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Daily consumption of a high-phenol extra-virgin olive oil reduces oxidative DNA damage in postmenopausal women

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Extra-virgin olive oils (EVOO), high in phenolic compounds with antioxidant properties, could be partly responsible for the lower mortality and incidence of cancer and CVD in the Mediterranean region. The present study aims to measure oxidative DNA damage in healthy human subjects consuming olive oils with different concentrations of natural phenols. A randomised cross-over trial of high-phenol EVOO (high-EVOO; 592 mg total phenols/kg) *v.* low-phenol EVOO (low-EVOO; 147 mg/kg) was conducted in ten postmenopausal women in Florence. Subjects were asked to substitute all types of fat and oils habitually consumed with the study oil (50 g/d) for 8 weeks in each period. Oxidative DNA damage was measured by the comet assay in peripheral blood lymphocytes, collected at each visit during the study period. Urine samples over 24 h were collected to measure the excretion of the olive oil phenols. The average of the four measurements of oxidative DNA damage during treatment with high-EVOO was 30 % lower than the average during the low-EVOO treatment ($P=0{\cdot}02$). Urinary excretion of hydroxytyrosol and its metabolite homovanillyl alcohol were significantly increased in subjects consuming high-EVOO. Despite the small sample size, the present study showed a reduction of DNA damage by consumption of an EVOO rich in phenols, particularly hydroxytyrosol.

Extra-virgin olive oil: Phenols: Hydroxytyrosol: Oxidative DNA damage: Cancer

In recent years, evidence has accumulated of a lower mortality and incidence of cancer and CVD in the Mediterranean area, with regard to the rest of Europe and other parts of the industrialised world (Owen *et al.* 2004). Various aspects of diet have been hypothesised as possible determinants of this effect, but the most characterising dietary habit in this area is the daily consumption of olive oil as the main fat used for seasoning and for food preparation. Other hypothesised health-enhancing ingredients, such as vegetables, fruit, whole grains, nuts, fish, and possibly red wine, are commonly consumed also in other parts of the Western world, and their benefit for reducing chronic diseases can be observed even within non-Mediterranean countries.

We know much less about the effects of different qualities of olive oils. This quality is defined by European regulations (European Commission, 2003), which state the requirements for each commercial type of olive oil: extra-virgin, virgin, olive oil, and finally olive-pomace oil. Even extra-virgin olive oils (EVOO) can differ in terms of their MUFA content, tocopherols, and a large variety of minor polar components, mainly phenols. The phenolic fraction includes hydroxytyrosol and its precursors that have potent antioxidant capacity due to their catecholic moiety (Montedoro *et al.* 1992*a*,*b*). It is noteworthy that olive oil phenols are absorbed and excreted by animals (Visioli *et al.* 2001) and man (Visioli *et al.* 2000*b*; Vissers *et al.* 2001). Researchers have focused their attention on the hypothesised reduction of oxidation of LDL by olive oil phenols, which could potentially reduce atherosclerosis; some results have been encouraging but others have not (Vissers *et al.* 2001; Gimeno *et al.* 2002; Moschandreas *et al.* 2002).

A possible role of hydroxytyrosol in protecting DNA from oxidative damage has been shown *in vitro* (Deiana *et al.* 1999) and only recently also *in vivo* (Weinbrenner *et al.* 2004). We therefore planned a pilot study to assess *in vivo*,

Abbreviations: EPIC, European Prospective Investigation into Cancer and Nutrition; EVOO, extra-virgin olive oil; high-EVOO, high-phenol extra-virgin olive oil; HVAlc, homovanillyl alcohol; low-EVOO, low-phenol extra-virgin olive oil.

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in subjects consuming their habitual diets, the antioxidant effects of an EVOO rich in naturally occurring phenols and compare it with a similar quality EVOO with a concentration of phenols in the lower range of what is commonly available on the Italian market.

Materials and methods

Study design

A randomised cross-over intervention trial of high-phenol EVOO (high-EVOO) *v.* low-phenol EVOO (low-EVOO) was planned, to evaluate their effects on oxidative DNA damage in healthy volunteers.

The present pilot study consisted of two intervention periods of 8 weeks each, the first in September–November 2002, and the second after an 8-week wash-out period, in January–March 2003. Participants were randomised to the sequence of consumption of the two study oils (high-EVOO in the first period and low-EVOO in the second period, or vice versa), and were asked to substitute all fats and oils usually consumed for seasoning and food preparation with the specifically selected study oils, and to consume at least 50 g daily of the assigned study oils in raw form in addition to the study oils necessary for cooking. Subjects were also asked to use the study oils when preparing the meals for other members of the family and when eating away from home. Therefore, study oils were provided at each visit in a quantity estimated to be sufficient for the whole family for a period of 2 weeks. Study participants and personnel were unaware of the treatment assignment. However, because of the very marked difference between the taste of a phenol-rich and a phenol-poor oil, at follow-up visits the study personnel had quite clear clues from volunteers concerning their study oil consumption. These perceptions were not disclosed to study subjects.

Apart from the fat substitution, subjects were instructed to stay on their habitual diet. Volunteers were seen at our clinic on ten occasions (at the baseline and thereafter every 2 weeks) for body measurements (height at the first visit, and weight, waist and hip circumferences), blood pressure and heart rate, blood drawing, 24 h urine collection and 24 h diet recall interview at the baseline and at each subsequent visit. In addition to these procedures, at the baseline visit subjects were asked to report their dietary and lifestyle habits by filling in the European Prospective Investigation into Cancer and Nutrition (EPIC) study self-administered food-frequency questionnaire and lifestyle questionnaire (Pala *et al.* 2003). At the last visit in each of the two periods (fifth and tenth visit), subjects were asked questions about the acceptance of the assigned treatment and about their participation in the trial.

During the whole study period, subjects were seen at the study clinic ten times; five times in the first study period and five times in the second. During the 8-week washout, they were asked to consume their habitual fats and oils.

Subjects

Flyers about the study were distributed and posted in our clinics and among women's health initiative groups. Selection criteria included being a healthy postmenopausal woman (no menstrual periods in the previous 12 months), non-smoker, with no history of treated hypercholesterolaemia and/or hypertension, no chronic use of aspirin or antioxidant vitamins, and without history of cancer, CVD, diabetes or other important chronic conditions. Criteria for eligibility also included residing in the Florence area, consuming most of the meals at home (no more than two meals per week outside home) and consuming less than two glasses red wine per d to avoid interference between the effects of red wine phenols and the olive oil phenols. Interested subjects were asked to contact us by telephone, and were asked specific questions checking for eligibility criteria. Out of thirty-five women who contacted us, thirteen were found eligible and twelve agreed to enter the trial. Selected women were invited to a meeting in which the study was described in detail, and printed material and informed consent forms were provided to the subjects. Individual appointments for the baseline visit were set up and volunteers were instructed to collect their urine during the 24 h before the visit. Three 1 litre, disposable containers were given for urine collection, together with a 1 ml vial of 40 μM-butylated hydroxytoluene to be poured into each container just before starting the urine collection. Subjects were requested to dispose of the first urine in the morning of the collection day and to collect subsequently each voided urine in the following 24 h, including the first urine on the following day. At each visit, subjects were given containers for the following urine collection.

Before starting the second phase of the trial (after 8 weeks washout), two women, both initially assigned to the low-phenol olive oil, discontinued their participation. Ten volunteers were therefore left for the analyses. The flow of study subjects is presented in Fig 1.

Dietary intake measurements

At baseline, dietary habits were measured by the EPIC food-frequency questionnaire. Frequencies of consumption of standard or specifically reported portions were transformed into weight (g) of daily consumed food and daily nutrient intakes (Pisani *et al.* 1997; Pala *et al.* 2003). In addition, at each of the ten visits, subjects were interviewed by the study dietitian about food and beverages consumed in the previous 24 h. Food consumption data were transformed into average daily consumption of food, beverages and nutrients, by means of the nutritional software Microdiet (University of Salford, UK). The software includes, in addition to UK food composition data, also the same Italian food tables applied to the EPIC food-frequency questionnaire (Salvini *et al.* 1998). The composition of the study oils was also entered into the software to allow estimation of daily intake of olive oil phenols during the two intervention periods.

Selection of study oils

Aiming to select a very high-phenol olive oil and one in the lower bound of possible concentrations, several batches of high-quality EVOO, originating from different parts of the Mediterranean basin, were selected and analysed. All selected oils were in line with commercial requirements for EVOO, showing very low acidity (range 0·1–0·8 %) and low total

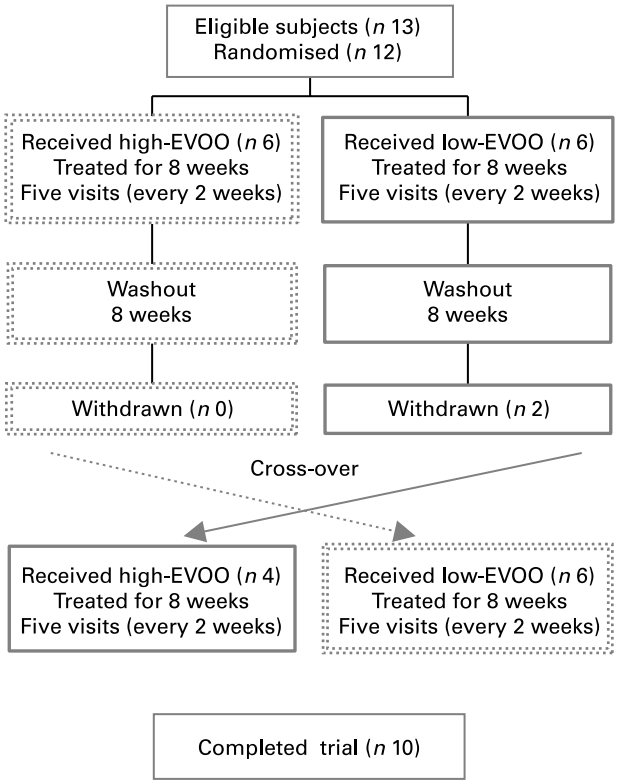

Fig. 1. Trial profile, summarising the flow of the study subjects. High-EVOO, high-phenol extra-virgin olive oil; low-EVOO, low-phenol extra-virgin olive oil. For details of subjects and procedures, see p. 743.

peroxides (1·8–12·3 meq O_2/kg). The concentration of MUFA ranged from 73·9 to 79·4 %, and α-tocopherol varied from 135 to 290 mg/kg. Total phenols ranged from 87 to 806 mg/kg and also the fraction of free hydroxytyrosol showed a very large variation (0·4–19 mg/kg). After organoleptic examination by study personnel to test the palatability of the samples, the final selection was made. A blend of Italian EVOO, predominantly from the southern Italian region of Puglia (mostly Coratina cultivar), was chosen as the high-phenol oil (total phenols 592 mg/kg), and a sample of a blend of EVOO mainly from the Liguria region was chosen as the low-phenol olive oil (total phenols 147 mg/kg). The main chemical characteristics of the selected olive oils are shown in Table 1.

To measure the total phenols in the study oils, extraction and HPLC–diode array detection–MS analysis were applied according to previous studies (Romani *et al.* 2001; Pinelli *et al.* 2003; Mulinacci *et al.* 2006*b*). The free and linked form of hydroxytyrosol were evaluated after the acidic hydrolysis of the phenolic olive oil fraction (Mulinacci *et al.* 2006*a*).

For the analysis of the fatty acids, a Fisons 8060 gas chromatograph equipped with a split-splitless injector and a flame ionisation detector was used. The fused silica column was an SP 2380, 30 m × 0·32 mm internal diameter, 0·20 μm film thickness (Supelco, Bellefonte, PA, USA). The column temperature was set at 165°C for 5 min, then programmed to 220°C at 5°C/min followed by 10 min isotherm. The injector and detector temperature was 220°C. The carrier gas (He) flow-rate was 0·6 ml/min. The fatty acids were analysed as methyl ester derivatives prepared by trans-esterification with a cold methanolic solution of potassium hydroxide (IUPAC standard method 2·301; IUPAC, 1987).

The tocopherols composition was determined by normal-phase HPLC with spectrofluorometric detection (Dieffenbacher & Pocklington, 1992). A Knauer K-501 pump equipped with a Jasco FP-1520 fluorescence detector (λ_{ex} 290 nm, λ_{em} 330 nm) and a silica column Spherisorb S5W, 250 mm × 4·6 mm internal diameter, 5 μm particle size was used. The elution was carried out in isocratic condition with *n*-hexane containing 1 % (v/v) isopropyl alcohol as the mobile phase. A sample of 250 mg oil was dissolved in 25 ml mobile phase in a volumetric flask before analysis.

Biological markers

Peripheral blood (30 ml) was collected from an antecubital vein at each visit. Blood was drawn in different vacutainers depending on the analyses to be performed. Samples for comet assay were sent within 3 h from collection to the appropriate laboratory. Additional samples drawn in EDTA and in lithium heparin were centrifuged at 3200 ***g*** for 20 min: plasma, buffy coat, and erythrocytes were separated into 1 ml vials and stored at −80°C in alarmed freezers until shipment to the appropriate laboratory or stored for future analyses. All analyses were carried out blinded to study identifiers by laboratory personnel.

Comet assay. One EDTA tube was brought within 3 h from collection to the laboratory for analyses of DNA damage. Lymphocytes were isolated utilising Lymphoprep separation medium (Gibco, UK). Blood samples of 3 ml were diluted 1:2 with PBS and layered on an equal volume of Lymphoprep medium in a centrifuge tube. After centrifugation at 1000 ***g*** for 20 min, gradient-separated lymphocytes were recovered, diluted 1:4 with PBS and centrifuged again at 1000 ***g*** for 10 min. The resulting cell pellets were re-suspended in PBS and counted in a Neubauer chamber.

Table 1. Composition of the selected study oils in terms of phenols and tocopherols (mg/kg) and fatty acids (%)

	Study oils	
	Low-EVOO	High-EVOO
Hydroxytyrosol (free form)	1·6	12·2
Tyrosol	2·6	14·6
Elenolic acid derivatives	2·4	6·7
Elenolic acid	13·5	47·9
3,4-DHPEA-EDA*	4·7	51·3
Total secoiridoids derivatives	114·8	351·4
Oleuropein aglycone	6·3	107·6
Luteolin	1·2	0·2
Total phenols	147·3	591·8
Total hydroxytyrosol†	15·4	157·3
α-Tocopherol	220·0	275·0
β-Tocopherol	1·1	2·0
γ-Tocopherol	1·4	11·9
Saturated fatty acids (%)	15·8	12·6
MUFA (%)	73·8	78·5
PUFA (%)	10·3	8·9

High-EVOO, high-phenol extra-virgin olive oil; low-EVOO, low-phenol extra-virgin olive oil.
* 3,4 Dialdehydic form of elenolic acid linked to hydroxytyrosol.
† Free and linked forms, evaluated after acidic hydrolysis.
For details of procedures, see p. 743.

Membrane integrity was assessed by the trypan blue exclusion method. The comet assay was used to measure both DNA breaks and oxidised bases. Samples of the lymphocyte suspension containing about 200 000 cells were further centrifuged at 250 ***g*** for 10 min, and the resulting pellets were re-suspended in low-melting point agarose, layered on microscopic slides and run through the comet assay as previously described (Giovannelli *et al.* 2003). Detection of oxidative DNA damage was carried out by means of the enzyme formamidopyrimidine DNA glycosylase, which introduces breaks at sites of oxidised purines such as 8-oxo-2′-deoxyguanosine.

The value of DNA damage (expressed as % DNA in the comet tail) obtained in slides without enzyme incubation estimated the basal number of DNA strand breaks, whereas specific DNA oxidative damage on purines was assessed for each subject by subtracting the percentage DNA in the comet tail found in the buffer-incubated slides from that obtained in the slides incubated with formamidopyrimidine DNA glycosylase.

Microscopic analysis was carried out by means of a Labophot-2 microscope (Nikon, Tokyo, Japan) provided with epifluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm). The images of fifty randomly chosen nuclei per slide were captured and analysed using a custom-made imaging software coupled with a CCD camera (model C5985; Hamamatsu, Sunayama-Cho, Japan). Each point was run in duplicate. Data were expressed as percentage DNA migrated in the tail, a parameter that is directly correlated with the number of breaks. Calibration of the method with γ-rays, which induce a known dose-dependent frequency of breaks, showed that 10 % DNA in the tail corresponds to about 1·3 breaks per 10^9 Da DNA.

In vitro *exposure to hydrogen peroxide*. In order to evaluate the response of lymphocytes to oxidative stress, samples of the isolated cells were exposed to H_2O_2 *in vitro*. Immediately after isolation, 400 000 cells from each subject were re-suspended in 1 ml PBS containing 25 μM-H_2O_2. This concentration of H_2O_2 was chosen on the basis of preliminary experiments in which the effect of different doses had been evaluated. The rationale for this choice was that the 25 μM concentration induced an increase in DNA damage, which was significant (+150 % above basal levels) and at the same time not overly strong, so that even weak protective effects of olive oil phenols could be detected. The incubation with H_2O_2 was conducted for 5 min at 4°C to inhibit DNA repair. After completing the incubation, the lymphocytes were centrifuged at 250 ***g*** for 10 min, and the resulting pellets were re-suspended in low-melting point agarose (two slides for each experimental point) to be run through the comet assay as described earlier. Only DNA strand breaks were measured in this set of experiments.

Plasma antioxidant capacity. The antioxidant capacity of plasma was evaluated according to a method based on the inhibition of the radical cation 2-2′-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS®) formation (Randox Laboratories Ltd, Crumlin, Co. Antrim, UK) (Miller *et al.* 1993).

Olive oil phenolics in human urine. At each visit, the 24 h urine samples collected by the volunteers and stabilised with butylated hydroxytoluene were weighed, then mixed together if collected in more than one container, and finally sampled in fifteen 10 ml vials and frozen at −20°C. Two vials for each subject were shipped in dry ice to the appropriate laboratory. For the quantification of total simple phenols in urine, 360 units β-glucuronidase (Sigma, St Louis, MO, USA) were added to 1 ml urine after thawing and incubated overnight at 37°C and pH 5. At the end of incubation, each sample was added with ^{2}H-labelled hydroxytyrosol (0·5 μg/ml) and α-naphthole (1 μg/ml), as internal standards for hydroxytyrosol and for its metabolite homovanillyl alcohol (HVAlc), respectively. After acidification with 0·3 M-HCl–acetonitrile (1:1, v/v), urine was extracted twice with three volumes of ethyl acetate; the organic phase was evaporated to dryness under N_2. The residue was derivatised with a mixture of bis-trimethylsilyl-trifluoro acetamide–pyridine (4:1, v/v) before the GC–MS analysis. Calibration curves were prepared using 1 ml samples of urine that did not contain the compounds under investigation. These samples were spiked with ^{2}H-labelled hydroxytyrosol (0·5 μg/ml) and α-naphthole (1 μg/ml) and increasing amounts (10–2000 ng/ml) of authentic hydroxytyrosol and its metabolites; each sample was then extracted and analysed, as already described. GC–MS analyses were performed on a CP-Syl8 fused silica capillary column (Varian Inc., Palo Alto, CA, USA) connected with a GCQ mass spectrometer (ThermoQuest, Petterson, CA, USA). Ions at *m/z* 216 for α-naphthole, at *m/z* 312 for HVAlc, 370 and 372 for natural and ^{2}H-labelled hydroxytyrosol respectively, were recorded. These ions are selected after mass spectra obtained from authentic standards (Caruso *et al.* 2001).

Routine examinations. At the first and last visits of each of the two treatment periods, a sample was also drawn for routine laboratory examination. These samples were assayed by standard procedures at the central laboratory of the Careggi Hospital (Florence, Italy).

Statistical analysis

Descriptive statistics were calculated to provide general information on the sample of volunteers.

To evaluate the effect of treatment, of period and to test the presence of carry-over, responses obtained during the first period (September–November 2002) were compared with those obtained in the second period (January–March 2003), considering the sequence of the treatment, i.e. 'low–high' corresponding to low-EVOO in the first period and high-EVOO in the second period and 'high–low' corresponding to high-EVOO in the first period and low-EVOO in the second period.

Indicated by y_{ijk}, the mean of the log-transformed values of the four responses observed on the kth subject in the period $j = 1, 2$ of group $i =$ Low, High, we used the usual parameterisation, based on Jones & Kenward (1989), $y_{ijk} = \mu + s_{ik} + \pi_j + \tau_i + \lambda_{ij-1} + \epsilon_{ijk}$, where π_j $(j = 1, 2)$ are the period effects; τ_i $(i =$ Low, High) are the direct treatment effects, $\lambda_{L,1}$ and $\lambda_{H,1}$ are the carry-over effect parameters; s_{ik} are the random subject effects with mean 0 and variance σ_s and ϵ_{ijk} are white-noise random error.

Exact non-parametric tests for two-period cross-over trials were applied (Jones & Kenward, 1989). Two-sample exact Wilcoxon rank-sum tests were applied to assess, for each outcome, the hypothesis of equality of carry-over effect H_0: $\lambda_1 = \lambda_2$, treatment effect H_0: $\tau_L = \tau_H \mid \lambda_1 = \lambda_2$ and period effect H_0: $\pi_1 = \pi_2 \mid \lambda_1 = \lambda_2$ and to derive point estimates

with 95 % CI for the exponential of the differences on treatment (the term $\exp(\tau_H = \tau_L)$ gives the geometric mean ratio by treatment group) and period (the term $\exp(\pi_1 - \pi_2)$ gives the geometric mean ratio by period). All analyses were carried out with STATA 7 (Stata Statistical Software release 7; StataCorp LP, College Station, TX, USA).

Ethical considerations

The study was approved by the Florence Health Authority's Ethics Committee. All study procedures conformed with the Declaration of Helsinki for medical research involving human subjects, as revised in 1983.

Results

The main baseline characteristics of study volunteers are shown in Table 2. The age range was 47–67 years; the average BMI was borderline normal (mean 25·1 (range 20·7–27·8) kg/m^2). The average daily energy intake was 9221 (SD 4224) kJ (2204 (SD 1015) kcal), and was characterised by a 32·9 % energy intake from fats, with olive oil as the most frequently consumed added fat (32·8 g/d *v.* 0·2 g seed oils, 0·6 g margarine and 0·6 g butter and 1·1 g other animal fats). Biochemical data measured at the baseline showed a mean cholesterol level of 5·88 (range 4·84–7·33) mmol/l. No statistically significant differences were observed between sequence of treatment for any of the considered variables (data not shown).

Table 3 shows the average values of the outcomes of interest, at the baseline and during high-EVOO and low-EVOO treatment. The intake of total olive oil phenols, as estimated by the repeated 24 h recalls (four measurements per subject during high-EVOO and four during low-EVOO), was approximately four times higher during the treatment with high-EVOO (mean 34·9 (SD 11·0) mg/d) as compared with the low-EVOO treatment (mean 9·7 (SD 3·4) mg/d), although the consumption of the study oil tended to be higher during the low-EVOO treatment (64·4 (SD 22·5) *v.* 53·3 (SD 16·1) g/d). Intake of free hydroxytyrosol was seven-fold during the high-EVOO. Baseline urinary excretion of hydroxytyrosol and of its metabolite HVAlc demonstrates that this Tuscan sample of women is habitually exposed to olive oil phenols: hydroxytyrosol excretion ranges from 116 to 3146 μg/d, and HVAlc from 25 to 212 μg/d. The increased intake of olive oil phenols affected the excretion of hydroxytyrosol and its metabolite HVAlc. Fig 2 shows the geometric means of cumulative urinary excretion of hydroxytyrosol and HVAlc, by treatment sequence and by period. Treatment and period effects were tested by exact non-parametric tests for two-period cross-over trials, on log-transformed data, and results are summarised in Table 4. The ratio of geometric means of the measurements showed a statistically significant increased

Table 2. Characteristics of the ten postmenopausal women at the baseline visit

(Mean values and standard deviations)

	Mean	SD	10th Percentile	90th Percentile
Age (years)	57·1	5·9	50·4	66·0
Weight (kg)	60·9	9·2	49·0	71·1
Height (cm)	155·3	8·8	1·42	1·66
BMI (kg/m^2)	25·1	2·2	21·8	27·6
Waist:hip ratio	0·8	0·1	0·76	0·90
Systolic blood pressure (mm/Hg)	122·0	14·9	107·5	145·0
Diastolic blood pressure (mm/Hg)	72·0	6·3	62·5	80·0
Pulse rate (frequency/min)	64·6	4·6	57·5	70·0
Average daily dietary intake*				
Energy intake				
KJ	9221	4244	4487	15 754
Kcal	2204	1015	1073	3543
Protein (% total energy)	15·6	2·3	12·5	18·9
Carbohydrates (% total energy)	46·4	5·4	41·0	57·0
Fats (% total energy)	32·9	4·6	26·3	38·2
Vitamins				
Vitamin C (mg)	159·7	72·4	82·9	269·1
Vitamin E (mg)	8·0	3·5	3·5	12·2
β-Carotene (μg)	4634·8	2701·8	1848·5	8853·6
Folates (μg)	307·8	129·2	168·0	485·0
Alcohol (g)	17·0	11·0	2·6	27·4
Added fats (g/d)				
Olive oil	32·8	19·4	11·1	61·6
Seed oils	0·2	0·1	0·1	0·4
Margarine	0·6	1·1	0·1	2·1
Butter	0·6	0·8	0·1	2·0
Other animal fat	1·1	1·1	0·0	2·6
Baseline blood levels				
Total cholesterol (mmol/l)	5·88	0·77	4·93	6·95
HDL-cholesterol (mmol/l)	1·92	0·38	1·46	2·49
LDL-cholesterol (mmol/l)	3·41	0·66	2·49	4·13
Triacylglycerol (mmol/l)	1·20	0·61	0·73	2·15
Glucose (mmol/l)	4·44	0·56	3·33	5·00

*Data obtained from the food-frequency questionnaire administered at the baseline visit.

For details of subjects and procedures, see p. 743.

Table 3. Summary of intakes, excretion, and biomarkers at baseline, and during high-phenol extra-virgin olive oil (high-EVOO) and low-phenol extra-virgin olive oil (low-EVOO) treatment

(Mean values and standard deviations)

	Baseline (*n* 10)		High -EVOO (*n* 40)		Low-EVOO (*n* 40)	
	Mean	SD	Mean	SD	Mean	SD
Intake estimates from 24 h diet recalls						
Olive oil (g/d)*	24·5	8·8	53·3	16·1	64·4	22·5
Total olive oil phenols (mg/d)	–†	–	34·9	11·0	9·7	3·4
Hydroxytyrosol (free form) (mg/d)	–†	–	0·7	0·3	0·1	0·1
Total hydroxytyrosol (free and linked forms) (mg/d)	–†	–	8·4‖	–	1·0‖	–
24 h urine excretion						
Hydroxytyrosol (μg/d)	682·0	924·6	1347·1‡	1007·2	880·1§	1451·7
Homovanillyl alcohol (μg/d)	73·8	54·6	444·1‡	509·0	150·7§	128·3
Study biomarkers						
Oxidised DNA bases (% DNA in comet tail)	11·9	7·67	5·6‡	5·1	6·5	5·1
Basal DNA breaks (% DNA in comet tail)	12·9	7·0	5·1‡	1·5	5·0	2·3
Plasma antioxidant capacity (mmol/l)	0·9	0·3	0·9‡	0·5	0·8	0·5

* Olive oil consumption is slightly underestimated since the study oil included in some home prepared recipes could not be included in this figure, but contributed to the estimate of phenol intake.
† No estimates of average phenol concentration in commonly consumed olive oils were available.
‡ *n* 39.
§ *n* 37.
‖ Estimated from average olive oil consumption and concentration of free and linked hydroxytyrosol in the study oils.
For details of subjects and procedures, see p. 743.

excretion of hydroxytyrosol (treatment effect 2·17; $P=0·01$), HVAlc (treatment effect 3·08; $P=0·019$), and total combined hydroxytyrosol and HVAlc excretion (treatment effect 1·94; $P=0·01$). Phenol excretion was not affected by period, and no carry-over was detectable for any of the outcomes (data not shown).

Fig 3 shows the geometric mean ratios of measured DNA oxidative damage by treatment sequence (high-EVOO *v.* low-EVOO) during the intervention period. Measurements were taken every 2 weeks. Although the effect was not constant over the treatment period, individual differences showed a reduction of damage mainly in the fourth and fifth measurement time (i.e. after 6 and 8 weeks of treatment).

Overall, the average of the four measurements during treatment with high-EVOO was always lower than the average during the low-EVOO treatment (Fig 4). The non-parametric tests (Table 4) showed a decreased DNA damage, with a 30 % significant reduction of oxidised bases, expressed as the ratio of the geometric means of the repeated measures (treatment effect 0·70; 95 % CI 0·56, 0·86; $P=0·019$); a non-significant period effect (lower damage in the winter period) was also shown. Restricting the analyses on measurements taken after 6 and 8 weeks, an even stronger result could be observed (treatment effect 0·53; 95 % CI 0·33, 0·76; data not shown). DNA breaks were not affected by treatment ($P=0·352$), but were strongly influenced by study period, with lower breaks in January–March (period effect 0·62; $P=0·01$). Treatment did not affect plasma antioxidant capacity, but this measure was also affected by period (period effect 0·64; $P=0·01$).

The response of lymphocytes to *in vitro* induced oxidative stress was evaluated at the first and fifth measurement time in both study periods (i.e. at baseline and after 8 weeks of treatment). No difference in the amount of DNA breakage induced by H_2O_2 was found between high-EVOO and low-EVOO treatment (data not shown).

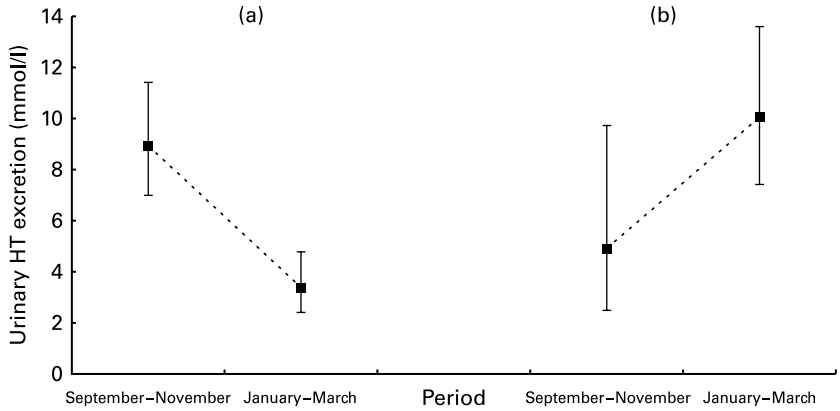

Fig. 2. Total combined urinary excretion of hydroxytyrosol and homovanillyl alcohol (mmol/l) in ten volunteer subjects by period and treatment sequence. (a) High-phenol extra-virgin olive oil (high-EVOO) in the first period and low-phenol EVOO (low-EVOO) in the second period; (b) low-EVOO in the first period and high-EVOO in the second period). Values are geometric means, with 95 % CI represented by vertical bars. For details of subjects and procedures, see p. 743.

Table 4. Summary of treatment and period effects during high-phenol olive oil (high-EVOO) and low-phenol olive oil (low-EVOO) intervention

(Geometric mean ratios and 95 % confidence intervals)

	Treatment effect	95 % CI	Period effect	95 % CI
Intake estimates from 24 h diet recalls				
Total olive oil phenols intake (mg/24 h)	3·56*	3·01, 4·20	0·99	0·83, 1·14
24 h urine excretion				
Hydroxytyrosol excretion (μg/24 h)	2·17*	1·33, 5·54	0·86	0·61, 1·71
Homovanillyl alcohol excretion (μg/24 h)	3·08*	1·49, 5·10	1·63	0·74, 2·74
Hydroxytyrosol and homovanillyl alcohol excretion (mmol/l)	1·94*	1·39, 4·06	0·84	0·50, 1·62
Study biomarkers				
Oxidised DNA bases (% DNA in comet tail)	0·70*	0·56, 0·86	0·77	0·63, 1·02
Basal DNA breaks (% DNA in comet tail)	0·96	0·84, 1·08	0·62*	0·53, 0·70
Plasma total antioxidant capacity (mmol/l)	1·09	0·94, 1·24	0·64*	0·55, 0·72

* $P<0·05$; exact non-parametric tests; Jones & Kenward (1989).
For details of subjects and procedures, see p. 743.

Discussion

Olive oil is the characterising ingredient of diets in the Mediterranean basin and the hypothesis that high intakes of olive oil could reduce cancer incidence is appealing (Willett 1997; Simopoulos, 2004; Visioli *et al.* 2004). Despite the limited number of subjects, the present cross-over trial was able to show a reduction in DNA oxidative damage in postmenopausal women consuming an EVOO with a high concentration of total phenols, and of the potent antioxidant hydroxytyrosol. In the high-EVOO the total content of hydroxytyrosol, both free and linked, was about ten-fold higher than in the low-EVOO.

The detection of phenols in EVOO is quite recent (Montedoro *et al.* 1992*a*,*b*) and various researchers have focused their attention on their effects (Visioli *et al.* 1999; Pirisi *et al.* 2000; Romani *et al.* 2001; Conte *et al.* 2002; Pinelli *et al.* 2003; Mulinacci *et al.* 2005*b*). Phenols comprise a large family of molecules and the most abundant in EVOO are tyrosol, hydroxytyrosol, and other complex secoiridoids, sharing the hydroxytyrosol or tyrosol nucleus. They are the major determinants of sensorial characteristics of EVOO and their concentration in the oil is related to several factors: cultivar and environmental characteristics, ripeness of the fruit, and time and type of milling process. Hydroxytyrosol has powerful antioxidant potentials (Papadopoulos & Bosku, 1991), protects LDL from oxidative damage (Visioli *et al.* 1995; Caruso *et al.* 1999; Ramirez-Tortosa *et al.* 1999; Fito *et al.* 2000) and decreases F_2-isoprostanes excretion (Visioli *et al.* 2000*a*). However, the effect on lipid oxidation was evidenced by some (Gimeno *et al.* 2002; Marrugat *et al.* 2004; Weinbrenner *et al.* 2004) but not all intervention trials (Vissers *et al.* 2001; Moschandreas *et al.* 2002).

The focus of our intervention was the effect of hydroxytyrosol on DNA oxidation, since a reduction of DNA oxidative damage is a mechanism underlying possible cancer prevention by olive oil. Mammalian cells are continuously attacked by oxygen radicals and such damage, if not appropriately counteracted, contributes to generation of DNA damage and somatic cell mutation that could direct the cell to a cancerous transformation (Halliwell, 2000; Marnett, 2000; Neumann *et al*. 2003; Saran *et al.* 2004). Deiana *et al.* (1999) showed an inhibition of the peroxynitrate-dependent DNA base modification *in vitro*. An *in vitro* study (Quiles *et al.* 2002) showed a lower DNA damage exerted by hydroxytyrosol on human prostate cells. A recent trial with three olive oils with different concentration of total phenols showed an effect on the oxidant–antioxidant status of human cells. In particular, 8-oxo-2′-deoxyguanosine in mitochondrial DNA was reduced by the intake of olive oil phenols in men consuming a very-low-antioxidant diet (Weinbrenner *et al.* 2004). Additionally,

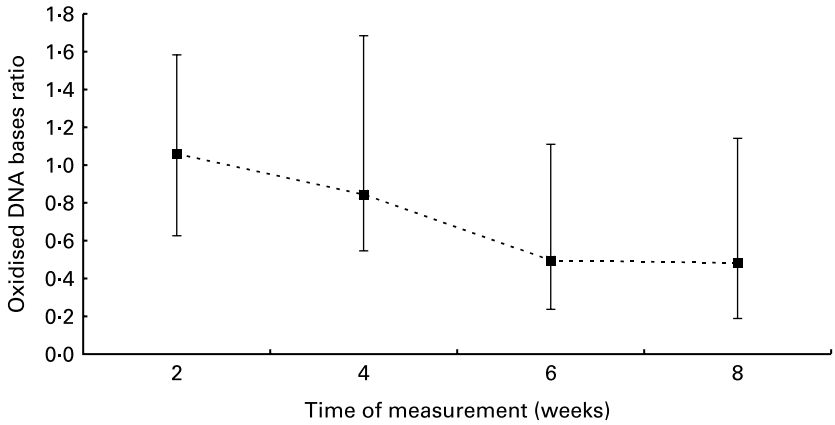

Fig. 3. Oxidised DNA bases in ten volunteer subjects, as measured by the comet assay: between high-phenol extra-virgin olive oil *v.* low-phenol extra-virgin olive oil treatment period, measured every 2 weeks. Values are geometric mean ratios, with 95 % CI represented by vertical bars. For details of subjects and procedures, see p. 743.

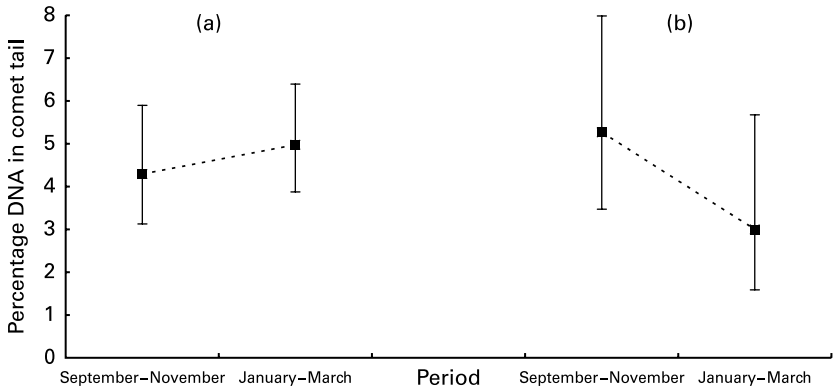

Fig. 4. Oxidised DNA bases (% DNA in comet tail) by period and treatment sequence. (a) High-phenol extra-virgin olive oil (high-EVOO) in the first period and low-phenol EVOO (low-EVOO) in the second period; (b) low-EVOO in the first period and high-EVOO in the second period. Values are geometric means, with 95 % CI represented by vertical bars. For details of subjects and procedures, see p. 743.

in a sample of 100 volunteers randomly selected from a large prospective study conducted in Europe – the EPIC Study (Palli *et al.* 2000) – a negative association was observed between amounts of olive oil consumption and DNA adduct formation.

As a measure of DNA damage we used single-cell microgel electrophoresis, or comet assay; this is a very sensitive method initially developed as a modification of the alkaline elution technique for the study of single- and double-strand DNA breaks (Singh *et al.* 1988). It involves embedding individual cells in agarose on a microscope slide and measuring the degree of migration of nuclear DNA upon electrophoresis in an alkaline buffer. The extent of migration is proportional to the number of breaks in the DNA molecule. A modification of the method has been described, that allows the detection of specific lesions on DNA, such as oxidation of either purines or pyrimidines, by the addition of endonucleases cleaving DNA at damaged sites (Collins *et al.* 1993). Thus, it is possible with the comet assay to study both DNA breaks and specific oxidative damage at the single-cell level. This procedure has been used to study basal oxidative damage in animal models (Giovannelli *et al.* 2000) and in different tissues and cells, including human lymphocytes (Pool-Zobel *et al.* 1997; Collins *et al.* 1998). In the present study, DNA bases were shown to be protected by olive oil phenols, although the result was not evident for DNA strand breaks.

The present study evidenced also a statistically significant period effect, when observing DNA breaks. These were higher at the first baseline, i.e. in the period immediately following the summer months, an association that is probably the result of exposure to UV radiation, as was also recently shown in other studies from our group (Giovannelli *et al.* 2002, 2005).

Compliance is a critical aspect of intervention trials. Subjects recruited for the present study were women in their fifties and sixties, a population group that, in Italy, tends to consume their meals at home and to be personally responsible for food preparation. In fact, the study results show a very strong compliance to the randomly assigned treatment; olive oil consumption doubled with regard to baseline estimates, resulting in a remarkable increase of urinary excretion of hydroxytyrosol and its metabolite, even in subjects assigned to the low-EVOO group. Although baseline excretion clearly demonstrates a chronic exposure to EVOO, their quality, in terms of phenol concentration, is currently unknown. The availability of a marker of olive oil phenol intake is particularly important in current research. Large epidemiological studies, such as the EPIC study, currently conducted in ten European countries, offer the possibility of measuring markers of dietary intakes of food, nutrients and even contaminants. Biological samples for almost half a million subjects across Europe were collected at the baseline. Such samples could even be used to characterise dietary patterns in different Mediterranean and European populations, not only in terms of fruit and vegetables, using circulating carotenoids as markers of intake (Al-Delaimy *et al.* 2005), but also in terms of types and quality of ingested olive oils.

Epidemiological studies based in the Mediterranean have shown a protection from cancer at higher levels of intake of olive oil (Martin-Moreno *et al.* 1994; La Vecchia *et al.* 1995; Trichopoulou *et al.* 1995; Braga *et al.* 1998; Franceschi *et al.* 1999). Unfortunately, due to the limited consumption of this type of fat in non-Mediterranean countries, such results have not been replicated and confirmed, to date, in other parts of Europe or of the Western world. Moreover, questionnaires so far adopted in such epidemiological studies have not allowed the exploration of the different roles of different olive oils types (i.e. olive oil *v.* EVOO). Most studies were designed when little was known about phenols in olive oils available on the market and not much detail was included in questionnaires. This could probably explain why Greek researchers (Trichopoulou *et al.* 2003) did not find a significantly protective effect of olive oil (subjects could be consuming a larger proportion of olive oil than EVOO), despite the general strong protection of the Mediterranean diet index evidenced by their analyses based on the Greek component of the EPIC study. Recently, re-interviewing approximately 2000 women from the Florence EPIC study, we observed that EVOO are used by most Florentine women for cooking and seasoning (G Masala and D Palli, unpublished results). This epidemiological finding is confirmed by the present intervention trial, showing a sustained excretion of hydroxytyrosol and of its metabolite HVAlc in baseline 24 h urine samples.

Two batches of EVOO, instead of simple olive oils with fixed amounts of phenols added, or, more extremely, *ad hoc* prepared supplements with known concentration of the phenols of interest, were used as treatment in the present study.

These alternative possibilities had been considered and discarded. First of all, we wanted as much as possible to approximate a 'real-life' situation. The Tuscan population is normally 'exposed' to olive oil and it is used to the consumption of high-quality EVOO. If we had supplemented a simple, non-EVOO with known quantities of antioxidants, subjects would have been forced to consume this low-quality olive oil for two periods of 8 weeks (about 2 months) each and we thought this approach was not ethical. We also decided against the use of capsules with hydroxytyrosol and other phenols, being interested in the effect of a complex mixture of phenols from real food. Moreover, a higher bioavailability of hydroxytyrosol as a natural component of EVOO was demonstrated by previous work; a higher recovery of hydroxytyrosol was observed when free hydroxytyrosol was administered as EVOO, as compared with the recovery measured when the vehicle of administration of free hydroxytyrosol was a refined olive oil or a yoghurt (Visioli *et al.* 2003).

Our finding that EVOO phenols protect from oxidative damage in healthy postmenopausal women needs to be reproduced in larger intervention studies on subjects of both sexes, in different age groups, and possibly also in populations not chronically exposed to EVOO, but offers, *in vivo*, new and strong evidence of the protection exerted by high-phenol olive oils in human subjects.

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