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Peripheral levels of glutathione and protein oxidation as markers in the development of Alzheimer's disease from Mild Cognitive Impairment

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Abstract

There is a great interest in the relationship between Mild Cognitive Impairment (MCI) and the progression to Alzheimer's disease (AD). Several studies show the importance of oxidative stress in the pathogenesis of AD. The purpose of this study was the link between oxidative damage, MCI and AD. It analysed protein carbonyls and erythrocyte glutathione system plasma levels of 34 subjects with MCI, 45 subjects with AD and 28 age-matched control subjects. The results showed an increase in protein modification, a decrease in GSH levels and GSH/GSSG ratio in AD and MCI patients compared to age-matched control subjects ($p < 0.05$). The present study shows that some peripheral markers of oxidative stress appear in MCI with a similar pattern to that observed in AD, which suggests that oxidative stress might represent a signal of the AD pathology. AD and MCI are biochemically equivalent. MCI does not necessarily need to progress to AD on a biochemical level.

Keywords: *Oxidative stress, Alzheimer's disease, Mild Cognitive Impairment, glutathione, protein carbonyls*

Introduction

Alzheimer's disease (AD) has a multifactorial pathogenesis and the challenge is to diagnose it early. A large amount of evidence shows that the risk of Mild Cognitive Impairment (MCI) patients' developing AD increases every year after the diagnosis. Some experts believe that the risk of MCI patients' progressing to AD is 10-times greater. Researchers are interested in understanding the relationship between MCI and the progression to the early stage of AD and whether MCI is an initial stage of AD or not [1].

MCI is a syndrome defined as a cognitive decline which is greater than that expected for an individual's age and education level but that does not interfere notably with activities of daily life and no conventional signs of dementia. It is, thus, distinct from dementia, in which cognitive deficits are more severe and widespread and have a substantial effect on daily functions [2]. AD is recognized as the most common form of dementia and is clinically associated with cognitive impairment, loss of language and motor skills and changes in behaviour [3]. AD is characterized by neuronal degeneration and by a loss of cholinergic neurons in the median forebrain [4]. Most of the MCI subjects will progress to dementia, especially Alzheimer's disease (AD), at a rate of

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10–15% per year, as compared to the normal ageing who converts at a rate of 1–2% per year [5]. Therefore, a better understanding of the similarities and differences between MCI and AD subjects is becoming increasingly important in the interest of developing early diagnostic and treatment methods for AD.

Reactive oxygen species (ROS) are constantly being produced in cells through normal metabolic processes. Oxidative stress occurs when the balance of oxidants within the cell exceeds the levels of antioxidants present. An increased level of ROS can lead to the damage of macromolecules within the cell; it is this damage to lipids, proteins and DNA that can rise to pathological consequences. There exists convincing evidence that oxidative stress and ROS play an important role in the aetiology and/or progression of a number of human diseases [6–12].

Several studies show the importance of oxidative stress in the pathogenesis of AD that means an imbalance between the formation and spread of ROS and antioxidant defences [13]. Protein oxidation is indexed in the AD brain by an increase in modified proteins. The initial origin of AD pathogenesis has not been determined though it has become evident that oxidative stress is implicated in the development of this disease. Studies have shown an increase in protein carbonyls in the hippocampus and parietal cortex, but not in the cerebellum, where there is less significant AD pathology [14]. In this context, it has been suggested that the levels of carbonyl reductase (CR), an enzyme that reduces a wide variety of quinones and aldehydes, are elevated in AD. CR is present in the cerebellum and medulla neurons, but whether it is present in the cerebral cortex or hippocampus is unknown [15]. Besides, protein oxidation occurs in the AD brain in relation to the histopathology of the disease, i.e. protein oxidation occurs in brain regions rich in β-amyloid peptide (hippocampus, inferior parietal lobule), but not in β-amyloid peptide-poor cerebellum [16].

The accumulation of oxidatively modified proteins disrupts cellular function either by a loss of catalytic ability or by an interruption of regulatory pathways and may reflect deficiencies in one or more parameters of a complex function that maintains a balance between the presence of a multiplicity of pro-oxidants, antioxidants and the repair, replacement or elimination of biologically-damaged proteins [17]. One of the greatest challenges in oxidation research today is the determination of oxidative stress *in vivo*. Because proteins are ubiquitous in all cells and tissues and are susceptible to oxidative modification, they can serve as useful markers of oxidative stress [18].

Glutathione (GSH) plays a key role as an essential cellular antioxidant in the defence of brain cells against oxidative damage induced by ROS. High intracellular GSH levels protect cells from ROS insults by non-enzymatically reacting with ROS. GSH is also able to enzymatically react with peroxides and to conjugate with oxidized products. GSH reacts with the oxidant hydrogen peroxide (H_2O_2) catalysed by glutathione peroxidase (GPx) and converts it to H_2O. During this detoxification, GSH is oxidized to glutathione disulphide (GSSG). GSSG derived from GPx is then reduced to GSH by the reaction catalysed by the glutathione reductase (GR) [19]. Under physiological conditions the reduced form of glutathione is 10–100-fold higher than it is in the oxidized form. The effects of oxidative stress in GSH/GSSG ratio tend to decrease by raising the concentration of oxidized glutathione or decreasing the reduced glutathione amount. However, in response to oxidative stress, cells maintain their glutathione redox state through different mechanisms. GR activity should be increased in order to eliminate the excess GSSG formed by oxidative stress. Only when oxidative stress becomes prolonged and cellular systems are no longer able to counteract the ROS-mediated insults does the amount of free GSH decrease, leading to irreversible cell degeneration and death [20]. GSH in the nucleus maintains the redox state of critical protein sulphydryls that is necessary for DNA repair and expression. Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism. Too high a concentration of GSSG may damage many enzymes oxidatively [21].

The aim of our study was to investigate the pattern of different parameters of protein damage as well as redox status (GSH/GSSG) in the MCI, AD patients and aged-matched healthy individuals. This study was conducted in order to test the hypothesis that elevated levels of protein oxidation and decreased levels of GSH are present in the peripheral blood of individuals with MCI, which would imply that free radical-mediated lipid and protein oxidation are an early event in the progression to AD from MCI.

On the basis of these studies, suggesting that oxidative imbalance may help to understand whether MCI is an early clinical stage of AD and/or whether a common pathogenesis between AD and MCI occurs, plasma levels of protein carbonyls and erythrocyte glutathione system were evaluated in peripheral blood specimens from patients with AD and MCI and compared with those from aged-matched healthy subjects.

Experimental procedures

Subjects

The present study was reviewed and approved by the Ethical Committee of the Hospital Universitario Clinico San Carlos of Madrid (Spain). It was carried out in patients with MCI ($n = 34$; mean age 76.6 ± 1.4 years) and free-living patients with Alzheimer's

disease ($n = 45$; mean age 79.7 ± 0.9 years), admitted to the Geriatrics Department (Hospital Universitario Clínico San Carlos, Madrid). Both groups of patients were compared to the healthy elderly individuals ($n = 28$; mean age 80.4 ± 2.0 years) admitted to the hospital for a routine evaluation of their health status. MCI diagnosis followed the criteria of Petersen et al. [5] when there was evidence of memory impairment, preservation of general cognitive and functional abilities and absence of diagnosed dementia. Clinical diagnosis of AD was ascertained by means of exhaustive medical, neurological, psychiatric and neuropsychological examinations. AD patients fulfilled criteria for probable AD according to the guidelines of the National Institute of Communicative Disorders and Stroke and the AD and Related Disorders Association, NINCDS-ADRDA [22]. All participants included in the study underwent the following cognitive and medical evaluations: (1) the Tinetti Assessment Tool—a simple, easily administered test that measures a patient's gait and balance [23]; (2) the Zarit Burden interview (ZBI)—an instrument for assessing the stress experienced by caregivers of elderly patients with dementia [24]; (3) Cognitive status—screened by the Mini-Mental State Examination (MMSE) [25]; (4) the Cambridge Cognitive Examination (CAMCOG) [26]; (5) memory was evaluated by Wechsler Memory Scale (WMS) and Rivermead Behavioral Memory Test (RBMT) [27]; (6) Neuropsychiatric Inventory (NPI)—to assess 10 behavioural disturbances occurring in dementia patients which were delusions, hallucinations, dysphoria, anxiety, agitation/aggression, euphoria, disinhibition, irritability/lability, apathy and aberrant motor activity [28].

For the patients without cognitive impairment, short evaluation of depression by the observational 'Scale and Geriatric Depression Scale of Yesavage' was carried out [29]. For the patients with dementia, the Cornell-Brown Scale for Quality of Life in dementia was performed [30].

The individuals with chronic disease and/or who were under drug treatment were excluded from the study. Informed consent was obtained from all participants or their caregivers.

Blood collection

Blood samples were taken from patients after an overnight fast. Blood was collected from the cubital vein (right arm) between 9:00–10:00 h and placed in 15 ml vacutainers that contained 0.2 ml of 5% EDTA as an anti-coagulant and as an antioxidant. Haemolysed samples were discarded. Blood was centrifuged at 800 *g* for 10 min and the plasma was removed. Erythrocytes were washed twice with an equal volume of 0.9% NaCl and then centrifuged for 10 min at 800 *g*. Plasma and pelletted red blood cells (RBC) were stored frozen at -80°C until analysis.

Quantification of protein carbonyl group

Plasma protein carbonyl group levels were evaluated following derivatization of the carbonyl group with dinitrophenylhydrazine (DNPH) assay, which leads to the formation of a stable dinitrophenyl hydrazone according to the method of Levine et al. [31]. Briefly, the plasma protein was precipitated by the addition of TCA and suspended in 2 M HCl containing 2% DNPH. After incubation for 1 h at 37°C, protein samples were washed with ethanol and ethyl acetate and precipitated with TCA. The precipitated protein was dissolved in 6 M guanidine hydrochloride solution and the optical density was read at 370 nm. Calculations were made using the molar extinction coefficient of 22 000 M^{-1} cm^{-1} and results were expressed as nmol carbonyls per milligram of protein. Protein determinations were performed according to the bicinchonic acid method [32].

Glutathione content

Erythrocyte glutathione levels were determined fluorimetrically according to Senft et al.'s [33] method and suitably modified. Briefly, erythrocytes were suspended in 2.5 ml of 0.1 M phosphate buffer (pH 8) containing 5 mM EDTA. Aliquots (1.5 ml) of the cell suspension were collected, deproteinized with 350 µl of a 25% solution of metaphosphoric acid, homogenized by gentle sonication at 0°C and then centrifuged for 10 min at 14 000 g. Aliquots (0.7 ml) of the supernatant were added to 3.3 ml of phosphate-EDTA buffer and then vortexed and 500 µl of this solution was transferred to the reaction mixture constituted by 4.4 ml of phosphate-EDTA buffer and 100 µl of ortho-phthalaldehyde (OPA, 1 mg/ml in methanol). After incubation at 25°C for 30 min, fluorescence was measured at $\lambda_{exc} = 350$ nm (band width 5 nm) and $\lambda_{em} = 420$ nm (band width 5 nm). Values were expressed as nmol/g haemoglobin. Haemoglobin levels were determined by the Drabkin assay [34].

Glutathione peroxidase (GPx) and glutathione reductase (GR) determination

Glutathione peroxidase (E.C. 1. 11. 1. 9). Activity was measured according to the Paglia and Valentine [35] method. GPx catalyses the oxidation of the 4 mM GSH by 0.68 mM cumene hydroperoxide in 50 mM phosphate buffer, pH 7.4, including 4 mM sodium azide (in order to inhibit catalase activity). The oxidized glutathione produced is converted to its reduced form in the presence of 0.22 mM NADPH, 1 mM EDTA and 0.702 IU GR. The concomitant oxidation of NADPH is followed by the decrease in

absorbance at 340 nm and 25°C. One enzyme unit is defined as the oxidation of 1 μmol NADPH per minute under assay conditions. The enzyme activity was expressed as units/g Hb (1 unit = 1 μmol of NADPH oxidized min^{-1} calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}).

Glutathione reductase (E.C. 1. 6. 4. 2). Activity was determined as described elsewhere [36]. The final concentrations of the reagents used in the assay were 50 mM phosphate buffer, pH 7.4, including 6.3 mM EDTA, 4 mM GSSG and 0.3 mM NADPH. The activity was measured by monitoring the oxidation of NADPH to $NADP^+$ during the reduction of oxidized glutathione (GSSG) by the decrease in absorbance at 340 nm and 25°C. The enzyme activity was expressed as units/g Hb (1 unit = 1 μmol of NADPH oxidized min^{-1} calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}).

The activities of GR and GPx were expressed as nmol NADPH/min g haemoglobin. The amount of haemoglobin in RBC, used to standardize the activities of enzymes, was measured by the Drabkin method [34].

Statistical analysis

Statistical analysis was performed using the one-way ANOVA design and the Newman Keuls test in the SPSS package program. All values were expressed as means ± standard error of the mean (SEM). Correlation coefficients were calculated using Pearson's procedure and *p*-values less than 0.05 were considered statistically significant.

Results

Figure 1 shows that carbonyl group measurements were significantly increased from 0.45 to 0.61 nmol/mg protein (35.60%) ($p < 0.05$) in the plasma of the MCI patients and to 0.66 nmol/mg protein (46.67%) ($p < 0.05$) in the Alzheimer's group vs the control group. No significant differences were found between the MCI and AD groups. These results indicate that protein damage is stimulated in mild cognitive impairment and in Alzheimer's disease.

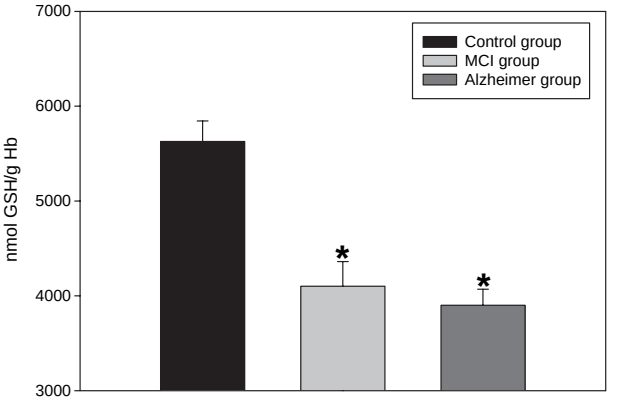

Figure 2. GSH levels in erythrocytes from control, MCI and Alzheimer's patients. Data represent the mean ± SEM. Differences from control, * $p < 0.05$ (Newman Keuls test).

As for the cellular redox status, the results showed a significant decrease in the content of reduced glutathione in erythrocytes from elderly patients with MCI and patients diagnosed with AD vs the control group ($p < 0.05$) (Figure 2). The reduction in GSH was similar in the AD group and the MCI group. However, there were no significant changes in the oxidized form of the glutathione between groups (Figure 3).

The GSH/GSSG ratio decreased significantly in the elderly patients with MCI and the patients diagnosed with AD vs the control group ($p < 0.05$) (Figure 4).

An inverse correlation was found between oxidized glutathione levels and GSH/GSSG ratio in the three groups and a positive correlation between reduced glutathione levels and GSH/GSSG ratio was only

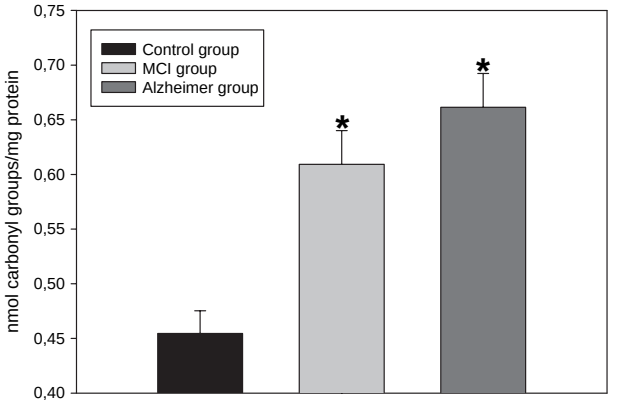

Figure 1. Plasma carbonyl groups from control, MCI and Alzheimer's patients. Data represent the mean ± SEM. Differences from control, * $p < 0.05$ (Newman Keuls test).

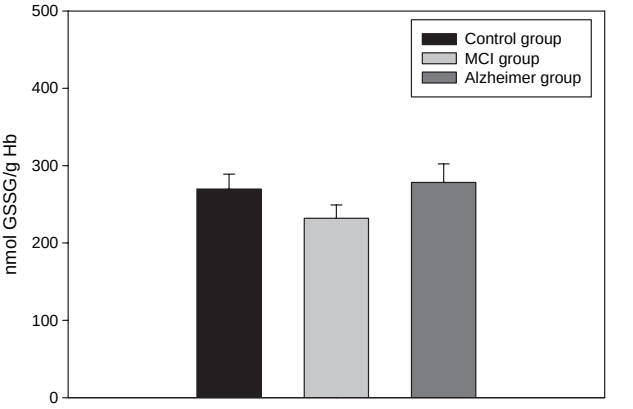

Figure 3. GSSG levels in erythrocytes from control, MCI and Alzheimer's patients. Data represent the mean ± SEM.

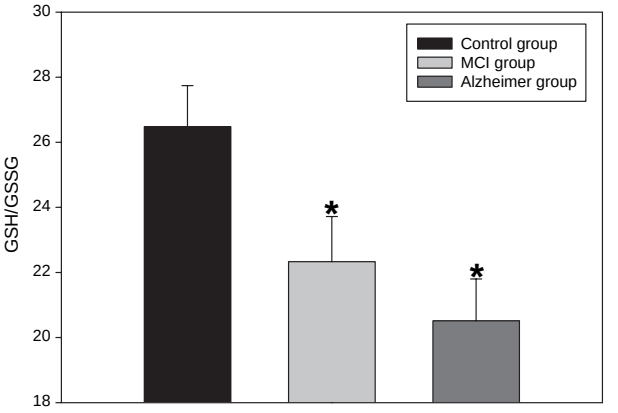

Figure 4. GSH/GSSG ratio in erythrocytes from controls, MCI and Alzheimer's patients. Data represent the mean ± SEM. Differences from control, * $p < 0.05$ (Newman Keuls test).

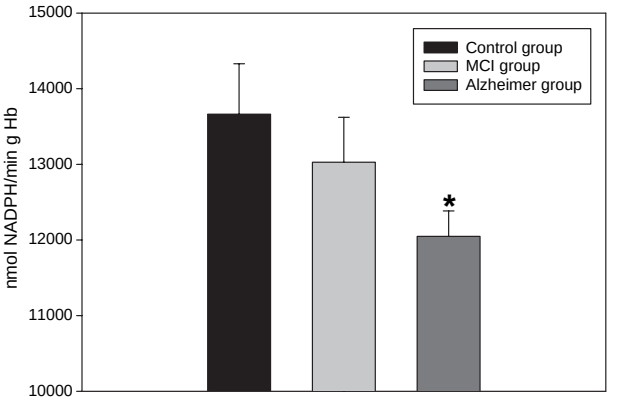

Figure 5. Glutathione peroxidase activity in erythrocytes from control, MCI and Alzheimer's patients. Data represent the mean ± SEM. Differences from control, * $p < 0.05$ (Newman Keuls test).

found in the MCI group, as shown by the Pearson's correlation coefficient (Table I).

Besides this, the results showed a decrease in the GPx activity in erythrocytes for elderly patients with MCI and patients diagnosed with AD vs the control group. This decrease was only significant in the AD patients ($p < 0.05$) (Figure 5). However, there were no significant changes in the GR activity of AD and MCI patients in comparison with the control group (Figure 6).

Discussion

Mild cognitive impairment (MCI) can be defined as an isolated deficit in recent memory [37,38] and might be considered an intermediate phase between normal ageing and Alzheimer's disease. Some researchers believe that MCI is in fact the earliest form of AD [1]. Many studies support the hypothesis that oxidative stress plays an important role in the pathogenesis of AD [39]. Cerebral tissue appears to be particularly vulnerable to free radical damage because of its low content of antioxidants, high content of polyunsaturated fatty acids of neuronal membranes and high oxygen requirements for its metabolism. Peripheral markers have been used to assess biochemical alterations associated with AD and MCI involved in its pathophysiology. For example, increased levels of 4-hydroxynonenal and F2-isoprostane have been found in cerebrospinal fluid and plasma of AD patients [40]. Therefore, the measurement of peripheral antioxidants is considered to be an appropriate way to look at oxidative stress [41–43].

The present study was conducted to evaluate peripheral indicators of oxidative stress in elderly subjects with MCI, patients with AD and normal elderly subjects for comparison. Blood is an important pool of antioxidant defences in the body and is also an easily available source for the study of the oxidant–antioxidant imbalance. In this study, the modification of several components of the antioxidant defence system of the organism in relation to the presence of MCI is shown.

Proteins are targets for ROS and the amount of protein modification has been reported to be correlated with the degree of oxidative stress. Protein modifications induced by oxidative stress include production of carbonyls which provides evidence for general oxidative modification of proteins. These moieties are chemically stable, which is useful for both their detection and storage [44]. Protein carbonyl content is actually the most commonly used marker of protein oxidation and the accumulation of protein carbonyls has been observed in several human diseases including diabetes, inflammatory bowel disease and arthritis [45].

Table I. Pearson correlation coefficient for GSSG vs GSH/GSSG ratio.

	GSSG vs GSH/GSSG ratio		GSH vs GSH/GSSG ratio	
	r	*p*	*r*	*p*
Control group	−0.743	<0.000 174*	0.281	<0.231
MCI group	−0.576	<0.000 568*	0.616	<0.000 177*
Alzheimer's group	−0.682	<0.000 000 341*	0.241	<0.115

*Significant correlation ($p < 0.05$).

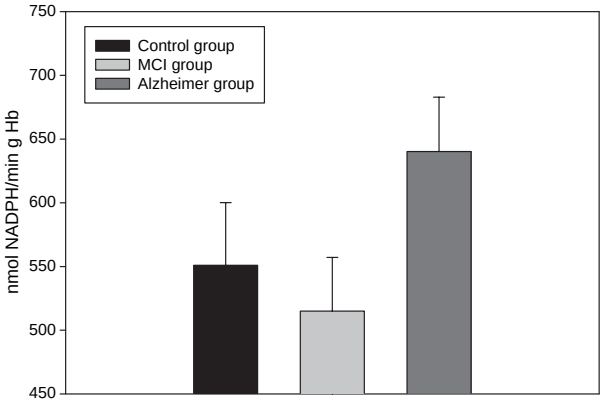

Figure 6. Glutathione reductase activity in erythrocytes from control, MCI and Alzheimer's patients. Data represent the mean ± SEM.

The usage of protein carbonyl groups as a marker may have some advantages in comparison with lipid peroxidation products because the formation of protein-bound CO groups seems to be a common phenomenon of protein oxidation and because of the relatively early formation and relative stability of oxidized proteins. It is known that cells degrade oxidized proteins within hours and days, whereas lipid peroxidation products are detoxified within minutes. Interestingly enough, the protein CO groups form early and circulate for longer periods in the patient's blood compared with other parameters of oxidative stress, such as malondialdehyde [44].

Previous studies have shown elevated levels of carbonyl species in different regions of the patient's brains with AD [15]. Moreover, an increase in protein carbonyls in the hippocampus and parietal cortex have been found, where the AD pathology is more evident [14].

We found significant elevated levels of protein carbonyls in the plasma from patients with AD, in comparison with the aged-healthy subjects. Moreover, we observed a similar increase (statistically significant) in this parameter for the MCI group. Our findings are in agreement with further studies which have demonstrated that protein oxidation levels were significantly high in plasma from AD subjects [46–48]. The proteins which were found oxidized have been suggested to be involved in inflammation processes in Alzheimer's disease. The authors suggest that these oxidized proteins in plasma may be useful as diagnostic biomarkers for AD [46].

The accumulation of oxidative modified proteins may reflect deficiencies in one or more parameters of the complex system that maintains the balance between the pro-oxidants and antioxidants and the elimination of biologically damaged proteins. We found that protein carbonyl contents were increased in MCI and AD groups vs control group, especially in the AD group with the highest increased percentage. This accumulation could be due to an excess of free radical generation or other oxidant species.

The cellular redox status depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG) and appears to be a critical determinant in cells. Under normal conditions, the reduced form predominates over the oxidized form. The balance of GSH and GSSG provides a dynamic indicator of oxidative stress *in vivo* [49]. In addition, GSH is involved in a number of other essential tasks including DNA synthesis and repair, protein synthesis, amino acid transport, enhancement of immune function and enzyme activation. Specific roles to the nervous system appear to include actions as a redox modulator of some ionotropic receptor currents and as a potential neurotransmitter. Due to such multiple roles in normal tissue, there is a considerable potential for alterations in GSH to be causally associated with the disease. It has been well established that a decrease of GSH concentration may be associated with ageing and the pathogenesis of many diseases, including Parkinson's disease, HIV, liver disease, cystic fibrosis and ageing [50]. Because blood glutathione concentrations may reflect glutathione status in other less-accessible tissues, measurements of both GSH and GSSG in blood have been considered essential as an index of glutathione status and a useful indicator of disease risk in humans [21].

The variations of glutathione, as an antioxidant, can be expressed in two ways: as a function of GSH concentration and as a function of the redox state of the GSH/GSSG ratio. In the present study, we observed significantly lower and close values of GSH in MCI and AD patients in comparison with controls ($p < 0.05$) and no significant changes in the GSSG levels. In addition, the ratio GSH/GSSG diminished significantly in the MCI and AD groups ($p < 0.05$). The ratio GSH/GSSG was negatively correlated with the GSSG levels within each group and positively correlated with the GSH levels only within the MCI group. Although we found a decrease in both the GSH concentration and GSH/GSSG ratio, the latter one is one of the most important indicators of oxidative stress and could be a potential risk factor for age-related diseases such as dementias [49,51].

Erythrocyte GPx antioxidant enzyme levels were significantly lower in AD patients in comparison with controls. Previous studies have reported a similar pattern in GPx activity of AD subjects when compared with aged-control individuals [52–55], in contrast with an increase in this activity [56,57].

The decrease in GSH content in erythrocytes from AD and MCI patients could be associated with reduced activities of glutamate cysteine ligase and

glutathione synthase [58]—the two enzymes involved in *de novo* GSH synthesis—because we have found no change in the amount of GSSG glutathione nor in the GR activity, suggesting that a decrease in *de novo* GSH synthetic capacity is responsible for the decline in GSH content in AD and MCI.

According to our data, we could establish the degree of oxidized proteins and GSH/GSSG ratio as the common features found in AD and MCI patients in terms of oxidant–pro-oxidant status since they showed the most differences with the aged-matched healthy controls in comparison with the rest of the markers determined.

We found that protein carbonyl contents were increased in MCI and AD groups vs control group. The current data indicate that increased oxidation in both groups of patients are not restricted to the brain. This accumulation could be due to an excess of free radical generation or other oxidant species. The increased protein modification may be due to the decreased GSH concentration which protects proteins against oxidation of the sulphydryl groups of the globin chains, denaturation of proteins and the formation of precipitates [59]. These findings indicate that stress response may cause some vital protein alterations and may form the pathogenic mechanisms of several neurodegenerative diseases. Glutathione plays an important role in the detoxification of ROS. It is reported that oxidative stress reduces GSH levels and leads to increased levels of ROS [60].

Our results show that a relationship between the modification of several components of the antioxidant defence system of the organism and the presence of MCI exists. Patients with MCI and patients with AD showed similarities in oxidative stress markers in comparison with the control group.

In conclusion, our present study shows that some peripheral markers of oxidative stress appear in MCI with a similar pattern to that observed in AD. A high free radical production occurring in MCI yields to a rapid consumption of blood antioxidants without a simultaneous activation of the antioxidant system. Subjects developing MCI and subsequently AD may have an inadequate antioxidant system unable to counteract the high-production of free radicals. The oxidative stress present in these patients might be produced by the accumulation of oxidative-damaged protein in plasma and the decrease of enzymatic and non-enzymatic antioxidants. The degree of oxidative stress shown in this work by the protein modification and the redox status might contribute to the pathology of AD. Finally, AD and MCI are biochemically equivalent, since they tend to be equally different from the control group and not different from each other. MCI does not necessarily progress to AD on a biochemical level.

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