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**Plasma antioxidant status, immunoglobulin G oxidation and lipid peroxidation  
in demented patients: relevance to Alzheimer disease and vascular dementia**

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## **Abstract**

A large body of evidence supports a role of oxidative stress in Alzheimer disease (AD) and in cerebrovascular disease. A vascular component might be critical in the pathophysiology of AD, but there is a substantial lack of data regarding the simultaneous behavior of peripheral antioxidants and biomarkers of oxidative stress in AD and vascular dementia (VaD).

Sixty-three AD patients, 23 VaD patients and 55 controls were included in the study. We measured plasma levels of water-soluble (vitamin C and uric acid) and lipophilic (vitamin E, vitamin A, carotenoids including lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ - and  $\beta$ -carotene) antioxidant micronutrients as well as levels of biomarkers of lipid peroxidation [malondialdehyde (MDA)] and of protein oxidation [immunoglobulin G (Ig G) levels of protein carbonyls and dityrosine] in patients and controls.

With the exception of  $\beta$ -carotene, all antioxidants were lower in demented patients as compared to controls. Furthermore, AD patients showed significantly higher Ig G dityrosine content as compared to controls. AD and VaD patients showed similar plasma levels of plasma antioxidants and MDA as well as a similar Ig G content of protein carbonyls and dityrosine. We conclude that, independent of its nature – vascular or degenerative - dementia is associated with the depletion of a large spectrum of antioxidant micronutrients and with increased protein oxidative modification. This might be relevant to the pathophysiology of dementing disorders, particularly in light of the recently suggested importance of the vascular component in AD development.

## **Introduction**

Alzheimer disease (AD) constitutes the most prominent cause of dementia in the elderly and is clinically characterized by memory dysfunction, loss of lexical access, spatial and temporal disorientation and impairment of judgment. The biochemical mechanism of the pathogenesis of this disease is still unknown, but attention is given to the possible implication of oxidative stress in the development of AD. In AD [1, 2], as in other neurodegenerative disorders [3-5], an impaired energy metabolism has been shown, and the impaired mitochondrial function may lead to an increased free radical-related damage affecting critical cellular key components [1-5].

An increase in DNA, lipid and protein oxidation products has been shown in blood and in post-mortem brain samples obtained from AD patients in comparison with controls [6]. A threefold increase in 8-oxo-dG has been found in mitochondrial DNA (mtDNA) isolated from cortical tissue of AD patients in comparison to nuclear DNA (nDNA) [7]. An increased 8-oxo-dG was also detected in nDNA from lymphocytes from AD patients as compared to control subjects [8]. This increase has been recently found to be inversely correlated to plasma levels of several antioxidant vitamins and micronutrients in AD patients [9].

Higher ascorbic acid and  $\beta$ -carotene blood levels have been found in association with a better memory performance among subjects older than 65 years [10]. On the other hand, lower plasma vitamin C and E (Sinclair et al., 1998) as well as lower serum  $\beta$ -carotene and vitamin A [11] levels have been found in demented patients than in healthy controls. Plasma vitamin C levels have been shown to be lower in AD patients as compared to controls even in the presence of an adequate dietary vitamin C intake [12], and AD patients showed significantly lower cerebrospinal fluid levels of vitamin E than controls [13].

It is not known, however, whether AD is associated with a specific plasma antioxidant profile as has been shown for other non-neurodegenerative diseases [14], as studies conducted so far assessed only few antioxidants rather than a broader spectrum of water-soluble and lipophilic antioxidant vitamins and

micronutrients. In light of oxidative stress involvement in AD, the importance of the former both in aging [15] as well as in cardio- and cerebrovascular diseases [16,17] should be also considered: free radical hyperproduction associated to these different processes and disease states, when concomitant, may act synergistically or additively not only in deteriorating outcome, but also in precipitating a subclinical condition allowing AD to be uncovered [18]. This idea is substantially corroborated by recent findings showing a tight relationship, in animal models, between cerebral hypoperfusion, oxidative stress and amyloidogenesis [19, 20] as well by the relationship found between oxidative stress and the metabolism of homocysteine, a risk factor for both AD and vascular dementia (VaD) [21].

In the present study, peripheral levels of a broad spectrum of antioxidant micronutrients as well as of biomarkers of protein and lipid oxidation in patients with AD and VaD as compared to healthy controls were measured. Since circulating immunoglobulins are the second most prevalent serum protein, and the carbonyl content of immunoglobulins G (Ig G) has been previously shown to be sensitive to dietary antioxidant supplementation [22,23], we chose the evaluation of the carbonyl and dityrosine content in Ig G rather than total oxidized proteins.

## **Subjects and Methods**

### *Subjects*

Eighty-six AD and VaD patients admitted to the Geriatric Day Hospital of the Department of Clinical and Experimental Medicine at the University Hospital of Perugia, Italy, were consecutively enrolled in the study. The diagnosis of AD or VaD was made on the basis of scores obtained to a full battery of cognitive, functional and behavioral tests as well as according to NINDS-AIREN [24] and NINCDS-ADRDA [25] criteria and to neuroradiologic findings. Sixty-three patients were diagnosed as having AD (46 female, 17 male,  $76.8 \pm 6.9$  years; MMSE  $20.4 \pm 3$ ) and 23 patients as having VaD (14 female, 9 male,  $78.0 \pm 6.5$  years; MMSE  $19.8 \pm 3$ ). Patients were compared to 55 healthy controls (36 female, 19 male,  $75.7 \pm 7.3$  years; MMSE  $28.7 \pm 1$ ).

For nutritional status assessment, blood cell count, plasma albumin, prealbumin, cholesterol, and triglycerides were measured. Data regarding dietary habits - as assessed by a food frequency questionnaire [26] -, the Body Mass Index (BMI) [weight Kg/(height m)<sup>2</sup>] and the Mini Nutritional Assessment (MNA) [27] were collected in all subjects.

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Subjects with anxiety or depression (evaluated by the Hamilton Anxiety Scale and the Geriatric Depression Scale, respectively) [28,29], history of smoking habit/alcohol abuse, major organ failure, malnutrition, dyslipidemia, alteration of protein metabolism as well as those taking iron or antioxidant supplements were excluded from the study. In order to exclude secondary causes of dementia, blood levels of vitamin B 12, folic acid and thyroid hormones were also determined.

#### *Blood sampling and antioxidant measurements*

The investigation conforms to the principles outlined in the Declaration of Helsinki.

After obtaining informed consent from subjects or their relatives, patients and controls underwent blood drawing in a sodium heparin tube. Blood was immediately centrifuged and plasma was stored frozen at -80°C until analysis. In order to preserve vitamin C, an aliquot of plasma was deproteinized with 10% metaphosphoric acid and the supernatant kept at -80°C until analysis.

Vitamin C and uric acid were measured by HPLC with electrochemical detection according to Kutnink et al. [30] with a Supelco C 18 column (250 mm x 4.6 mm i.d) and a Supelco C 18 guard column (20 mm x 4.6 mm i.d.).

Vitamin A and vitamin E were measured, after extraction with ethanol and hexane, by HPLC with UV detection at 280 nm with a Waters Symmetry C 8 column (150 mm x 4.6 mm i.d.) [31].

Carotenoids including lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ - and  $\beta$ -carotene were detected at 450 nm using a Suplex, pKb 100 5- $\mu$ m particles column (Supelco, Bellafonte, PA) according to Stahl et al. [32].

### *Measurement of protein oxidation*

On thawing of plasma samples, Ig G were isolated from plasma and protein content was measured using bicinchoninic acid (BCA) method as previously described [22].

Protein carbonyls were assessed by ELISA using the method of Carty et al. [22]. Absorbance was measured spectrophotometrically at 490 nm. Carbonyl content was calculated from the standard curve and expressed as nmol carbonyl per mg of Ig G.

The analysis of protein [dityrosine](#) was performed by [reverse-phase](#) HPLC with UV detection at 240 nm connected in series with fluorescence at Ex 320 nm/Em 400 nm. [Oxidised amino acid](#) content of isolated Ig G was determined against a standard curve of 50-250 µM for dityrosine [33].

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### *Measurement of lipid peroxidation*

Plasma levels of malondialdehyde (MDA) were measured by HPLC as previously described [34] using a fluorescence detector (Ex 513 nm/Em 550 nm), with a 5 µm endcapped C 18 reversed phase column.

### *Statistical analysis*

Statistical analysis was performed with the program SPSS 11.0 (Chicago, IL). All data are presented as mean  $\pm$  SD. Nonparametric 1-way analysis of variance (Kruskal Wallis) with the use of Dunn posttest was used for comparisons among groups. Linear regression was used for correlations between parameters.

## **Results**

There was no significant difference between groups regarding age, years of education, BMI and MNA scores. Biochemical indexes of nutritional status and dietary intake did not differ among groups.

AD and VaD patients showed lower mean levels of plasma non-enzymatic antioxidants as compared to controls (Table 1). Since considering the ratio between plasma vitamin E/ vitamin A/ and total cholesterol did not alter results, data are expressed as total plasma levels of these compounds.

AD and VaD patients showed significantly decreased plasma levels of the water-soluble vitamin C and uric acid, of the lipophilic vitamin E and vitamin A, and of the carotenoids lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene and  $\alpha$ -carotene as compared to controls; among biomarkers of oxidative stress, only the content of dityrosine in Ig G was found to be significantly higher ( $p < 0.01$ ) in AD patients as compared to controls; although a trend towards higher levels of dityrosine was also observed in VaD subjects compared to controls ( $6.3 \pm 1.7 \mu\text{M}$  in VaD patients vs.  $5.1 \pm 1.6 \mu\text{M}$  in controls;  $p = 0.06$ ), it did not reach statistical significance (Table 1). A significant inverse association was found between plasma lycopene and MDA levels ( $r = -0.53$ ,  $p < 0.0001$ ).

## Discussion

To our knowledge, there are no studies showing peripheral levels of a broad spectrum of both water-soluble and lipophilic antioxidant micronutrients as well as of biomarkers of free radical-induced damage to lipids and Ig G in patients with dementia. Our main finding is that plasma antioxidants are similarly depleted and Ig G oxidation products are similarly increased in AD and VaD. The potential implications of these results are related to the recently suggested importance of a vascular component in AD development [35-37]. In advanced age, as in the case of the patients included in our study, the differential diagnosis between AD and VaD is mainly made on the base of clinical features. This is due to the fact that the prevalence of unspecific subcortical vascular lesions seen on neuroimaging studies increases with advancing age and is high in AD [38]. For this reason, it is suggested the impaired oxidant/antioxidant balance observed in this study to be similar in AD and VaD patients might be at least partly related to the vascular component of both diseases [35-39]. On the other hand, the observation of a condition of oxidative stress occurring as a

pre-symptomatic feature in the process of neurodegeneration [40,41] has to be taken into account. In this prodromal stage of AD, called “mild cognitive impairment” [42], plasma antioxidants have been interestingly shown to be depleted to a similar extent as of in AD [43].

It is unlikely that antioxidant depletion in demented patients is due to malnutrition, based on thorough nutritional assessment performed in all subjects. Studies have suggested that demented subjects are malnourished - particularly in the final phase of the disease -, but lower plasma antioxidant levels have been recently shown in the early AD stages in well-nourished subjects [44,12].

Non-enzymatic antioxidants, particularly dietary antioxidants such as vitamin C, vitamin E,  $\beta$ -carotene and polyphenols, exert positive effects on cognitive performance [45,2]. Therefore, it has been suggested that a balanced diet containing high amounts of antioxidant-rich fruits and vegetables might constitute an efficient way of decreasing the incidence and the prevalence of dementia in the elderly [46,47].

The plasma non-enzymatic antioxidant profile of demented patients shows that the major components of the antioxidant defense system are affected in this condition. Vitamin C represents the major water-soluble antioxidant in human plasma and is able to prevent lipid peroxidation even in the presence of bleomycin-detectable free iron [48], known to play a pathophysiological role in metal-mediated oxidative stress in AD [49]. Vitamin E, on the other hand, is the most powerful chain-breaking antioxidant, found to be inversely associated with cognitive performance in the elderly [50].

The antioxidant potential of carotenoids and retinol (vitamin A) is gaining increasing attention with respect to dementing disorders [44,9], as carotenoids such as lycopene have been shown to be efficient quenchers of singlet molecular oxygen and scavengers of peroxy radicals [51]. High serum levels of carotenoids were found to be associated with less severe cerebral white matter lesions in the Rotterdam Scan Study [52]. In the present study, we found a strong inverse association between plasma levels of lycopene and MDA. Interestingly, plasma levels of lycopene



in AD patients were also found to be inversely associated with DNA oxidative damage as assessed by the content of 8-oxo-dG in nDNA of lymphocytes [9].

Among biomarkers of oxidative stress, Ig G levels of dityrosine but not of carbonyls were shown to be significantly higher in demented patients as compared to controls. Increased brain protein oxidation has been found in AD as well as in other neurodegenerative disorders [53].

In one study, the total amount of oxidatively modified proteins measured by HPLC was found to be increased in plasma from AD patients as compared to controls [54]. In the present study, the choice of evaluating the carbonyl and dityrosine content in Ig G rather than total oxidized proteins was based upon a number of considerations. First of all, and bearing in mind that targets of protein oxidation are determined by proximity to the source of reactive oxygen species, relative concentration and size, circulating immunoglobulins are the second most prevalent serum protein. Albumin carries lipids, thereby rendering difficult the discrimination between primary and secondary oxidation. Furthermore, the carbonyl content of Ig G has been previously shown to be sensitive to dietary antioxidant supplementation [22,23]. Finally, Ig G have a half life of 15 days, making them a good [temporal](#) indicator of oxidative stress. While no studies on dityrosine in serum or plasma of patients with AD are known yet, dityrosine has been found in plaques from AD patients [55]. In one study, dityrosine and 3-nitrotyrosine levels were found to be elevated in the hippocampus and neocortical regions of AD brains and in ventricular cerebrospinal fluid, reaching quantities five- to eight-fold greater than mean concentrations in brain and ventricular cerebrospinal fluid of cognitively normal subjects [56]. In the same study, interestingly, also uric acid - a proposed peroxynitrite scavenger - was decreased globally in the AD brains and ventricular cerebrospinal fluid.

The results of this study show that dementia, apparently independent of its vascular or degenerative origin, is associated with the depletion of a large spectrum of antioxidant micronutrients and with increased protein oxidative modification. This might be relevant to the

pathophysiology and prevention of dementing disorders, particularly in light of the recently suggested importance of the vascular component in AD development.

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Table 1. Plasma levels of vitamin C, uric acid, vitamin A, vitamin E, lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, levels of protein carbonyls and dityrosine in immunoglobulin G, and plasma levels of malondialdehyde in Alzheimer Disease patients, Vascular Dementia patients and in controls. Data are given in mean  $\pm$  S.D.

<b>Compound (units)</b>	<b>Alzheimer Disease</b> (n = 63)	<b>Vascular Dementia</b> (n = 23)	<b>Controls</b> (n = 55)
Vitamin C ( $\mu\text{mol/L}$ )	25.9 $\pm$ 8.9 ***	26.6 $\pm$ 11.3 ***	52.4 $\pm$ 16.4
Uric acid ( $\mu\text{mol/L}$ )	199.0 $\pm$ 52.0 ***	193.6 $\pm$ 46.6 ***	312.9 $\pm$ 82.3
Vitamin A ( $\mu\text{mol/L}$ )	2.0 $\pm$ 0.38 ***	2.0 $\pm$ 0.37 ***	2.7 $\pm$ 0.3
Vitamin E ( $\mu\text{mol/L}$ )	37.8 $\pm$ 5.8 ***	36.4 $\pm$ 4.7 ***	50.2 $\pm$ 10.2
Lutein ( $\mu\text{mol/L}$ )	0.36 $\pm$ 0.18 ***	0.34 $\pm$ 0.1 ***	0.67 $\pm$ 0.35
Zeaxanthin ( $\mu\text{mol/L}$ )	0.06 $\pm$ 0.02 ***	0.07 $\pm$ 0.05 ***	0.15 $\pm$ 0.08
$\beta$ -Cryptoxanthin ( $\mu\text{mol/L}$ )	0.17 $\pm$ 0.14 ***	0.17 $\pm$ 0.12 **	0.41 $\pm$ 0.42
Lycopene ( $\mu\text{mol/L}$ )	0.61 $\pm$ 0.22 *	0.61 $\pm$ 0.23 *	0.78 $\pm$ 0.36
$\alpha$ -Carotene ( $\mu\text{mol/L}$ )	0.06 $\pm$ 0.03 **	0.06 $\pm$ 0.03 **	0.12 $\pm$ 0.12
$\beta$ -Carotene ( $\mu\text{mol/L}$ )	0.57 $\pm$ 0.28	0.53 $\pm$ 0.27	0.55 $\pm$ 0.34
Protein carbonyls ( $\mu\text{mol/L}$ )	4.3 $\pm$ 1.6	3.7 $\pm$ 1.4	4.0 $\pm$ 1.5
Dityrosine ( $\mu\text{mol/L}$ )	7.3 $\pm$ 2.4 *	6.3 $\pm$ 1.7	5.1 $\pm$ 1.6
Malondialdehyde ( $\mu\text{mol/L}$ )	0.25 $\pm$ 0.07	0.24 $\pm$ 0.11	0.28 $\pm$ 0.14

\* $p < 0.01$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$  vs controls