

# Antioxidant defences and oxidative stress markers in erythrocytes and plasma from normally nourished elderly Alzheimer patients

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## Abstract

**Objectives:** to investigate blood markers of oxidative stress, and enzymatic and non-enzymatic antioxidants in normally nourished elderly people with Alzheimer's disease.

**Design:** case-control study.

**Subjects:** twenty patients with Alzheimer's disease and 23 elderly control subjects, living at home, free from disease and not undergoing any treatment known to have a strong influence on blood oxidative stress markers or antioxidant defence systems.

**Methods:** we performed a nutritional evaluation, including anthropometric and biological measures and a 3-day dietary record. We determined concentrations of antioxidant vitamins ( $\alpha$ -tocopherol, retinol) and malondialdehyde in plasma and erythrocytes. We also measured erythrocyte enzymatic activities of glutathione peroxidase and copper-zinc superoxide dismutase.

**Results:** the two groups were similar in age, body mass index, dietary record and serum albumin concentration. After adjustment for age, sex and cardiovascular co-morbidity, mean plasma concentration of  $\alpha$ -tocopherol was lower in those with Alzheimer disease than in control subjects ( $15 \pm 3.5$  mg/l compared with  $18.2 \pm 3.5$ ;  $P=0.002$ ), as was the mean plasma concentration of retinol ( $0.54 \pm 0.2$  mg/l *vs*  $0.7 \pm 0.2$ ;  $P=0.014$ ). The mean concentration of free plasma malondialdehyde was higher in those with Alzheimer's disease ( $0.70 \pm 0.2$  mmol/l *vs*  $0.5 \pm 0.1$ ;  $P=0.036$ ). In Alzheimer disease patients, free plasma malondialdehyde concentrations were inversely correlated with levels of  $\alpha$ -tocopherol ( $P=0.002$ ) and retinol ( $P=0.025$ ). Erythrocyte levels of vitamins and enzymatic activities were similar in the two groups.

**Conclusion:** lower plasma concentrations of  $\alpha$ -tocopherol and retinol in normally nourished elderly patients with Alzheimer's disease than in controls could suggest that these antioxidant vitamins had been consumed as a result of excessive production of free radicals.

**Keywords:** Alzheimer's disease, case-control study, oxidative stress, vitamin A, vitamin E

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## Introduction

The central nervous system is particularly vulnerable to free radical damage owing to the high brain oxygen consumption, its abundant polyunsaturated fatty acid content, and the relative paucity of antioxidant enzymes, compared with other tissues [1]. Several observations, mainly based on neuropathological studies, have emphasized the oxidative stress hypothesis for early-onset Alzheimer's disease (AD) and AD in old age [2]. In some neuropathological studies, markers of protein oxidation, DNA (mainly mitochondrial) oxidation and lipid peroxidation have been described, suggesting the occurrence of an oxidative stress [2].

Increased levels of trace elements such as iron, mercury and aluminium, which are potent catalysts for free radical generation, have been found in the brain of AD patients [2, 3]. Moreover, genetic abnormalities of transferrin C2, associated with defective binding of iron and aluminium, are associated with a higher rate of AD [4]. Conversely, epidemiological studies have shown a possible protective effect of moderate consumption of alcohol, an effect attributed to the scavenger properties of polyphenols for iron and aluminium [5, 6]. The oxidative stress hypothesis of AD has been further strengthened by demonstration of a beneficial effect of antioxidant treatment with  $\alpha$ -tocopherol or selegiline in AD patients [7].

However, the co-occurrence of a systemic oxidative stress with brain oxidative stress during AD remains hypothetical [8]. In a 20-year longitudinal study, higher plasma levels of two main antioxidants, ascorbic acid and  $\beta$ -carotene, were significant predictors of better memory performance [9]. Similarly, in a non-demented aged population, plasma  $\alpha$ -tocopherol concentrations were positively correlated with cognitive functioning in a cross-sectional study [10].

Case-control studies in AD have shown contradictory results. An increased activity of the anti-oxidant enzyme glutathione peroxidase (GPX) has been observed in plasma [11] and in erythrocytes [12] of AD patients compared with controls. In addition, the activity of the erythrocyte copper-zinc superoxide dismutase in AD patients was found to be either lower than [13] or similar to [11] that in controls. Zaman *et al.* found plasma concentrations of antioxidant vitamins such as  $\alpha$ -tocopherol, retinol and carotenoids to be lower in AD and multi-infarct dementia [14]. However, a previous report found carotenoid levels to be higher in AD than in controls, while retinol levels were similar [15]. These studies did not include nutritional evaluation of AD patients and, in particular, did not investigate the possibility of low dietary intake of antioxidant compounds. This could limit the interpretation of data on oxidative stress. Plasma vitamin C was found to be lower in AD patients than in controls despite an adequate vitamin C intake, independent of the living conditions of the patients [16]. Another confounding factor is that ageing

itself seems to be associated with increased oxidative stress [17–21], although not, apparently, in healthy centenarians [17, 22].

The aim of this work was to compare the red blood cell and plasma concentrations of enzymatic and non-enzymatic antioxidants, and the markers of oxidative stress in weight-stable and clinically stable community-living AD patients and elderly control subjects.

## Methods

### Subjects

We recruited patients from the outpatient memory centre. They were diagnosed with probable (or possible in four of them) dementia of Alzheimer's type according to NINCDS/ADRDA criteria [23]. They all fulfilled the criteria of the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders, 3rd edition, revised*, for AD [24]. Patients with signs of fronto-temporal dementia or dementia with Lewy bodies were not included, in order to focus the study only on those with AD. Patients had previously been followed up in the memory centre for at least 6 months and each had a brain computed tomography scan.

Inclusion of the patients was on the basis of their weight stability. Twenty-five patients with AD were thus included. Five were later excluded: one due to a subsequent refusal, one due to an incidental pneumonia and two due to a marked weight loss observed at the second visit. Thus, we investigated 20 patients (mean age 80.1 years, SD 5.6, range 72–90). Seven presented behavioural disturbances but there was no evidence of disturbances of nutritional behaviour. The mean duration of AD was 3.9 years (range 2–9). Nineteen were on cholinergic therapy.

We compared this group with 23 control subjects (mean age 76.0 years, SD 6.9, range 62–87) who were relatives of patients attending the geriatric centre. Control subjects were free from any memory complaint, central neurological disease or symptom. They were asked to participate after their weight stability has been checked. Initially we recruited 28 control subjects, but five of them later withdrew their informed consent.

AD patients and controls were included after giving their written informed consent, and, if necessary, their surrogate's co-consent. All subjects lived at home.

During the first visit, both patients and controls had a medical examination. We excluded subjects with signs of Parkinson's disease, diabetes mellitus, renal insufficiency or an infectious or inflammatory disease during the previous 2 months. Four out of 20 AD patients and nine out of 23 control subjects had treated hypertension, and ischaemic heart disease was present in five AD patients and in nine controls. Five AD patients and five controls were taking an angiotensin-converting

enzyme inhibitor or calcium antagonist. Apart from this, no patients nor control subjects were taking any drugs that could influence the indices under investigation, particularly levels of vitamins A, E or C. In addition, none was a current smoker. All subjects had undergone a Mini-Mental State Examination (MMSE) [25].

### Nutritional evaluation

On two consecutive visits, 1 month apart, AD patients and controls underwent a medical nutritional examination. At the first visit we confirmed that they had not had any appreciable weight loss (<4% of their body weight) over the previous 3 months. We then recorded anthropometric measures such as weight, height and mid-arm circumference and body mass index. Controls and patients were asked to complete a 3-day dietary record the week before the second visit, with the help of their caregiver when necessary. The subjects were asked to record exhaustively the foods and beverages ingested and to report their exact amount.

At the second visit, weight stability was confirmed, dietary records collected and blood sampled in a non-fasting state. Concentrations of serum albumin (normal range 35–45 g/l) and acute phase protein (C-reactive protein, normal range <5 mg/l) were determined by latex-immuno nephelometry (BNA, Behring, Rueil-Malmaison, France). Serum albumin was used as a marker of protein balance, while a normal C-reactive protein concentration confirmed that no inflammatory process was occurring. The 3-day dietary record was analysed using the Nutrisoft Bilnut program (Nutrisoft, Tours, France) which allows the estimation of the amount of nutrient intake including lipid, protein, carbohydrate, alcohol, vitamins and minerals.

### Measurement of erythrocyte and plasma antioxidants and markers of oxidative stress

Blood was collected in Vacutainer tubes (Becton-Dickinson, Meylan, France) with EDTA for antioxidant enzyme activities and with heparin for malondialdehyde (MDA) and vitamin concentration determinations. For MDA and antioxidant enzyme assays, the Vacutainers were immediately put on ice, then centrifuged at 3000 *g* for 15 min at 4°C and immediately analysed. Owing to the lack of standardization of these analyses, a standard curve was determined each day.

### Determination of enzymatic activity

Erythrocyte superoxide dismutase and GPX activities were determined using Randox test combinations (Randox, Crumlin, UK). The activities were measured enzymatically at 37°C on a Kontron spectrometer at 505 and 305 nm, respectively, as previously reported [26, 27]. Randox provided standards.

### MDA assays

We analysed total plasma and erythrocyte MDA (free and bound forms), which are the catabolites of unsaturated fatty acid oxidation, by a modified method [28]. Basic hydrolysis was first used to release MDA bound to amino groups. Secondly, the protein-free extract obtained after acid treatment reacted with thiobarbituric acid, and the MDA/thiobarbituric acid adduct was separated from interfering chromogens by high performance liquid chromatography using a C18 $\mu$  Bondapack column and an apparatus from Waters (Ontario, Canada). Quantification was by fluorimetric detection. MDA concentrations were computed by reference to a calibration curve prepared using assays of tetraethoxypropane (Aldrich-Chemic, Steiheim, Germany).

### Vitamin analysis

We measured plasma and erythrocyte  $\alpha$ -tocopherol and retinol as previously described [29]. Briefly, 500  $\mu$ l of washed and lysed red blood cells or 100  $\mu$ l of plasma were mixed with absolute ethanol containing 0.1% ascorbic acid, and a mixture of retinol acetate and tocopherol acetate was used as internal standard. The vitamins were subsequently extracted in hexane. This extract was concentrated and dissolved in methanol. High performance liquid chromatography separations were done on a C18 $\mu$  Bondapak 3.9 $\times$ 300 mm column (Waters) with a 1.5% deionized water/methanol mixture as the mobile phase. Monitoring was with a spectrophotometric detector (Waters  $\lambda$  Max-481) at 290 nm. Vitamins were quantified using appropriate extinction coefficients with standards obtained from Sigma Chimie (Saint-Quentin Fallavier, France).

### Statistical analysis

The baseline clinical characteristics and the nutritional parameters in AD patients and in control subjects were compared using the  $\chi^2$  test for categorical variables. For numerical data, ANOVA tests were performed except when the variances of the distributions were significantly different. In this case, we used the non-parametric Kruskal–Wallis test. Multiple regressions were used to compare the data of oxidative stress markers and antioxidant vitamins and enzymes in blood from AD patients and control subjects, adjusted for sex, age and co-morbidity such as treated hypertension and ischaemic heart and vascular disease. These latter co-morbidity conditions and cardiovascular treatments were entered into the model since a previous study had documented a link between them and, respectively, an increased [19] or a decreased oxidative stress [30]. We calculated correlation coefficients between antioxidant vitamins and oxidative stress markers in both AD patients and controls.

## Results

The nutritional indices under investigation were statistically similar in the AD patients and the control subjects (Tables 1 and 2). The mean body mass indices of AD patients and controls were in the overweight range, and no patient or control had a body mass index  $<18 \text{ kg/m}^2$ . The absence of any significant inflammatory process in patients and controls was confirmed by the normal C-reactive protein values observed.

The dietary intakes of energy, proteins, fat, alcohol and micronutrients such as carotene, retinol and  $\alpha$ -tocopherol were statistically similar in the two groups (Table 2). However, the distribution of the results of retinol intake assessment was very scattered, thus limiting the value of the statistical comparison. Carbohydrates, proteins, lipids and alcohol accounted for 48, 16, 33 and 3% respectively of total energy intake in the AD group and 47, 21, 30 and 3% of the total energy intake in controls. AD patients and controls ate raw fruit or vegetables and cooked fruit or vegetables at least once a day, with the exception of one AD patient who failed to eat cooked vegetables on 1 day from the 3-day record. Among AD patients, nine out of 20 subjects did not drink any wine, compared with 10 out of 23 subjects in the control group. Few subjects drank tea (six in each group). The percentages of subjects who ate fried or smoked food on one occasion were similar in the two groups (fried food was eaten by four in the AD group and three controls, smoked food by none in the AD group and one control).

**Table 1.** Characteristics of patients with Alzheimer's disease and controls

	Patients ( <i>n</i> =20)	Controls ( <i>n</i> =23)
Mean age, years ( $\pm$ SD)	80 $\pm$ 6	76 $\pm$ 7
Sex ratio (men : women)	4 : 16	7 : 16
Mean value ( $\pm$ SD)		
Body mass index, $\text{kg/m}^2$	26 $\pm$ 6	25 $\pm$ 3
Mid arm circumference, cm	27 $\pm$ 3	26 $\pm$ 2
Serum albumin, g/l	39 $\pm$ 5	39 $\pm$ 2
C-reactive protein, mg/l	10 $\pm$ 4	10 $\pm$ 5

**Table 2.** Mean dietary intake in patients with Alzheimer's disease and controls according to the 3-day dietary record

	Mean intake ( $\pm$ SD), by group	
	Patients ( <i>n</i> =20)	Controls ( <i>n</i> =23)
Energy, kcal/kg/day	33 $\pm$ 14	27 $\pm$ 8
Protein, g/kg/day	1.3 $\pm$ 0.4	1.4 $\pm$ 0.8
Fat, g/kg/day	1.2 $\pm$ 0.5	0.9 $\pm$ 0.3
Alcohol, g/kg/day	0.17 $\pm$ 1	0.12 $\pm$ 0.02
Retinol, $\mu\text{g/day}$	807 $\pm$ 1022	505 $\pm$ 653
Carotene, $\mu\text{g/day}$	2575 $\pm$ 1833	2673 $\pm$ 1847
$\alpha$ -Tocopherol, mg/day	7.0 $\pm$ 3	7 $\pm$ 4.0

The mean MMSE of AD patients was 14.2 ( $\pm$ 4.6). In control subjects the MMSE was in the normal range (mean 29.0  $\pm$  0.1).

Plasma  $\alpha$ -tocopherol and retinol levels were significantly lower in AD patients than in controls and there was a trend for higher plasma concentrations of free MDA in AD patients (Table 3). However, the distributions of these indices overlap each other (Figure 1). The values of plasma  $\alpha$ -tocopherol retinol and free MDA were similar in possible and probable AD patients. The plasma  $\alpha$ -tocopherol concentration was not significantly correlated with the mean  $\alpha$ -tocopherol intake, nor was plasma retinol concentration correlated with retinol or carotene intake.

There was a significant inverse correlation between plasma  $\alpha$ -tocopherol and free MDA ( $P=0.002$ ), and between plasma retinol and free MDA ( $P=0.025$ ) in AD patients (Figure 2). Conversely, in controls, no such trend was found (Figure 2).

Erythrocyte enzymatic activities were similar, as were erythrocyte vitamin concentrations and MDA levels (Table 3). There was no significant difference in plasma concentrations of any oxidative stress markers or defences according to age, sex or history of treated hypertension or cardiovascular ischaemic disease.

## Discussion

In this case-control study, plasma  $\alpha$ -tocopherol and retinol concentrations were lower in AD patients than in elderly control subjects. Similarly, a trend towards higher

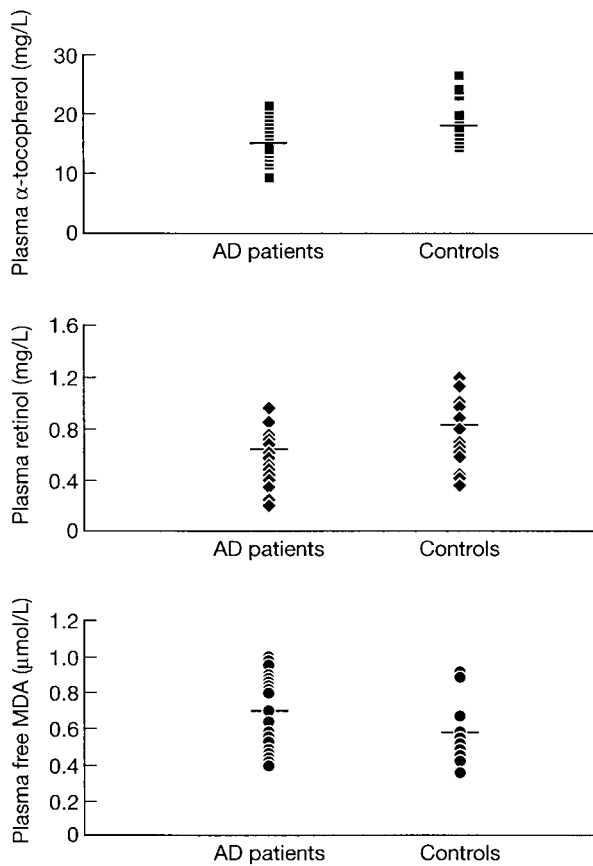
**Table 3.** Plasma and erythrocyte antioxidant vitamins and markers of oxidative stress, and erythrocyte antioxidant enzyme activities in patients with Alzheimer's disease and controls

	Mean level ( $\pm$ SD), by group	
	Patients	Controls
$\alpha$ -Tocopherol		
Plasma, mg/l	15.1 $\pm$ 3.5 <sup>a</sup>	18.2 $\pm$ 3.6
Erythrocyte, nmol/gHb	19.1 $\pm$ 11.2	28.0 $\pm$ 15.9
Retinol		
Plasma, mg/l	0.54 $\pm$ 0.19 <sup>b</sup>	0.71 $\pm$ 0.24
Erythrocyte, nmol/gHb	1.26 $\pm$ 0.54	1.06 $\pm$ 0.48
Plasma MDA, $\mu\text{mol/l}$		
Free	0.69 $\pm$ 0.19 <sup>c</sup>	0.57 $\pm$ 0.13
Total	1.15 $\pm$ 0.56	1.06 $\pm$ 0.33
Erythrocyte MDA, nmol/gHb		
Free	9.8 $\pm$ 4	9.8 $\pm$ 4.50
Total	14.0 $\pm$ 5.0	17.0 $\pm$ 5.8
Erythrocyte activity, U/gHb		
Glutathione peroxidase	32 $\pm$ 10	33 $\pm$ 10
Superoxide dismutase	850 $\pm$ 220	763 $\pm$ 200

<sup>a</sup> $P=0.002$  versus control subjects; <sup>b</sup> $P=0.014$  versus control subjects;

<sup>c</sup> $P=0.036$  versus control subjects.

MDA, malondialdehyde.



**Figure 1.** Individual plasma concentrations of  $\alpha$ -tocopherol, retinol and free malondialdehyde (MDA) in Alzheimer disease (AD) patients and controls.

plasma concentrations of free MDA, inversely correlated with plasma levels of both  $\alpha$ -tocopherol and retinol, was observed in AD, suggesting a deficiency in  $\alpha$ -tocopherol and retinol or their consumption as an antioxidant secondary to an excessive production of free radicals. Since the oxidative stress analysis was made on plasma and erythrocytes but not on brain extracellular space, we can only speculate that oxidative stress can occur in the brain.

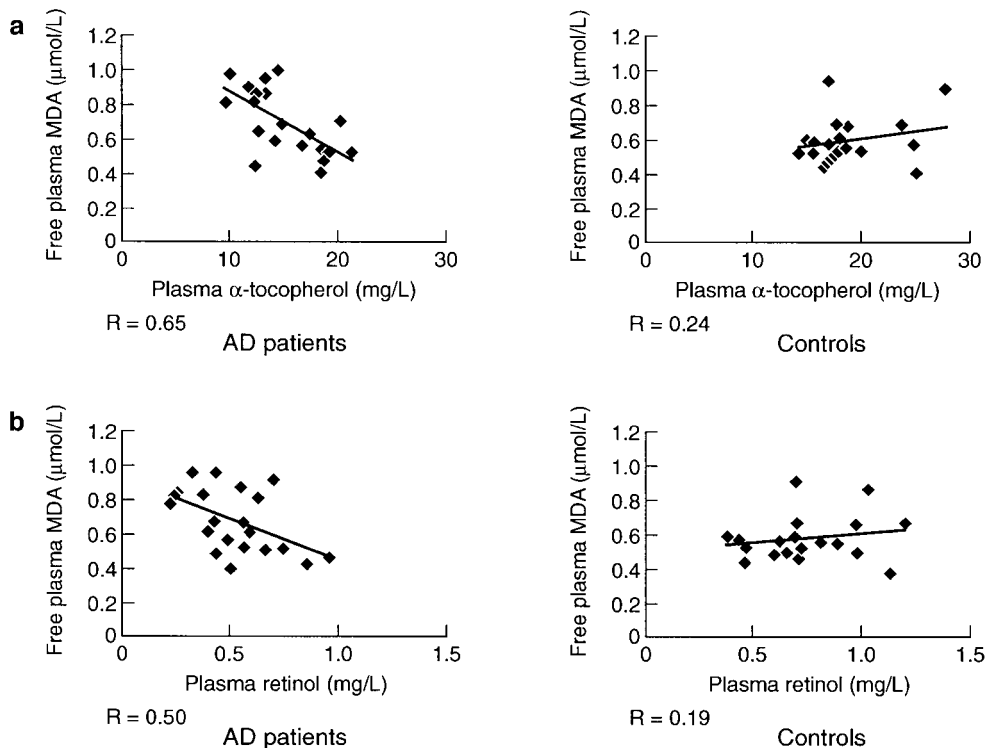
Erythrocyte GPX antioxidant enzyme levels were similar in AD patients and controls, in contrast to those reported previously [12]. In that study, the increase in erythrocyte GPX activity was interpreted as an adaptive change secondary to an increased superoxide dismutase activity in response to an excessive free radical generation. In a study comparing institutionalized Alzheimer subjects and institutionalized control subjects, erythrocyte GPX activity was similar in both groups, but plasma GPX activity was found to be increased in the demented group [11]. The authors suggested that the increase in plasma GPX activity reflected an increase in oxygen radical formation in the extracellular space. This is consistent with the fact that in our study only plasma defences and markers were altered in AD compared with control subjects.

We did not find any threshold value of plasma vitamin A or E levels between AD patients and control elderly subjects. The mean plasma vitamin levels did not differ greatly between the two groups, which contrasts with the results of Zaman and co-workers' study comparing a newly institutionalized demented population with subjects living at home [14]. A possible limitation of the latter case-control study could have been the impact of co-morbidity in both AD and control subjects. In the present study, the subjects were free of diseases known to affect strongly antioxidant defences and levels of oxidative stress, and we found no effect of cardiovascular ischaemic diseases or treated hypertension on the level of oxidative stress markers. Therefore, we suggest that the observed plasma levels of antioxidant vitamins and lipoperoxidation products (MDA) reflect the presence of plasma oxidative stress in AD patients.

Lower levels of plasma antioxidant vitamins in AD patients could have been the consequence of lower intakes of these nutrients. Here, the consumption of antioxidant vitamins under investigation did not differ between AD patients and controls, even for retinol, which seems to be higher in AD than in controls due to the scatter of this measure. Until now, the impact of AD on nutritional behaviour in the early stages or before the onset of the first symptoms of the disease has not been described. Weight loss has been observed during the course of this disease [31], and could precede the first symptoms of the disease [32]. However, both patients and controls in our study appeared to be normally nourished and their weight was stable. The energy intakes of AD patients were within the values required for their weight balance, consistent with a previous report [33]. Their lipid, protein and carbohydrate intakes were in accordance with the French recommended dietary allowances [34], thus defining a normal diet. All subjects in our study seemed to receive adequate amounts of vitamin A [35] but low amounts of vitamin E compared with the recommended dietary allowances [35].

A 3-day dietary record is probably not an adequate estimation of vitamin balance since the observation time is short. However, our results are in accordance with those of Hallfrisch *et al.* [36], who reported a decline of vitamin E intake with age in both men and women in a large cohort. In contrast to us, they demonstrated a strong correlation between intakes and plasma concentrations of vitamins A and E. The possibility of an imbalance of dietary antioxidants was explored in a previous study which showed a decrease in plasma vitamin C in AD patients despite an adequate intake [16]. It is thus possible that the lower concentrations of vitamins A and E that we found among AD patients were not the consequence of a lower intake, but were due to their consumption secondary to an excessive production of free radicals.

One of the consequences of diminished plasma vitamin A and E levels could be impaired immune function [37]. This could be a risk factor for a higher rate



**Figure 2.** Correlation curves between **a**  $\alpha$ -tocopherol and **b** retinol and free plasma malondialdehyde (MDA) in Alzheimer disease (AD) patients and controls.

of infectious diseases and subsequent weight loss, but no study has confirmed this hypothesis. However, in institutionalized AD patients, malnutrition has been related to the previous number of infectious events [38]. The positive impact of pharmacological vitamin E supplementation on function in AD, with no effect on cognitive functioning, has been reported [7], but its effect on weight maintenance and co-morbidity was not described. Thus, further studies should be conducted before any recommendation is made on the pharmacological use of vitamin E in AD.

### Key points

- Normally nourished elderly patients with Alzheimer's disease had lower plasma concentrations of  $\alpha$ -tocopherol and retinol than in controls.
- It may be that these antioxidant vitamins had been consumed as a result of excessive production of free radicals.

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