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Independent and Interactive Association of Blood Antioxidants and Oxidative Damage in Elderly People

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Oxidative stress is recognized as one of the major contributors to the increased risk of several diseases. Many recent population studies have established a close link between antioxidant defense and lowered risk of morbidity and mortality from cancer and heart disease, but little is known about the cooperative interactions of antioxidants. We examined the cross-sectional independent and interactive association of serum lipid-soluble antioxidant levels and free radical scavenging enzymes to serum malondialdehyde (MDA) levels, as a marker of oxidative damage. The participants were 160 nonsmoker institutionalized elderly. Upper tertile values of erythrocyte-superoxide-dismutase (E-SOD) constituted the strongest-associated single compound with a 74% decreased risk of high MDA. Upper tertiles of carotenoids and α-tocopherol independently showed a similar lowering of risk of about 57%. The highest tertiles of lycopene and either β-carotene or α-tocopherol simultaneously reveal a higher decreased risk for oxidative damage (74 and 71%, respectively), very similar to those in the upper tertiles of all these three vitamins (75%). This study represents one of the few attempts to date to understand the interactive effect between antioxidants and suggests that lipid-soluble antioxidants act not individually, but rather cooperatively with each other. The efficacy of this interaction is more effective when lycopene is present.

Keywords: Lycopene; β-Carotene; α-Tocopherol; Antioxidant enzymes; Oxidative stress; Biochemical markers

INTRODUCTION

Oxygen free radicals are involved in the pathogenesis of several diseases including, atherosclerosis, inflammatory diseases, cancer, and certain neurological disorders,[1,2] either as the primary cause of the disease or as a secondary phenomenon.[3]

Oxidative damage occurs as a consequence of an imbalance between the formation and inactivation of oxygen free radicals. This process leads to the destruction of membrane lipids and production of lipoperoxides and their products, such as aldehydes. Inactivation and removal of these reactive oxygen species depend on relations involving a wide spectrum of antioxidative defense mechanisms. The capacity of defense is determined by a dynamic interaction between individual components, which comprises vitamins E and C, carotenoids, metabolites such as glutathione and uric acid and antioxidant enzymes. Among these enzymes, the most important are superoxide dismutase (CuZn-SOD; EC 1.15.1.1), glutathione peroxidase (GSH-Px; EC 1.11.1.9), and catalase (CAT; EC 1.11.1.6). In the last few years, attention has been paid to examining the relation of decreased antioxidant–vitamin or enzyme status with markers of oxidative damage to lipids and to DNA.[4,5] A recent study suggests an age-related increase in lipid peroxidation not linked to a decline in the antioxidant defense system.[6] Also, many studies have evaluated whether antioxidants afford protection against heart disease, cancer, or neurological disorders.[7–9]

Malondialdehyde (MDA) is the most abundant individual aldehyde generated by free radical attack on polyunsaturated fatty acids of cell membranes. Excessive production of MDA could combine with

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free amino groups of proteins and alter biological properties of proteins and DNA.[10] Several studies have established a close link between increased lipoperoxide production and various clinical conditions,[11–13] such as oxidative stress, myocardial infarction, stroke, rheumatic diseases, diabetes mellitus, maxillary sinusitis, renal disease, allergic inflammation, or liver problems. Thus, MDA appears to be a promising noninvasive biomarker for free radical damage.

However, very limited information exists concerning the relationship of blood levels of antioxidants with oxidative damage. The aim of this study was to assess the association of serum lipid-soluble antioxidants (α-tocopherol, retinol, lycopene, and β-carotene), antioxidant enzymes (GSH-Px and SOD), and plasma MDA as a biomarker for oxidative stress, including the possible effects of age, alcohol, plasma lipids, and Body Mass Index (BMI). This study quantifies the effects of these factors and examines the possible interaction between them since different lipid-soluble antioxidants appear to act synergistically,[14] as part of a prospective study on the association between diet, blood antioxidants status, biomarkers of oxidative damage, and disease. It is interesting to note that synergism between antioxidants has been rarely considered in previous population studies.

MATERIAL AND METHODS

Population

The sample group was established on the basis of a randomly selected group of 160 nonsmoking elderly (60 men and 100 women), aged 60–80 years (mean age of 73.5 and 73.8 years for men and women, respectively) recruited in seven nursing homes in different areas of Asturias (Northern Spain).

A medical history was obtained for each subject prior to enrollment. We excluded subjects with a history of cancer or cardiovascular disease (as they were end-points in our prospective study), those taking anti-inflammatory agents, antidepressants, and thyroid hormones (as these medications might interfere with oxidative stress), alcoholics, current smokers, or those taking vitamin or mineral supplements. Other common diseases such as diabetes or the intake of anti-hypertensive drugs that might alter the oxidant/antioxidant balance were not excluded as there were no differences between the tertiles of MDA in proportion with the participants with these diseases (data not shown). Subjects were randomly selected from the nursing homes' registry of residents. The participation rate was about 85%. All participants were mentally and physically capable of participating in the initial protocol of the study and gave their consent. The study design was approved by the Committee on Ethical Research of the Oviedo University Hospital.

In a preliminary interview, the subjects were informed of the objective of the study; after they agreed to participate, a personal appointment was made to collect data. A questionnaire checked by a nurse at the examination corroborates that they were nonsmokers and were not taking the above-mentioned drugs. Also provided was the information on drinking habits.

To calculate BMI, height was registered by using a stadiometer exact to 1 mm (Año-Sayol, Barcelona, Spain). Subjects were barefoot, in an upright position, and with the head positioned in the Frankfort horizontal plane. Weight was measured with a weighing machine with 500 g precision (Seca, Hamburg, Germany).

Blood Measurements

For each subject, 30 ml of whole blood was withdrawn by venipuncture after a 12 h fast. Samples were collected into heparin-treated tubes. The blood was centrifuged at 1000*g* for 15 min to separate plasma and serum. The buffy coat was removed and the remaining erythrocytes were drawn from the bottom, washed three times in cold saline solution (9.0 g/l NaCl) and hemolyzed by addition (1/6, by vol.) of doubly distilled water containing 5 ml/l Triton X-100, followed by vigorous vortex-mixing and storage on ice for 10 min. Membrane-free hemolysate was obtained by centrifugation at 10,000*g* for 5 min. For assay of SOD and GPx, hemoglobin was extracted from hemolysate by addition of an equal volume of chloroform/ethanol (15/25, by vol.). All the aliquots were frozen at −80°C until analyzed.

Analytical Methods

The serum samples were thawed up and mixed well. 250 μl samples were diluted in a 4 ml centrifuge-tube with 250 μl bidist water and deproteinized by adding 500 μl ethanol followed by mixing for 10 s on a Vortex mixer. From a dispenser, 1000 μl of *n*-hexane was added and the tubes closed by a polyethylene stopper. After mechanical shaking for 10 min, the tubes were centrifuged at an equivalent force of 2000*g*. From the clear supernatant, 400 μl was pipetted into Eppendorf tubes, which were placed in a Speed-Vac-Concentrator and evaporated to dryness at ambient temperature under reduced pressure. The residue was dissolved completely in 100 μl ethanol/dioxane (1:1) by shaking for 10 min with a mixer for Eppendorf tubes, then 150 μl acetonitrile was added and mixed again. This sample extract solution was now ready for injection into

the HPLC. The efficiency of this extraction procedure was tested by adding known amounts of retinol, α-tocopherol, β-carotene, and lycopene to aliquots of some serum samples ($n = 8$). After extraction and analysis, the values obtained in supplemented and unsupplemented samples were compared. The retinol, α-tocopherol, and carotenoids concentrations obtained in this way were between 93 and 104% of the expected values.

Retinol, β-carotene, α-tocopherol, and lycopene were separated and quantified using an Alliance HPLC equipment (Waters) with an Xterra RP18 column (4.6 × 250 mm, Waters). Extracted samples (100 μl) were injected and eluted in isocratic conditions with 5% water and 95% of a mixture of acetonitrile/tetrahydrofuran/methanol/water (684:220:68:28), at a flow rate of 1.5 ml/min. Detection was performed in the range 230–550 nm with a photodiode array detector (Water 996). Peaks were identified according to their absorption spectra and quantified by area integration in chromatograms extracted at the wavelength giving maximum absorbance for every compound (325 for retinol, 456 for β-carotene, 293 for α-tocopherol, and 474 nm of lycopene). Vitamin standards used for peak identification and quantitation were purchased from Sigma.

The activity of CuZn-SOD and GPx in both plasma and hemolysate were spectrophotometrically determined. The SOD activities of both plasma and red blood cells were measured with the SOD-525 assay test (Byoxytech, Oxis International S.A., France). Red blood cells glutathione peroxidase (Se-GPx) was evaluated with the colorimetric reagent set GPx-340 (Byoxytech, Oxis International S.A., France) and for the measurement of the plasma form of GPx, we used the immunoenzyme technique pl-GPx-EIA Assay (Byoxytech, Oxis International S.A., France).

MDA concentrations were determined using the colorimetric assay of lipid peroxidation LPO-586 (Byoxytech, Oxis International S.A., France). This assay kit uses the reaction of a chromogenic reagent with MDA alone, without interferences with 4-hydroxyalkenals (hydrochloric acid solvent procedure), in aqueous samples at 45°C. One molecule of MDA reacts with two molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm.[15]

Serum total cholesterol and triglycerides were measured using enzymatic methods.[16,17] The hemoglobin assay (Sigma cat.no. 541-2) is based on the colorimetric cyanometahemoglobin method. The specific enzyme activities were expressed as U/g Hb.

Statistical Methods

Statistical analyses were performed by using the SAS statistical software (SAS Institute, Cary, NC, USA; release 6.10). Goodness of fit to normal distributions was investigated by probit plots and the Kolmogorov test. As the distribution of all variables was skewed, the natural logarithm of each value was used in the statistical tests. For descriptive purposes, mean and median values of the whole population are presented on untransformed variables.

Intergroup comparisons of continuous variables were made using Student's *t*-test.

In order to estimate the association between antioxidant variables and MDA, the Pearson rank correlation was applied.

We examined the relation between plasma MDA and blood antioxidant levels in two ways: by comparing categories (lowest tertile with highest levels) and by using the antioxidants enzymes and lipid-soluble antioxidants as continuous variables. Study participants were classified into tertiles of plasma MDA levels. Distribution of lipid-soluble and enzymes antioxidants across tertiles of MDA was evaluated by calculating the means of these variables for each tertile. To test for linear trend across MDA categories a linear regression model was used and, in case of ordinally scaled variables, the Cochran–Mantel–Haenszel statistics were applied.

As absolute plasma levels of lipid-soluble antioxidants are known to vary to a large extent with plasma lipids, the means and all analyses with these variables were adjusted for plasma lipids.[18]

Logistic regression models were used to derive the odds ratios for the highest antioxidant levels of having a high MDA level. Alcohol intake, BMI, and plasma lipids were included as covariates. The conventional probability value (0.05) for significance was used in the interpretation of results.

RESULTS

Characteristics of the study population by sex are presented in Table I. A greater proportion of the men than women consumed wine. The male wine consumers drank more wine than female drinkers. On an average, the men had lower BMI than the women. No significant differences were observed for plasma MDA, enzymes, or serum lipid-soluble antioxidants (after adjusting for plasma lipid levels) between sexes. Therefore, analyses were performed for men and women together.

To illustrate the direction and strengths of the associations, the correlation coefficients between plasma MDA levels and enzymes and lipid-soluble antioxidants are given in Table II. Essentially, we observed that plasma MDA concentration was inversely associated with E-SOD and with serum lycopene adjusted to lipids. A positive relationship between E-SOD with either P-SOD or erythrocyte-GPx (E-GPx) and between E-GPx with plasma-GPx

TABLE I Characteristics of the study population by sex

	Men ($n = 60$)	Women ($n = 100$)
Age (years)	73.5 ± 5.4	73.8 ± 5.3
Alcohol consumer (%)	26.7	17.5
Wine (ml/day)[†]	200 ± 172*	78.4 ± 61.0
BMI (kg/m^2)	27.2 ± 4.7**	30.1 ± 4.8
Cholesterol (mmol/l)	5.3 ± 1.0	5.5 ± 0.9
Triglycerides (mmol/l)	1.23 ± 0.73	1.32 ± 0.55
E-SOD (ku/g Hb)	22.9 ± 6.7	25.3 ± 9.4
P-SOD (u/ml)	33.0 ± 9.6	34.8 ± 9.3
E-GPx (u/g Hb)	34.0 ± 14.5	34.6 ± 17.7
P-GPx (u/l)	122 ± 94.1	110 ± 102
MDA (μmol/l)	2.01 ± 1.41	1.88 ± 1.72
Retinol (μmol/l)	1.20 ± 0.43	1.19 ± 0.40
Retinol/adjusted lipid	1.29 ± 0.46	1.25 ± 0.43
α-tocopherol (μmol/l)	19.1 ± 19.4	21.7 ± 9.1
α-tocopherol/adjusted lipid	20.0 ± 8.5	22.5 ± 9.2
β-carotene (nmol/l)	129 ± 117	176 ± 133**
β-carotene/adjusted lipid	139 ± 128	187 ± 149**
Lycopene (nmol/l)	181 ± 153	160 ± 118
Lycopene/adjusted lipid	193 ± 163	168 ± 125

Values are means ± SD. *$p \leq 0.01$; **$p \leq 0.001$. [†]Only alcohol drinkers.

(P-GPx) was found. A positive correlation between all plasma lipid-soluble antioxidant levels was observed.

Mean values of age, BMI, wine intake, plasma lipids, antioxidants enzymes, and serum lipid-soluble antioxidants by tertiles of plasma MDA levels are presented in Table III. Except for P-GPx, levels of enzymes and lipid-soluble antioxidants were lowest for those in the highest tertile of plasma MDA, but a significantly declining trend from the lowest to the highest tertile was only seen for E-SOD, P-SOD, lycopene-adjusted lipids, and BMI.

We examine the differences in mean plasma MDA levels when lipid-soluble antioxidants and enzymes are considered alone or when various combinations of antioxidants are taken into consideration.

First, we present data on plasma MDA for those in the highest tertile of a single compound (lipid-soluble antioxidants or enzyme) (Table IV). In this case, subjects in the highest tertile of E-SOD presented the lowest mean levels of MDA. Logistic regression analysis was used to explore the relative importance of each independent selected variable in having the highest plasma MDA levels (highest tertile) after adjusting for BMI, alcohol intake, and plasma lipids (only for lipid-soluble antioxidants). High E-SOD was the strongest protector single compound for the highest MDA levels. Those with the greatest levels of E-SOD had a 74% lower risk of being in the highest tertile of plasma MDA. The upper tertiles of lycopene, β-carotene, or α-tocopherol were inversely associated with having higher MDA levels, with a lowered risk of about 57%.

The effect on plasma MDA levels and the odds of being in the highest tertile of plasma MDA for those in the upper tertiles of various antioxidants simultaneously are presented in Table V. The combination of the highest levels of β-carotene and α-tocopherol together did not substantially improve either mean plasma MDA values or protection against being in the highest tertile of plasma MDA. Subjects with higher levels of lycopene and either β-carotene or α-tocopherol simultaneously showed lowered mean values of plasma MDA and a lower risk for high MDA (74 and 71%, respectively) than those in the upper tertile of only one of these factors. For those with higher levels of all these three antioxidants simultaneously (lycopene, β-carotene, or α-tocopherol) we did not observe a greater protective effect (75%) than when lycopene was combined with only one of them.

DISCUSSION

Measuring the degree of oxidative stress that a subject is undergoing is not in wide clinical use, in part because no standardized method has been accepted as measuring the oxidative stress status of humans. HPLC-based methods remain the gold standard for the analysis of products of lipid peroxidation because of the superior chemical specificity, but these methods take much longer, are more expensive, and so are not adapted to screening studies. Even when HPLC methods are more specific, we believe the photometric method we used complies with the criteria of good analytical practices.[15] Other studies reporting total plasma MDA measured by HPLC do report a group mean similar to ours, 1.92 μM,[19] 1.62 μM.[20]

TABLE II Pearson correlation coefficients between MDA, lipid-soluble antioxidants, and antioxidant enzymes

	MDA	E-SOD	P-SOD	E-GPx	P-GPx	Retinol	α-Tocopherol	β-Carotene	Lycopene
Age	−0.09	−0.07	0.03	0.10	−0.02	0.11	0.07	0.08	0.04
E-SOD	−0.35***								
P-SOD	−0.13	0.10							
E-GPx	−0.08	0.22**	0.20**						
P-GPx	0.05	−0.13	0.00	0.17*					
Retinol	−0.01	0.00	0.03	0.00	−0.07				
α-Tocopherol	−0.08	0.13	0.03	−0.06	0.00	0.60***			
β-Carotene	−0.03	−0.04	−0.08	0.10	−0.04	0.45***	0.51***		
Lycopene	−0.15*	−0.02	−0.01	0.06	−0.06	0.28***	0.36***	0.41***	

All variables were $\log_e$ transformed before correlation. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

TABLE III Mean values for age, BMI, consumption of wine, plasma lipids, plasma-soluble antioxidants* and enzymes by tertiles of the plasma concentrations of the marker of oxidative stress malondialdehyde (MDA)

	MDA			
	$<0.82\,\mu g/l$ ($n = 53$)	$0.82–2.47\,\mu g/l$ ($n = 54$)	$>2.47\,\mu g/l$ ($n = 53$)	p-value for trend
Age (years)	74.2 ± 5.3	73.1 ± 5.0	72.5 ± 6.6	0.12
Wine (ml/day)	203 ± 197	191 ± 205	92.9 ± 132	0.16
BMI (kg/m^2)	29.1 ± 4.8	28.4 ± 5.3	27.1 ± 4.4	0.02
Cholesterol (mmol/l)	5.4 ± 0.4	5.3 ± 1.2	5.3 ± 0.9	0.78
Triglycerides (mmol/l)	1.17 ± 0.4	1.24 ± 0.8	1.33 ± 0.6	0.23
E-SOD (ku/g Hb)	29.1 ± 9.5	24.1 ± 6.6	21.4 ± 5.7	0.001
P-SOD (u/ml)	37.2 ± 13.6	31.5 ± 7.5	32.8 ± 8.4	0.04
E-GPx (u/g Hb)	36.2 ± 15.8	34.1 ± 17.1	31.5 ± 13.1	0.13
P-GPx (u/l)	85.1 ± 91.4	129 ± 107	115 ± 81.9	0.13
Retinol (μmol/l)	1.24 ± 0.43	1.14 ± 0.42	1.21 ± 0.45	0.31
α-tocopherol (μmol/l)	21.1 ± 8.6	20.9 ± 10.2	19.5 ± 7.7	0.31
β-carotene (nmol/l)	163 ± 121	152 ± 135	152 ± 165	0.43
Lycopene (nmol/l)	195 ± 171	156 ± 132	156 ± 135	0.05

Values are means ± SD. * Adjusted to plasma lipids.

In general, the mean values observed in this study were slightly lower than those reported in others.[7,21–23] A possible explanation that has been pointed out by other authors[23] is a less-favorable biological status in subjects living in institutions compared to free-living elderly populations. However, we cannot confirm this point because no comparative data are available for elderly in our country. On the contrary, previous studies in our community comparing institutionalized with non-institutionalized elderly people,[24,25] reveal no differences in mean nutrient intake or blood parameters between these two groups. Therefore, we believe rather, that data cannot be easily compared with other values given in the literature because of the scarce data for elderly, the variety of assay methods and population used and the lack of unit standardization. This is specially true for β-carotene, and we have found that mean values given by other authors vary considerably: from 350 to 1950 μg/l.[26,27]

We found that moderate intake of wine, usually defined as one to three glasses per day, produces a reduction in plasma MDA levels. This is in accordance with studies on the biological mechanisms of alcohol that have identified red wine to be a concentrated source of dietary phenolic acids and polyphenols that contribute to a general antioxidant capacity,[28] and with an intervention study that found an increase in total plasma antioxidant capacity after supplementation of the diet with red wine in moderate amounts.[29]

We introduce BMI as covariate as we found it negatively correlated with MDA. We did not find data in the literature regarding this relation. A possible explanation could be that those elderly with a higher BMI could have been following a diet with a higher consumption of vegetables (and therefore of carotenes) and a lower intake of saturated fat and calories as has been seen before.[30] In this sense, other studies have found a positive association of some antioxidant vitamin concentrations

TABLE IV Plasma MDA levels (μmol/l) and odds ratios of being in the highest tertile of plasma MDA levels, adjusted for alcohol intake and BMI, for single antioxidants

Status of the explanatory variables		MDA†	OR (95% CI)
High E-SOD	>24.1 (ku/g Hb)	1.27 ± 1.16**	0.26** (0.10–0.63)
	≤24.1 (ku/g Hb)	2.34 ± 1.50‡	
High P-SOD	>35.6 (u/ml)	1.99 ± 1.50	1.04 (0.48–2.25)
	≤35.6 (u/ml)	2.14 ± 1.59‡	
High E-GPx	>35.2 (u/g Hb)	1.98 ± 1.65	0.92 (0.43–1.97)
	≤35.2 (u/g Hb)	2.15 ± 1.53‡	
High P-GPx	>102.9 (u/l)	2.19 ± 1.49	0.97 (0.46–2.07)
	≤102.9 (u/l)	2.04 ± 1.58‡	
High lycopene¶	>202 (nmol/l)	1.68 ± 1.63	0.44* (0.19–0.97)
	≤202 (nmol/l)	2.05 ± 1.34‡	
High α-tocopherol¶	>24.1 (μmol/l)	1.74 ± 1.33	0.42* (0.17–0.94)
	≤24.1 (μmol/l)	2.03 ± 1.59‡	
High β-carotene¶	>170 (nmol/l)	1.65 ± 1.47	0.43* (0.17–0.97)
	≤170 (nmol/l)	2.10 ± 1.48‡	
High retinol¶	>1.39 (μmol/l)	2.01 ± 1.60	0.96 (0.44–2.05)
	≤1.39 (μmol/l)	2.13 ± 1.60‡	

*$p \le 0.05$; **$p \le 0.001$. † Values are means ± SD. ‡ Average value of the lowest two tertiles of the population. ¶ Adjusted for plasma lipids.

TABLE V Plasma MDA levels (μmol/l) and odds ratios of being in the highest tertile of plasma MDA levels, adjusted for plasma lipids, alcohol intake and BMI, for selected combinations of antioxidants

Status of the explanatory variables	MDA[†]	OR (95% CI)
High α-tocopherol and high β-carotene ($N = 29$)	1.74 ± 1.40 2.01 ± 1.49[‡]	0.38* (0.14–1.00)
High lycopene and high α-tocopherol ($N = 17$)	1.23 ± 1.30* 2.08 ± 1.50[‡]	0.29* (0.08–1.00)
High lycopene and high β-carotene ($N = 20$)	1.17 ± 1.27** 2.10 ± 1.48[‡]	0.26* (0.08–0.83)
High β-carotene, α-tocopherol and lycopene ($N = 15$)	1.36 ± 1.40 2.03 ± 1.50[‡]	0.25* (0.07–0.97)

*$p \leq 0.05$; **$p \leq 0.01$. [†] Values are means ± SD. [‡] Average value of the lowest two tertiles of the population.

and BMI,[31,32] which may explain the inverse association with MDA.

We found that those subjects with lower levels of oxidative damage had higher levels of the antioxidant enzymes E-SOD. Our results were consistent with the findings of laboratory studies on the role of antioxidants protecting cells against oxidative damage.[33] Reports in the literature concerning the influence of these free radicals scavenging enzymes on plasma MDA levels in healthy subjects are scarce. Different studies have described that even when a significant effect on enzymatic activity was found after supplementation with antioxidant nutrients, a significant change in plasma lipid peroxidation was not observed.[23] However, in these studies the effect after supplementation is basically on GPx, not on SOD which in our study has been shown to have the highest inverse relationship with plasma MDA.

In our analysis, higher levels of lycopene, β-carotene, and α-tocopherol were associated with a lower risk of having higher levels of plasma MDA (a linear relationship was only found for lycopene). However, retinol did not have this effect despite the substantial singlet oxygen scavenging ability of retinol.[34]

Carotenoids have been shown to exert antioxidant properties *in vitro*,[35] but there is little direct evidence that they protect biological structures against free radicals *in vivo*. Data concerning the antioxidant effects of lycopene in humans are rather sparse. Intervention trials conducted to determine if consumption of carotenoid-rich vegetables would influence the antioxidants status conclude that tomato juice reduced plasma thiobarbituric acid reactive substances (TBARS)[36] and the susceptibility of lymphocyte DNA to oxidative damage,[37] while carrot juice and spinach powder had no effect on lipid peroxidation.[38] Also, recent population studies have established a close link between dietary intake of tomatoes, a major source of the carotenoid lycopene, and a lower risk of cancer and coronary heart disease.[39,40]

We observed a significant inverse association of β-carotene and α-tocopherol on plasma MDA levels when they were treated as discrete variables. Most of the studies confirm the health benefits of lycopene, but this is not so with β-carotene or α-tocopherol and benefits have not always been demonstrated. It has been shown that β-carotene plays a part in improving the antioxidant capacity of the cell and in protecting DNA from oxidative damage only at relatively low concentrations but loses this capacity at higher concentrations.[41] Also, in a recent study β-carotene was found to increase the generation of superoxide.[42] In many other studies, β-carotene was inversely correlated with certain cancers,[7,8] myocardial infarction,[43] or with lipid peroxidation.[44,45]

In previous studies, α-tocopherol has been shown to reduce the MDA concentrations,[11,44] but activities of α-tocopherol as an antioxidant are rather complicated. It has been reported that this vitamin does not always act as such a potent antioxidant as expected, showing that it may also cause oxidation, this is the so-called tocopherol-mediated-peroxidation.[46]

Considering that data in the literature concerning the synergistic effect of the possible combination of blood antioxidants on levels of oxidative stress markers in the human population are scarce, we examined if the different combination of lipid-soluble antioxidants might improve their effectiveness on oxidation. The most prominent and suggestive finding in our study was the synergistic interaction between antioxidants when lycopene was present. Our data reveal that the effect of the highest levels of both β-carotene and α-tocopherol was a slightly greater, but very close to the single compounds. Nevertheless, the inverse association of single carotenoids and α-tocopherol with oxidative damage was greater in subjects who had high serum levels of both lycopene and β-carotene or both lycopene and α-tocopherol. This enhanced effect, after combining lycopene with one of the other antioxidants was not further improved when the highest tertiles of β-carotene and α-tocopherol occurred together with the highest tertile of lycopene (upper levels of lycopene, β-carotene, and α-tocopherol).

Several *in vitro* studies reveal interactions between antioxidants.[47,48] A recent study comparing several carotenoids, α-tocopherol, and their mixtures regarding their capacity to inhibit lipid peroxidation found the highest protection for a single compound being given by lycopene. Also, they conclude that

lycopene was responsible for a synergistic effect of carotenoids and α-tocopherol.[49] Furthermore, a prospective study on antioxidants vitamins and cancer mortality also found a synergism of carotene and vitamin A.[50]

The activity of an antioxidant is determined by its chemical reactivity towards free radicals, the fate of its radical form, mobility, and the location in the biomembranes.[51] Therefore, this difference might provide shielding effects that account for the synergistic properties.

This study represents one of the few attempts to date to understand the interactive effect of antioxidants in the modulation of oxidative stress in a human population. Our data provide some evidence for a decreased risk of oxidative damage in those with high E-SOD, lycopene, β-carotene, or α-tocopherol and suggest that radical scavenging antioxidants act *in vivo* not individually, but rather cooperatively or even synergistically when lycopene is present.

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