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RESEARCH ARTICLE

Comparison of Extracellular and Intracellular Blood Compartments Highlights Redox Alterations in Alzheimer's and Mild Cognitive Impairment Patients

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Abstract: ***Background:*** Many studies suggest oxidative stress as an early feature of Alzheimer's Disease (AD). However, evidence of established oxidative stress in AD peripheral cells is still inconclusive, possibly due to both, differences in the type of samples and the heterogeneity of oxidative markers used in different studies.

Objective: The aim of this study was to evaluate blood-based redox alterations in Alzheimer's Disease in order to identify a peculiar disease profile.

Method: To that purpose, we measured the activity of Superoxide Dismutase, Catalase and Glutathione Peroxidase both in the extracellular and the intracellular blood compartments of AD, MCI and control subjects. The amount of an open isoform of p53 protein (unfolded p53), resulting from oxidative modifications was also determined.

Results: Decreased SOD, increased GPx activity and higher p53 open isoform were found in both AD and MCI plasma compared to controls. In blood peripheral mononuclear cells, SOD activity was also decreased in both AD and MCI, and unfolded p53 increased exquisitely in younger AD males compared to controls.

***Conclusion*:** Overall, these data highlight the importance of considering both extracellular and intracellular compartments, in the determination of antioxidant enzyme activities as well as specific oxidation end-products, in order to identify peculiar blood-based redox alterations in AD pathology.

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1. INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia in elderly population, is a progressive and neurodegenerative disorder characterized by irreversible cognitive and physical deterioration. Although the exact mechanism of AD is still unclear, it is now well recognized that multiple etiological factors, including genetic and environmental factors, and general lifestyle can contribute to the development of the disease [1].

Although the "AD amyloid cascade hypothesis" has dominated thinking, modelling, diagnosis and drug development efforts for almost twenty years [2], accumulating evidence underlined oxidative stress as an early event in AD development [3-5], preceding even plaques and tangles formation, as well as the clinical manifestation of AD [3, 6, 7]. Different end-products of peroxidation including malondialdehyde [8], peroxynitrite [9, 10], carbonyl groups [11], and advanced glycosylation end-products (AGEs) [8] have been described in AD brain and cerebrospinal fluid (CSF) [12-14]. In addition, decrease of antioxidant defense mechanisms in different brain regions, including the hippocampus, has been found in AD [15-18].

The possibility that inefficiencies of free radicals detoxifying enzymes and production of oxidative end-products can be early events contributing to AD development, brings up the idea that those enzymes might be also investigated as potential biomarkers of the disease. On the other hand, an evident and quantifiable level of oxidative stress in AD peripheral cells and fluids still has to be unequivocally demonstrated. At this regard, a recent meta-analysis study reported that peripheral total protein oxidative damage is not a feature of AD [19], while specific oxidized end-products may highlight peculiar redox changes in AD. Accordingly, oxidation of low density lipoproteins was found considerably increased

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in AD as demonstrated by different studies [20-24], likely reflecting a pro-oxidative environment targeting more specifically a lipid fraction rather than a generalized pattern of proteins. No differences in term of oxidative-markers, such as 4-hydroxy-2-nonenal (HNE), 3-NitroTyrosine (3NT) and Protein Carbonyl (PC), were found in immortalized lymphocytes derived from either sporadic AD or healthy subjects [25]. It is worth noting, however, that the same AD lymphocytes showed an increased nitration at the tyrosine residues of the p53 protein that modifies its tertiary structure towards an unfolded conformation, pointing out this modified form of p53 as a useful biomarker of the presence of ongoing oxidative stress [25, 26]. These findings suggest that AD peripheral cells may show fine and specific redox state alterations.

Oxidative stress is widely invoked in conditions ranging from inflammation and aging to complex diseases, and the specificity of the oxidative stress response in a given disease is generally very poorly understood. In addition limited insights into the reciprocal influences between extracellular and intracellular compartment in the redox changes are available.

This study was aimed to clarify the potential usefulness of measuring redox alterations in peripheral blood as potential biomarkers for AD pathology. In particular, we chose to study redox potential alterations by a double approach: i) measuring both the activity of three enzymes: Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx), involved in ROS/RNS progressive detoxification and the amount of unfolded p53 isoform, resulting from peroxynitrite modifications [25], and ii) evaluating both the extracellular and the intracellular blood compartment, to identify which one of them best highlights an altered redox profile in Alzheimer.

2. MATERIALS AND METHODS

2.1. Subjects

43 patients with Mild Cognitive Impairment (MCI), 53 individuals with probable dementia of Alzheimer's type (AD) and 44 cognitively healthy subjects (CHS) were enrolled at the Unit of Neurology of two different Hospitals: Álvarez Buylla Hospital and Cabueñes Hospital of Asturias (Spain). Table **1** reports the demographic and clinical profile of the three groups of participants. Subjects received a diagnosis of probable or possible AD according to NINCDS/ ADRDA criteria [27], whereas MCI diagnosis followed the criteria of Petersen when there was evidence of memory impairment, preservation of general cognitive and functional abilities and absence of diagnosed dementia [28]. Healthy voluntaries recruited from routine controls had to meet the following criteria: 1) no history of past or current psychiatric or neurologic disorders, and 2) a score higher than 26 in the Mini-Mental State Examination (MMSE). All patients underwent neuroimaging and neuropsychological assessment, such as the Folstein version of MMSE. In addition, depending the patient's clinical profile other tests of personalized assessment were performed. None of the subjects studied was taking antioxidant supplements. Also, subjects with acute comorbidities were excluded from the study. The research protocol was approved by the Clinical Research Ethical Committee of Asturias (n. 31/09; 98/10). All patients or their families provided written informed consent for the participation in this study. Blood samples were collected in the morning and centrifuged immediately. Plasma and peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Little Chalfont, United Kingdom), aliquoted and stored at -80°C until measurement.

Table 1. Demographic and clinical profile of the subjects. Demographic and clinical variables and genotype frequency of the ApoE polymorphisms of all the subjects. N: number; M: male; F: female; L.O.I.: length of illness; MMSE: Mini-Mental State Examination. Data are expressed as mean ± standard deviation (SD). For the genotype frequency values are expressed as number (%).

	CHS	MCI	AD
n (M; F)	44 (24; 20)	43 (23; 20)	53 (13 ; 40)
Mean age ± SD (years)	71.02 ± 8.49	75.12 ± 6.36	77.72 ± 7.73
MMSE	29.20 ± 0.90	26.47 ± 2.15	22.57 ± 3.17
CDR	0	0.5	1
Disease onset		71.7±6.49	71.67 ±8.27
L.O.I (months)		24.5 ± 14.36	26.8±13.25
ε2/ε3	ND	0%	0.03%
ε3/ε3	ND	76%	53%
ε3/ε4	ND	16%	26%
ε2/ε4	ND	0%	0%
ε4/ε4	ND	0.03%	13%

Abbreviations: N, number of individuals; M, male; F, female; SD, standard deviation; MMSE, Mini Mental State Examination; CHS, age-matched cognitively healthy subjects; MCI, Mild Cognitive Impairment; AD, Alzheimer's Disease; L.O.I., Length of Illness.

2.2. Superoxide Dismutase Activity

SOD activity was measured following the inhibition of epinephrine oxidation, according to McCord method [29]. 25 µg of PBMC's protein extracts or 10 µl of plasma and 5 µl of epinephrine 0,1 M (Sigma Aldrich, St Louis, MO, USA) were added to a final volume of 200 µl of G buffer (0.05 M glycine and 0.1 M NaCl at pH 10.34), and the reaction was monitored measuring the decrease of absorbance at 480 nm. The activity of purified SOD enzyme (3000 U/mg Sigma Aldrich, St Louis, MO, USA) was also measured in each experiment as a positive control. Data were expressed as Units/ml for plasma and Units/mg of protein using the molar extinction coefficient of 4.02 at 480 nm.

2.3. Catalase Activity

Catalase activity was measured monitoring the decomposition of H_2O_2 according to Shangari and O'Brien [30]. In particular 20 µg of PBMC's protein extracts or 5 µl of plasma were incubated in a final volume of 100 µl of substrate (65µM hydrogen peroxide in 6.0 mM PBS buffer pH 7.4) at 37°C for 60 s. The enzymatic reaction was stopped by the addition of 100 µl of 32.4 mM ammonium molybdate (Sigma Aldrich, St Louis, MO, USA) and measured at 405nm. The results were extrapolated by a standard curve (ranging from 12U/ml to 0,25 U/ml) performed with purified CAT enzyme (20100 U Sigma Aldrich, St Louis, MO, USA), and expressed as Units/ml for plasma and Units/mg of protein for PBMCs.

2.4. Glutathione Peroxidase Activity

The assay was performed in accordance with Awasthi *et al*. [31], measuring NADPH oxidation at 340nm in the reaction that involved oxidation of glutathione reduced (GSH) to glutathione oxidized (GSSG) followed by its reduction by glutathione reductase (GR). 20 µg of PBMC's protein extracts or 5 µl plasma were mixed with a reaction mix (final volume 200 µl) containing 50 mM PBS with 0.4 mM EDTA, pH 7.0; 1.0 mM sodium azide solution; 1.0 mg β – NADPH; 100U/ml GR, 200 mM GSH and 10µl of 0.042% hydrogen peroxide. The activity of purified GPx enzyme (116 U/mg) was also measured in each experiment as positive control. All the reagents were purchased by Sigma Aldrich, St Louis, MO, USA. GPx activity was measured as nmol NADPH oxidized to $NADP^+$/mg protein or ml plasma/min by using the molar extinction coefficient of 0.00622 at 340nm, and the data were expressed as U/ml or U/mg.

2.5. Protein Expression of Antioxidant Enzymes

SOD3, GPX3 and CAT protein amounts were measured in plasma of CHS, MCI and AD groups by using commercial Elisa kit (Cloud-Clone Corp., Houston, USA). Results were extrapolated by standard curves and each samples have been performed at least in triplicate. Data were expressed as pg/ml for SOD3 and as ng/ml for GPX3 and CAT, as suggested by the commercial kit.

2.6. p53 Unfolded Isoform Analysis

p53 unfolded isoform was analysed by direct Enzyme-Linked Immunosorbent Assay (ELISA) using the conformational antibody PAb240 (Neomarkers-Lab Vision,Fremont, CA, USA). This antibody recognizes an epitope masked when the protein is in its native conformation and accessible only when the tertiary structure is unfolded [25, 26, 32, 33]. Briefly, 100 µg of non-denaturated protein extracts, quantified with Bradford protein assay, or control peptide at different concentration ranging from 125 ng to 7,8 ng were coated on a plate overnight at 4°C. Anti-p53 Pab 240 antibody (1:150) was incubated for 2 h at 37°C, followed, after washing, by 1h at RT of secondary anti-mouse antibody conjugated with peroxidase. Finally, 100 µl of TMB (3,3,5,5-tetramethylbenzidine) substrate was added and the reaction was stopped with 100 µl of sulphuric acid 2 M. Optical density (OD) was measured at 450 nm. Results were extrapolated by standard curve and expressed as ng of unfolded p53/100 µg of protein or ng of unfolded p53/ ml.

2.7. Statistical Evaluation

All the assays were performed in triplicate. Data were expressed as the mean value ± 95 % confidence interval (CI) or as the mean value ± S.E.M, according to the experiment. Statistical significance of differences was determined by one way ANOVA, followed by the Bonferroni test. Student's *t*-test was also used to compare values in different groups. For the correlation analysis a parametric method was used and data were analysed by simple linear regression. Differences were considered significant for a p-value <0.05.

3. RESULTS

3.1. Antioxidant Enzymatic Activity in Plasma from AD, MCI and Control Groups

Enzymatic activity of SOD, CAT and GPx was evaluated in the extracellular compartment [ext] derived from 53 AD, 43 MCI and 44 age-matched cognitively healthy subjects (CHS). All the data are reported in Fig. (**1**). When considering SOD and GPx enzymatic activity, both AD and MCI group showed a similar trend when compared to the CHS cohort: SOD[ext] was significantly lower (MCI 251,55 ± 23,49; AD 254,05 ± 20,59 *vs.*. CHS 550,31 ± 33,35 p<0,0001 and p<0,001 respectively) (Fig. **1A**), while GPx[ext] activity was found higher (MCI 232,81 ± 7,4; AD 226,06 ± 8,01 *vs.*. CHS 176,94 ± 8,71 p<0.0001 and p<0.001 respectively) (Fig. **1C**). Differently, CAT[ext] activity resulted lower in the AD plasma than in CHS samples (AD 22,52 ± 1,22 *vs.*. CHS 30,82 ± 1,64 p<0.001), while MCI CAT[ext] values were not significantly different from those of controls (Fig. **1E**). We also evaluated the protein expression of extracellular antioxidant enzymes. In particular SOD3, GPX3 and extracellular CAT protein amounts were measured in the plasma of people from CSH, AD and MCI groups, and no significant difference was found among the three groups (Fig. **2**).

To understand whether gender could affect the activity of extracellular enzymes, male and female sub-groups were also examined. In CHS group, SOD[ext] activity was significantly lower in women (W) compared to men (M) (CHS M 610,3 ± 45 *vs.* CHS W 476,2 ± 45 p <0.05), while GPx[ext] and CAT[ext] activity were not influenced by gender. In MCI and AD groups, no substantial sex-related differences

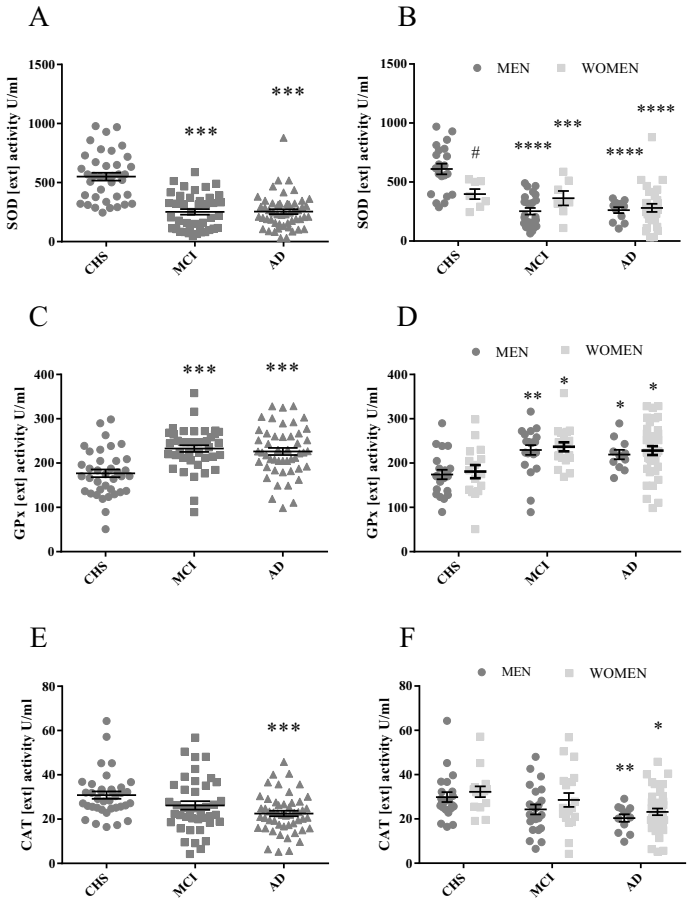

Fig. (1). Antioxidant Enzyme activity in plasma derived from AD, MCI and control groups. The activity of SOD (A), GPx (C) and CAT (E) enzymes were assessed in plasma derived from MCI, AD and CHS as reported in method section. SOD[ext] activity: MCI, AD p<0,0001 *vs*. CHS; GPx[ext] activity : MCI, AD p<0,0001 *vs*. CHS; CAT[ext] activity: AD p<0,0001 *vs.* CHS. SOD (B), GPx (D) and CAT (F) enzymes were also analysed in function of gender (MEN/WOMEN). SOD[ext] activity: MCI men (M) p<0,00001; MCI women (W) p<0.0001 *vs*. respective CHS, AD M and AD W p<0,00001 *vs.* respective CSH; CHS W p<0,05 *vs.* CHS M (#); GPx[ext] activity : MCI M p<0.001and MCI W p<0.05 *vs*. respective CHS; AD M and AD W p<0,05 *vs*. respective CHS; CAT[ext] activity: AD M p<0.001 and AD W p<0,05 *vs*. respective CHS.

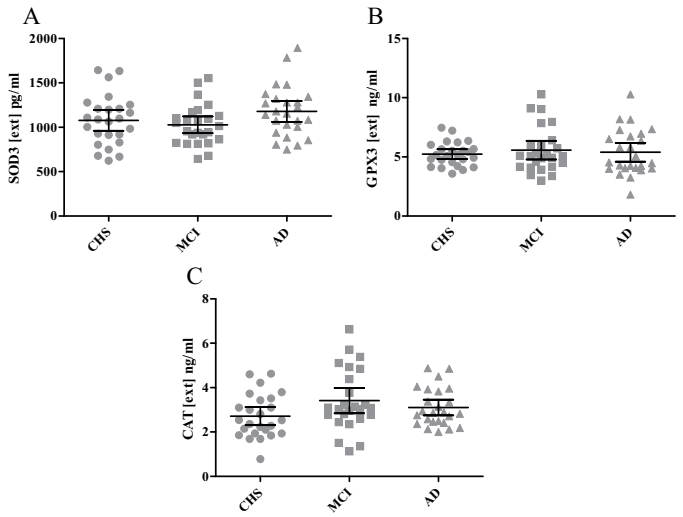

Fig. (2). Superoxide dismutase, catalase and glutathione peroxidase expression.
Antioxidant enzyme analyzed by using sandwich enzyme immunoassays for *in vitro* quantitative measurement of SOD3 (A), GPX3 (B) and extracellular catalase (C) in human plasma derived from MCI, AD and CHS. Results were extrapolated by standard curves and samples have been analyzed at least in triplicate. No differences have been found between the three groups for each enzyme.

were found for SOD [ext], GPx [ext] and CAT[ext] activity when compared with CHS, although GPX[ext] in MCI men reached a higher statistical significance than in women (Fig. **1B**,**D**,**F**). Similarly, CAT[ext] in AD male displayed a lower *p*-value then AD female when compared with CHS. Such differences could be probably due to either the dispersion of values (*i.e.* MCI), or the numerical discrepancy in gender subgroups (*i.e.* AD: 13 M; 40 F).

3.2. Antioxidant Enzymatic Activity in PBMCs from AD, MCI and Control Groups

Antioxidant enzymatic activities were also studied in PBMC extracts [int] derived from AD, MCI and CHS subjects (Fig. **3A**,**C**,**E**) in order to evaluate the possibility that changes in the extracellular redox equilibrium may either induce or be associated to an intracellular redox change in the immune system [34] and this, on turn, might be relevant for the development of Alzheimer's disease. Significant differences were found for SOD[int] activity, which was significantly lower in MCI and AD samples than in CHS, (MCI 26,09 ± 2,16; AD 23,36 ± 2,37 *vs.* CHS 37,44 ± 2,77 $p<0.001$, and $p<0.0001$ respectively) (Fig. **3A**). In contrast, CAT[int] and GPx[int] activities did not significantly change among the three groups (Fig. **3C**, **E**). These results were also analysed as a function of gender. We found that SOD[int] in men MCI showed a significantly lower activity compared to CHS ($p<0.001$), while that in women MCI did not change. Instead, in the AD group, only women SOD[int] exhibited a significant reduction with respect to the analogous CHS, probably due to the greater dispersion of value observed in the male group (Fig. **3B**). No differences were found when gender subdivision was applied to the analysis of GPx[int] and CAT[int] activity (Fig. **3D**,**F**).

3.3. Unfolded p53 in Plasma and PBMCs from AD, MCI and Control Groups

In order to evaluate an early-formed oxidative end-product, we focused on the nitrated isoform of p53 protein, which undergoes to a conformational change due to the steric hindrance of 3-nitrotyrosines and can be detected using conformational specific monoclonal antibodies [25, 26]. Unfolded p53 in plasma and PBMCs derived from AD, MCI and CHS was measured by ELISA immunoassay using PAb240 antibody, that specifically recognizes an epitope that is masked when the protein is in its native conformation, and becomes accessible only when the tertiary structure is un-

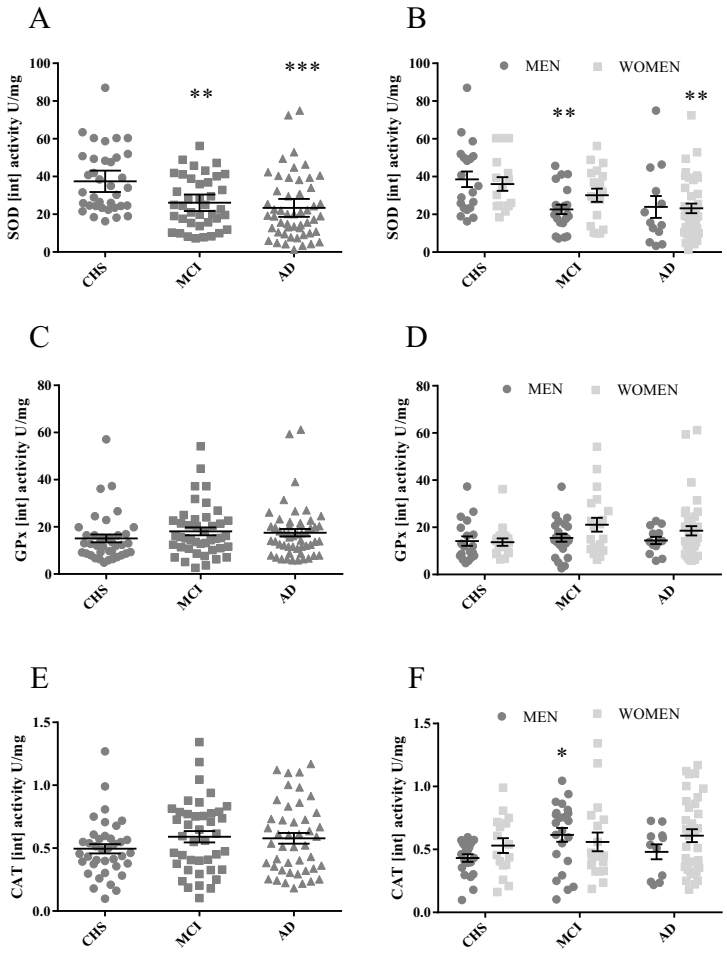

Fig. (3). Antioxidant Enzyme activity in PBMCs derived from AD, MCI and control groups. The activity of SOD (A), GPx (C) and CAT (E) enzymes were assessed in PBMCs derived from MCI, AD and CHS as reported in method section. SOD[int] activity: MCI $p<0,001$ and AD $p<0,0001$ *vs.* CHS respectively. SOD (B), GPx (D) and CAT (F) enzymes were also analysed in function of gender (MEN/WOMEN). SOD[int] activity: MCI M $p<0.001$ *vs.* CHS; AD W $p<0,001$ *vs.* CHS; CAT[int] activity: MCI M $p<0.05$ *vs.* CHS.

folded [32, 33]. As shown in (Fig. **3**), plasma levels of unfolded p53 were increased in both MCI and AD groups as compared to CHS, being anyway higher in samples from AD subjects (unfolded p53[ext]: MCI 5.9 ± 011, AD 6.2 ± 0.13 *vs.* CHS 5.2 ± 0,10 $p<0.001$ and $p<0.0001$ respectively) (Fig. **4A**). However, PBMC levels of unfolded p53 were higher only in the AD group when compared with CHS (Fig. **4C**) (unfolded p53[int]: AD 18.05 ± 0.5 *vs.* CHS 15.2 ± 0,5 $p<0.0001$), confirming our previous results [25, 35-37]. The gender analysis of p53[ext] showed no differences between men and women in MCI. Both sexes showed increased levels of unfolded p53 compared with the corresponding control group ($p<0.05$), although male AD showed higher p53[ext] levels than female AD subjects compared to the analogous CHS group ($p<0.001$) (Fig. **4B**). The male AD group highlighted better also the differences with CHS group in the intracellular compartment (Fig. **4D**). In fact p53[int] showed in the male AD group a higher statistical significance with CHS ($p<0.0001$) than the female one ($p<0.05$).

3.4. Redox Equilibrium Potential, Age and Cognitive Status

We then attempted to highlight a possible relationship between the progression of the disease and the extracellular and intracellular redox equilibrium. Correlation studies between either the antioxidant enzyme activities (Fig. **5**) or unfolded p53 (Fig. **6**) and the cognitive status, measured as MMSE score, or the age, were performed. Among antioxidant enzymes, only SOD activity showed a significant correlation with both the cognitive status and the age of subjects. Both SOD[ext] and SOD[int] activity statistically well correlated with MMSE score (SOD[ext] p=0,0004, r^2=0,09; SOD[int] p=0,0051, r^2= 0,06) (Fig. **5B**,**F**), and the age (SOD[ext] p=0,027, r^2= 0,04; SOD[int] p=0,005, r^2 0,06) (Fig. **5A**,**E**). We also subdivided the cohort of subjects in two specific age intervals (≤75 years and > 75 years), and within these two groups further subdivision by gender was performed. Interestingly in the women CHS group, SOD[ext] activity was lower in the group over 75 years old than in the younger one (Fig. **5C**). As shown in Fig. (**5C**,**D**) the decrease in the activity of SOD[ext] observed in MCI and AD with respect to CHS was statistically more impressive in the younger group than in the older one independently of gender. However, when considering the gender, SOD[ext] activity was significantly reduced in MCI men > 75 years old, but not in the women of the same age. When considering intracellular SOD activity, a significant reduction can be observed in young men MCI and women AD over 75 years old, when compared to their respective CHS group (Fig. **5H**,**G**). This result could be due to the high dispersion of values within the AD younger group and the discrepancy among the number of subjects within each groups (AD ≤ 75 years 16 (9 F; 7M); AD > 75 years 37 (31 F; 6 M)).

We then analysed the possible correlation between unfolded p53 and both the cognitive status and the age of subjects. Only the amount of p53[int] showed a significant inverse correlation with age ($p<0,02$, r^2=0,04) (Fig. **5E**), while unfolded p53[ext] did not correlate when all age is considered (data not shown). However, a statistically significant inverse correlation between unfolded p53[ext] and age was found when a subgroup, represented by younger men (≤75 years) was considered (p=0,05 r^2=0,16) (Fig. **6A**). In particular, in both men MCI and AD ≤75 years, unfolded p53[ext] was found statistically higher when compared with the analogue CHS (Fig. **6D**), while only AD men of this age showed higher amounts of unfolded p53[int] (Fig. **6H**).

The increased expression of unfolded p53 [int] was significantly correlated with dropping of cognitive decline ($p<0,0001$, r^2=0,13) (Fig. **6F**); while no statistical significant correlation was found between MMSE and unfolded p53[ext] when all groups of age were considered (data not shown). However, when the same specific subgroup previously reported (men ≤75 years) was analysed, a good inverse correlation between unfolded p53[ext] and MMSE score was found (p=0,001, r^2=0,28) (Fig. **6B**).

4. DISCUSSION

This study identified peculiar alterations in the antioxidant defence systems regulating the extracellular and intracellular redox state in the context of cognitive decline diseases. In particular, it highlighted significant differences between the extracellular and the intracellular blood compartments in AD and MCI patients when compared to healthy subjects. In the extracellular compartment, the antioxidant cascade, involving SOD, CAT and GPx enzymes, was found impaired in both AD and MCI. We observed a drastic reduction in SOD activity and a significant increase in GPx activity in both groups of patients. Also a significant reduction in CAT enzymatic activity was found in the AD group and a trend of decrease in CAT activity was also found in MCI plasma samples, although no statistically significant. These three investigated antioxidant activities work in concert to prevent an excess of ROS/RNS production: SOD catalyses the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (H_20_2), which is further degraded by CAT and GPx to water. It is well established that ROS/RNS can affect SOD enzyme and compromise its activity [38-40]. Thus, the decreased activity of SOD[ext] (also known as SOD3 to distinguish it from cytoplasmic SOD1 and mitochondrial SOD2), can be explained as the result of a long lasting exposure to pro-oxidative environment. In fact, no differences were found in term of SOD3 expression between the three groups (Fig. **2**). These data are in good agreement with those reported by Padurariu *et al*. [41], although other authors did not find differences in the SOD activity in plasma from AD patients [19, 42]. Interestingly, the female control group had a lower SOD3 activity than men, that further decreased with the age (CHS W > 75 years *vs.* CHS W ≤75 years); while no differences in term of gender and age where found in the AD and MCI groups. In this regard, Bellanti *et al*. [43], demonstrated that estrogens influenced circulating redox balance by affecting cellular antioxidant enzyme system. In particular the authors demonstrated in women a linear relationship between the circulating estrogen levels and SOD activity.

Although CAT and GPx catalyze the same reactions, their pathophysiological role is quite different. GPx is recognized to be the major enzyme for H_2O_2 detoxification under normal conditions [44]. Since no differences were found in the amount of GPX3 protein (Fig. **2**) among the three groups,

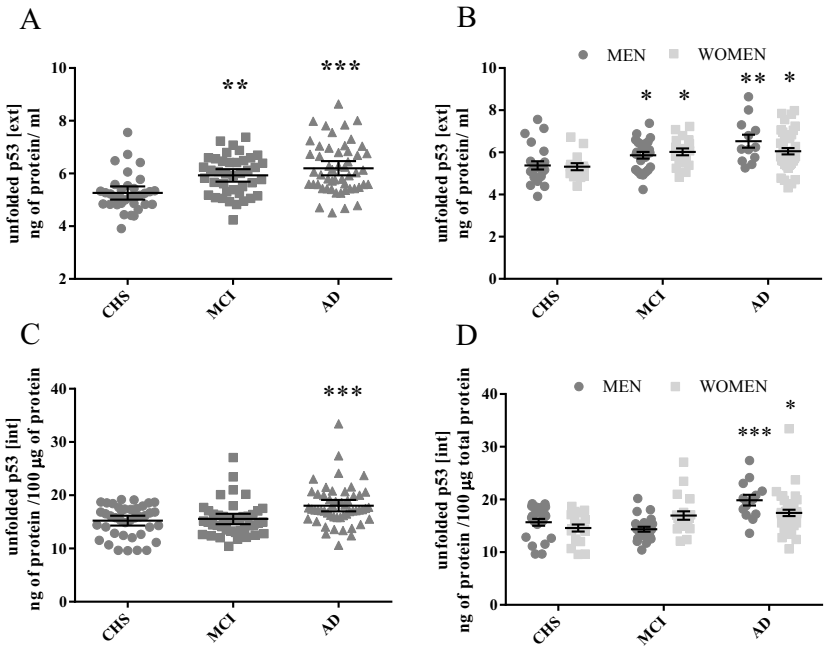

Fig. (4). Unfolded p53 in plasma and PBMCs derived from AD, MCI and control groups. Unfolded p53 amount were measured in plasma (A) and PBMCs (C) derived from MCI, AD and CHS as reported in method section. Unfolded p53[ext]: MCI p< 0,001 and AD p<0,0001 *vs.* CHS respectively; unfolded p53[int]: AD p<0,0001 *vs.* CHS. Data analysed in function of gender (MEN/WOMEN) of p53[ext] (B) and p53[int] (D). Unfolded p53[ext]: MCI M and MCI W p<0,05 *vs.* respective CHS; AD M p<0,001and AD W p<0.05 *vs.* respective CHS; unfolded p53[int]: AD M p<0.0001 and AD W p<0,05 *vs.* CHS.

the increase of its activity observed in AD and MCI subjects could be interpreted as an adaptive attempt to counteract an excess of H_20_2 and/or $ONOO^-$ in plasma. Interestingly, Sies *et al.* [45], demonstrated that exquisitely the extracellular GPx isoform (one of the eight different GPx isoforms, also known as GPx3), is able to reduce peroxynitrite ($ONOO^-$). Differently, catalase plays a significant role in protecting cells against severe oxidative stress [46]. Having one of the highest rates of turnover known, this enzyme acts counteracting and balancing the continual production of hydrogen peroxide. The decrease in the extracellular catalase activity (which is one of the 5 different CAT isoforms) observed only in AD subjects would be consistent with an impairment in these patients in the removal of H_2O_2 that in turn could lead to a microenvironmental redox state perturbation. Our results are consistent with those previously reported by Puertas *et al.* [42], who also observed a significant decreased CAT activity in AD subjects, although in that study was also reported a decreased GPx activity. On the other hand, other studies showed either an increase or no changes of CAT and GPx activity in AD [6, 47-49], indicating the existence of controversy on this point.

When considering the intracellular compartment, SOD (SOD1, SOD2), but not GPx or CAT activity, was significantly reduced in PBMCs of both AD and MCI patients. This result confirm our previous findings in immortalized lymphocytes derived from familiar and sporadic AD [25], and suggest SOD activity impairment to be an early event in the development of the disease.

The differences between the extracellular and the intracellular blood compartments observed in this study can be explained, at least partially, taking into account that each compartment is endowed with its own antioxidant enzyme isoforms, encoded by different genes [50], and that the two compartments can be differently perturbed by endogenous and/or exogenous oxidants.

The extracellular and intracellular redox equilibrium influences the structure, stability, and function of macromolecules that reside within each compartment. Among them, both p53 protein structure and activity have been suggested to be particularly affected by the redox state [26, 32]. We have previously demonstrated a positive correlation between SOD activity and nitrated-p53 isoform in immortalized B-lymphocytes derived from AD and from control subjects [25]. Here we have found that a high amount of unfolded p53 isoform occurs in both MCI and AD plasma, while in the intracellular compartment only AD showed increased unfolded p53 levels. It is noteworthy that the major differences of extracellular and intracellular unfolded p53 observed in AD subjects, compared with the CHS group, were found in male subjects under 75 years old. This enhancement in AD might be due to a progressively higher pro-oxidant intracellular environment that favors unfolded p53 formation. However, we cannot exclude the influence of a severely compromised extracellular redox state (as observed in AD) since both compartments may influence each other in regulating and maintaining the redox steady-state and in modulating the redox signaling or perturbing normal cellular processes [34]. In this regard Vignini *et al.* [51], demonstrated that an increased nitrosative stress occured in AD compared with matched control subjects, and interestingly, AD males showed a higher peroxynitrite (ONOO-) production (25% more than AD women) that leaded to protein nitration.

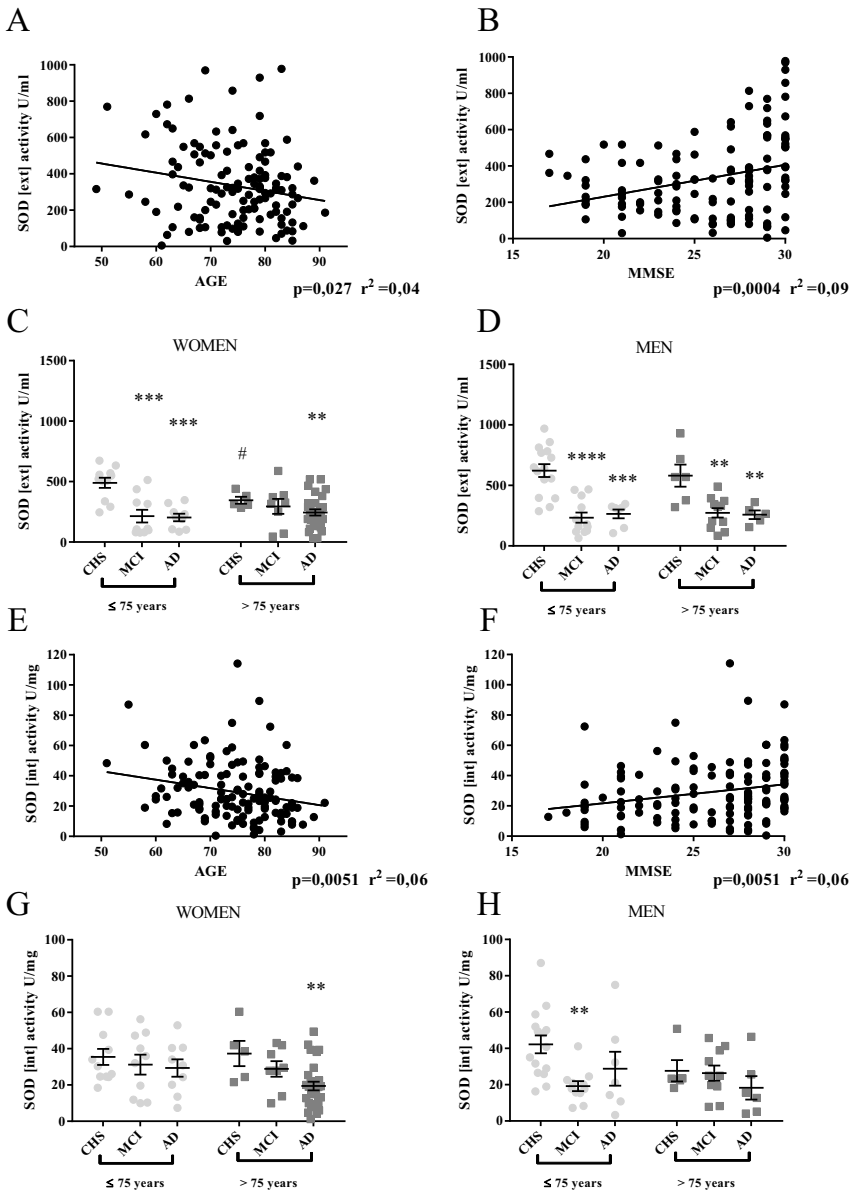

Fig. (5). Correlation among SOD activity with cognitive decline or age. The values of SOD[ext] activity (A,B) and SOD[int] (E,F) of all samples (MCI, AD and CHS) were correlated with the age (A,E) and with the corresponding values of MMSE score (B-F). SOD[ext]/AGE p=0.027 r^2=0.04 (y=-5.03x+708.3); SOD[ext]/MMSE p=0.0004 r^2=0.09 (y=17.59x-121.3); SOD[int]/AGE p=0.0051 r^2=0.06 (y=-0.56x+71.01); SOD[int]/MMSE p=0.0051 r^2=0.06 (y=1.25x-3.37). Data analysed in function of gender and two specific age interval (≤75 years and > 75 years). SOD[ext] ≤75: MCI (M/W p<0,00001/p<0.0001), AD (M/W p<0,0001) *vs.* CHS; SOD[ext]>75: MCI (M p<0,001), AD (M/W p<0,001) *vs.* CHS (C-D); CHS W ≤75 p<0,05 *vs.* CHS W >75 (#); SOD[int]≤75: MCI (M p<0,001) *vs.* CHS; SOD[int]>75 AD (W p<0,001) *vs.* CHS (G-H).

Whether or not the intracellular nitrated unfolded p53 could constitute a useful biomarker to discriminate between the early and late stage of the disease will be further demonstrated with an ongoing follow up study on the MCI group.

To further understand which of the examined parameters could better characterize the AD- related blood changes, the antioxidant enzyme activities as well as the amount of unfolded p53 were correlated with both cognitive decline and the age of enrolled subjects. Among antioxidant enzymes, only SOD activity correlated with either MMSE score and age both extra and intra-cellularly. Also unfolded p53[int] inversely correlated with MMSE and age. On the other hand a significant positive correlation between MMSE and age with blood circulating unfolded p53 was found only for male subjects under 75 years old. Therefore, the nitrated open p53 isoform and impaired SOD activity might be considered likely early biomarkers of peripheral oxidative stress occurring in AD.

CONCLUSION

The complexity of Alzheimer's disease (AD) and its long prodromal phase are challenges for early diagnosis. However, it is worth noting the importance to study biomarkers, in particular, in the preclinical and early phases reflecting the

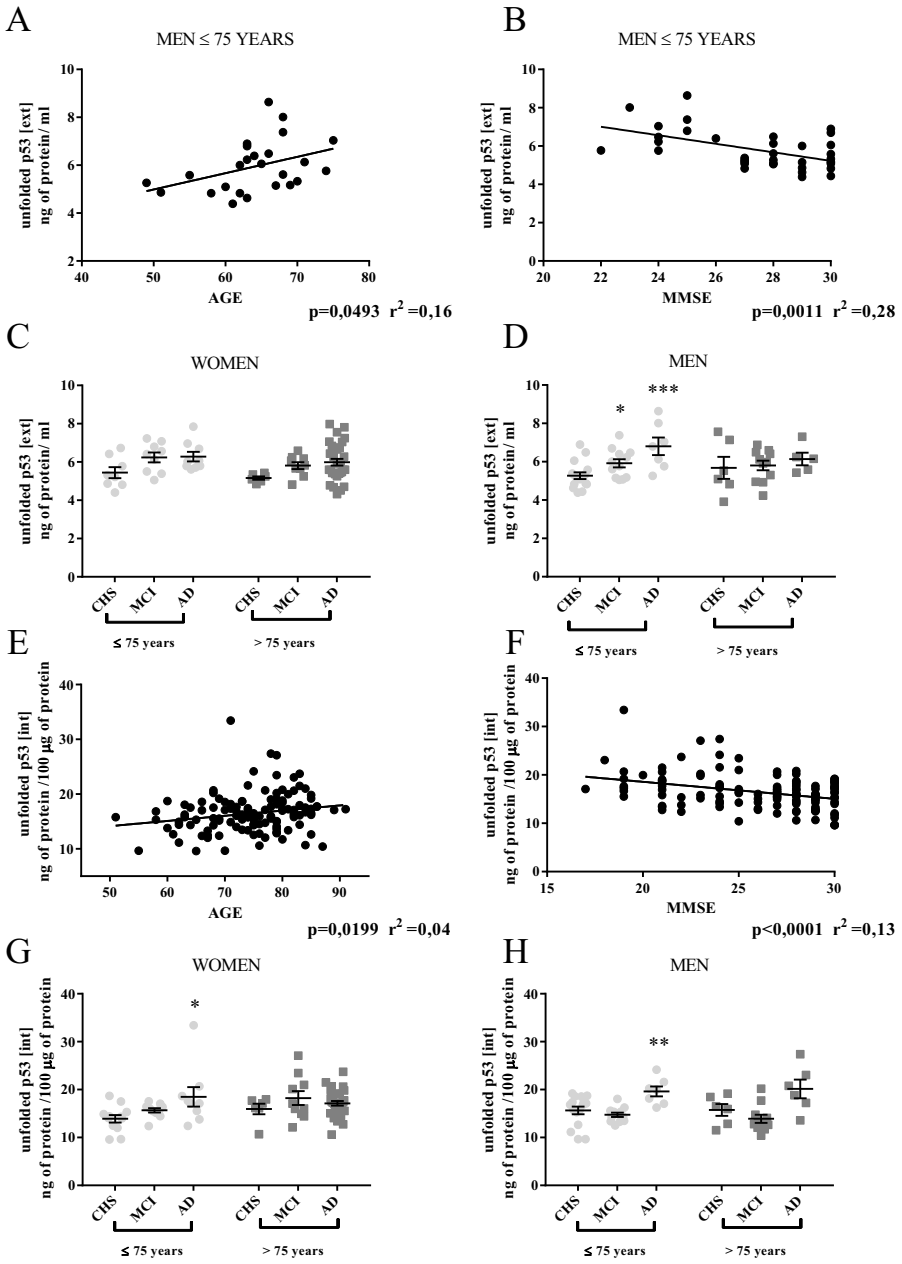

Fig. (6). Correlation among unfolded p53 with cognitive decline or age. The values of unfolded p53[ext] (A,B) and unfolded p53[int] (E,F) of all samples (MCI, AD and CHS) were correlated with the age (A,E) and with the corresponding values of MMSE score (B,F). Unfolded p53 M≤75 [ext]/AGE p=0.05 r^2=0.16 (y=0.07x+1.574); unfolded p53 M≤75 [ext]/MMSE p=0.0011 r^2=0.28 (y=-0.22x+11.91); unfolded p53[int]/AGE p=0.02 r^2=0.04 (y=0.084x+10.14); unfolded p53[int]/MMSE p<0.0001 r^2=0.13 (y=-0.29x+23.95). Data analysed in function of gender and two specific age interval (≤75 years and > 75 years). Unfolded p53[ext] ≤75: MCI,AD (M) p<0.05, p<0.0001 *vs.* CHS (C-D); unfolded p53[int]≤75: ADI (M/W p<0,001/p<0.05) *vs.* CHS (G-H).

pathological features of the disease. Blood-based biomarkers potentially provide a minimally invasive approach for this purpose. With regard to this topic, our data underscore the importance of studying both extracellular (plasma) and intracellular (PBMC) blood compartments in term of antioxidant enzymatic activities, as well as specific oxidation end-products, in order to identify peculiar redox alterations representative of dynamic events occurring in cognitive decline diseases.

LIST OF ABBREVIATIONS

AD	=	Alzheimer's Disease
CAT	=	Catalase
CHS	=	cognitively healthy subjects
CSF	=	cerebrospinal fluid
GPx	=	Glutathione Peroxidase
MCI	=	Mild Cognitive Impairment

MMSE = Mini-Mental State Examination

SOD = Superoxide Dismutase

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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