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RESEARCH ARTICLE

CHRONIC TOXIC-METAL POISONING AND NEURODEGENERATIVE DISEASES

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ABSTRACT

Background: Many epidemiological studies describe the role of toxic metals in the complex etiology of neurodegenerative diseases (ND): multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disese (AD), amyotrophic lateral sclerosis (ALS) Urine-sample analysis after provocation testing with chelating agents allows quantification of the toxic-metal burden in human subjects, including those affected by ND. Our objective was to compare the toxic-metal levels in the urine of patients with ND and those of healthy controls from the same geographical area (Italy), and to evaluate the effect of chelation therapy using calcium disodium ethylenediaminetetra acetic acid ($CaNa_2EDTA$, or EDTA) to eliminate the chronic poisoning caused by toxic metals.

Methods and Findings: Inductively coupled plasma mass spectrometry (ICP-MS) was used to evaluate the levels of 21 toxic metals (Al, Sb, As, Ba, Be, Bi, Cd, Ce, Gd, Pb, Hg, Ni, Pd, Pt, Te, Tl, Th, Sn, Ti, W, U) in the urine samples of 1147 patients who underwent the EDTA chelation test: 671 were affected by ND (MS, PD, AD, and ALS), 138 by non-ND (e.g. fibromyalgia, rheumatoid arthritis, peripheral neuropathies), and 338 were healthy controls (HC). Study design: before the start of chelation therapy, the patients underwent a "chelation test" to verify the possible toxic-metal burden. Thereafter, their toxic-metal burden was periodically monitored for more than one year. Our patients did not display the presence of Bi and Ti, while Be, Pt, Te, and Sn were present in only a small number of patients; Ab, Ba, and Th were more present, but to a lesser extent compared with Al, As, Cd, Cs, Gd, Pb, Tl, W, U, Hg, Ni, Pd. Interestingly, Be, Pd, Pt, Te, Tl, Th, Sn, W, and U were never present in MS patients, but were present in other ND patients. Poisoned patients affected by diseases were more numerous than HC. Except for As, toxic-metal presence was significantly more elevated in the ND patients. Repeated EDTA chelation therapy was able to remove all toxic metals with no adverse effects. One important limitation was that the source of intoxication was not always evident.

Conclusions: Our results underline the important relationship between the chronic body burden of some toxic metals and the presence of ND. Chelation therapy using EDTA can remove the toxic-metal burden.

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INTRODUCTION

It is difficult to establish how and when neurodegenerative diseases (ND) begin. The intricate etiologies of multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) appear to be multifactorial. Many studies focus on the damage to the central nervous system (CNS) caused by toxic substances, in particular the role of toxic metals that can lead to neurotoxicity - e.g. aluminum (Al) (Exley, 2014) - therefore representing a danger for the CNS. Acute exposure to lead (Pb) can increase the secretion of certain cytochemokines through microglial activation, and induce subsequent bystander neuronal death via caspase-3 activation (Kumawat *et al*., 2014). The exposure of mice to Pb enhances the expression of amyloid beta and phosphorylated tau proteins in the hippocampus, thus contributing to the impairment in spatial learning and memory at different life stages (Liu *et al*., 2014). Exposure to Pb at each stage of pregnancy has adverse effects on the neurobehavioral development of human neonates (Liu *et al*., 2014). Moreover, exposure to Pb is considered responsible for neuropsychological and functional decline in humans (Mason *et al*., 2014). The toxicity and neurodegenerative effects of mercury (Hg) on humans have recently been reported (Carocci *et al*., 2014), as well as the neurotoxic activity in the rat cerebral cortex of methylmercury (MeHg) via excessive induction of reactive oxygen species (ROS) (Liu *et al*., 2014). A relationship has been established between MeHg exposure via seafood consumption and the risk of developmental neurotoxicity (Sheehan *et al*., 2014). Finally, toxicity mechanisms induced by organic Hg species in cultured human astrocytes have also been noted (Pieper *et al*., 2014). The neurotoxic effects of cadmium (Cd) are associated with both biochemical changes of the cell and functional changes of the CNS (Wang and Du, 2013). In particular, significant modifications induced by Cd on enzyme activities in the brain regions of rat offspring (frontal cortex, hippocampus,

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hypothalamus, pons, and cerebellum) have been demonstrated (Stolakis *et al*., 2013). Neurobehavioral changes in mice induced by Nickel (Ni) have been related to aerobic metabolism deficiency provoked by this toxic metal (He *et al*., 2013). In addition, exposure of mouse neuroblastoma cell lines to Ni induces oxidative damage in mitochondrial DNA (Xu *et al*., 2011). Occupational exposure to solid wastes has recently been associated with the development of toxic neuropathies (Ekor and Odewabi, 2014). Arsenic (As), Pb, Hg, and thallium (Tl) are considered hazardous agents of industrial benefit. In particular, occupational-induced neuropathies are often secondary to low-level, long-term (chronic) exposure to toxic agents, and are related to the dose and duration of exposure, and to host factors. Likewise, taxi drivers have been shown to be extremely vulnerable to toxic metals: they are seen to have increased blood levels of Hg, As, Pb, and Cd compared to controls (Brucker *et al*., 2015). In the past, acute intoxication due to toxic metals, e.g. Pb or Al, in exposed workers was treated by chelation therapy for some days after blood and urine tests. Chronic intoxication is highlighted in the urine possibly only by submitting the patients to preventive chelation therapy to remove toxic metals from their organs. In fact, toxic metals bind to the chelating agents, are eliminated by the kidneys, and recovered in the urine samples (Brucker *et al*., 2015). In the present study we examined the relationship between chronic metal intoxication and the development of the most common neurodegenerative diseases. We submitted ND patients to an evaluation of their toxic-metal body burden. To do so we carried out the "chelation test", e.g. the intravenous infusion of the chelating agent calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$, or EDTA), followed by recovery of patient urine to evaluate its toxic-metal content. (Ferrero, 2016)

MATERIALS AND METHODS

Subjects

We studied 1147 doctor's office patients, age ranging from 18 to 82 years, who all chose to start chelation therapy. Some were affected by ND, while others were unaffected by any known disease, although they had previously been exposed to environmental or occupational heavy metals, who decided to undergo chelation therapy and acted as controls. Finally, we evaluated patients affected by non-neurodegenerative diseases (non ND) (e.g. fibromyalgia, rheumatoid arthritis, peripheral neuropathies). Each patient provided written informed consent. All procedures involving human participants followed Declaration of Helsinki guidelines, and were approved by Milan University's Ethics Advisory Committee (number 64/14). Personal data were recorded and analyzed in an anonymous format. All patients selected and enrolled for the study received chelation therapy once a week.

Study design

Before the start of chelation therapy, the patients were subjected to a "chelation test" (see below) to verify their possible toxic-metal burden. All patients underwent chelation therapy for almost three months. After ten applications, the patients underwent another chelation test to assess body-burden modifications; indeed, they were continuously monitored for toxic-metal burden throughout chelation therapy.

Chelation test

EDTA (2g), diluted in 500mL physiological saline (Farmax srl, Brescia, Italy), was slowly (over 2 hours) administered intravenously to subjects that were invited to collect urine samples before and after initial intravenous EDTA treatment. Urine collection following chelation treatment lasted 12 hours. Urine samples were accurately enveloped in sterile vials and sent to the Laboratory of Toxicology (Doctor's Data Inc., St. Charles, IL, USA) for analysis as previously reported (Ferrero, 2016). Samples were acid-digested with certified metal-free acids (digestion takes place in a closed-vessel microwave digestion system), diluted with ultrapure water, and examined via inductively coupled plasma mass spectrometry (ICP-MS), a reliable new method for reducing interference that uses collision/reaction cell methods coupled with ion-molecule chemistry,. Urine standards, both certified and in-house, were used for quality control and data validation. To avoid the potentially great margin of error due to fluid intake and sample volume, results were reported in micrograms (μg) per g of creatinine. When the first "chelation test" showed toxic-metal burden the patients initiated weekly chelation therapy.

Chelation therapy

When ten chelation therapy applications had been carried out, the patients underwent a further chelation test to verify their toxic-metal burden. For each patient a minimum of 3 mineralograms (value of toxic-metal burden in urine samples) to a maximum of seven mineralograms were performed. Each patient underwent from 30 to 70 chelation applications.

Toxic-metal analysis

Twenty-one toxic metals were analyzed: Al, antimony (Sb), As, barium (Ba), beryllium (Be), bismuth (Bi), Cd, cesium (Cs), gadolinium (Gd), Pb, Hg, Ni, palladium (Pd), platinum (Pt), tellurium (Te), Tl, thorium (Th), tin (Sn), titanium (Ti), tungsten (W) and uranium (U). Gadolinium was frequently used as a contrast agent in magnetic resonance imaging to diagnose ND.

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA), with the solution type as the main factor. Post hoc comparisons were made using Tukey's honestly significant difference test (HSD).

RESULTS

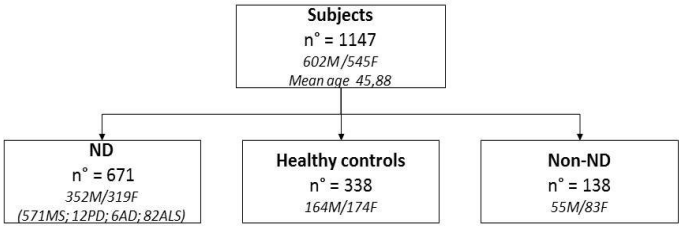
Patient characteristics

As shown in Figure 1, the patient population was classified as ND, non-ND, and healthy controls (HC (i.e. not affected by ND, non-ND, or other known diseases). The population was represented by non-significant distribution between men and women; mean patient age was 45.88. The majority of ND patients was affected by MS.

Toxic-metal burden in patient urine samples following chelation test

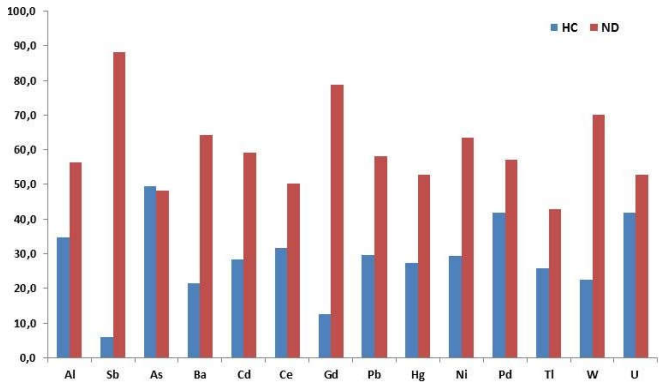
Patient urine samples collected before the chelation test did not reveal toxic-metal content (data not shown). The values of

toxic-metal burden assessed in the urine samples taken from patients following chelation test are shown in Table 1. The cut-off represents the threshold limit values of toxic metals, with higher values indicating toxicity. Those patients whose metal values were >cut-off are reported in the first line and indicated as A for each toxic metal in the total population (TP = all patients examined). The percentage of intoxication by each toxic metal with respect to the TP is shown line 2. Lines 3 and 4 respectively show the mean and the standard error of the mean (SEM) of the metal level values >cut-off. Lines five and six show the number of ND patients burdened with each toxic metal, and the percentage of these patients vs. A.



M/F=male/female

Figure 1. Characteristics of enrolled subjects



HC=healthy controls
ND=patients affected by neurodegenerative diseases

Figure 2. Percentage of patients affected by toxic-metal burden

Lines 7 and 8 show the mean value of toxic-metal levels >cut-off and the SEM in ND patients. Lines 9 and 10 show the number of MS patients affected by toxic-metal burden (i.e. levels of toxic metals >cut-off) and their percentage vs. A. Lines 11 and 12 report the mean values and SEM of toxic-metal levels >cut-off in MS patients. Lines 13 and 14 show the number of patients with a burden related to each toxic metal, and their percentage vs. A in non-ND patients. Lines 15 and 16 show the mean values of toxic-metal levels >cut-off and the SEM in non-ND patients. Lines 17 and 18 show the number of patients affected by toxic-metal burden relative to each toxic metal, and their percentage vs. A in HC. Finally, lines 19 and 20 report the mean values and SEM of toxic-metal levels >cut-off in HC. Patients affected by Al burden totaled 271, representing 24% of the TP. The cut-off value for Al is 25 µg/g creatinine measured in urine samples, and the mean value of Al>cut-off was 116.92±7.10. Neurodegenerative-disease patients affected by Al burden totaled 166 (61% of A), with a mean value of Al>cut off =58.53±5.81. Of the ND patients affected by Al burden, the majority were MS patients: 143 (53% of A), with a mean value of Al>cut-off=56.86±5.62. Non-ND patients affected by Al burden totaled 21 (8% of A), with a mean value of Al>cut-off=45.01±4.76. Patients classified as HC affected by Al burden totaled 84% (31% of A), with a mean value of Al>cut-off=59.51±3.80. Indeed, more than the 50% of the population affected by Al burden was represented by ND patients, particularly by MS patients. However, both HC and non-ND patients were also affected by Al burden. Twenty patients were affected by Sb burden, representing 2% of TP. The cut-off value for Sb is 0.30 µg/g creatinine in urine samples, and the mean value of Sb>cut-off was 0.58±0.13. Neurodegenerative disease patients affected by Sb burden totaled 18, (90% of A), with a mean value of Sb>cut-off =0.08±0.01. Once again, the most highly represented ND was MS: 13 patients (65% of A), with a mean value of Sb>cut off= 0.07±0.003. Only one non-ND patient was affected by Sb burden (5% vs. A) with the value of Sb>cut-off=0.03. Only one HC patient was affected by Sb burden (5% vs. A), with a level of Sb>cut-off=0.06. Neurodegenerative-disease patients were those mostly affected by Sb burden.

Patients affected by As burden totaled 118 (10% of TP). The cut-off value for As is 108.00 µg/g creatinine in urine samples, with a mean value of As>cut-off=359.29±33.08. Sixty-five ND patients were affected by As (55% of A), with a mean value of As>cut-off=77.11±7.27. Fifty-seven patients had MS (48% of A), with a mean value of As>cut-off=77.95±8.04. Non-ND patients affected by As burden totaled 9 (8% of A), with a

Table 1. Toxic-metal burden in urine samples of patients following chelation test

	Cut off	N° pz>cutoff (A)	% TP	Mean (>cutoff)	SEM	N° Pz>cutoff ND	% Vs A	Mean (>cutoff)	SEM	N° Pz>cutoff MS	% Vs A	Mean (>cutoff)	SEM	N° Pz>cutoff non ND	% Vs A	Mean (>cutoff)	SEM	N° Pz>cutoff HC	% Vs A	Mean (>cutoff)	SEM
Aluminum	*25,00*	271	23,6	116,92	7,10	166	61	58,53	5,81	143	53	56,86	5,62	21	8	45,01	4,76	84	31	59,51	3,80
Antimony	*0,30*	20	1,7	0,58	0,13	18	90	0,08	0,01	13	65	0,07	0,03	1	5	0,03	nd	1	5	0,06	nd
Arsenic	*108,00*	118	10,3	359,29	33,08	65	55	77,11	7,27	57	48	77,95	8,04	9	8	51,92	6,96	44	37	130,78	12,20
Barium	*7,00*	23	2,0	45,04	4,39	18	78	3,85	0,63	6	26	3,05	0,65	2	9	1,98	nd	3	13	0,99	nd
Beryllium	*1,00*	3	0,3	1,71	nd	1	33	0,04	nd	0	0	0,00	nd	0	0	0,00	nd	2	67	0,07	0,10
Bismuth	*10,00*	0	0,0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	nd
Cadmium	*0,80*	580	50,6	3,38	0,14	374	64	3,31	0,19	327	56	3,08	0,15	59	10	2,59	0,07	147	25	2,89	0,11
Cesium	*9,00*	206	18,0	16,48	1,15	113	55	9,09	0,56	10	5	8,78	0,53	32	16	10,62	0,50	61	30	9,93	0,50
Gadolinium	*0,30*	425	37,1	53,62	2,60	354	83	52,47	5,79	88	21	58,83	6,62	31	7	10,33	1,67	40	9	4,68	0,67
Lead	*2,00*	635	55,4	27,52	1,09	406	64	29,86	2,49	357	56	22,92	1,56	65	10	20,27	0,67	164	26	24,10	0,73
Mercury	*3,00*	65	5,7	5,12	0,64	37	57	1,54	0,21	32	49	1,55	0,22	11	17	1,71	0,21	17	26	1,63	0,19
Nickel	*10,00*	152	13,3	18,41	1,49	102	67	9,14	0,80	86	57	8,80	0,75	11	7	7,20	0,33	39	26	8,20	0,49
Palladium	*0,30*	128	11,2	0,75	0,07	75	59	0,16	0,03	0	0	nd	nd	3	2	nd	nd	50	39	0,23	0,03
Platinum	*1,00*	2	0,2	310,69	nd	1	50	nd	nd	0	0	nd	nd	0	0	nd	nd	1	50	nd	nd
Tellurium	*0,80*	1	0,1	1,00	nd	1	100	nd	nd	0	0	nd	nd	0	0	nd	nd	0	0	nd	nd
Thallium	*0,50*	70	6,1	1,22	0,15	39	56	0,28	0,04	0	0	nd	nd	12	17	0,56	0,07	19	27	0,34	0,04
Thorium	*0,03*	11	1,0	0,12	0,04	6	55	0,010	0,001	0	0	nd	nd	2	18	nd	nd	3	27	nd	nd
Tin	*9,00*	5	0,4	503,12	nd	5	100	nd	nd	0	0	nd	nd	0	0	nd	nd	0	0	nd	nd
Titanium	*15,00*	0	0,0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	0,00	0	0	0,00	0,00
Tungsten	*0,40*	75	6,5	0,93	0,11	53	71	0,25	0,03	0	0	nd	nd	11	15	0,22	0,03	11	15	0,20	0,03
Uranium	*0,03*	132	11,5	0,09	0,01	77	58	0,02	0,001	0	0	nd	nd	6	5	0,01	0,001	49	37	0,02	0,001

mean value of As>cut-off=51.92±6.96. Patients classified as HC affected by As burden totaled 44 (37% of A), with a mean value of As>cut-off=130.78±12.20. Twenty-three patients were affected by Ba burden (2% of TP). The cut-off value for Ba is 7.00 µg/g creatinine, and the mean value of Ba>cut-off was=45.04±4.39. Eighteen ND patients were affected by Ba burden (78% of A), with a mean value of Ba>cut-off=3.85±0.63. Of the ND patients, 6 were affected by MS (26% of A), with a mean value of Ba>cut-off=3.05±0.65. Only 2 patients (9% of A) affected by Ba burden were non-ND patients, with a mean of Ba levels>cut-off=1.98. Three HC patients were affected by Ba burden (13% of A), with a mean of Ba levels >cut-off=0.99. Only 3 patients were affected by Be burden. The cut-off value for Be is 1.00 µg/g creatinine, and the mean value of Be>cut off was 1.71. One ND patient and two HC displayed Be intoxication.

No patient was affected by Bi intoxication

Patients affected by Cd burden totaled 580 (51% of TP). The cut-off value for Cd is 0.80 µg/g creatinine, and the mean value of Cd>cut-off was=3.38±0.14. Neurodegenerative disease patients affected by Cd burden totaled 374 (64% of A), with a mean value of Cd>cut-off=3.31±0.19. Most of the ND patients were affected by MS, (327 = 56% of A), with a mean value of Ba>cut-off=3.08±0.15. Non-ND patients affected by Cd burden totaled 59 (10% of A), with a mean value of Cd>cut-off=2.59±0.07. Finally, HC totaled 147 (25% of A), with a mean value of Cd>cut-off=2.89±0.11. Patients affected by Cs burden totaled 206 (18% of TP). The cut-off value for Cs is 9.00 µg/creatinine, and the mean value of Cs>cut-off was=16.48±1.15. Neurodegenerative-disease patients affected by Cs burden totaled 113 (55% of A), with a mean value of Cs>cut-off=9.09±0.56. Of the ND patients, 10 were affected by MS (5% of A), with a mean value of Cs>cut-off=8.78±0.53. Non-ND patients affected by Cs burden totaled 32 (16% of A), with a mean value of Cs>cut-off=10.62±0.50. Healthy controls with Cs burden totaled 61 (30% of A), with a mean value of Cs>cut-off=9.93±0.50. Patients affected by Gd intoxication totaled 425 (37% of TP). The cut-off value for Gd is 0.30µg/g creatinine, and the mean value of Gd>cut-off was=53.62±2.60. Most of the patients affected by Gd burden suffered from ND, (354 = 83% of A), with a mean value of Gd>cut-off=52.47±5.79. Of the ND patients, 88 were affected by MS (21% of A), with a mean value of Gd>cut-off=58.83±6.62. Non-ND patients affected by Gd burden totaled 31 (7% of A), with a mean value of Gd>cut-off=10.33±1.67. Of the HC, 40 were affected by Gd burden (9% of A), with a mean value of Gd>cut-off=4.68±0.67. Patients affected by Pb intoxication totaled 635 (55% of TP). The cut-off value for Pb is 2.00µg/creatinine, and the mean value of Pb>cut off was 27.52±1.09. Neurodegenerative-disease patients affected by Pb burden totaled 406 (64% of A), with a mean value of Pb>cut-off=29.86±2.49. The majority of ND patients affected by Pb burden suffered from MS, (357 = 56% of A), with a mean value of Pb>cut off=22.92±1.56. Non-ND patients affected by Pb burden totaled 65 (10% of A), with a mean value of Pb>cut-off=20.27±0.67. Healthy controls affected by Pb burden totaled 164 (26% of A), with a mean value of Pb>cut-off=24.10±0.73.

Patients affected by Hg intoxication totaled 65 (6% of TP). The cut-off value for Hg is 3.00µg/g creatinine, and the mean value of Hg>cut off was 5.12±0.64. Neurodegenerative-disease patients affected by Hg burden totaled 37 (57% of A), with a mean value of Hg>cut-off=1.54±0.21. Of the ND patients, 32 were affected by MS (49% of A), with a mean value of Hg>cut-off=1.55±0.22. Non-ND patients bearing Hg burden totaled 11 (17% of A), with a mean value of Hg>cut-off=1.71±0.21. Healthy controls bearing Hg burden totaled 17 (26% of A), with a mean value of Hg>cut-off=1.63±0.19. Patients affected by Ni intoxication totaled 152 (13% of TP). The cut-off value for Ni is 10.00µg/g creatinine, and the mean value of Ni>cut-off was 18.41±1.49. Patients with ND affected by Ni burden totaled 102 (67% of A), with a mean value of Ni>cut-off=9.14±0.80. Patients with MS affected by Ni burden totaled 86 (57% of A), with a mean value of Ni>cut-off=8.80±0.75. Non-ND patients affected by Ni burden totaled 11 (7% of A), with a mean value of Ni>cut-off=7.20±0.33. Healthy controls bearing Ni burden totaled 39 (26% of A), with a mean value of Ni>cut-off=8.20±0.49. Patients affected by Pd intoxication totaled 128 (11% of TP). The cut-off value for Pd is 0.30µg/g creatinine and the mean value of Pd>cut-off was 0.75±0.07. Patients with ND affected by Pd burden totaled 75 (59% of A), with a mean value of Pd>cut-off=0.16±0.03. It is interesting to note that no MS patient was affected by Pd intoxication. Non-ND patients affected by Pd burden totaled only 3 (2% of A). Healthy controls affected by Pd burden totaled 50 (39% of A), with a mean value of Pd>cut-off=0.23±0.03. Only 2 patients were affected by Pt intoxication: one ND patient and one HC. The cut-off value for Pt is 1.00µg/g creatinine, and the mean value of Pt>cut-off was 310.69. Only 1 patient was affected by Te intoxication. This patient suffered from ND. The cut-off value for Te is 0.80µg/g creatinine, and the value of Te>cut-off was 1. Patients affected by Tl intoxication totaled 70 (6% of TP). The cut-off value for Tl is 0.50µg/g creatinine, and the mean value of Tl>cut-off was 1.22±0.15. Patients with ND affected by Tl burden totaled 39 (56% of A), with a mean value of Tl>cut-off=0.28±0.04. No MS patients presented Tl intoxication. Non-ND patients affected by Tl intoxication totaled 12 (17% of A), with a mean value of Tl>cut-off=0.56±0.07. Healthy controls affected by Tl burden totaled 19 (27% of A), with a mean value of Tl> cut-off=0.34±0.04. Patients affected by Th intoxication totaled 11 (1% of TP). The cut-off value for Th is 0.03µg/g creatinine, with a mean value of Th>cut-off=0.12±0.04. Patients with ND affected by Th burden totaled 6 (55% of A), with a mean value of Th>cut-off=0.01±0.001. No MS patient was affected by Th intoxication. Non-ND patients affected by Th burden totaled 2 (18% of A), while HC totaled 3 (27% of A). Patients affected by Sn burden totaled 5. The cut-off value for Sn is 9.00µg/g creatinine, and the mean value of Sn>cut-off was 503.12. All patients affected by Sn burden were ND patients, but no MS patient was present. No patient was affected by Ti intoxication, whose cut off value is 15.00µg/g creatinine.

Patients affected by W intoxication totaled 75 (7% of TP). The cut-off value for W is 0.40µg/creatinine, and the mean value of W>cut-off was 0.93±0.11. Neurodegenerative-disease patients affected by W burden totaled 53 (71% of A), with a mean value of W>cut-off=0.25±0.03. No MS patients presented W intoxication. Non-ND patients with W burden totaled 11 (15% of A), with a mean value of W>cut-off=0.22±0.03. Healthy controls affected by W burden totaled 11 (15% of A), with a mean value of W>cut-off=0.20±0.03. Patients affected by U intoxication totaled 132 (12% of TP). The cut-off value for U is 0.03µg/g creatinine, and the mean value of U>cut off was 0.09±0.01. Neurodegenerative-disease patients affected by U burden totaled 77 (58% of A), with a mean value of U>cut-off=0.02±0.001.

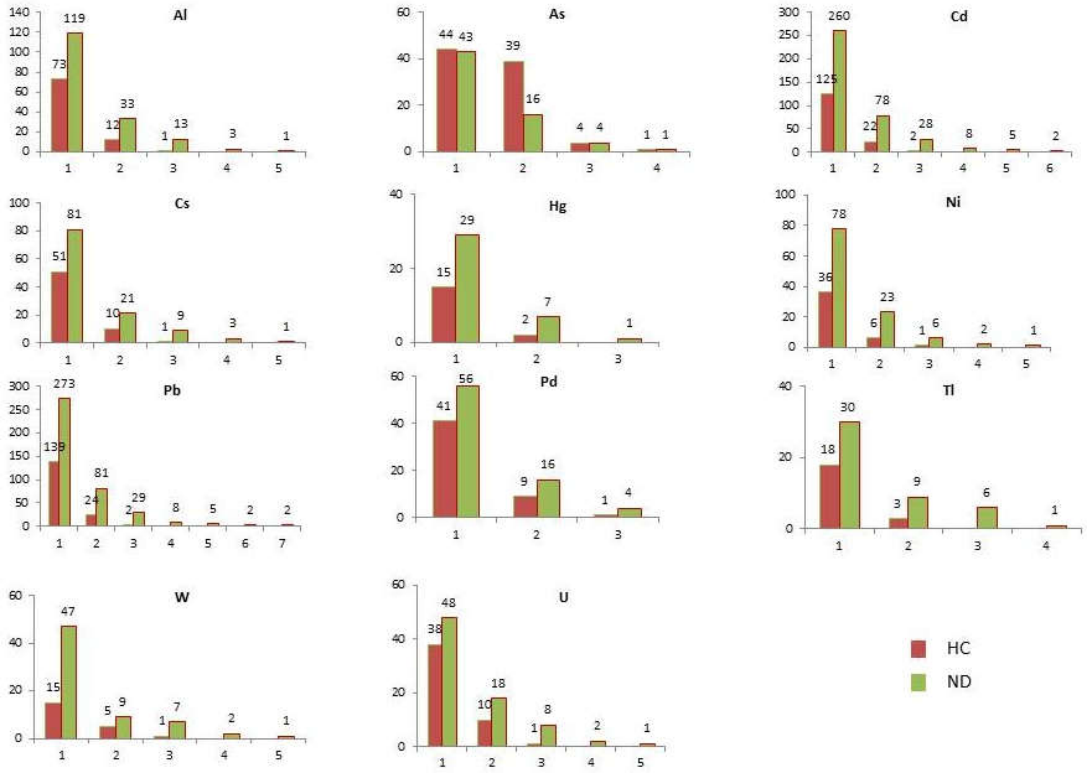


Figure 3. Number of poisoned patients as revealed by mineralogram (i.e. measurement of toxic-metal burden in urine samples under EDTA challenge)
1-7= number of performed mineralograms:
1=after chelation test
2=after 10 chelation applications
3=after 20 chelation applications
4=after 30 chelation applications
5=after 40 chelation applications
6=after 50 chelation applications
7=after 60 chelation applications
Number of patients affected by toxic-metal burden as revealed by urine mineralograms are reported in *italics*

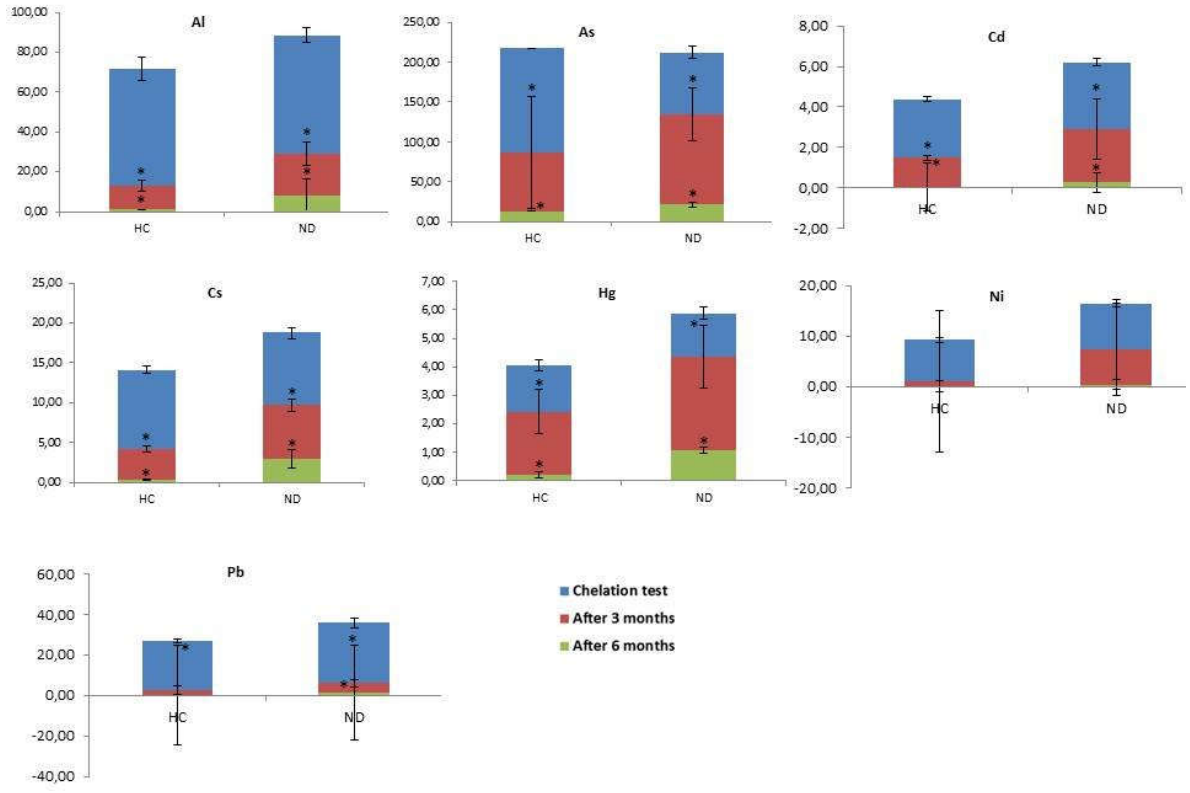


Figure 4. Al, As, Cd, Cs, Hg, Ni, and Pb, , , , and levels evaluated in urine samples of the examined subjects following chelation test (blue) and after 3 months (red) and 6 months (green) of chelation therapy and expressed as meas ± SEM of µg/g creatinine. The studied subjects were healthy controls (HC) and patients affected by neurodegenerative diseases (ND). *P<0.05 vs. blue

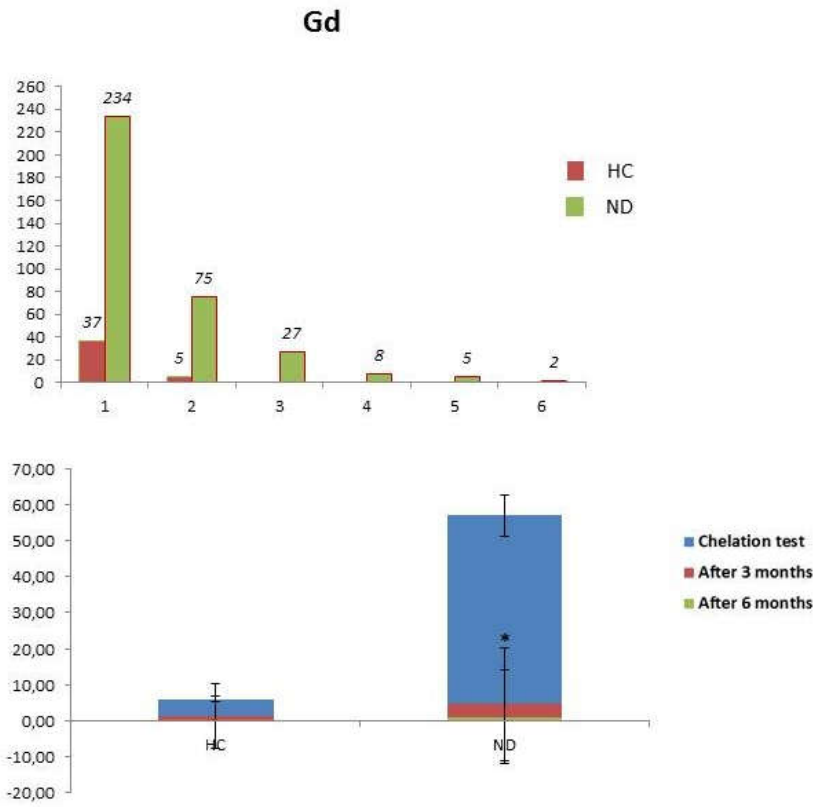


Figure 5.

No MS patients were affected by U intoxication. Non-ND patients affected by U burden totaled 6 (5% of A), with a mean value of U>cut-off=0.01±0.001. Healthy controls affected by U burden totaled 49 (37% of A), with a mean value of U>cut-off=0.02±0.001. It needs to be noted that some patients were poisoned by various toxic metals, while others were poisoned only by one or two specific toxic metals.

Percentage of patients affected by each toxic metal burden vs. total poisoned population

We examined the number of poisoned patients in relation to each toxic metal, highlighting those patients affected by ND, those unaffected by ND, and non-ND (i.e. HC) patients. Figure 2 shows the percentage of ND and HC patients affected by toxic-metal burden with respect to the total population bearing toxic metals.

We did not consider Be, Bi, Pl, Te, Th, Sn, and Ti due to the inconsistent number of patients bearing these toxic metals. Except for As, all ND patients presented a more elevated toxic-metal burden compared with HC.

Reduction of poisoning following chelation therapy

Figure 3 shows the number of poisoned patients who spontaneously underwent numerous chelation therapy applications. Evaluation refers to the more important toxic metals used to determine patient poisoning, as reported in Table 1. The number of mineralograms performed is reported for each toxic metal. Mineralograms express the levels of burden, measured in µg toxic metal/g creatinine in the urine samples, which exceed the cut-off. This is indicated by progressive numbering (after 1) in relation to the chelation therapies (number x 10) carried out: number 1 is the result of the chelation test; 2=10 chelation applications; 3=20 chelation applications; 4=30 chelation applications; 5=40 chelation applications; 6=50 chelation applications; 7=60 chelation applications. The values reported relate to ND patients and HC. The numbers in italics represent the poisoned patients. Neurodegenerative-disease patients affected by Al poisoning were more numerous than HC, as evaluated by the chelation test. The number of Al poisoned patients fell significantly both in ND patients and in HC after 10 chelation therapy applications, and decreased even further following 20 or more chelation therapy applications. The 5th mineralogram revealed the presence of Al in the urine samples in only a few ND patients and HC, who displayed levels of Al only slightly higher than those of cut-off values. Arsenic levels were equally elevated in the ND and HC populations. Ten chelation therapy applications were able to significantly reduce the number of ND poisoned patients, but not of HC. The 4th mineralogram demonstrated the presence of As in only one ND patient and in one HC.

The number of patients poisoned by Cd was significantly more elevated in ND patients than in HC. The poisoned patients progressively decreased following numerous chelation therapy applications. The number of patients poisoned by Cs was more elevated in ND patients than in HC. The decrease in Cs levels was similar to those of Cd following chelation therapy. Neurodegenerative-disease patients affected by Hg, as well as by Ni poisoning, were more than HC; the number of poisoned patients fell dramatically following numerous chelation therapy applications. Neurodegenerative-disease patients affected by Pb poisoning were more than HC, and their number reduced progressively following chelation therapy. Analogous results were obtained in Pd poisoned patients.

There were more ND patients poisoned by Tl than HC. Decrease of Tl occurred after about 30 chelation therapy applications. The number of ND patients poisoned by W was more elevated than HC; W disappeared after about 40 chelation therapy applications. The number of ND patients poisoned by U was not as elevated compared to HC. The number of poisoned patients progressively decreased following numerous chelation therapy applications.

Levels of the more represented toxic metals (Al, As, Cd, Cs, Hg, Ni, and Pb) in the urine samples of patients after the chelation test and following different amounts of chelation therapy

The patients who underwent chelation therapy displayed a significant reduction of toxic-metal levels in urine samples. Figure 4 shows values concerning Al, As, Cd, Cs Hg, Ni, and Pb after the chelation test, and their reduction after 3 and 6 months, respectively, of chelation therapy. Chelation therapy was able to significantly remove Al burden after 3 and 6 months of treatment both in ND patients and in HC. Analogously, As, Cd, Cs, Hg, and Pb levels were significantly reduced by chelation therapy lasting 3 months, and then reduced after 6 months with respect to the values obtained following chelation test. The exception concerns the significance of Pb reduction in HC, which was evident after 6 months of chelation therapy but not after 3 months. Nickel levels were reduced after 3 and 6 months of chelation therapy, but this reduction was not significant with respect to the values obtained following chelation test. Indeed, the Ni burden remained mostly elevated in one patient only: high SEM values did not render significant the statistical evaluation.

DISCUSSION

To date, only a small number of commercial chemicals have been tested and listed as developmental neurotoxicants. Moreover, an increasing number of epidemiological, clinical, and experimental studies suggest an association between the exposure to toxicants or drugs during the perinatal period and the development of metabolic-related diseases and neurotoxicity later in life (Fox *et al*., 2012). Among neurotoxicants, exposure to inorganic Hg, as well as MeHg, and to Pb have been associated with brain-development deficits and neurodegeneration. Moreover, trace elements such as Pb, MeHg, and As (and, to a lesser extent, Cd) have been described as paradigms of developmental neurotoxicants (Grandjean and Herz, 2014). For decades, autophagy, i.e. the process that under certain circumstances delivers cytoplasmic components to the lysosomes for degradation, has been considered a cell-death pathway. However, it has recently been shown to have a survival function through the clearing of protein aggregates and damaged cytoplasmic organelles in response to a variety of stress conditions. Increasing evidence in the literature reveals that the induction of autophagy might contrast neurodegeneration (Radad *et al*., 2012). In this context, exposure to some heavy metals, such as Cd, Pb, and MeHg, has been associated with the autophagic dysfunction present in PD and in AD (20). Metals are frequently used in industry, and represent the major source of exposure to toxins in workers. Essential metals, such as iron (Fe), copper (Cu), and manganese (Mn), have physiological roles, but high levels of such metals can create many health risks. In fact, Mn and Fe are known to be involved in the neuropathology of PD (Kwakye *et al*., 2016). Moreover, Fe and Mn can provoke oxidative stress and neurodegeneration (Farina *et al*., 2013). The CNS is particularly vulnerable to metals. The brain readily accumulates metals, which, under physiological conditions, are incorporated into the metalloproteins required for neuronal health and energy homeostasis. However, an excess of essential metals, or exposure to toxic non-essential metals (Al, As, Pb, Hg) due to occupational metal exposure, can induce neurotoxicity (Caito and Aschner, 2015). Toxic-metal poisoning can affect the CNS following not just acute but also chronic intoxication. Indeed, Pb, Hg, and Cd have been shown to remain in the brain for long periods of time, possibly becoming responsible for chronic neurotoxicity (Gilani *et al*., 2015).

Due to the important neurological implications related to toxic-metal poisoning in humans, we studied the levels of toxic-metal burden in patients affected by ND, and compared them to the levels measured in healthy controls. Moreover, we studied the usefulness of chelation therapy using EDTA in patients affected by toxic-metal poisoning. Trace elements in scalp hair samples from patients with relapsing-remitting MS have already been measured, showing a different distribution when compared with controls (Tamburo *et al*., 2015). The measurement of trace elements in hair is usually adopted in children with autism spectrum disorders and communication disorders (Skalny *et al*., 2016). In the recent past, the severity of autism has been associated with toxic-metal body burden (Adams *et al*., 2009), and the significant elevation of the trace element Cu, and of the toxic elements Pb and Hg has been observed in the hair of autistic children (Lakshmi Priya and Geetha, 2011). However, hair represents the least used method of body burden detoxification, and reveals only the temporary presence of toxic substances. In humans, poisons are eliminated mainly by the kidneys, and by the gut after oral contamination. The deposition of toxic metals in human organs can last many years, and it is necessary to provoke the elimination of such metals with chelating agents. We studied the possible poisoning of the patients using the chelation test with EDTA (Ferrero, 2016). We have already shown the involvement of Al in neurotoxicity; we demonstrated that Al levels were significantly higher in ND patients than in healthy subjects, and that treatment of these patients with EDTA chelation therapy significantly reduced Al intoxication (Fulgenzi *et al*., 2014). Moreover, we showed that prolonged treatment with EDTA, together with the antioxidant cellfood, can efficiently eliminate Al intoxication (Fulgenzi *et al*., 2015). In the present study, we also examined the possible poisoning of neurodegenerative-disease and non-neurodegenerative-disease patients and healthy controls with all the metals that are considered toxic for humans. We studied 1147 patients, 671 of whom were affected by ND. In each patient we evaluated the efficacy of long-term chelation therapy to remove toxic-metal poisoning. Our results highlight many important points that require consideration.

All patients were affected, at different percentages with respect to the total population, by toxic-metal burden: ND and non-ND patients, and HC, as shown by the measurement of 21 toxic metals in urine samples following EDTA (chelation test) (Table 1). Interestingly, some known toxic metals were never shown to be responsible for human intoxication e.g. Bi and Ti, whereas Be, Pt, Te, and Sn were responsible for only a small number of cases of intoxication. The transdermal delivery of Bi, a heavy metal, and Al salts induced an atypical primitive neuroectodermal tumor in a chronically exposed patient, whose intracellular accumulation was detected by elemental

microanalysis (Roncati *et al*., 2015). Heavy metals, such as Bi and Hg, appear to accumulate during aging in human spinal interneurons, as well as in a few α-motoneurons. Toxicant-induced dysfunction of spinal interneurons could lead to the damage or loss of α-motoneurons, especially in susceptible individuals. As recently reported, this could manifest in a spectrum of motoneuron disorders, which include ALS/motor-neuron disease, sarcopenia, benign fasciculation syndrome, and amyotrophy in MS (Pamphlett and Jew, 2016). However, Bi intoxication appears very rarely in humans following abuse of Bi salts for the treatment of peptic ulcers, dyspepsia, and chronic gastritis (Erden *et al*., 2013). Acute or prolonged exposure to Ti dioxide nanoparticles can induce toxicity on human cerebral cell lines at particularly low doses, and is comparable to Ti levels detected in laboratory animals undergoing intranasal treatment with Ti dioxide nanoparticles associated to neurotoxic effects (Coccini *et al*., 2015). However, no Ti intoxication has been reported in humans. Platinum, which in our study was responsible for just 2 intoxications, is used to treat tumors; its antitumoral use has increased our knowledge regarding the toxic side-effects of this metal (Köppen *et al*., 2015). Beryllium was responsible for just three intoxications in the present study**;** Be, which is deposited in the atmosphere (36), was recently detected in the urine and blood of the adult population of Northern France, following occupational and environmental exposure (Nisse *et al*., 2016). Tellurium was present in only 1 patient in our study. Only a small number of occupational cases of Te intoxication have previously been reported; the use of nanoparticles has highlighted this fact, although the dissolved metal seems to be more toxic than the nanoparticles themselves (Bruneau *et al*., 2015). Tin (Sn) exists naturally in the soil, and can be ingested through the consumption of vegetables, fruit, fish, or meat. The ingestion of naturally occurring Sn is not harmful to humans. However, it can combine with chloride, sulfur, and oxygen, and be transformed into inorganic Sn. Inorganic Sn is generally non-toxic, except in the case of excessive ingestion, because of its limited absorption by the gastrointestinal tract, and its rapid elimination from the body. Organotin compounds, especially dimers and trimers, are commonly used in industry and agriculture as stabilizers, catalysts, and biocides. They can be absorbed through inhalation, ingestion, or dermal exposure, and are excreted via the biliary or renal systems. In contrast to inorganic Sn compounds, organic Sn compounds are highly toxic, and MRI evidence of leukoencephalopathy in workers exposed to organotin with amnestic disorders has been shown (Lee *et al*., 2016). We demonstrated that 11 patients were affected by Th intoxication: Th is used in agriculture (Avelar *et al*., 2016). Finally, Sb, the prenatal exposure to which was previously analyzed in a cohort of pregnant women from an urban center (Fort *et al*., 2014), was present in 20 of the studied patients. Barium, whose toxicity has already been described (Bhoelan *et al*., 2014), was present in 23 of our patients. All of the other toxic metals were found in an elevated number of patients.

Interestingly, Be, Pd, Pt, Te, Tl, Th, Sn, W, and U were never present as toxicants in SM patients, although they were present in other ND patients. Toxic metals were present in ND and non-ND patients, and also in HC. However, except for As, their presence was always more elevated in patients affected by diseases than in controls. The most elevated levels of Gd were found in SM patients, who underwent numerous MRI scans to control demyelinating areas. Gadolinium is a heavy metal with the atomic number 64 that belongs to the lanthanide family. It was recently demonstrated that Gd-based contrast agents are highly toxic, and that Gd is deposited in the dentate nucleus, globus pallidus, and pulvinar region of the thalamus (Kanda *et al*., 2016). A spectrum of adverse effects and toxicity caused by Gd that are not only related to the brain have also been reported (Ramalho *et al*., 2016). Chelation therapy with EDTA is known to be able to remove all of the toxic metals studied. Patient intoxication was drastically reduced or eliminated as a result of numerous chelation therapy applications due to the fact that the patients analyzed were affected by "chronic" intoxication. Therefore, repeated chelation therapy was useful to progressively eliminate the toxic-metals deposited in the body. The treatment was safe for every patient; ND patients were advised to use integrators, such as cellfood (Fulgenzi *et al*., 2014) and glutathione. Neurological symptoms improved, as previously reported for Al intoxication (Fulgenzi *et al*., 2014; Fulgenzi *et al*., 2015). EDTA chelation therapy is known to be useful in the treatment of cardiovascular disease, and metal contamination has been shown to have an important link with atherosclerosis (Peguero *et al*., 2014; Solenkova *et al*., 2014). The use of EDTA for secondary prevention in post-myocardial infarction patients has been suggested when the patient has been chronically exposed to toxic metals (Lamas and Issa, 2016; Aneni *et al*., 2016). Why are toxic metals present as contaminants also in HC? Why are they unable to provoke ND or cardiovascular disease in such patients? A possible explanation lies in the fact that ND patients have problems eliminating toxic metals.

Many of them displayed low levels of glutathione (GSH), which is produced intracellularly from three amino acids (glutamate, cysteine, and glycine). Glutathione is oxidized to GSH disulfide (GSSG) by glutathione peroxidase, and then regenerated as GSH following reaction with GSH reductase (Jacob *et al*., 2013). Glutathione plays a key role in cell resistance against oxidative damage by providing the enzymes involved in ROS metabolism with reducing equivalents, by eliminating potentially toxic oxidation products, and by reducing oxidized protein thiols (Aoyama and Nakaki, 2013). The availability of GSH under oxidative conditions is ensured by GSH recycling and biosynthetic pathways, which can be upregulated when oxidative stress occurs. The measurement of GSH and its disulfide forms (GSSG) in blood and their ratios is considered an index of whole-organism oxidative status, and is a useful indicator of disease risk in humans (Rossi *et al*., 2006). Recently, Aoyama and Nakaki showed that GSH function disorder is implicated in the etiology of some ND, such as AD, PD, ALS, progressive supranuclear palsy, Huntington's disease, and MS (Aoyama and Nakaki, 2013). According to this study, the ND patients examined in our previous paper showed lower GSH levels at baseline than controls (Fulgenzi *et al*., 2014). We also previously reported the case of a patient affected by MS, who displayed an altered GSH/GSSH ratio in relation to the genetic variant, resulting in a lack of expression of the gene for one of the two most relevant human isoenzymes of cytosolic glutathione S transferase (Fulgenzi *et al*., 2012). Comparison of toxic-metal concentrations in the urine of athletes and sedentary subjects living in the same area suggests that physical activity can counteract, at least in part, the cumulative effect of a toxic environment by increasing the urinary excretion of toxic metals in people that exercise regularly (Lerena *et al*., 2012). Patient lifestyle is therefore important to avoid possible future re-intoxication, e.g. smoking cessation, and the elimination of contaminated food or drugs. In conclusion, our results show that toxic-metal poisoning can

be implicated in the complex etiology of neurodegenerative diseases.

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S1 Appendix

EDTA dose

A recent trial assessed that in stable post-myocardial-infarction patients the combination of oral high-dose vitamins and EDTA chelation therapy, compared with a double placebo, reduced clinically important cardiovascular events to an extent that was both statistically significant and of potential clinical relevance (55). Chelation infusion contents in these patients contained a variable dose of ethylenediaminetetraacetic acid up to a maximum of 3g depending on estimated glomerular filtration rate, and were Na_2EDTA based. A regimen of 40 infusions was carried out. In the past, the protocol to remove acute Pb intoxication due to accidental dramatic inhalation of toxic metal in factory workers in Italy was based on one chelation therapy application once a day for three consecutive days. Acute intoxication is, at present, rare. In our experience, 2g EDTA once a week in adults (infused slowly in around two hours) is a safe dose that excludes side-effects and can, when administered on numerous occasions, correct toxic-metal burden in patients affected by chronic intoxication. Moreover, we do not suggest the addition of vitamins, such as vitamin C, in the EDTA solution. Indeed, in 2009 a French group showed that EDTA chelation therapy, without added vitamin C, decreased oxidative DNA damage and lipid peroxidation (56). Vitamins and integrators can be successfully used orally after chelation therapy. Patient plasma electrolytes were always well maintained at physiologic values (data not shown). Analyzed blood parameters showed: total cholesterol (TC), total antioxidant capacity (TAC), and serum active vitamin B12 (Vit B12) improved significantly, and serum reactive oxygen species (ROS) and homocysteine levels dropped significantly after six months of chelation therapy, in accordance with previously reported results (30).

S2 Further patient characteristics

Several patients affected by ND had previously been treated with conventional drugs, and had spontaneously interrupted therapy around two months before starting chelation treatment (MS patients, who suspended the use of immunosuppressant or immunomodulatory drugs). Around 65% were cigarette smokers, or were exposed to passive smoking. Some of them (40%) were ND patients who had smoked around 20 cigarettes per day for 30-40 years before the onset of ND. Before joining this study, 98% of the smokers discontinued cigarette use, or cut down to 1-2 cigarettes per day. A small percentage (2%) of patients refused to stop smoking, and were thus declined entry to our chelation therapy protocol. These patients were excluded from our study even when they displayed severe poisoning after chelation test (data not reported). The toxic metals mostly seen to be present in smokers after chelation test were Pb and Cd. Other important considerations relate to the previous infectious diseases reported in the anamnestic data of ND patients. We found 15% of MS patients to be positive for IgG antibodies against the Epstein Barr virus following western blot (immunoblotting) analysis: association has been shown with cytomegalovirus positivity.

S3 Gd elimination

Both ND patients and HC who underwent magnetic resonance imaging (MRI) with Gd as a contrast agent showed Gd poisoning. The ND patients affected by Gd poisoning were more numerous than HC owing to the use of MRI as a diagnostic tool in ND patients. Figure 5 shows that Gd was significantly reduced only after 6 months of repeated chelation therapy in ND patients. The reduction of Gd levels in HC patients was not significant due to the limited values of Gd intoxication.

S4 Which mechanism explains the usefulness of chelation therapy?

We previously showed that EDTA is able to modulate in vitro the activation of human umbilical vein endothelial cells induced by TNFα. This can ameliorate endothelial cell function, preventing the development of atherosclerosis and partially removing the endothelial activation provoked by toxic metals (57). In the same paper, we showed the distribution of labeled EDTA in the CNS (57).

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