

| | |
|--|--|
| Manuscript Number: | BIOM-D-13-00347R1 |
| Full Title: | HOMEOSTASIS OF METALS IN THE PROGRESSION OF ALZHEIMER'S DISEASE |
| Article Type: | Original |
| Keywords: | Alzheimer's disease; mild cognitive impairment; metal homeostasis; inter-element relations |
| Corresponding Author: | Jose-Luis Gomez-Ariza, Dr University of Huelva Huelva, SPAIN |
| Corresponding Author Secondary Information: | |
| Corresponding Author's Institution: | University of Huelva |
| Corresponding Author's Secondary Institution: | |
| First Author: | Raul Gonzalez-Dominguez |
| First Author Secondary Information: | |
| Order of Authors: | Raul Gonzalez-Dominguez Tamara Garcia-Barrera, Dr Jose-Luis Gomez-Ariza, Dr |
| Order of Authors Secondary Information: | |
| 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. |
| Response to Reviewers: | Answers to the reviewers remarks: Main points: 1. There seems to be a large age difference between the average of the AD patients and the healthy controls (81.4 compared to 74). Although there is a reasonable amount of variation in these groups, the age difference is substantial. Due to many age-related factors at that age, which can include changes to diet, exercise, other illnesses, etc, there could be many, non-AD-related, reasons for the differences in biometals. The authors did not offer any explanation of why the difference in age here or how that could factor into the study. One way of determining whether it is likely to have an effect is to plot the values for each healthy control for each metal and determine if there appears to be any age-related change across the healthy control group. If not, then the authors can probably rule out major age-related changes between 74 and 81. As recommended, the concentrations of each metal were plotted versus the age of the |

healthy controls, and no significant differences were observed. This result has been commented in the new version of the manuscript.

2. I am not really happy with the term 'fractionation' for what the authors have performed with the blood samples. The use of the word fractionation generally implies that a sample is broken into a number of 'fractions'. Instead here the authors have simply spun the samples and taken the pellet and supernatant. I am not sure how or why they use the term high molecular weight and low molecular weight? Or even the justification for this particular separation. Molecular weight of what, proteins, metal-binding proteins, other molecules? How do they know that there is a separation of proteins rather than simply spinning out cell debris?

We have included a new paragraph in the Introduction section to justify this particular separation procedure and clarify the terms low and high molecular weight fractions.

3. The whole 'fractionation' process also could potentially contribute to the differences between metals across the groups. This is also supported by the fact that the LMW fractions have considerably higher variation than the HMW fractions and the authors show more difference between groups in the LMW fractions. The authors need some form of quality control or positive control, perhaps spiking with a non-biological metal or rare isotope metal to demonstrate that they have produced consistent fractions across each group. The authors also need to add the centrifugation as rcf or list the model of centrifuge.

The fractionation procedure has been previously validated using an aqueous solution of bovine serum albumin 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 checked. This experience has been included in the new version of the manuscript. The model of the centrifuge was listed in the Materials and Methods section.

4. Have the authors confirmed that filtering samples through a 0.45 um filter is not affecting the subsequent metal readings?

It was confirmed that the filtering of samples does not affect to metal levels in final extracts.

5. On page 9, the authors say that changes to aluminium and manganese in AD patients highlights their importance in the disease. This statement is not true. Simply because there are changes in metals does not prove that they have an important role in the disease as there can be many reasons including secondary effects that explain these changes.

This sentence was deleted.

Minor points:

The last sentence in the abstract needs to be re-written. "Therefore, this study may contribute to deepen into underlying pathological mechanisms related to metals in Alzheimer's disease" This should probably read "Therefore, this study may contribute to our understanding of the pathological mechanisms related to metals in Alzheimer's disease .."

The sentence was re-written as proposed.

The last sentence of paragraph one in the introduction doesn't make sense. "These imbalances have multiple origins that can be related to increased exposure to metals (Charlet et al. 2012), but in addition to complex impairments in homeostatic mechanisms controlling transport and interactions of important biometals (Kozlowski et al. 2012)."

The sentence was re-written.

HOMEOSTASIS OF METALS IN THE PROGRESSION OF ALZHEIMER'S DISEASE

^{a,b,c}Raúl González-Domínguez, ^{a,b,c}* Tamara García-Barrera, ^{a,b,c}* José Luis Gómez-Ariza

^aDepartment of Chemistry and CC.MM. Faculty of Experimental Science. University of Huelva. Campus de El Carmen. 21007 Huelva. SPAIN; ^bCampus of Excellence International ceiA3. University of Huelva. SPAIN; ^cResearch Center of Health and Environment (CYSMA). University of Huelva. Campus de El Carmen. 21007 Huelva. SPAIN

Corresponding authors:

José Luis Gómez-Ariza. Tel: +34959219968, fax: +34 959 219942, e-mail address: ariza@uhu.es

Tamara García-Barrera. Tel: +34959219962, fax: +34 959 219942, e-mail address: tamara@dqcm.uhu.es

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

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 β amyloid peptides ($A\beta$) in form of senile plaques, as well as hyperphosphorylation of τ 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 β amyloid deposition and hyperphosphorylation of τ 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. 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 (Gerhardsson et al. 2011), 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 (*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 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). Sera was divided into aliquots in Eppendorf tubes and frozen at -80 ° 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.

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 |

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 $1 \mu\text{g L}^{-1}$. Platinum sampling and skimmer cones were employed, with a sampling depth of 7 mm. The forward power was set at 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 ^{27}Al , ^{51}V , ^{53}Cr , ^{55}Mn , ^{57}Fe , ^{63}Cu , ^{64}Zn , ^{65}Cu , ^{66}Zn , ^{78}Se , ^{82}Se , ^{95}Mo , ^{98}Mo , ^{103}Rh , ^{112}Cd and ^{114}Cd 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. Samples were centrifuged in a centrifuge model Eppendorf 5804R.

SAMPLE PREPARATION

For non-denaturing protein precipitation from serum samples, 300 μl of cold acetone (-20°C) was dropwise added 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 4°C 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 $1 \mu\text{g L}^{-1}$ 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 μL of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v). Mineralization was carried out at 400W, ramping from room temperature to 150°C in 10 minutes, and maintaining this temperature for other 10 minutes. 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 μm 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.

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 | TOTAL | 4.05 \pm 0.682 | 4.91 \pm 1.27 | 5.18 \pm 1.36 | \uparrow AD/MCI vs. HC |
| | HMM | 3.57 \pm 0.748 | 4.56 \pm 1.21 | 4.76 \pm 1.17 | \uparrow AD/MCI vs. HC |
| | LMM | 0.324 \pm 0.495 | 0.442 \pm 0.350 | 0.777 \pm 0.451 | \uparrow AD vs. HC/MCI |
| V | TOTAL | 0.0598 \pm 0.0138 | 0.0642 \pm 0.00663 | 0.0599 \pm 0.0122 | N.S. |
| | HMM | 0.0580 \pm 0.0153 | 0.0584 \pm 0.0121 | 0.0611 \pm 0.0291 | N.S. |
| | LMM | <LOD | <LOD | <LOD | N.S. |
| Cr | TOTAL | 0.212 \pm 0.0857 | 0.223 \pm 0.0356 | 0.207 \pm 0.0813 | N.S. |
| | HMM | 0.222 \pm 0.0881 | 0.229 \pm 0.0313 | 0.190 \pm 0.0625 | N.S. |
| | LMM | <LOD | <LOD | <LOD | N.S. |
| Mn | TOTAL | 1.09 \pm 0.670 | 0.585 \pm 0.339 | 0.665 \pm 0.320 | \downarrow AD/MCI vs. HC |
| | HMM | 1.04 \pm 0.681 | 0.586 \pm 0.339 | 0.635 \pm 0.335 | \downarrow AD/MCI vs. HC |
| | LMM | <LOD | <LOD | <LOD | N.S. |
| Fe | TOTAL | 988.3 \pm 303.3 | 991.9 \pm 321.3 | 879.7 \pm 300.9 | N.S. |
| | HMM | 997.8 \pm 473.3 | 977.4 \pm 539.0 | 880.2 \pm 397.8 | N.S. |
| | LMM | 0.445 \pm 0.126 | 0.580 \pm 0.188 | 0.535 \pm 0.187 | \uparrow AD/MCI vs. HC |
| Cu | TOTAL | 1058.5 \pm 221.7 | 1037.5 \pm 180.7 | 1126.8 \pm 264.2 | N.S. |
| | HMM | 1070.8 \pm 265.8 | 1025.8 \pm 156.5 | 1134.6 \pm 301.3 | N.S. |
| | LMM | 5.49 \pm 2.13 | 5.47 \pm 2.63 | 7.34 \pm 3.90 | \uparrow AD vs. HC |
| Zn | TOTAL | 910.5 \pm 160.0 | 857.1 \pm 142.3 | 815.0 \pm 157.8 | \downarrow AD vs. HC |
| | HMM | 915.9 \pm 158.9 | 834.7 \pm 138.6 | 803.6 \pm 153.8 | N.S. |
| | LMM | 2.04 \pm 0.612 | 1.98 \pm 0.995 | 2.15 \pm 1.07 | N.S. |
| Se | TOTAL | 122.9 \pm 24.14 | 126.6 \pm 19.82 | 120.5 \pm 31.12 | \downarrow AD vs. MCI |
| | HMM | 123.3 \pm 24.64 | 131.8 \pm 20.64 | 118.3 \pm 29.08 | \downarrow AD vs. MCI |
| | LMM | 4.05 \pm 2.39 | 2.63 \pm 2.61 | 2.90 \pm 1.55 | \downarrow MCI vs. HC |
| Mo | TOTAL | 1.06 \pm 0.415 | 1.35 \pm 0.644 | 1.02 \pm 0.484 | N.S. |

| | | | | | |
|-----------|--------------|---------------|---------------|---------------|------|
| | HMM | 1.07±0.173 | 1.26±0.146 | 1.07±0.194 | N.S. |
| | LMM | <LOD | <LOD | <LOD | N.S. |
| Cd | TOTAL | 0.0640±0.0111 | 0.0899±0.0221 | 0.0799±0.0142 | N.S. |
| | HMM | 0.0672±0.0656 | 0.0863±0.0662 | 0.0751±0.0774 | N.S. |
| | LMM | <LOD | <LOD | <LOD | N.S. |

<LOD below limit of detection

N.S. non significant

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 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 vs. HC | ↑AD vs. HC | ↑AD vs. HC |
| | Zn | ↑AD/MCI vs. HC | ↑AD/MCI vs. HC | ↑AD/MCI vs. HC |
| | Cu | - | - | ↑AD vs. HC |
| Mn | Al, V, Cr, Fe, Cu, Zn, Se, Mo, Cd | ↓AD/MCI vs. HC | ↓AD/MCI vs. HC | - |
| Cu | Zn | ↑AD vs. HC | - | - |
| | Se | - | - | ↑AD vs. HC |

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).

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 vs. HC |

| | | |
|-----------------|-----------------------------|----------------|
| <i>Al-HMM</i> | <i>Fe,Zn,Se-TOTAL</i> | ↑AD vs. HC |
| <i>Al-LMM</i> | <i>Mn,Fe,Zn-TOTAL</i> | ↑AD vs. HC |
| <i>Mn-TOTAL</i> | <i>Al,Cu,Zn,Se-HMM</i> | ↓AD/MCI vs. HC |
| <i>Mn-HMM</i> | <i>Al,Fe,Cu,Zn,Se-TOTAL</i> | ↓AD/MCI vs. HC |

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 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.

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

| element | comparison |
|----------------|-------------------|
| Al | ↑AD vs. HC |
| Fe | ↑AD/MCI vs. HC |
| Cu | ↑AD vs. HC |
| Se | ↓MCI vs. HC |

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 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 β -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. demonstrated that accumulation of aluminum in brain of AD patients starts significantly in the later phase (Rao et al. 1999), 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 and 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 and 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, 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 melanotransferrin (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, 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 Figure 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.

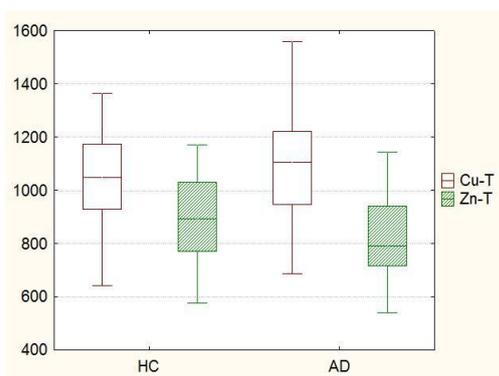


Fig. 1 Levels of copper and zinc ($\mu\text{g L}^{-1}$) in healthy controls and Alzheimer disease patients

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.

ACKNOWLEDGEMENTS

This work was supported by the projects CTM2012-38720-C03-01 from the Ministerio de Ciencia e Innovación and P008-FQM-3554 and P009-FQM-4659 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de Educación for a predoctoral scholarship. The authors also thank to Dr. Alberto Blanco and Carlos Salgado from Hospital Juan Ramón Jiménez for providing serum samples.

REFERENCES

- Ahmed SS, Santosh W (2010) Metallomic profiling and linkage map analysis of early Parkinson's disease: A new insight to aluminum marker for the possible diagnosis. *PLoS One* 5:e11252
- Aschner M, Guilarte TR, Schneider JS, Zheng W (2007) Manganese: recent advances in understanding its transport and neurotoxicity. *Toxicol Appl Pharmacol* 221:131-147
- Basun H, Forsell LG, Wetterberg L, Winblad B (1991) Metals and trace-elements in plasma and cerebrospinal-fluid in normal aging and Alzheimers-disease. *J Neural Transm-Park* 3:231-258
- Baum L, Chan IHS, Cheung SKK, Goggins WB, Mok V, Lam L, Leung V, Hui E, Ng C, Woo J, Chiu HFK, Zee BCY, Cheng W, Chan MH, Szeto S, Lui V, Tsoh J, Bush AI, Lam CWK, Kwok T (2010) Serum zinc is decreased in Alzheimer's disease and serum arsenic correlates positively with cognitive ability. *Biometals* 23:173-179
- Berthon G (1996) Chemical speciation studies in relation to aluminium metabolism and toxicity. *Coord Chem Rev* 149:241-280
- Bocca B, Alimonti A, Bomboi G, Giubilei F, Forte G (2006) Alterations in the level of trace metals in Alzheimer's disease. *Trace Elem Electroly* 23:270-276
- Bolognin S, Messori L, Zatta P (2009) Metal ion physiopathology in neurodegenerative disorders. *Neuromol Med* 11:223-238

- Bonda DJ, Lee HG, Blair JA, Zhu X, Perry G, Smith MA (2011) Role of metal dyshomeostasis in Alzheimer disease. *Metallomics* 3:267-270
- Bowman AB, Kwakye GF, Hernández EH, Aschner M (2011) Role of manganese in neurodegenerative diseases. *J Trace Elem Med Biol* 25:191-203
- Bush AI, Pettingell WH, Multhaup G, Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE (1994) Rapid induction of Alzheimer A beta amyloid formation by zinc. *Science* 265:1464-1467
- Bush AI, Tanzi RE (2002) The galvanization of β -amyloid in Alzheimer's disease. *Proc Natl Acad Sci USA* 99:7317-7319
- Charlet L, Chapron Y, Faller P, Kirsch R, Stone AT, Baveye PC (2012) Neurodegenerative diseases and exposure to the environmental metals Mn, Pb, and Hg. *Coord Chem Rev* 256:2147-2163
- Choi J, Rees HD, Weintraub ST, Levey AI, Chin LS, Li L (2005) Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. *J Biol Chem* 280:11648-11655
- Crapper DR, Krishnan SS, Dalton AJ (1973) Brain aluminum distribution in Alzheimer's disease and experimental neurofibrillary degeneration. *Science* 180:511-513
- De Leo ME, Borrello S, Passantino M, Palazzotti B, Mordente A, Daniele A, Filippini V, Galeotti T, Masullo C (1998) Oxidative stress and overexpression of manganese superoxide dismutase in patients with Alzheimer's disease. *Neurosci Lett* 250:173-176
- Egana JT, Zambrano C, Nunez MT, Gonzalez-Billault C, Maccioni RB (2003) Iron-induced oxidative stress modify tau phosphorylation patterns in hippocampal cell cultures. *Biometals* 16:215-223
- El-Ahmady O, El-Maraghy A, Ibrahim A, Ramzy S (1995) Serum copper, zinc, and iron in patients with malignant and benign pulmonary diseases. *Nutrition* 11:498-501
- Erikson KM, Syversen T, Steinnes E, Aschner M (2004) Globus pallidus: A target brain region for divalent metal accumulation associated with dietary iron deficiency. *J Nutr Biochem* 15:335-341
- Frausto da Silva JJR, Williams RJP (2001) *The Biological Chemistry of the Elements*. Oxford University Press, Oxford
- Garrick MD, Dolan KG, Horbinski C, Ghio AJ, Higgins D, Porubcin M, Moore EG, Hainsworth LN, Umbreit JN, Conrad ME, Feng L, Lis A, Roth JA, Singleton S, Garrick LM (2003) DMT1: A mammalian transporter for multiple metals. *Biometals* 16:41-54

- Gerhardsson L, Lundh T, Minthon L, Londos E (2008) Metal concentrations in plasma and cerebrospinal fluid in patients with Alzheimer's disease. *Dement Geriatr Cogn Disord* 25:508-515
- Gerhardsson L, Lundh T, Londos E, Minthon L (2011) Cerebrospinal fluid/plasma quotients of essential and non-essential metals in patients with Alzheimer's disease. *J Neural Transm* 118:957-962
- Han X (2010) Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochim Biophys Acta* 1801:774-783
- Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* 5:228-234
- Hegde ML, Shanmugavelu P, Vengamma B, Rao TS, Menon RB, Rao RV, Rao KSJ (2004) Serum trace element levels and the complexity of inter-element relations in patients with Parkinson's disease. *J Trace Elem Med Biol* 18:163-171
- Ihara Y, Hayabara T, Sasaki K, Fujisawa Y, Kawada R, Yamamoto T, Nakashima Y, Yoshimune S, Kawai M, Kibata M, Kuroda S (1997) Free radicals and superoxide dismutase in blood of patients with Alzheimer's disease and vascular dementia. *J Neurol Sci* 153:76-81
- Jolly DH, Poittrinal P, Millart H, Kariger E, Blanchard F, Collery P, Choisy H (1993) Blood zinc, copper, magnesium, calcium, aluminum and manganese concentrations in patients, with or without Alzheimer-type dementia. *Trace Elem Med* 10:192-195
- Ke Y, Qian ZM. (2007). Brain iron metabolism: Neurobiology and neurochemistry. *Prog Neurobiol* 83:149-173
- Kiziler AR, Aydemir B, Guzel S, Alici B, Ataus S, Tuna MB, Durak H, Kilic M (2010) May the level and ratio changes of trace elements be utilized in identification of disease progression and grade in prostatic cancer? *Trace Elem Electroly* 27:65-72
- Kovatsi L, Toulou K, Tsolaki M, Kazis A (2006) Cerebrospinal fluid levels of calcium, magnesium, copper and zinc in patients with Alzheimer's disease and mild cognitive impairment. *Trace Elem Electroly* 23:247-250
- Kozlowski H, Luczkowski M, Remelli M, Valensin D (2012) Copper, zinc and iron in neurodegenerative diseases (Alzheimer's, Parkinson's and prion diseases). *Coord Chem Rev* 256:2129-2141
- Lavados M, Guillón M, Mujica MC, Rojo LE, Fuentes P, Maccioni RB (2008) Mild cognitive impairment and Alzheimer patients display different levels of redox-active CSF iron. *J Alzheimers Dis* 13:225-232

- Loef M, Schrauzer GN, Walach H (2011) Selenium and Alzheimer's disease: A systematic review. *J Alzheimers Dis* 26:81-104
- Maccioni RB, Muñoz JP, Barbeito L (2001) The molecular bases of Alzheimer's disease and other neurodegenerative diseases. *Arch Med Res* 32:367-381
- Malavolta M, Giacconi R, Piacenza F, Santarelli L, Cipriano C, Costarelli L, Tesei S, Pierpaoli S, Basso A, Galeazzi R, Lattanzio F, Mocchegiani E (2010) Plasma copper/zinc ratio: an inflammatory/nutritional biomarker as predictor of all-cause mortality in elderly population. *Biogerontology* 11:309-319
- McGregor SJ, Brock JH, Halls D (1991) The role of transferrin and citrate in cellular uptake of aluminium. *Biol Met* 4:173-175
- McKahn G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology* 34:939-944
- Mecocci P (2004) Oxidative stress in mild cognitive impairment and Alzheimer disease: a continuum. *J Alzheimers Dis* 6:159-163
- Morris JC, Storandt M, Miller JP, McKeel DW, Price JL, Rubin EH, Berg L (2001) Mild cognitive impairment represents early-stage Alzheimer disease. *Arch Neurol* 58:397-405
- Muñiz CS, Fernández-Martin JL, Marchante-Gayón JM, Alonso IG, Cannata-Andía JB, Sanz-Medel A (2001) Reference values for trace and ultratrace elements in human serum determined by double-focusing ICP-MS. *Biol Trace Elem Res* 82:259-272
- Mustak MS, Rao TSS, Shanmugavelu P, Sundar NMS, Menon RB, Rao RV, Rao KSJ (2008) Assessment of serum macro and trace element homeostasis and the complexity of inter-element relations in bipolar mood disorders. *Clin Chim Acta* 394:47-53
- Nischwitz V, Berthele A, Michalke B (2008) Speciation analysis of selected metals and determination of their total contents in paired serum and cerebrospinal fluid samples: An approach to investigate the permeability of the human blood-cerebrospinal fluid-barrier. *Anal Chim Acta* 627:258-269
- Núñez MT, Urrutia P, Mena N, Aguirre P, Tapia V, Salazar J (2012) Iron toxicity in neurodegeneration. *Biometals* 25:761-776
- Omar RA, Chyan YJ, Andorn AD, Poeggeler B, Robakis NK, Pappolla MA (1999) Increased expression but reduced activity of antioxidant enzymes in Alzheimer's disease. *J Alzheimers Dis* 1:139-145

- Pérez-Parajón J, Blanco-González E, Cannata JB, Sanz-Medel A (1989) A critical appraisal of the speciation of aluminium in serum by ultrafiltration. *Trace Elem Med* 6:41-46
- Perry G, Cash AD, Srinivas R, Smith MA (2002) Metals and oxidative homeostasis in Alzheimer's disease. *Drug Develop Res* 56:293-299
- Rao JKS, Rao RV, Shanmugavelu P, Menon RB (1999) Trace elements in Alzheimer's disease brain: a new hypothesis. *Alzheimers Rep* 2:241-246
- Reitz C, Brayne C, Mayeux R (2011) Epidemiology of Alzheimer disease. *Nat Rev Neurol* 7:137-152
- Religa D, Strozzyk D, Cherny RA, Volitakis I, Haroutunian V, Winblad B, Naslund J, Bush AI (2006) Elevated cortical zinc in Alzheimer disease. *Neurology* 67:69-75
- Rivera-Mancía S, Ríos C, Montes S (2011) Manganese accumulation in the CNS and associated pathologies. *Biometals* 24:811-825
- Roskams AJ, Connor JR (1990) Aluminium access to the brain: a role for transferrin and its receptor. *Proc Natl Acad Sci USA* 87:9024-9027
- Sayre LM, Perry G, Atwood CS, Smith MA (2000) The role of metals in neurodegenerative diseases. *Cell Mol Biol* 46:731-741
- Seiler HG, Sigel A, Sigel H (1994) *Handbook on metals in clinical and analytical chemistry*. Marcel Dekker, New York
- Smith Q, Rabin O, Chikhale E (1997) Delivery of metals to brain and the role of the blood-brain barrier. In: Connor J (ed) *Metals and Oxidative Damage in Neurological Disorders*. Plenum Press, New York, pp 113-130
- Smith MA, Zhu X, Tabaton M, Liu G, McKeel DW, Cohen ML, Wang X, Siedlak SL, Dwyer BE, Hayashi T, Nakamura M, Nunomura A, Perry G (2010) Increased iron and free radical generation in preclinical Alzheimer disease and mild cognitive impairment. *J Alzheimers Dis* 19:363-372
- Squitti R, Lupoi D, Pasqualetti P, Dal Forno G, Vernieri F, Chiovenda P, Rossi L, Cortesi M, Cassetta E, Rossini PM (2002) Elevation of serum copper levels in Alzheimer's disease. *Neurology* 59:1153-1161
- Squitti R, Quattrocchi CC, Salustri C, Rossini PM (2008) Ceruloplasmin fragmentation is implicated in 'free' copper deregulation of Alzheimer's disease. *Prion* 2:23-27
- Squitti R, Ghidoni R, Scrascia F, Benussi L, Panetta V, Pasqualetti P, Moffa F, Bernardini S, Ventriglia M, Binetti G, Rossini PM (2011) Free copper distinguishes mild cognitive impairment subjects from healthy elderly individuals. *J Alzheimers Dis* 23:239-248

- Srivastava RAK, Jain JC (2002) Scavenger receptor class B type I expression and elemental analysis in cerebellum and parietal cortex regions of the Alzheimer's disease brain. *J Neurol Sci* 196:45-52
- Suzuki KT (2005) Metabolomics of selenium: Se metabolites based on speciation studies. *J Health Sci* 51:107-114
- Templeton DM (1999) Biomedical aspects of trace element speciation. *Fresenius J Anal Chem* 363:505-511
- Tomljenovic L (2011) Aluminum and Alzheimer's disease: After a century of controversy, is there a plausible link? *J Alzheimers Dis* 23:567-598
- Vincent JB, Love S (2012) The binding and transport of alternative metals by transferrin. *Biochim Biophys Acta* 1820:362-378
- Vural H, Sirin B, Yilmaz N, Eren I, Delibas N (2009) The role of arginine-nitric oxide pathway in patients with Alzheimer disease. *Biol Trace Elem Res* 129:58-64
- Zapatero MD, de Jalon AG, Pascual F, Calvo ML, Escanero J, Marro A (1995) Serum aluminum levels in Alzheimer's disease and other senile dementias. *Biol Trace Elem Res* 47:235-240
- Zatta P, Cervellin D, Mattiello G, Gerotto M, Lazzari F, Gasparoni G, Gomirato L, Mazzolini G, Scarpa G, Zanoboni V, Pilone G, Favarato M (1993) Plasma multielemental analysis in Alzheimer's disease and multi-infarctual dementia. *Trace Elem Med* 10:85-89
- Zatta P, Kiss T, Suwalsky M, Berthon G (2002) Aluminium(III) as a promoter of cellular oxidation. *Coord Chem Rev* 228:271-284
- Zheng W, Xin N, Chi ZH, Zhao BL, Zhang J, Li JY, Wang ZY (2009) Divalent metal transporter 1 is involved in amyloid precursor protein processing and A β generation. *Faseb J* 23:4207-4217

Figure

[Click here to download high resolution image](#)

