

## Short communication

# Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects

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Previous studies indicate that regular consumption of a diet rich in fruits and vegetables is associated with a lower risk for age-related diseases. The aim of the present study was to evaluate whether the often-reported age-related decrease of plasma antioxidants in man depends on differences in dietary intake or on other age- and gender-related factors. In this observational case-control study, thirty-nine community-dwelling healthy subjects aged 65 years and older consuming high intakes of fruits and vegetables daily (HI) and forty-eight healthy subjects aged 65 and older consuming low intakes of fruit and vegetables daily (LI) were enrolled. Plasma levels of retinol, tocopherols, carotenoids and malondialdehyde (MDA) as well as content of protein carbonyls in Ig G were measured. Plasma levels of retinol, tocopherols and carotenoids were significantly higher in group HI than in group LI subjects independent of age and gender. MDA levels were inversely correlated with vitamin A and  $\alpha$ -carotene. Protein carbonyls were inversely correlated with  $\gamma$ -tocopherol. In the elderly, a higher daily intake of fruits and vegetables is associated with an improved antioxidant status in comparison to subjects consuming diets poor in fruits and vegetables. Modification of nutritional habits among other lifestyle changes should be encouraged to lower prevalence of disease risk factors in later life.

### Ageing: Antioxidants: Nutrition: Prevention

In developed countries, it is estimated that the percentage of subjects aged 60 years and older will increase from 19% currently to almost 40% in year 2050 (Tinker, 2002). Important with this trend is that the older population is itself ageing: according to the US Census Bureau, the group of very old people (aged 80 years and older) is projected to grow as much as eight to ten times on the global scale between 1950 and 2050. Most chronic diseases manifest in this later stage of life. Until the 1970s, it was thought that disease risk could not significantly increase after a certain advanced age. Therefore, there should be no benefit in changing habits including diet after 80 years of age (Lasher *et al.* 2000). To date it is well known that the incidence of several age-related and nutrition-dependent degenerative diseases keeps increasing even in the eldest portion of the population.

Previous epidemiologic studies have consistently shown that diet has been implicated in the incidence of six out of ten of the leading causes of death of Americans (Department of Health and Human Services, 1988), and that it plays a crucial role in the prevention of age-related chronic diseases (Darnton-Hill *et al.* 2004) as well as in the control of cardiovascular risk factors such as LDL cholesterol (Djoussé *et al.* 2004). Bioactive compounds like antioxidants, mostly contained in fruits and vegetables (Lampe, 1999), play a particularly important role in the protection against oxidative and nitrative stresses. These

processes have been associated with the ageing process and the pathophysiology of age-related illnesses (Liu *et al.* 2002). Antioxidants might fall below desired values with increasing age, likely due to age-related biological and socio-economic changes associated with critical risk factors for malnutrition (Robertson & Montagnini, 2004). Several studies indicate a correlation between an inadequate antioxidant status and an increased risk for or poor outcome of several age-related diseases – including CVD, dementia, osteoporosis, cancer and degenerative diseases of the eye (van't Veer *et al.* 2000).

The implication of individual antioxidants in disease prevention has been examined in various supplementation trials. The results, however, are not as clear-cut as those obtained for fruits and vegetables, and are often disappointing. The preventive effect against chronic degenerative diseases, in fact, is much stronger and consistent for antioxidant-rich fruits and vegetables than for single compounds (Willett, 1994; Gaziano *et al.* 1995; Joshipura *et al.* 1999, 2001; Liu *et al.* 2000, 2001; Bazzano *et al.* 2002; John *et al.* 2002; Mozaffarian *et al.* 2003; Steffen *et al.* 2003; Yusuf *et al.* 2004).

Malnutrition and a poor antioxidant status of the organism are common in the elderly. It is not known, however, whether healthy older subjects consuming high amounts of fruits and vegetables exhibit a good antioxidant status correlated with decreased

amounts of biomarkers for oxidative stress. In the present study, plasma levels of lipophilic antioxidants, of malondialdehyde (MDA) as a marker of lipid peroxidation, as well as the carbonyl content of Ig G as a marker of protein oxidation were measured in well-characterized healthy elderly subjects consuming diets rich or poor in fruits and vegetables.

## Subjects and methods

### Subjects

Eighty-seven healthy community-dwellers recruited by advertising in Umbria, a region of central Italy, were included in the study after giving informed consent (forty males, forty-seven females; median age 70.5, range 65–102). Subjects underwent physical/instrumental examination to assess clinical conditions and identify deficiencies of the major organs. For this purpose, an electrocardiogram, two consecutive measurements of blood pressure, as well as a series of cognitive, functional and behavioural tests were performed. Routine laboratory parameters were also determined in all subjects including blood cell count, plasma albumin, prealbumin, cholesterol and triacylglycerols. Data regarding dietary habits, BMI and the Mini Nutritional Assessment (Guigoz & Vellas, 1999) were collected in all subjects.

Subjects who were not fully healthy, smokers, subjects with malnutrition, dyslipidaemia, BMI <18 or >30, as well as subjects consuming more than one and less than four portions of fruit and vegetables per day (see later) and those taking medications and/or antioxidant/vitamin/iron supplements were excluded from the study.

Subjects were asked to self-complete a qualitative food-frequency questionnaire (Winkler & Döring, 1998) modified to assess intake of fruits and vegetables, in order to identify those individuals consuming a diet rich in fruits and vegetables [ $\geq 4$  portions/d = >350 g/d, group HI (high intake)] and those consuming a diet poor in fruits and vegetables [0–1 portion/d = 0–100 g/d, group LI (low intake)]. All subjects included in the HI group had a mixed fruit and vegetable intake consisting of equal amounts of fruits and vegetables or of amounts of vegetables exceeding that of fruits by one portion. The diets of all subjects were varied and 'mixed colour', particularly for vegetables.

### Blood sampling and measurements

The investigation conforms to the principles outlined in the Declaration of Helsinki. After giving informed consent, subjects underwent blood drawing. Blood was collected in a heparinized tube and immediately centrifuged. Plasma was stored frozen at  $-80^{\circ}\text{C}$  until analysis.

Carotenoids including lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene, and  $\alpha$ - and  $\beta$ -carotene were analysed by HPLC with UV/vis detection at 450 nm according to Stahl *et al.* (1993). A second UV/vis detector was connected in series and set at 325 and 292 nm for quantitation of retinol (vitamin A), and  $\alpha$ - and  $\gamma$ -tocopherol (vitamin E), respectively. Recovery from the column was >90% for each micronutrient. The calibration curves were linear from 0 to 1000 nmol/l for all carotenoids, with correlation coefficients >0.99. The intra- and inter-assay precision varied between 5 and 15%.

For the measurement of protein oxidation, Ig G were isolated from plasma and protein content was measured using the bicinchoninic acid method (Carty *et al.* 2000). Protein carbonyls were assessed by ELISA following the method of Carty *et al.* (2000). The CV described for this method is 4.9%, the intra-assay variation for ten samples measured in triplicate is 1.2% and the recovery of carbonyls in the samples is 76%. Carbonyl content was calculated from a standard curve and expressed as nmol carbonyl per mg of Ig G.

Plasma levels of MDA were measured after protein precipitation and derivatization by HPLC with fluorescent detection (Merck-Hitachi, Darmstadt, Germany) (excitation 515 nm, emission 553 nm) using a commercial kit consisting of columns and necessary reagents (Chrom-Systems Instruments and Chemicals GmbH, Munich, Germany). The CV described for this method is <3.4%, the calibration curves are linear from 0.01 to at least 10  $\mu\text{mol/l}$ , and the recovery of MDA from the column is 99%.

### Statistical analysis

Statistical analysis was performed with the program Statistical Package for the Social Sciences version 11.5 (SPSS, Chicago, IL, USA). All data are presented as means and standard deviations, unless otherwise specified. The Mann-Whitney test was first used to compare, between groups, the levels of the different not normally distributed analytes. To correct for age and gender, a linear general model was used to compare the levels of the different analytes in the LI and HI groups. Age and gender were introduced in the model as covariates. As the significances obtained with the two tests were similar (with the exception of protein carbonyls, see later), only the results obtained with the linear general model are presented, being corrected for age and gender. Correlations between parameters were examined by Spearman's correlation. Significance was accepted if the null hypothesis was rejected at the  $P < 0.05$  level.

## Results and discussion

According to the food-frequency questionnaire, thirty-nine subjects were included in the HI group (nineteen males, twenty females, 74.7 (SD 11.2) years) and forty-eight subjects in the LI group (twenty-one males, twenty-seven females, 77.6 (SD 12.7) years). All subjects included in the HI group had a mixed fruit and vegetable intake consisting of equal amounts of fruits and vegetables or of amounts of vegetables exceeding that of fruits by one portion. The diets of all subjects were varied and 'mixed colour', particularly for vegetables.

Group subjects did not differ in any of the major variables measured for the assessment of the nutritional status (Table 1).

As shown in Table 2, mean levels of all antioxidant micronutrients and vitamins were lower in LI subjects compared to HI subjects. Therefore, the main result of the present study is that the major lipophilic antioxidants of the human organism are significantly higher in healthy subjects consuming a diet rich in fruit and vegetables compared to subjects with low intakes of fruits and vegetables. Importantly, after correction for the variables age and gender, differences were statistically significant for  $\alpha$ - and  $\gamma$ -tocopherol, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene and  $\beta$ -carotene. Plasma levels of the latter, in agreement with previous results (Al-Delaimy *et al.* 2004), appear to be higher in the Italian population compared to other European countries.

**Table 1.** Serum levels of total cholesterol (mg/dl), triacylglycerols (mg/dl) and albumin (g/dl) as well as BMI values of healthy subjects consuming a high daily intake of fruit and vegetables (HI subjects) and of healthy subjects consuming a low daily intake of fruit and vegetables (LI subjects)†

Parameter	HI subjects (n 39)		LI subjects (n 48)	
	Median	Range	Median	Range
Total cholesterol	165	112–191	161	136–198
Triacylglycerols	152	123–192	158	125–168
Albumin	3.9	2.9–4.9	3.8	3.3–5.1
BMI	23.0	19–26	22.5	19–26

† For details of procedures, see p. 641.

Previous studies have shown that plasma levels of lipophilic antioxidants are dependent on physiologic factors including age and gender (Brady *et al.* 1996; Al-Delaimy *et al.* 2004). The age and gender differences of antioxidant profiles shown in previous studies (Brady *et al.* 1996; Mecocci *et al.* 2000) might be epiphenomena of the major influence exerted instead by fruit and vegetable intake.

In addition, we observed that plasma MDA levels were significantly higher in LI subjects than in HI subjects. Furthermore, MDA levels were found to be inversely correlated, in the LI group, with vitamin A ( $\rho = -0.45$ ,  $P = 0.003$ ) and  $\alpha$ -carotene ( $\rho = -0.47$ ,  $P = 0.005$ ). When analysing the differences between groups in protein carbonyls, values were significantly higher in the LI group as compared to the HI group, but the difference was lost after correction for age and gender. Protein carbonyls were inversely correlated with  $\gamma$ -tocopherol ( $\rho = -0.46$ ,  $P = 0.02$ ). The present results suggest that, no matter how old an individual is, a long-term balanced diet with high contents of fruits and vegetables prevents at least in part the formation of bio-products of free radical damage. In a recent study from Dragsted *et al.* (2004), forty-three healthy male and female non-smokers were randomized to a 25 d intervention study with either diet devoid of fruits and vegetables, a diet containing 600 g fruits and vegetables per day, or with a vitamin- and mineral-supplement pill. In this study, markers of oxidative damage to

**Table 2.** Plasma levels of lipophilic antioxidants and vitamins and of malondialdehyde, and content of protein carbonyls in Ig G in healthy subjects consuming a high daily intake of fruit and vegetables (HI subjects) and in healthy subjects consuming a low daily intake of fruit and vegetables (LI subjects)†

Parameter	HI subjects (n 39)		LI subjects (n 48)	
	Mean	SD	Mean	SD
$\alpha$ -Tocopherol ( $\mu\text{mol/l}$ )	24.9	5.4	19.2**	2.2
$\gamma$ -Tocopherol ( $\mu\text{mol/l}$ )	1.6	0.6	1.4*	0.5
Retinol ( $\mu\text{mol/l}$ )	2.1	0.6	2.0	0.4
Lutein ( $\mu\text{mol/l}$ )	0.54	0.2	0.53	0.2
Zeaxanthin ( $\mu\text{mol/l}$ )	0.13	0.04	0.10*	0.06
$\beta$ -Cryptoxanthin ( $\mu\text{mol/l}$ )	0.36	0.3	0.23*	0.1
Lycopene ( $\mu\text{mol/l}$ )	0.99	0.2	0.68**	0.2
$\alpha$ -Carotene ( $\mu\text{mol/l}$ )	0.11	0.05	0.09	0.09
$\beta$ -Carotene ( $\mu\text{mol/l}$ )	0.90	0.3	0.51**	0.2
Malondialdehyde ( $\mu\text{mol/l}$ )	0.20	0.1	0.30*	0.2
Protein carbonyls (nmol/mg)	0.82	0.30	0.94	0.24

Mean values were significantly different: \* $P < 0.01$ , \*\* $P < 0.001$ .

† For details of procedures, see p. 641.

**Table 3.** Observed correlations between age and lipophilic antioxidant micronutrients and vitamins with relative  $\rho$  and  $P$  values in healthy subjects consuming a high daily intake of fruits and vegetables (HI subjects, n 39) and in healthy subjects consuming a low daily intake of fruits and vegetables (LI subjects, n 48)†

Parameter	$\rho$ value	$P$ value
Age/vitamin A, LI subjects	0.53	<0.001
Age/vitamin A, HI subjects	0.39	<0.05
Age/zeaxanthin, LI subjects	0.37	<0.02
Age/ $\beta$ -cryptoxanthin, LI subjects	0.32	<0.05
Age/ $\beta$ -cryptoxanthin, HI subjects	0.51	<0.01
Age/ $\beta$ -carotene, LI subjects	0.42	<0.01

† For details of procedures, see p. 641. Only statistically significant results are reported.

proteins and lipids did not change after the intervention in either group, probably due to the short period of the study.

Finally, associations found between age and antioxidants both in LI and HI subjects are shown in Table 3. In the Women's Health Study, Liu *et al.* (2000) have reported a risk of incident atherosclerotic disease among older women that was approximately 30% less in women who ate five to ten servings of fruits and vegetables per day in comparison with those consuming two to five servings per day. *The World Health Report 2002* (World Health Organization, 2002) suggests that increasing individual fruit and vegetable consumption could contribute to lowering the worldwide burden of disease for IHD and ischaemic stroke by 30 and 19%, respectively (World Health Organization, 2002).

In conclusion, we show in the present study that in the elderly, in the absence of disease, a higher intake of fruits and vegetables is associated with higher lipophilic antioxidant levels as well as with the presence of lower levels of biomarkers of lipid peroxidation.

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