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Complex Profile of Altered Heavy Metals Accumulation in Multiple Sclerosis, a Relationship with Copper and Zinc Homeostasis

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Abstract

BACKGROUND: Multiple sclerosis (MS) is a demyelinating inflammatory disease of the central nervous system white matter. Both of environmental and genetic factors have been implicated in its pathogenesis. Heavy metals generate free radicals causing alteration in genetic material and blood-brain barrier damage. In addition, intracellular accumulation of certain heavy metals can trigger autoimmune reaction against myelin proteins and nerves cytoskeletal proteins.

AIM: We aimed to analyse complex profile of altered heavy metals accumulation in multiple sclerosis and relationship with copper and zinc homeostasis.

METHODS: The present study was carried out in the period between September 2019 and May 2021 on 86 MS Egyptian patients and 86 age and gender matched healthy controls. Whole blood levels of lead (Pb), mercury (Hg), and Cadmium (Cd) in microgram/liter (μg/L) in addition to Zinc (Zn) and Copper (Cu) in milligram/liter (mg/L) were quantitated using the Agilent ICP-MS-MS. The current study also discussed physiological-toxic metals interactions in these patients.

RESULTS: We demonstrated significant relations between toxic heavy metals levels and MS suggested by significantly higher levels of Pb, Hg, and Cd and significantly lower levels of Zn and Cu as well as Cu/ Zn in MS patients than controls. Besides, it could be assumed that; physiological heavy metals homeostasis limits the accumulation of toxic heavy metals that share absorption and transport binding sites, suggested by the significant negative correlations between whole blood levels of Cu and both of Hg and Pb.

CONCLUSION: A complex profile of altered elements rather than a single element imbalance in MS pathogenesis is suggested.

Introduction

Multiple sclerosis (MS) is a demyelinating inflammatory disease of the central nervous system white matter with a global spread. Both environmental factors and genetic predisposition are believed to contribute to MS pathogenesis [1]. Heavy metals are major environmental pollutants, they have no known biological functions yet and related to many health problems. Heavy metals toxicity has been shown to be affected by individual susceptibility, genetic factor, nutrition, and health [2].

In addition to production of free radicals, Pb can traverse endothelial cells of the blood brain barrier. It can replace calcium ions in the calcium-ATPase pumps. In addition, it can interfere with deoxyribonucleic acid transcription [3]. Lead can affect both the central and peripheral nervous systems, causing axon degeneration, and myelin sheath destruction [4].

All Hg forms are known to be toxic for humans. Mercury can alter the mitochondrial function with subsequent production of reactive oxygen species (ROS). Experimental models have shown that Hg exposure has resulted in triggering the autoimmune response with immune complex formation and autoantibody synthesis [5].

The previous studies suggested that Cd indirectly participates in oxidative stress via Fenton reaction. Cd may lead to immune dysfunction. In addition, Cd is implicated in the pathogenesis of neurological abnormalities, neonatal cerebral edema, and cerebral hemorrhage in animal experimental studies [6].

Due to its key role in the regulation of immune system, Zn could influence cytokine production through Matrix Metallo-proteinase control and stabilizing the association of the myelin basic protein with brain myelin membranes. Even a mild Zn deficiency can weaken the function of the immune system and lead to oxidative stress [7].

Since Cu is involved in the synthesis of the myelin sheath, a deficiency may cause myelinopathy. Cu/Zn has a well-known relationship, particularly in the management of oxidative stress. Increased ROS development and subsequent neurodegeneration are thought to be linked to its disruption [8], [9].

Monitoring the human tissue toxic metals level has been continued for the last couple of decades in developed countries. However, in Egypt, toxic metals level monitoring in human tissues has been rarely measured in recent years. Our study aims to identify chronic toxicity of Hg, Cd, and Pb as well as copper (Cu) and zinc (Zn) dyshomeostasis as possible triggers for MS among Egyptians. In addition, raising the attention to the possibility of environmental toxins involvement in the pathogenesis of autoimmune and neurological diseases in Egyptian population.

Materials and Methods

Patient selection

A cross-sectional study of 86 patients with established MS diagnosis according to the revised MacDonald's criteria, any age and both genders, and 86 age and gender matched healthy individuals. Patients were recruited from Kasr Al Ainy MS Research Unit during their follow-up visits. The control participants were not related to the MS patients. From both groups, participants with neurological diseases other than MS, as well as debilitating renal and liver diseases, were not eligible. Participants taking supplements containing Zn and other elements were also excluded.

Sample collection

In addition to participants' demographic data, Three milliliters of venous blood were collected in duplicate in clean polyethylene tubes, containing heparin as an anticoagulant.

Chemicals and reagents

All reagents were of high-quality grade unless specified otherwise. All were purchased from Merck (Merck KGaA, Darmstadt, Germany), and used as received. All solutions were prepared with the use of deionized water (18 MΩ-cm at 25°C) obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All the laboratory ware was washed by soaking them in 20 percent v/v (HNO_3) reagent for at least 4 h, rinsing 3 times with deionized water, according to the United States environmental protection agency method 200.8 and drying in a laminar flow hood [10].

Merck also supplied the ICP-MS-MS standards (Merck KGaA, Darmstadt, Germany). The tuning solution, which contained 10 ng/mL Li, Y, Co, Tl, and Ce in 1 percent HNO_3, was made from single-element stock standards and was used to optimize ICP-MS-MS parameters prior to each analytical run. Internal standards of V, Rh, and Bi were used to correct for instrumental drift. All internal standards were added online in the form of a 1 μg/mL multi-element solution in 1% HNO_3. V corrects for Mn, Rh is suitable for Cd, and Bi was added to correct for Hg and Pb.

The synthetic matrix solution used to prepare intermediate standards was an aqueous solution of NH_4 OH, 2% w/v, H_4EDTA, 0.25% w/v, 7.5 g/l NaCl, and 0.5 g/l $CaCl_2$. The default method for the determining metals in whole blood was originally based on a method provided by Agilent Technologies, which we later updated, tested, and approved. Our current method employs an aqueous solution of n-butanol, NH_4OH, H_4EDTA, and Triton X-100, with internal standard solution added as a blood diluent. This diluent is an excellent blood solvent as the calibration standards are partially matrix-matched to blood specimens by the addition of sodium and calcium chloride.

Instrumentation

Microwave system (Analytical Jean) was used to digest blood samples. The Agilent 8800 ICP-MS-MS Triple Quad was used for metals quantitation (Agilent Technologies, Tokyo, Japan). In order to minimize interference, In the Agilent 8800 ICP-MS-MS, the Octupole Reaction System was pressurized with helium to use "collision mode." The ratio Au/Hg = 20/200 was optimized for accurate Hg determination to obtain the highest signal, that means maximizing the fraction of Hg that remains in solution.

Sample preparation

For blood digestion, 3 mL of the sample were mixed with 2 mL of H_2O_2 and 5 mL of HNO_3. Gold was added up to 200 ng/mL. All digestions were carried out in PTFE digestion vessels. The digestion program for eight vessels consisted of an initial power ramp from 0 to 800 W over 10 min, followed by a 20-min continuous power stage at 800 W and a 15-min final cooling stage without power.

After digestion, vessels are allowed to cool down for 24 h before opening, to minimize the loss of volatile analytes, such as Hg. The resulting digested sample was transferred to a 15 mL volumetric flask and made up to that volume with the prepared stock solution.

Statistical analysis

Data were coded and entered using the Statistics Package for the Social Sciences version 26

(IBM Corp., Armonk, NY, USA). In quantitative data, the mean, standard deviation, median, minimum, and maximum were used to summarize the data, while in categorical data, the frequency (count) and relative frequency (percentage) were used to summarize the data. The non-parametric Kruskal–Wallis and Mann–Whitney tests were used to compare quantitative variables [11].

To compare categorical data, the Chi-square (2) test was used. When the expected frequency is <5, the exact test was used instead [12]. The Spearman correlation coefficient was used to evaluate correlations between quantitative variables [13]. Statistical significance was defined as $p < 0.05$.

Results

Demographic data for all participants

The total sample aged between 14 and 52 years. Median age in the MS group was 33.63 ± 7.46 while in the control group it was 31.71 ± 6.32 (Table 1). In MS group, there were 33 males (38.4%) and 53 females (61.6 %) with a female dominant ratio of 1.6. In the control group, there were 29 males (33.7%) and 57 females (66.3%) (Table 2). There were no statistically significant differences between the two studied groups regarding age, gender or residence. In MS patients, Table 3 demonstrates significantly higher whole blood Pb level in males.

Comparative whole blood heavy metals levels in the two studied groups

Figure 1 demonstrates elevated whole blood levels of Pb, Hg, and Cd in cases compared to controls with highly statistically significant differences ($p < 0.001$). Median whole blood levels of Pb, Hg, and Cd in μg/L in MS group were 44.135 (12.254–133.150), 0.873 (0.116–4.201), and 1.250 (0.245–4.215), respectively. Median whole blood levels of Pb, Hg and Cd in μg/L in the control group were 18.605 (3.324–55.321), 0.439 (0.025–1.731), and 0.406 (0.098–1.221), respectively.

Disease condition and its relation to heavy metals level

In MS group, (79.1%, N: 68) of patients followed the relapsing remitting MS (RRMS) course. 13 patients (15.1%) were secondary progressive MS (SPMS) and only five patients (5.8%) were primary progressive MS (PPMS) (Table 4). Diagnosis of MS was at the age of 26 (12–47). The median expanded disability status scale (EDSS) of studied cases was 3.5 (1–6).

Table 1: Ages of both studied groups

Age	Cases					Controls					p value
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	
	33.63	7.46	33.00	14.00	52.00	31.71	6.32	32.00	19.00	50.00	0.068

Non-parametric Mann–Whitney test. Data represented in mean±standard deviation (SD). p≤0.05 is statistically significant. p<0.01 is highly statistically significant.

Table 2: Gender and residence of the two studied groups

	Cases		Control		p value
	Count	%	Count	%	
Gender					
Female	53	61.6	57	66.3	0.525
Male	33	38.4	29	33.7	
Residence					
Rural	32	37.2	37	43.0	0.437
Urban	54	62.8	49	57.0	

Chi-square test. Data represented as count and (%). p≤0.05 is statistically significant. p<0.01 is highly statistically significant.

Table 3: Relations between whole blood Pb, Hg and Cd level and gender in MS group

Gender	Mean	SD	Median	Minimum	Maximum	p-value
Pb level						
Female	47.154	27.956	36.690	12.254	110.850	0.016
Male	65.250	33.356	56.180	21.110	133.150	
Hg level						
Female	1.295	0.994	0.951	0.202	4.171	0.742
Male	1.189	0.900	0.859	0.116	4.201	
Cd level						
Female	1.290	0.808	1.118	0.246	3.581	0.074
Male	1.597	0.859	1.431	0.245	4.215	

p≤0.05 is statistically significant SD: standard deviation.

Table 4: Types of multiple sclerosis in the case group

	Cases	
	Count	%
Multiple sclerosis type		
SPMS	13	15.1
PPMS	5	5.8
RRMS	68	79.1

Data represented as count and (%), SPMS: Secondary progressive multiple sclerosis, PPMS: Primary progressive multiple sclerosis, RRMS: Relapsing remitting multiple sclerosis.

Table 5 presents correlations between toxic heavy metals of study and MS onset and EDSS. MS onset was earlier in patients with higher Pb levels with a significant negative correlation (r. −0.215, $p = 0.047$). EDSS was higher in patients with higher Cd level with a statistically significant positive correlation (r. 0.420, $p < 0.001$).

Table 5: Spearman correlation between whole blood Cd, Pb, and Hg level and age of MS onset and EDSS

Disease onset	Cd	Pb	Hg
Correlation Coefficient	0.045	–0.215–	0.089
p-value	0.680	0.047	0.414
Number	86	86	86
EDSS			
Correlation Coefficient	0.420	0.147	–0.013–
p-value	<0.001	0.178	0.905
Number	86	86	86

p≤0.05 is statistically significant.

Physiological heavy metals of study and physiological-toxic metals interactions

Figure 2 shows significantly lowered levels of Zn ($p = 0.003$), Cu ($p < 0.001$) and Cu/Zn ($p = 0.017$) in MS patients when compared to controls. Blood levels of Zn

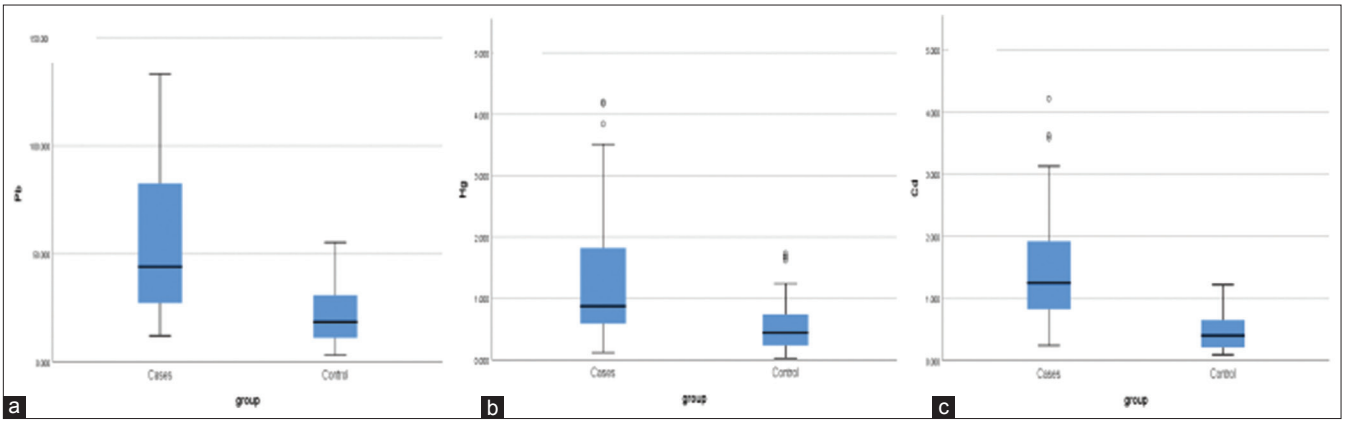

Figure 1: (a) Median whole blood Pb levels in both studied groups (p < 0.001), (b) median whole blood Hg levels in both studied groups (p < 0.001) and (c) median whole blood Cd levels in both studied groups (p < 0.001)

and Cu in mg/L in MS group were 4.357 (0.355–11.714) and 0.666 (0.115–8.769), respectively. Blood levels of Zn and Cu in mg/L in the control group were 5.124 (3.158–8.526) and 0.874 (0.597–1.848), respectively. Cu/Zn was 0.147 (0.021–4.270) in cases. On the other hand, it was 0.174 (0.093–0.361) in control group.

Table 6 presents the correlations between both physiological heavy metals of study and all of Pb, Hg, and Cd.

Table 6: Spearman correlation between Zn, Cu, and other heavy metals of study in MS group

	Zn	Cu
Cd		
Correlation Coefficient	–0.173–	0.159
p value	0.112	0.144
Number	86	86
Pb		
Correlation Coefficient	–0.132–	–0.216–
p-value	0.226	0.046
Number	86	86
Hg		
Correlation Coefficient	0.013	–0.304–
p-value	0.903	0.005
Number	86	86
Cu		
Correlation Coefficient	–0.128–	
p-value	0.240	
Number	86	

p≤0.05 is statistically significant.

We observed that the lower levels of Cu and Zn were accompanied by higher levels of Pb, Hg and Cd. However, these negative correlations were only significant between Cu and both of Pb (r.−0.216−, p = 0.046) and Hg (r. −0.304−, p = 0.005). Although higher Cu levels were associated with the lower Zn levels, this correlation was not statistically significant.

Discussion

MS is the most prevalent non-traumatic disabling disease in young adults. MS is thought to occur when an environmental agent or event interacts with a genetic predisposition to immune dysfunction. Humans are exposed to heavy metals in various ways, including food and water consumption, inhalation of contaminated air and occupational exposure. Most heavy metals are toxic to the body even at minimal concentrations.

Ideally, neither children nor adults should have any heavy metals in their bodies. Although the Pb, Hg and Cd levels in most MS patients in the present study was lower than the current threshold for toxicity (as set by the United States Centers for Disease Control and Prevention since 1991 and by the WHO). There are no known safe blood Pb, Cd, and Hg levels. The range and

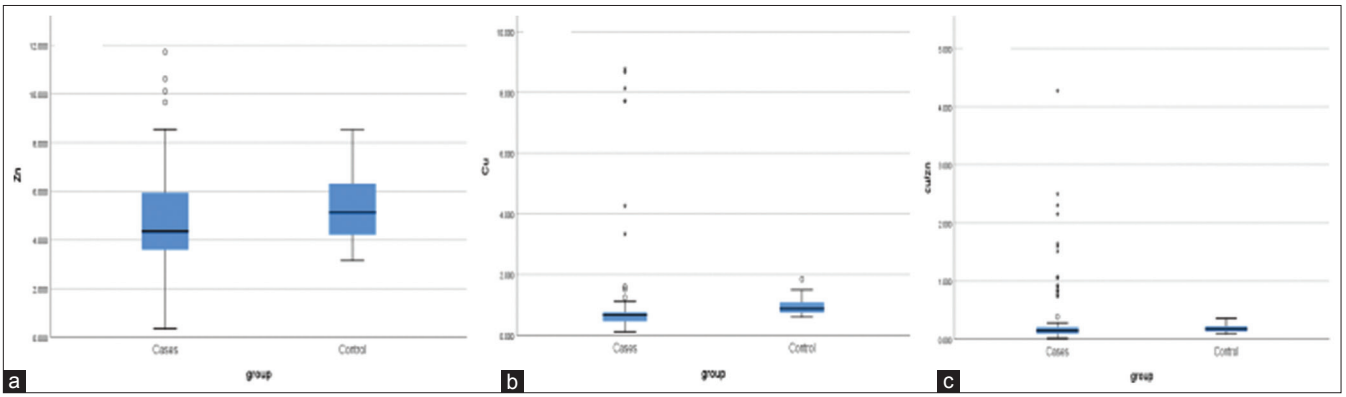

Figure 2: (a) Median whole blood Zn levels in both studied groups (p = 0.003), (b) median whole blood Cu levels in both studied groups (p < 0.001) and (c) Cu/Zn in both studied groups (p = 0.017). (non-parametric Mann–Whitney test)

severity of symptoms and adverse effects in both adults and children increases as exposure increases [6].

Recent studies started investigating the role of imbalance of single or multiple heavy metals in the pathogenesis of MS. In Egypt, although facing heavy water and air pollution problems, toxic metals level monitoring in human tissues has been rarely measured in recent years.

Relative differences in heavy metals levels in different studies are most likely due to variations in study area, population and measurement methods. However, measurement method differences do not have a great impact on the comparative result as long as the procedure has been the same for patients with MS and healthy controls.

Jakubowski *et al*. [14], Becker *et al*. [15], Batáriová *et al*. [16], Aliomrani *et al*. [3], and Paknejad *et al*. [17] previously supported our results regarding significantly higher blood Pb level in males than females. Males have greater blood Pb levels than females due to their higher RBC count, higher exposure and gender-related variations in Pb metabolism [18]. On the other hand, Razavi *et al*. [19] reported non-significant relation between levels of Pb in blood and gender. Paknejad *et al*. [17] observed gender based difference regarding Cd level. In our study, the difference was not sufficient enough to be statistically significant.

Unlike the current work, Tamburo *et al*. [20] found higher levels of all of Pb, Cd, Cu, Zn as well as Cu/Zn in female's hair compared to males. Hair samples, though considered a useful predictor of body levels of some toxic metals and essential minerals, are subject to external contamination, which could explain higher metals levels in females due to more frequent use of potentially contaminated hair preparations.

Dehghanifiroozabadi *et al*. [21] findings were compatible with our work regarding the significantly higher blood Pb level in MS patients when compared to controls. Despite the fact that patients in the current study had considerably higher levels of Pb, there is extensive dispute about its contribution to MS pathogenesis. Alizadeh *et al*. [22] and Aliomrani *et al*. [23] found no significant difference in serum Pb concentrations between patients and controls.

In Iran, Aliomrani *et al*. [3] as well as Paknejad *et al*. [17] agreed with our results regarding higher Cd in MS patients. However, their studied Pb level showed non-significant difference between patients and controls. In accordance to the current work, Attar *et al*. [24] demonstrated that Serum Hg level in MS patients was significantly higher than in the control group.

Elberry *et al*. [25] quantitated blood levels of Pb, Hg and Cd in 50 Cases with a diagnosis of definite, probable or possible MS and 50 healthy controls at El-Hussein University Hospital, Cairo, Egypt. MS group possessed higher levels of both Pb and Hg than controls, which was constituent with our results. However, Cd levels did not show a significant difference. This partial disagreement could be explained that we recruited only patients with established MS diagnosis according to revised MacDonald's criteria.

Bredholt and Frederiksen [7], in their systematic review and meta-analysis, previously approved our results regarding significantly lower Zn levels in MS patients than healthy controls. Palm and Hallmans [26] and Ghazavi *et al*. [27] indicated lower Zn levels in MS patients than controls, which was constituent with our study results. Regarding Cu *et al*. [26] found no statistically significant difference between cases and controls, while it was higher in MS patients in Ghazavi *et al*. [27] study. Both were not constituent with the present study results.

Our study results were close to Nashmi *et al*. [9]. They found a significant increase in levels of Pb and Cd in patients with MS patients than controls. Furthermore, Zn and Cu levels in MS patients were considerably lower than in healthy controls. Palm and Hallmans [26] and Reeves and Chaney [28] attributed lower Zn and Cu in MS patients, respectively, to incomplete absorption, which is a reasonable explanation for ours especially with the competition of high Pb, Hg, and Cd for Metallothionein in MS group.

According to Smith *et al*. [29], Cu concentrations in RBCs were significantly lower in MS patients compared to controls, so as to RBC Cu/Zn. Both were close to our results. Unlike the current work, Tamburo *et al*. [20] found non-significant difference between scalp hair levels of Pb, Cd, Cu, Zn, as well as Cu/Zn in RRMS patients and healthy controls and found. Difference in study population and environmental factors is of concern.

In our study, the abnormal levels of all the five measured metals as well as Cu/Zn in MS group compared to controls were in the same line with Alimonti *et al*. [30], who measured 26 elements in four groups of subjects; Alzheimer's disease, Parkinson disease, MS and healthy individuals. They found that, in MS, up to 13 parameters were different. Our results, supported by those finding, strongly suggests a complex profile of altered elements rather than a single element imbalance, which is in keeping with the multifactorial etiology of MS [30].

Regarding disease condition and its relation to heavy metals level, Hamdy *et al*. [31] previously supported our statistics considering the most common course of MS and the median age of onset. Razavi *et al*. [19] and Nashmi *et al*. [9] also reported RRMS as the most common course.

Dehghanifiroozabadi *et al*. [21] demonstrated that the mean age at onset of the disease was 29.41 ± 7, which was comparable to the present study. According to Razavi *et al*. [19] study, the mean duration

of disease was about 4 years. With reference to mean age (33.73 ± 1.34) of their studied cases, their age of onset seems comparable to that of the current study. The mean EDSS was lower than in ours. The relative underdiagnoses of the disease milder presentations could provide an explanation for the relatively higher EDSS in our study.

Razavi *et al*. [19] reported higher Pb levels in patients with longer disease duration. This matched with the negative correlation between Pb level and age of MS onset in our study. Because Pb is utilized as a defective building block in place of calcium, it could accumulate and bind to bone with time [32], [33]. In terms of EDSS, Razavi *et al*. [19] found significant difference in Pb level in MS patients' blood, which was not constituent with the current work.

The physiological-toxic heavy metals interactions in our study are not fully understood. However, Pb, Hg, Cd, Zn, and Cu are well known to combine with metallothionein. Therefore, it could be assumed that decrease in Pb, Hg, and Cd toxicity may necessitate the presence of other divalent metallic ions capable of competing for binding sites associated with absorption and transport [28], [34]. Furthermore, a competition for glutathione binding is also suggested [35].

In his study regarding the influence of a high Cu diet on the absorption of Hg and Cd in rats. Grosicki [36] demonstrated a beneficial role of dietary Cu supplements in decreasing the bioavailability of Hg through the gastrointestinal tract. In addition, non-significant difference in Cd bioavailability in the presence of Cu supplements was found. However, Noël *et al*. [37] confirmed the inhibitory capability of Cu on Cd accumulation, contrary to the current results.

To the best of our knowledge, the current study was the first one conducted in Egypt, quantitating all of Hg, Pb, Cd, Cu, and Zn in whole blood in a considerable sample size covering all the spectrum of confirmed MS. This fact could make the statistical tests more powerful. In addition, the Agilent 8800 ICP-MS-MS is a quick semi-quantitative and quantitative method of analysis with high precision, allowing analysis of tiny samples with low analyte concentration and simultaneous detection of several elements.

Conclusion and Recommendations

Conclusively, this study suggested the accumulation of the toxic metals (Pb, Hg and Cd) in MS patients at the expense of Zn and Cu. Our results support a complex profile of altered elements rather than a single element imbalance in MS pathogenesis. Furthermore, a possible link between toxic heavy metals levels and both the onset and severity of MS is suggested.

Further ante-mortem and cadaveric studies using CSF analysis and neural tissues, on a larger number of participants are needed to confirm the uneven distribution of trace elements and their prevalence to white matter in MS patients. Our findings encourage more environmental studies in Egypt to make a match between biological and environmental monitoring. In this context, special consideration should be given to the toxic metals these patients are chronically exposed. Further clinical trials are needed to assess the potential protective effect of both Cu and Zn supplementation to help decrease absorption of toxic heavy metals. Chelation therapy clinical trials are to be considered in MS patients.

Statements and Declarations

Authors contribution

Samar Ramadan Mohamed, Usama Mohamed El-Barrany and Ahmed Elshatory contributed to the study conception and design. Material preparation and data collection were performed by Samar Ramadan Mohamed, Hend A. Fadl and Islam M. Tork. Data analysis were done by Samar Ramadan Mohamed, Islam M. Tork, Omar, T. M. Y., Usama Mohamed El-Barrany and Ahmed Elshatory. The first draft of the manuscript was written by Samar Ramadan Mohamed and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The data analyzed during the current study are not publicly available as participants privacy could be compromised, but are available from the corresponding author on reasonable request.

Ethical approval

All procedures followed the principles outlined in the Declaration of Helsinki. The Ethical Committee of the Medical University of Cairo approved the study protocol (Approval Code: MD-140-2019).

Consent to participate

Informed consent was obtained from all study participants prior to their participation.

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