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# **FRUIT AND VEGETABLE INTAKE AND ITS RELATIONSHIP TO DIETARY ANTIOXIDANT CAPACITY AND MARKERS OF OXIDATIVE STRESS: A GENDER-RELATED STUDY**

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## ABSTRACT

**Objectives** Oxidative stress plays a detrimental role in the development of chronic diseases. Fruit and vegetables contain several phytonutrients (carotenoids, polyphenols) which exert antioxidant effects. Aim of the study was to investigate gender differences in fruit and vegetable intake and the relationship to plasma levels of carotenoids and total antioxidant capacity (pTAC). We studied also sex differences in the relationship between fruit and vegetable intake and plasma levels of lipid hydroperoxides, as well as of oxidized low-density lipoprotein (ox-LDL).

**Research Methods & Procedures:** This study included 83 healthy subjects (35 men and 48 women, mean age was  $40 \pm 10$  years) . Dietary intake of carotenoids and total antioxidant capacity (dTAC) were evaluated on the basis of a 15-day food frequency questionnaire. Plasma levels of  $\beta$ -carotene, lutein and pTAC were studied. Moreover, levels of plasma lipid hydroperoxides and ox-LDL were evaluated using ferrous oxidation-xylenol orange 2 (FOX2) assay and a monoclonal antibody-based ELISA procedure , respectively.

**Results** Dietary habits were gender-related with a higher intake of fruit and vegetables ( $p < 0.05$ ) and of  $\beta$ -carotene ( $p < 0.001$ ) in women than in men. Mean values of plasma lutein and beta-carotene were higher in women compared with men. Mean values of ox-LDL and lipid hydroperoxides were higher in men than in women ( $p < 0.05$ ). Significant negative correlations were established between the individual values of ox-LDL vs levels of lutein vs  $\beta$ -carotene and vs pTAC values in plasma in both groups. Individuals belonging to the tertile with the highest daily FV intake or highest daily dTAC showed lowest levels of plasma ox-LDL. In each category gender related differences were observed with male subjects showing higher levels of ox-LDL than female subjects. Moreover, lower levels of plasma  $\beta$  -carotene were observed in males in each tertile of daily FV intake compared to females.

**Conclusions.** Based on the data obtained, we confirm that a high fruit and vegetable consumption exerts a positive effect on antioxidant defenses and decreases oxidative damage of plasma lipoproteins on both sexes. The results suggest that the protective effect can be found to a higher extent in women than in me.

Sex-based differences are present in many chronic diseases. Thus, a higher consumption of antioxidant-rich fruit and vegetables should be recommended in effort to prevent diseases in which sex-related differences in oxidative stress play a considerable role.

## INTRODUCTION

According to the World Health Organization (WHO)<sup>1</sup>, daily intake of adequate amounts of plant foods is inversely associated with the development of chronic diseases, such as cardiovascular diseases<sup>2</sup>. The WHO recommends eating  $\geq 400$  g of fruits and vegetables (FV) per day, not counting starchy tubers<sup>3</sup>. It also estimates that in more than half of the countries of the European Region the consumption is lower than 400 g per day of fruit and vegetables, and in one third of the countries the average intake is less than 300 g per day. Gender differences in dietary intakes and eating behaviors have been reported. Literature data suggest that women consume more fruit and vegetables, legumes, and whole foods. Men tend to have more fat and protein rich foods and to drink more wine, beer, spirits, and sweet carbonated drinks<sup>4,5</sup>.

Among molecular mechanisms involved in the protective effects exerted by a high intake of plant foods, it has been proposed that phytochemicals contained in fruit and vegetables play a key role<sup>6,7</sup>. In particular some phytochemicals behave as antioxidants, modulate gene expression and prevent oxidative stress which is involved in the pathogenesis and progression of several chronic diseases, including cardiovascular diseases<sup>7-9</sup>, cancer<sup>10</sup> and ageing-associated diseases. Among phytochemicals contained in fruits and vegetables, the nutritional roles of carotenoids<sup>11</sup> and polyphenols have been mainly investigated<sup>7</sup>. Additionally, fruit and vegetables are an important source of vitamin C, fiber, and magnesium, nutrients that have been negatively associated with oxidative stress<sup>12-14</sup>.

Circulating markers of oxidative stress and inflammation are known to play a complex role in the development of age-related chronic diseases<sup>15</sup>. Lipid peroxidation of plasma high density lipoproteins (HDL) and low density lipoproteins (LDL) occurs *in vivo*. In particular the attention given to plasma levels of oxidized low density lipoproteins (ox-LDL) is supported by their physio-pathological roles<sup>16</sup>. In fact, ox-LDL are related to atherosclerotic events<sup>16</sup> and are considered a marker for cardiovascular diseases<sup>16-17</sup>. Among antioxidant extracellular molecules, a key role is played by paraoxonase-1 (PON1). The enzyme associated to HDL exerts an antioxidant activity by protecting *in vitro* both lipoproteins and biological membranes against lipid peroxidation<sup>18,19</sup>, therefore there is growing interest in the dietary factors that modulate PON1 expression and activity<sup>20,21</sup>. Previous studies have shown that a high intake of fruit and vegetable is related to lower levels of oxidative stress markers, such as ox-LDL and F<sub>2</sub>-isoprostanes in adolescents, middle aged men and in women<sup>12,13,22-25</sup>. Gender related differences have been less investigated. A growing interest

is devoted to sex as an important biological variable in biomedical research <sup>26-29</sup>. Gender differences are present in many chronic diseases. Males tend to suffer from myocardial infarctions earlier than females, and a woman's risk of cardiovascular disease increases after menopause<sup>27</sup>. The interest in investigating nutritional aspects and gender-related differences in the effect of dietary intake of fruit and vegetables on plasma lipid peroxidation, is also supported by recent studies which have demonstrated gender-related differences in the production and metabolic deactivation of reactive oxygen metabolites mainly in animal models<sup>27</sup>. Few studies have described sex differences in response to diet or antioxidant therapy in human subjects<sup>29</sup>.

The aim of our study was to investigate gender related relationships between fruit and vegetable (FV) intake, total dietary antioxidant capacity (dTAC) and markers of antioxidants/oxidative stress in plasma of 83 healthy subjects (35 males and 48 females). Plasma levels of carotenoids (lutein and  $\beta$ -carotene), widely used as biomarkers for fruit and vegetable intake <sup>30</sup>, were evaluated. We thought it of interest also to study the effect of fruit and vegetable intake and dietary antioxidants on the activity of the antioxidant enzyme PON1. Plasma levels of ox-LDL and lipid hydroperoxides, widely used to investigate lipid peroxidation of plasma lipids<sup>17</sup>, were evaluated as biochemical parameters of oxidative stress.

The relationship between dietary habits and plasma levels of markers of antioxidant/oxidative stress of the participants also was studied by a multivariate statistical analysis including partial least squares regression (PLS) and principal component analysis (PCA). PLS and PCA are widely used in various disciplines including medicine and nutritional sciences, especially when a large number of predictors is necessary<sup>31</sup>.

## **MATERIALS AND METHODS**

### **SUBJECTS**

There were 83 healthy subjects enrolled in the study. Volunteers were recruited in the Polytechnic University of Marche (UNIVPM), Italy. 35 of them were males and 48 were females. The mean age was  $40 \pm 10$  years and mean body mass index (BMI) was  $23.1 \pm 2.0$  kg/m<sup>2</sup> (Table 1). Inclusion criteria for subjects were: not taking vitamins, minerals, or other types of supplements during the previous two months; no-smoking; body mass index (BMI) within the normal range according to the World Health Organization criteria ( $18.5$ – $25.5$  kg/m<sup>2</sup>) and normal biochemical and

haematological profile (serum cholesterol < 6.8 mmol/L, serum triacylglycerols < 2.8 mmol/L, glucose < 6.11 mmol/L).

Exclusion criteria were: diagnosed diseases such as allergies, cancer, diabetes, obesity, hypertension, mental diseases, gastrointestinal or renal diseases, as well as intake of drugs related to these pathologies, alcohol consumption > 30 g/day, vegetarian diet.

None of the female subjects was pregnant or lactating. The study was conducted according to the Declaration of Helsinki and all procedures were approved by the Ethics Committee of the “Azienda Ospedaliero-Universitaria Ospedali Riuniti” Ancona (Italy) (Protocol number 211525). All subjects gave informed consent.

## **DIETARY ASSESSMENT**

Each subject was asked to complete a 15-day dietary record to evaluate energy and nutrient intake. Dietary data were estimated by the software “MètaDieta”, (MetaDieta software version 3.1, METEDA, Ascoli Piceno, Italy) validated by the Italian Association of Dietetic and Clinical Nutrition” in 2009. Food composition tables were derived from the European Institute of Oncology (Milan, Italy). Fruit and vegetable intake was assessed from data of the quantitative questionnaire, which included evaluation of fresh fruits and fresh or cooked vegetables. Fruit juice was not considered in this study. Serving size information was provided for each food group and subjects ticked a box representing how many servings they consumed on an average day. A portion of fruit corresponded to 150 g, 50 g for salad and 250 g for vegetables (Larn 2014)<sup>32</sup>. Daily food consumption was estimated as frequency x portion x size for each consumed food item.

The MètaDieta software also contains an antioxidant database (USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of selected foods, Release 2 (2010). The method used to evaluate ORAC levels was developed by Prior et al.<sup>33</sup> and measures both hydrophilic (H-ORAC) and lipophilic ORAC (L-ORAC) for water soluble and fat soluble antioxidant compounds. The USDA Database contains Total ORAC (T-ORAC) values for 326 food items. The values of total dietary antioxidant capacity (dTAC) are expressed as mmol Trolox equivalent/100 g (mmol TE/100 g)<sup>34</sup>.

## **BLOOD SAMPLES AND EVALUATION OF PLASMA LIPIDS AND GLYCEMIA**

Fasting blood samples (10 mL) were collected from each subject by venipuncture from the antecubital vein: 5 mL were placed in tubes containing heparin while 5 mL were placed in tubes without any anticoagulant and centrifuged at 1500 g for 10 min at 4

°C for serum separation. Plasma and serum aliquots were prepared and stored at -80 °C until analysis.

Serum glucose, triacylglycerols (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) were analyzed by commercial kits (Chemadiagnostica, Jesi, Italy). Low density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula<sup>35</sup>.

## **PLASMA LEVELS OF CAROTENOIDS**

Carotenoids ( $\beta$ -carotene and lutein) were quantified in plasma of subjects by high-performance liquid chromatography (HPLC) system, using a single dilution step after extraction with propanol (1:5, v/v) and vigorous vortexing of 250  $\mu$ L of extraction mixture. This mix was centrifuged for 2 min at 20,000 g at 4 °C. Forty microlitres of supernatant were injected into the HPLC with electrochemical detector (ECD) by Shiseido (Tokyo, Japan), using a pre-separation concentrating column 50  $\times$  2.0mm ID 5  $\mu$ m, separation C18 column 150  $\times$  2.0 mm ID 3  $\mu$ m, and a post-separation reducing column CQR 20  $\times$  2.0 mm, all from Shiseido. For each carotenoid quantified, two mobile phases were used. Mobile phase 1 for loading and concentrating the sample (50 mM sodium perchlorate in methanol/water 95:5, v/v) was the same for both molecules, while mobile phase 2 was 50 mM sodium perchlorate in methanol/isopropanol (80:20, v/v) for lutein and 50 mM sodium perchlorate in methanol/ isopropanol (98:2, v/v) for  $\beta$ -carotene. Moreover, flow rate was 200  $\mu$ L/min for phase 1 in both analyses. Flow rates for phase 2 were 300 and 80  $\mu$ L/min for  $\beta$ -carotene and lutein, respectively. Total chromatographic run times and retention times were 24 min/12.3 min for  $\beta$  carotene and 21 min/9.8 min for lutein, as previously described <sup>36</sup>.

## **BIOMARKERS OF OXIDATIVE STRESS.**

**Lipid hydroperoxides** The levels of lipid hydroperoxides were determined in plasma samples using ferrous oxidation-xylenol orange 2 (FOX2) assay as previously described <sup>19</sup>. The levels of lipid hydroperoxides were quantified using a stock solution of t-butyl hydroperoxide. The results are shown as  $\mu$ mol of lipid hydroperoxides for L of plasma.

**Ox-LDL** Oxidized LDL were determined in plasma by a sandwich ELISA procedure using the murine monoclonal antibody mAB-4E6 as the capture antibody, and a peroxidase conjugated antibody against oxidized apolipoprotein B bound to the solid phase (ox-LDL, Mercodia AB, Uppsala, Sweden). Intra and inter-assay CVs were 2.82% and

7.29%, respectively. As LDL-C is considered a major determinant of absolute ox-LDL levels, plasma values of ox-LDL (U/L) were adjusted by the plasma levels of LDL-C (mmol/L) by calculating their ratio (units of ox-LDL per mmol of LDL-C), in agreement with Zuliani et al<sup>37</sup>.

**Plasma Total Antioxidant capacity** Plasma total antioxidant capacity (pTAC) was assessed using the oxygen radical absorbance capacity (ORAC) assay adapted for semi-automated measurement on a 96-well microplate reader (Synergy HT; BioTek, Winooski, VT, USA)<sup>38</sup>. The oxygen radical absorbance capacity of plasma samples employs the oxidative loss of the intrinsic fluorescence of fluorescein induced by the free radical initiator 2,2'-azobis(2-amidinopropane)hydrochloride. Fluorescein fluorescence decay shows a lag or retardation in the presence of antioxidants, related to the antioxidant capacity of the sample. Trolox was used as a reference antioxidant for calculating the ORAC values. Results are expressed as  $\mu\text{mol}$  Trolox equivalents /L (mmol TE/L).

**Plasma Paraoxonase-1 (PON1) activity** Paraoxonase activity of PON1 was measured in plasma using paraoxon as substrate<sup>19</sup>. The basal assay mixture included 5 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl, 4 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$  and 1.0 mmol/L paraoxon. Paraoxon hydrolysis was spectrophotometrically monitored for 8 min (every 15 s) at 412 nm. Non-enzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. One unit of PON1 paraoxonase activity is equivalent to 1 nmol of paraoxon hydrolyzed/min/mL.

## STATISTICAL ANALYSIS

Values of nutrient intake and biochemical markers are shown as mean  $\pm$  standard deviation (SD). Statistical differences were calculated by using Student T test. A p of 0.05 was considered statistically significant. Pearson correlation coefficients and p-value were used to show correlations and their significance using the software Microcal Origin 5.0, OriginLab.

To assess the association between fruit and vegetable consumption, dTAC and biochemical markers, we categorized the participants by tertiles using the Excell program.

Data analysis was also carried out through partial least squares regression (PLS) and principal component analysis (PCA) using the software Simca p8.0



One of the goals of PCA is to reduce the number of variables to enable visualization of the information in the multivariate data set. In PCA, a linear combination of the original variables is constructed to obtain principal components while preserving the largest possible variation in X. Scatter plots are used to visualize object similarities and clustering tendencies, whereas loading plots reveal the contributions of the original variables. In our study, the variables used in the principal component analysis were BMI, energy intake, FV intake, dietary  $\beta$ -carotene, dTAC, plasma levels of lipid hydroperoxides, plasma levels of ox-LDL, plasma PON1 activity, pTAC, plasma levels of lutein and  $\beta$ -carotene. PLS can analyze data with strongly collinear, noisy, and numerous X-variables (X BLOCK), and also simultaneously model several response variables (Y) (Y BLOCK). The objective of partial least squares regression is to find the relations between two blocks (X and Y). In our study X BLOCK contains variables associated with diet of the subjects (FV intake, dietary  $\beta$ -carotene, dTAC), while Y BLOCK contains variables associated with levels of antioxidant/oxidant status in plasma of the subjects (lipid hydroperoxides, pTAC, ox-LDL, lutein and  $\beta$ -carotene). The joint score vectors (t, u) were obtained from a PLS fit using X BLOCK (represented by t) and Y BLOCK (represented by u) and plotted against each other.

## RESULTS

Anthropometric and clinical data of participants are summarized in Table 1. Plasma lipids (TC and LDL-C) and glucose levels were lower in women compared with men. Higher values of HDL-C and of the ratio HDL-C/LDL-C were observed in women than in men ( $p<0.05$ ).

Table 1 – Anthropometric data and plasma lipids

	<b>TOTAL SUBJECTS (n=83)</b>	<b>MEN (n=35)</b>	<b>WOMEN (n=48)</b>
<b>Age (years)</b>	40 $\pm$ 10	41 $\pm$ 8	38 $\pm$ 11
<b>BMI (kg/m<sup>2</sup>)</b>	23.1 $\pm$ 2.0	24.3 $\pm$ 1.5	22.2 $\pm$ 1.9*
<b>TC (mmol/L)</b>	4.6 $\pm$ 0.5	4.8 $\pm$ 0.4	4.4 $\pm$ 0.5*
<b>HDL-C (mmol/L)</b>	1.5 $\pm$ 0.3	1.4 $\pm$ 0.2	1.6 $\pm$ 0.3*
<b>LDL-C (mmol/L)</b>	3.0 $\pm$ 0.5	3.2 $\pm$ 0.4	2.6 $\pm$ 0.5*
<b>HDL-C/LDL-C ratio</b>	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2*
<b>Glucose (mmol/L)</b>	4.8 $\pm$ 0.4	5.1 $\pm$ 0.42	4.6 $\pm$ 0.4*

Values are expressed as mean  $\pm$  S.D

\* $p<0.05$  vs men

**Dietary habits** The assessment of dietary habits has shown gender-related differences with lower energy intake and lower protein and lipid intake in women (Table 2).  $\beta$  - carotene intake was in agreement with previous studies in Italian subjects <sup>39</sup>. The study of micronutrient intake showed a higher intake of vitamin C ( $p < 0.05$ ) and  $\beta$  - carotene ( $p < 0.001$ ) and higher dTAC in women compared to men but the difference was not statistically significant (Table 2).

Table 2- Dietary habits

	<b>MEN (n=35)</b>	<b>WOMEN (n=48)</b>
<b>FOOD</b>		
Fruits and Vegetable (portions)	2.4 $\pm$ 0.7	3.0 $\pm$ 1.1*
Meat (n.portions/day)	0.9 $\pm$ 0.1	0.7 $\pm$ 0.29*
Fish (n.portions/day)	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1
<b>COMPONENTS</b>		
Energy (Kcal/day)	1778.3 $\pm$ 77.6	1480.5 $\pm$ 219.4**
Proteins (g/day)	70.0 $\pm$ 4.2	57. 1 $\pm$ 8.3**
Available Carbohydrate (g/day)	221.0 $\pm$ 14.9	190.5 $\pm$ 18.6
Total Fiber (g/day)	20.1 $\pm$ 1.36	20.3 $\pm$ 2.1
Lipids (g/day)	59.8 $\pm$ 1.3	51.2 $\pm$ 9.0*
Total saturated (g/day)	17.2 $\pm$ 1.9	13.1 $\pm$ 3.5*
Total monounsaturated (g/day)	24.6 $\pm$ 0.8	23.3 $\pm$ 3.4
Total polynsaturated (g/day)	6.7 $\pm$ 0.7	5.6 $\pm$ 0.8
Total W3 (g/day)	1.05 $\pm$ 0.07	1.02 $\pm$ 0.09
Total W6 (g/day)	5.3 $\pm$ 0.9	4.4 $\pm$ 0.7
Vitamin C (mg/day)	65.3 $\pm$ 16.0	76.7 $\pm$ 7.3*
Vitamin E (mg/day)	7.9 $\pm$ 0.7	8.1 $\pm$ 0.7
Thiamine (mg/day)	0.77 $\pm$ 0.13	0.63 $\pm$ 0.10
Folic Acid (mcg/day)	193.5 $\pm$ 16.6	174.8 $\pm$ 29.8
$\beta$ –Carotene (mg/day)	2.3 $\pm$ 1.8	3.2 $\pm$ 1.4**
dTAC (mmol TE/day)	5.3 $\pm$ 1.6	5.9 $\pm$ 1.1

\*  $p < 0.05$  vs men

\*\* $p < 0.001$  vs men

Values are expressed as mean  $\pm$  S.D

**Plasma levels of carotenoids and total antioxidant capacity** The average values of plasma levels of  $\beta$ -carotene and lutein were in agreement with those reported by other authors<sup>40</sup> (Table 3). Higher levels of carotenoids were observed in plasma of women compared with men (Table 3). pTAC was higher in women but the difference was not statistically significant.

### **Biochemical markers of lipid peroxidation and paraoxonase activity**

Levels of ox-LDL ranged between 5.1 and 66.0 U / ml with a mean value of  $34.4 \pm 12.1$  U / mL ; levels of hydroperoxides ranged between 0.76  $\mu$ mol / L and 3.08  $\mu$ mol /L with a mean value of  $1.57 \pm 0.47$   $\mu$ mol /L. The average values of ox-LDL and lipid hydroperoxides were in agreement with values reported by other authors in a healthy population<sup>37</sup>. Both markers of lipid peroxidation (ox-LDL and lipid hydroperoxides) were higher in men than in women (Table 3). The activity of paraoxonase-1 was not significantly different in the two groups of subjects (Table 3).

TABLE 3 – Plasma levels of carotenoids, total antioxidant capacity (pTAC), biochemical markers of lipid peroxidation and paraoxonase-1 activity

PLASMA LEVELS	MEN ( n=35)	WOMEN ( n=48 )
$\beta$ - Carotene ( $\mu$ g/mL)	$0.36 \pm 0.19$	$0.47 \pm 0.22^{**}$
Lutein ( $\mu$ g/mL)	$0.24 \pm 0.07$	$0.28 \pm 0.09^*$
pTAC (mmol TE/L)	$18.10 \pm 1.82$	$18.43 \pm 3.74$
ox -LDL(U/mL)	$37.05 \pm 6.16$	$32.49 \pm 11.5^*$
Lipid hydroperoxides ( $\mu$ mol/L)	$1.73 \pm 0.24$	$1.50 \pm 0.29^*$
Paraoxonase activity (U/mL)	$82 \pm 31$	$72 \pm 35$

*Values are expressed as mean  $\pm$  S.D*

\*p<0.05 vs men

\*\*p<0.001 vs men

### **Correlations between plasma lipids and markers of lipid peroxidation**

A positive correlation was established between levels of ox-LDL and lipid hydroperoxide in the whole group of subjects ( $r = 0.42$ ;  $n = 83$ ;  $p < 0.005$ ). A positive correlation was also established between levels of ox-LDL vs LDL-C levels ( $r = 0.71$ ;  $n = 83$ ;  $p < 0.001$ ) and vs TC ( $r = 0.51$ ;  $n = 83$ ;  $p < 0.001$ ). Conversely, levels of ox-LDL were inversely related to HDL-C levels ( $r = -0.41$ ;  $n = 83$ ;  $p < 0.05$ ). No correlation was established between levels of ox-LDL and paraoxonase-1 activity (data not shown).

### **Correlations between dietary habits, carotenoid intake and plasma levels of carotenoids**

To investigate whether dietary intake of fruit and vegetables is related to levels of phytochemicals in plasma we analyzed correlations between dietary carotenoid intake and dTAC versus plasma levels of  $\beta$ -carotene, lutein and pTAC values. As summarized in Table 4, both in male and female subjects a significant positive correlation was established between daily intake of fruit and vegetables and daily intake of  $\beta$ -carotene vs plasma levels of  $\beta$ -carotene. Even levels of plasma lutein positively correlated with daily intake of fruits and vegetables in both groups of subjects. The results also showed a positive relationship between daily consumption of fruits and vegetables and dietary intake of  $\beta$ -carotene vs pTAC values. No significant correlation was observed between dTAC and pTAC in the whole group and by gender.

### **Correlations between dietary habits, plasma carotenoids and markers of lipid peroxidation**

As reported in table 4 both in male and female subjects, a significant negative correlation was observed between daily intake of fruit and vegetables, daily intake of  $\beta$ -carotene and dTAC vs plasma levels of markers of lipid peroxidation (ox-LDL and lipid hydroperoxides).

Moreover significant correlations were established between individual values of markers of lipid peroxidation (ox-LDL and lipid hydroperoxides) vs plasma levels of lutein vs  $\beta$ -carotene and vs pTAC values of subjects included in the study (Table 5).

To better investigate the effect of dietary habits on markers of oxidative stress in male and female subjects, we reported the levels of plasma ox-LDL by sex-specific tertile of daily fruit and vegetable intake and daily dTAC. As shown in Figure 1 and Figure 2, male or female subjects belonging to the tertile with highest daily fruit and vegetable intake or highest daily dTAC showed lowest levels of plasma ox-LDL. In each category, gender related differences were observed, male subjects showed higher levels of ox-

LDL compared to female subjects (Figure 1 and Figure 2). As summarized in figure 3, lower levels of plasma  $\beta$ -carotene were observed in males in each tertile of fruit and vegetable intake.

The relationship between dietary habits, markers of antioxidant/oxidative stress in plasma was investigated using partial least squares regression (PLS) and principal component analysis (PCA).

As summarized in Figure 4A, X BLOCK includes variables related to dietary habits (FV intake, dietary  $\beta$ -carotene and dTAC) and Y BLOCK includes variables associated to antioxidant/oxidative stress in plasma (lipid hydroperoxides, ox-LDL, pTAC, lutein and  $\beta$ -carotene). X-loadings and Y-loadings, that are the linear coefficients that link the terms to the x or y scores, respectively, are also reported.

Figure 4B, showing the graph obtained by PLS analysis, confirms the general correlation among dietary factors (FV intake, dietary  $\beta$ -carotene and dTAC), levels of antioxidants (pTAC, plasma levels of  $\beta$ -carotene and lutein) and lipid peroxidation markers (plasma levels of ox-LDL and lipid hydroperoxides) in plasma.

Moreover, a principal component analysis was carried out. This method reduces data dimensions because the new low-dimensional variables are used instead of the original high-dimensional ones. Figure 5A and Figure 5B show the scatter plot of the principal components (PC1 and PC2) after PCA and loading plot, respectively, considering the first two components. PC1 components have the highest data variance, with a variance contribution ratio of 41%, followed by PC2 components with variance contribution rates of 11%. Therefore, the first two principal components reflect the majority of the information (which explains 52% of the total variance).

The results revealed that male and females have a different trend, mainly due to higher FV intake and higher concentrations of plasma antioxidants ( $\beta$ -carotene, lutein and pTAC) in females and higher plasma levels of markers of lipid peroxidation (plasma lipid hydroperoxides and ox-LDL) in males.

TABLE 4 - Correlations between dietary factors, plasma antioxidants and markers of lipid peroxidation

	PLASMA LEVELS				
	$\beta$ -Carotene	Lutein	pTAC	Ox-LDL	Lipid hydroperoxides
<b>Fruit and Vegetable portions</b>					
Total subjects (n=83)	0.44**	0.40**	0.35**	- 0.71**	-0.37*
Male (n=35)	0.38*	0.42*	0.42*	- 0.65**	-0.34*
Female (n=48)	0.42*	0.34*	0.31*	-0.76**	-0.40*
<b>Dietary beta-carotene</b>					
Total subjects(n=83)	0.37**	0.31*	0.34*	-0.41*	-0.37*
Male (n=35)	0.35*	0.34*	0.39*	- 0.43*	-0.33*
Female(n=48)	0.33*	0.29*	0.32*	- 0.39*	-0.39*
<b>dTAC</b>					
Total subjects(n=83)	0.31*	0.32*	0.1	-0.51**	-0.42*
Male(n=35)	0.29	0.34*	0.24	-0.53**	-0.42*
Female(n=48)	0.30*	0.31*	0.04	-0.47**	-0.35*

\*\*p< 0.001, \*p<0.05

Table 5 : Correlations between plasma antioxidants and markers of lipid peroxidation

	PLASMA ANTIOXIDANT		
	$\beta$ -Carotene	Lutein	pTAC
<b>Ox-LDL</b>			
Total subjects (n=83)	-0.30*	-0.52**	- 0.37**
Male (n=35)	-0.26	-0.48*	-0.36*
Female (n=43)	-0.28*	-0.50**	-0.31*
<b>Lipid hydroperoxides</b>			
Total subjects(n=83)	-0.31*	-0.40**	-0.31*
Male (n=35)	- 0.26	-0.37*	-0.29*
Female(n=43)	- 0.29*	-0.35*	-0.28*

\*p<0.05

\*\* p<0.001

FIGURE 1 - Plasma levels of ox-LDL in sex-specific tertiles of daily fruit and vegetable intake. Males (■): T1 (n=12, 0.46-1.93), T2 (n=11, 1.9-2.86), T3 (n=12, 2.86-5.80). Females (□): T1 (n=16, 1.10-2.3), T2 (n=16, 2.30-3.33), T3 (n=16, 3.33-5.80)

\*  $p < 0.05$  vs male subjects belonging to tertile with lowest daily fruit and vegetable intake (T1)

#  $p < 0.05$  vs female subjects belonging to tertile with lowest daily fruit and vegetable intake (T1)

§  $p < 0.05$  for the comparison between male vs female subjects belonging to the same tertile

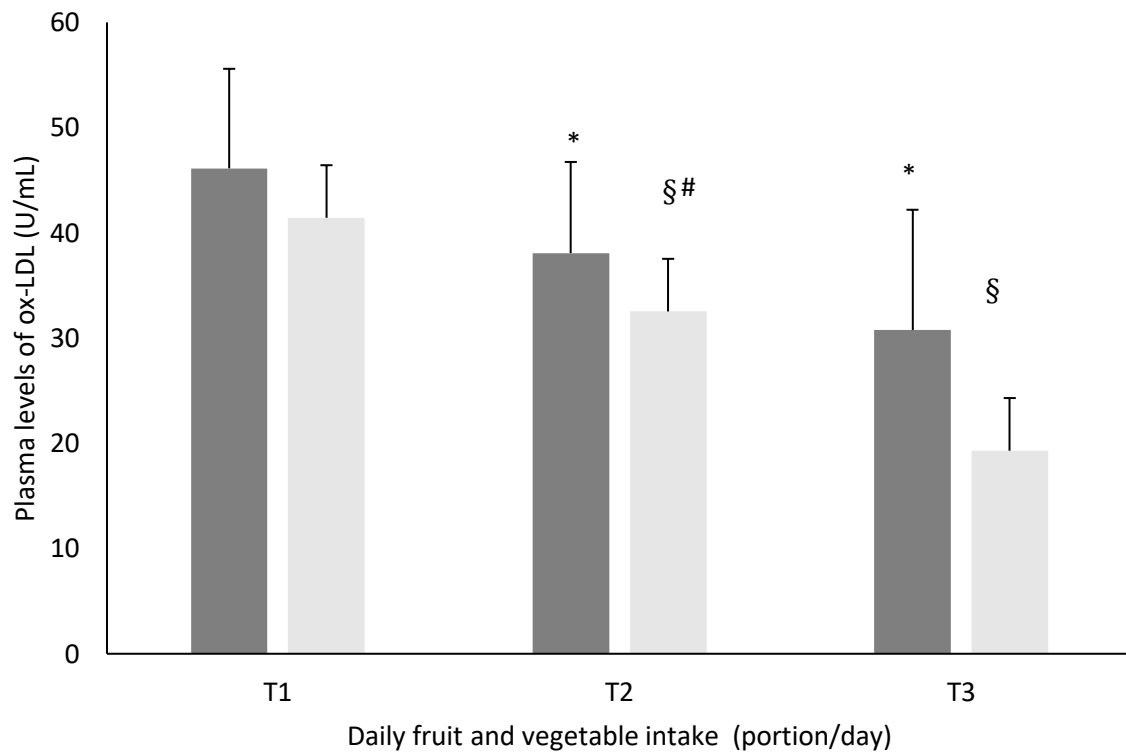


FIGURE2- Plasma levels of ox-LDL in sex-specific tertile of daily d-TAC. Males (■) : T1 (n=12, 2.34-4.63), T2(n=11, 4.36-6.29), T3 (n=12, 6.29-9.60). Females (□) : T1 (n=16, 3.02-5.18), T2 (n=16, 5.18-6.61), T3 (n=16, 6.61-9.65).

\* p<0.05 vs male subjects belonging to tertile with lowest daily d-TAC (T1)

# p<0.05 vs female subjects belonging to tertile with lowest daily d-TAC (T1)

§ p<0.05 for the comparison between male vs female subjects belonging to the same tertile

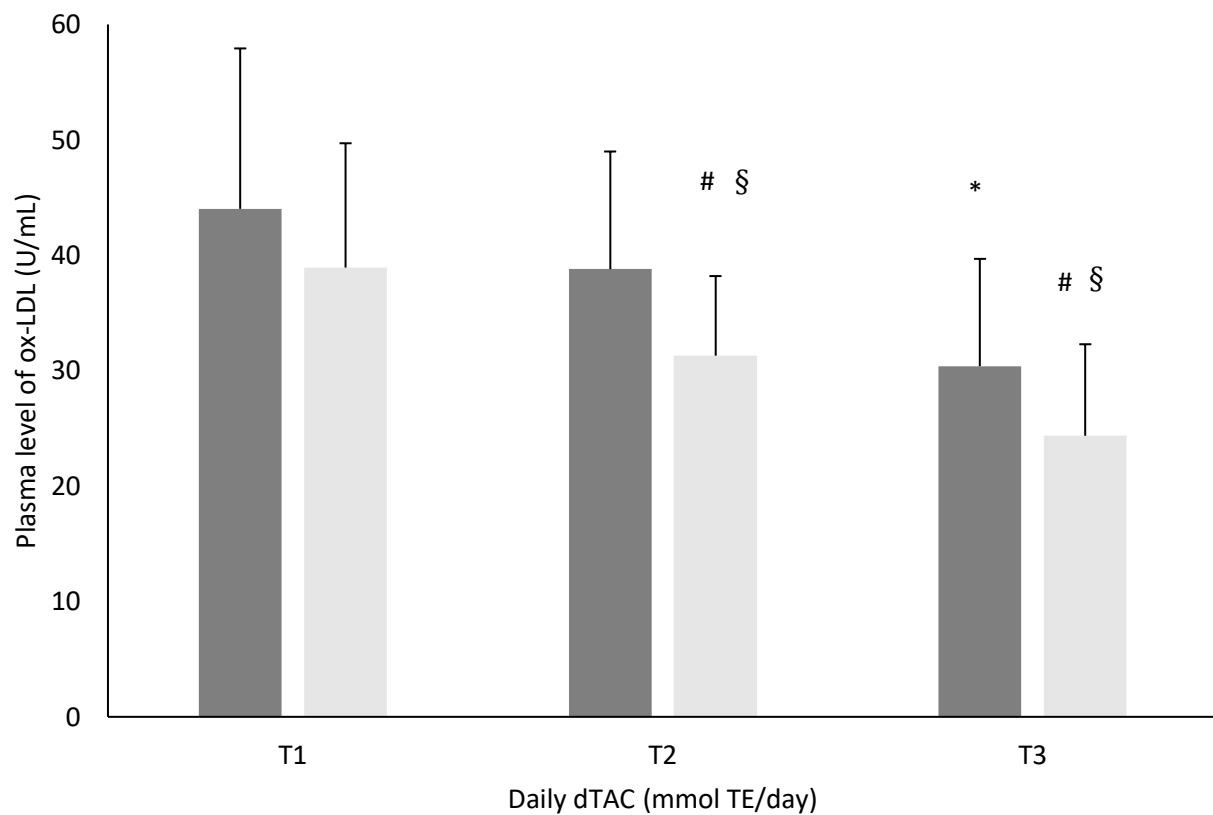




FIGURE 3: Plasma levels of beta-carotene in sex-specific tertiles of daily fruit and vegetable intake. Males (■): T1 (n=12,0.46-1.93), T2 (n=11,1.9-2.86),T3 (n=12, 2.86-5.80). Females (□): T1 (n=16, 1.10-2.3), T2(n=16, 2.30-3.33),T3 (n=16, 3.33-5.80)

\* p<0.05 vs male subjects belonging to tertile with lowest daily fruit and vegetable intake (T1)

# p<0.05 vs female subjects belonging to tertile with lowest daily fruit and vegetable intake (T1)

§ p<0.05 for the comparison between male vs female subjects belonging to the same tertile

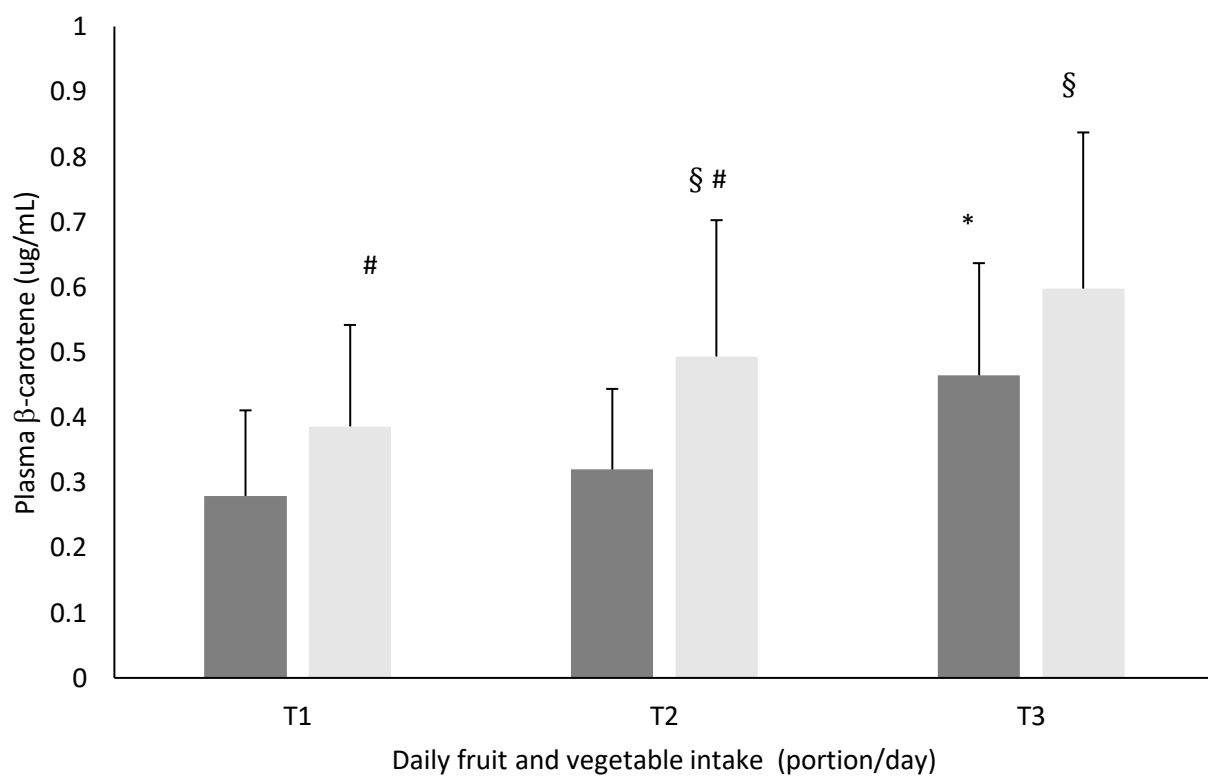


FIGURE 4: The data analysis through partial least squares regression (PLS) among dietary factors (X BLOCK) and plasma factors (Y BLOCK).

A) Variables included in X BLOCK and Y BLOCK and their X-loadings and Y-loadings.

B) Scatterplot joint score vectors. The joint score vectors (t, u) obtained from an PLS fit using X BLOCK (represented by t) and Y BLOCK (represented by u) are plotted against each other

A

DIETARY FACTORS	X BLOCK	
	Variable	Loading
	Fruit and vegetable intake	0.652
	$\beta$ -Carotene	0.580
	dTAC	0.489
PLASMA FACTORS	Y BLOCK	
	Variable	Loading
	Lipid Hydroperoxides	-0.29
	pTAC	0.21
	ox-LDL	-0.48
	Lutein	0.29
	$\beta$ -Carotene	0.28

B

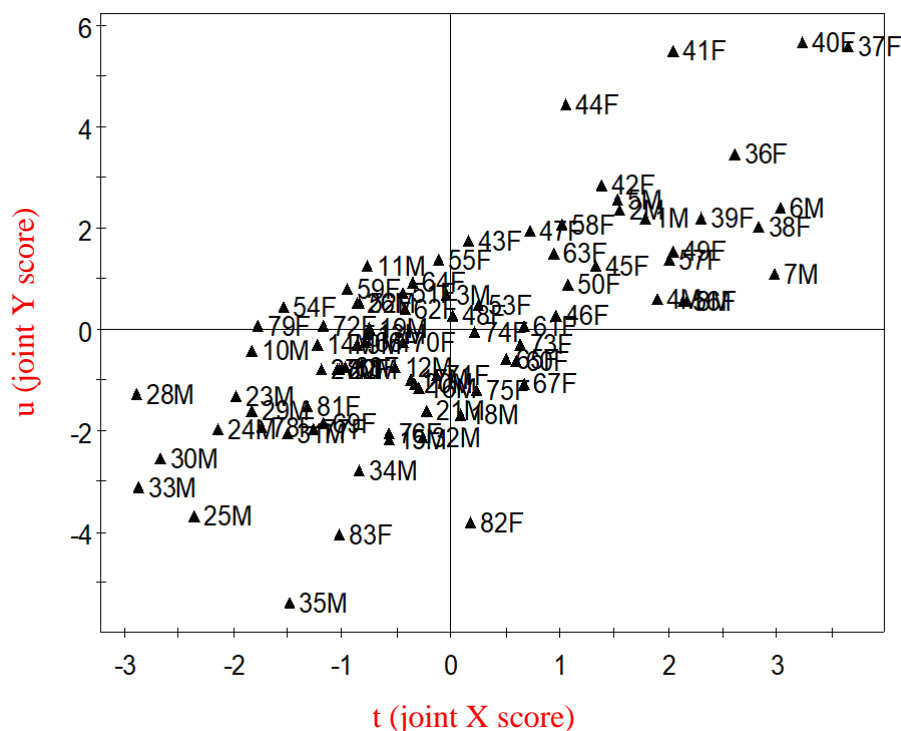
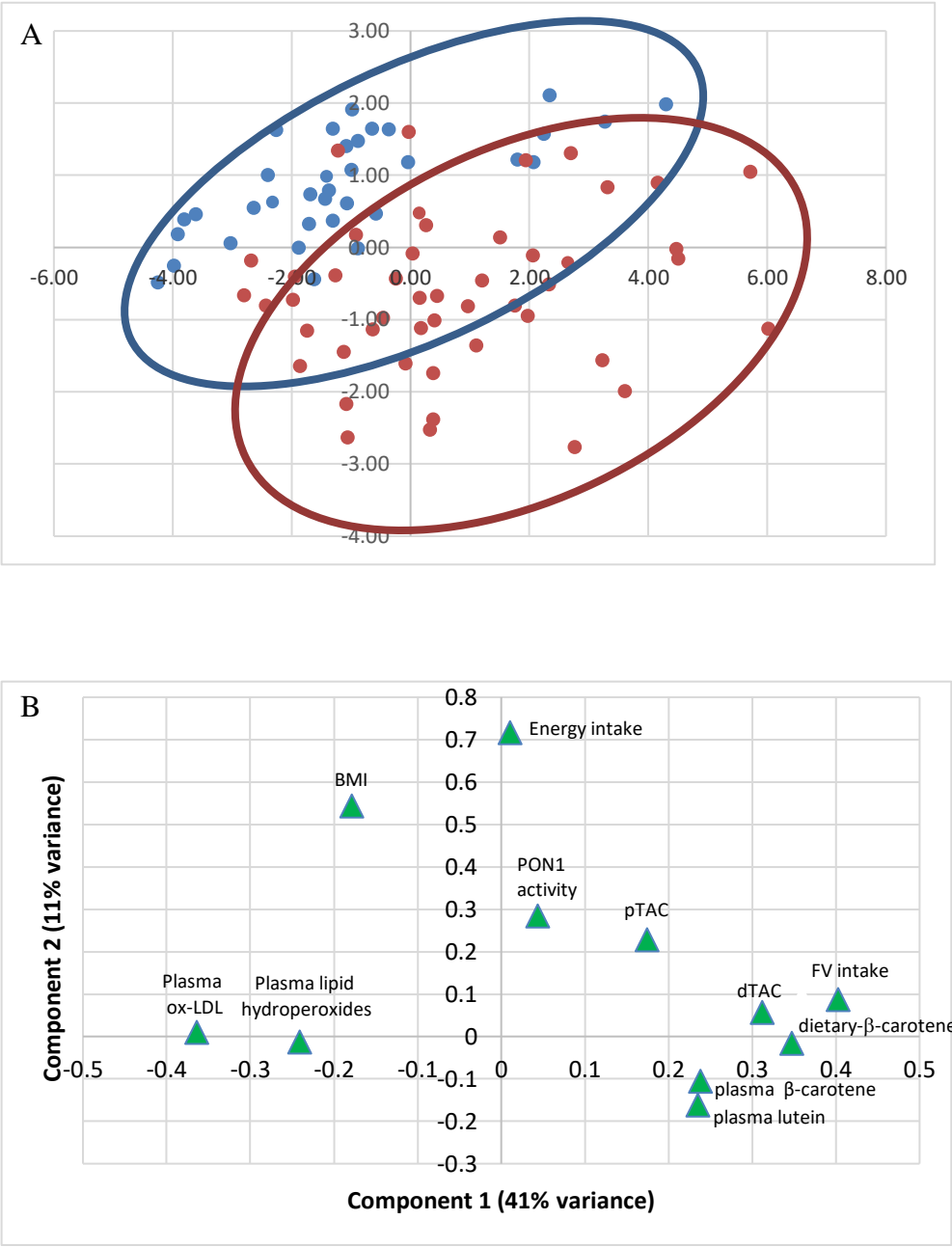


FIGURE 5: Principal component analysis (PCA): A) Scatter plot of scores and loadings (B) considering the first two components ( ● Male subjects ; ● Female subjects)



## DISCUSSION

In this study, we confirmed gender related differences in dietary habits<sup>4</sup>. Higher intakes of fruit and vegetables, vitamin C and  $\beta$ -carotene were observed in women compared to men. In the whole group of subjects and in subgroups, fruit and vegetables intake was positively associated with levels of carotenoids and TAC in plasma. Our results are in agreement with previous studies, which have demonstrated that fruit and vegetable intake and related nutrients are associated with lower levels of oxidative stress<sup>22-25</sup>. In detail, the negative significant correlations established, both in males and females, between dietary carotenoids intake and dTAC vs plasma levels of markers of lipid peroxidation (ox-LDL and lipid hydroperoxides) of subjects included in the study confirm a protective role of dietary lipophilic antioxidants against lipid peroxidation of LDL. We demonstrated that higher levels of plasma carotenoids were associated with lower values of oxidized LDL. Our results agree with previous studies which have demonstrated that lutein and  $\beta$ -carotene are molecules able to inhibit lipid peroxidation<sup>40-41</sup>. Previous studies have also shown that levels of carotenoids in plasma correlate with carotenoid levels associated with LDL. In fact, due to their hydrophobic nature, carotenoids are transported by lipoproteins. Other authors have demonstrated that carotenoid intake is related to lower lipid oxidation and lower oxidative damage to DNA in middle-aged men<sup>41</sup>. A relationship has also been shown<sup>41</sup> between a high fruit and vegetable intake and an increased resistance of plasma lipoproteins to oxidation due to a higher level of plasma antioxidants, which exert an inhibitory role against lipid peroxidation. Ox-LDL play an atherogenic role and are able to induce a pro-inflammatory status by activating the nuclear factor-kappa-B, a redox-sensitive and pro-inflammatory transcriptional factor<sup>42</sup>. Our results confirm that antioxidant rich-foods, expressed by dTAC values can have a protective role against lipid peroxidation of LDL and could prevent inflammatory pathways triggered by ox-LDL.

Our study has also demonstrated that the gender-related differences in dietary habits reflect in significant differences in markers of lipid peroxidation (ox-LDL and lipid hydroperoxides). Within each category of fruit and vegetable tertile or dietary TAC tertile, levels of ox-LDL were significantly higher in males compared to females. These results are likely related to the lower levels of carotenoids in the plasma of males with respect to females observed in each tertile. These results were confirmed by a multivariate statistical analysis. In fact, partial least squares regression (PLS) revealed a relationship between dietary habits and plasma levels of markers of antioxidant/oxidative stress of subjects. Principal component analysis (PCA) has

confirmed a different trend, mainly due to higher FV intake and higher concentrations of plasma antioxidants in females and higher plasma levels of markers of lipid peroxidation in males.

Dietary and genetic factors influence digestion and absorption of carotenes. A wide variability in response to ingested  $\beta$ -carotene has been previously reported <sup>43</sup>.

Our data contribute to this discussion and suggest gender-related potential effects, which deserve to be better investigated in both sexes.

The correlation coefficient between fruit and vegetable intake and ox-LDL was higher ( $r = -0.71$ ) compared to the coefficients calculated for the relationship between dietary  $\beta$ -carotene or dTAC vs ox-LDL ( $-0.41$  and  $-0.51$  respectively, Table 4). Oxidative damage of LDL occurs *in vivo* and is modulated by exogenous and endogenous factors. Regarding this aspect, the role of dietary factors, as mentioned above, is fundamental with carotenoids being the key factor. However, in addition to carotenoids, other nutrients and/or bioactive molecules such as fibers, vitamin C, vitamin E and polyphenols could be involved in the modulatory effect shown by fruit and vegetable consumption. Most polyphenols have low bioavailability and are detected in small amounts both in plasma and tissues<sup>44</sup>. Cooperation among antioxidants with different chemical characteristics may work in an integrated and complementary network and a synergistic effect has been proven *in vitro* <sup>6,7</sup>. No correlation between dTAC and pTAC was observed in the present study. The contribution of dTAC to pTAC is a matter of debate. Several factors such as bioavailability of bioactive molecules and methods to evaluate pTAC could explain the results. In our experimental conditions, dTAC was evaluated using the ORAC assay which measures the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxy radical-induced oxidation. Peroxy radicals are the predominant free radicals found in lipid oxidation in foods and biological systems under physiological conditions. Hence, ORAC values are considered by some to be of biological relevance as a reference for antioxidant effectiveness. Other mechanisms can be involved in the antioxidant effects exerted by dietary bioactive molecules such as chelating effects, modulator of cellular signaling processes and control of gene expression contributing to oxidative stress<sup>45</sup>. Many dietary antioxidants work in a network, and the total amount of antioxidants derived from the combinations of individual antioxidants is considered a better concept than individual dietary antioxidants. Therefore, databases containing levels of phytochemicals and total antioxidant capacity of several foods have been developed to estimate antioxidant vitamin intake from diet and dietary supplements<sup>46-48</sup> and to assess the beneficial effects of dietary antioxidants. Although the

applicability of dTAC data and its *in vivo* physiological relevance is debated, it has been demonstrated that dTAC represents a relevant tool in epidemiological studies. In fact, it has been demonstrated that an increase of dTAC is associated with a lower risk for ischemic stroke in an Italian cohort<sup>49</sup> and lower inflammatory status<sup>50</sup> in middle-aged people.

Regarding PON1, several studies have demonstrated a protective effect against cardiovascular diseases and a decrease of its activity has been observed in several pathological conditions<sup>19,51,52</sup>, therefore particular attention has been devoted to dietary factors that may modulate PON1 activity or expression<sup>20,21</sup>. We observed that PON1 activity was not related to fruit and vegetable intake in subjects included in the study. In addition, plasma levels of ox-LDL were not correlated with PON1 activity and gender-related differences were not observed. Interventional studies have reported that polyphenols and anthocyanin-rich diets can positively modulate PON1 activity and/or expression<sup>20,21</sup>. Other authors failed to confirm these results. The contradictory results are likely related to the complex interconnections between fruit and vegetable chemical composition, lifestyle and genetic PON1 isoforms.

The protective effect exerted by PON1 against lipid peroxidation of LDL and biological membranes has been widely demonstrated *in vitro*<sup>18-19</sup>. In the present study, plasma levels of ox-LDL were negatively correlated with HDL-C but they were not correlated with PON1 activity and gender-related differences were not observed. The correlation between PON1 activity and levels of circulating ox-LDL *in vivo* is debated. PON1 is not the only extracellular antioxidant enzyme in the blood. Other components associated to HDL such as platelet acetyl factor-hydrolase, lecithin-cholesterol acyltransferase and ApoA1 could have an impact in preventing formation of oxidized LDL in the circulation<sup>18</sup>.

## Conclusions

Based on data obtained, it is possible to confirm that a diet rich in fruit and vegetable antioxidants may improve plasma levels of carotenoids and the antioxidant status. Increased consumption of fruit and vegetables increases the plasma antioxidant capacity in both sexes. However, sex-specific differences in oxidative stress markers of lipid peroxidation have been observed and the protective effect exerted by dietary carotenoids can be found to a higher extent in females compared to males. Sex differences are present in many chronic diseases. Dietary recommendation towards a higher consumption of antioxidants should be highlighted in prevention and/or

treatment of diseases in which sex-related differences in oxidative stress play a considerable role.

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