Journal of Trace Elements in Medicine and Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Lead, cadmium and mercury in cerebrospinal fluid and risk of amyotrophic lateral sclerosis: A case-control study

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ARTICLE INFO

Article history:
Received 16 September 2016
Accepted 31 December 2016

Keywords:
Amyotrophic lateral sclerosis
Cerebrospinal fluid
Environment
Lead
Cadmium
Mercury

ABSTRACT

Exposure to neurotoxic chemicals such as pesticides, selenium, and heavy metals have been suggested to play a role in the etiology of amyotrophic lateral sclerosis (ALS). We assessed exposure to lead, cadmium, and mercury in 38 ALS patients (16 men and 22 females) and 38 hospital-admitted controls by using their cerebrospinal fluid (CSF) content as biomarker. We determined CSF heavy metal levels with inductively coupled plasma sector field mass spectrometry, according to a methodology specifically developed for this biological matrix. ALS patients had higher median values for Pb (155 vs. 132 ng/L) but lower levels for Cd (36 vs. 72 ng/L) and Hg (196 vs. 217 ng/L). In the highest tertile of exposure, ALS odds ratio was 1.39 (95% CI 0.48–4.25) for Pb, 0.29 (0.08–1.04) for Cd and 3.03 (0.52–17.55) for Hg; however, no dose-response relation emerged. Results were substantially confirmed after conducting various sensitivity analyses, and after stratification for age and sex. Though interpretation of these results is limited by the statistical imprecision of the estimates, and by the possibility that CSF heavy metal content may not reflect long-term antecedent exposure, they do not lend support to a role of the heavy metals cadmium, lead and mercury in ALS etiology.

1. Introduction

The etiology of amyotrophic lateral sclerosis (ALS), an extremely severe neurodegenerative disease which selectively affects both the upper and the lower motor neurons, is still unknown, though in the so-called familial form (around 10% of cases) a few gene mutations appear to play a major role [1,2].

The etiology of the disease is still largely unknown, and environmental factors, alone or in the presence of particular genetic backgrounds, are believed to play a major role in it [2–7]. These environmental factors include neurotoxic chemicals, such as pesticides, the metalloid selenium and a few heavy metals [4,8,9]. Among the latter, lead, cadmium, and mercury have been implicated in ALS etiology following toxicological and epidemiological observations [8,10,11]. However, such evidence linking exposure to these metals to ALS is not consistent, and major methodological issues, particularly concerning exposure assessment and the potential for bias due to unmeasured confounding have been raised concerning these investigations [9]. In this study, we aimed at assessing a biomarker of heavy metal antecedent exposure, its cerebrospinal fluid (CSF) content, and its potential relation with ALS risk.

2. Methods

2.1. Study population

The study population (composed of 38 ALS cases and 38 controls) and details of case identification, their clinical characteristics and sampling procedure have been previously described in detail [12]. Briefly, all cases: resided in the Emilia-Romagna region, northern Italy; received a diagnosis of clinically definite or probable ALS using the revised El Escorial Criteria [13] at the ALS Center in Modena, from May 1998 to April 2011; underwent lumbar puncture during diagnostic evaluation; and had at least 1 mL CSF available when the present study was designed. Twelve of these patients

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http://dx.doi.org/10.1016/j.jtemb.2016.12.012
0946-672X/

(generally the youngest ones) also received extensive genetic screening, showing no positivity for the major genes involved in familial ALS (namely SOD1, C9ORF72, FUS, TDP43). The controls: were patients admitted to the same hospital between 1999 and 2010; resided in the Emilia-Romagna region; underwent lumbar puncture due to suspected neurological disease; were subsequently found not to be affected by neurological disease; and had a CSF sample of 1 mL or more available in September 2011. We randomly selected among these eligible persons 38 individuals, matched 1:1 to ALS cases on age (±10 years, generally ± 5 years) and gender. These individuals were referred to neurological examination due to headache (n = 17), paresthesias (n = 5), diplopia (n = 6), vertigo (n = 3) and other signs and symptoms (n = 7).

Study participants underwent lumbar puncture for CSF sampling after providing their informed consent, and use of samples for research purposes was later approved by the Modena Ethical Committee. Around 6 mL of CSF were obtained from each participant using an ultraclean procedure, and the sample was immediately stored at −80 °C in polypropylene tubes. When the study was designed, a 1-mL aliquot was transported deep frozen in dry ice to the Munich Helmoltz Zentrum laboratory, and kept continuously frozen until use. When the analytical phase was started, the samples were slowly thawed in a refrigerator at 4 °C, vortexed and then analysed.

2.2. Laboratory analyses

We determined the CSF levels of Cd, Hg and Pb using a previously described methodology [14–16]. Briefly, we slowly thawed the samples in a refrigerator to 4 °C, vortexed and subsequently diluted samples 1:10 with Milli-Q water, containing ^{103}Rh as internal standard at a final concentration CSF of 1 μg/L in the diluted CSF samples. Element concentrations of CSF samples were determined using inductively coupled plasma–mass spectrometry: An NexIon, ICP-MS instrument (Perkin Elmer, Rodgau-Jügesheim Germany) was employed for ^{111}Cd, ^{202}Hg, and ^{208}Pb determination. Sample introduction was carried out using the instrumentís peristaltic pump connected to a Micromist nebulizer with a cyclone spray chamber. The RF power was set to 1250 W, the plasma gas was 15 L Ar/min, whereas the nebulizer gas was approximately 0.9 L Ar/min after daily optimization.

These determination methods had been validated by regular laboratory intercomparison studies (GEQUAS quality control scheme and participation in certification campaign of IRMM/EU) and by regular analysis of adequate certified reference materials. Along the analysis of samples several certified reference materials (CRM) were analysed (expressed as certified value/detected value). For Cd: ERM-BD-150: 11.4 ± 2.9 μg/kg/11.4 ± 0.5 μg/kg (corresponding to 31 ng/L Cd in measurement solution); ERM-DB-001: 125 ± 7 μg/kg/123 ± 2.9 μg/kg (corresponding to 330 ng/L Cd in measurement solution); BCR-635: 6.6 ± 0.6 μg/L/6.57 ± 0.16 μg/L. For Pb: ERM-BD-151: 207 ± 14 μg/kg/204.4 ± 2 μg/kg (corresponding to 2 μg/L Pb in measurement solution), ERM-DB-001: 2.14 ± 2 mg/kg/2.34 ± 0.19 mg/kg (corresponding to 23 μg/L Pb in measurement solution); BCR-635: 210 ± 24 μg/L/220 ± 5 μg/L. For Hg: ERM-BD-150: 0.0603 ± 0.0178 μg/kg/0.073 ± 0.0131 μg/kg (corresponding to 1 ng/L Hg in measurement solution), ERM-BD-151: 0.052 ± 0.04 μg/kg/0.51 ± 0.001 μg/kg (corresponding to 5 ng/L Hg in measurement solution). The limit of quantification of the determination method was 18 ng/L for Cd and 20 ng/L for Pb and Hg, each related to undiluted CSF. Coefficient of variation was for Cd 2.1%, for Pb 2.7%, and for Hg 1.9% (n = 10, each).

According to IUPAC recommendations, accuracy should be derived from comparison with CRM. For Cd, accuracy was derived from CRM ERM-BD 150 as the measurement concentration (~30 ng/L) was about the lower concentrations in CSF. Accuracy for Cd was determined at 100% (11.4 μg/kg/11.4 μg/kg). For, Hg, accuracy was derived from CRM ERM-BD 151 since measurement concentrations in digests were approximately the same as in low concentrated diluted CSF samples. Accuracy for Hg was determined at 98% (0.52 μg/kg/0.51 μg/kg). Lead accuracy was derived from the same CRM. For Pb, accuracy was determined at 99% (207 μg/kg/204.4 μg/kg).

Table 1
Distribution of cerebrospinal fluid heavy metals concentration (ng/L) in ALS cases and controls.

	Cases					Controls				
	10th	25th	50th	75th	90th	10th	25th	50th	75th	90th
Pb										
All	38.3	70.1	155	351	553	40.9	69.5	132	497	4130
Males	38.3	62.1	155	339	2200	40.9	76.6	116	689	7750
Females	41.3	87.1	150	351	472	41.7	56.5	143	463	1370
Cd										
All	13.6	24.6	35.9	66.8	94.3	14.5	22.0	71.6	105	258
Males	16.7	24.1	32.3	73.5	117	19.0	24.0	66.1	120	259
Females	13.6	24.6	38.0	58.4	83.4	13.0	22.0	71.6	82.9	188
Hg										
All	80.2	125	196	264	402	26.0	32.5	217	634	1280
Males	51.4	97.7	150	221	269	21.7	28.0	156	654	1280
Females	97.1	168	230	391	705	31.1	32.7	247	599	993

2.3. Data analysis

We carried out data analysis independently for each of the three heavy metals under examination based on CSF concentration tertiles computed from the controls. Using unconditional logistic regression models, we computed the odds ratio (OR) for ALS with its 95% confidence interval (CI) for each of the highest two concentration tertiles, compared with the lowest tertile. We fit crude models, along with models adjusted for sex and age (in years, continuous), and then models additionally adjusted for total CSF selenium content ([units], continuous). We also computed a *P* value for trend based on the continuous values of heavy metal exposure. We eventually carried out several sensitivity analyses: removing from data analysis controls characterized by specific symptoms and signs (i.e., the 17 subjects suffering from headache, the 5 affected by paresthesias and the 6 affected by diplopia); adjusting for the other two remaining metals investigated; using log-transformed values or winsorized values by setting data exceeding the 95th percentile to the 95th percentile; fitting conditional logistic regression models based on the age- and sex-based matching; and assigning exposure category on the basis of pre-specified fixed cutpoints of CSF metal content instead of tertiles.

3. Results

The newly-diagnosed ALS cases included 16 men and 22 women, with a mean age of 55 years (range 30–76 years), in 7 cases characterized by bulbar onset disease and in 31 by spinal onset ALS. The 38 sex- and age-matched controls had a mean age of 52 years, ranging from 30 to 85 years. Table 1 and Fig. 1 summarize the distribution of heavy metals with reference to their median concentration, and their 10° and 90° percentiles. Median CSF concentrations in cases compared with controls were higher for lead, slightly lower for mercury and considerably lower for cadmium, with an interquartile range systematically wider for all the three metals.

Odds ratios (ORs) of ALS according to heavy metal CSF concentrations are shown in Table 2. The odds of ALS were greater in the highest tertile of CSF Pb concentration than in the lowest tertile (OR = 1.4 in both multivariable-adjusted models), though confidence interval were rather wide (e.g., 95% CI, 0.46–4.17 in age- and

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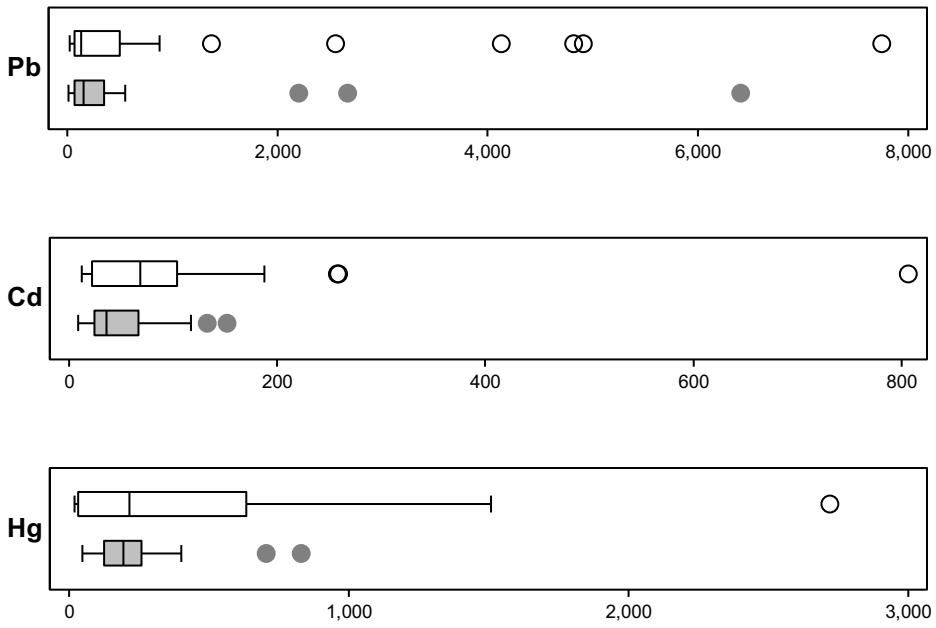

Fig. 1. Plot of CSF heavy metal concentrations, as ng/L, in controls (white) and ALS cases (gray).

Table 2
Odds ratio (OR) with 95% confidence intervals (CI) of amyotrophic lateral sclerosis associated with cerebrospinal fluid content of heavy metals.

Exposure tertiles	Cases/ controls	Crude analysis		Multivariate analysis 1[a]		Multivariate analysis 2[b]	
		OR	95% CI	OR	95% CI	OR	95% CI
Pb							
Lowest (<86.7)	11/12	1.00		1.00		1.00	
2nd (86.7–196)	10/13	0.84	0.26–2.68	0.79	0.24–2.57	0.87	0.25–2.99
3rd (>196)	17/13	1.43	0.48–4.25	1.39	0.46–4.17	1.43	0.46–4.41
Cd							
Lowest (<31.0)	16/12	1.00		1.00		1.00	
2nd (31.0–82.5)	16/13	0.92	0.32–2.63	0.95	0.33–2.76	1.17	0.37–3.67
3rd (>82.5)	6/13	0.35	0.10–1.18	0.29	0.08–1.04	0.24	0.06–0.89
Hg							
Lowest (<79.3)	3/12	1.00		1.00		1.00	
2nd (79.3–329)	29/13	8.92	2.15–37.07	12.41	2.69–57.31	12.63	2.62–60.75
3rd (>329)	6/13	1.85	0.38–9.08	3.03	0.52–17.55	3.12	0.51–19.03

[a] Adjusted for sex and age.
[b] Adjusted for sex, age, and total selenium.

sex-adjusted results). However, there was no appreciable trend of the OR across tertiles, since it was below the unity in the middle category of exposure, and this was confirmed by the trend analysis based on continuous levels of exposure. Concerning Cd exposure, OR decreased in the middle and highest tertiles, and the analysis for linear trend based on continuous values confirmed an inverse association between exposure and risk. OR associated with tertile of Hg exposure showed a strongly increased but very statistically unstable OR, with no evidence of any dose-response relation both in this analysis and in that based on continuous values of exposure. In this latter trend analysis, OR was 1 for both Pb and Hg, while for Cd it was 0.99 (95% CI 0.98–1.00).

Results did not substantially change for any of the elements investigated when in multivariate analysis we adjusted for the remaining two heavy metals, for either inorganic or organic CSF selenium content instead of overall selenium level, or in stratified analysis according to sex or age group (using 50 years as cutpoint – data not shown). Results also showed little variation when we used log-transformed values or winsorized values, using conditional logistic regression instead of the unconditional one, assigning exposure category on pre-specified CSF heavy metal cutpoints, or removing control subjects characterized by specific symptoms or signs (data not shown).

4. Discussion

Overall, our results failed to show an association between heavy metals exposure, as reflected by CSF content, and increased risk of ALS. This was substantially true for all the heavy metals investigated in this study, since there was no detectable dose-response trend of increased ALS risk across tertiles or continuous values of exposure, and this was confirmed in both stratified and sensitivity analyses. However, while lack of association was clearly evident for Cd and appears to rule out any association of the disease under study with such exposure, results for Pb and Hg are more difficult to interpret. For Pb, there was a slight increase in the top category of exposure, though the relative risk estimate was statistically unstable and the analysis based on continuous values did not suggest an association. In adjusted analyses of Hg, those in the middle tertile of exposure had 12 times the odds of ALS compared with those in lower tertile, but in the highest tertile, the OR was much smaller. This pattern and the absence of linear dose-response association in the analy-

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ses of continuous Pb concentration provide little support for a real etiological association.

The literature about the involvement of Pb, Cd and Hg in ALS etiology is conflicting, though some suggestive evidence particularly for Pb have been provided. All the three elements have the toxicological potential for damaging neural cells, through mechanisms ranging from glutamate-induced neurotoxicity, oxidative stress, protein misfolding, altered RNA and protein synthesis and aggregation, and they may induce inflammation, axonal and mitochondrial transport dysfunction, and cell death thus providing biological plausibility for an association with ALS [8]. In addition, there is evidence that these metals may cross the blood-brain and blood-CSF barriers, particularly in diseased individuals, and/or may undergo retrograde axonal transport, and thus reaching the target tissues for neurodegeneration [8,17]. In addition, none of these three heavy metals has a peculiarly selective toxicity for the motor neurons among the various neural cells, a distinctive feature of another neurotoxicant (which however also is an essential element) implicated in ALS etiology, selenium. Finally, the epidemiologic evidence linking Pb, Cd and Hg to ALS etiology is still sparse and rather conflicting [8], though at least for Pb most of the recent reports highlighted a possible association with disease risk [18–23]. One human study, carried out in Sweden, has specifically investigated CSF trace elements content in ALS patients, showing higher levels of Pb, Cd and lower concentration of Hg in cases compared with controls [18].

The lack of substantial association we found for the heavy metals we investigated might be due error in the measurement of long-term antecedent exposure by CSF heavy metal levels, both in cases and in controls. In general, concentrations of a chemical and particularly of heavy metals in the CSF and more generally in the central nervous system has the strong advantage, as compared with peripheral biomarkers of exposure such as their blood [24] and toenail [25] content, to reflect levels of the contaminant levels in a compartment closely related to the pathological process characterizing ALS and more generally neurodegenerative disease [12,26,27]. However, two major issues arise: the ability of Pb, Cd and Hg to accumulate near the blood-CSF barrier and/or to cross it, and the long-term persistence of an increased content of these metals in biomarkers, with particular reference to CSF level, after the ending of a subject's overexposure. Unfortunately, both these issues are not entirely clear. For lead, there is evidence in the human that the CSF content may reflect plasma (though not whole blood) Pb concentration [28,29], while such evidence for mercury and cadmium is anecdotal or missing, respectively [30]. However, it has been shown that all these three heavy metals, also depending on their speciation particularly for Hg [31], may accumulate in the blood-brain and particularly in the blood-CSF barrier at the choroid plexus, the organ which secretes CSF [32], reaching *in loco* high concentrations. Though this might represent a mechanism protecting the CSF and more generally the central nervous system against heavy metals [33], such a high content in the choroid plexus may also have adverse proteomic effects and relevant neurotoxicological implications on the choroid plexus itself or the brain [34–36]. In addition, there is evidence that blood-CSF barrier is altered in nearly half the ALS patients, and this abnormality might in turn increase the transfer of contaminants from the blood into the CSF [8,37,38]. Concerning the persistence of an increased CSF heavy metal content following the end of environmental overexposure, we were unable to find any evidence from the literature for the heavy metals here examined, thus hampering the evaluation of our results. Overall, if misclassification of exposure occurred for any of the heavy metals, it may have hampered the detection of an association between with ALS risk in the current study, likely for an excess time window between exposure ending and disease onset. This highlights the need of either prospective studies (though very difficult to perform if encompassing CSF sampling, also due to disease rarity) or further studies assessing long-term sources of exposure though retrospective dietary assessment or evaluation of occupational history. It is also possible that disease progression may alter trace element content in body tissues, altering nutritional status and trace element distribution in the various compartments, as suggested by some observations of a direct association between blood and spinal cord Pb content and ALS stage [24,39]. However, it must be noted that our study was based only on newly-diagnosed ALS patients, thus reducing the risk of reverse causation.

The present study also suffered from a limited statistical power, due to the sample size, as also reflected by the wide confidence intervals of the estimates. This is in turn due to ALS being a rare disease, and in addition usually not requiring CSF sampling during its diagnostic process, and to the difficulty of finding CSF from potentially matched controls unaffected by neurological disease. Therefore, and under the hypothesis of a role of Pb in ALS etiology, it is possible that a larger study population is needed to capture statistically stable estimates for slightly increased risk associated to that exposure, possibly only in the highest exposure category. It is also possible that higher levels of exposure, for Pb but also for Hg, are needed to identify an association with the disease, under the hypothesis that ALS is a multifactorial disease with various different factors potentially playing a role in its etiology and that in our study population such high levels of exposure were not adequately represented. This might be also supported by a comparison of our results with those obtained in 20 control subjects from another Italian setting, who showed substantially similar CSF Cd concentrations but much higher Hg and Pb levels (four to six times) compared with our controls [40].

We did not identify reasons to hypothesize the occurrence of selection bias in our study populations, with reference to both cases and controls. CSF sampling was occasionally needed during the diagnostic process of patients later found to be affected by ALS, with no apparent association whatsoever with variables (such as occupational status, smoking and residence) potentially associated with heavy metal exposure. This was also true for the control populations, which included patients reporting signs and symptoms not strongly associated *a priori* with the exposures of interest. In addition, study results were substantially confirmed after excluding specific subgroups of controls through sensitivity analyses.

In conclusion, we found little evidence of an increased ALS risk associated with CSF Pb, Cd and Hg content, with no dose-response relation. These findings do not add support to the hypothesis that these heavy metals are involved in disease etiology, though they must be evaluated with caution since the biomarker we used in the study might have been unable to reflect long-term antecedent exposures.

Funding sources

This work was supported by the Fondazione Pietro Manodori of Reggio Emilia (to Dr. Vinceti) and by the US National Institute of Health grant no. R21ES024700 (to Dr. Weuve).

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Please cite this article in press as: M. Vinceti, et al., Lead, cadmium and mercury in cerebrospinal fluid and risk of amyotrophic lateral sclerosis: A case-control study, J Trace Elem Med Biol (2016), http://dx.doi.org/10.1016/j.jtemb.2016.12.012