum between Mn substituted rats and control rats allows account about formation of Mn complexes *in vivo* after Mn exposure. Supplementary Figure S2 shows examples of native SEC-chromatograms for serum of the feeding study as well as the injection study (serum one hour after injection and four days after injection, i.e. time point of sacrifice of animals). Chromatograms were evaluated by PeakFit™ software for identification of the different peak fractions according to the molecular weight of serum proteins as well as for determination of retention times and peak areas. The first peak was differentiated into three major HMM fractions, namely 700-350 kDa (fraction A), 350-150 kDa (fraction B) and 100-60 kDa (fraction C). One more LMM fraction assumed for compounds of 150-100 Da (fraction E) could be separated. Although this fraction appears to be really small due to low intensities, the applied software allows accurate alignment of peaks. This is illustrated by the zoom out in Figure S2. According to the retention times of laboratory made Mn standards, fraction A could be assigned to α-2-Macroglobulin (alpha2M), fraction B to γ-Globulin (γ-Glob), fraction C to

transferrin (Tf), fraction D to citrate, fraction E to amino acids (i.e. glutamic acid, cysteine) and fraction F to inorganic Mn. The SEC-fraction assignment is summarized in Table 1. It has to be mentioned that the applied standards are possible binding partners of Mn according to obtained retention times and literature. Of course, other molecules, which were not examined, might also be eluting in the same time frame as SEC only allows setting eluting time frames due to its limited resolution. However, it is still the most suitable separation method for the inherently very labile Mn-species. Important in this study, thus, was to distinguish between high molecular mass compounds (HMM) and low molecular mass compounds (LMM), over all Mn-citrate. These were separated to our convenience by the applied method. Additionally, a further LMM fraction as well as inorganic Mn could accordingly be separated. Serum samples were analyzed by SEC-ICP-MS and respective peak areas were used to calculate Mn concentrations for each SEC-fraction with regard to total Mn concentration in serum.

| | *SEC fraction* | *Size* | *Assignment* |
|---|---|---|---|
| **HMM** | Fraction A | 700-350 kDa | α-2-Macroglobulin |
| | Fraction B | 350-150 kDa | γ-Globulin |
| | Fraction C | 100-60 kDa | Transferrin |
| **LMM** | Fraction D | 280-170 Da | Citrate |
| | Fraction E | 150-100 Da | Amino acids |
| | Fraction F | <60 Da | Inorganic Mn |

**Table 1 Assignment of SEC fractions from SEC-ICP-MS**
According to retention times of Mn standards, SEC peaks were assigned to five possible Mn carriers and inorg. Mn in serum (fraction A-F).

*Formation of serum Mn-species depends on exposure conditions*

A promising approach for investigations in Mn-related exposure scenarios is the speciation of Mn compounds in serum, which has also been carried out to understand transport procedures

of Mn in blood and at or across neural barriers (BBB blood brain barrier, BCB blood cerebrospinal fluid barrier).[26] Mn-transport across BBB seems to be dependent of the oxidation state of Mn, and uptake rates for Mn-citrate were found to be substantially higher compared to Mn(II) or Mn(III)-transferrin.[27] This was also observed by Yokel and Crossgrove [28] as well as by Nischwitz et al., who investigated the permeability of the BCB by means of Mn-speciation.[29] All in all, there are indices for a carrier-mediated uptake of LMM Mn compounds (especially Mn-citrate), which is believed to lead to Mn accumulation in the brain.[30] The results obtained herein for Mn-species in serum might enlarge these different studies on Mn-speciation. For analysis of differences in SEC-fractions, partial least square means were calculated after log transformation and differences were calculated after Tukey-Kramer adjustment. The model also included the interaction effect described by *SEC-fraction x Experiment*, where *SEC-fraction* means fractions A-F and *Experiment* means control or Mn-exposed. Subchronic Mn exposure (feeding study): As shown in the box plot in Figure 1 the main effects came from the LMM fractions D, E (citrate, amino acids) and from the inorganic Mn fraction F. These fractions were also statistically significant compared to control serum fractions (D: $p<0.05$, E: $p<0.001$ and F: $p<0.01$), which was not the case for the HMM SEC-fractions (fractions A, B and C), (corresponding least square means can be found in Supplementary Table S1). The results of the two-way ANOVA of the feeding study are shown in Supplementary Figure S3 and Table S3, which underlined the results from Figure 1. Focusing on the interaction effect it became obvious that again the LMM SEC-fractions as well as the inorganic Mn fraction were significantly different in Mn-fed versus control animals (fraction D: $p=0.0467$, E: $p<0.0001$ and F: $p=0.0027$) while the HMM fractions were not (fraction A: $p=1.0000$, B: $p=0.9999$ and C: $p=0.4848$).

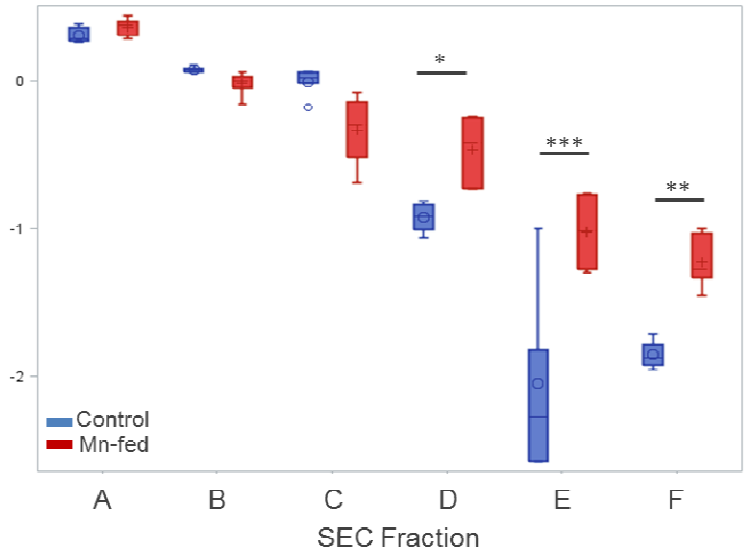


**Figure 1 Least square means comparison of SEC-fractions in the feeding study**
**The interaction analysis revealed that the main effect for formation of Mn-species came from fractions D, E and F (citrate, amino acids and inorganic Mn), which showed significance compared to control samples (fraction D: *p<0.05, fraction E: ***p<0.001, fraction F: **p<0.01). Fractions A-C were not significant and therefore less important for formation of Mn-species in serum after subchronic feeding.**

Acute Mn exposure (injection study): The application of a general linear model with repeated measures revealed an x-fold increase in all serum Mn-species one hour after injection, which was significant compared to control for all serum fractions ($p<0.0001$), as shown in Figure 2A. Four days after the injection, Mn concentration of serum SEC fractions seemed to be balanced except for fractions C and E (transferrin and amino acids), which were still significantly increased (C: $p<0.05$ and E: $p<0.01$) when compared to control samples as shown in Figure 2B. (Corresponding least square means can be found in Supplementary Table S2).

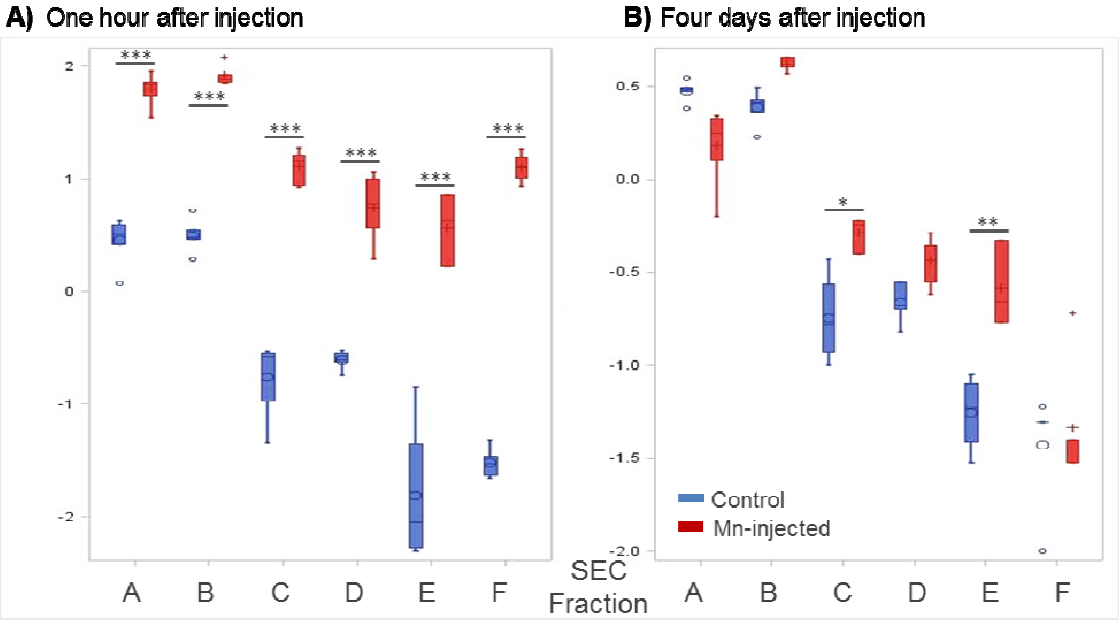


**Figure 2 Least square means comparison of SEC-fractions in the injection study**
**A) One hour after injection: all fractions show significance between serum of control and Mn-injected animals (***p<0.001).**
**B) Four days after injection: while fractions A, B, D and F showed no significance between serum of control and Mn-injected animals, fractions C and E were significant indicating main effects of these fractions (C: *p<0.05, E: **p<0.01).**

Hence, we found the LMM SEC fraction E (amino acids) to play a crucial role for Mn transportation in serum both under subchronic as well as under acute exposure. This fraction was significantly increased in the feeding study as well as one hour and four days after injection in serum of Mn-treated rats as shown in the interaction analysis (Figure 1 and 2). LMM compounds including Mn bound to amino acids were also described by Michalke et al. in CSF. By application of CZE-ICP-MS (capillary zone electrophoresis-ICP-MS) they identified Mn-histidine, Mn-fumarate, Mn-malate, inorganic Mn, Mn-oxalacetate, Mn-alpha-ketoglutarate, Mn-carrying NAD and Mn-adenosine with the far most abundant species being Mn-citrate.[31] For Mn-citrate we herein found some interesting observations by comparison of the different Mn exposure times. This fraction was significantly increased during subchronic Mn exposure, i.e. in the feeding study (Figure 1), as well as one hour after injection (Figure

2A). However, one hour after injection, all SEC fractions were increased significantly due to the high Mn concentrations in serum after *i.v.* injection. Interestingly, four days after injection, there was no significant difference in the citrate fraction between control and Mn-exposed serum samples (Figure 2B). The formation of Mn-citrate in serum seems to be predominant only after a certain time or occurring Mn exposure, but not due to a short intervention. The other way around might be the case regarding Mn-transferrin: this fraction showed no significant interaction effect due to Mn-feeding (Figure 1). However, one hour after Mn-injection transferrin showed a strong significant increase and still was significantly increased four days after injection when compared to control (Figure 2). Hence, it seems as if transferrin is a first response for transportation of Mn after acute exposure[30] and passes this task to other serum compounds after a prolonged exposure, where other transporters like citrate become more important. Regarding the HMM fractions A and B, no significant differences between control and Mn-exposed groups were observed during subchronic feeding or four days after injection of Mn. Those results diminish the importance of these Mn-compounds in understanding the formation and distribution of Mn-species in serum during the respective Mn exposure. Furthermore, the inorganic Mn fraction was – aside from one hour after injection – only significantly increased due to feeding of Mn (Figure 1). Therefore, only a prolonged impact of Mn on the body might show increased inorganic Mn levels in serum in Mn-speciation.

It is clear that Mn can promote neurodegenerative mechanisms and that the transport across neural barriers is influenced by the Mn-species in serum as different compounds are able to bind Mn and transport it to and into the brain. Though the majority of Mn in serum in exposed rats was still bound to HMM compounds (approximately 90% in all three exposure situations), in summary, we observed a decrease in HMM compounds and an increase in

LMM compounds (Figure 3). This increase was the strongest under subchronic oral exposure to Mn (3-fold compared to control) and weakest one hour after Mn injection. Consequently, after a certain time of Mn-exposure it might come to a translocation of Mn towards LMM compounds in serum and transportation of Mn in this form also to or across neural barriers might be possible.

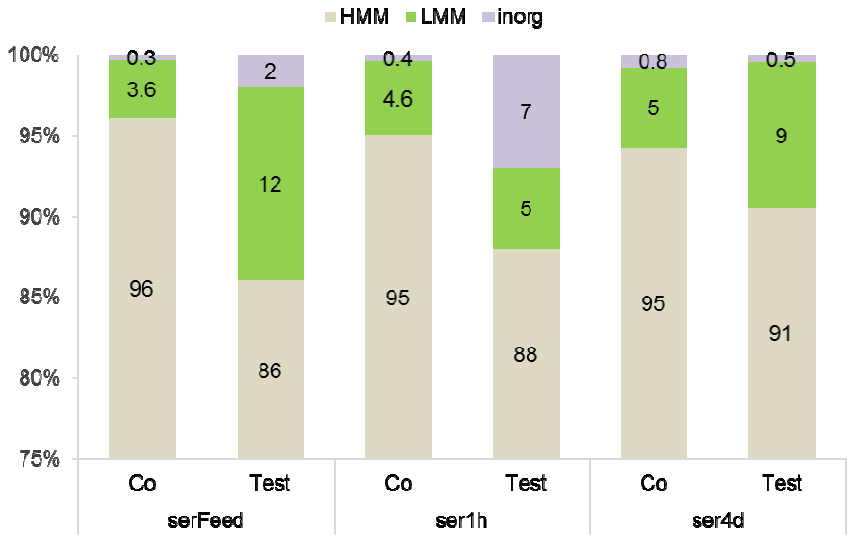


**Figure 3 Percentage of Mn bound to HMM, LMM and inorganic Mn in serum**
The majority of Mn was bound to HMM in serum in all three exposure scenarios (serumFeed: feeding, ser1h: one hour after injection, ser4d: four days after injection). A decrease in HMM and an increase in LMM was observed, which was the strongest due to chronic feeding of Mn.

*Correlation of serum Mn-species with brain metabolites gives information about brain status*

Due to the occurring need for reliable measurement parameters of Mn body status, which is not solely based on total Mn concentrations in serum, correlation of different biological variables becomes of great research interest. Most studies contain correlation of total Mn concentrations in blood or tissue with Mn in air or with various biological parameters.[32] For example, Montes et al. correlated blood Mn and prolactin concentrations in blood in Mexican subjects living near a mine and mineral processing plant. They observed an increase in serum

prolactin levels with increase in blood Mn levels as a sign of disturbed dopamine synthesis in brain.[24] Nevertheless, the authors clearly stated that Mn levels in blood should be taken carefully, because blood Mn and actual concentrations in tissues seem not to be concordant. As a new promising approach, we herein correlated serum Mn-species as well as total Mn concentrations in serum and brain with most important brain metabolites determined by ESI-ICR/FT-MS (experimental procedure and results for determination of brain Mn concentrations is shown in Supplementary Information and Figure S4). These most important metabolites were gained by OPLS-DA according to VIP values (variable in projection) above 1.5. Analysis of metabolome data from the feeding study revealed 553 important masses with 292 being responsible for control and 261 masses being responsible for Mn-fed group discrimination (Figure 4A). In the injection study, 1377 masses, with 527 for control and 850 for treated rats, were found as most important for the one and other group (Figure 5A). The results from correlation analysis are displayed in correlation heatmaps, where a strong green color means strong positive correlation and a strong red color means strong negative correlation between variable (SEC-fraction or total Mn concentration) and brain metabolite.

Based on the correlation heatmap of the feeding study (Figure 4B), total concentration of Mn in serum was merely positively and even partially negatively correlated with those brain metabolites, which were defining the Mn-fed group according to VIP>1.5. On the opposite, total Mn in brain as well as LMM compounds in serum, like SEC-fraction E (amino acids), F (inorganic Mn) and above all D (citrate), showed a strong positive correlation to the important masses of the treated group. On the other hand, the HMM fraction A showed only partial positive correlation while fraction B and C were obtained as strong negatively correlated with the brain metabolites of the treated group. In the figure, the zoom out of the important metabolites defining the treated group displays correlations of four important

metabolites: PGH2, 15(S)-HETE, PGB1 and GSSG. As published in Fernsebner et al.[18], these metabolites were increased in brain due to Mn-feeding and can be seen as markers of oxidative stress or lipid peroxidation and are therefore indicating inflammation in brain tissue. We found these markers to be strongly positively correlated with total Mn concentrations in brain as well as with serum SEC-fractions D and F (Mn-citrate and inorganic Mn).

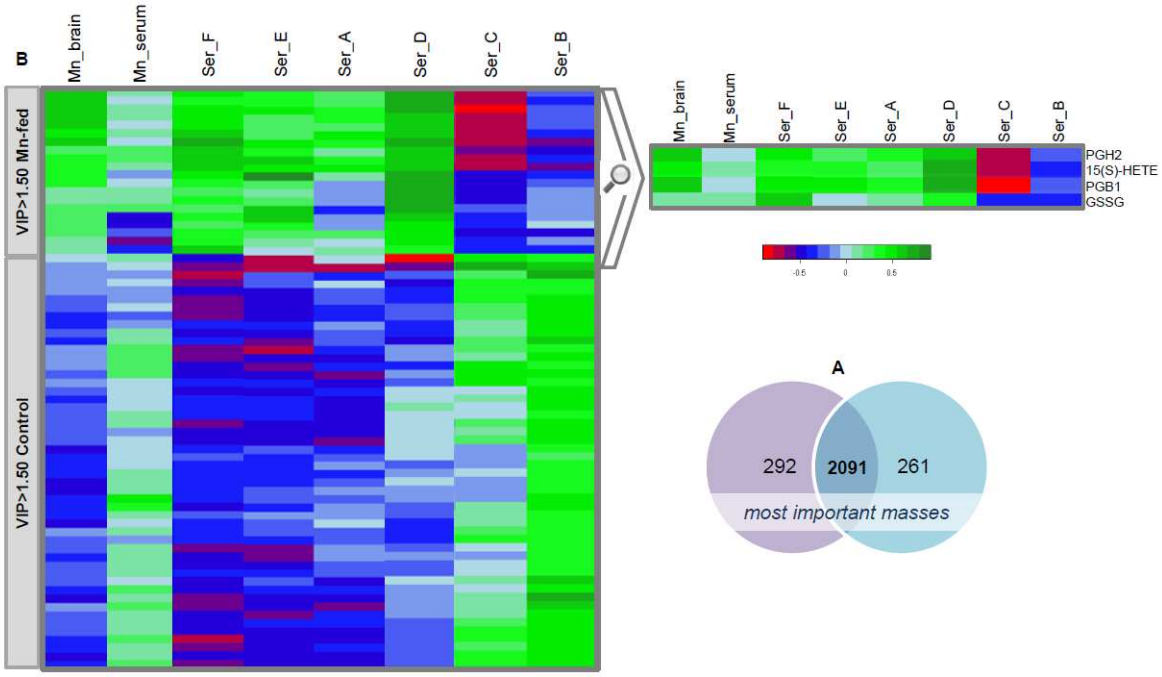


**Figure 4 ESI-ICR/FT-MS data and correlation heatmap of the feeding study**

**A Venn Diagram of most important masses:** Based on the OPLS-DA of ESI- ICR/FT-MS data all masses with VIP>1.50 (variables important in projection) which defined one or the other group (control vs. Mn-fed) were termed as most important masses (total 2091 masses). In sum, 292 masses were defining the control group and 261 masses defining the Mn-fed group.

**B Correlation heatmap of the feeding study:** Total Mn concentrations in brain and serum as well as serum SEC-fractions (A-F) were correlated with most important known metabolites of control and Mn-fed group (VIP>1.50, grey charts). Dark green color means strong positive correlation and dark red color means strong negative correlation. Total Mn concentrations in brain as well as serum LMM SEC fractions D, E and F (citrate, amino acids and inorganic Mn) showed strong positive correlation with the Mn-fed group describing metabolites. The opposite was case for serum HMM fractions B, C and partly A as well as for total Mn concentration in serum. The zoom out represents the correlation with four major metabolites defining the Mn-fed group, which are markers of oxidative stress in the brain tissue as published in Ref. 18. Total Mn concentration in serum was not correlated with those markers whereas overall serum SEC fraction D (citrate) was strongly positive correlated.

The correlation heatmap of the injection study (Figure 5B) showed that total Mn concentration in serum was not correlated with the test group defining metabolites. In comparison, total Mn concentrations in brain as well as serum SEC fractions B and D (γ-globulin, citrate) were positively correlated with these brain metabolites. Fractions C (transferrin) and E (amino acids) showed similar correlation behavior, which was only partially positive with the metabolites of the Mn-injected group. Interestingly, fraction F (inorganic Mn) here was only positively correlated with some important metabolites of the injected group while HMM fraction A (α-2-Macroglobulin) was found to be strongly negatively correlated with the metabolites defining the Mn-injected group. Among the brain metabolites of Mn-treated rats we also found PGB1 and 15(S)-HETE to be increased in the brain of the treated rats compared to control - as observed in the feeding study. Additionally, Cer(d38:2) and ResolvinD2 were found to be unique in the Mn-injected rat brains as shown in Figure 5C. Ceramides such as Cer(d38:2) belong to the class of sphingolipids and were reported to serve as second messenger in modulating enzymes of the sphingolipid metabolism, which regulate apoptosis, cellular senescence and also inflammatory reactions.[33] ResolvinD2 is formed during sequential oxygenation of docosahexaenoic acid as anti-inflammatory response to inflammation of brain tissue.[34] As the zoom out of the heatmap in Figure 5B shows, total Mn concentrations in brain as well as SEC serum fractions B, D and E (γ-globulin, citrate and amino acids) showed a strong positive correlation with these metabolites indicative of neural inflammation. However, total Mn concentrations in serum as well as SEC fraction F (inorganic Mn) were only correlated with ResolvinD2 but not with the other mentioned metabolites. Therefore, under acute exposure, Mn-citrate, Mn bound to amino acids but also HMM compounds like transferrin seemed to be correlated to changes in brain metabolites.

This type of correlation method seemed very powerful for making a clear statement about the importance of knowledge of Mn-species in serum in clarification of the brain neurological status due to Mn exposure. The most important outcome of this way of correlation study was the clear demonstration that total Mn concentration in serum was obviously not correlated with the important brain metabolites of Mn-exposed animals (Figure 4B, Figure 5B). This is again an evidence for total Mn concentration in serum being diagnostically less conclusive for determination of brain status under Mn exposure.

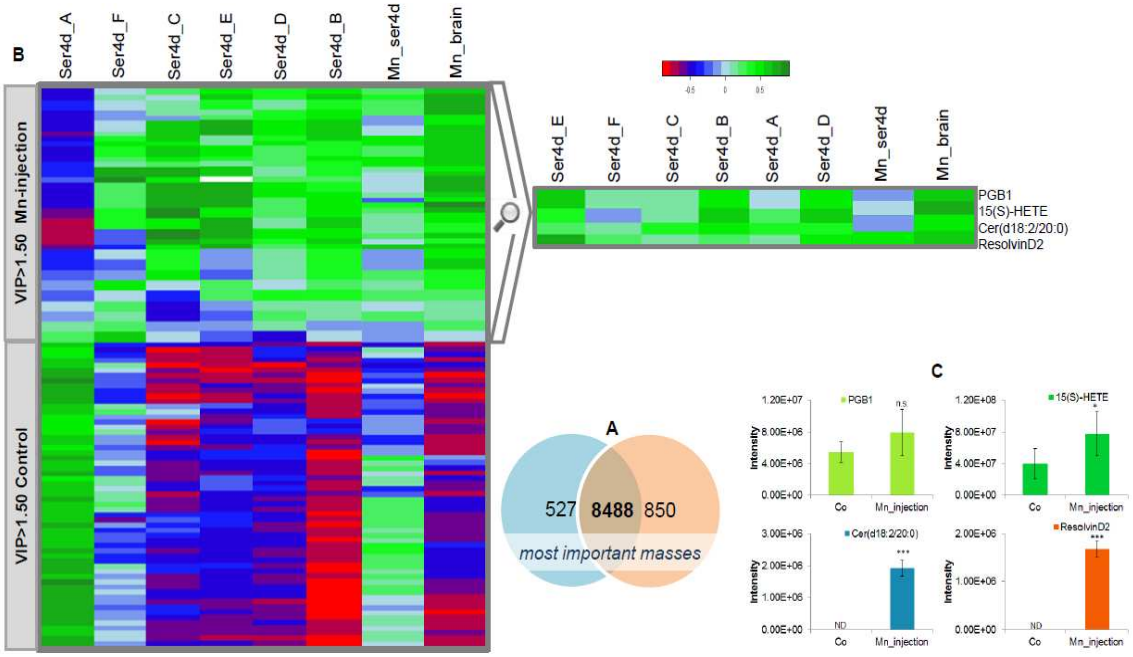


**Figure 5 ESI-ICR/FT-MS data and correlation heatmap of the injection study**

**A Venn Diagram of most important masses:** Based on the OPLS-DA of ESI-ICR/FT-MS data all masses with VIP>1.50 (variables important in projection) which defined one or the other group (control vs. Mn-injected) were termed as most important masses. In sum, 527 masses were defining the control group and 850 masses defining the Mn-fed group (in total 8488 masses detected).

**B Correlation heatmap of the injection study:** Mn concentrations in brain and serum as well as serum SEC-fractions (4 days after injection) were correlated with most important known metabolites of control and Mn-injected group (VIP>1.50, grey charts). Dark green color means strong positive correlation and dark red color means strong negative correlation. Total Mn concentration in brain as well as SEC fractions B and D (γ-globulin and citrate) showed strong positive with correlation with almost all brain metabolites defining the exposed group. SEC fractions C and E (Tf and amino acids) how similar correlation , which was mainly positive with the brain metabolites of the treated group. Total Mn concentration in serum was merely correlated with these metabolites. The zoom out also represents the correlation with four major metabolites defining the Mn-injected group, which are prominent markers of oxidative stress in the brain tissue (**C**). These were mainly positively correlated with SEC fractions B, D and E (γ-globulin, citrate, amino acids).

**C Markers of oxidative stress and neuroinflammation:** PGB1, 15(S)-HETE, Cer(d38:2) and ResolvinD2 were found to be increased in Mn-exposed rat brains as sign of oxidative stress and neural inflammation. *p<0.05, ***p<0.001 (unique in Mn-injected rats), n.s.: not significant, ND: not detected

Gaining knowledge about LMM species like Mn-citrate in serum might therefore be indicative of the brain inflammatory status. This could be of great concern in determination of body Mn status for example in exposed persons. The importance of Mn-speciation was also stated by Michalke et al.[17], where analysis of paired human serum and CSF samples revealed a "switching concentration" of total Mn in serum. Above that concentration, Mn in serum and CSF were correlated with Mn-citrate in serum. With focus on the results herein, the knowledge of both, Mn-concentration and exposure conditions is important to understand Mn-species formation in serum. By knowledge of the serum Mn-species, in turn, the degree of progression of Mn towards brain accumulation or even inflammation might be assessed as shown by our correlation study.

## Conclusion

This study presents a powerful approach combining Mn-speciation in serum (SEC-ICP-MS) with analysis of brain metabolites (ESI-ICR/FT-MS) in two different Mn exposure scenarios with respect to Mn transport mechanisms in serum related to conditions in the brain. First, it became clear that total Mn concentration in serum does not reflect the body Mn status under non-acute situations and is therefore not suitable for medical diagnosis of exposed persons. Secondly, formation of Mn-species in serum seems to depend on time of exposure, where Mn-transferrin plays an important role for transportation under acute exposure and Mn-citrate/-amino acids after a certain time of exposure. Finally, determined serum Mn-species showed different correlation behavior with brain metabolites including markers of neuroinflammation. The results from the combinatory study of metallomics with metabolomics enlarge the speciation study, as certain serum Mn-species might influence the

progression of neuroinflammation in different manner – dependent on exposure conditions. Our findings stress the need for Mn-speciation in investigation of Mn-exposure, when information about the neurological status should be drawn before disease onset. The applied combined approach should therefore be tested in paired human serum/CSF samples of healthy and exposed individuals to find suitable reference data.

**Author Contributions:** K.N., B.M., and P.S.-K. designed the study concept; K.N. performed research; K.N., B.M. evaluated metal-related data; K.N.; P.S.-K., A.W., B.K. analyzed FT/ICR-MS data; M.L. carried out statistical analysis; J.Z. carried out animal experiments. All authors contributed to discussion and have given approval to the final version of the manuscript.

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**Supporting Information** Total concentrations of Mn in serum, examples of SEC chromatograms, tables with least square means, and procedure for determination of Mn concentrations in brain. This information is available free of charge via the Internet at http://pubs.acs.org/.

**Abbreviations:** L/HMM, Low/High Molecular Mass; SEC-ICP-MS, Size Exclusion Chromatography; ICR/FT-MS, Ion Cyclotron Resonance Fourier transform Mass Spectrometry

## References


(1) Gao, G.-x., Wu, Y.-h., Guo, Y.-p., LI, Y., and CANG, E.-z. (2003) Survey on chronic occupational hazards in welders. *Chin. J. Ind. Med. 16*, 107-107.

(2) Lucchini, R. G., Guazzetti, S., Zoni, S., Donna, F., Peter, S., Zacco, A., Salmistraro, M., Bontempi, E., Zimmerman, N. J., and Smith, D. R. (2012) Tremor, olfactory and motor changes in Italian adolescents exposed to historical ferro-manganese emission. *Neurotoxicology 33*, 687-696.

(3) Finkelstein, M. M., and Jerrett, M. (2007) A study of the relationships between Parkinson's disease and markers of traffic-derived and environmental manganese air pollution in two Canadian cities. *Environ. Res. 104*, 420-432.

(4) Ferraz, H. B., Bertolucci, P. H., Pereira, J. S., Lima, J. G., and Andrade, L. A. (1988) Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology 38*, 550-553.

(5) Chen, P., Parmalee, N., and Aschner, M. (2014) Genetic factors and manganese-induced neurotoxicity. *Front. Genet. 5*, 265.

(6) Exil, V., Ping, L., Yu, Y., Chakraborty, S., Caito, S. W., Wells, K. S., Karki, P., Lee, E., and Aschner, M. (2014) Activation of MAPK and FoxO by manganese (Mn) in rat neonatal primary astrocyte cultures. *PLoS One 9*, e94753.

(7) Aschner, M., Erikson, K. M., Hernandez, E. H., and Tjalkens, R. (2009) Manganese and its Role in Parkinson's Disease: From Transport to Neuropathology. *NeuroMol. Med. 11*, 252-266.

(8) Chen, C. J., and Liao, S. L. (2002) Oxidative stress involves in astrocytic alterations induced by manganese. *Exp. Neurol. 175*, 216-225.

(9) HaMai, D., and Bondy, S. C. (2004) Oxidative basis of manganese neurotoxicity. *Ann. N. Y. Acad. Sci. 1012*, 129-141.

(10) Jenner, P. (2003) Oxidative stress in Parkinson's disease. *Ann. Neurol. 53 Suppl 3*, S26-36; discussion S36-28.

(11) Koller, W. C., Lyons, K. E., and Truly, W. (2004) Effect of levodopa treatment for parkinsonism in welders: A double-blind study. *Neurology 62*, 730-733.

(12) Herrero Hernandez, E., Discalzi, G., Dassi, P., Jarre, L., and Pira, E. (2003) Manganese Intoxication: The Cause of an Inexplicable Epileptic Syndrome in a 3 Year Old Child. *Neurotoxicology 24*, 633-639.

(13) Hernandez, E. H., Discalzi, G., Valentini, C., Venturi, F., Chio, A., Carmellino, C., Rossi, L., Sacchetti, A., and Pira, E. (2006) Follow-up of patients affected by manganese-induced Parkinsonism after treatment with CaNa 2 EDTA. *Neurotoxicology 27*, 333-339.

(14) Aschner, M., Guilarte, T. R., Schneider, J. S., and Zheng, W. (2007) Manganese: recent advances in understanding its transport and neurotoxicity. *Toxicol. Appl. Pharmacol. 221*, 131-147.

(15) Michalke, B., and Fernsebner, K. (2014) New insights into manganese toxicity and speciation. *J. Trace Elem. Med. Biol. 28,* 106–116.

(16) Michalke, B., Halbach, S., and Nischwitz, V. (2007) Speciation and toxicological relevance of manganese in humans. *J. Environ. Monit. 9*, 650.

(17) Michalke, B., Lucio, M., Berthele, A., and Kanawati, B. (2013) Manganese speciation in paired serum and CSF samples using SEC-DRC-ICP-MS and CE-ICP-DRC-MS. *Anal. Bioanal. Chem. 405*, 2301-2309.

(18) Fernsebner, K., Zorn, J., Kanawati, B., Walker, A., and Michalke, B. (2014) Manganese leads to an increase in markers of oxidative stress as well as to a shift in the ratio of Fe(II)/(III) in rat brain tissue. *Metallomics 6*, 921-931.

(19) Takeda, A., Sawashita, J., and Okada, S. (1998) Manganese concentration in rat brain: manganese transport from the peripheral tissues. *Neurosci. Lett. 242*, 45-48.

(20) Schmitt-Kopplin, P., Gelencser, A., Dabek-Zlotorzynska, E., Kiss, G., Hertkorn, N., Harir, M., Hong, Y., and Gebefügi, I. (2010) Analysis of the unresolved organic fraction in atmospheric aerosols with ultrahigh-resolution mass spectrometry and nuclear magnetic resonance spectroscopy: Organosulfates as photochemical smog constituents†. *Anal. Chem. 82*, 8017-8026.

(21) Wägele, B., Witting, M., Schmitt-Kopplin, P., Suhre, K., and Provart, N. J. (2012) MassTRIX Reloaded: Combined Analysis and Visualization of Tran-Scriptome and Metabolome Data. *PLoS ONE 7*, e39860.

(22) Crossgrove, J., and Zheng, W. (2004) Manganese toxicity upon overexposure. *NMR Biomed. 17*, 544-553.

(23) Smith, D., Gwiazda, R., Bowler, R., Roels, H., Park, R., Taicher, C., and Lucchini, R. (2007) Biomarkers of Mn exposure in humans. *Am. J. Ind. Med. 50*, 801-811.

(24) Montes, S., Riojas-Rodriguez, H., Sabido-Pedraza, E., and Rios, C. (2008) Biomarkers of manganese exposure in a population living close to a mine and mineral processing plant in Mexico. *Environ. Res. 106*, 89-95.

(25) Zheng, W., Kim, H., and Zhao, Q. (2000) Comparative toxicokinetics of manganese chloride and methylcyclopentadienyl manganese tricarbonyl (MMT) in Sprague-Dawley rats. *Toxicol. Sci. 54*, 295-301.

(26) Bowman, A. B., Kwakye, G. F., Hernandez, E. H., and Aschner, M. (2011) Role of manganese in neurodegenerative diseases. *J. Trace Elem. Med. Biol. 25*, 191-203.

(27) Zheng, W., Aschner, M., and Ghersi-Egea, J. F. (2003) Brain barrier systems: a new frontier in metal neurotoxicological research. *Toxicol. Appl. Pharmacol. 192*, 1-11.

(28) Yokel, R. A., and Crossgrove, J. S. (2004) Manganese toxicokinetics at the blood-brain barrier. *Res. Rep. Health Eff. Inst.*, 7-58; discussion 59-73.

(29) Nischwitz, V., Berthele, A., and Michalke, B. (2008) Speciation analysis of selected metals and determination of their total contents in paired serum and cerebrospinal fluid samples: An approach to investigate the permeability of the human blood-cerebrospinal fluid-barrier. *Anal. Chim. Acta 627*, 258-269.

(30) Diederich, J., Brielmeier, M., Schwerdtle, T., and Michalke, B. (2012) Manganese and iron species in Sprague-Dawley rats exposed with MnCl2 center dot 4H(2)O (i.v.). *Microchem. J. 105*, 115-123.

(31) Michalke, B., Berthele, A., Mistriotis, P., Ochsenkuhn-Petropoulou, M., and Halbach, S. (2007) Manganese speciation in human cerebrospinal fluid using CZE coupled to inductively coupled plasma MS. *Electrophoresis 28*, 1380-1386.

(32) Hoet, P., Vanmarcke, E., Geens, T., Deumer, G., Haufroid, V., and Roels, H. A. (2012) Manganese in plasma: A promising biomarker of exposure to Mn in welders. A pilot study. *Toxicol. Lett. 213*, 69-74.

(33) Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A., and Obeid, L. M. (1995) Role of ceramide in cellular senescence. *J. Biol. Chem. 270*, 30701-30708.

(34) Farooqui, A. A., Horrocks, L. A., and Farooqui, T. (2007) Modulation of inflammation in brain: a matter of fat. *J. Neurochem. 101*, 577-599.