**An approach for manganese biomonitoring using a manganese carrier switch in serum from transferrin to citrate at slightly elevated manganese concentration**

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**Abbreviations:**

CSF = cerebrospinal fluid

CE = capillary electrophoresis

CNS = central nervous system

ESI-FT-ICR-MS = electrospray ionization Fourier transform ion cyclotron resonance

 mass spectrometry

ICP-DRC-MS = inductively coupled plasma–dynamic reaction cell–mass spectrometry

ICP-OES = inductively coupled plasma optical emission spectrometry

LMM = low molecular mass

LoD Limit of Detection

Milli-Q-water ultra pure water with resistivity (typically 18.2 MΩ·cm at 25 °C).

MMT = methyl-cyclopentadienyl-manganese-tricarbonyl

Mn = manganese

Mn(C) = total Mn concentration in cerebrospinal fluid

Mn-Cit(S) = Mn-citrate in serum

Mn(S) = total Mn in serum

Mn-Tf(S) = Mn-transferrin concentration in serum

NB = neural barrier

PD = Parkinson disease

SEC = size exclusion chromatography

UF = ultrafiltration

**Abstract**

After high-dose-short-term exposure (usually from occupational exposure) and even more under low-dose long term exposure (mainly environmental) manganese (Mn) biomonitoring is still problematic since these exposure scenarios are not necessarily reflected by a significant increase of total Mn in blood or serum. Usually, Mn concentrations of exposed and unexposed persons overlap and individual differentiation is often not possible. In this paper Mn speciation on a large sample size (n=180) was used in order to be able to differentiate between highly Mn-exposed or low or unexposed individuals at low total Mn concentration in serum (Mn(S)). The whole sample set consisted of three subsets from Munich, Emilia Romagna region in Italy and from Sweden. It turned out that also at low total Mn(S) concentrations a change in major Mn carriers in serum takes place from Mn-transferrin (Mn-Tf(S)) towards Mn- (Mn-Cit(S)) with high statistical significance (p < 0.000002). This carrier switch from Mn-Tf(S) to Mn-Cit(S) was observed between Mn(S) concentrations of 1.5 µg/L to ca. 1.7 µg/L. Parallel to this carrier change, for sample donors from Munich where serum and cerebrospinal fluid were available, the concentration of Mn beyond neural barriers - analysed as Mn in cerebrospinal fluid (Mn(C)) - positively correlates to MnCit (S) when Mn(S) concentration was above 1.7 µg/L. The correlation between Mn-Cit(S) and Mn(C) reflects the facilitated Mn transport through neural barrier by means of Mn-citrate. Regional differences in switch points from Mn-Tf(S) to Mn-Cit(S) were observed for the three sample subsets. It is currently unknown whether these differences are due to differences in location, occupation, health status or other aspects. Based on our results, Mn-Cit(S) determination was considered as a potential means for estimating the Mn load in brain and CSF, i.e. it could be used as a biomarker for Mn beyond neural barrier. For a simpler Mn-Cit(S) determination than size exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS), ultrafiltration (UF) of serum samples was tested for suitability, the latter possibly being a preferred choice for routine occupational medicine laboratories. Our results revealed that UF could be an alternative if methodical prerequisites and limitations are carefully considered. These prerequisites were determined to be a thorough cleaning procedure at a minimum Mn(S) concentration > 1.5 µg/L, as at lower concentrations a wide scattering of the measured concentrations in comparison to the standardized SEC-ICP-MS results were observed.

1. **Introduction**

Manganese (Mn) is an essential element for humans. It has been widely reported in literature that in general its concentration is low. However after increased exposure at elevated concentration it causes detrimental health effects. Following (excessive) exposure Mn can accumulate in basal ganglia which may cause severe neurological disorders, one of the worst being manganism, a disease with symptoms similar but different to Parkinson´s disease (PD) (1-3). However, neurological effects have been observed at exposures considerably lower than those in the past being associated with manganism (4-9). A long series of studies with many participants have unravelled the neurotoxic mechanisms of Mn which have been partly summarized in several reviews (e.g. (5,7,10)).

Mn concentrations in plasma and serum, but also in saliva, erythrocytes, urine and hair have been extensively evaluated for use in Mn exposure assessment with only limited success (11,12). Total Mn levels were serving as an indicator of *recent* Mn exposure only on a group comparison basis. This was explained by homeostatic mechanisms regulating levels within a narrow range and precluding a direct relationship between external exposure and levels within the body (12). Another approach was considered in differentiated distribution and speciation of manganese.

In a series of investigations it was previously shown that the distribution of Mn-species differ between serum and cerebrospinal fluid (CSF) (13-17). In serum Mn was found mainly associated to proteins like transferrin and only at minor amounts to small carriers like citrate whereas contrarily in CSF mainly low molecular mass (LMM) Mn-species were found of which Mn-citrate was identified as the most important Mn-species. These findings were in accordance with others who suggested a LMM Mn-carrier into the brain, independent from transferrin (18,19). Further, Yokel et al. (9) and Aschner et al. (7) reported about Mn-citrate to be transported at increased rates across neural barriers (NB) in rats.

Specifically our investigation from 2013 (20) revealed results which possibly could be an appropriate means for Mn biomonitoring: Both, total Mn concentration of serum (Mn(S)) and total Mn concentration in CSF (Mn(C)) showed a very strong positive correlation to the concentration of Mn-transferrin in serum as long as total Mn(S) remained at a physiological, low concentration range, i.e. below ca. 1.6 µg/L. In turn when total Mn(S) exceeds ca. 1.6 µg/L, both, total Mn(S) and Mn(C) disclose a positive linear relationship to Mn-citrate in serum (Mn-Cit(S)). This link between total Mn(C) and Mn-Cit(S) at increased Mn concentration in serum is specifically important since - in contrast to serum - CSF samples usually are not available (and can only be obtained after neurological indication drawn from neurologists). Therefore the above reported correlation between Mn(C) and Mn-Cit(S) at elevated Mn(S) could be used to estimate an increased risk for internal Mn exposure of the brain beyond NB, in this case via Mn in CSF, by analysing Mn-Cit(S). But for these previous experiments only a limited number of samples was available. Consequently a confirmation of our findings from (20) with an increased sample size was desirable before Mn-Cit(S) could be proposed as a biomarker.

The previous experiments were conducted mainly by size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS), with a 2-D identification by CE-ICP-MS and ESI-FT-ICR-MS (20).

However, routine application of SEC-ICP-MS has drawbacks, such as time-consuming column cleaning. This is limiting sample throughput and increasing sample storage time prior analysis with elevated risk of sample degradation. These disadvantages, aside from high operating costs that are caused by prolonged runtimes, make SEC-ICP-MS less applicable for occupational routine laboratories. The use of ultrafiltration units at low cutoff (e.g. a 5 kDa) and subsequent total Mn determination in the filtrate could be a practical alternative where the LMM ultrafiltrate (< 5 kDa) from serum roughly represents the Mn-Cit(S) fraction (16). A mandatory prerequisite however was a rigorous pre-cleaning of the UF-tubes and filters by a seven-step procedure and overall very careful handling to avoid any Mn contamination. Preliminary investigations however revealed low reproducibility and low statistical power at serum Mn concentrations below 1.2 µg/L. Therefore, the aim of this work was in a first step to confirm the linear relationships previously found only on a limited set of samples from the Munich area now with a significantly increase of the data set up to 180 samples from three different areas (from Munich, Germany, from the Emilia Romagna region in Italy, and from Sweden). In a second step the reliability of ultrafiltration versus SEC under practical conditions for occupational health laboratories was elucidated. Specific questions to be answered were “are the same results gained in SEC and UF?”, and if so, “under which conditions”? (i.e. simpler procedure of pre-cleaning of tubes, or concentration dependence of performance).

**2. Materials and methods**

**2.1. Chemicals**

Standard compounds for retention time determination as well as for mass calibration of the SEC column were purchased from Sigma-Aldrich, Deisenhofen, Germany, as: Blue dextran: 2000 kDa, α-2-macroglobuline: 609 kDa, arginase: 107 kDa, transferrin: 78 kDa, albumin: 68.5 kDa, β-lactoglobuline: 36.5 kDa, lysozyme: 14.3 kDa, Metallothionein: 7 kDa, l-thyroxine: 777 Da, N,N´-bis(t-BOC)-l-cystine: 440.5 Da, citric acid: 192.5 Da, inorganic MnCl2.

TRIS, HNO3, HCl (suprapure), NH4-acetate (NH4Ac) and acetic acid (HAc) were from Merck, Darmstadt, Germany. HNO3 was purified by subboiling destillation. Argonliqu and NH3 were purchased from Air-Liquide, Krefeld, Germany. Argonliqu was vaporized at the tank providing Ar gas. The TSK SEC-gel (230 – 450 mesh) was purchased from Tosoh Bioscience GmbH, Stuttgart, Germany.

**2.2. Standards, samples and sample preparation**

Mn-protein stock standards (1 mg powder /ml) were prepared by dissolving each compound in 10 mL TRIS-HAc buffer (10 mM, pH 7.4). Stock solution of MnCl2 was prepared by dissolving 100 mg/L (related to Mn). Mn – citrate stock solution was prepared by mixing a solution of 1 g/L citrate with a MnCl2 solution (5 mg/L) using a volumetric ratio of 4+1 (v:v), resulting in a Mn-citrate stock concentration of 1 mg Mn/L. Analogous, Mn-albumin and Mn-transferrin stock solutions were prepared by mixing 1 g/L protein solution with 5 mg/L MnCl2 solution (4+1, each), resulting in 1 mg Mn/L for each compound. Stock solutions were aliquoted before being stored in the dark at –20 oC. No destabilization of standard compounds was observed using these conditions. Working solutions were prepared daily by appropriate dilution with NH4Ac, 10 mM, pH 7.4.

Single standards and the analysis of standard mixtures were used to achieve information on SEC retention times.

**2.3. Samples**

A set of paired serum and CSF samples was drawn from patients at the Department of Neurology of the Technical University Munich. These sample donors had unspecific neurological complaints, like headache, dizziness or various sensory symptoms. CSF and serum samples were collected for diagnostic purpose and handled as described previously (20). In short terms: CSF was collected from each individual by standardized lumbar puncture and serum was obtained from blood drawn from the cubital vein directly after the spinal tab. Thus CSF and serum constituted “paired samples”. In the case of unremarkable CSF test results from the hospital clinical chemistry laboratory, CSF and serum samples were considered to origin from neurologically healthy individuals. Only sample pairs from such individuals were used in the exercise. After patients consented to the use of their samples for scientific investigations, the previously aliquoted, frozen-stored samples were thawed at 4 oC in the refrigerator, vortexed and were ready for further analysis.

In order to increase the sample size both for comparing SEC with UF and correlations of species concentrations in serum with total concentrations, respectively, serum samples from Sweden and Italy were added to the sample set from Munich. The Swedish samples had been drawn in analogous manner as described above. In a research project on Mn in welding fume (*Speciation of manganese compounds in welding fumes and welders’ blood*, funded by AFA Insurance, Stockholm, Sweden (No. 090227)) staff at the Department of Occupational and Environmental Medicine at Örebro University Hospital (Örebro, Sweden) collected samples from welders and non-welding controls at their workplaces.The Department of Diagnostic, Clinical and Public Health Medicine, University of Modena (Modena, Italy) randomly sampled blood from eligible subjects from each sex- and age-specific subgroup of Modena residents aged between 35 and 70 years, considered as general population of the Emilia Romagna region, Italy. The recruitment of subjects to be included in the study was approved by the Modena province Ethical Committee. All samples were collected after the donors had consented to their use in a scientific investigation

* 1. **Size exclusion chromatography (SEC)**

Established SEC separation methods from previous experiments were applied using a Knauer 1100 Smartline inert Series gradient HPLC system with two serially installed SEC columns: Biobasic 300mesh column (300x8mm ID, Thermo, separation range 700 – 5 kDa) and a 250x10 mm ID Kronlab column filled with TSK-HW40S (separation range 100 – 2000 Da). This column combination provided separation of various Mn-proteins from each other and from Mn-citrate as well as the latter from inorganic Mn. NH4Ac (250 mM, pH 7.4) was used as the eluent at a flow-rate of 0.75 mL/min. In previous experiments species stability was proven on this separation system by reinjection experiments (20). Mass balance for samples were typically between 90 and 110 %, specifically being similar for standard compounds (Mn-Tf: 98 %, Mn-citrate: 108 %, inorganic Mn: 102 %).

* 1. **Columns mass calibration**

The mass calibration in the serially connected columns was performed using protein and LMM standards with known molecular weights. The mobile phase was the same as for samples (c.v. above). Retention times (RT) were determined by peak maxima in UV and for Mn/Fe-proteins additionally by ICP-MS detection. Retention times followed two calibration curves for the two columns (20): From 14 min (void, > 700 kDa) to 20 min (~5 kDa) according to the equation “ln(kDa)= -0.557 x RT +14.551 (r²=0.999) and from 21 min (> 2 kDa) to 28 min (55Mn2+, 56Fe2+) according to the equation “ln(kDa)= -0.4339 x RT +8.439 (r²=0.9936).

**2.6. Ultrafiltration (UF)**

Cleaning procedure for UF device before use:

Floater and ultrafiltration tube were put separately into pre-cleaned vessels where they were completely covered with 1% HNO3 (ca. 10 mL). Vessels with floater or tube (in HNO3) were subject to an ultrasonic bath for 15 min. Subsequently, floaters and tubes were removed and thoroughly rinsed with Milli-Q water. This cleaning procedure was repeated in total four times (each time with fresh HNO3) and a fifth time with only Milli-Q water. The performance of the cleaning procedure was checked by analysing aliquots of the final cleaning step for total Mn contents with ICP-DRC-MS. The cleaned ultrafiltration units were thoroughly shaken to remove remaining water drops and then were directly used.

Filtration procedure:

Diluted serum samples (1:2; 1.0 mL final volume) were added into Centrisart ultrafiltration tubes (cellulosetriacetate, 5 kDa molecular weight cut-off, Sartorius, Göttingen, Germany). After insertion of the floater the tube was allowed to stand for 5 minutes for completely wetting the membrane before centrifugation started at 1000 g, 5 minutes at 4°C (Biofuge 17 RS, Heraeus Sepatech, Osterode, Germany) followed from 2000 g for 75 minutes. The total volumes of permeate (about 0.5 – 0.7 mL) were transferred to vials for ICP-DRC-MS with a pipette and directly used for determination of total Mn contents.

**2.7. ICP-DRC-MS parameters**

A NexIon ICP-MS, Perkin Elmer (Rodgau-Jügesheim, Germany), was employed for total and chromatography determination of 55Mn and 56Fe using the dynamic reaction cell (DRC) mode.

During SEC coupling the column effluent passed the UV detector and was subsequently introduced to a Meinhard nebulizer in a cyclone spray chamber. The RF power was set to 1250 W, the plasma gas was 15 L Ar /min. The nebulizer gas was optimized and typically set to 0.94 L Ar/min. The dwell time was 350 ms. NH3 was applied as DRC gas at 0.58 ml/min. According to manufacturer´s data on interference reduction for iron determination and previous own investigations on Mn determination DRC mode with NH3 was preferred over KED mode (21). The DRC band pass (q) was set to 0.45.

**2.8. Quality control, precision, recovery**

**2.8.1. ICP-OES**

For quality control regarding total Mn and Fe determination an ICP-OES „Optima 7300” (Perkin Elmer) was used in parallel to ICP-DRC-MS for serum samples. Sample introduction was performed by the instrument´s peristaltic pump at 1.0 mL/min and a seaspray nebulizer that fitted into a cyclon spray chamber. The measured spectral element lines were: Mn: 257.610 nm and Fe 259.941 nm.

The RF power was set to 1000 W, the plasma gas was 15 L Ar /min, whereas the nebulizer gas was 0.60 L Ar/min. Regularly after ten measurements three blank determinations and a control determination of certified standards (CPI International, Palo Alta, CA, USA) were performed.

Additional quality control was performed for total Mn and Fe determination by analyzing control material “human plasma control ClinCheck 8853-8858” from Recipe, Munich. Control material was reconstituted as indicated on the flask label with Milli-Q water before measurements.

**2.8.2. Precision and recovery of SEC-ICP-DRC-MS**

Recipe “human plasma control ClinCheck 8853-8858” was analyzed in six independent measurements by SEC-ICP-DRC-MS for precision determination. The Mn peaks were quantified with respect to a Mn calibration curve (0, 0.5, 1, 5, 10 µg/L) and peak concentrations were subsequently summed up for total Mn and recovery calculation. The sum of eluted and quantified peaks were related to the total Mn determination (= 100%).

Additionally, a laboratory serum pool sample was analysed six times “as is” and after addition of 10 µg/L Mn standard for precision and recovery determination. Mn peaks were quantified with respect to the Mn calibration curve and peak concentrations were summed up for total Mn and recovery calculation.

**2.8.3.** **Precision and recovery of UF followed from ICP-DRC-MS**

Six aliquots of Recipe “human plasma control ClinCheck 8853-8858” were handled for UF in analogy to regular samples (see above). The ultrafiltrate fractions as well as the remaining HMM fractions were collected and Mn was measured by ICP-DRC-MS. Ultrafitrate concentrations were taken for precision calculation. The sums of both fractions per sample were compared to the total Mn determination (= 100%) for recovery calculation.

**2.9. Statistics**

Different linear regressions have been calculated to measure the strength of the linear relationship between the quantitative variables, objects of our study (22). The goodness of the fit has been evaluated using the square of Pearson correlation coefficient (r²). Linear regression equations were calculated as Y=a+bX. The significances of linear relationships were calculated applying the “Student´s t-test”. Moreover we report the p-value in order to test the significance. The level we have chosen as significant is p<0.05.

Additionally, a multiple regression model has been built up in order to study the dependence between the variable Mn-Cit(S) (response variable) and Mn(S) and whether the relationship is different for the three different sample sets (Munich, Sweden and Emilia Romagna/Italy). The multiple regression model is: Mn-Cit(S) = a + b1 \* Mn(S) +b2 \* D-[Emilia Romagna Region] + b3 \* D-[Sweden] where D-[Munich] was randomly chosen as reference point.

1. **Results and Discussion**
	1. **Results on Quality control experiments**

Comparison of ICP-OES to ICP-DRC-MS: **Comparable results were obtained from both determination systems: ICP-OES values ranged between 99 – 102 % compared to ICP-DRC-MS values, which were regarded as 100%.”**

Mn and Fe determination by analyzing control material “human plasma control ClinCheck 8853-8858” from Recipe: Total determinations of Recipe ClinCheck resulted in 15.8 ± 0.7 µg/L for Mn and 1208 ± 35 µg/L for Fe. The target mean values provided by Recipe were 15.1 µg/L (target range: 12.1 – 18.1 µg/L) for Mn and 1165 µg/L (target range: 932 – 1398 µg/L) for Fe. The recoveries calculated for the determined Mn or Fe concentrations were 104.6 % for Mn or 103.5 % for Fe, each related to target mean values.

Precision and recovery of SEC-ICP-DRC-MS: The serial precision from measurements of the Recipe ClinCheck aliquots (n=6) was 2 or 8 % (Fe or Mn), the recovery for both elements was 99 %. The serial precision of serum pool aliquot measurements (n=6) was 3 or 10 % (Fe or Mn), the recovery of the native sample was 93 % at 0.56 µg Mn/L or 103.5 % at 1209 µg Fe/L The recovery of the 10 µg Mn/L spike (n=6) into the serum pool sample was 108 %. Diederich et al. (23) reported 85-117 % recovery Mn for serum samples using partly the same separation gels as in this work. These values are practically coincident with our findings.

Precision and recovery of UF followed from ICP-DRC-MS: The precision of UF/ ICP-DRC-MS measurements for the Recipe ClinCheck aliquots (n=6) was 6 % (Mn), the recovery (filtrate + pellet fraction) was 119 % at 15.1 µg Mn/L. The recovery of the 10 µg Mn/L spike (n=6) into the control sample was 89 %. These values were considered sufficient to use the method for subsequent analysis of our serum samples, although the Mn concentration of the Recipe material was higher than in most samples analyzed in this study.

**3.2 Paired samples: Investigations on correlations between Mn(C) and Mn-species in serum**

For investigating the correlations between Mn(C) and different Mn species in serum the subgroup of paired serum/CSF samples from Munich (n=39) was used. It was shown that both, total Mn(C), but also total Mn(S) correlated well with Mn-citrate from serum at elevated Mn(S) concentrations above 1.9 µg/L, whereas these correlations disappeared below 1.45 µg/L Mn(S) concentration. In turn, at this lower, physiological Mn(S) concentration, Mn(C) correlated to Mn-Tf(S) as demonstrated in table 1. The results are supported by our previous findings (20) which were based on 24 samples. Although samples from the former study were taken at different time, both studies, the former and this one, were based on analogously derived samples from the same region (Munich) and thus strengthening the results by increasing the sample size. The results of both studies showed high squared linear regression coefficients but in the present study lower values of the respective slopes “b” were calculated (of regression equation) compared to the previous paper, as shown in Table 1.

The still small sample size of paired samples, caused by the limited availability of such paired samples, might be considered as a limitation. However, the finding that Mn(C) correlates with MnCit(S) with higher Mn(S) concentration is supported by the animal experiments from Diederich et al. (23). Their experiments showed that i.v. injection of a MnCl2 solution into the cubital vein of rats resulted in a strong increase of Mn-Tf in rat serum after 1 h, however after 4 days LMM-Mn-compounds, notably MnCit, but not Mn(III)-Tf, were increased in rat brain.

The findings are also in accordance with former reports that in contrast to protein-bound-Mn-complexes, Mn(II) present as Mn-Cit complexes enters the brain much more rapidly than Mn(III)-Tf (24). Further, Yokel and Crossgrove conducted experiments to determine the influx transfer coefficients (K*in*) into rat brain for Mn2+, Mn-Cit and Mn-Tf and found values in the order K*in* -MnCit > K*in* -Mn2+ > K*in* -Mn-Tf (9,23).

Overall, the results from animal experiments (9,23,25) and from analysis of paired serum/CSF samples (16,20) , this study] suggest that Mn-Cit(S) is transported across the neural barrier into brain, which can explain the found correlation between Mn-Cit(S) and Mn(C).

Therefore, an investigation about Mn-species in serum is supposed to be of interest.

**3.3. Investigations on correlations between Mn-species in serum versus total Mn(S)**

In the previous study with a limited sample number available (n=24) it was found that total Mn concentration in serum correlated best to the serum Mn-Tf(S) fraction only when total Mn(S) was below ca. 1.5 - 1.6 µg/L. It was shown that at higher concentrations there was no significant influence of Mn-Tf(S) on total Mn(S) (20). In turn, total Mn(S) correlated well with Mn-Cit(S) at elevated concentration above 1.6 µg/L (20). This switch from Mn-Tf(S) to Mn-citrate(S) as an Mn-carrier was considered to be a possible means for biomonitoring Mn in CSF. However, the sample size was small in that work and could therefore only be considered as indicative. Now these preliminary findings were repeated with a considerably increased sample size (n = 180, of which n = 50 having Mn(S) > 1.9 µg/L) as the whole set of serum samples was used.

We found that at elevated Mn(S) concentration the positive correlations of Mn-Cit(S) with Mn(S) were confirmed, showing high values of squared linear regression coefficients but approximately only half the value of respective slopes *“b”* compared to the previous paper (20) as shown in Table 1 . This difference to the previous investigation is not completely clear but might be explained by the low sample number in the earlier study.

***TABLE 1***

We found that at Mn(S) concentrations below 1.45 µg/L, Mn(S) and Mn(C) are completely uncorrelated to Mn-Cit(S) (both *r2* < 0.02, table 1) thus qualitatively confirming the result from the previous study.

Additionally, at such lower, physiological Mn concentrations in serum (< 1.45 µg/L), Mn(S) (and Mn(C), see above) correlated positively to Mn-Tf(S) (c.f. table 1). Overall, the previous preliminary findings were now confirmed with a considerable sample number. Figure 1 shows these correlations.

**Figure 1**

It is clearly seen in figure 1 that Mn-Tf(S) correlates well to Mn(S) with a high r2 value at low Mn(S) concentrations (i.e. < 1.45 µg/L), whereas at these low Mn(S) concentrations Mn-Cit(S) shows no correlation with Mn(S), having *r2* close to zero. At Mn(S) concentrations > 1.9 µg/L the situation is different. Now Mn-Cit(S) correlates well with Mn(S) with a high *r2* value, but Mn-Tf(S) has no correlation, demonstrated by *r2* close to zero. It seems that at low concentrations Mn is carried by Mn-Tf in serum but at elevated concentration it is mainly carried by Mn-Cit.

These results, shown in figure 1, must be considered as an important finding because a significant (for p-values cf. table 1) and concentration dependent change in human Mn metabolism is confirmed now using an increased sample size: Already at a slightly elevated Mn serum concentration the Mn-transport switches from proteins as Mn-carriers, i.e. Mn-transferrin – being well controlled by the Tf-receptor (TfR) shuttle at NB - to Mn-citrate, which is a Mn-carrier whose transport is facilitated across NB and that is even enriched in CSF and the brain (16,23,26).

For elucidating this switch point more precisely the regression lines of Mn-Tf(S) vs. Mn(S) for both ranges, data points with Mn(S) < 1.45 µg/L and data points with Mn(S) > 1.9 µg/L, were calculated in figure 1. In analogy the regression lines for Mn-Cit(S) vs. Mn(S) (both for Mn(S) < 1.45 µg/L and > 1.9 µg/L) were computed. Data points with Mn(S) between 1.45 and 1.9 µg/L were ignored for these calculations as they were expected to be too close to the switch point and it was not clear at the beginning to which regression line they correspond to. For improved and clearer graphical demonstration the regression lines from figure 1 were transferred into figure 2 (for an improved graphical demonstration without re-plotting the respective data points) and zoomed to the important concentration range of Mn(S) where the changes in relationships between Mn-Tf(S), Mn-Cit(S) and Mn(S) appeared. The switch point subsequently was calculated as intercept point of the respective slopes from Mn-Tf(S)- Mn(S) data and from the respective slopes of Mn-Cit(S) - Mn(S) data.

**Figure 2**

As seen in figure 2 the switch point from Mn-Tf(S) to Mn-Cit(S) may vary individually but it is generally located in the range of ca. 1.5 – 1.9 µg/L (Mn(S): In the previous study (20) the switch point was found to be in the range 1.5 – 1.6 µg/L Mn(S) whereas in this study we report a Mn(S) concentration of 1.51 µg/L derived from Mn-Tf(S) or 1.66 µg/L derived from Mn-Cit(S) based on the intersection of respective regression lines, both averaged to 1.58 µg/L. This is within the previously found range.

The significances of the above correlation coefficients were calculated. As could be expected from figure 1, both the correlations and their significance supported the above finding. The values are listed in table 1.

The change of the Mn carrier from Mn-Tf(S) to Mn-Cit(S) at increased Mn(S) concentration of ca. 1.6 µg/L, monitored in the whole serum sample set (n=180) combined with the findings that Mn-Cit is enriched across NB, reported e.g. by references (9,23,27) for paired serum/CSF samples and for rat brain, can explain the important correlation we found between Mn(C) and Mn-Cit(S) in the paired sample set from Munich at Mn(S) concentrations higher than 1.6 µg/L.

It is currently unknown whether high Mn-Cit(S) is correlated with Mn(C) for other groups than those who showed up at the neurological department with unspecific neurological complaints (but with unremarkable neurological results and assigned as “neurologically healthy”, having mostly unremarkable Mn(S) concentrations in the range reported for non-exposed individuals (28)), but Mn-Cit(S) might become a potential biomarker for increased risk of high Mn concentration beyond the neural barriers. In contrast to Mn(S) and Mn in blood, Mn-Cit(S) is of higher importance as it is a ligand that is known to have facilitated transport through the neural barriers and correlates well with Mn(C) in the Munich subgroup, even though it is presently not known whether a high concentration of Mn-Cit(S) for any potential subgroup actually implies higher Mn(C) concentrations detected among the neurologically healthy Munich patients with neurological complaints.

An interesting question is whether the apparent switch from Mn-Tf(S) to Mn-Cit(S) and the correlation of the latter to Mn(C) is due only to an increase of current exposure or whether it might be due also to an increased cumulative exposure over a prolonged period of time. From human samples analyzed in this study, we presently cannot give a conclusive answer as no exposure data were available. However, from our previous investigationswith exposed rats it appears that after acute (low-dose) exposure by i.v. injection of inorganic Mn, in serum 1 h after the injection, first Mn-binding by Mn-Tf(S) (ca. 50 fold increase) and to a lesser extent by Mn-Cit(S) (8 fold increase) occurs, whereas 4 days later Mn-Tf(S) concentration reverted to baseline values (23,29) but Mn-Cit(S) was still significantly elevated (1.7 fold, p<0.001 (29)). Furthermore, 4 days after Mn-injection, in rat brain only insignificant variations of Mn-proteins were observed, but contrary, Mn-Cit and inorganic Mn were significantly elevated (1.7 fold, p<0.001). In that paper by Neth et al. (29) the importance of LMM-Mn-species was even more pronounced in a seven weeks longing low dose feeding trial. In this trial HMM-Mn-species from rat brain were practically not affected, but Mn-Cit was increased both in serum and brain (3.2 or 1.6 fold, p<0.05). From these findings those authors concluded that in acute exposure situation mainly a Mn-Tf and some Mn-Cit increase is observed as a first response (c.f. 1h), but the response is shifting comparatively quick towards Mn-Cit (c.f. 4 days) and an increased cumulative exposure over a prolonged time period (c.f. 7 weeks feeding) seems to be expressed by an increase of Mn-Cit but is not reflected in Mn-Tf variation (29).

Our result that at increased Mn(S) concentration a switch occurs from transferrin to a “non-transferrin molecule” appears to have a parallel in the case of iron transport in serum, specifically with respect to iron overload and iron toxicity (30): In this paper the authors come to the conclusion that the so-called “non-transferrin bound iron” might correspond to loosely bound low molecular weight ligands, presumably iron citrate.

Our findings regarding the switch of the Mn-carrier in serum samples are related to the whole serum sample set, which consisted of the three subsets from Munich (n=39), the Emilia Romagna region in Italy (n=52) and from Sweden (n=89). However, as regional differences might occur between the three subsets we applied the multiple regression model to the three subsets and determined the correlations and switch points for each subset. It turned out that 97% of the total variation of Mn-Cit(S) can be explained by the multiple regression model. Examining the regression coefficients we can first deduce that on average, for the same level of total Mn(S) the level of Mn-Cit(S) in Emilia-Romagna-Region is -0.921 µg/L lower than in Munich. Contrary, for the same level of Mn(S) the level of Mn-Cit(S) in Sweden is on average 0.289 µg/L higher than in Munich. The variables that are contributing to the model are: Mn(S) (p= 0.000649) and D-Emiglia-Romagna Region (p= 4.8\*10-120). In any case the model suggests to keep the variable “Emilia Romagna Region” and Munich as a significant contributions to the model (p= 4.8\*10-120 and p= 0.000649, respectively; whereas the variable “Sweden” appeared to be not significant: p < 0.145). As no paired samples were available from Italy or Sweden, it was not possible to study the consequences of these regional/occupational differences on Mn(C).

Switch points for each subset were calculated additionally as described above: They were found at Mn(S) concentrations of 1.31 µg/L (Swedish samples), 1.26 µg/L (Munich samples) and 2.14 µg/L (Emilia Romagna samples). The reason for the higher value of the switch point for the “Emilia Romagna samples is presently unclear.

Therefore, when elucidating the increased risk of Mn transport to brain it is advisable to also take regional factors into account. Mn-Cit(S) determination appears to be necessary as being indicated by the differences in intercepts and switch points together with the identical slopes of correlation equations.

Since Mn is a competitor to Fe, specifically when binding to Tf and citrate (31,32)we analysed total Fe in serum (Fe(S)), Fe-transferrin in serum (Fe-Tf(S)) and Fe-citrate in serum (Fe-Cit(S)). These Fe-species practically showed the same mean values for serum samples, irrespective of whether the Mn serum samples were below 1.45 or above 1.9 µg Mn/L. This is shown in table 2.

Table 2

There was no correlation between Mn(S) and Fe(S) or Fe-species, respectively. Both the slopes “*b*” and *r*2 determined with Pearson´s linear regression analysis were close to zero for both Mn(S) ranges, below (< 1450 ng/L) and above (>1900 ng/L) the switch, see Table 2. This lacking correlation is simply explained by the fact that Fe concentration in serum is approximately a thousand-fold higher compared to Mn. The regular Fe-saturation of e.g. Tf is at approximately 25-30% (33,34)**,** which means that approximately 70% of the binding sites are still available for Mn binding. Aschner quantified the available binding sites for Mn to be 50 µM, corresponding to 2750 µg Mn/L (31). An increase by 1 µg/L starting from a physiological “normal” Mn(S) concentration of ca. 1 µg/L, across the switch point (1.58 µg/L) up to 2 µg/L is an insignificant increase regarding the 2750 µg Mn/L of available binding sites. Therefore transferrin saturation by Mn seems not plausible. For citrate no values about metal saturation are reported. It is known that transferrin can carry only three-valent cations (e.g. Fe(III) or Mn(III)), but Mn is supposed to be taken up and initially present in the divalent state in serum (32). Thus it might be speculated whether the oxidation process via ceruloplasmin of Mn(II) to Mn(III) could be a limiting factor. If so, the additional Mn(II) could use another highly accessible carrier: citrate. A Mn citrate complex was detected in CSF in our former work (20). Previous studies from Grzybowski et al*.* (35), addressing the nature of Mn-citrate species in aqueous solutions, suggested the presence of species such as [Mn(II)Cit]2-, [Mn(II)Cit]-, and [Mn(II)Cit], which are thought to be more mobile and possibly more readily absorbable by biological tissues (36).

However, to the best of our knowledge there are presently no reports in literature about limits in Mn(II) oxidation rates in body fluids. On the contrary, Critchfield et al.report about a higher Mn-Tf after Mn(II) exposure to MMT from combustion, which was explained by Mn(II) to Mn(III) oxidation in blood within 1-5 hours (37). So far the reason why Mn switches from Tf(S) to citrate (S) is not explained. It is presumably related to the similar switch for iron from Fe-Tf(S) to Fe-Citr(S).

**3.** 4.Requirements for ultrafiltration of Mn-Cit(S)

As reported above, Mn-Cit(S) possibly reflects Mn-load to the body compartment beyond neural barriers and therefore Mn-Cit(S) is of interest for biomonitoring. The SEC-ICP-MS speciation technique may be problematic for occupational routine laboratories due to high operating costs combined with prolonged analysis times. Therefore the use of ultrafiltration units, for example at a 5 kDa cutoff and subsequent total Mn determination in the filtrate was tested and methodical limits were elucidated.

The evaluation of Mn concentrations determined from UF fractionation from serum samples compared to the Mn concentrations determined for LMM by SEC-ICP-MS revealed that multiple cleaning of UF tubes and inserts was mandatory. As opposed to the cleaning protocol of Nischwitz et al. (38) only 1% HNO3, and as final cleaning step, Milli-Q-water were used for the cleaning cycles. When applying 4 x HNO3 + 1 x Milli-Q-water, the determined Mn concentration was typically below LoD (50 ng/L) and less than 2% of the cleaned tubes showed was contaminated with detectable Mn up to 100 ng/L (Figure 3 A).

**Figure 3**

The comparison of UF vs. LMM-SEC results from serum with Mn(S) in the range 1200-1700 ng/L showed that the average Mn concentration determined in the UF filtrate usually matched the concentration of the LMM-SEC fraction (sum of Mn-citrate + possibly small amounts of inorganic Mn) for all samples Mn(S). The recovery calculated on mean values ranged from 97 to 106 % (Figure 3 B).

A closer look however reveals that individual samples were drastically scattered, notably more for lower Mn(S) concentrations. This limits the usefulness of the approach for individual samples. Specifically it turned out that for Mn(S) concentrations below < 1300 ng/L (corresponding to Mn-LMM ≤ 200 ng/L) the recovery of the UF-fractions ranged between 0 and 320% (for graphical reasons a zoomed version is plotted between 50 – 150% in Fig. 3 b), which is surely an inacceptable performance. At higher Mn(S) concentration results were considerably improving. At Mn(S) concentrations ranging from 1400 to 1500 ng/L (corresponding to Mn-LMM concentrations of ca. 200 – 500 ng/L) or even higher at Mn(S) > 1600 ng/L (corresponding to Mn-LMM > 600 ng/L) the individual concentration values were only little scattered around the SEC-LMM target valves, resulting in a recovery of 106 ± 16% (1400 to 1500 ng/L) or 104 ± 15% (Mn(S) > 1700 ng/L). Better performance than nearly quantitative recovery with 15% RSD around SEC-LMM target values could not be achieved.

Nevertheless, the described UF procedure can be useful for occupational health monitoring laboratories, which mostly lack regularly evaluated HPLC-ICP-MS coupling techniques, as long as the validity limits of the procedure are strictly considered, which is rigorous pre-cleaning of UF tubes (with contamination control) and application of this UF method only for serum samples with Mn(S) > 1500 ng/L.

This conclusion is supported also by the results from Nischwitz et al. (38) who found that ultrafiltration provided a suitable size-fractionation of various elements including Mn, although they determined a mean recovery of 150 %. This elevated recovery was explained by the low Mn concentrations being reported below 1 µg/L, which is actually below the herein reported validity limit of 1500ng/L for this procedure. Furthermore, the quality control results (see above) derived from the analysis of the Recipe control material support the usefulness of UF/ICP-DRC-MS, specifically at higher Mn concentration.

The search for a reliable and distinctive biomarker for Mn exposure is still of big concern in occupational and also environmental medicine. Therefore, e.g. Cowan et al. started an evaluation of possible Mn-biomonitoring matrices applying a cross-sectional design (39). They included concentrations of Mn, Fe and Fe-metabolic proteins in saliva, plasma, erythrocytes, urine and hair from controls and Mn-exposed smelters as well as airborne Mn levels to reliably distinguish Mn-exposed subjects from the general, Mn-unexposed healthy population. As an important outcome Mn/Fe ratios were considered as a means to get workers roughly differentiated from controls but on a group assessment basis only. A couple of individuals of both groups were erroneously classified to the wrong group.

Smith et al. found Mn in urine less suitable for determining internal exposure levels, especially because the primary route of excretion is via bile to feces (>95%) (11). They also report about limitations for total Mn in blood as an exposure biomarker since this biomarker was highly dependent on the exposure parameters. Mn storage in bone and later remobilization was assumed as the reason and thus no estimation of historical Mn accumulation was possible (7). Possibly also a secondary exposure from recycled Mn from red blood cells must be taken into consideration. Analogously to iron, Mn might be trapped in red blood cells and might be released when the cell dies.

Total Mn concentrations in plasma and serum have been extensively evaluated for use in Mn exposure assessment also with limited success. Hoet et al. (40) compared plasma Mn values from welders with those of controls and found a 33 % increase for welders. A crucial exposure limit value from air at workplace was determined at 10 µg/m3. This is considerably lower than, for example, the Swedish limit value TLV for respirable Mn at 100 µg/m3. However, these findings were not consistent during the investigated working days, leading to the conclusion that total Mn serum levels may serve reasonably well as an indicator of recent but not historical Mn exposure and additionally only for group comparisons (e.g., welders vs. controls). The important individual exposure assessment was regarded as problematic due to large variations between individuals. This is supported by a study from Baker et al.(41) where Mn in plasma and urine were reported to exhibit major variance within individuals and a single sample might not be representative for the sample donor´s Mn concentration in these matrices. Further biomonitoring matrices for Mn detection that have been investigated are bone, hair and nail clippings, though all resulted in an inconsistent data set without a clear cut between exposed/unexposed persons (42-44). Pre-cleaning of samples was indispensable for removing attached Mn contamination (45) but even then the keratin-based matrices (hair, nails) often did not allow the distinction between exogenously and metabolically incorporated Mn (7). The above described limitations of the traditional, but inadequate exposure assessment methods bear the potential of bias in epidemiologic studies. This together with the need for Mn biomonitoring from occupational and environmental medicine and the well-known neurotoxic potential of Mn fuels research and discussion on the best Mn biomarker, an issue which highlights the relvance of the analytical methodologies which may adequately assess and predict CNS Mn levels.

Our finding that increased exposure at the neural target site beyond neural barriers is reflected in a specific Mn-compound –Mn-citrate - in serum above a switch point is possibly an important step forward, all the more as regional differences are seen as well. Mn-speciation is a prerequisite for this differentiation and the simpler UF-method may help occupational routine laboratories to handle this analytical approach as long as contamination control and validity limits are not ignored.

1. **Conclusion**

Mn exposure in neurologically relevant tissues or organs such as the central nervous system is not necessarily associated with an increase of total Mn in blood or serum. Mn concentrations of exposed and unexposed persons often overlap widely and individual differentiation is often not possible (11,40). A very rapid distribution of Mn to target organs is considered to be the main reason. In this study we showed with an increased sample size that at slightly elevated total Mn(S) concentration, which however is close to the Mn(S) range of unexposed persons and far below values of exposed ones, a change in Mn carriers towards Mn-citrate takes place. Parallel to this carrier change, we have shown that Mn beyond the neural barrier, i.e. Mn(C), for an especially studied subset correlates to this second carrier Mn-citrate(S) at higher Mn(S) concentration, reflecting the facilitated transport of Mn into the brain. The latter has been confirmed also in rat brain after single low dose exposure (23). Mn-Cit(S) determination thus lends itself for estimating a Mn load in the CSF, i.e. offers a function as a biomarker for Mn beyond the neural barrier. For a simpler Mn-Cit(S) determination ultrafiltration of serum samples could be a suitable choice e.g. for routine occupational medicine laboratories, provided that methodical prerequisites and limitations (cleaning procedure, Mn(S) > 1.5 µg/L) are carefully considered.

**Conflict of interest:**

None of the authors state any conflict of interest

**Legends:**

**Table 1**

Slopes and squared linear Pearson´s regression coefficients r2 of important Mn-species each correlated to Mn(S) are shown and differentiated according to low and elevated Mn(S) concentrations. The significances of linear relationships were calculated applying the “Student´s t-test” and the p-values are reported in order to test the significance.



Table 2

Slopes and Pearson´s squared linear regression coefficients r2 of important Fe-species each correlated to Mn(S) are shown and differentiated according to low and elevated Mn(S) concentrations. The significances of linear relationships were calculated applying the “Student´s t-test” and the p-values are reported in order to test the significance.



**Figure 1 A:** Linear regression of Mn-Tf(S) and Mn-Cit(S) versus Mn(S) for those data where Mn(S) < 1.45 µg.

**Figure 1B:** Linear regression (Mn-Tf(S) vs. Mn(S) and Mn-Cit(S) vs. Mn(S)) plotted for Mn(S) concentrations above 1.90 µg/L.



**Figure 2:**

Zoomed view on the regression lines (calculated in fig. 1) at the important Mn(S) concentration range; “a”: switch point (1.51 µg/L) calculated from Mn-Tf(S) intercepts, or at “b”: switch point (1.66 µg/L) calculated from Mn-Cit(S) intercepts, in average 1,58 µg/L.



**Figure 3 a:** Mn concentrations in purge solutions after each purge cycle from floaters. Purge cycles 1-4: with 1 % HNO3, cycle 5 (and 6, only shown for proof that after 5 cycles no further changes were seen) with Milli-Q-water.

**Figure 3 b:** Recovery of UF compared to serum LMM fraction determined by SEC-ICP-DRC-MS (set to 100%).



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