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Pharmacology

Acetylcholinesterase inhibitors in Alzheimer's disease influence Zinc and Copper homeostasis

R. Giacconi[a,*], C. Giuli[b], T. Casoli[c], M. Balietti[c], L. Costarelli[d], M. Provinciali[a], A. Basso[a], F. Piacenza[a], D. Postacchini[b], R. Galeazzi[d], P. Fattoretti[c], L. Nisi[a], P. Fabbietti[e], R. Papa[f], M. Malavolta[a]

[a] Advanced Technology Center for Aging Research, IRCCS INRCA, Ancona, Italy
[b] Geriatrics Operative Unit, IRCCS INRCA, Fermo, Italy
[c] Center for Neurobiology of Aging, IRCCS INRCA, Ancona, Italy
[d] Clinical Laboratory & Molecular Diagnostics, IRCCS INRCA, Ancona, Italy
[e] Unit of Geriatric Pharmacoepidemiology, IRCCS INRCA, Ancona, Italy
[f] Centre of Socio-Economic Gerontological Research, IRCCS INRCA, Ancona, Italy

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ABSTRACT

Background: Alzheimer's disease (AD) is the most common age-related neurodegenerative disease. An altered homeostasis of Zinc (Zn) and Copper (Cu), as well as a dysregulated expression of Zn-regulatory proteins have been previously described in AD. Acetylcholinesterase inhibitors (AChEI) are commonly used as AD treatment to improve cognitive function, but their effect on Zn homeostasis is still unexplored.

Objectives: The aims of this study were to define the metal dyshomeostasis in AD patients, to investigate AChEI influence on Zn homeostasis and inflammation, and to analyze the relationship between cognitive impairment at two-year follow-up and metal concentrations, considering AChEI use.

Methods and Results: 84 Healthy Elderly (HE) and 95 AD patients were enrolled (62 AchEI user and 33 AchEI naïve). HE showed similar plasma Zn and Cu concentrations and Cu/Zn ratio in comparison to AChEI users, but significantly higher Zn level, as well as lower Cu amount and Cu/Zn ratio than AChEI naïve patients. Moreover, AChEI users had increased Zn plasma level, reduced Cu amount, Cu/Zn ratio, and IL1β concentration and lower Zip2 lymphocytic expression vs. naïve patients. A multiple linear regression analysis showed that the MMSE score decline after two-year follow-up was reduced by AChEI therapy and was positively associated with plasma Zn decrease over time.

Conclusion: Our data revealed that AChEI use may affect peripheral Zn and Cu homeostasis in AD patients, decrease Cu/Zn ratio demonstrating a general reduction of inflammatory status in patients under AChEI treatment. Finally, AChEI influence on circulating Zn could be implicated in the drug-related slowdown of cognitive decline.

1. Introduction

Increasing evidence suggests that Alzheimer's disease (AD) pathogenesis involves not only neurons impairment, but also neuroimmunological mechanisms and systemic inflammation [1,2].

In the central nervous system, inflammation and degenerative phenomena (e.g. protein aggregation, defective protein degradation, oxidative stress and mitochondrial dysfunction) have been related to the altered homeostasis and distribution of some metals, such as Zinc (Zn) and Copper (Cu) [3]. Peripherally, a recent meta-analysis showed higher Cu and lower Zn serum concentrations in AD patients than healthy controls [4]. Cu is essential to human health, but Cu overload has been associated with AD development [5,6]; moreover, Cu exposure enhances inflammatory response and reduces amyloid-beta (Aβ)

Abbreviations: AChEI, Acetylcholinesterase inhibitors; AD, Alzheimer's disease; ADL, Activities of Daily Living; Aβ, amyloid beta; CRP, C-reactive protein; Cu, Copper; GDS, Geriatric Depression Scale; IADL, Instrumental Activities of Daily Living; IL10, Interleukin-10; IL1β, Interleukin-1β; IL6, Interleukin-6; MMSE, Mini Mental State Examination; MT2A, metallothionein-2A; NSAIDs, nonsteroidal anti-inflammatory drugs; TNFα, Tumor Necrosis Factor-α; Zip2, Zinc Transporter SLC39A2; Zip3, Zinc Transporter SLC39A3; Znt1, Zinc Transporter SLC30A1; Zn, Zinc

* Corresponding author at: Advanced Technology Center for Aging Research, IRCCS INRCA, Via Birarelli 8, 60121 Ancona, Italy.
E-mail address: r.giacconi@inrca.it (R. Giacconi).

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clearance in the brain [7]. An altered Zn metabolism was also reported to contribute to the dysregulation of peripheral immune cells [8,9] and, consequently, to systemic inflammation [10,11].

Currently, acetylcholinesterase inhibitors (AChEI) are the most common treatment for AD with various degree of efficacy in patients' memory and cognitive functions [12]. These drugs have been shown to reduce systemic pro-inflammatory cytokine production [13,14] via the cholinergic anti-inflammatory pathway [15], to exert anti-inflammatory effects in peripheral blood mononuclear cells of AD patients [16,17], and to have inhibitory effects on Aβ oligomer-induced inflammation *in vitro* and *in vivo* by inhibiting microglial activation [18].

The main purpose of this study was to better define the presence of metal dyshomeostasis in AD patients and investigate the possible influence of AChEI use on Zn metabolism and inflammation. Furthermore, we evaluated the relationship between cognitive decline, measured as MMSE score change, at two-year follow-up and plasma metal changes.

2. Material and methods

2.1. Subjects

All the subjects (84 Healthy Elderly (HE) and 95 AD (62 AchEI user and 33 AchEI naïve) were enrolled at the Evaluation of Alzheimer's Unit of Geriatrics Operative Unit at the IRCCS INRCA Hospital in Fermo (Italy). The study protocol of My Mind Project (grant. N. 154/GR-2009-1584108) complied with the principles of the Declaration of Helsinki (code SC/12/301) and was approved by the local ethics committee (IRCCS INRCA Bioethics Advisory Committee, Ancona, Italy). Written informed consent was obtained from all subjects. All participants underwent a complete clinical, physical, neuropsychological, and functional evaluation. The status of HE was defined as the absence of relevant cognitive diseases, while the diagnosis for possible or probable AD was carried out by using the DSM-IV or NINCDS-ADRDA criteria [19]. The inclusion criteria were age 65 years or older and, for AD patients, the involvement of a caregiver and at least 4-month AChEI use; the exclusion criteria were serious medical or psychiatric conditions and sensorimotor deficits, severe AD, and neurodegenerative disorders other than AD. Laboratory parameters, plasma metal concentration and the comprehensive clinical and neuropsychological assessments were collected at baseline and after two years; all the other data were determined only at baseline. Sixty-nine % of AchEI users were treated with rivastigmine (n = 43), 27% with donepezil (n = 17), and 4% with galantamine (n = 2); 45% of AchEI naïve subjects was treated with memantine (n = 15), while 55% (n = 18) was not pharmacologically treated for dementia. In addition to AChEI and glutamate antagonists, the use of the following drugs was also registered: benzodiazepines, antidepressant, anticoagulants, antihypertensives, non-steroidal anti-inflammatory drugs (NSAIDs), lipid lowering medications.

2.2. Laboratory determinations

Venous peripheral blood samples were collected after an overnight fast. Serum albumin was measured by an enzymatic colorimetric test on Modular automated clinical chemistry analyzers (Roche-Hitachi). Serum concentration of high sensitive C-reactive protein (CRP) was determined by amplified immunonephelometry assay (CardioPhase hsCRP-Dade Behring Inc Deerfield, IL).

Plasma and lymphocytes were stored at −80 °C in the Biological Bank of IRCCS INRCA until use.

2.3. Neuropsychological and functional assessment

The neuropsychological test battery was previously described in Giuli et al. [20].

Global cognitive functions of HE and AD patients were assessed by the Mini Mental State Examination (MMSE) [21], a commonly used psychometric screening assessment that has a maximum score of 30 points, grouped into seven categories. Lower scores indicate more severe cognitive impairment. MMSE was corrected for age and education [22]. Mood status was evaluated using the 30 items version of the Geriatric Depression Scale (GDS-30 items) [23] developed to identify depressive symptoms in older adults. Total score was obtained adding one point for each answer indicating depression: a score > 10 suggests depression. Functional status was assessed by the Activities of Daily Living (ADL) [24] and Instrumental Activities of Daily Living (IADL) scales [25].

2.4. Trace elements determination in plasma

Plasma Zn and Cu levels were measured by a Thermo XII Series ICP-MS device (Thermo Electron Corporation, Waltham, MA, USA) in accordance to Malavolta et al. [26].

2.5. Extraction of total RNA and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from thawed lymphocytes using the RNeasy kit (Qiagen,Germany) and quantified by NanoDrop spectrophotometer. cDNA synthesis from total RNA was performed using i-Script reverse transcriptase (Biorad, Hercules, CA) and the cDNA was used in real-time PCR assay to detect the expression levels of β-actin housekeeping gene as well as MT2A, SLC39A2 (Zip2), SLC39A3 (Zip3), and SLC30A1 (Znt1). For gene expression analysis, 55 AD patients were randomly selected (35 AChEI user and 20 AChEI naïve; 35 females and 20 males; age range 74–80 years). User and naïve subjects were matched for age, gender, education, GDS, ADL, IADL, and MMSE value.

The primers used are reported in Table 1S (Supplementary material). 1 μg of cDNA was amplified in a total volume of 20 μL containing iQ SYBR Green Supermix (Biorad, Hercules, CA) on a BioRad iQ5 Optical Real Time PCR, employing a primer concentration of 150 nM (β-Actin), 200 nM (MT2A), 300 nM (Zip2) and 200 nM (Zip3 and Znt1). Assays for each transcript were carried out as duplicates. Any inefficiencies in RNA input or reverse transcription were corrected by normalization to the housekeeping gene. Relative amounts of the target mRNAs were calculated based on the comparative CT method [ΔΔCt (Cycle Threshold)].

2.6. Plasma cytokine assays

IL-1β, TNF-α, IL-10 and IL-6 were measured by Quantikine Immunoassay RD System in 64 AD patients (44 AChEI user and 20 AChEI naïve; 42 females and 22 males; age range 70–89 years). User and naïve subjects were matched for age, gender, education, GDS, ADL, IADL, and MMSE score.

2.7. Statistical analysis

Results were reported as mean ± Standard Error of the Mean (SEM) or as percentages for continuous and categorical variables, respectively. For continuous variables, normal distribution was verified by the One-sample Kolmogorov–Smirnov test. Log transformation of non-normally distributed variables was adopted. Differences among groups were performed by One-way Analysis of Variance (continuous variables) or by Pearson's χ^2 test (categorical variables). Adjustment for non-balanced variables were applied. LSD *post-hoc* tests were also performed.

A multiple linear regression model was used for evaluating the associations among (i) MMSE score, plasma Zn level, lymphocytic expression of MT2A, Zip2, Zip3, and Znt1 of AD patients at baseline, (ii)

Table 1
Characteristics of studied cohorts.

	HE n = 84	AD n = 95	P value
Age (years)	72.9 ± 0.070	77.5 ± 0.055	< 0.0001
Females (%)	80%	67%	NS
MMSE	28.1 ± 0.012	20.2 ± 0.038	< 0.0001
GDS	7.8 ± 0.058	8.4 ± 0.060	NS[§]
ADL	5.98 ± 0.002	5.23 ± 0.011	< 0.0001[§]
IADL	7.98 ± 0.003	3.42 ± 0.021	0.0001[§]
Education (years)	9.3 ± 0.055	5.0 ± 0.035	< 0.0001*
Albumin (g/dl)	4.27 ± 0.003	4.19 ± 0.004	NS[§]
CRP (pg/ml)	0.24 ± 0.004	0.34 ± 0.005	NS[§]
Zn (μM)	12.87 ± 0.021	12.88 ± 0.020	NS[§]
Cu (μM)	17.13 ± 0.032	17.24 ± 0.034	NS[§]
Cu/Zn ratio	1.36 ± 0.004	1.36 ± 0.003	NS[§]

MMSE: Mini Mental State Examination; **GDS**: Geriatric Depression Scale; **ADL**: Activities of Daily Living; **IADL**: Instrumental Activities of Daily Living; **CRP**: C-reactive protein; **NS**: Not significant; *correction for age; [§]correction for age and education.

lymphocytic expression of MT2A, Zip2, Zip3, and Znt1, and plasma metal concentrations of AD patients at baseline, (iii) MMSE score, plasma Zn level and Cu/Zn ratio changes during the two-year follow-up; (iv) MMSE score changes during the two-year follow-up and drug use at baseline. Data analyses were performed by SPSS Statistics version 20.0.0 (IBM, IL, USA).

3. Results

3.1. Participant's characteristics

Clinical and demographic characteristics of HE and AD subjects are reported in Table 1. HE subjects were significantly younger and had a significantly higher level of education than AD patients ($p < 0.0001$); MMSE score, ADL and IADL values were significantly lower in AD vs. HE subjects ($p < 0.0001$). No significant differences were found for gender, GDS, serum albumin and CRP levels, plasma Zn and Cu concentrations, and Cu/Zn ratio. However, when the AD group was divided on the basis of AChEI treatment, HE showed similar plasma Zn concentration to AChEI treated (13.03 ± 0.21 vs. 13.20 ± 0.23 μM), but significantly higher values than AChEI naïve patients (13.03 ± 0.21 vs. 12.24 ± 0.31 μM, $p < 0.05$); moreover, AChEI users had significantly increased Zn level vs. AChEI naïve subjects (13.20 ± 0.23 vs. 12.24 ± 0.31 μM, $p < 0.05$) (Fig. 1A). The slight difference in plasma Zn level between AChEI user and AChEI naive patients may depend on the small sample size, but also on the lower plasma Zn variability reported in women (67% in our cohort) [27]. Drug naïve patients showed also significantly higher plasma Cu level and Cu/Zn ratio vs. AChEI users and HE (Cu level, 18.22 ± 0.38 vs. 16.69 ± 0.53 μM and 18.22 ± 0.38 vs. 16.19 ± 0.38 μM, respectively; Cu/Zn ratio, 1.45 ± 0.06 vs. 1.24 ± 0.04 and 1.45 ± 0.06 vs. 1.28 ± 0.04, respectively) (Fig. 1 B and C). Characteristics of AChEI naïve and user patients are reported in Table 2S of Supplementary material. No differences were observed for age, gender, MMSE score, GDS, ADL, IADL, education, serum albumin and CRP levels, and drug use.

To further analyze the effect of AChEI and exclude any possible influence of other drugs used in AD treatment (i.e. glutamate antagonists), the AChEI naïve group was subdivided into memantine-treated and memantine-free subjects –no other glutamate antagonist was prescribed in our cohort. The comparison of basal and follow-up plasma Zn, Cu and Cu/Zn ratio showed no significant differences between the subgroups, thus excluding any interference on metal homeostasis of memantine (Table 3S of Supplementary materials).

3.2. Lymphocytic MT2A and Zn transporters gene expression and plasma cytokine concentration in AD patients in relation to AChEI treatment

To better understand the mechanism involved in the modulation of plasma Zn level by AChEI, the expression of Zn responsive genes was analysed in lymphocytes of the subgroups of AChEI users and naïve patients. AChEI users showed significantly lower Zip2 expression than drug naïve patients ($p < 0.05$), while MT2A, Zip3 and Znt1 gene expression evidenced no differences (Fig. 2A). Increased Zip2 mRNA level in whole AD group was associated with lower MMSE score in a linear regression analysis ($\beta = -0.698$; $p < 0.05$; Table 4S Supplementary materials), suggesting a possible implication of this zinc transporter in cognitive decline.

Plasma IL1β concentration of AChEI users was significantly lower as compared to AChEI naïve subjects ($p < 0.05$), whereas no differences were observed for TNFα, IL10 and IL6 (Fig. 2B).

3.3. Main predictors of cognitive decline in AD patients during two-year follow-up

A multiple linear regression analysis showed a positive association between cognitive decline and plasma Zn level changes after the two-

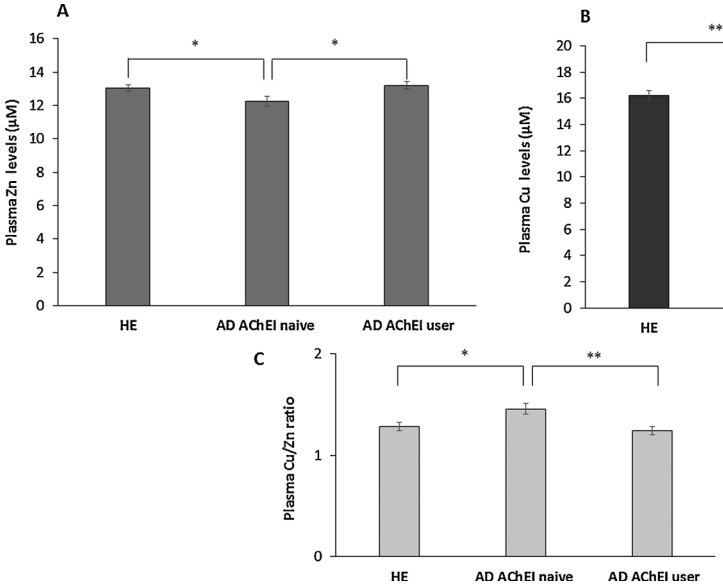

Fig. 1. Baseline plasma concentrations of Zinc (Zn) (A) and Copper (Cu) (B) and Cu/Zn ratio (C) in Healthy Elderly (HE) and Alzheimer's disease (AD) patients subdivided in acetylcholinesterase inhibitors (AChEI) naive and user. AChEI naïve patients showed significantly lower Zn and higher Cu levels and significantly higher Cu/Zn ratio vs. AChEI users and HE. ANCOVA analysis adjusting for age, gender and education was applied; *$p < 0.05$; **$p < 0.01$.

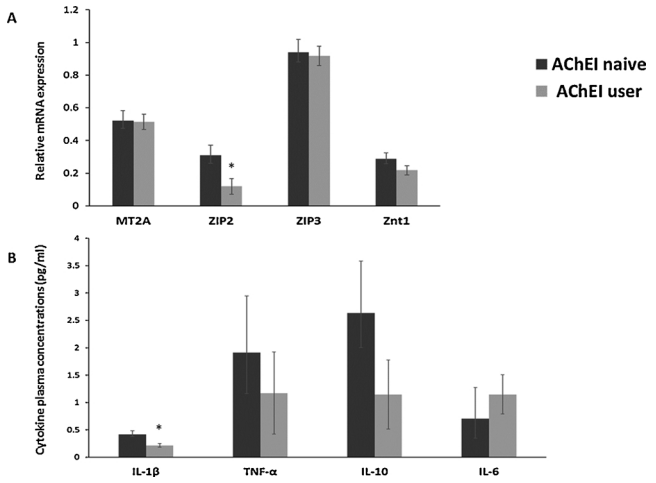

Fig. 2. Baseline gene expression of MT2A and Zinc transporters Zip2, Zip3 and Znt1 in lymphocytes (A) and plasma concentrations of cytokines of Alzheimer's disease patients in relation to acetylcholinesterase inhibitors (AChEI) treatment (B). AChEI users showed significantly lower Zip2 expression and plasma IL-1β level vs. naïve patients. ANOVA analysis was applied; *$p < 0.05$.

Table 2
Multivariate linear regression analysis for variables independently associated with MMSE score decline in AD patients during the two-year follow-up.

Predictors	Standardized Beta Coefficients	Unstandardized Coefficients		P value
		B	Std. Error	
Age	0.218	1.048	0.573	NS
Gender	0.295	14.848	6.467	NS
Duration of dementia	0.069	0.067	0.118	NS
GDS	−0.050	−0.213	0.528	NS
ADL	−0.059	−1.254	2.988	NS
IADL	0.155	1.832	1.693	NS
Education (years)	−0.076	−0.554	0.940	NS
Albumin	0.138	10.422	8.959	NS
CRP	−0.136	−6.552	6.102	NS
Cu/Zn ratio changes	0.114	0.118	0.146	NS
Zn changes	0.283	0.444	0.214	0.043
AChEI	0.254	13.064	6.157	0.029
Benzodiazepines	0.023	1.829	9.501	NS
Antidepressants	0.107	6.412	7.390	NS
Anticoagulants/ Antiplatelets drugs	−0.134	−6.396	8.055	NS
Antihypertensives drugs	0.317	15.123	5.855	0.046
NSAIDs	0.238	11.598	8.307	NS
Lipid lowering medications	−0.055	−3.303	7.115	NS

Cognitive decline was defined as delta between 24-month and baseline MMSE score (delta-MMSE). Cu/Zn ratio and Zn concentration changes were defined as delta between 24-month and baseline values. **MMSE**: Mini Mental State Examination; **GDS**: Geriatric Depression Scale; **ADL**: Activities of Daily Living; **IADL**: Instrumental Activities of Daily Living; **CRP**: C-reactive protein; **AChEI**: acetylcholinesterase inhibitors; **NSAIDs**: nonsteroidal anti-inflammatory drugs; **NS**: Not significant. Gender was categorized as follows: 1 = males and 0 = females.

year follow-up in whole AD group; moreover, the cognitive status changes was dependent on AChEI and antihypertensives use at baseline (Table 2). In details, higher is the plasma Zn level reduction and more exacerbated is the cognitive deterioration, while AChEI and antihypertensives assumption slows down the MMSE score decline. No association was found with age, gender, GDS, ADL, IADL, duration of dementia, education, serum albumin and CRP levels, Cu/Zn ratio changes and other drugs. In Fig. 1 of supplementary materials (Fig. 1S) the AChEI effect on MMSE score change during the two-year follow-up was reported.

4. Discussion

Abnormal homeostasis of some trace metals has been observed in AD patients, especially regarding Zn and Cu metabolism [4,28,29]. In our study, dividing the AD cohort on the basis of AChEI use, HE showed higher plasma Zn and lower Cu values than AChEI naïve patients and similar plasma Zn and Cu concentrations to AChEI treated patients. These results could explain, at least in part, the discrepancies observed in literature [4,30], and suggest the importance of taking into account the AChEI use for an unbiased evaluation of circulating Zn and Cu concentrations in AD. Of course, other factors might be involved in literature heterogeneity, as, for instance, sex-related changes in body composition that influence blood trace elements in the elderly population [31], ethnicity or socio-economic status [32]. Our cohort was composed prevalently by females and had only Caucasian subjects, mainly with rural origin, low/medium schooling and employment, as well as an homogeneous life style (e.g. Mediterranean diet, negligible percentage of smokers, moderate alcohol use). Thus, our results need to be reproduced in other cohorts to increase their robustness, but certainly represent a first, key step. AChEI represent a recognized dementia treatment and in patients with moderate to severe AD, their constant use is associated with cognitive and functional benefits, as well as with prolonged independence and reduced mortality [33,34]. Their clinical efficacy is based on acetylcholinesterase inhibition, leading to an increase of acetylcholine levels at synapses. Indeed, deficits in the cholinergic system, such as decreased activity of choline acetyltransferase, reduction of presynaptic markers of cholinergic transmission, and loss of neurons from the nucleus basalis of Meynert have been demonstrated in AD [35]. However, AchEI treatment has been demonstrated to influence also the glutamatergic system. Glutamate induced apoptosis and loss of viability were prevented in cultured neurons by exposure to AchEI [36]. In addition, rats treated with rivastigmine showed a significant increase of expression of the glutamate reuptake carrier rEAAC1 in hippocampus, thus decreasing the concentration of glutamate in the synaptic cleft [37]. Therefore, the glutamatergic system may be modulated by AChEI exposure and this regulation can be considered an additional beneficial effect of these drugs in the treatment of AD. With regard AChEI anti-inflammatory role we observed that AChEI users had decreased Cu/Zn ratio and reduced plasma IL-1β level as compared to AChEI naïve patients, and it is known that high values of Cu/Zn ratio strongly correlate with systemic inflammatory biomarkers [26,38]. These results strengthen the anti-inflammatory properties previously described for these drugs [16]. Furthermore, the AChEI influence on circulating Zn suggests their potential role in improving immune functions. Indeed, subclinical Zn deficiency is common in the elderly and is responsible for immune system disturbances and premature T lymphocyte apoptosis [39,40], thus leading to a low degree of inflammation [9]. Since AD is characterized by a systemic chronic inflammatory status [41] and in AD patients peripheral immune cells perturbations have been documented [41], the AChEI capacity to ameliorate Zn status and inflammatory condition may be part of their therapeutic mechanism. Moreover, Zn is a cofactor of antioxidant enzymes such as Cu/Zn Superoxide dismutase, increases the activity of glutathione, catalase and reduces that of inducible nitric acid synthase and NADPH oxidase [42], thus, not surprisingly, metal dyshomeostasis may lead to abnormal redox status and contribute to AD progression [43].

Some evidence demonstrates that inflammation may cause a Zn transfer from serum to liver, with an increment of the hepatic level [44,45], while oxidative and/or nitrosative stresses, which are relevant in AD [46], can trigger the Zn release from metalloproteins and its relocation from cell compartments or brain regions [47]. Therefore, AChEI, through their anti-inflammatory [16,17] and antioxidant

proprieties [46], could favor Zn redistribution from liver or brain to peripheral blood. However, further research is needed to clarify these mechanisms.

Investigations on animal models showed that AChEI inhibit microglial activation induced by Aβ oligomers and reduce Aβ plaque burden ameliorating cognitive impairment [18,48]. An increased expression of Zn transporters has been found in AD human cortex [49], as well as in the brain of aged *Octodon degus*, a natural animal model for AD; this increase is associated with Aβ accumulation and neurodegeneration [50]. Besides, the interaction of Aβ with extracellular Zn promotes the Aβ uptake into the dentate granule cells of rat normal brain, leading to short-term memory loss [51], while the abnormal Zn influx into the dentate gyrus affects object recognition memory via attenuated long-term potentiation induction [52]. These findings support the importance to strictly regulate intracellular and extracellular Zn to guarantee synaptic plasticity and cognitive function. Interestingly, we found that Zip2 expression in lymphocytes from AD patients was negatively associated with MMSE score. Zip2 is a plasma membrane-localized zinc transporter that regulates Zn uptake and represents the most sensitive gene to Zn deficiency condition [53,54]. Therefore, the lower expression of Zip2 in peripheral lymphocytes and the concomitant higher plasma Zn level in AChEI user vs. AChEI naïve AD patients may endorse the idea that these drugs contribute to regulate Zn homeostasis as part of their positive effect. For instance, in AD, a chronic increased frequency of CD4 + T cells activation was reported, thus promoting a pro-inflammatory status and worsen the disease progression [55]. Since CD4 + T cell activation requires an increased Zn influx [56,57], Zip2 downregulation by AChEI might diminish the phenomenon.

Finally, we observed that the MMSE score decline after two-year follow-up was reduced by AChEI therapy and was positively associated with Zn changes. This result suggests, for the first time, that a Zn decrease over time may promote cognitive decline and disease progression. Adequate Zn intake is crucial for proper cognitive functions in elderly subjects [58] and a Zn decrement during aging is common, contributing to immune dysfunction and peripheral low grade chronic inflammation [11]. This last condition is largely associated with neuroinflammation as the age-related abnormal blood-brain barrier permeability allows the entrance in the central nervous system of peripheral soluble factors and immune circulating cells [10,41]. Therefore, AChEI therapy, regulating Zn homeostasis, might counteract these phenomena and delay AD progression.

A limitation of the present investigation may be the relatively small sample size of AChEI user and naïve AD patients, therefore the effects of AChEI on Zn homeostasis regulation in lymphocytes should be further investigated in larger populations as well as in AD animal models to better clarify if AChEI can modulate cerebral Zn homeostasis and how this affects cognitive function.

5. Conclusions

In summary, the present study revealed, for the first time, the influence of AChEI on peripheral metal homeostasis (increasing plasma Zn level and reducing plasma Cu amount and Cu/Zn ratio) of AD patients. Moreover, a Zn decrease after two-year follow-up favors cognitive decline in AD patients. These findings provide a basis for future studies on the effect of AChEI in regulating immune function and their capability to modulate Zn transporter expression in the brain. These drugs might regulate Zn intracellular accumulation and synaptic release, reducing Aβ formation and delaying AD neurodegeneration.

Conflict of interest

The authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jtemb.2019.06.001.

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