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Selenium and selenium species in the etiology of Alzheimer's dementia: The potential for bias of the case-control study design

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ABSTRACT

Several human studies imply that the trace element selenium and its species may influence the onset of neurological disease, including Alzheimer's dementia (AD). Nevertheless, the literature is conflicting, with reported associations between exposure and risk in opposite direction, possibly due to biases in exposure assessment.

After conducting a cohort study that detected an excess AD risk associated with higher levels of inorganic-hexavalent selenium in subjects with mild cognitive impairment (MCI), we investigated the relation between selenium and AD using a case-control study design. We determined cerebrospinal fluid levels of selenium species in 56 MCI participants already included in the cohort study, considered as referents, and in 33 patients with established AD.

AD risk was inversely correlated with inorganic selenium species and with the organic form bound to selenoprotein P. Selenium bound to other organo-selenium species was positively correlated with AD risk, suggesting compensatory selenoprotein upregulation following increased oxidative stress. The finding of an increased AD risk associated with inorganic-hexavalent selenium from the cohort study was not replicated.

This case-control study yielded entirely different results than those generated by a cohort study with a partially overlapping participant population, suggesting that case-control design does not allow to reliably assess the role of selenium exposure in AD etiology. This inability appears to be due to exposure misclassification, falsely indicating an etiologic role of selenium deficiency likely due to reverse causation, and involving most selenium species. The case-control design may instead lend insights into the pathologic process underlying disease progression.

1. Introduction

There is consensus that the trace element selenium (Se), whose exposure is generally around 20–100 μg/day in most populations worldwide, is an element of considerable interest from both a toxicological and a nutritional perspective, with a very narrow range of safe exposure [1–3]. However, the range of exposure considered to be safe differs across studies and regulatory agencies, and recent evidence has highlighted the likely occurrence of side effects at exposure levels previously deemed to be safe [2,4–6], while failing to demonstrate

Abbreviations: AD, Alzheimer's dementia; CI, confidence interval; CSF, cerebrospinal fluid; IQR, interquartile range; LOD, limit of detection; MCI, mild cognitive impairment; OR, odds ratio; Se(IV), selenite; Se(VI), selenate; Se-Cys, selenocysteine-bound Se; Se-GPX, glutathione-peroxidase-bound Se; Se-HSA, human serum albumin selenium-bound Se; Se-Met, selenomethionine-bound Se; Se-SELENOP, selenoprotein P-bound Se; Se-TXNRD, thioredoxin reductase-bound Se

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beneficial effects in chronic disease prevention [7–9]. Among the various diseases ascribed to altered Se status are neurodegenerative diseases, and specifically Alzheimer's dementia (AD), a possible connection currently attracting considerable attention [10–13]. AD is the most common neurodegenerative dementia. Its neuropathological hallmarks are senile amyloid plaques and neurofibrillary tangles, which can be estimated in vivo by means of cerebrospinal fluid (CSF) biomarkers beside imaging data. The clinical diagnosis of AD can be corroborated by biomarkers suggestive of amyloidosis (i.e. senile plaques), which are decreased CSF levels of Aβ1-42 (β-amyloid) and cortical binding of amyloid PET ligand. Other CSF biomarkers suggestive of neurodegeneration are increased levels of total tau protein and of phosphorylated tau [14,15].

There is some evidence for a role of Se in AD etiology [10,12,13,16,17]. However, the effects of Se on AD risk have been suggested to be either beneficial or adverse [11,18–22], as with other neurodegenerative diseases [10,23–30]. The use of Se in AD therapy has also been recently considered, though at extremely high doses [31]. Overall, these results mirror those yielded by the laboratory studies, which have provided biological plausibility for both a toxic and a beneficial role of Se on AD pathophysiology, mainly due to the complex and conflicting effects on oxidative stress by selenium species and selenoproteins [32–39].

Most of the human studies dealing with the issue of selenium and neurodegenerative disease, such as Alzheimer's dementia, amyotrophic lateral sclerosis and Parkinson's disease, have been characterized by a case-control design [12,13,16]. They have been based in most cases on peripheral indicators of exposure (such as serum/plasma selenium levels), and not on a central nervous system indicator such as cerebrospinal fluid, a choice which may have substantial methodological implications and may lead to exposure misclassification [40–43]. Most of these case-control studies showed no association or an inverse correlation between this element and AD risk, although positive associations were also occasionally reported [12,13,16]. In addition, none of these case-control studies has included a full speciation analysis of Se chemical forms. This is a particularly relevant issue, taking into account the sharp differences in biological activities of Se species [2,39,40,44], and their uneven distribution in the human body as well as in environmental sources, including diet [45–47]. In addition, the case-control approach characterizing these studies might suffer from exposure misclassification and reverse causality, therefore yielding biased effect estimates.

Only two cohort studies, one experimental and the other one non-experimental, have assessed the relation between selenium exposure and AD risk. A randomized controlled trial carried out within the large selenium and vitamin E cohort intervention study (SELECT) showed little effect on Alzheimer's dementia risk by 200 μg/day organic selenium supplementation, therefore being considered to be substantially negative [48]. The other cohort study had a nonexperimental design, and was carried out in a cohort of Italian participants affected by mild cognitive impairment (MCI) [21]. In that study, we assessed if baseline levels of the various selenium chemical forms, as detected in cerebrospinal fluid, were associated to AD risk. We found that among all the selenium species tested, the inorganic hexavalent form (selenate) was the only one positively and strongly associated with subsequent dementia occurrence.

To explain these conflicting results between case-control and cohort studies, a number of hypotheses may be put forward: one is the misclassification of long-term selenium exposure due to alterations in nutritional status or metabolism in these patients following disease progression, the different reliability of peripheral versus central indicators of selenium exposure, and the relevance of exposure to single selenium species to determine its health effects, particularly in the central nervous system [2,23,27,40,49,50]. To investigate these methodological issues and particularly to assess the potential biases related to the case-control design, we carried out a case-control study partially overlapping with our previous cohort investigation.

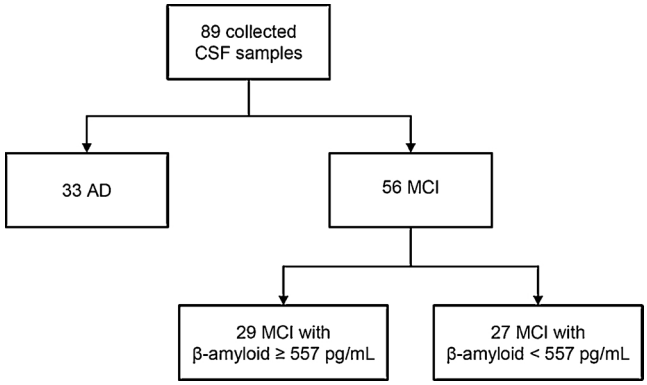

Fig. 1. Flowchart of the case-control study on selenium species in the cerebrospinal fluid (CSF) of patients with Alzheimer's dementia (AD) and with mild cognitive impairment (MCI).

2. Materials and methods

2.1. Study participants

The flowchart reported in Fig. 1 shows the study we undertook after the Modena Ethics Committee approval (No. 84/2015). Written consent to use CSF for scientific research purposes was obtained from all the patients involved in the study before sampling. We considered as eligible for our original cohort study a consecutive series of subjects who received a clinical diagnosis of either amnestic MCI (single domain or multiple domain) or non-amnestic MCI of non-vascular origin admitted 2008–2014 to the Modena and Reggio Emilia Neurology Memory Clinic of Policlinico University Hospital (formerly Sant'Agostino-Estense) according to Peterson's criteria revisions proposed by Winblad et al. [51]. These criteria include: (i) presence of memory complaints by participant or informant; (ii) cognitive and functional status not consistent with a diagnosis of dementia (as defined by DSM IV); (iii) cognitive deficits on memory testing together with report of decline over time by participant or informant; (iv) intact functional status based on with Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL) questionnaires. We further restricted participation in this study to the 56 MCI subjects who underwent lumbar puncture, if required for diagnostic purposes, and had 1 ml of cerebrospinal fluid available for research purposes. In the present study, these MCI participants constituted the referent group. In addition to them, we recruited 33 subjects who in the 2008–2014 period at the same clinic had received a clinical diagnosis of AD according to the criteria for probable Alzheimer Disease of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) available at the time [21,52,53], underwent lumbar puncture for diagnostic purposes and had 1 ml of cerebrospinal fluid still available for research purposes. These AD patients represent the case group.

We had routine blood tests available, along with the neurological and neuropsychological examinations and brain MRI for all participants at the time of either MCI and AD diagnosis. Analytical determinations routinely performed in the cerebrospinal fluid and available for this study were levels of Aβ1-42 (β-amyloid) as well as the tau protein, as total tau (t-tau) and phosphorylated tau (p-tau). APOE ε4 allele status was available in 64 participants.

2.2. Analytical determinations

The sampling of cerebrospinal fluid and selenium speciation refers to Mandrioli, Michalke et al. 2017. Briefly, standardized lumbar punctures were performed minimizing the risk of biological and chemical contamination [54]. After collection, we transported

cerebrospinal fluid to the adjacent laboratory within 30 min, centrifuged (15 min, 2700 g, ~20 °C) and aliquoted samples into polypropylene storage tubes. Cerebrospinal fluid β-amyloid, t-tau, and p-tau 181 were measured as previously described [53]. The remaining anonymized aliquots were immediately stored at minus 80 °C and transported in dry ice to the element speciation laboratory at the Helmholtz Zentrum München.

We determined total selenium by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS). Moreover, the following selenium species – selenite (Se(IV)), selenate (Se(VI)), selenomethionine-bound Se (Se-Met), selenocysteine-bound Se (Se-Cys), thioredoxin reductase-bound Se (Se-TXNRD), glutathione-peroxidase-bound Se (Se-GPX), selenoprotein P-bound Se (Se-SELENOP) and human serum albumin-bound Se (Se-HSA) – were determined in cerebrospinal fluid samples by ion exchange chromatography (IEC) coupled with ICP-DRC-MS, according to [42,55]. For total Se determination, cerebrospinal fluid samples were diluted 1/10 with Milli-Q water + 1 μg/L Rh as the internal standard, whereas for Se-speciation, we used a Knauer 1100 Smartline inert Series gradient HPLC system with an ion exchange column AS-11 (250 × 4 mm I.D.) from Thermo Fischer Scientific Inc. (Sunnyvale, CA, USA) for species separation. Samples (undiluted cerebrospinal fluid, a´ 20 μL) were determined in duplicate. Mobile phases were A = 3.33 mM Tris-HAc buffer, 5% methanol, pH 8.0, and B = 10 mM Tris-HAc buffer, 500 mM ammonium acetate, 5% methanol, pH 8.0, using gradient elution as specified in Mandrioli, Michalke et al. 2017. The experimental settings for ICP-DRC-MS (NexIon 300 D, Perkin Elmer) were as follows: radio frequency power: 1250 W; plasma gas flow: 15 L Ar/min; auxiliary gas flow: 1.05 L Ar/min; nebulizer gas flow: 0.92 L Ar/min; daily optimized, dwell time 300 ms; ions monitored: ^{77}Se, ^{78}Se, ^{80}Se, ^{103}Rh; DRC reaction gas: CH_4 reaction at 0.58 mL/min; DRC rejection parameter q: 0.6. Five-point calibration curves between blanks and 5000 ng Se/L were linear with very good r^2 for monitored Se isotopes (> 0.999881). Data files from selenium chromatograms were processed with the PeakFitTM software for peak area integration. The limit of detection (LOD) was 19.5 ng Se/L for all Se-species. Accuracy of selenium determination and selenium species quantification was checked by analyzing control materials and a certified reference material: quality control (QC) for total Se determination was performed by analyzing control materials 'human serum' and 'urine' from Recipe, Munich. Accuracy values were 98.4 ± 3.8% (serum) and 102.1 ± 5.4% (urine).

The certified reference material NIST 1950 (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for QC regarding total Se, Se-SELENOP, Se-GPX and Se-HSA. Accuracy values were 103 ± 5.1% (Se-SELENOP, target value = 100%: 50.2 ± 4.3 μg/kg), 93 ± 3.1% (Se-GPX, target value = 100%: 23.6 ± 1.3 μg/kg), and 97 ± 1.7% (Se-HSA, target value = 100%: 28.2 ± 2.6 μg/kg).

2.3. Data analysis

For our analysis, we used half of the threshold of values which fell below the limit of detection (LOD) [56,57]. Most participants had Se species above the LOD (96%, 87%, 100%, 98%, 30%, 54% and 99% for Se(IV), Se(VI), Se-SELENOP, Se-Met, Se-Cys, Se-GPX and Se-HSA, respectively). We used linear regression analysis to assess the correlation between log-transformed cerebrospinal fluid concentrations and either β-amyloid or p-tau at baseline, excluding from the analysis subjects having values below the LOD. We computed a crude and adjusted odds ratio of AD by using bivariate and multivariable logistic regression analysis. These were respectively used in the entire study population and in subgroups according to sex, age and APOE ε4 status, as well as to a β-amyloid cutoff of 557 pg/mL, which was specifically validated for our laboratory in agreement with the literature [58,59]. In such analysis, exposure to Se and Se species was considered either as dichotomous (plus/minus the median as computed in referents only) or in terms of continuous variables. In multivariable analysis, we adjusted for potential factors hypothesized or known to be associated with exposure and/or to the outcome, such as age, sex, education (years) and length (years) of storage of the cerebrospinal fluid sample. We ran an additional multivariable analysis by adding β-amyloid and p-tau to the model, in order to test the association between Se and AD independently of changes in these biomarkers of amyloidosis and neurodegeneration.

3. Results

Table 1 summarizes the characteristics of our case (AD) and referent (MCI) participants at diagnosis, and the concentrations of Se, Se species, and biomarkers of amyloidosis and neurodegeneration in cerebrospinal fluid. Some characteristics differed across the two populations, namely age, education and APOE ε4 status: all these factors were thoroughly checked in the analysis. Concerning biomarkers, MCI subjects showed higher levels of overall Se, inorganic Se and HSA-Se, while there was a minor difference concerning summed organic Se species between the two groups. When looking at the single organic selenium compounds, however, Se-SELENOP levels were higher, while Se-Met and Se-GPX concentrations were lower in MCI patients compared with AD cases. Levels of β-amyloid were lower in AD patients compared with MCI subjects, while the opposite was true for t-tau and p-tau. Results were broadly consistent in subgroup analyses according to sex and age group (Supplemental Tables S1–S2). When results from AD patients were compared to each subgroup of referents (according to β-amyloid level), results were comparable to those obtained in dealing with the entire referent population. However, MCI participants with lower β-amyloid levels had higher levels of overall Se, inorganic hexavalent Se (Se(VI)), Se-HSA and neurodegeneration markers t-tau and p-tau, while the levels of organic Se and those of the two major constituents of this category, SELENOP and Se-Met, were lower (Table 1).

In multiple regression analysis (Table 2), there was little evidence of any association of β-amyloid with Se species, with the exception of a positive relation with Se-SELENOP and particularly Se-Met and Se-Cys in MCI participants. On the other hand, there was only a slighter association with Se-Met in AD patients. Limited evidence of an association between Se species and p-tau emerged, with the exception of a slight association of organic Se and particularly Se-SELENOP and (less precisely) Se-Met in AD subjects. Overall, Se was very slightly and positively associated with β-amyloid and p-tau in the AD group, as it was with p-tau in MCI subjects.

Odds ratios (ORs) for AD according to median values of overall Se and single Se species levels are reported in Table 3, using as a cutpoint the median level computed for the corresponding MCI population, i.e. all referents in the two subgroups identified according to the β-amyloid value. Both crude estimates and those adjusted for sex, age, years of sample storage and education are reported. ORs associated with overall Se were less than 0.5 in both crude (0.43, 95% CI 0.18–1.08) and adjusted (0.46, 95% CI 0.17–1.22) analysis, and they further decreased for inorganic Se, due to low ORs for both tetravalent Se (Se(IV)) and hexavalent Se (Se(VI)). ORs for organic Se were close to the unit (adjusted OR 0.96, 95% CI 0.33–2.77), but this was due to opposite patterns for Se-SELENOP (0.44, 95% CI 0.15–1.28) and for the remaining organic forms Se-Met, Se-Cys and Se-GPX, characterized by very high ORs. The OR for Se-HSA was 0.31 in the adjusted analysis (95% CI 0.11–0.87). These estimates were substantially confirmed when the referent population was limited to participants with higher β-amyloid cerebrospinal fluid concentrations. These were less likely to be affected by subclinical Alzheimer's disease compared with MCI participants with lower β-amyloid levels, except for ORs for overall organic Se (well above the unit) and for Se-SELENOP, which were not lower but above the unit. After stratifying the analysis according to APOE ε4 status, effect estimates were statistically more unstable due to the limited number of subjects in each category (Supplemental Table S3). Results for overall Se were not markedly different, while the OR for inorganic

Table 1
Characteristics of the study population and distribution of levels of Se species (as μg Se/L cerebrospinal fluid) and β-amyloid, total (t-tau) and phosphorylated (p-tau) tau proteins (pg/mL) in the cerebrospinal fluid of the study population.

	AD		MCI		MCI with β-amyloid ≥ 557 pg/mL		MCI with β-amyloid < 557 pg/mL	
	N	(%)	N	(%)	N	(%)	N	(%)
Total	33	(100)	56	(100)	29	(100)	27	(100)
Sex								
Men	16	(48.5)	30	(53.6)	16	(55.2)	14	(51.9)
Women	17	(51.5)	26	(46.4)	13	(44.8)	13	(48.1)
Age at entry								
< 65 years	21	(63.6)	24	(42.9)	14	(48.3)	10	(37.0)
≥65 years	12	(36.4)	32	(57.1)	15	(51.7)	17	(63.0)
Education								
< 8 years	6	(18.2)	18	(32.1)	11	(37.9)	7	(26.0)
8–12 years	12	(36.4)	16	(28.6)	6	(20.7)	10	(37.0)
≥13 years	15	(45.4)	22	(39.3)	12	(41.4)	10	(37.0)
APOE ε4								
Non-carriers	11	(33.3)	21	(37.5)	16	(55.2)	5	(18.5)
Carriers	14	(42.4)	18	(32.1)	6	(20.7)	12	(44.4)
Missing	8	(24.3)	17	(30.4)	7	(24.1)	10	(37.1)
	50th	(IQR)	50th	(IQR)	50th	(IQR)	50th	(IQR)
Total Se	3.68	(2.91 - 4.31)	4.17	(3.17 - 4.62)	4.16	(3.73 - 4.51)	4.40	(3.62 - 5.08)
Inorganic Se	0.44	(0.34 - 0.60)	0.64	(0.46 - 0.77)	0.63	(0.48 - 0.73)	0.69	(0.46 - 0.91)
Se(IV)	0.34	(0.24 - 0.42)	0.41	(0.32 - 0.63)	0.41	(0.33 - 0.55)	0.45	(0.30 - 0.66)
Se(VI)	0.12	(0.06 - 0.23)	0.14	(0.09 - 0.32)	0.12	(0.09 - 0.26)	0.20	(0.11 - 0.40)
Organic Se	1.84	(1.19 - 2.25)	1.82	(1.20 - 2.29)	1.84	(1.37 - 2.40)	1.60	(1.03 - 2.18)
Se-SELENOP	1.52	(0.84 - 1.91)	1.58	(1.07 - 2.01)	1.63	(1.16 - 2.04)	1.45	(0.94 - 1.87)
Se-Met	0.18	(0.10 - 0.23)	0.14	(0.08 - 0.22)	0.17	(0.10 - 0.23)	0.12	(0.06 - 0.16)
Se-Cys	0.01	(0.01 - 0.08)	0.01	(0.01 - 0.01)	0.01	(0.01 - 0.01)	0.01	(0.01 - 0.01)
Se-GPX	0.05	(0.01 - 0.12)	0.01	(0.01 - 0.08)	0.01	(0.01 - 0.09)	0.01	(0.01 - 0.06)
Se-HSA	1.26	(0.86 - 1.52)	1.54	(1.13 - 1.83)	1.53	(1.08 - 1.80)	1.60	(1.16 - 1.97)
Unknown species	0.12	(0.04 - 0.36)	0.25	(0.14 - 0.39)	0.28	(0.12 - 0.38)	0.24	(0.15 - 0.43)
β-amyloid	452	(385 - 499)	596	(449 - 798)	789	(691 - 1012)	441	(370 - 509)
t-tau	597	(440 - 791)	372	(220 - 619)	255	(198 - 374)	511	(304 - 769)
p-tau	96	(77 - 118)	69	(50 - 88)	56	(48 - 80)	86	(62 - 128)

Abbreviations: AD, Alzheimer's dementia, IQR, interquartile range; MCI, mild cognitive impairment; Se(IV), selenite; Se(VI), selenate; Se-SELENOP, selenoprotein P-bound Se; Se-Met, selenomethionine-bound Se; Se-Cys, selenocysteine-bound Se; Se-GPX, glutathione-peroxidase-bound Se; Se-HSA, human serum albumin selenium-bound Se.

Table 2
Linear regression analysis of cerebrospinal fluid Se species versus log-transformed values of AD pathology biomarkers (β amyloid and phosphorylated (p-tau) tau protein as dependent variables) in the 33 AD and the 56 MCI study participants. Adjusted for sex, age at entry and years of storage.

	33 AD study participants		56 MCI study participants	
Se species	β	95% CI	β	95% CI
β-amyloid				
Total Se	0.08	(-0.02 to 0.17)	0.03	(-0.10 to 0.15)
Inorganic Se	0.03	(-0.32 to 0.37)	−0.25	(-0.65 to 0.15)
Se(IV)	−0.00	(-0.64 to 0.64)	−0.06	(-0.72 to 0.61)
Se(VI)	0.13	(-0.60 to 0.86)	−0.80	(-1.72 to 0.13)
Organic Se	0.09	(-0.06 to 0.24)	0.18	(0.01 to 0.34)
Se-SELENOP	0.11	(-0.05 to 0.27)	0.17	(-0.01 to 0.35)
Se-Met	0.59	(-0.19 to 1.37)	2.31	(0.84 to 3.78)
Se-Cys	0.34	(-2.18 to 2.87)	2.94	(-0.75 to 6.63)
Se-GPX	−0.00	(-0.39 to 0.39)	0.39	(-1.62 to 2.39)
Se-HSA	0.08	(-0.10 to 0.26)	0.01	(-0.22 to 0.24)
Unknown species	0.13	(-0.29 to 0.56)	−0.11	(-0.66 to 0.45)
p-tau				
Total Se	0.11	(-0.08 to 0.29)	0.09	(-0.04 to 0.23)
Inorganic Se	−0.05	(-0.69 to 0.60)	−0.08	(-0.52 to 0.35)
Se(IV)	−0.45	(-1.63 to 0.72)	−0.53	(-1.23 to 0.16)
Se(VI)	0.28	(-1.11 to 1.66)	0.24	(-0.73 to 1.20)
Organic Se	0.28	(0.01 to 0.54)	0.10	(-0.08 to 0.28)
Se-SELENOP	0.37	(0.09 to 0.64)	0.13	(-0.08 to 0.33)
Se-Met	0.67	(-0.82 to 2.17)	0.11	(-1.59 to 1.81)
Se-Cys	0.80	(-3.70 to 5.31)	−2.32	(-8.62 to 3.98)
Se-GPX	−0.08	(-1.06 to 0.91)	−0.12	(-2.68 to 2.45)
Se-HSA	−0.00	(-0.35 to 0.34)	0.04	(-0.22 to 0.30)
Unknown species	−0.16	(-0.97 to 0.64)	0.74	(0.18 to 1.30)

Abbreviations: AD, Alzheimer's dementia; MCI, mild cognitive impairment; Se(IV), selenite; Se(VI), selenate; Se-SELENOP, selenoprotein P-bound Se; Se-Met, selenomethionine-bound Se; Se-Cys, selenocysteine-bound Se; Se-GPX, glutathione-peroxidase-bound Se; Se-HSA, human serum albumin selenium-bound Se.

Table 3
Crude and adjusted[a] odds ratios (ORs) of AD according to cerebrospinal fluid Se species levels, according to different referent groups. Selenium exposure status defined as 0 (below or equal) and 1 (above) with reference to the median value in the control (MCI) participants.

	All referents		Referents with β-amyloid ≥557 pg/mL		Referents with β-amyloid < 557 pg/mL	
	OR	95% CI	OR	95% CI	OR	95% CI
Crude analysis						
Total Se	0.43	(0.18 - 1.08)	0.54	(0.19 - 1.50)	0.25	(0.08 - 0.77)
Inorganic Se	0.32	(0.12 - 0.83)	0.34	(0.12 - 1.01)	0.25	(0.08 - 0.77)
Se(IV)	0.38	(0.15 - 0.95)	0.35	(0.12 - 1.01)	0.30	(0.10 - 0.89)
Se(VI)	0.53	(0.22 - 1.29)	0.88	(0.32 - 2.38)	0.35	(0.12 - 1.02)
Organic Se	1.06	(0.45 - 2.51)	0.99	(0.37 - 2.69)	1.66	(0.59 - 4.63)
Se-SELENOP	0.65	(0.27 - 1.56)	0.53	(0.19 - 1.47)	1.29	(0.47 - 3.58)
Se-Met	2.47	(1.00 - 6.13)	1.12	(0.41 - 3.04)	2.14	(0.74 - 6.16)
Se-Cys	3.06	(1.20 - 7.79)	2.62	(0.88 - 7.81)	3.67	(1.12 - 12.03)
Se-GPX	3.56	(1.40 - 9.02)	3.28	(1.14 - 9.47)	3.88	(1.31 - 11.47)
Se-HSA	0.32	(0.12 - 0.83)	0.30	(0.10 - 0.88)	0.25	(0.08 - 0.77)
Unknown species	0.57	(0.24 - 1.38)	0.47	(0.17 - 1.30)	0.62	(0.22 - 1.73)
Adjusted[a] analysis						
Total Se	0.46	(0.17 - 1.22)	0.55	(0.17 - 1.84)	0.23	(0.06 - 0.81)
Inorganic Se	0.37	(0.14 - 1.01)	0.36	(0.11 - 1.16)	0.28	(0.08 - 0.98)
Se(IV)	0.45	(0.17 - 1.20)	0.38	(0.12 - 1.14)	0.37	(0.11 - 1.23)
Se(VI)	0.64	(0.25 - 1.62)	0.97	(0.33 - 2.81)	0.24	(0.07 - 0.86)
Organic Se	0.96	(0.33 - 2.77)	0.97	(0.28 - 3.39)	2.17	(0.51 - 9.22)
Se-SELENOP	0.44	(0.15 - 1.28)	0.33	(0.09 - 1.20)	1.12	(0.28 - 4.48)
Se-Met	3.15	(1.15 - 8.63)	1.47	(0.49 - 4.42)	2.35	(0.69 - 7.93)
Se-Cys	4.00	(1.36 - 11.77)	3.58	(1.03 - 12.46)	5.81	(1.41 - 23.96)
Se-GPX	4.05	(1.44 - 11.38)	3.64	(1.16 - 11.41)	4.53	(1.30 - 15.74)
Se-HSA	0.31	(0.11 - 0.87)	0.34	(0.11 - 1.04)	0.13	(0.03 - 0.56)
Unknown species	0.57	(0.21 - 1.54)	0.51	(0.16 - 1.59)	0.62	(0.19 - 1.98)

Abbreviations: AD, Alzheimer's dementia; MCI, mild cognitive impairment; Se(IV), selenite; Se(VI), selenate; Se-SELENOP, selenoprotein P-bound Se; Se-Met, selenomethionine-bound Se; Se-Cys, selenocysteine-bound Se; Se-GPX, glutathione-peroxidase-bound Se; Se-HSA, human serum albumin selenium-bound Se.
[a] Adjusted for sex, age at entry, years of storage and years of education.

Se was much lower in APOE ε4 carriers, particularly in the adjusted analysis. In such analysis and in the latter subgroup, ORs for Se-Met, Se-Cys and Se-GPX were considerably increased compared with non APOE ε4 carriers, and this also increased the overall OR for organic Se. The OR associated with Se-HSA was also higher in the APOE ε4 carriers.

Adjusted ORs of AD for 1-unit continuous increase in exposure to Se and Se species are reported in Table 4, also taking into account the two subgroups of referents defined according to β-amyloid values and APOE ε4 carrier status. Results showed a pattern generally comparable to the aforementioned estimates referring to dichotomous exposure categories based on the median value, showing high consistency between these two analyses. The only difference according to referent subgroup was the higher OR associated to Se-Cys when MCI subjects in the lower β-amyloid category were considered as referents. A further breakdown of the study population according to sex or age group and based on continuous Se levels (Supplemental Tables S4–S5) yielded little evidence of substantial differences, with some exceptions. Women had lower ORs for overall and inorganic Se, and a higher OR for organic Se compared with men, while differences across sexes were even increased when single Se species were considered (such as Se(IV), Se-SELENOP, Se-Cys and Se-GPX). Older subjects had higher ORs for AD compared with younger participants for most Se categories and species, and this was particularly true for organic Se. Comparable results were obtained when OR calculations were based on dichotomous exposure categories, i.e. above or below the median level of selenium and of the single selenium species (data not shown).

We eventually performed a calculation of the ORs for AD through an additional adjustment in the multivariable analysis, i.e. for β-amyloid and p-tau alongside sex, age, storage time and education (Supplemental Table S6). The results of such adjusted model were substantially the same as those computed without this additional adjustment and are reported in Table 3, except for a considerably higher OR for organic Se (1.94, 95% CI 0.51–7.41) driven by very high ORs for Se-Met, Se-Cys and Se-GPX (but not Se-SELENOP).

4. Discussion

We found that a case-control approach to assess the relation between Se status in the central nervous system and AD risk, including participants with established AD and referents with MCI, showed an inverse association between overall Se exposure in the central nervous system and the disease. This was also true when exposure assessment was limited to inorganic Se, while risk positively correlated with exposure to some Se species, most of which bound to selenoproteins. These results were markedly different and even opposite to those generated by a longitudinal study carried out in part of this study population, since in the follow-up of the referent group (MCI participants) we had found a positive relation between baseline levels of inorganic hexavalent Se and subsequent dementia occurrence, and no relation for the other Se species [21]. Therefore, the comparative assessment of these results shows the potential for bias of case-control studies assessing Se status at the time of the study, despite the use of a central-nervous-system indicator and a comprehensive analysis of all Se chemical forms, and independently of the likelihood of AD-related pathological changes in the referent population we used.

These findings suggest that progression to AD modifies the levels of Se species in cerebrospinal fluid, and that these changes markedly influence the assessment of selenium-related relative risk, incorrectly indicating an inverse association between exposure and AD. Assuming therefore that case-control study design generates misleading results on the relation between Se and AD, we may hypothesize that previous case-control studies on this issue, independently of the referent population and the biomarker being used (blood, urine and cerebrospinal fluid Se levels) or diet itself, most likely suffered from reverse causation. Accordingly, such bias may have incorrectly suggested Se deficiency as underpinning disease status, based on the lower levels of overall Se detected in AD patients, a hypothesis which in turn generated interest in Se supplementation to prevent AD [24,25,60]. As a result, a longitudinal study design appears to be needed to investigate AD etiology as

Table 4
Adjusted[a] odds ratios (ORs) of AD according to increasing levels (0.1 μg/L) of cerebrospinal fluid Se species, according to the different referent populations.

	All referents		Referents with amyloid ≥557 pg/mL		Referents with amyloid < 557 pg/mL	
	OR	95% CI	OR	95% CI	OR	95% CI
All subject						
Total Se	0.95	(0.90 - 1.00)	0.95	(0.89 - 1.02)	0.93	(0.87 - 0.99)
Inorganic Se	0.85	(0.72 - 1.01)	0.90	(0.72 - 1.11)	0.77	(0.62 - 0.96)
Se(IV)	0.77	(0.60 - 1.00)	0.78	(0.56 - 1.07)	0.72	(0.53 - 0.99)
Se(VI)	0.86	(0.63 - 1.18)	1.04	(0.71 - 1.53)	0.65	(0.43 - 0.96)
Organic Se	0.98	(0.91 - 1.05)	0.96	(0.87 - 1.05)	1.00	(0.91 - 1.09)
Se-SELENOP	0.92	(0.85 - 1.00)	0.89	(0.80 - 1.00)	0.93	(0.84 - 1.03)
Se-Met	1.87	(1.08 - 3.26)	1.55	(0.84 - 2.85)	2.33	(1.09 - 4.97)
Se-Cys	2.18	(1.08 - 4.41)	1.76	(0.81 - 3.80)	3.23	(1.14 - 3.51)
Se-GPX	1.72	(1.08 - 2.75)	1.72	(0.97 - 3.03)	1.90	(1.03 - 3.51)
Se-HSA	0.92	(0.84 - 1.02)	0.95	(0.85 - 1.06)	0.86	(0.75 - 0.99)
Unknown species	0.85	(0.66 - 1.09)	0.89	(0.66 - 1.18)	0.84	(0.64 - 1.11)
APOE ε4 carriers						
Total Se	0.93	(0.84 - 1.03)	0.88	(0.66 - 1.19)	0.93	(0.84 - 1.03)
Inorganic Se	0.70	(0.48 - 1.00)	0.02	(0.00 - 12.81)	0.73	(0.51 - 1.04)
Se(IV)	0.65	(0.40 - 1.05)	0.16	(0.01 - 3.14)	0.69	(0.43 - 1.09)
Se(VI)	0.66	(0.38 - 1.13)	0.66	(0.28 - 1.57)	0.67	(0.39 - 1.16)
Organic Se	0.99	(0.88 - 1.11)	1.37	(0.82 - 2.27)	0.97	(0.86 - 1.10)
Se-SELENOP	0.92	(0.79 - 1.07)	1.04	(0.70 - 1.56)	0.89	(0.75 - 1.06)
Se-Met	1.95	(0.75 - 5.09)	1.36	(0.29 - 6.40)	2.37	(0.72 - 7.80)
Se-Cys	4.04	(0.76 - 21.60)	2.42	(0.27 - 21.96)	–	–
Se-GPX	1.54	(0.84 - 2.81)	1.57	(0.58 - 4.22)	1.80	(0.74 - 4.42)
Se-HSA	0.90	(0.76 - 1.08)	0.80	(0.51 - 1.25)	0.94	(0.76 - 1.16)
Unknown species	0.83	(0.55 - 1.25)	1.20	(0.53 - 2.75)	0.75	(0.48 - 1.18)
APOE ε4 non-carriers						
Total Se	0.96	(0.87 - 1.07)	0.94	(0.84 - 1.06)	1.26	(0.73 - 2.16)
Inorganic Se	0.87	(0.64 - 1.20)	0.91	(0.65 - 1.29)	0.90	(0.48 - 1.69)
Se(IV)	0.84	(0.55 - 1.30)	0.87	(0.53 - 1.38)	1.26	(0.49 - 3.25)
Se(VI)	0.82	(0.42 - 1.58)	0.95	(0.46 - 1.95)	0.30	(0.03 - 2.65)
Organic Se	0.99	(0.88 - 1.11)	0.91	(0.78 - 1.07)	0.92	(0.59 - 1.44)
Se-SELENOP	0.94	(0.82 - 1.09)	0.89	(0.74 - 1.06)	0.78	(0.43 - 1.42)
Se-Met	0.91	(0.77 - 1.08)	1.30	(0.43 - 3.96)	–	–
Se-Cys	1.75	(0.59 - 5.44)	1.70	(0.55 - 5.28)	3.31	(0.01 - too high)
Se-GPX	0.70	(0.14 - 3.39)	0.67	(0.14 - 3.18)	0.18	(0.00 - 80.99)
Se-HSA	1.04	(0.90 - 1.22)	1.04	(0.88 - 1.22)	1.32	(0.64 - 2.70)
Unknown species	0.58	(0.29 - 1.14)	0.48	(0.21 - 1.08)	–	–

Abbreviations: AD, Alzheimer's dementia; MCI, mild cognitive impairment; Se(IV), selenite; Se(VI), selenate; Se-SELENOP, selenoprotein P-bound Se; Se-Met, selenomethionine-bound Se; Se-Cys, selenocysteine-bound Se; Se-GPX, glutathione-peroxidase-bound Se; Se-HSA, human serum albumin selenium-bound Se.

[a] Adjusted for sex, age at entry, years of storage and years of education.

related to exposure to Se and its species.

Our findings also appear to confirm the findings and the hypothesis from the two studies investigating selenoprotein P levels in cerebrospinal fluid and post-mortem tissues from AD patients [61,62]. Rueli et al. found elevated selenoprotein P levels in choroid plexus and cerebrospinal fluid of AD patients compared with controls [61], consistent with previous results by Bellinger et al. obtained in post-mortem brain cortex specimens [62]. As the authors suggested, those findings could reflect a compensatory response to oxidative stress characterizing dementia progression through the upregulation of Se-containing and non-Se-containing antioxidant enzymes [61,62], although increased levels of selenoproteins found in AD patients might alternatively be associated with harmful effects *per se* [62], as suggested for other diseases [4,9,37,63,64]. Bellinger et al. and Rueli et al. therefore concluded that the increased selenoprotein P levels found in cases could reflect the upregulation of this antioxidant enzyme accompanying disease progression [61,62]. Accordingly, the high ORs associated with some organic Se species in our study cannot be interpreted as indicating excess AD risk associated with these forms. Rather, they appear to be a reverse-causation effect, not least because in the cohort study [21], no change in organic Se levels could be detected at baseline in MCI participants who later progressed to AD. The increased organic Se levels we observed in AD patients may have been a consequence of oxidative stress accompanying (and possibly favoring) dementia progression, and leading to selenoprotein upregulation. Consistent with these findings and hypotheses, increased selenoprotein activity or Se levels in blood or cerebrospinal fluid in AD patients have been reported in other works [61,65–72], while other studies yielded different results [13,16]. In addition, in post-mortem brain samples of participants in the Chicago Memory and Aging Project, a positive association between Se brain levels and neurofibrillary tangle severity, one of the neuropathological fingerprints of Alzheimer's disease, has also emerged [20]. In this study, the higher Se content may be a consequence of disease progression too, or alternatively it may have etiologic relevance. Accordingly, Bellinger et al. found evidence of an association between immunoreactivity to selenoprotein P and intraneuronal neurofibrillary tangles, and of co-localization of amyloid-beta protein and selenoprotein P in post-mortem brain tissues from AD patients [62].

Our results highlight the relevance of Se speciation in addressing the relation between Se and AD [21]. Growing evidence has been provided to show how speciation may influence the relation of exposure to heavy metals and other trace elements, Se in particular, with neurodegenerative disease [40,73]. Se species have different toxicological and nutritional activities, due to relevant differences in biological reactivity and function [39,44,50,74] not yet entirely elucidated but under active investigation [23,27,40,42,75]. To the best of our knowledge, the present study is the first case-control investigation of the specific relation between the full spectrum of Se species and AD risk. The relevance of Se speciation to the investigation of the involvement of Se in neurodegenerative disease etiology and progression has been recently

highlighted [21,28,30,40,43,54,76]. Interestingly, however, none of the Se species avoided the risk of bias due to reverse causation in our population, since the ORs in the present case-control study markedly differ from those generated by our cohort investigation for almost all inorganic and organic species, and for overall Se as well [21].

Our study was based on a central-nervous-system indicator of Se exposure, cerebrospinal fluid levels, and not of peripheral biomarkers such as selenium concentrations in serum, plasma, urine or nails, or an assessment of its dietary intake. We used cerebrospinal fluid levels due to evidence of complex regulatory systems in selenium exchange between blood and the central nervous system, while for some Se species, namely the inorganic ones, no correlation may exist between these two compartments [41,42,77–80]. This may be related to the specific features of the transfer and metabolism of Se species across the blood-brain barrier, and the relative independence of their central-nervous-system levels [41,42,77–80]. Therefore, the use of a target tissue in addressing the neurological effects of Se may allow to avoid serious exposure misclassification arising from the use of circulating Se levels as a proxy of brain Se content. The limitations inherent in using peripheral biomarkers of exposure have been extensively addressed [1,9,39,81,82]. Unfortunately, no indicator reflecting very long-term Se exposure, in addition to exposure to specific Se species, has been identified so far. Three case-control studies have used cerebrospinal fluid levels to assess the relation between selenium exposure and AD, generally finding lower levels in patients compared with controls [18,68,83], consistent with our findings.

In our study, we used MCI participants as referents instead of 'healthy subjects'. This was the case for mainly ethical reasons, since lumbar puncture may be part of the standard diagnostic process for these subjects, so that cerebrospinal specimens become available. The possibility that this referent population may not be adequate in reflecting 'control' levels of Se species in cerebrospinal fluid must be considered. For this reason, we also restricted our assessment by using only part of the referent group, the one in the highest category of cerebrospinal fluid β-amyloid, for which subclinical progression of Alzheimer's disease was less likely to have occurred. The lack of substantial changes in relative risk estimates for AD indicated that bias due to an incorrect choice of the control group is not likely to have occurred.

The reasons underlying the lower Se status found in our AD patients compared with referents are difficult to evaluate. We speculate that an impairment of nutritional status, as may occur even slightly in AD patients [84], could have been the source of decreased intake, and therefore of the lower overall Se status we detected. In addition, disease progression status may affect Se delivery to the brain and/or impair its transport, excretion and utilization, and these changes may affect Se species unevenly. Findings from case-control studies recruiting subjects defined as 'healthy' and carriers of subjective memory complaints, mild cognitive impairment, and AD found decreasing blood Se levels across these subgroups of increasing disease severity [85–88], suggesting impairments in nutrient status already at early disease stages [88,89]. As previously mentioned, conversely, increased selenoprotein levels in blood, brain and cerebrospinal fluid have been detected in studies carried out in established AD [16,21,61], possibly due to oxidative stress-driven upregulation of antioxidant enzymes such as selenoproteins determining higher levels of organic-bound Se and even overall Se [36,39,61,62]. Overall, it is therefore possible that AD risk associated with Se species may be biased in opposite directions, depending on the balance between impaired Se intake or metabolism and selenoprotein upregulation.

In our study, we found that ORs for AD were not substantially modified for overall Se and inorganic Se by an additional adjustment for biomarkers of amyloidosis and neurodegeneration, i.e. β-amyloid and p-tau. These are two parameters we did not account for in the main analysis, since they could be intermediate factors in any relation between selenium and dementia onset. Conversely, the OR associated with organic Se was increased compared to that found in the less adjusted analysis. This further suggests that, even at the same levels of neurodegeneration biomarkers, Alzheimer's disease is associated with an upregulation of selenoproteins in the central nervous system, possibly as a consequence of increased oxidative stress [90–92]. We also observed differences in the ORs associated with some Se species according to parameters such as sex, age and APOE ε4 carriership, a factor potentially interacting with or influencing Se status [93,94]. Among these, we found a high OR associated with organic Se in the oldest subjects, opposite to what we detected in the youngest participants.

Some limitations may well have affected the results of the present study. First, the study size was rather small, due to both the limited number of MCI and AD patients needing cerebrospinal fluid sampling for clinical purposes and the analytical complexity of Se speciation analyses. This clearly increased the statistical imprecision of our effect estimates, as reflected by their confidence intervals [95]. Another limitation is inherent in the nonexperimental nature of our study: this is the possibility of unmeasured confounding due to other chemicals of nutritional and/or toxicological importance covariating with Se species. However, experimental studies encompassing Se administration are likely to be impossible for ethical reasons, due to the serious adverse effects arising from trials encompassing selective administration of organic or inorganic Se species [4,5,9,96]. Therefore, only nonexperimental cohort studies may offer future opportunities to further investigate this issue, in the attempt to check for potential confounders. Alternatively, secondary analyses of Se trials such as that recently published for SELECT [48] may be of strong interest to test the effects of single Se chemical forms (or sources, as in the case of selenized yeast) in AD etiology. The association of Se status with the etiology of chronic disease such as cardiovascular disease, cancer and diabetes has been substantially elucidated, as is demonstrated by the large number of studies including randomized trials and the consistency of the results [4,7,9]. However, the relation of Se status with neurodegenerative disease including AD still needs to be elucidated, based on recent experimental and nonexperimental human studies and by laboratory investigations.

5. Conclusions

This case-control study yielded entirely different results than those generated by a recent cohort study with a partially overlapping participant population, suggesting that the case-control design does not allow to reliably assess the role of selenium exposure in Alzheimer's dementia etiology. This inability appears to be due to exposure misclassification, falsely indicating an etiologic role of selenium deficiency likely due to reverse causation, and involving both inorganic and organic selenium species. The case-control study design may instead lend insights into the pathologic process underlying disease progression, suggesting the occurrence of selenoprotein upregulation.

Conflict of interest

None.

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Appendix A. Supplementary data

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