CHARACTERIZATION OF THE METAL PROFILE IN SERUM DURING THE PROGRESSION OF ALZHEIMER'S DISEASE

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Metal dyshomeostasis is closely related to Alzheimer's disease, so the characterization of the metal profile in these patients is of special interest for studying associated neurodegenerative processes and to discover potential markers of disease. An analytical approach, based on non-denaturing precipitation of proteins, has been optimized for the fractionation of high molecular mass (HMM) and low molecular mass (LMM) metal-species from serum, which were subjected to multielemental analysis by inductively coupled plasma mass spectrometry (ICP-MS). This methodology was applied to healthy controls, Alzheimer's disease (AD) and mild cognitive impairment (MCI) patients in order to study the progression of dementia. Thus, it was found that some metals, such as iron, copper, zinc and aluminum, suffer progressive changes along the advance of neurodegeneration, suggesting that these imbalances could be related to the decline of cognitive functions. On the other hand, elements as manganese, lithium or vanadium allow discriminate between controls and diseased subjects, both AD and MCI, but no differences were found between these two clinical stages of dementia, so they could be considered as precursors in the early development of neurodegenerative failures. In addition, it should be noted the important role that low molecular mass fractions of iron, copper, aluminum and cobalt appear to play in the pathogenesis of Alzheimer. Finally, correlation analysis indicated that these metal abnormalities can be interrelated, participating in common processes as oxidative stress, altered homeostasis and uptake into brain, and impaired glucose metabolism.

KEYWORDS. Alzheimer's disease, mild cognitive impairment, metals, species, disease progression

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, with prevalence around 20% at age 80 (Yesavage, 2002). Major histopathological features associated with AD are senile plaques and neurofibrillary tangles, related to aberrant processing of amyloid precursor protein (APP) leading to the deposition of amyloid β, and hyperphosphorylation of tau protein, respectively (Maccioni, 2001). However, this simplistic view is rather incomplete, since AD is a multifactorial disorder in which many altered cellular processes are involved, such as oxidative stress (Markesbery, 1999), neuroinflammation (Akiyama, 2000), impairments in energy metabolism (Ferreira, 2010), and others. Some of these neuropathological changes are also found in individuals with mild cognitive impairment (MCI), normally considered as a preclinical stage of AD (Morris, 2001). In this sense, abnormal levels of tau and Aβ in cerebrospinal fluid have been found to be useful markers of incipient AD (Hansson, 2006), as well as oxidative stress, one of the earliest changes in disease pathology, that also occurs in mild cognitive impairment (Mecocci, 2004). Thus, comparative studies between MCI and AD patients are of great importance to follow the progression of neurodegeneration, as well as for understanding the pathogenesis of disease in order to discover potential biomarkers for early diagnosis.

Metals play a crucial role in the development of Alzheimer`s disease, involving both toxic and essential elements (Duce, 2010; Bonda, 2011). Special attention has been paid on homeostasis of iron, copper and zinc, which are involved ~~implicated~~ in interactions with the major protein components of AD (Kozlowski, 2009). These metals promote Aβ aggregation and plaque formation (Bush, 2002), and consequently, accumulation of these ions is found in amyloid deposits (Lovell, 1998). Moreover, they have been related to metabolism of amyloid precursor protein and its processing by secretases (Hesse, 1994; Bush, 1994), and to a lesser extent they participate ~~there are also reports that indicate their participation~~ in tau abnormalities, promoting its phosphorylation (Egana, 2003) and inducing aggregation (Zhou, 2007). Metal-induced oxidative stress is other mechanism that can lead to profound neurodegenerative processes in AD (Perry, 2002). Disruption of metal homeostasis can produce oxidative stress by the formation of reactive oxygen species (ROS), most of which are catalyzed by iron or copper, but also the redox inactive metals, such as cadmium, arsenic and lead, present toxic effects via bonding to sulphydryl groups of proteins and depletion of glutathione (Valko, 2005; Jomova, 2011). Among these toxic metals, aluminium has been traditionally involved in AD pathogenesis since Crapper et al. demonstrated an elevated level of this metal in brain samples from histopathological confirmation of AD patients (Crapper, 1973). Possible roles of aluminium in the etiology of AD come from its ability to induce oxidative stress, β-pleated sheet formation, as well as production, polymerization and aggregation of Aβ protein, but this linkage remains still controversial (Gupta, 2005; Tomljenovic, 2011). On the other hand, relationships between AD and protective elements have also been studied. Thus, the involvement of selenium in AD due to its anti-oxidative function has been recently reviewed (Loef, 2011), as well as the neuroprotective role of zinc against Aβ cytotoxicity, despite the neurotoxic properties previously mentioned (Cuajungco, 2003).

Taking into account the importance of metals in Alzheimer's disease, numerous authors have measured metal concentrations in brain tissue (Religa, 2006), plasma (Zatta, 1993) and cerebrospinal fluid (Rao, 1999). Moreover, paired CSF and plasma samples have been studied in order to investigate the leakage of metals through the blood–cerebrospinal fluid barrier (Gerhardsson, 2011). However, although total concentration of metals can be a valuable information, a more comprehensive approach involves the characterization of their interactions with biomolecules (Templeton, 1999). Metals can be mainly present as free ions and complexed with low molecular mass ligands, or in form of metalloproteins. This distinction between high molecular mass (HMM) and low molecular mass (LMM) species is very important, since finally affects to biological activity or toxicological potential of the element, and their mobility across

different biological compartments. Elemental speciation is normally carried out by hyphenated techniques based on separation of species and later metal-specific detection, being the size exclusion chromatography (SEC) coupled online with inductively coupled plasma mass spectrometry (ICP-MS) the most widespread tool for the molecular size characterization of metal species (Das, 1996). However, this approach presents several drawbacks including long time of analysis, potential transformation of species during the separation step and loss of integrity of the metal-ligand binding. As an alternative, ultrafiltration (UF) has been employed to determine the size distribution of species by a simpler and faster method. Thus, Nischwitz et al. employed both SEC (Nischwitz, 2008) and UF (Nischwitz, 2010) for size fractionation of Mn, Fe, Cu, Zn, Mg and Ca species in serum and CSF samples in order to investigate the permeability of the human blood-CSF barrier. Improved performance was found for UF compared to SEC with respect to contamination, species stability and mass balances, with a considerably reduction of the total time of analysis.

This work focus on the analysis of different elements (Li, Al, V, Cr, Mn, Fe, Co, Cu, Zn, Se, Mo, Cd, Pb) in human blood serum samples by inductively coupled plasma mass spectrometry (ICP-MS). In order to distinguish between high and low molecular mass species, proteins were precipitated in non-denaturing conditions, and metals concentrations were evaluated both in the supernatant (LMM) and the precipitate (HMM). This methodology was applied to Alzheimer's disease and mild cognitive impairment patients in order to discover potential markers of disease and to study the progression of neurodegenerative process.

EXPERIMENTAL

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). For ICP-MS analysis, standard solutions employed were multielemental calibration standard 2A and single elemental standard of mercury–from Agilent, and ICP standards of rhodium and molybdenum from Merck. Bovine serum albumin standard (purity 96 %) was obtained from Sigma-Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

Blood samples were obtained by venipuncture of the antecubital region after 8 hours 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 minutes to allow clot retraction, and centrifuged (3500 rpm for 10 minutes). The serum was divided into aliquots in Eppendorf tubes and frozen at -80 ° C until analysis. Subjects of the study, 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 (McKahnn, 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.

Table 1. Clinical characteristics of healthy controls, MCI and AD patients

	AD (n=30)	**MCI (n=16)**	**HC (n=30)**
Age (years)	80.9±4.5	75.9±5.7	74.0±5.7
Sex (male/female)	12/18	10/6	13/17

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. Additionally, 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 μg L^{-1}. Platinum sampling and skimmer cones were employed, with a sampling depth of 7 mm. The forward power was set at 1500W, and the gas flow rates were fixed at 15 L min^{-1} for plasma gas, 1 L min^{-1} for auxiliary gas, 1 L min^{-1} for carrier gas, 0.15 L min^{-1} for makeup gas and 3.5 mL min^{-1} for helium. Isotopes monitored were ^{7}Li, ^{29}Al, ^{51}V, ^{53}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{63}Cu, ^{64}Zn, ^{65}Cu, ^{66}Zn, ^{78}Se, ^{82}Se, ^{95}Mo, ^{98}Mo, ^{103}Rh, ^{112}Cd, ^{114}Cd and ^{208}Pb, with a dwell time of 0.3s per isotope.

A MARS microwave oven (CEM Matthews, NC, USA) was used for the mineralization of samples in PFA Teflon vessels.

SAMPLE PREPARATION

Serum samples were submitted to protein precipitation under non-denaturing conditions according to classical protocols for protein purification (Scopes, 1994; Simpson, 2004). For this purpose, 300 μl of cold acetone (-20ºC) was added dropwise to 150 μl of serum, and kept for 10 minutes in an ice bath. During this time, the mixture was sporadically vortexed, and then, the precipitate was removed by centrifugation (10000rpm, at 4ºC for 5 minutes). The supernatant, containing low molecular mass (LMM) species, was taken to dryness under nitrogen stream, and reconstituted in 750 μl of water, with 1 μg L^{-1} of rhodium as internal standard. On the other hand, precipitate was subjected to microwave assisted acid digestion for the determination of metal content in the high molecular mass (HMM) fraction. For this purpose, the precipitate was introduced into the microwave vessel together with 500 μL of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v). The mineralization was carried out at 400W, ramping from room temperature to 150ºC in 10 minutes, and maintaining this temperature for other 10 minutes. Then, extracts were made up to 2 mL with water, adding 1 μg L^{-1} of rhodium aqueous solution. Before analysis, samples were filtered through 0.45 μm pore size filters of PTFE.

In addition, total metal content of serum (TOTAL) was determined in diluted samples as previously described (Muñiz, 2001). In this way, serum was five-fold diluted with ultrapure water and rhodium solution was added to reach a final concentration of 1 μg L^{-1}.

In order to check the integrity of metal-protein bindings during the sample treatment procedure, an aqueous solution of bovine serum albumin at 50 mg ml^{-1}, containing copper and zinc, was processed in the same way, and precipitate, supernatant and diluted solution were analyzed by ICP-MS.

STATISTICAL ANALYSIS

Statistical calculations were made with 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 pairwaise comparisons to find the differences between groups. Spearman's correlation coefficients were also obtained in order to interrelate the metals levels found in the different fractions (LMM, HMM and total serum). Only p values below 0.05 were regarded as statistically significant.

In addition, a classification analysis was performed by partial least squares discriminant analysis (PLS-DA), in order to visualize the statistical discrimination between groups (scores plots) and to confirm the metals differentiating patients from matched controls (loadings plots). For this, data was processed with SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden).

RESULTS

ASSESSMENT OF SAMPLE TREATMENT PROCEDURE

One of the most important challenges when working with metallic species is maintaining the metal-ligand binding integrity. Thus, stability of species during the analytical procedure must be verified to guarantee that there are no transformations or metal release, in order to study the *in vivo* distribution of metals among different size fractions. The protocol employed for protein precipitation works with non-denaturing conditions, but the integrity of the metal-protein unions during this procedure has not been studied to our knowledge. For this purpose, a standard solution containing 50 mg mL^{-1} of bovine serum albumin (similarly to albumin in human serum) was treated as previously described, and the different extracts (TOTAL, HMM, LMM) were analyzed for copper and zinc content (Table 2).

Table 2. Concentrations and RSDs for Cu and Zn in BSA extracts (n=5).

		concentration (μg L^{-1})	RSD	ratio LMM/TOTAL
Cu	**TOTAL**	931.8	6.7%	3.2%
	HMM	889.5	2.9%	
	LMM	30.06	9.6%	
Zn	**TOTAL**	605.3	9.9%	2.3%
	HMM	597.2	4.5%	
	LMM	14.06	13.4%	

Reproducible results were obtained by ICP-MS analysis, as indicates the low relative standard deviations calculated from five replicates. Only for LMM fractions the RSD exceeded 10%, probably due to the lower metal concentrations and losses in the evaporation step by splashing. On the other hand, when metal concentrations from LMM extract and the whole serum are compared, it is observed that the released metal fraction is very low, below 5%, so it can be concluded that disassembly of metal-protein complex during precipitation is insignificant.

METAL CONCENTRATIONS IN SERUM SAMPLES

Concentrations of metals in the different fractions (TOTAL, HMM and LMM) are listed in Table 3, referred to total volume of serum. In addition, results from statistical comparisons between groups by Mann-Whitney U test are also presented, indicating the statistical significance (p value against N.S., which means non-significant) and the groups in which are found the differences.

Table 3. Serum concentrations of metals (expressed as mean ± SD, in μg L^{-1}) and statistical pairwaise comparisons by Mann-Whitney U test.

	Healthy	Mild cognitive	Alzheimer	Statistics

		control	impairment	disease	
Li	**TOTAL**	3.36±1.34	2.95±1.14	3.04±1.18	N.S.
	HMM	<LOD	<LOD	<LOD	N.S.
	LMM	3.17±0.721	2.85±1.15	3.12±1.06	N.S.
Al	**TOTAL**	4.05±0.810	4.91±1.23	5.18±1.46	p<0.01 (HC-MCI) p<0.001 (HC-AD)
	HMM	3.57±0.710	4.56±1.17	4.76±1.34	p<0.001 (HC-MCI) (HC-AD)
	LMM	0.324±0.433	0.442±0.333	0.777±0.474	p<0.001 (HC-AD) p<0.01 (MCI-AD)
V	**TOTAL**	0.0613±0.0154	0.0668±0.0110	0.0673±0.0132	N.S.
	HMM	0.0559±0.0155	0.0710±0.0162	0.0798±0.0344	N.S.
	LMM	<LOD	<LOD	<LOD	N.S.
Cr	**TOTAL**	0.218±0.0919	0.224±0.0346	0.200±0.0788	N.S.
	HMM	0.196±0.0886	0.215±0.0877	0.190±0.0827	N.S.
	LMM	<LOD	<LOD	<LOD	N.S.
Mn	**TOTAL**	1.16±0.734	0.570±0.333	0.618±0.346	p<0.001(HC-MCI) (HC-AD)
	HMM	1.10±0.609	0.584±0.367	0.656±0.319	p<0.001 (HC-MCI) (HC-AD)
	LMM	<LOD	<LOD	<LOD	N.S.
Fe	**TOTAL**	1001.2±283.91	976.61±316.38	854.27±298.54	N.S.
	HMM	994.35±574.61	988.55±714.83	876.77±495.02	N.S.
	LMM	0.400±0.117	1.10±0.371	1.30±0.457	p<0.001 (HC-MCI) (HC-AD)
Co	**TOTAL**	0.294±0.130	0.244±0.0842	0.285±0.203	N.S.
	HMM	0.264±0.158	0.247±0.115	0.262±0.141	N.S.
	LMM	0.0255±0.0679	0.0383±0.0800	0.0500±0.0129	N.S.
Cu	**TOTAL**	1050.1±203.92	1034.1±175.13	1114.8±260.93	N.S.
	HMM	1005.9±228.28	1017.4±150.23	1120.6±332.63	N.S.
	LMM	5.46±1.95	5.55±2.56	8.89±6.03	N.S.
Zn	**TOTAL**	899.7±161.7	844.6±146.4	809.06±144.9	p<0.05 (HC-AD)
	HMM	901.6±328.3	840.6±87.69	811.9±150.3	N.S.
	LMM	1.86±0.582	2.32±0.870	2.27±1.15	N.S.
Se	**TOTAL**	118.5±26.84	123.7±15.40	112.8±25.76	p<0.05 (MCI-AD)
	HMM	124.2±24.49	131.2±14.09	115.6±28.10	p<0.01 (MCI-AD)
	LMM	4.25±2.42	2.68±2.53	2.87±1.84	p<0.05 (HC-MCI) (HC-AD)
Mo	**TOTAL**	1.17±0.548	1.22±0.654	0.977±0.388	N.S.
	HMM	1.18±0.210	1.26±0.141	0.958±0.224	N.S.
	LMM	<LOD	<LOD	<LOD	N.S.
Cd	**TOTAL**	0.0910±0.0320	0.0917±0.0218	0.0951±0.0392	N.S.
	HMM	0.0908±0.0923	0.0904±0.0798	0.0933±0.0989	N.S.
	LMM	<LOD	<LOD	<LOD	N.S.
Pb	**TOTAL**	0.0454±0.0308	0.0545±0.0306	0.0496±0.0353	N.S.
	HMM	0.0453±0.0308	0.0528±0.0296	0.0506±0.0361	N.S.
	LMM	<LOD	<LOD	<LOD	N.S.

N.S. non significant
<LOD below limit of detection

The serum concentration of manganese was significantly higher in healthy controls, in both TOTAL and HMM-fraction. For zinc (TOTAL and HMM) was observed a decline along the

progression of dementia (HC< MCI< AD), while for aluminum was found the opposite behavior in all the extracts. For selenium, significant lower concentrations were found in AD patients respect to HC and MCI (TOTAL and HMM), while selenometabolites (LMM-fraction) were low in both MCI and AD patients compared to HC. Finally, labile iron increases markedly in both MCI and AD subjects.

Other metals also presented altered levels in relation to neurodegeneration, although these differences were not statistically significant, probably due to dispersion of experimental data because the high biological variability in serum concentrations of metals (Goullé, 2005). In this way, lithium was slightly increased in healthy controls and vanadium was reduced in these same individuals. On the other hand, molybdenum was decreased in AD patients when compared with both HC and MCI. Increase in the LMM fraction of cobalt and copper is observed along the development of dementia. Finally, iron and copper (TOTAL and HMM) showed progressive changes, decreasing and increasing with the advance of neurodegeneration, respectively.

CORRELATION ANALYSIS

Spearman´s correlation coefficients (r) were calculated in order to understand the interdependency of metals in the organism (Table 4). For metals present in only one fraction (HMM or LMM), correlations were obtained according to the concentration found in the whole diluted serum (TOTAL).

Table 4. Correlations between serum metals ($p<0.05$)

		correlation coefficients (r)
Li		Al-TOTAL (r = 0.30), Co-TOTAL (r = 0.27),
Al	**TOTAL**	Li (r = 0.30)
	HMM	Mo (r = 0.30)
	LMM	Mn (r = -0.27), Fe-HMM (r = -0.25), Co-LMM (r = 0.37), Cu-LMM (r = 0.29)
V		Cr (r = 0.76), Se-TOTAL (r = 0.38), Cd (r = 0.42), Zn-HMM (r = 0.44)
Cr		V (r = 0.76), Se-TOTAL (r = 0.35), Cd (r = 0.40), Zn-HMM (r = 0.32)
Mn		Co-TOTAL (r = 0.36), Zn-TOTAL (r = 0.33), Al-LMM (r = -0.27)
Fe	**TOTAL**	Zn-TOTAL (r = 0.28),
	HMM	Mo (r = 0.39), Al-LMM (r = -0.25)
	LMM	Co-LMM (r = 0.25), Cu-LMM (r = 0.33)
Co	**TOTAL**	Li (r = 0.27), Mn (r = 0.36), Cd (r = 0.24)
	HMM	Mo (r = 0.32)
	LMM	Cd (r = 0.24), Al-LMM (r = 0.37), Fe-LMM (r = 0.25)
Cu	**LMM**	Al-LMM (r = 0.29), Fe-LMM (r = 0.33)
Zn	**TOTAL**	Mn (r = 0.33), Fe-TOTAL (r = 0.28), Se-TOTAL (r = 0.36)
	HMM	V (r = 0.44), Cr (r = 0.32), Cd (r = 0.29)
Se	**TOTAL**	V (r = 0.38), Cr (r = 0.35), Zn-TOTAL (r = 0.36)
Mo		Al-HMM (r = 0.30), Fe-HMM (r = 0.39), Co-HMM (r = 0.32)
Cd		V (r = 0.42), Cr (r = 0.40), Co-TOTAL (r = 0.24), Zn-HMM (r = 0.29), Co-LMM (r = 0.24)

As can be observed, a lot of correlations were obtained, indicating the complex biochemistry of elements and their interrelated homeostasis.

CLASSIFICATION ANALYSIS

Partial least squares discriminant analysis (PLS-DA) provides statistical models that allow visualizing groupings and trends between different groups of samples through representation of scores plots. In Fig. 1A is represented the sores plot considering both AD and MCI patients, as well as healthy controls. Samples from AD and HC could be discriminated, but the cloud of points for MCI individuals (circled in Fig. 1A) partially overlap with the other two groups.

However, when only AD and HC are included in the analysis, clearer separation is obtained (Fig. 1B). In addition, the representation of loadings plots of this second statistical model allows discovering potential discriminant markers (Fig. 1C). Since directions in the scores plot correspond to directions in the loadings plot, identification of variables that separate the groups can be obtained by easy inspection of corresponding loadings plot (Trygg, 2007). In this way, aluminum (TOTAL, HMM and LMM), copper (TOTAL and LMM), vanadium (HMM) and LMM-fractions of iron and cobalt, clustered in the left side of the plot, could be considered overexpressed in AD samples. On the other hand, iron, manganese and zinc (TOTAL and HMM), as well as selenium (TOTAL, HMM and LMM), found in the opposite corner, are reduced in these samples, which confirms results previously provided by Mann-Whitney tests.

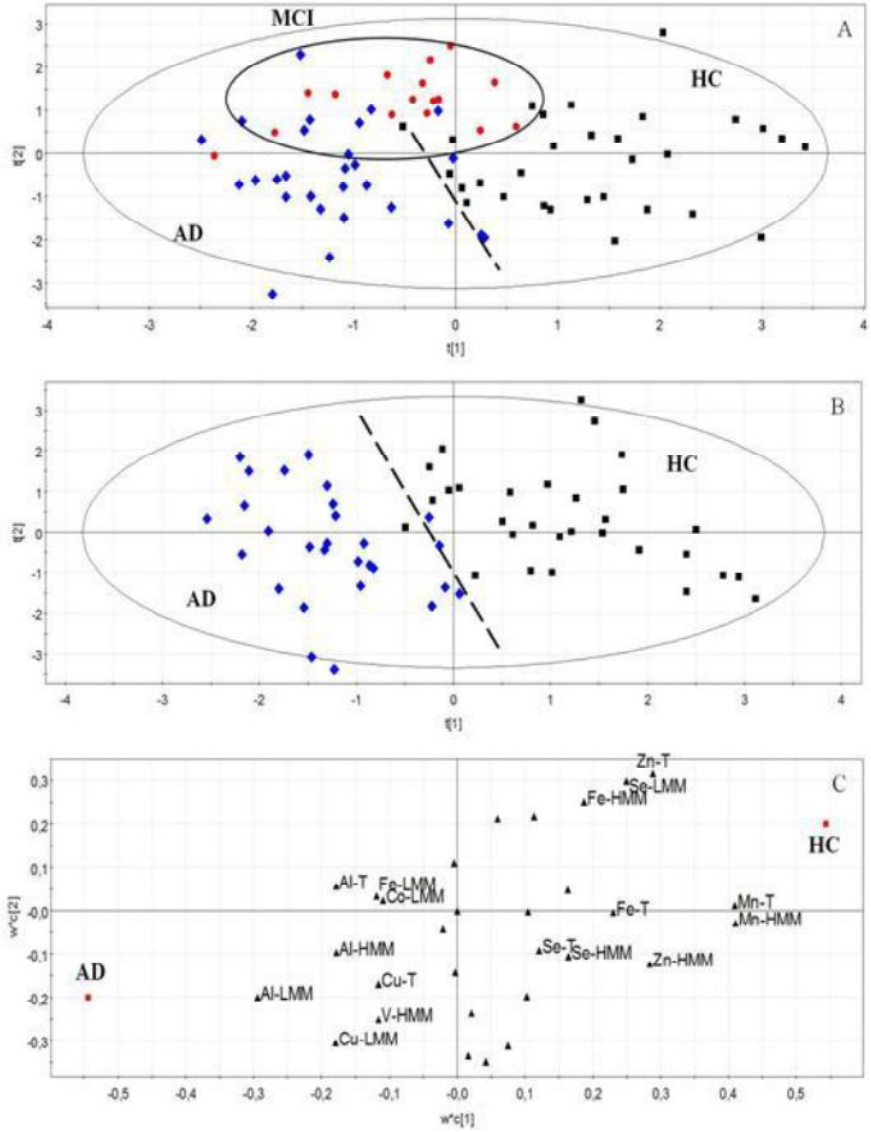

Fig. 1. PLS-DA results of metal analysis. (A) Scores plot for three groups comparison (HC, MCI and AD); (B) Scores plot for two groups comparison (HC and AD); (C) Loadings plot for two groups comparison (HC and AD).

DISCUSSION

Neurodegenerative processes in Alzheimer's disease and mild cognitive impairment are related to metal imbalances, as denotes the altered levels of different elements in serum samples. Moreover, complex interdependency in the homeostasis of these metals is demonstrated based on the calculated correlation coefficients.

The findings obtained in this study allow relating three major biological transition metals (copper, zinc and iron) with the progression of Alzheimer's disease. Considering total content and HMM fraction, the presence of copper in serum tends to increase when patients become progressively cognitively impaired, while iron and zinc decrease along the development of disease. As stated in introduction, hosmeostatic alterations in brain levels of these metals have been implicated in the pathogenesis of AD (Kozlowski, 2009), which is finally reflected in peripheral biofluids. Thus, total content of iron and zinc have been previously found significantly decreased in patients with AD compared to age matched controls in plasma (Vural, 2010) and serum (Baum, 2010), and copper was increased in serum samples (Squitti, 2002). Moreover, few works also reported the involvement of zinc in the early development of dementia, which suggests a significant decrease of serum Zn in MCI compared to controls (Dong, 2008), but higher CSF levels than in AD patients (Kovatsi, 2006). This metal malfunction metabolism has been related to an abnormal flux of metals across the blood-brain barrier (BBB) and the choroid plexus, from blood and CSF respectively, which lead to their accumulation in brain and finally results in biological damage (Yokel, 2006). In this sense, it must be remarked the cross-interactions in homeostasis of different metals and their uptake into brain (Smith, 1997). As an example, transferrin (Tf) presents the ability to bind several metals, such as Fe, Mn, Zn, Cr, Co, Cd, V and Al (Vincent, 2012), acting potentially as transporter agent via tranferrin receptor (Tf-R). On the other hand, LMM metallic species can enter into the brain through nonspecific transporters, as the divalent metal transporter 1 (DMT-1), implicated in the uptake of Fe and Mn, and to a lesser extent Zn, Cu, Co, Cd, and Ni (Garrick, 2003). This interrelationship of metal homeostasis can be confirmed considering the calculated correlation coefficients in Table 4. Thus, levels in the total and/or HMM fractions of Fe, Zn, Mn, Al, Co, Mo and Cd presented positive correlations among them, indicating a possible common route for their biological regulation. Among these interrelated elements, aluminum and manganese showed significant imbalances in the analyzed sera (Table 3). Aluminum was increased in serum from AD and, to lesser extent, MCI patients compared to healthy controls, supporting the hypothesis for the contribution of Al in AD pathology. Accumulation of aluminum has been previously reported in brain (Crapper, 1973; Corrigan, 1993), plasma (Basun, 1991, Zatta, 1993) and serum (Zapatero, 1995) of AD patients, but it is the first time that is associated with MCI, and therefore, with the early development of neurodegenerative failures. In the case of manganese, a two-fold increase is found in controls respect to diseased subjects. Manganese is closely related to Parkinson's disease, but confuse results can be found about this element in plasma and CSF samples from Alzheimer's disease patients (Basun, 1991; Jolly, 1993; Gerhardsson, 2008). However, its potential involvement in AD seems clear, due to the close relationship in the homeostasis of iron and manganese and their transport across the BBB (Aschner, 1990). Moreover, overexpression of mitochondrial Mn superoxide dismutase enzyme (MnSOD) has been described in human AD brains, but with a reduced activity (Omar, 1999), which support the deregulated metabolism of this element.

Dyshomeostasis of redox-active metals and production of oxidative stress has been also linked to Alzheimer's disease pathology (Huang, 2004). Alterations between labile and non-labile metals have been suggested to play a pivotal role in metal-evoked oxidative stress, due to the ability of free metals to produce reactive radicals, resulting in oxidative damage. In this sense, low molecular mass fractions of iron, cobalt, copper and aluminum have been found to be progressively increased in Alzheimer's disease. The release of free iron in AD has been associated with impairments in proteins that regulate its metabolism, such as decreased ability of ferritin to retain iron (Friedman, 2011), as well as reduced transferrin content in brain and weak expression of tranferrin receptor in neurons, resulting in non-tranferrin bound iron (NTBI) uptake into the brain (Núñez, 2012). Thus, accumulation of redox-active iron in brain was reported in preclinical AD patients and tended to increase with progression of disease (Smith, 2010). This fact is in accordance with our results in serum, suggesting that an imbalance in iron homeostasis could be a precursor of neurodegenerative processes. A similar dysfunction is

found for copper, due to ceruloplasmin fragmentation in serum of AD patients, leading to free copper deregulation (Squitti, 2008). Elevated labile copper has been found in brain (James, 2012) and serum (Squitti, 2006) of AD patients, and, similarly to iron, non-ceruloplasmin copper correlates with dementia, allowing distinguish MCI patients (Squitti, 2006; Squitti, 2011). Only known function of cobalt is its integral part of vitamin B12, but in form of free ion is able to generate reactive oxygen species, as copper and iron. Thus, the increased values of Co levels that we observe in LMM fraction agrees with the reported increasing of this element in blood (Bocca, 2006) and cerebrospinal fluid (Rao, 1999). Finally, LMM-aluminum was also elevated, but unlike iron, copper and cobalt, it is not a redox-active metal, although competes with other metals, principally iron, for binding to transferrin, leading to release of these metals and increasing free radical damage (Zatta, 2002). Based on this competitive mechanism of transport it could be explained the negative correlations of free aluminum with iron and manganese (HMM and TOTAL), as part of the homeostatic changes generating oxidative stress. On the other hand, positive correlation coefficients interrelates LMM fractions of Fe, Cu, Co and Al, showing the multiplicity of routes associated to oxidative damage in Alzheimer's disease.

Alterations in selenium levels may be also related to oxidative stress due to its antioxidant properties. Thus, total selenium and HMM fraction were significantly reduced in AD samples, while the decrease in selenometabolites was found in both AD and MCI patients, which is consistent with numerous studies regarding analysis of total selenium or measurement of selenoproteins activity (Loef, 2011). In addition, selenium was positively correlated with other metals such as vanadium, chromium and zinc, all of them implicated in glucometabolic disorders (Wiernsperger, 2010). The involvement of abnormal energy metabolism in AD is well known, including impaired glucose homeostasis, increased oxidative stress and insulin resistance (Correia, 2012). Thus, beside the mentioned changes in levels of zinc and selenium, hypometabolism could be behind the increase of vanadium in serum in AD and MCI patients (Table 3).

Finally, it has been observed small changes of lithium and molybdenum concentrations in serum. Total lithium was reduced in both AD and MCI serum samples, consistent with previous results in plasma (Zatta, 1993) from AD patients. Metabolism of this element was related to cobalt and aluminum, as revealed the correlation coefficients (Li/Al $r = 0.30$, Li/Co $r = 0.27$), which could denotes its neuroprotective potential against aluminum toxicity (Bhalla, 2010) and its ability to enhance folate and B12 transport into cells (Schrauzer, 2002). On the other hand, lower concentrations were found for Mo in AD compared to MCI and HC. This essential element has been associated with AD only once (Bocca, 2006), but its role is still unclear.

CONCLUSIONS

The involvement of metals in pathogenesis of Alzheimer's disease makes necessary the characterization of the metal profile to understand their role in neurodegeneration. For this purpose, it has been demonstrated the usefulness of a high throughput approach based on non-denaturing protein precipitation for size fractionation of metal species into low and high molecular mass fractions (LMM and HMM). Alterations in total concentrations of metals, as well as metalloproteins or free species, could be related to the development of Alzheimer. The most significant changes were observed in manganese, aluminum, selenium and zinc, but also were found important alterations related to copper and iron homeostasis, and the LMM fractions of several elements. In addition, results confirm the hypothesis of MCI as a preclinical stage of AD, regarding the progression in serum levels of several metals along the advance of dementia, such as iron, copper, zinc and aluminum. Complementarily, correlation analysis allowed studying metal imbalances in a more comprehensive manner, since metabolism of most of elements could be interrelated. In this sense, metals and AD seem to be linked principally through altered homeostasis of metals and their uptake into brain, oxidative stress and impaired glucose metabolism.

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