

# Homeostasis of metals in the progression of Alzheimer's disease

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**Abstract** In order to study the involvement of metals in the progression of Alzheimer's disease, serum samples from patients with Alzheimer and mild cognitive impairment were investigated. For this purpose, metal content was analyzed after size-fractionation of species and then, inter-element and inter-fraction ratios were computed. In this way, the analysis allowed discovering changes that could be used as markers of disease, but also provided a new insight into the interactions in the homeostasis of elements in neurodegeneration and its progression. Aluminum and labile forms of iron and copper were increased in demented patients, while manganese, zinc and selenium were reduced. Interestingly, levels of

different elements, principally iron, aluminum and manganese, were closely inter-related, which could evidence a complex interdependency between the homeostasis of the different metals in this disorder. On the other hand, imbalances in metabolism of copper, zinc and selenium could be associated to abnormal redox status. Therefore, this study may contribute to our understanding of the pathological mechanisms related to metals in Alzheimer's disease.

**Keywords** Alzheimer's disease · Mild cognitive impairment · Metal homeostasis · Inter-element relations

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## Introduction

Metal and metalloid elements play important roles in biological systems, regulating and participating in numerous cellular processes. In particular, equilibrium of metals has been demonstrated to be critical in central nervous system, where they are essential for diverse biological functions, such as enzymatic activities, mitochondrial function, myelination, neurotransmission, and others. Abnormal metabolism of metal ions in brain can result in levels outside the normal physiological range, usually by misallocation or lack of specific metal binding proteins, which finally leads to biological damage (Frausto da Silva and Williams 2001). In this sense, metal dyshomeostasis has been linked to the pathogenesis of several

neurodegenerative disorders, including Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis or prion protein disease (Sayre et al. 2000; Bolognin et al. 2009). These imbalances may have multiple origins such as exposure to metals (Charlet et al. 2012) or complex impairments in mechanisms controlling their homeostasis (Kozlowski et al. 2012).

Alzheimer's disease (AD) is a multifactorial disorder characterized by an insidious onset and progressive decline of cognitive functions, representing up to 70 % of all cases of dementia among the elderly (Reitz et al. 2011). Key hallmarks of this type of dementia are aberrant processing of amyloid precursor protein (APP) leading to deposition of  $\beta$  amyloid peptides (A $\beta$ ) in form of senile plaques, as well as hyperphosphorylation of  $\tau$  protein, responsible for the formation of neurofibrillary tangles (Maccioni et al. 2001), in which homeostasis of metals is closely involved. Thus, the essential metal ions iron, copper and zinc are able to interact with the major protein components of AD, promoting processing of APP by secretases (Bush et al. 1994), senile plaque formation (Bush and Tanzi 2002), and hyperphosphorylation of tau protein (Egana et al. 2003). In addition, abnormal accumulation and distribution of different metals that may elicit oxidative stress and macromolecular damage have been also associated with AD (Perry et al. 2002). On the other hand, mild cognitive impairment (MCI) is of great interest to discover changes in the onset of neurodegeneration, since it is thought that represents a preclinical stage of AD (Morris et al. 2001). In this sense, previous findings support the contribution of different cellular processes in disease pathogenesis, such as oxidative stress (Mecocci 2004), lipid dysfunction (Han 2010) or  $\beta$  amyloid deposition and hyperphosphorylation of  $\tau$  protein (Hansson et al. 2006), which are already present in MCI patients. Moreover, regarding metals, their homeostasis appear to be also related to the progression of AD, considering levels of calcium and zinc in cerebrospinal fluid (Kovatsi et al. 2006) or altered concentrations of redox-active metals in serum, brain and CSF (Smith et al. 2010; Lavados et al. 2008; Squitti et al. 2011). Therefore, the study of metal levels can provide valuable information about changes occurring in organisms during neurodegenerative processes, as well as about the involvement of these metals in the progression of disease. However, a more comprehensive approach involves the characterization of their

interactions with biomolecules. Metals can be mainly present as labile ions and complexed with low molecular mass ligands, or in form of metalloproteins. This distinction between low molecular mass (*LMM*) and high molecular mass (*HMM*) species is very important, since it finally affects to biological activity or toxicological potential of the element, and their mobility across different biological compartments.

Alternatively, knowledge about inter-relationships of metals in the organism may allow a deeper understanding of complex mechanisms controlling their homeostasis than simple total content analysis. The simpler manner to bring out these interactions is measuring elemental ratios, either for single elements between different biological compartments or by inter-elemental comparisons. Regarding inter-compartmental ratios, the study of paired serum and CSF samples has been proposed for the investigation of permeability of the human blood-CSF barrier (Nischwitz et al. 2008), since transport pathways of metals and their species to the brain are of special interest in neurodegenerative disorders. Thus, Gerhardsson et al. (2011) applied this methodology to determine CSF-plasma quotients in Alzheimer's disease patients, in order to discover the possible leakage of metals through this barrier, and they found altered ratios of some elements in subjects with AD as compared with the controls, but no differences with increased duration and/or severity of the disease. On the other hand, element-to-element ratios can provide additional information since, in living systems, there is a complex interdependency between the levels of elements to maintain homeostasis (Seiler et al. 1994). Thus, the effect of changes in a single element concentration could be not restricted to this element alone, but the total element distribution pattern in the system could be affected. In this sense, it has been previously shown that ratios of trace elements are useful for the assessment of inter-element relations occurring in Parkinson's disease (Hegde et al. 2004), bipolar mood disorders (Mustak et al. 2008), or prostatic cancer (Kiziler et al. 2010).

The aim of this work is to assess the complex role that metals play in pathology of Alzheimer's disease and its clinical precursor, mild cognitive impairment. For this purpose, the metal profile of serum was characterized by elemental speciation using a procedure based on protein precipitation in non-denaturing conditions for the analysis of high molecular mass

**Table 1** Clinical characteristics of patients (AD and MCI) and healthy controls

	AD (n = 25)	MCI (n = 15)	HC (n = 25)
Age (years)	81.4 ± 4.6	75.9 ± 5.5	74.0 ± 5.1
Sex (male/ female)	10/15	9/6	12/13

(*HMM*, principally metalloproteins) and low molecular mass (*LMM*, labile complexes) fractions. Secondly, element-to-element ratios were evaluated in order to deepen into the correlated homeostasis of the different metals during neurodegeneration. Finally, fraction-to-fraction ratios were computed to determine if there are imbalances in metabolism of single elements regarding their distribution across different species.

## Materials and methods

### Reagents and samples

Acetone (Trace Analysis Grade), nitric acid (purity 67–69 %, Trace Metal Grade) and hydrogen peroxide (purity 30–32 %, Optima Grade) were purchased from Fisher Scientific (Leicestershire, UK). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Blood samples were obtained by venipuncture of the antecubital region after 8 h of fasting. All samples were collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system, previously cooled in refrigerator. The samples were immediately cooled and protected from light for 30 min to allow clot retraction, and centrifuged (3,500 rpm for 10 min). Sera was divided into aliquots in Eppendorf tubes and frozen at  $-80^{\circ}\text{C}$  until analysis. Subjects, whose clinical characteristics are shown in Table 1, were recruited by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain). Patients were newly diagnosed of sporadic Alzheimer's disease (AD) according to the criteria of the NINCDS-ADRDA (McKahn et al. 1984), and only subjects that had not yet received any type of medication were included in the study. In the mild cognitive impairment (MCI) group were enrolled individuals with cognitive decline, but who not meet the NINCDS-ADRDA requirements for a possible or probable diagnosis of Alzheimer. Finally, healthy controls (HC) were studied by

neurologists to confirm the absence of neurological disorders, whom have not more than two reported cases of Alzheimer's disease in their families.

In spite of the substantial averaged age difference between AD patients (81.4 y) and the healthy controls (74 y), the plots of different metal concentrations versus the age of healthy controls do not exhibit significant difference (results not shown). Therefore age-related changes can be rule-out.

The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of University of Huelva. In addition, all persons gave informed consent for the extraction of peripheral venous blood.

### Instrumentation

Elemental analysis was performed by inductively coupled plasma mass spectrometry, using the Agilent 7500ce collision/reaction cell system (Agilent Technologies, Tokyo, Japan), with helium of high-purity grade (>99.999 %) as collision gas. Instrumental conditions were optimized by using a tuning aqueous solution containing Li, Co, Y, and Tl at  $1\text{ }\mu\text{g L}^{-1}$ . Platinum sampling and skimmer cones were employed, with a sampling depth of 7 mm. The forward power was set at 1,500 W, and the gas flow rates were fixed at  $15\text{ L min}^{-1}$  for plasma gas,  $1\text{ L min}^{-1}$  for auxiliary gas,  $1\text{ L min}^{-1}$  for carrier gas,  $0.15\text{ L min}^{-1}$  for makeup gas and  $3.5\text{ mL min}^{-1}$  for helium. Isotopes monitored were  $^{27}\text{Al}$ ,  $^{51}\text{V}$ ,  $^{53}\text{Cr}$ ,  $^{55}\text{Mn}$ ,  $^{57}\text{Fe}$ ,  $^{63}\text{Cu}$ ,  $^{64}\text{Zn}$ ,  $^{65}\text{Cu}$ ,  $^{66}\text{Zn}$ ,  $^{78}\text{Se}$ ,  $^{82}\text{Se}$ ,  $^{95}\text{Mo}$ ,  $^{98}\text{Mo}$ ,  $^{103}\text{Rh}$ ,  $^{112}\text{Cd}$  and  $^{114}\text{Cd}$  with a dwell time of 0.3 s per isotope.

A MARS microwave oven (CEM Matthews, NC, USA) was used for the mineralization of samples in PFA Teflon vessels. Samples were centrifuged in a centrifuge model Eppendorf 5804R.

### Sample preparation

For non-denaturing protein precipitation from serum samples, 300  $\mu\text{L}$  of cold acetone ( $-20^{\circ}\text{C}$ ) was dropwise added to 150  $\mu\text{L}$  of serum, and kept for 10 min in an ice bath. During this time, the mixture was sporadically vortexed, and then, the precipitate was removed by centrifugation (10,000 rpm, at  $4^{\circ}\text{C}$  for 5 min). The supernatant, containing low molecular mass (*LMM*) species, was taken to dryness under nitrogen stream, and reconstituted in 750  $\mu\text{L}$  of water,

with  $1 \mu\text{g L}^{-1}$  of rhodium as internal standard. On the other hand, the precipitate was subjected to microwave assisted acid digestion for the determination of metal content in the high molecular mass (*HMM*) fraction. For this, precipitate was introduced into the microwave vessel together with  $500 \mu\text{L}$  of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v). Mineralization was carried out at 400 W, ramping from room temperature to  $150^\circ\text{C}$  in 10 min, and maintaining this temperature for other 10 min. Then, extracts were made up to 2 mL with water, adding  $1 \mu\text{g L}^{-1}$  of rhodium. Before analysis, samples were filtered through  $0.45 \mu\text{m}$  pore size filters of PTFE.

This fractionation procedure was validated using an aqueous solution of bovine serum albumin standard containing copper and zinc. Reproducible and accurate results were obtained by ICP-MS analysis, and the integrity of the metal-protein bindings during the sample treatment procedure was demonstrated.

Finally, total metal content of serum (*TOTAL*) was determined in diluted samples as previously described (Muñiz et al. 2001). In this way, serum was five-fold diluted with ultrapure water and rhodium solution was added to reach a final concentration of  $1 \mu\text{g L}^{-1}$ .

### Statistical analysis

Statistical calculations were made in STATISTICA 8.0 software (StatSoft, Tulsa, USA). Non parametric methods were used since most of the variables showed a skewed distribution (checked by normal probability plots) and variances were not homogeneous (checked by Levene's test). Thus, group comparison was conducted using Kruskal–Wallis one-way analysis of variance, and when significant effects were observed, Mann–Whitney U test was carried out for pairwise comparisons to find the differences between groups. Only p values below 0.05 were regarded as statistically significant.

## Results

### Element concentrations

Concentrations of metals for healthy controls, MCI and AD patients in the different extracts (*TOTAL*, *HMM* and *LMM*), referred to total volume of serum, are given in Table 2. Statistically significant changes

according to Mann–Whitney U test are also listed, indicating the groups in which differences are found.

As can be observed, significant differences were found in the concentrations of aluminum, manganese, iron, copper, zinc and selenium between the groups of study. Thereby, serum aluminum increases as patients become progressively cognitively impaired, from control to mild cognitive impairment and finally, Alzheimer's disease. This trend is notably remarkable for the *LMM*-fraction, which only represents about 10 % of the total, but doubles its concentration in AD patients compared to controls. On the other hand, total zinc showed the opposite behavior, since suffers a decline along the progression of dementia. For Zn-*HMM* fraction, a similar trend is observed (although statistically non significant), while no changes occurs in the *LMM*-species. In the case of manganese, it can be found the most prominent differences along with those already mentioned for aluminum, although with reverse trend, since its concentration in serum is considerably reduced in demented subjects, both MCI and AD ones. However, this is only observed in total serum and the fraction associated to proteins, because it was not detected manganese in the *LMM* fraction. Low molecular mass iron species were markedly increased in both MCI and AD subjects, as well as labile copper in AD; while their *TOTAL* and *HMM* levels showed progressive changes, decreasing and increasing with the advance of neurodegeneration, respectively. Finally, depletion of selenium is found in AD patient with respect to mild dementia regarding major fractions (*TOTAL* and *HMM*), but in the case of selenometabolites (*LMM*) the reduction is observed in both MCI and AD patients compared to controls.

### Inter-element correlations

Metal concentrations (Table 2) were further analyzed to obtain element-to-element ratios for each fraction in order to understand the inter-relations of elements. Those ratios that showed significant changes between the study groups are listed in Table 3, which allows discovering the effect of alterations of single elements on the homeostasis of the rest in each level of structural organization. This complementary study of metal interactions according their distribution in different fractions is essential, since the form in which elements are present in the organism finally affects their biological roles and properties (Templeton 1999).

**Table 2** Concentrations of metals in serum (expressed as mean  $\pm$  SD, in  $\mu\text{g L}^{-1}$ ) and statistical comparisons by Mann–Whitney U test

		Healthy control	Mild cognitive impairment	Alzheimer's disease	Comparison
Al	<i>TOTAL</i>	4.05 $\pm$ 0.682	4.91 $\pm$ 1.27	5.18 $\pm$ 1.36	$\uparrow$ AD/MCI versus HC
	<i>HMM</i>	3.57 $\pm$ 0.748	4.56 $\pm$ 1.21	4.76 $\pm$ 1.17	$\uparrow$ AD/MCI versus HC
	<i>LMM</i>	0.324 $\pm$ 0.495	0.442 $\pm$ 0.350	0.777 $\pm$ 0.451	$\uparrow$ AD versus HC/MCI
V	<i>TOTAL</i>	0.0598 $\pm$ 0.0138	0.0642 $\pm$ 0.00663	0.0599 $\pm$ 0.0122	N.S.
	<i>HMM</i>	0.0580 $\pm$ 0.0153	0.0584 $\pm$ 0.0121	0.0611 $\pm$ 0.0291	N.S.
	<i>LMM</i>	<LOD	<LOD	<LOD	N.S.
Cr	<i>TOTAL</i>	0.212 $\pm$ 0.0857	0.223 $\pm$ 0.0356	0.207 $\pm$ 0.0813	N.S.
	<i>HMM</i>	0.222 $\pm$ 0.0881	0.229 $\pm$ 0.0313	0.190 $\pm$ 0.0625	N.S.
	<i>LMM</i>	<LOD	<LOD	<LOD	N.S.
Mn	<i>TOTAL</i>	1.09 $\pm$ 0.670	0.585 $\pm$ 0.339	0.665 $\pm$ 0.320	$\downarrow$ AD/MCI versus HC
	<i>HMM</i>	1.04 $\pm$ 0.681	0.586 $\pm$ 0.339	0.635 $\pm$ 0.335	$\downarrow$ AD/MCI versus HC
	<i>LMM</i>	<LOD	<LOD	<LOD	N.S.
Fe	<i>TOTAL</i>	988.3 $\pm$ 303.3	991.9 $\pm$ 321.3	879.7 $\pm$ 300.9	N.S.
	<i>HMM</i>	997.8 $\pm$ 473.3	977.4 $\pm$ 539.0	880.2 $\pm$ 397.8	N.S.
	<i>LMM</i>	0.445 $\pm$ 0.126	0.580 $\pm$ 0.188	0.535 $\pm$ 0.187	$\uparrow$ AD/MCI versus HC
Cu	<i>TOTAL</i>	1,058.5 $\pm$ 221.7	1,037.5 $\pm$ 180.7	1,126.8 $\pm$ 264.2	N.S.
	<i>HMM</i>	1,070.8 $\pm$ 265.8	1,025.8 $\pm$ 156.5	1,134.6 $\pm$ 301.3	N.S.
	<i>LMM</i>	5.49 $\pm$ 2.13	5.47 $\pm$ 2.63	7.34 $\pm$ 3.90	$\uparrow$ AD versus HC
Zn	<i>TOTAL</i>	910.5 $\pm$ 160.0	857.1 $\pm$ 142.3	815.0 $\pm$ 157.8	$\downarrow$ AD versus HC
	<i>HMM</i>	915.9 $\pm$ 158.9	834.7 $\pm$ 138.6	803.6 $\pm$ 153.8	N.S.
	<i>LMM</i>	2.04 $\pm$ 0.612	1.98 $\pm$ 0.995	2.15 $\pm$ 1.07	N.S.
Se	<i>TOTAL</i>	122.9 $\pm$ 24.14	126.6 $\pm$ 19.82	120.5 $\pm$ 31.12	$\downarrow$ AD versus MCI
	<i>HMM</i>	123.3 $\pm$ 24.64	131.8 $\pm$ 20.64	118.3 $\pm$ 29.08	$\downarrow$ AD versus MCI
	<i>LMM</i>	4.05 $\pm$ 2.39	2.63 $\pm$ 2.61	2.90 $\pm$ 1.55	$\downarrow$ MCI versus HC
Mo	<i>TOTAL</i>	1.06 $\pm$ 0.415	1.35 $\pm$ 0.644	1.02 $\pm$ 0.484	N.S.
	<i>HMM</i>	1.07 $\pm$ 0.173	1.26 $\pm$ 0.146	1.07 $\pm$ 0.194	N.S.
	<i>LMM</i>	<LOD	<LOD	<LOD	N.S.
Cd	<i>TOTAL</i>	0.0640 $\pm$ 0.0111	0.0899 $\pm$ 0.0221	0.0799 $\pm$ 0.0142	N.S.
	<i>HMM</i>	0.0672 $\pm$ 0.0656	0.0863 $\pm$ 0.0662	0.0751 $\pm$ 0.0774	N.S.
	<i>LMM</i>	<LOD	<LOD	<LOD	N.S.

<LOD below limit of detection

N.S. non significant

The most important findings are found again for aluminum and manganese. For aluminum, results suggest that ratios Al/Fe and Al/Se were higher in AD compared to healthy controls in all the fractions analyzed, while for Al/Zn the increase is also present in mild impairment. Moreover, a significant increase is found in Al/Cu, but only for *LMM*-fraction. On the other hand, ratios involving manganese with most of the other elements, in both *TOTAL* and *HMM* fractions, were reduced in diseased patients. Finally, copper could be correlated to zinc (*TOTAL*) and

selenium (*LMM*), according to the increased ratios found in AD patients respect to controls.

Furthermore, due to the importance that aluminum and manganese appears to play regarding their concentrations (Table 2) and element-to-element ratios (Table 3), the inter-element ratios between different fractions were calculated (Table 4).

Ratios between aluminum and most of the other elements were increased in AD compared to healthy controls, while for manganese a decrease is observed in both MCI and AD patients, corroborating and

**Table 3** Altered element-to-element ratios (A/B) in the different fractions (*TOTAL*, *HMM*, *LMM*)

A	B	<i>TOTAL</i>	<i>HMM</i>	<i>LMM</i>
Al	Fe, Se	↑AD versus HC	↑AD versus HC	↑AD versus HC
	Zn	↑AD/MCI versus HC	↑AD/MCI versus HC	↑AD/MCI versus HC
	Cu	–	–	↑AD versus HC
Mn	Al, V, Cr, Fe, Cu, Zn, Se, Mo, Cd	↓AD/MCI versus HC	↓AD/MCI versus HC	–
Cu	Zn	↑AD versus HC	–	–
	Se	–	–	↑AD versus HC

complementing data shown in Table 3. Therefore, it could be concluded that inter-element correlations occur not only between structurally analogue species, but also between the different size fractions, indicating the complex biochemistry of elements.

#### Inter-fraction correlations

Shifts in homeostasis of elements, principally redox-active ones, may have profound cellular consequences related to release of labile metals leading to increased production of free radicals. For this reason, *LMM/TOTAL* and *HMM/TOTAL* ratios were calculated in order to evaluate the possible role of these homeostatic imbalances in the development of Alzheimer's

disease. In Table 5 is observed that only *LMM*-to-*TOTAL* ratios showed significant changes along the progression of dementia. Thus, *LMM*-fractions of aluminum, iron and copper were increased in relation to their total concentration in MCI and/or AD compared to controls. On the other hand, the ratio for selenium was decreased in MCI patients.

#### Discussion

The involvement of metals in the development of Alzheimer's disease is an emerging hypothesis in the last years (Bonda et al. 2011), with serious implications for diagnosis and therapeutic considerations. Thus, determination of multi-elemental content in blood, cerebrospinal fluid and brain has been often proposed for the study of AD (Basun et al. 1991; Zatta et al. 1993; Religa et al. 2006; Bocca et al. 2006; Gerhardsson et al. 2008). However, these works are usually focused on the determination of individual trace element concentrations, but there is limited information concerning to interactions in elemental homeostasis, which would allow a deeper understanding of underlying pathological mechanisms. In this work, the combined analysis of metal content by size-fractionation, as well as inter-element and inter-fraction relations provided a new insight into the interdependency in the homeostasis of elements in neurodegeneration and its progression, as discussed below.

#### Aluminum

Increased aluminum levels have been traditionally linked to pathogenesis of Alzheimer's disease by its accumulation in brain (Crapper et al. 1973), which is

**Table 4** Altered element-to-element ratios (A/B) between the different fractions (*TOTAL*, *HMM*, *LMM*)

A	B	Comparison
Al- <i>TOTAL</i>	Zn, Se- <i>HMM</i>	↑AD versus HC
Al- <i>HMM</i>	Fe, Zn, Se- <i>TOTAL</i>	↑AD versus HC
Al- <i>LMM</i>	Mn, Fe, Zn- <i>TOTAL</i>	↑AD versus HC
Mn- <i>TOTAL</i>	Al, Cu, Zn, Se- <i>HMM</i>	↓AD/MCI versus HC
Mn- <i>HMM</i>	Al, Fe, Cu, Zn, Se- <i>TOTAL</i>	↓AD/MCI versus HC

**Table 5** Altered elemental ratios between *LMM*-fraction and total serum concentration

Element	Comparison
Al	↑AD versus HC
Fe	↑AD/MCI versus HC
Cu	↑AD versus HC
Se	↓MCI versus HC



also reflected in the peripheral system (Zatta et al. 1993; Zapatero et al. 1995). Involvement of aluminum overload in development of AD is related to its neurotoxic effects, triggering the formation of A $\beta$ -sheets and neurofibrillary tangles, interfering with neurotransmission, and inducing oxidative damage (Tomljenovic 2011). However, investigations into aluminum toxicity require speciation studies, since its metabolism and influence on physiological processes depends on the chemical form (Berthon 1996). Experimental evidence has suggested that at equilibrium, around 90 % of total aluminum in plasma is carried by proteins, while the rest is complexed to low molecular weight ligands (Pérez-Parajón et al. 1989). In this sense, experimental results presented in Table 2 show a similar pattern in both controls and MCI patients, with around 10 % of aluminum distributed in *LMM*-fraction. However, this percentage was raised to 15 % for AD patients, which is corroborated by increased *LMM*-to-*TOTAL* ratio (Table 5), supporting the hypothesis of an altered homeostasis in AD leading to the accumulation of labile aluminum probably due to oversupply of this element. In addition, when aluminum concentrations in the different fractions are carefully examined, it is observed that in whole serum as well as protein-bound species there is only a slight increase in both mild and severe dementia, but for labile fraction a much more pronounced increase occurs, exclusively in AD compared to controls. Similarly, Rao et al. (1999) demonstrated that accumulation of aluminum in brain of AD patients starts significantly in the later phase, which could be related to the marked increase of *Al-LMM* at this stage found in our study. Therefore, it could be concluded that neurotoxicity induced by aluminum in AD must be mainly due to low molecular mass species.

Moreover, the abnormal aluminum concentrations affected the distribution of other elements, as reflects the changes in element-to-element ratios (Tables 3, 4), disturbing the elemental homeostasis in serum. Among these cross-interactions, it must be remarked the close relationship between aluminum and iron. Aluminum is known to be co-transported on the organism with the Fe-transferrin complex, which is involved in its cellular uptake (McGregor et al. 1991). In particular, this mechanism is especially important for the entry of this element into the brain, principally through the blood–brain barrier (BBB) by transferrin-receptor mediated endocytosis (TfR-ME), competing with iron for

transferrin binding and its assimilation (Roskams and Connor 1990). In addition, this inter-related homeostasis of iron and aluminum seems to be also behind the oxidative stress induced by the latter since, although aluminum is not a redox-active metal, competition with iron for binding to transferrin provokes its release, exacerbating free radical damage by Fenton reaction (Zatta et al. 2002). Thus, this mis-metabolism concerning aluminum and iron, related to abnormal flux of metals across the blood–brain barrier and induced oxidative stress, is reflected in Alzheimer's disease by increased *Al/Fe* ratios in the different fractions (Table 3), as well as between them (Table 4), evidencing the complex biochemistry of these elements in this disorder. On the other hand, increased ratios of *Al/Zn* (*TOTAL*, *HMM*, *LMM*) and *Al/Cu* (*LMM*) were also found in AD, indicating an altered redox status. This finding has been previously associated with other neurological disorders such as Parkinson's disease (Ahmed and Santosh 2010) and bipolar mood disorders (Mustak et al. 2008), in which the increase in aluminum was related to altered homeostasis of these elements, by increasing paramagnetic oxidant elements like Cu, and by decreasing Zn, an antioxidant metal. The possible nexus between Al, Cu and Zn could be related to the expression of superoxide dismutase (Cu, Zn-SOD), a key enzyme to protect the organism against oxidative damage, which plays an essential role in AD pathogenesis (Ihara et al. 1997; Choi et al. 2005), and whose activity is altered by aluminum induced toxicity (Zatta et al. 2002). Finally, *Al/Mn* and *Al/Se* ratios were also disturbed in Alzheimer's disease and mild cognitive impairment, but this will be discussed in next sections.

### Manganese

Serum manganese levels were significantly lowered in diseased subjects (MCI and AD) compared to controls, which could be exclusively related to down-expression of Mn-containing proteins, since low molecular weight species were not observed. Although manganese is an essential trace element, it is susceptible of promoting neurotoxicity because it possesses mechanisms to enter and accumulate into CNS, inducing disruption of mitochondrial metabolism, oxidative stress, altered glutamate and dopamine metabolism, among others (Rivera-Mancía et al. 2011). Thus, brain accumulation of manganese has been traditionally linked to Parkinson's disease, but its potential link

with other neurodegenerative disorders such as Alzheimer's disease is being investigated (Bowman et al. 2011). In this sense, few works reported increased cortical Mn in Alzheimer's disease (Srivastava and Jain 2002; Religa et al. 2006), but confusing results can be found about this element in CSF and blood fluids (Basun et al. 1991; Jolly et al. 1993; Zatta et al. 1993; Bocca et al. 2006; Gerhardsson et al. 2008). Nevertheless, there are evidences about the involvement of manganese homeostasis in AD development as key constituent of clue enzymes in the central nervous system. In this way, over-expression of mitochondrial superoxide dismutase (Mn-SOD) has been described in human AD brains (De Leo et al. 1998), but with reduced activity as response to oxidative stress (Omar et al. 1999), as well as reduced arginase activity together with decreased manganese levels in plasma in relation to altered arginine-NO pathway (Vural et al. 2009). Complementarily, the important role of manganese in AD is demonstrated based on the effect that presents over homeostasis of other elements (Tables 3, 4), many of them closely related to AD. Interactions of these metals could be related to common nonspecific mechanisms of transport, as well as their uptake into brain (Smith et al. 1997), since manganese is predominantly transported by the divalent metal transporter 1 (DMT-1) and transferrin receptors (Aschner et al. 2007). Transferrin presents the ability to bind numerous metals (Vincent and Love 2012), while several metallic species can be transported by DMT-1 (Garrick et al. 2003), so competition for the same carrier transport systems could explain the interdependency between these elements. Therefore, reduction in serum manganese could be considered as a precursor in the progression of Alzheimer's disease, since it allows discriminate between controls and diseased subjects, but not between the two clinical stages of dementia studied (MCI and AD), which would contribute to early neurodegenerative failures related to down-expression of Mn-containing enzymes, such as altered antioxidant defenses or ammonia detoxification, among others.

## Iron

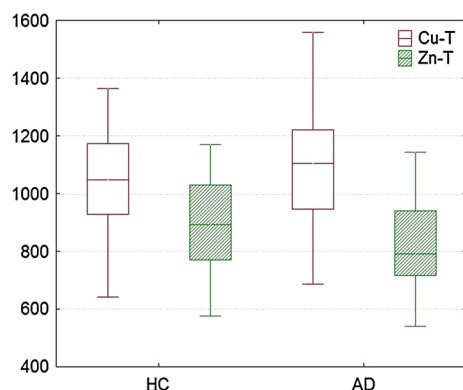
Unlike previous findings for other metals, a different trend is observed for iron depending on the fraction in which is associated. Serum total iron and the protein bound fraction suffer a slight decrease along the

development of disease, not statistically significant, but with important biological relevance, since is accompanied by an increase of the *LMM* fraction in AD and MCI patients. The altered iron metabolism in Alzheimer's disease comes from both genetic and protein related factors, such as hemochromatosis protein (HFE) mutation, over-expression of melano-transferrin (MTf) or decreased ability of ferritin to retain iron, inducing its accumulation into brain (Ke and Qian 2007) and which is finally reflected in reduced circulating levels of total iron (Basun et al. 1991; Baum et al. 2010). In addition, regulation of iron is closely related to homeostasis of other metals, as it has been described in previous sections, normally due to the competitive mechanisms of transport. Thus, iron deficiency has been proposed as a risk factor for metal toxicity, due to enhanced absorption and brain accumulation (Erikson et al. 2004). On the other hand, an excess of labile iron was found in demented patients (Table 2), which correlates with the advance of cognitive impairments as reflects the higher *LMM/TOTAL* ratio in AD patients (Table 5). It is known that disruption in homeostasis of redox-active metals can produce oxidative stress by imbalances between their labile and non-labile forms (Perry et al. 2002), which is particularly important for iron in AD pathogenesis (Smith et al. 2010; Lavados et al. 2008). In this context, non-transferrin bound iron (NTBI) uptake into the brain was proposed as the primary mechanism by which neurons acquire iron in AD, based on global decrease of transferrin in brain and weak neural expression of transferrin receptor (Núñez et al. 2012). Moreover, increased divalent metal transporter has been found in the cortex and hippocampus of APP/PS1 transgenic mouse model (Zheng et al. 2009), which is implicated in the uptake of iron, but also manganese, copper, zinc and other metals, in form of low molecular mass species (Garrick et al. 2003). Therefore, based on these experimental results, altered homeostasis of iron could represent a primary factor inducing abnormal flux of metals into the brain by altering the common pathways of metal management, triggering a cascade of deleterious events that finally leads to neuronal death.

## Copper and zinc

Copper plays a basic role as active centre in proteins related to oxidase and oxygenase activities, electron transfer and controlling the level of oxygen radicals,





**Fig. 1** Levels of copper and zinc ( $\mu\text{g L}^{-1}$ ) in healthy controls and Alzheimer disease patients

while zinc is involved in antioxidant and detoxification processes, among others. Thus, serum copper and zinc can be considered as two important markers of redox status. In this study, regarding total content and *HMM* fraction, serum copper tends to increase as patients become progressively cognitively impaired, while zinc suffers a decrease along the development of disease, as it was previously demonstrated by other authors (Basun et al. 1991; Squitti et al. 2002; Baum et al. 2010). This altered homeostasis of copper and zinc is outlined in box-plots represented in Fig. 1, which show the clear imbalance in these essential elements associated with AD. However, a more valuable indicator of disruption in copper and zinc homeostasis is the Cu/Zn ratio (El-Ahmady et al. 1995; Malavolta et al. 2010), which can inform about the interdependency of these elements in oxidative stress situations. Thereby, increased Cu/Zn ratio was found in AD patients (Table 3), due mainly to significantly decreased zinc in these patients, which could be responsible for decreased blood antioxidant capacity.

Taking into account labile species, zinc did not allow discriminating between the different groups of study, but Cu-*LMM* was markedly increased in AD subjects. Moreover, as in the case of iron, increased *LMM/TOTAL* ratio (Table 5) is an indicator of free copper deregulation, which has been previously related to ceruloplasmin fragmentation in serum of AD patients (Squitti et al. 2008).

## Selenium

The relevance of selenium in Alzheimer's disease relies on its important role in antioxidant and redox regulation, existing considerable data about negative

correlations between cognitive decline and selenium levels and selenoproteins activity (Loef et al. 2011). Thus, reduced levels of this element were found in the different fractions along the development of neurodegeneration (Table 2), as well as increased ratios between different metals and selenium (Table 3), indicative of protective response against oxidative stress. In addition, it is important to note that selenium homeostasis appears to be partially deregulated in AD, as revealed decreased *LMM-to-TOTAL* ratio (Table 5). Selenium metabolism starts with its assimilation and transformation into selenide, the common intermediate in pathways for selenoprotein synthesis. Then, selenide is transformed to selenometabolites that, through different routes, are finally incorporated into proteins (Suzuki 2005). In this sense, the trend found towards the accumulation of selenoproteins against the free forms in AD could indicate a regulative mechanism in response to oxidative stress, in order to maintain the level of essential selenoenzymes.

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