NeuroToxicology 38 (2013) 25–32

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Cerebrospinal fluid of newly diagnosed amyotrophic lateral sclerosis patients exhibits abnormal levels of selenium species including elevated selenite

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ARTICLE INFO

Article history:
Received 1 March 2013
Accepted 23 May 2013
Available online 31 May 2013

Keywords:
Selenium
Selenite
Selenoprotein-P
Amyotrophic lateral sclerosis
Case–control study
Cerebrospinal fluid
Risk
Environment
Speciation analysis

ABSTRACT

Exposure to selenium, and particularly to its inorganic forms, has been hypothesized as a risk factor for amyotrophic lateral sclerosis (ALS), a fast progressing motor neuron disease with poorly understood etiology. However, no information is known about levels of inorganic and some organic selenium species in the central nervous system of ALS patients, and recent observations suggest that peripheral biomarkers of exposure are unable to predict these levels for several Se species including the inorganic forms. Using a hospital-referred case–control series and advanced selenium speciation methods, we compared the chemical species of selenium in cerebrospinal fluid from 38 ALS patients to those of 38 reference neurological patients matched on age and gender. We found that higher concentrations of inorganic selenium in the form of selenite and of human serum albumin-bound selenium were associated with increased ALS risk (relative risks 3.9 (95% confidence interval 1.2–11.0) and 1.7 (1.0–2.9) for 0.1 μg/L increase). Conversely, lower concentrations of selenoprotein P-bound selenium were associated with increased risk (relative risk 0.2 for 1 μg/L increase, 95% confidence interval 0.04–0.8). The associations were stronger among cases age 50 years or older, who are postulated to have lower rates of genetic disease origin. These results suggest that excess selenite and human serum albumin bound-selenium and low levels of selenoprotein P-bound selenium in the central nervous system, which may be related, may play a role in ALS etiology.

1. Introduction

Selenium (Se), an essential trace mineral, has both nutritional and toxicological properties, with a broad range of biological activities which strongly depend on its chemical form. Inorganic species of Se are generally more toxic and of lower nutritional value than the organic species (Vinceti et al., 2009; Valdiglesias et al., 2010; Nogueira and Rocha, 2011), in some cases exhibiting entirely different effects (Borella et al., 1996; Hoefig et al., 2011; Nogueira and Rocha, 2011; Bodnar et al., 2012; Bitencourt et al., in press), and more generally, each Se compound has specific and distinctive biological properties. Unfortunately, most human studies have investigated the overall Se content of body tissues without assessing the different chemical species of the metalloid. Moreover, the levels of Se and of specific Se compounds in the different body tissues may considerably vary and are frequently uncorrelated, hampering or even precluding assessment of their concentrations in target organs and compartments of relevance for specific diseases on the basis of peripheral indicators of exposure (such as blood or toenails Se) or dietary intake of the metalloid (Behne et al., 2010; Dennert et al., 2011; Vinceti et al., 2012b). This may be particularly true when attempting to assess involvement of Se species in the etiology of human neurological diseases: a recent Se speciation studies has found that while peripheral (blood) levels of some organic Se compounds tend to reflect central nervous

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0161-813X
http://dx.doi.org/10.1016/j.neuro.2013.05.016

system (CNS) levels, no such correlation exists for other organic species such as selenoprotein P, overall Se content and the inorganic forms selenate and selenite (Solovyev et al., 2013), confirming previous observations on overall Se (Michalke et al., 2009). Other observations appear to confirm the independence of brain selenoprotein P compared with its plasma circulating levels (Scharpf et al., 2007).

Amyotrophic lateral sclerosis (ALS), a severe degenerative motor neuron disease of largely unknown etiology apart from the few cases of genetic origin, is among the diseases whose etiology is hypothesized to be related to overexposure to selenium (Vinceti et al., 2012a). This hypothesis was first proposed based on observation of a cluster of ALS cases in a seleniferous area of South Dakota, and an excess incidence of ALS in an Italian population consuming drinking water with high concentrations of the inorganic Se species selenate (Kilness and Hochberg, 1977; Vinceti et al., 1996, 2010b). Consistent with the hypothesis is the demonstration of Se-induced neurotoxicity in laboratory studies (Ammar and Couri, 1981; Nogueira et al., 2003; Maraldi et al., 2011; Estevez et al., 2012) and its selective toxicity on motor neurons in species such as swine (Casteignau et al., 2006; Raber et al., 2010; Vinceti et al., 2012a), generally showing a much higher or even an exclusive toxicity of inorganic species compared with the organic ones. Moreover, recent epidemiologic observations suggest deleterious effect of Se on neurological end-points such as visual evoked potentials (Saint-Amour et al., 2006) and, even at low doses of exposure, neonatal neurological and behavioral development (Yang et al., 2013).

We conducted a case–control study to examine the hypothesis that Se species and particularly the inorganic ones are associated with ALS risk by using a CNS biomarker of exposure, cerebrospinal fluid (CSF), which appears to play a key role in assessing exposure to etiopathogenetic and therapeutic factors in this disease (Tarasiuk et al., 2012; von Neuhoff et al., 2012; Wilson et al., 2013; Winer et al., 2013).

2. Methods

2.1. Study participants

ALS patients were recruited from a case series of residents of the Emilia-Romagna region, northern Italy, who were diagnosed with clinically definite or clinically probable ALS using the revised El Escorial Criteria (Georgoulopoulou et al., 2011) at the ALS Center of the Modena University Neurological Department from May 1998 to April 2011, and who underwent lumbar puncture during diagnostic procedures. This group comprised 72 consecutive patients with sporadic ALS, some of whom were enrolled in a previous study (Mandrioli et al., 2006). Thirty-eight patients had at least 1 mL CSF available when the present study was designed and composed the enrolled cases. We recently performed genetic screening including all the major genes that are implicated in ALS (SOD1, C9ORF72, FUS, TDP43) in 12 of these patients, generally the younger ones, with none testing positive. We were not allowed to perform genetic analysis in the remaining patients because of lack of informed consent and/or available blood samples.

The control population consisted of patients residing in the Emilia-Romagna region who were admitted to the same department between 1999 and 2010, inclusive, and underwent lumbar puncture because of suspected but later unconfirmed neurological disease, and had a sample of at least 1 mL of CSF still available in September 2011. Among these individuals, we randomly selected 38 subjects matched 1:1 to ALS cases on age (±10 years, in most cases ±5 years) and gender. Signs or symptoms that led to neurological examination and lumbar puncture were: headache ($n = 17$), paresthesias ($n = 5$), diplopia ($n = 6$), vertigo ($n = 3$) and other ($n = 7$). All control patients were subsequently discharged from hospital without a diagnosis of a major disease; final diagnosis was primary headache in 13 individuals, and other diagnoses with negative instrumental tests in the remaining 25 individuals.

Informed consent for diagnostic lumbar puncture was obtained from all patients, and utilization of the CSF specimens for the present study was approved by the Modena Ethical Committee.

2.2. Sample collection

Approximately 6 mL of CSF were collected by lumbar puncture from each patient and immediately stored at −80 °C in polypropylene tubes. A 1 mL aliquot was transported by air courier deep frozen in dry ice to the Munich laboratory, and kept continuously frozen until use. For analysis, samples were slowly thawed in a refrigerator at 4 °C, vortexed and subsequently analyzed.

2.3. Chemicals

Suprapure grade chemicals were used throughout. Selenite, selenate, selenomethionine (Se-Met), selenocystine (Se-Cys), thioredoxin reductase (EC 1.8.1.9.)-bound selenium (Se-TrxR), glutathione peroxidase (EC 232-749-6)-bound selenium (Se-GPx), human serum albumin (HSA) and Tris buffer were ordered from Sigma–Aldrich, Deisenhofen, Germany. Certified Se and Rh stock standards (1000 mg/L) were purchased from CPI International, Santa Rosa, CA, USA. Ammonium acetate (NH_4Ac) and acetic acid (HAc) were obtained from Merck, Darmstadt, Germany. Ar_{liq} and methane (99.999% purity) were purchased from Air Liquide, Gröbenzell, Germany. Selenite and selenate stock solutions were prepared at a concentration of 1000 mg Se/L by dissolving in Milli-Q water (18.2 MΩ cm, Milli-Q system, Millipore, Bedford, MA, USA). HSA was prepared at a concentration of 1000 mg/L. Preparation of Se-HSA was performed by mixing 10 mg Se/L selenite with this stock solution and incubating for at least 14 days. Working standards of Se species were prepared daily from their stock standard solutions by appropriate dilution with Milli-Q H_2O. Selenoprotein P-bound Se (SePP) is not commercially available as a standard compound, but it can be prepared from serum using affinity chromatography (AFC): The AFC-SePP fraction was purified by a mass-calibrated size exclusion chromatography (SEC) column, where the SePP fraction eluted at a RT calculated for 61.8 kDa.

2.4. Selenium speciation

We determined total Se and the Se species selenite, selenate, Se-Met, Se-Cys, Se-TrxR, Se-GPx, SePP and Se-HSA in the CSF samples using high pressure liquid chromatography (HPLC) coupled with inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) according to methodologies previously established for biological matrices, specifically for CSF (Michalke and Berthele, 2011; Solovyev et al., 2013). Se speciation was conducted by strong anion exchange (SAX)-ICP-DRC-MS (Michalke and Berthele, 2011). The SAX separation followed Xu et al. (2008) but was slightly modified for baseline separation of close eluting peaks.

A Knauer 1100 Smartline inert Series gradient HPLC system was connected to an anion exchange column ProPac SAX-10 (250 × 2 mm I.D.) from Thermo (Dionex Idstein, Germany) for species separation. The sample volume was 100 μl. The mobile phases were: eluent A: 10 mM Tris–HAc, pH 8.0; and eluent B: A + 500 mM NH_4Ac, pH 8.0. Gradient elution expressed as %-eluent A: 0–3 min 100%; 3–10 min 100–60%; 10–23 min 60–45%; 23–26 min 45–43%; 26–28 min 43–0%; 28–52 min 0%; 52–60 min 100%. The flow rate was 0.20 mL min^{-1}. For internal standardization the column effluent was mixed with 1 μg/L Rh (final

concentration, total flow rate: 0.25 mL min^{-1}) and directed to ICP–MS.

Total Se was analyzed by flow injection (FI)-ICP-DRC-MS (Michalke et al., 2009). A Knauer 1100 Smartline inert Series HPLC system equipped with vacuum degasser and an electronic valve with a 25 μl injection loop (Perkin Elmer, Rodgau-Jügesheim, Germany) was directly coupled to ICP-DRC-MS. The flow rate was 1 mL min^{-1} of 50% eluents A and B, each, from SAX separation. CSF-samples were diluted 1:1.1 by adding 1 μg/L Rh (final conc.) as internal standard.

The experimental settings chosen for ICP-DRC-MS (Perkin Elmer NexIon) after optimization were: radio frequency power: 1250 W, plasma gas flow: 15 L Ar/min auxiliary gas flow: 1.05 L Ar/min, nebulizer gas flow: 0.98 L Ar/min, daily optimized, dwell time 300 ms, ions monitored: ^{78}Se, ^{80}Se, ^{103}Rh, DRC reaction gas: CH_4 reaction at 0.58 mL min^{-1}, DRC rejection parameter q: 0.6.

Peak quantification from FI-quantification and from chromatograms was done by comparing peak areas with peak area calibration curves from FI-ICP-DRC-MS. Standard addition method was used for standard-retention-time matched identification of Se species and as QC means in quantification. Species identity was further confirmed using a 2D approach of SAX-capillary electrophoresis (CE)-ICP-DRC-MS: SAX fractions were analyzed with CE-ICP-DRC-MS. In both serially applied separation techniques (SAX, CE) the observed peaks matched retention- or migration times of respective standard compounds, in accordance to a parallel investigation (Solovyev et al., 2013). Species identification was regarded as acceptable when species matched the standard compounds with both chromatography/electrophoretic techniques (match in first and second technique). Details of these procedures have been published elsewhere (Solovyev et al., 2013). Rh and Se data files were exported from the NexIon software and processed with Peakfit™ software for peak area integration. For each sample (or standard) a quotient of Se-peak area to Rh-peak area was calculated and taken as the result corrected for the internal standard (Rh).

In these analyses, in addition to well-known Se organic and inorganic species, we detected 13 peaks attributable to extremely small amounts of Se of unknown form; these were added to known inorganic and organic species to compute total Se. No detectable trace of Se-Cys was found in any sample. Limit of detection for all Se species in CSF was 0.01 μg/L, lower than that we obtained in a recent pilot study in neurologically healthy German individuals (Solovyev et al., 2013).

2.5. Quality control

For total Se determination, quality control was performed by analyzing the control materials "human serum" and "urine" from RECIPE, Munich, Germany. Control materials were reconstituted as indicated on flask labels and the resulting solutions were diluted 1/50 (serum) or 1/10 (urine) with Milli-Q water before measurements. Se concentrations were found to be 62 ± 3 μg/L (serum, n = 3) and 24 ± 3 μg/L (urine, n = 3), compared to manufacturer's target mean values of 62 and 23 (range: 16–30) μg/L, respectively.

In a preceding inter-laboratory comparison our SePP quantification was compared to the immuno assay method for SePP determination by Hollenbach et al. (2008). There the reliability of our SePP determination was verified: The immunologically determined SePP concentration in a pooled CSF sample prepared for the inter-laboratory comparison was 0.54 nM/L (±9.5%) SePP (expressed as total protein). According to literature data regarding SePP (10 Se atoms per SePP and molecular weight MW(SePP) = 61 kDa (Ballihaut et al., 2012)) this SePP-protein concentration refers to 0.43 ± 0.04 μg Se/L at SePP. This value corresponded well to the SAX-ICP-DRC-MS value from our method at 0.41 ± 0.01 μg Se/L at SePP peak: Thus our method was verified by an independent reference method.

Besides, the good concordance of our Se species concentrations in CSF and serum from this and previous papers with some available Se-species concentrations in literature underlines further that Se species determinations with our method are at least overall OK. This is the more important as with the same method cases and controls were measured and the differences between both are the relevant issue in this paper.

For recovery determination during SAX-ICP-DRC-MS single Se species (10 μg Se/L) were analyzed and peak Se concentrations were quantified and related to the injected Se amounts (=100%). Analogously, CSF samples were quantified for total Se (=100%) before injection and compared to the sum of eluted and quantified chromatographic peaks. Recoveries were 95 ± 9% for CSF, 106 ± 10% for GPx, 97 ± 8% for selenite, 102 ± 8% for TrxR, 89 ± 8% for Se-HSA, and 84 ± 13% for SePP, the latter lower recovery being explained by stability problems accompanied by an increasing selenate signal, as expected (Michalke and Berthele, 2011).

2.6. Statistical analysis

Analyses were conducted for each Se species and for total inorganic Se (sum of selenite and selenate), total organic Se (sum of SePP, Se-GPx, Se-TrxR, Se-Met and Se-HSA), and total Se (sum of inorganic, organic and unknown forms). Inclusion of Se-HSA in the organic category was based on the presumption that nearly all of this Se was organic, most likely in the form of Se-Met (Xu et al., 2008), though trace amounts of inorganic Se in this compound cannot be entirely excluded (Haratake et al., 2008).

We tested differences in distribution of Se species in cases and controls using the Wilcoxon signed-rank test. We estimated the relative risk (RR) of ALS, as expressed by the odds ratio, associated with a one-unit increase in single Se species or categories using conditional logistic regression models. Some Se species distributions had outliers at high values, which was a concern as they could act as high leverage points and be unduly influential in regression models, especially given the small sample size. We used two approaches to address such outliers: (1) diagnostics were conducted to identify highly influential observations, assessed by delta beta statistics (Hosmer and Lemeshow, 2000) and observations with high influence statistics (delta beta > 0.1) were omitted and the model was refit and (2) the models were fit using Se species concentration values that were winsorized by setting data exceeding the 95th percentile to the 95th percentile. Both sets of results are presented. Analyses were repeated in the subgroup of matched pairs in which the case was diagnosed at age 50 or older. Sensitivity analyses were conducted by selectively omitting from analyses control patients with specific symptoms and signs leading to neurological examination (specifically, the 17 subjects suffering from headache, the 5 with paresthesias and the 6 with diplopia).

3. Results

The 38 ALS cases included 16 men and 22 women, with mean age of 55.5 years (range 30.7–76.4 years), who underwent lumbar puncture during their diagnostic process for neurological signs and symptoms of uncertain origin. Seven of these patients were later diagnosed as having ALS with bulbar onset and 31 as having spinal onset of the disease, in all cases at a very early clinical stage. The 38 age- and gender-matched controls had mean age 52.6 years (range 30.2–85.5 years).

Table 1 summarizes the distribution of the inorganic and organic Se species we determined. Of the inorganic forms, selenite – but not selenate – levels were shifted toward higher values in

Table 1
Distribution of Se species (μg/L) in newly diagnosed amyotrophic lateral sclerosis cases ($n = 38$) and controls ($n = 38$).

Se species	Percentile		
	5th	50th	95th
Selenite			
Controls	0	0.026	0.180
Cases	0	0.051	0.169
Selenate			
Controls	0	0	0.089
Cases	0	0	0.089
Selenomethionine[a]			
Controls	0	0	0.305
Cases	0	0	0
Thioredoxin reductase-bound Se			
Controls	0	0.050	0.389
Cases	0	0.043	0.214
Glutathione peroxidase-bound Se			
Controls	0	0.021	0.164
Cases	0	0.026	0.120
Human serum albumin-bound Se			
Controls	0	0.087	0.258
Cases	0	0.127	0.359
Selenoprotein P-bound Se			
Controls	0.036	0.856	8.121
Cases	0.093	0.577	2.196
Total inorganic Se			
Controls	0	0.034	0.180
Cases	0	0.059	0.277
Total organic Se			
Controls	0.053	1.084	8.284
Cases	0.287	0.765	2.508
Total Se[b]			
Controls	0.140	1.100	8.380
Cases	0.320	0.765	2.660

[a] Only four nonzero values of this compound detected.
[b] Sum of total inorganic and total organic Se and unknown forms.

cases, as shown by median values. Levels of total organic Se were lower among cases than among controls, and this was particularly true for SePP. Overall, levels of organic Se compounds were higher than levels of inorganic compounds; as a result, total Se was lower among cases than among controls. Since only four subjects had detectable Se-Met levels, this species was not analyzed separately in subsequent analyses. Median values in controls were roughly comparable to CSF Se species levels recently detected in a series of 24 patients with unspecific neurological complaints from the Department of Neurology of the Technical University Munich (Solovyev et al., 2013), which reported median levels of total Se = 0.861, SePP = 0.474, Se-GPx = 0.036, Se-TrxR = 0.035, Se-HSA = 0.068, selenite = 0.046, and selenate lower than the detection limit (all concentrations expressed as μg/L).

In conditional logistic regression models omitting gross outliers from analysis, the relative risk (RR) of ALS increased with increasing selenite content of CSF but not selenate content (Table 2). The RR increased with increasing Se-HSA concentration and decreased with increasing SePP concentration. The RR similarly decreased with increasing total organic Se, both unadjusted and adjusted for inorganic Se, and with increasing total Se. In analyses restricted to case–control pairs in which the case was diagnosed at age 50 or older, associations were generally stronger. All patients omitted as outliers had delta beta statistics (indicating the change in the beta coefficient when the observation is omitted) that were grossly large compared to those for other observations (for example, delta betas of 0.13 and 0.45 for selenite, whereas the other 74 observations had values between 0 and 0.036), and were above the 95th percentile of their Se species distributions. Comparing included subjects to patients excluded as outliers, median time from onset to lumbar puncture was 15 *vs.* 13 months, respectively, and median survival after diagnosis was 44 *vs.* 45 months, respectively.

Results were similar when conducting the analyses using winsorized distributions of the Se species (Table 3), but with estimates generally closer to the null value, with the exception of analyses for total inorganic Se among the older age at diagnosis subsample, which showed stronger associations in the winsorized analysis.

In sensitivity analyses selectively omitting subgroups of control subjects with specific neurological symptoms and signs, no substantially different results emerged.

4. Discussion

Overall Se content in body tissues such as blood, nails, brain and spinal cord of ALS patients has previously been investigated (Mitchell et al., 1991; Moriwaka et al., 1993; Ince et al., 1994; Bergomi et al., 2002), but the growing awareness of the different toxicological and physiological roles of the various Se species

Table 2
Relative risks (RR) of amyotrophic lateral sclerosis associated with 1 μg/L[a] increase in cerebrospinal fluid concentration of Se species: analysis removing observations with extremely high influence statistics.[b]

Se species	All subjects (38 cases, 38 controls)			Cases diagnosed ≥ 50 years (28 cases, 28 controls)		
	RR (95% CI)	*P*-value	#Cases/#controls	RR (95% CI)	*P*-value	#Cases/#controls
Selenite	3.9 (1.2–11.0)	.02	36/36	8.2 (1.5–46.2)	.02	26/26
Selenate	0.9 (0.2–4.2)	.93	38/38	3.8 (0.4–39.6)	.26	27/27
Thioredoxin reductase-bound Se	1.0 (0.9–1.1)	.13	37/37	1.1 (1.0–1.2)	.12	26/26
Glutathione peroxidase-bound Se	1.0 (0.9–1.1)	.47	38/38	1.0 (0.9–1.2)	.91	28/28
Human serum albumin-bound Se	1.7 (1.0–2.9)	.05	37/37	2.2 (1.0–4.7)	.04	27/27
Selenoprotein P-bound Se	0.2 (0.04–0.8)	.03	37/37	0.1 (0.01–0.8)	.03	27/27
Total inorganic Se						
Crude	1.7 (0.8–3.5)	.18	36/36	2.9 (1.0–8.4)	.06	26/26
Adjusted for organic Se	1.9 (0.8–4.8)	.17	36/36	4.1 (0.8–21.3)	.09	26/26
Total organic Se						
Crude	0.3 (0.1–1.0)	.04	36/36	0.2 (0.03–0.95)	.04	26/26
Adjusted for inorganic Se	0.3 (0.1–0.9)	.04	36/36	0.1 (0.02–0.96)	.05	26/26
Total Se	0.3 (0.1–0.9)	.03	37/37	0.3 (0.1–1.1)	.08	27/27

[a] Unit increase is 0.1 μg/L for selenate, selenite, total inorganic Se and human serum albumin bound-Se and 1 μg/L for all other compounds.
[b] Results obtained from conditional logistic regression models. To reduce the influence of extreme outliers in the distributions of some Se species, observations with unusually high influence statistics (delta beta > 0.1) were omitted, resulting in smaller sample sizes for some analyses.

Table 3
Relative risks (RR) of amyotrophic lateral sclerosis associated with 1 μg/L[a] increase in cerebrospinal fluid concentration of Se species: analysis using winsorized variables.[b]

Se species	All subjects (38 cases, 38 controls)			Cases diagnosed ≥50 years (28 cases, 28 controls)		
	RR (95% CI)	*P*-value	#Cases/#controls	RR (95% CI)	*P*-value	#Cases/#controls
Selenite	1.9 (0.8–4.6)	.13	38/38	3.9 (1.1–13.0)	.03	28/28
Selenate	0.9 (0.2–4.4)	.88	38/38	1.9 (0.3–13.6)	.54	28/28
Thioredoxin reductase-bound Se	1.0 (0.9–1.0)	.37	38/38	1.0 (0.9–1.1)	.65	28/28
Glutathione peroxidase-bound Se	1.0 (0.9–1.1)	.73	38/38	1.0 (0.9–1.2)	.58	28/28
Human serum albumin-bound Se	1.5 (0.9–2.4)	.11	38/38	1.9 (1.0–3.6)	.05	28/28
Selenoprotein P-bound Se	0.3 (0.08–0.8)	.02	38/38	0.2 (0.06–0.9)	.03	28/28
Total inorganic Se						
Crude	1.4 (0.7–2.9)	.30	38/38	3.2 (1.1–9.4)	.04	28/28
Adjusted for organic Se	1.7 (0.8–3.9)	.18	38/38	4.6 (1.1–20.2)	.04	28/28
Total organic Se						
Crude	0.4 (0.2–0.9)	.03	38/38	0.4 (0.2–1.1)	.07	28/28
Adjusted for inorganic Se	0.4 (0.2–0.9)	.03	38/38	0.3 (0.1–1.0)	.05	28/28
Total Se	0.5 (0.2–1.0)	.05	38/38	0.5 (0.2–1.1)	.09	28/28

[a] Unit increase is 0.1 μg/L for selenate, selenite, total inorganic Se and human serum albumin bound-Se and 1 μg/L for all other compounds.
[b] Results obtained from conditional logistic regression models. To reduce the influence of extreme outliers in the distributions of some Se species, Se variables were winsorized by setting values above the 95th percentile to the 95th percentile.

makes such an approach inadequate. Unlike previous investigations, we assessed whether specific Se compounds and particularly the inorganic species represented a risk factor for ALS, prompted by suggestive findings of epidemiologic studies (Kilness and Hochberg, 1977; Vinceti et al., 1996, 2010b) and animal and laboratory investigations (Vinceti et al., 2009; Maraldi et al., 2011; Estevez et al., 2012). To the best of our knowledge, the present study is the first on Se speciation in ALS and more generally in human diseases, among the large number of epidemiologic studies on health effects of Se (Vinceti et al., 2009; Dennert et al., 2011). Our results indicate the importance of Se speciation, despite the analytical complexity and cost (Borella et al., 1996; Vinceti et al., 2009; Valdiglesias et al., 2010; Nogueira and Rocha, 2011); RR estimates for total Se were opposite to those for selenite or total inorganic Se, confirming recent results on total CSF Se from Norwegian ALS patients (Roos et al., 2013), and thus an analysis based on total Se would have led to opposite conclusions. This indicates that findings may be seriously misleading if assessment is based on overall Se content of body tissues and not specific Se species, highlighting the need to focus on Se homeostasis, speciation and metabolism when investigation the relation between this metalloid and CNS diseases (Vinceti et al., 2012a; Steinbrenner and Sies, in press).

Use of CSF, a nervous system indicator of growing interest in ALS (Sussmuth et al., 2008; Gladman et al., 2012; Mendonca et al., 2012; Tarasiuk et al., 2012; von Neuhoff et al., 2012; Wilson et al., 2013; Winer et al., 2013), as a biomarker of exposure rather than peripheral indicators such as blood or toenails is an additional strength of the present study. In our recent study on the relation among CSF and other indicators in neurologically healthy subjects (Solovyev et al., 2013), positive associations between CSF and blood levels were found only for Se-GPx, Se-TrxR and Se-HSA, while there was little or no correlation between CSF and blood levels for other organic forms such as SePP, for selenite and selenate (correlation coefficients −0.03 and 0.23, respectively, unpublished data by N.S. and B.M.), and total Se. Similarly, SePP in the brain has been found to be independent of plasma circulating levels (Scharpf et al., 2007). These findings indicate the inadequacy of studies of inorganic Se not based on biomarkers of exposure directly associated with target tissues. However, CSF is very rarely used as a biomarker in epidemiologic studies, since it can be collected only following specific medical indications, making it infeasible to use not only in population-based but also in most hospital-based investigations.

Previous studies on CNS Se content in ALS patients, which have generally shown higher overall Se levels in brain and spinal cord compared with controls (Mitchell et al., 1991; Moriwaka et al., 1993; Ince et al., 1994; Markesbery et al., 1995) and highly variable GPx activity (Przedborski et al., 1996), were conducted in deceased subjects, and may therefore have suffered from the potential inadequacy of post-mortem tissues in reflecting antecedent Se content and enzyme activities. These studies were also limited by small sample size and potential selection bias.

Our results indicate major abnormalities of Se compounds in ALS, and support the hypothesis that inorganic Se and specifically selenite may play a key role in the neurodegenerative process characterizing ALS, in line with epidemiologic evidence implicating overexposure to environmental Se (Kilness and Hochberg, 1977) and to drinking water with high content of inorganic Se as selenate (Vinceti et al., 1996, 2010b). Evidence supporting biological plausibility of a relation between Se species, and selenite in particular, and ALS has been provided from observations in swine indicating that a few organic and particularly inorganic Se species selectively kill motor neurons (Raber et al., 2010; Vinceti et al., 2012a), a nematode model in which selenite specifically affected motor function (Estevez et al., 2012) and recent *in vitro* observations (Maraldi et al., 2011). Inorganic Se forms and selenite in particular are well recognized in laboratory studies to be considerably more toxic than organic species (Benko et al., 2012; Peyroche et al., 2012; Vinceti et al., 2013), particularly for neurotoxic effects (Ammar and Couri, 1981; Tsunoda et al., 2000), even though they are retained in humans (Thomson et al., 1993) and animals (Vinceti et al., 2009) at a considerably lower rate than organic compounds. Selenite, and other Se species (Zheng et al., 2012), can induce oxidative stress and mitochondrial damage (Belyaeva and Saris, 2011; Ma et al., 2011), and both free radical damage and mitochondrial abnormalities have been implicated in ALS etiopathogenesis (Rossi et al., 2012). Selenite *in vitro* has also been shown to induce other biochemical alterations suggested to occur in ALS, such as copper/zinc superoxide-dismutase (SOD1) translocation into mitochondria and increased levels of reactive oxygen species and of inducible nitric oxide synthase, with enhanced susceptibility to Se toxicity by nervous cells compared with other cell types (Maraldi et al., 2011). Low-dose Se exposure in humans may also induce DNA damage (Karunasinghe et al., 2012) and P38-P53 activation (Chen et al., 2010) as confirmed by laboratory studies using selenite (Rudolf et al., 2008), and these mechanisms are among those suggested as involved in ALS etiopathogenesis (Kim and Choi, 2010; Coppede, 2011; Hanada et al., 2013). Finally, several studies have suggested an inhibitory effect of selenite on inflammatory

process (Rusolo et al., 2013; Shanu et al., 2013), which in turn may influence the relative distribution of selenium among body tissues (Maehira et al., 2002), and inflammation has been recently suggested to play a potential role in ALS etiology and pathogenesis (Evans et al., 2013). Therefore, the possibility of a link between altered Se compounds content in CNS and neuroinflammation in ALS etiology merits further consideration.

In the previous epidemiologic study on consumers of drinking water with high Se content in the Reggio Emilia area (Vinceti et al., 1996), excess ALS risk was associated to high levels of Se as selenate, the usual form of the metalloid in underground waters (Vinceti et al., 2010b), while in the present study the increased concentrations were found only for selenite. However, toxicity of these two inorganic Se species, whose dietary intake is usually very low, is comparable in some studies though not in all, with general higher toxicity for selenite (Spyrou et al., 1996; Weiller et al., 2004; Lunoe et al., 2011; Maraldi et al., 2011; Alturkmani et al., 2012; Benko et al., 2012; Tsai et al., 2013). Moreover, a conversion between these two inorganic Se forms, *i.e.* from the hexavalent species (selenate) to the tetravalent one (selenite), has been shown to occur as a chemical or a biological process (Hageman et al., 2013) and it might also occur in the human (Gammelgaard et al., 2012), though limited information on this issue is available.

The levels of selenite found in our study of CSF from ALS patients were close to those (0.316 μg/L) found in an *in vitro* study to induce apoptosis in cultured mouse cortical neurons (Xiao et al., 2006). Toxic effects of selenite appear to be highly dependent on cell type (Maraldi et al., 2011) and possibly, in nervous cells, on the brain region involved (Zia and Islam, 2000). An additional mechanism suggested to link excess Se to ALS risk is non-specific incorporation of Se into proteins (Bell, 2009).

The cause of the elevated CSF selenite content detected in our ALS patients may be attributable to several causes, such as excess exposure to the compound from environmental sources, abnormal and enhanced localization of this compound in the CNS, and variations in response and repair pathways to selenite-induced DNA damage (Manikova et al., 2012) or alteration of its accumulation, detoxification or toxicity (Sun et al., 2013), with the possible involvement of genetic factors. About the latter, an interplay between excess selenite exposure and genetics might occur in sporadic ALS according to the 'low-penetrance susceptibility model,' which hypothesizes some involvement of genetic as well as environmental factors even in the non-familial form of the disease (Sabatelli et al., 2013). Unfortunately, we did not have available in our patients peripheral indicators of Se exposure such as levels of blood or toenails Se species, which might have helped, despite the limited ability of these biomarkers in reflecting exposure to inorganic Se compared with organic (Vinceti et al., 2001, 2009), to assess the possible environmental origin of the excess CNS selenite content we detected. Ascertainment of historical residence of study patients through the Revenue Agency of the Ministry of Finance, which maintains records of historical residence nationwide and could be accessed for research purposes by one of us (Vinceti et al., 2012c), allowed us to rule out that any of the study subjects had resided in Reggio Emilia area where drinking water with high selenite content was distributed in the 1970s and 1980s (Vinceti et al., 2010a). However, we did not have available any specific information about personal sources of drinking water consumption and other potential environmental or occupational exposures of study subjects to Se species.

Our observation of an inverse association between ALS risk and levels of organic Se compounds and particularly SePP, with the exception of Se-HSA, may imply a deficiency in antioxidant response to free radical damage (Mandrioli et al., 2006) or more generally a higher risk of neurodegeneration due to a deficiency of proteins such as the antioxidant enzyme GPx (Wirth et al., 2010) and of SePP (Caito et al., 2011), which is considered a major source of Se for Se-dependent enzyme synthesis and also has antioxidant properties itself (Scharpf et al., 2007; Meplan et al., 2009). Reasons for decreased organic Se levels in CSF of our ALS patients are unclear, since they cannot be ascribed to a low Se supply, due to the higher amount of inorganic Se available. Two possibly interrelated hypotheses may be advanced: the role of unknown genetic or non-genetic factors in reducing the antioxidant response, or the ability of pro-oxidant Se species, including selenite, to reduce expression of Se containing enzymes (Tallandini et al., 1996; Branco et al., 2012; Medeiros et al., 2012), possibly through the induction of oxidative stress (Suryo Rahmanto et al., 2012). On the other hand, in other investigations selenite has been shown to increase brain GPx activity as well as lipid peroxidation (Glaser et al., 2010), the latter effect being possibly the cause of the former, and in human and *in vitro* studies it appears to increase SePP levels (Meplan et al., 2009; Hoefig et al., 2011).

Recent animal and human studies investigating the relation of SePP with neurological or other diseases have yielded sharply different results (Bellinger et al., 2008, 2012; Yang et al., 2011; Raman et al., 2012; Takata et al., 2012), not unexpectedly in selenium research, making it difficult to assess the biological significance of our findings. The hypothetical role of SePP in CNS diseases is intriguing and controversial, ranging from beneficial as suggested by animal studies (Raman et al., 2012) to potentially deleterious in human neurodegenerative diseases (Bellinger et al., 2008, 2012). Scharpf et al. investigated the distribution of SePP in the human CNS: they found very uneven localization, with high SePP immunoreactivity in spinal, cranial and particularly pyramidal motor neurons, and detected evidence supporting a crucial role of SePP expression and activity in human brain (Scharpf et al., 2007).

We observed an unexpected positive association between Se-HSA and ALS risk. The exact nature of Se-HSA is unclear, but available evidence suggests that it is mostly composed of organic Se (Xu et al., 2008), though the occurrence of inorganic forms cannot be ruled out (Haratake et al., 2008). Despite the lack of information about the biological properties of this species of the metalloid, we consider this finding worth further investigation in future human studies on the role of Se in neurodegeneration. Se-HSA might act as long-term storage of the metalloid for further selenoprotein synthesis, thus suggesting the possibility that the decreased SePP levels found in cases could be a consequence, on a hypothetical basis, of a decreased Se re-mobilization and availability from Se-HSA. Excess Se exposure in Chinese individuals has been associated with preferential localization of Se in the Se-HSA fraction, while in Se-deficient or adequate subjects the greatest Se amount was in the SePP fraction (Gu et al., 1998).

We found a stronger positive association between CSF selenite and ALS risk among older subjects. A possible reason for enhanced risk with older age may be the decreased role of genetic factors supposed to be more important in triggering earlier onset disease (Chio et al., 2012), indicating a greater role of environmental variables in older subjects.

We must recognize limitations of the present study, including the possibility that the disease process influenced the content of Se species in CSF, and possibly in different ways for different compounds. The possibility of reverse causality is inherent in studies conducted with biomarkers in patients suffering from disease; however, in the present study CSF sampling was conducted at a very early stage of the disease, *i.e.* during the clinical process leading to the its first diagnosis, thus decreasing the risk of disease-induced alterations in metabolism or intake. Furthermore, the extent to which inorganic Se species, which are not synthesized by the organism but rather absorbed, may reflect long-term environmental overexposure is unknown, due to a lack

of studies on their persistence or distribution in CSF or other CNS areas. We selected as control subjects neurological patients whose diagnosis was benign or null after their diagnostic process; however, we cannot entirely rule out selection bias in the referent population, possibly mediated by some risk factors affecting their conditions that are correlated with differential exposure to Se species. However, there is no *a priori* plausibility to such a hypothesis, and our sensitivity analyses were unable to identify trends supporting this possibility. Selection bias is unlikely to have occurred in the ALS patient group, as they were selected among a consecutive patient series admitted to the same hospital as control patients and from the same underlying population, which was furthermore quite homogeneous as to genetic and environmental lifestyle factors. ALS patients undergoing lumbar puncture for CSF sampling during the diagnostic process were more likely to be younger and characterized by pyramidal signs than ALS cases not undergoing this procedure who were therefore not included in the present study; however, there is no reason to suspect an association between these clinical features and alterations in single Se species, and excess CSF selenite content was confirmed (and even enhanced) in our older study group. Finally, study size was not large enough to yield statistically precise risk estimates, as reflected by wide confidence intervals surrounding the RRs, and the data included outliers, which we attempted to deal with by using multiple approaches to the analysis.

In conclusion, our results indicate a direct relation between ALS risk and the concentration of selenite in CSF of newly diagnosed ALS patients, as well an inverse association with the organic Se form SePP which might be a related phenomenon, supporting the hypothesis that overexposure to selenite may be an etiological risk factor in the disease. Selenite may trigger neurodegenerative effects through its powerful toxicity, which appears to be unique among toxic chemicals, being highly specific toward motor neurons in some animal studies.

Conflict of interest

None declared.

Acknowledgements

Financial support to this study was provided by the Pietro Manodori Foundation of Reggio Emilia, the National, Modena and Reggio Emilia sections of the Italian Amyotrophic Lateral Sclerosis Association (AISLA) and the Local Health Unit of Reggio Emilia to Dr. Vinceti, and by the US National Institute of Health to Dr. Crespi (grant NIH UL1TR000124).

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