RESEARCH ARTICLE

Polyphenol-rich foods exhibit DNA antioxidative properties and protect the glutathione system in healthy subjects

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Scope: Polyphenols (ingested via food items) can decrease DNA, and oxidative damage of proteins and lipids. However, polyphenol effects in healthy populations have not been well defined. The aim of this study was to assess the relationship between urinary total polyphenol excretion (TPE), a biomarker of total polyphenol intake (TPI), polyphenol-rich foods, and oxidative stress biomarkers in healthy adults of different ages participating in the cross-sectional PAScual MEDicina study.

Methods and results: Urinary TPE was determined by Folin–Ciocalteau method in spot urine samples of 81 participants (46 women), classified into three age groups: 18 to 39, 40 to 54, and 55 to 72 years of age. TPI was quantified from 3-day dietary records using the Phenol-Explorer database. Urinary TPE increased with age ($p < 0.001$). Urinary TPE was inversely associated with urinary 8-hydroxydeoxyguanosine (8-OHdG; $p<0.001$) and erythrocyte-oxidized glutathione concentrations ($p < 0.05$). A negative association between urinary 8-OHdG and daily intake of polyphenols from vegetables and fermented beverages such as red wine was observed.

Conclusion: Urinary TPE increased with age and may reflect attenuation of oxidative damage. These results could explain the beneficial effects in healthy individuals of a diet rich in vegetables and moderate red wine; food items typical of the Mediterranean diet.

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1 Introduction

Numerous epidemiological studies have shown several important links between diet and the occurrence of chronic diseases. Evidence is available to link several nutrients, minerals, food groups, and dietary patterns with an increased, or decreased, risk of diseases [1]. Oxidative stress is involved in the pathogenesis of several chronic diseases [2]. Oxidative stress

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Abbreviations: CAT, catalase; **CVD**, cardiovascular disease; **FRAP**, ferric-reducing ability of plasma; **GAE**, gallic acid equivalent; **GPx**, glutathione peroxidase; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **Nrf2**, nuclear factor erythroid 2-related 2; **8-OHdG**, 8-hydroxydeoxyguanosine excretion; **ORAC**, oxygen radical absorbance capacity; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances; **TPE**, total polyphenol excretion; **TPI**, total polyphenol intake

culminates due to an imbalance between prooxidants and antioxidants and a consequent excessive production of reactive oxygen species (ROS), which contribute to oxidative damage of lipids in cellular membranes as well as in proteins and DNA [3]. Endogenous antioxidant enzymes can prevent intracellular ROS concentrations from reaching levels at which damage occurs. Further, important exogenous dietary antioxidants such as some vitamins, selenium, or phytochemicals are also effective against oxidation and offer protection against oxidative damage and physiological benefit accrues [4]. However, the evidence from animal and in vitro studies to date is not sufficient to predict such an in vivo protective effect of food or food constituents in humans [5]. As such, the measurement of biomarkers of oxidative damage can be used to evaluate the effects of various dietary compounds, such as polyphenols.

Polyphenols are a heterogeneous group of naturally occurring molecules distributed in fruits, vegetables, nuts, seeds, wine, tea, cocoa, and coffee. Clinical and epidemiological studies provide evidence that polyphenol-rich foods and polyphenol-rich diets have protective effects against chronic diseases [6]. Polyphenols have a wide range of biological activities beyond their direct antioxidant activity. Various mechanisms have been proposed to explain their biological activity, including regulation of signal transduction and modulation of redox-sensitive transcription factors [7]. To establish the health-benefit effects of dietary polyphenol consumption, it is essential to have quantitative information regarding their dietary intake [8]. A realistic option is to assay total urinary phenols as a first approach to the study of the polyphenol intake-excretion balance. The data may, then, be related to measured physiological antioxidant phenomena and, hence, to the potential health benefits of dietary-derived polyphenols [8–10]. The 24 h urinary total polyphenol excretion (TPE) and spot urinary TPE have been validated in intervention studies as biomarkers of dietary polyphenol-rich food ingestion [8–10]. The level of protection provided by dietary polyphenols would differ, depending on the population at cardiovascular disease (CVD) risk. However, this has not been well defined in a healthy population nor has the urinary TPE been explored in relation to age.

We hypothesized that polyphenol-rich food consumption results in high levels of urinary TPE which, in turn, can be used as a marker of protection against oxidative damage conferred by polyphenol intake, in healthy participants of different ages. The objective of current study was to assess, in healthy human subjects segregated with respect to age, the relationships between urinary TPE, polyphenol-rich food intake, and biomarkers of oxidative stress.

2 Materials and methods

2.1 Subjects and design

The PAScual MEDicina (PASMED) study is a cross-sectional study aimed at defining the concentrations of circulating intermediate metabolites of various CVD risk biomarkers, and their distributions in healthy volunteers. The study was conducted in the Hospital Universitari Sant Joan de Reus (Spain) from March to July 2008 and from February to July 2009. Eligible subjects included men and women aged 18 to 75 years who were nonsmokers, with no other major CVD risk factors, not using any medications and/or vitamin supplements, and ostensibly healthy according to clinical history as well as physical and biochemical examination.

Participants provided written informed consent prior to enrolment into the trial and eligibility or exclusion was assessed by the attending physician, followed by a screening visit. The study protocol was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (08-01-31/proj1). The study protocol was in accordance with the Declaration of Helsinki of 1975 (revised 1983) and good clinical practice guidelines of the International Conference of Harmonization (ICH GCP).

2.2 Sample size

To calculate the sample size, we took as a reference the SD of LDL-cholesterol measurement. In our laboratory, this value is 21.47 mg/dL. The sample of 82 subjects provides an accuracy of $\pm$4.67 mg/dL LDL-cholesterol assuming an alpha risk of 0.95. We included at least ten participants (five women and five men) within each arbitrary 10-year age group, i.e. 18 to 29, 30 to 39, 40 to 49, 50 to 59, 60 to 69, and $>$70 years of age.

2.3 Measurements

2.3.1 Clinical history

All participants completed a detailed clinical history to provide sociodemographic data; family and personal history of illness; use of medications; level of ultraviolet exposure; and psychological, socioeconomic, and occupational status.

2.3.2 Physical examination

All data were collected by trained study personnel. Height and weight were measured while participants were wearing lightweight clothing and no shoes, using a calibrated balance and a well-mounted stadiometer, respectively. Waist circumference was measured as the point between the last rib and the iliac crest, at the end of normal exhalation. With the subject seated, blood pressure was measured three times at 1-min intervals using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain) and the mean value used in the statistical analyses.

2.3.3 Dietary intake and physical activity

Participants were provided with a questionnaire incorporating a 3-day 24-h food record (two work days and a holiday or weekend) and the Minnesota Leisure Time Physical Activity

Questionnaire validated in Spanish [11]. Trained dieticians explained how to complete these questionnaires.

2.3.4 Collection of blood and urine sample

Fasting blood samples were collected by venipuncture and the plasma (or serum) used to determine standard biochemistry analytes, biomarkers of oxidative stress, lipid profile, inflammation, endothelial dysfunction, antithrombotic activity, and insulin resistance. To minimize variability of analysis, the blood samples and supernatants of centrifuged spot urine samples were stored at –80°C in the central laboratory's Biobanc-REUS-IISPV (http://bancmb@grupsagessa.com) and thawed just prior to batched analyses.

2.3.5 Oxidative stress biomarkers

The extent of oxidative injury and the antioxidant capacity of plasma and enzymatic antioxidants were analyzed. Plasma was used to measure oxidized LDL by a monoclonal antibody-based immunoassay kit (Mercodia AB, Uppsala, Sweden); 8-isoprostan was analyzed using a commercial ELISA kit (Cayman Chemical Company, Ann Arbor, MI); 8-hydroxydeoxyguanosine (8-OHdG) was measured in urine using a competitive enzyme-linked immunosorbent assay (New 8-OHdG check, Japan Institute for the Control of Aging, Shizuoka, Japan) [12]. Carbonyl groups were measured by binding to dinitrophenylhydrazine and subsequent spectrophotometry [13]. Ferric-reducing ability of plasma (FRAP) was measured as the ability of plasma to reduce Fe^{3+} to Fe^{2+}. The Fe^{2+} forms a colored complex with 2,4,6-tripyridyl-s-triazine and which is measured by spectrophotometry [14]. Oxygen radical absorbance capacity (ORAC) and trichloroacetic acid ORAC ($ORAC_{TCA}$; plasma without protein) were measured as the antioxidant's capacity to scavenge peroxyl radicals of 2,2′-azobis(2-amidinopropane induced by dihydrochloride. The reaction was measured by fluorimetry [15]. Reduced glutathione (GSH) and oxidized glutathione (GSSG) in erythrocytes and plasma were also measured using fluorimetry [15] as were the levels of malondialdehyde and malondialdehyde-like substances (measured as thiobarbituric acid reactive substances; TBARS) [17]. Serum vitamin C was measured using a fluorimetric technique with a commercial ascorbic acid kit (Biovision Inc. San Francisco Bay Area, CA). The method used for the measurement of superoxide dismutase (SOD) activity in erythrocytes was based on the auto-oxidation of epinephrine, the product of which was measured by spectrophotometry [18]. Glutathione peroxidase (GP_x) in erythrocytes and plasma was determined via a spectrophotometric method that monitors the rate at which $NADP^+$ or NADPH is converted by the butyl hydroperoxide oxidation of GSH to GSSG and its subsequent reduction to GSH by glutathione reductase [19]. Catalase (CAT) in erythrocytes was measured via a spectrophotometric method based on the rate of hydrogen peroxide breakdown [20].

2.3.6 Determination of total polyphenol concentration in urine sample

To avoid degradation in the measurement of TPE, the urine samples were thawed in ice-water over 3 h. The analyses were performed immediately with the Folin–Ciocalteau method using an Oasis® MAX 96-well plate cartridge for solid phase extraction, as described by Medina-Remón et al. [9]. TPE was expressed as milligram of gallic acid equivalent (GAE) per gram creatinine. Creatinine measurement was used to adjust for variations in analyte concentration in urine. For analysis of creatinine in urine samples, the Jaffé alkaline picrate method was adapted to 96-well thermomicrotitre plates [9, 21].

2.3.7 Determination of total polyphenol intake

Total polyphenol intake (TPI) was quantified according to Phenol-Explorer database (http://www.phenol-explorer.eu) using the 3-day dietary record maintained by each participant.

2.4 Statistical analysis

Analyses were performed by available data only. Data were expressed as the mean and SD for variables with normal distribution. The geometric mean and antilog SD were used to describe log-transformed variables with normal distribution. The median and interquartile ranges were used to describe log-transformed variables that had nonnormal distribution. The Kolmogorov–Smirnov test was used to verify the distributions of the variables. The Mann–Whitney and Kruskal–Wallis tests were used for comparison of nonpaired and nonnormally distributed samples. ANOVA and Student *t*-tests were used for comparison of nonpaired and normally distributed samples. Pearson correlation coefficients were calculated for relationships between TPI and urinary TPE, between TPI and polyphenol from plant foods, between oxidative stress biomarkers and urinary TPE, and between oxidative stress biomarkers and TPI. Stepwise multivariate linear regression analyses were used to assess relationships between TPI and polyphenols from plant foods, between urinary TPE and oxidative stress biomarkers, and between polyphenols from plant food and oxidative stress biomarkers. The level of statistical significance was set at $p < 0.05$. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (17.0 version; IBM Corp., Armonk, NY, USA).

3 Results

3.1 Characteristics of subjects

From 117 eligible volunteers, 82 were enrolled in the PASMED study and, finally, data from 81 were analyzed. The

35 participants excluded during the screening process were because they did not fulfill all the inclusion criteria. The one volunteer excluded after enrolment was because the blood sample was not available. Thus, 81 participants entered the study; 46 women with a mean (SD) age of 44.6 (15.8) years and 35 men aged 42.3 (16.4) years. Participants were ostensibly healthy with no known CVD risk factors.

We classified our subjects into three age groups (18 to 39 years, 40 to 54 years, and 55 to 72 years) to evaluate the influence of age on urinary TPE and on oxidative stress biomarkers. Table 1 summarizes the mean values of oxidative stress biomarkers segregated according to age. In our study sample, the values of plasma-oxidized LDL, plasma 8-isoprostan, erythrocyte GSH, plasma GSSG, and plasma GSSG/GSH ratio significantly increased with age; mainly between the 18- to 39-year and the 40- to 54-year age groups. However, values of 8-OHdG, plasma ORAC, erythrocyte GSSG, erythrocyte GSSG/GSH ratio, plasma GSH, plasma FRAP, and erythrocyte GPx decreased significantly with age; especially between 18- to 39-year and 40- to 54-year age groups.

3.2 Urinary TPE and TPI

Overall, the mean (SD) total urinary TPE was 67.82 (1.83) mg GAE/g creatinine (range: 22.07 to 336.43). Segregated by gender, these values were 78.30 (1.68) and 55.84 (1.94) mg GAE/g creatinine in females and males, respectively ($p = 0.007$). Table 1 also summarizes the mean urinary TPE of study participants segregated according to age. A significant increase of TPE with age was observed ($p < 0.001$).

The mean daily TPI in the study population was estimated as 1564.56 (676.38) mg GAE/person/day (range: 422.02 to 3311.61 mg GAE/person/day). Segregated by gender, these values were 1600.24 (635.35) and 1496.61 (733.64) mg GAE/person/day in females and males, respectively. The differences were not statistically significant. TPI increased with age ($p < 0.001$). This variability in daily TPI among participants was also reflected in urinary TPE excretion. A significant Pearson correlation was observed between daily TPI and urinary TPE ($r = 0.281$, $p = 0.012$). Table 2 summarizes the plant food consumption and the polyphenol intake

Table 1. Oxidative stress biomarkers and urinary TPE segregated according to age groups

	18–39 years, $N = 34$	40–54 years, $N = 22$	55–72 years, $N = 25$	p^a	p^b	p^c	p^d
Plasma-oxidized LDL; U/L[a)]	55.45 (11.51)	75.87 (23.41)	86.03 (16.72)	<0.001	<0.001	<0.001	0.024
Plasma 8-isoprostan; pg/mL[b)]	1.61 (1.32)	3.59 (3.03)	4.54 (2.36)	<0.001	<0.001	<0.001	0.865
8-OHdG; ng/mg creatinine[b)]	85.69 (1.53)	22.57 (2.66)	25.63 (2.89)	<0.001	<0.001	<0.001	0.006
Carbonyl groups; nmol/mg protein[b)]	0.67 (1.76)	0.66 (1.69)	0.71 (1.83)	0.253	0.290	0.444	0.104
Plasma ORAC; μmol TE/mL[a)]	29.75 (5.89)	23 (7.75)	22.44 (14.12)	<0.001	0.001	<0.001	0.322
Plasma $ORAC_{TCA}$; μmol TE/mL[a)]	1.07 (0.44)	1.05 (0.29)	1.08 (0.27)	0.946	0.980	0.987	0.946
Erythrocyte GSH; μmol/g Hb[a)]	3.51 (0.88)	4.45 (1.2)	4.6 (0.75)	<0.001	0.003	<0.001	0.254
Erythrocyte GSSG; μmol/g Hb[a)]	0.74 (0.26)	0.57 (0.22)	0.51 (0.20)	0.001	0.027	0.001	0.653
Erythrocyte GSSG/GSH ratio[b)]	0.24 (1.76)	0.11 (1.61)	0.13 (1.67)	<0.001	0.001	<0.001	0.593
Plasma GSH; nmol/mL[c)]	9.06 (2.39)	7.35 (4.68)	4.39 (5.9)	<0.001	0.002	<0.001	0.862
Plasma GSSG; nmol/mL[a)]	22.58 (3.71)	33.37 (7.5)	37.34 (4.19)	<0.001	<0.001	<0.001	0.482
Plasma GSSG/GSH ratio[b)]	2.43 (1.50)	5.56 (2.11)	5.12 (2.76)	<0.001	<0.001	<0.001	0.006
Plasma TBARS; nmol/mL[a)]	1.01 (0.26)	0.97 (0.22)	1.07 (0.21)	0.365	0.856	0.624	0.380
Serum vitamin C; nmol/mL[a)]	291.65 (158.44)	245.24 (212.01)	352.98 (118.97)	0.075	0.287	0.039	0.091
Plasma FRAP; μmol TE/mL[a)]	0.56 (0.11)	0.50 (0.09)	0.45 (0.08)	<0.001	0.097	<0.001	0.249
Erythrocyte SOD; U/g Hb[a)]	1831.44 (722.72)	2004.95 (498.74)	1620.08 (271.33)	0.025	0.104	0.480	0.002
Erythrocyte GPx; U/g Hb[b)]	122.33 (1.30)	61.88 (1.32)	72.17 (1.41)	<0.001	<0.001	<0.001	0.241
Plasma GPx; U/L[b)]	159.42 (1.35)	148.42 (1.44)	149.10 (1.29)	0.038	0.034	0.034	0.633
Erythrocyte CAT; mmol/min/g Hb[a)]	234.63 (55.75)	237.59 (35.03)	252.31 (44.31)	0.347	0.974	0.372	0.572
Urinary TPE; mg GAE/g creatinine[b)]	46.14 (1.55)	69.7 (1.67)	110.12 (1.68)	<0.001	0.007	<0.001	0.016

Data are presented as a) mean (SD); b) geometric mean (antilog SD); c) median (IQR = interquartile range).
p^a: two-tailed test of significance between three age groups; p^b: two-tailed test of significance between 18- to 39-year-old group and 40- to 55-year-old group; p^c: two-tailed test of significance between 18-to 39-year-old group and 55- to 72-year-old group; p^d: test significance between 40- to 55-year-old group and 55- to 72-year-old group.

Table 2. Daily plant food intake and polyphenol intake from plant food sources segregated according to age groups

	Food intake; g portion/person/day						
Plant foods	18–39 years, $N = 34$	40–54 years, $N = 22$	55–72 years, $N = 25$	p^a	p^b	p^c	p^d
Cereals and tubers[a)]	190.85 (1.50)	243.85 (1.62)	219.16 (1.73)	0.597	0.310	0.868	0.482
Olive oil[b)]	41.00 (2.80)	45.64 (3.7)	41.76 (3.27)	0.583	0.590	0.960	0.779
Vegetables[b)]	208.05 (19.79)	302.75 (33.08)	302.52 (28.16)	0.012	0.040	0.044	0.992
Legumes[a)]	21.68 (2.32)	21.44 (3.29)	26.41 (2.34)	0.052	0.581	0.016	0.129
Fruits[b)]	230.77 (34.18)	449.93 (46.50)	421.66 (48.34)	<0.001	0.002	0.006	0.871
Nuts[a)]	6.43 (3.97)	10.71 (2.31)	9.11 (2.19)	0.302	0.135	0.592	0.302
Chocolate[a)]	10.26 (2.50)	7.51 (2.33)	7.10 (1.98)	0.006	0.076	0.002	0.153
Fruit juice[c)]	0.5 (66.67)	0.00 (66.67)	2.72 (79.17)	0.508	0.556	0.470	0.261
Coffee[b)]	76.03 (13.44)	149.17 (15.17)	88.39 (13.22)	0.002	0.003	0.608	0.057
Fermented alcoholic beverages[a)]	68.95 (2.57)	60.11 (10.62)	116.02 (2.20)	0.049	0.291	0.010	0.379
	Polyphenol intake; mg GAE/person/day						
Plant foods	18–39 years ($n = 34$)	40–54 years ($n = 22$)	55–72 years ($n = 25$)	p^a	p^b	p^c	p^d
Cereals and tubers[a)]	19.60 (2.46)	47.40 (2.07)	46.31 (2.30)	<0.001	0.001	0.001	0.790
Olive oil[b)]	20.82 (1.44)	23.99 (2.1)	22.12 (1.61)	0.405	0.410	0.783	0.823
Vegetables[b)]	246.70 (41.42)	367.49 (58.6)	409.31 (58.27)	0.071	0.254	0.099	0.922
Legumes[a)]	6.61 (3.38)	10.12 (4.55)	11.17 (3.67)	0.059	0.533	0.011	0.260
Fruits[b)]	428.03 (61.87)	769.12 (95.49)	650.07 (88.53)	0.009	0.012	0.140	0.570
Nuts[a)]	29.93 (4.62)	47.03 (2.63)	39.48 (2.40)	0.353	0.157	0.621	0.357
Chocolate[a)]	118.14 (2.45)	93.12 (2.76)	93.06 (2.26)	0.013	0.118	0.004	0.159
Fruit juice[c)]	0.9 (26.05)	0.00 (29.83)	4.89 (47.48)	0.508	0.587	0.412	0.287
Coffee[b)]	202.77 (35.85)	397.83 (40.47)	235.73 (35.25)	0.002	0.003	0.608	0.057
Fermented alcoholic beverages[a)]	51.32 (3.25)	71.23 (9.78)	135.48 (2.34)	0.021	0.299	0.003	0.324
Total polyphenols[b)]	1196.70 (90.45)	1967.90 (132.50)	1687.59 (140.19)	<0.001	<0.001	0.012	0.268

Data are presented as a) mean (SD); b) geometric mean (antilog SD); c) median (IQR = interquartile range).
p^a: two-tailed test of significance between three age groups; p^b: two-tailed test of significance between 18- to 39-year-old group and 40- to 55-year-old group; p^c: two-tailed test of significance between 18- to 39-year-old group and 55- to 72-year-old group; p^d: test significance between 40- to 55-year-old group and 55- to 72-year-old group. In fermented beverages category, red wine represents 73%, beer 8.8%, and cava/champagne 14.4% of total. GAE = gallic acid equivalent.

from plant food sources, segregated according to age. Vegetable and fruit consumption increased significantly between the 18- to 39- and 40-year to 54-year age groups ($p = 0.04$, $p = 0.002$) but no differences were observed between the 40- to 54-year and 55- to 72-year age groups. Coffee consumption increased significantly between the 18- to 39-year and 40- to 54-year age groups ($p = 0.003$) but it decreased again between the 40- to 54-year and 55- to 72-year age groups. Fermented beverage consumption was not significantly different between the 18- to 39-year and 40-to 54-year age groups, but a significant increase was observed starting with the 55- to 72-year age group ($p = 0.01$). We observed a significant decrease in chocolate consumption with age ($p < 0.006$).

Males consumed significantly more cereals and tubers ($p = 0.001$), olive oil (including virgin olive oil; $p = 0.048$), and fermented beverages than females ($p < 0.001$). Women consumed significantly more coffee than men ($p = 0.02$).

TPI was correlated with polyphenols from fruits ($p < 0.001$), vegetables ($p < 0.001$), coffee ($p < 0.001$), fermented beverages ($p = 0.013$), and olive oil ($p = 0.031$). Based on these Pearson correlations, we applied a stepwise multivariate linear regression analysis in which TPI was the dependent variable and polyphenol intake from fruits, vegetables, coffee, fermented beverages, and olive oil were the independent variables. Table 3 summarizes the standardized coefficients from stepwise multivariate model that showed that polyphenol intake from fruits is the largest contributor to TPI, followed by vegetables, coffee, and fermented beverages.

3.3 Urinary TPE and oxidative stress biomarkers

Stepwise multivariate linear regression analyses were performed with oxidative stress biomarkers as dependent variables and urinary TPE as principal independent variable. The models included other variables that can influence oxidation such as age, gender, physical activity, stress, ultraviolet exposure, and vitamin C intake as independent variables. As shown in Table 4, urinary 8-OHdG and erythrocyte GSSG exhibited an inverse association with urinary TPE. Other oxidative stress biomarkers tested showed no significant association. However, Pearson correlations showed significant negative relationships between urinary TPE and plasma FRAP

Table 3. Multivariate linear regression analysis with TPI (mg GAE/day) as the dependent variable and polyphenol intake from food sources as independent variables

Polyphenol source; mg GAE/day	β	SE	Beta	*p*	95% CI
From fruits	1.008	0.069	0.653	<0.001	0.869–1.146
From vegetables	819.457	80.396	0.460	<0.001	658.131–980.783
From coffee	0.751	0.160	0.209	<0.001	0.429–1.073
From fermented beverages	155.606	47.480	0.145	0.002	60.331–250.881

TPI = total polyphenol intake; β = nonstandardized coefficient (regression line coefficient); SE = standard error; Beta = standardized coefficient; *p* = two-side test of significance; CI = confidence interval; GAE = gallic acid equivalent.

($r = -0.432$, $p<0.001$), erythrocyte SOD ($r = -0.253$, $p < 0.05$), erythrocyte GPx ($r = -0.446$, $p < 0.001$), and plasma GPx ($r = -0.239$, $p<0.05$). However, the association was positive for GSH ($r = 0.257$, $p < 0.05$).

TPI, as assessed via the 3-day dietary record, showed a negative Pearson correlation with 8-OHdG and erythrocyte GSSG. However, there were no statistically significant associations with these oxidative stress biomarkers when assessed using multivariate linear regression analysis.

Stepwise multivariate linear regression analyses with oxidative stress biomarkers (urinary 8-OHdG and erythrocyte GSSG) as dependent variables and polyphenol intake from fruits, vegetables, coffee, and fermented beverages as independent variables were carried out. We chose these independent variables because they were those that showed a positive association with TPI. Urinary 8-OHdG exhibited an inverse association with polyphenol intake from vegetables ($\beta = -0.403$, $p = 0.006$, 95% CI = –0.687 to –0.120) and fermented beverages ($\beta = -0.209$, $p = 0.017$, 95% CI = –0.380 to –0.039). However, there were no statistically significant associations between polyphenol intake from plant food items and erythrocyte GSSG.

4 Discussion

The present study confirmed our hypothesis that, in healthy subjects of different ages, a high intake of dietary polyphenols would be reflected in a high urinary excretion, which would be related to changes in oxidative stress biomarkers as indicated by a significant inverse association with urinary 8-OHdG excretion and with erythrocyte GSSG concentrations.

Polyphenols can exert their effects, *in vitro*, by inactivating harmful free radicals, and by chelating divalent metal ions. They are also thought to prevent oxidation of food-associated lipids prior to consumption, which could reduce the prooxidant burden. However, it is suggested that polyphenols *in vivo* do not appear to be present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity, i.e. they are present in the circulation and tissues only in nano- to low-micromolar ranges and are predominantly present as conjugated metabolites. However, recent studies indicate that polyphenols may induce upregulation of endogenous antioxidant enzymes *in vivo* and exert an indirect antioxidant effect. Inductive or signaling effects may occur at concentrations much lower than required for effective radical scavenging [6, 22]. Polyphenols are capable of affecting two major redox-sensitive nuclear transcription factors, erythroid 2-related 2 (Nrf2) and Kappa B, which mediate antioxidant and inflammatory signaling [6, 22, 23]. Arola-Arnal et al. [24], proposed the influence of polyphenols on microRNA expression as a new mechanism of action. Hollman et al. [6], suggested that polyphenols should be considered as "versatile bioactive molecules" rather than mere antioxidants, and their role in gene transcriptional regulation need to be explored in more detail.

We observed that urinary 8-OHdG excretion was significantly, and inversely, associated with intake of polyphenols

Table 4. Multivariate linear regression analysis with oxidative stress biomarkers (urinary 8-OHdG and erythrocyte GSSG) as dependent variables and urinary TPE, age, gender, physical activity, stress, ultraviolet exposure, and vitamin C as independent variables

Variable	β	SE	Beta	*p*	95% CI
8-OHdG					
Age	−0.015	0.002	−0.511	<0.001	−0.020 to –0.011
Urinary TPE; mg GAE/g creatinine	−0.797	0.139	−0.444	<0.001	−1.074 to –0.521
Erythrocyte GSSG					
Age	−0.004	0.002	−0.232	0.071	−0.008 to 0.000
Gender; 1 = women; 2 = men	−0.196	0.059	−0.379	0.001	−0.313 to –0.079
Work and home stress	−0.062	0.028	−0.232	0.032	−0.118 to –0.005
Urinary TPE; mg GAE/g creatinine	−0.273	0.133	−0.276	0.044	−0.539 to –0.007

TPE = total polyphenol excretion; β = nonstandardized coefficient (regression line coefficient); SE = standard error; beta = standardized coefficient; CI = confidence interval; *p* = two-sided test of significance; GAE = gallic acid equivalent.

from vegetables and fermented beverages (mainly red wine). Thus, in healthy individuals, ingestion of some protective foods is associated with oxidative biomarker reduction even before these markers become relevant in assessing risk-factor status or in subclinical manifestations of CVD. However, the protective effects of polyphenols from foods would depend on their mechanisms of absorption, bioavailability, and metabolism [6, 25].

We need to take into account that assays for measuring oxidative stress have several shortcomings. Measurement of 8-OHdG is the commonest method of assessing DNA damage. Urinary 8-OHdG has been used to assess whole-body DNA damage. Measurements of urinary excretion rates of 8-OHdG alone should be interpreted with caution. Despite these limitations, 8-OHdG continues to be the most frequently studied biomarker in the measurement of endogenous oxidative DNA damage [26, 27]. Recently, an interventional study in young adult men showed that a moderate red wine intake significantly decreased 8-OHdG in DNA isolated from peripheral blood leukocytes [28, 29]. Di Wang et al. [29] also demonstrated that dietary supplementation with polyphenol extract from black tea was, possibly, a useful agent against functional disturbance caused by environmental xenobiotics. The mechanism proposed was through maintaining DNA double-helix architecture and mitigating oxidative stress. They suggested that the most plausible molecular mechanism was that tea polyphenols can up-regulate the activation of Nrf2.

Our present study demonstrated that age is related to changes of certain oxidative stress biomarkers. Urinary 8-OHdG was highest in the youngest subjects, and decreased with increasing age. Tamura et al. [31] also observed a significant inverse correlation between urinary 8-OHdG and age. However, their findings were based only on children and adolescents. Our results represent physiological changes associated with normal aging.

The glutathione system is an antioxidant system that helps to protect cells from ROS. We observed that individuals with higher TPE have lower levels of GSSG, which would indicate a greater capacity for detoxification of the glutathione system. In our study, concentrations of erythrocyte GSH also correlated positively and significantly with urinary TPE; albeit not statistically significantly on multivariate regression analysis. Urquiaga et al. [29] observed that moderate red wine consumption produced an increase in erythrocyte GSH. Also, Ya-Chen Y et al. [32] observed an induction of glutathione synthesis by flavonoids mediated via the Nrf2 pathway and protection against oxidative stress. Using Pearson correlation, we observed an inverse, and significant, association between urinary TPE and plasma FRAP, erythrocyte SOD, erythrocyte, and plasma GPx. In general, the antioxidant defense system seeks to maintain, or restore, redox homeostasis. To this end, endogenous enzymes and exogenous antioxidants function interactively and often synergistically [33]. The reduction of these enzymes could be a consequence of this attempt to regulate the body's redox balance. The studies of Covas et al. [34] and Estruch et al. [35] also reported that a steady consumption of a polyphenol-rich diet decreased scavenger enzymes such as SOD and GPx. The implication is that a regular diet rich in exogenous antioxidants could reduce the dependence on endogenous antioxidant defenses, probably by effects at the transcriptome level. Our data provide information on redox homeostatic regulation in a healthy population consuming its habitual diet. This is of considerable interest because, currently, there are data only on populations exposed to a high intake of polyphenols, or with a high degree of associated oxidative stress.

Our study also demonstrated that age, gender, and stress are related to erythrocyte GSSG. Further studies are needed to establish the mechanisms underlying these associations.

In the present study, the mean consumption of total polyphenol from all dietary constituents was estimated as 1564.56 mg GAE/person/day. TPI is documented as being higher than any other known dietary antioxidant, including dietary vitamin C, vitamin E, and carotenoid intake [36]. TPI, according to the observations of Medina-Remón et al. [36], ranged from 122.96 to 3298.17 mg/day in an elderly Mediterranean population considered at high risk of CVD. Also, Saura-Calixto et al. [37] estimated the TPI as 1171 mg/person/day in those consuming the Spanish Mediterranean diet. In our study participants, the mean consumption was 350.05 g/day fruits, 263.18 g/day vegetables, 103.00 g/day coffee, and 80.56 g/day fermented beverages and, in this ranking order, constituted the most important contributors to the TPI. Our results are similar to those of Medina-Remón et al. [36].

Of considerable note is our novel finding that age is a factor associated with the increase in TPE. This association was also observed by Medina-Remón et al. [36] but which had been established in an elderly population. Instead, the population we studied had a wide age range from 18 to 72 years. This enabled us to observe a significant increase in TPE with age. This association could be related to an increase in the consumption of vegetables, fruits, coffee, and moderate intake of fermented beverages with age, which are the greatest contributors to TPI. However, we observed that 40- to 54-year-old age group had a higher consumption of total polyphenol than the 55- to 72-year-old group while the urinary TPE excretion was higher in 55- to 72-year-old group. We observed that the 40- to 54-year-old group consumed more coffee but the 55- to 72-year-old group consumed more fermented beverages. Age alters the metabolism of common dietary polyphenols each of which has differing intrinsic activities, absorption, metabolism, and urinary elimination [38].

Our participants are healthy individuals with a low risk of CVD, and represent an optimal sample to identify relationships between polyphenols, diet, and oxidative stress. Such information represents an important phase in the understanding of the role of polyphenols in oxidative stress prevention and in optimizing dietary advice for the general population. Intervention studies would be necessary to confirm these intriguing data.

One limitation of our study is that urinary TPE is an indirect biomarker of overall circulating polyphenol status. Also, the study did not focus on clinical outcomes. Our objective was to assess, in general, the relationships between polyphenol-rich foods and oxidative stress biomarkers. To obtain this insight, we needed to determine the urinary TPE rather than individual polyphenols. However, as we have found associations between food groups and oxidative stress biomarkers, future studies are warranted to determine specific polyphenols and to identify specific biomarkers of each polyphenol-rich food. Further, their roles in signaling pathways need to be identified. Such information would be a valuable in future recommendations for dietary improvements and/or fortified food selection.

In conclusion, urinary TPE increased with age and may reflect an attenuation of oxidative damage. These results could potentially explain the beneficial effects in healthy individuals of a dietary intake rich in vegetables, and moderate red wine; typical food items of the Mediterranean diet.

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