Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans

Anja Machowetz,[*,1] Henrik E. Poulsen,[†,1,2] Sindy Gruendel,[*] Allan Weimann,[†] Montserrat Fitó,[‡] Jaume Marrugat,[‡] Rafael de la Torre,[§] Jukka T. Salonen,[‖] Kristiina Nyyssönen,[‖] Jaakko Mursu,[‖] Simona Nascetti,[¶] Antonio Gaddi,[¶] Holger Kiesewetter,[††] Hans Bäumler,[††] Hany Selmi,[††] Jari Kaikkonen,[‡‡] Hans-Joachim F. Zunft,[*] Maria-Isabel Covas,[‡] and Corinna Koebnick[*]

[*]German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany; [†]Department of Clinical Pharmacology, Rigshospitalet, Copenhagen, Denmark; [‡]Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d'Investigació Mèdica, Barcelona, Spain; [§]Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica, Barcelona, Spain; [‖]Research Institute of Public Health, University of Kuopio, Kuopio, Finland; [¶]Centro per lo Studio dell'Arteriosclerosi e delle Malattie Dismetaboliche "GC Descovich," Dipartimento di Medicina Clinica e Biotecnologia Applicata, Policlinico S. Orsola-Malpighi, Bologna, Italy; [††]Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Germany; and [‡‡]Oy Jurilab, Kuopio, Finland

ABSTRACT **High consumption of olive oil in the Mediterranean diet has been suggested to protect DNA against oxidative damage and to reduce cancer incidence. We investigated the impact of the phenolic compounds in olive oil, and the oil proper, on DNA and RNA oxidation in North, Central, and South European populations. In a multicenter, double-blind, randomized, controlled crossover intervention trial, the effect of olive oil phenolic content on urinary oxidation products of guanine (8-oxo-guanine, 8-oxo-guanosine and 8-oxo-deoxyguanosine) was investigated. Twenty-five mililiters of three olive oils with low, medium, and high phenolic content were administered to healthy males (n=182) daily for 3 wk. At study baseline the urinary excretion of 8-oxo-guanosine (RNA oxidation) and 8-oxo-deoxyguanosine (DNA oxidation) was higher in the Northern regions of Europe compared with Central and Southern European regions (P=0.035). Urinary excretion of the 8 hydroxylated forms of guanine, guanosine, deoxyguanosine and their nonoxidized forms were not different when comparing olive oils with low, medium, and high phenolic content given for 2 wk. Testing the effect of oil from urinary 8-oxo-deoxyguanosine changes from baseline to post-treatment showed a reduction of DNA oxidation by 13% (P=0.008). These findings support the idea that ingestion of olive oil is beneficial and can reduce the rate of oxidation of DNA. This effect is not due to the phenolic content in the olive oil. The higher DNA and RNA oxidation in Northern European regions compared with that in Central and Southern regions supports the contention that olive oil consumption may explain some of the North-South differences in cancer incidences in Europe.—Machowetz, A., Poulsen, H. E., Gruendel, S., Weimann, A., Fitó, M., Marrugat, J., de la Torre, R., Salonen, J. T., Nyyssönen, K., Mursu, J., Nascetti, S., Gaddi, A., Kiesewetter, H., Bäumler, H., Selmi, H., Kaikkonen, J., Zunft, H. J-F., Covas, M-I., Koebnick, C. Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. *FASEB J.* 21, 45–52 (2007)**

Key Words: phenolic compounds · DNA and RNA damage

FREE RADICALS AND OTHER REACTIVE OXYGEN species (ROS) generated *in vivo* by mechanisms such as aerobic metabolism, cytochrome P450, or respiratory burst can cause a serious imbalance between production of ROS and antioxidative defense of the body, termed oxidative stress (1, 2). Nucleic acids are among the targets, and oxidative stress leads to a plethora of mutagenic DNA lesions in purines, pyrimidines, and deoxyribose, including single- and double-strand breaks (2, 3). Therefore, accumulation of mutations from oxidative DNA damage is considered a crucial step in human carcinogenesis (4, 5). The most abundant DNA modification appears to be the hydroxylation of guanine in the 8-position to 8oxodG (2, 6); urinary excretion of 8oxodG is advocated as a biomarker of oxidative stress to DNA, measuring the rate of the whole body DNA oxidation (7). Although valid theoretically, it should be acknowledged that the origin of the lesions are presumed to be DNA but that the exact origin still is unknown. Other guanine modifications, 8oxoGua and 8oxoGuo, also represent urinary biomarkers of nucleic acid oxidation (8). Due to differences in the sugar portion, it is possible to differentiate between RNA and

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Clinical Pharmacology Q7642, Rigshospitalet, Copenhagen University Hospital, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark. E-mail: hepo@rh.dk

doi: 10.1096/fj.06-6328com

TABLE 1. *Criteria for an ideal biomarker of oxidative stress*

- It should be predictive of development of the disease or condition under investigation (example: lipid peroxidation in plasma should predict atherosclerotic events or cardiovascular death, DNA oxidation should predict cancer).
- It should reflect biological event(s) that can be related to the pathogenesis of the disease or condition.
- It should be stable over short periods (weeks, months) in stable individuals.
- It should produce identical results when the same sample is measured in different laboratories.
- The sample from which it is measured should be stable upon storage.
- The biomarker should relate to immediate events within short periods of time or should reflect integration of events over a well-defined period.
- Preferentially, the biomarker measurement should be noninvasive or measurable in an easily available biological specimen (example: urine, sputum) or in minimally, invasively obtainable biological specimen (example: blood or plasma).
- The cost of sample analysis should be low and it should be possible to perform a large number of analyses within a reasonable time.

DNA oxidation, but the oxidized base can originate from either nucleic acid. Criteria for valid biomarkers are given in **Table 1**.

In Mediterranean countries the incidence of several cancers, including breast, large bowel, ovary, endometrium, and prostate cancer, is lower than in Northern Europe (9). An association between high consumption of olive oil and decreased risk of breast (10), colon and rectum (11), oral, pharyngeal, laryngeal (12), and esophageal cancer (13) has been established. The fatty acid profile of olive oil is characterized by a high monounsaturated fatty acid content, predominantly oleic acid (14). Moreover, olive oil contains a remarkable variation of phenolic compounds (*e.g.*, oleuropein and its derivate hydroxytyrosol), generally believed to possess beneficial health effects (15, 16).

Data about the effects of phenolic compounds in olive oil on oxidative DNA damage from controlled human intervention trials are scarce. We performed a multicenter, double-blind, randomized, crossover, controlled intervention trial in Southern, Central, and Northern European countries to investigate the impact of olive oil consumption on DNA and RNA oxidation and the effects of the phenolic content of olive oils. In a randomized sequence we administered three olive oils that differed only in the phenolic content to healthy male subjects and measured 8oxoGua, 8oxoGuo, and 8oxodG in urine as biomarkers for DNA and RNA oxidation. We also included analysis of their corresponding nonoxidized forms, which could be markers of disturbances in the nucleotide pool with relevance for pathological processes (17–19).

MATERIALS AND METHODS

Subjects

Healthy nonsmoking male volunteers aged 20 to 60 years were recruited by advertisements in local media in six centers of five European Countries [Denmark, Finland, Germany (two centers), Italy and Spain]. Exclusion criteria were smoking, dieting, a high physical activity level (leisure time activity>3000 kcal/wk), regular use of aspirin, antioxidant supplements or any other drug with established antioxidant properties, lipid lowering drugs, and having any of the following diseases: obesity (body mass index>32 kg/m^2), hyperlipidemia and/or hypercholesterolemia (total serum cholesterol>8 mmol/L), diabetes, hypertension, celiac, or other intestinal diseases, any condition limiting mobility, or conditions that could affect compliance.

A total of 344 subjects were recruited, of whom 200 fulfilled the inclusion criteria and participated in the intervention trial. During the intervention 18 subjects dropped out of the study because of lack of compliance (n=4), secondary pathological events (n=4), and personal reasons, which did not allow them to continue (n=10). A total of 182 men from Southern (n=54), Central (n=70), and Northern (n=58) Europe completed the study.

Study design

The EUROLIVE study is a multicenter clinical intervention trial with a randomized, crossover, double-blind design. The power calculation of the study was based on an oxidation lag time of LDL, which was a more variable primary end point in the study than the primary end point nucleic acid oxidation. Subjects were advised to replace a part of their usual uncooked fat intake by a daily dose of 25 μl olive oil. Three types of olive oil containing different concentrations of phenolic compounds were tested. Subjects were randomly assigned to one of three treatment sequences (**Fig. 1**). A 2 wk run-in period before the trial and two washout periods of 2 wk each between the olive oil administrations were performed. During the overall study period, participants were instructed to abstain from their habitual olive oil intake. The local Ethics Committees of each trial center approved the study protocol. All participants gave written informed consent before starting the study.

Olive oils

The olive oils used in the intervention study were prepared especially for the trial. The selected oils were produced from

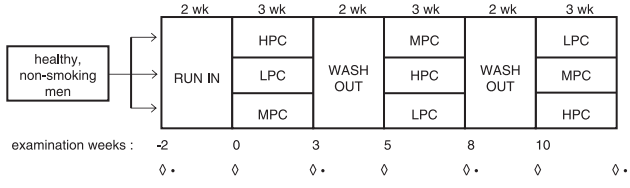

Figure 1. Study design. 24 h urine sampling (◇); anthropometry; 3 day estimated food record (●). LPC, low phenolic content; MPC, medium phenolic content; HPC, high phenolic content.

Picual olives (Andalucia, Spain) from the same cultivar, soil, and harvest. The three types of olive oil used in the trial were virgin, common, and refined. The chosen virgin olive oil contained the highest concentration of phenolic compounds with 366 mg/kg of caffeic acid equivalent (high phenolic content, HPC). Fatty acid composition and vitamin E content of the virgin olive oil and of harvests from virgin olive oils submitted to refinement were measured to ensure a selection of refined olive oil with the closest nutrient content to the previously selected virgin oil. An adjustment of vitamin E to values similar to those present in the virgin olive oil was also performed. During refinement the phenolic compounds are lost. Thus, this refined olive oil with 2.7 mg/kg of caffeic acid equivalent was the low phenolic content olive oil (LPC). By reconstituting refined with extra virgin oil in a 2:1 parts ratio, respectively, a common olive oil was obtained with a moderate phenolic content (MPC, 164 mg/kg). The olive oil predominantly contained the following phenolic compounds: oleuropein (65%), ligstroside aglycones (18%), hydroxytyrosol (11%), tyrosol (4%), and flavonoids (1%). Analysis of all olive oils showed no significant differences in their fatty acid profile, α-tocopherol, squalene, and β-sitosterol content but differed in their content of phenolic compounds. During the trial, codes for each type of olive oil were assigned. Except for the study coordinator, all investigators and subjects were blinded. Coded 25 μl olive oil containers were prepared at each study center and delivered to the participants at the beginning of each administration period.

Anthropometry

Anthropometric data included measurements of weight, height, and waist and hip circumference, which provide the basis for calculation of body mass index and waist-to-hip ratio. Measurements were carried out according to anthropometric standard operating procedures (20).

Dietary assessment

Dietary intake was recorded using a 3 day estimated food record at baseline and during the last days of each intervention period. Instructions on how to record intake and advice on the specific dietary recommendations for the study design were given by a nutritionist. Dietary recommendation included limiting consumption of vegetables or pulses (2 servings/day), fruits or juices (3 servings/day), black tea or coffee (2 cups/day), chocolate (2 bars/day), wine (2 glasses/day), and beer (1 L/day) in order to avoid an excessive intake of antioxidants and phenolic compounds. Energy consumption and intake of macro- and micronutrients were calculated according to national food and nutrient databases.

Urine sampling

Urine samples were obtained from the subjects at baseline and before and at the end of the intervention period (at weeks –2, 0, 3, 5, 8, 10, and 13). For 24 h urine collection, volunteers were advised to discard the first specimen of the first day and from then on to collect all specimens for 24 h, including the first specimen of the next day. The volume of the 24 h urine collections was recorded and was well mixed before performing aliquots. For phenolic marker analyses, 0.5 μl of 0.5 M sodium bisulfite was added to 25 μl urine aliquot. Samples were stored at −80°C until analyses.

DNA and RNA oxidation analysis

Oxidative DNA and RNA damage was assessed by measuring 8oxodG, 8oxoGuo, and 8oxoGua in urine and their corresponding nonoxidized forms by using HPLC/tandem mass spectrometry with a coefficient of variation below 10% (8). The compounds measured are stable when urine is kept unthawed at –20°C, and upon thawing they can be made stable by removal of proteins in the urine by filter centrifugation.

Phenolic marker

For evaluation of subjects' compliance, tyrosol, hydroxytyrosol, and 3-*o*-methylhydroxytyrosol as phenolic markers were determined in 24 h urine samples. Measurements were carried out on a Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph coupled to a tandem mass spectrometry detector system consisting of an HP5980 gas chromatograph, an HP5973 mass-selective detector, and an HP7683 series injector as described elsewhere. The intra- and interassay coefficients for tyrosol, hydroxytyrosol, and 3-*o*-methylhydroxytyrosol were 4.7%, 1.3%, 6.0%; and 3.8%, 3.0%, 6.6%, respectively (21).

Statistical analyses

Baseline data are shown as geometric mean and SEM. Calculated changes are given as mean ± SEM and percentage changes as median. Baseline differences between subjects in Southern, Central, and Northern Europe and between olive oil administration sequence groups were estimated using 1-way ANOVA. The effect of olive oil consumption and the effect of phenolic compounds in olive oil were estimated using a mixed linear model (PROC MIXED procedure). To assess the effect of olive oil consumption, calculated changes of the run-in period (Δwk0 to wk –2) and averaged changes of each olive oil intervention period (Σ[Δwk3 to wk0; Δwk8 to wk5; Δwk13 to wk10]/3) were used as dependent variables. The effect of different types of olive oil on DNA oxidation was estimated using calculated differences in periods with low (LPC), medium (MPC), and high (HPC) phenolic content olive oil as dependent variable (Δwk3 to wk0 of each period). In the final model, corresponding baseline values, age, administration sequence, and change in fat intake from baseline were included as fixed effects. European area (Southern, Central, and Northern Europe) and treatment (no olive oil and olive oil) or phenolic content (LPC, MPC, and HPC) were chosen as covariates and subjects as random factor. All biomarkers were log-transformed due to skew distribution. Statistical significance was defined as $P < 0.05$ for a 2-sided test. All analyses were performed using SAS 8.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline characteristic, nutrient intake, DNA and RNA oxidation markers, and phenolic compound excretion in Southern, Central, and Northern Europe at baseline

At baseline, European subjects slightly but significantly differed in body mass index and waist-to-hip ratio (**Table 2**). While body mass index tended to increase from Northern to Southern Europe, waist-to-hip ratio was highest in Central Europe and lowest in Southern Europe.

Baseline dietary habits also differed between European areas. Subjects from Southern Europe had a lower

TABLE 2. *Baseline characteristics, nutrient intake, and urinary phenolics of the study population in Northern, Central, and Southern Europe*[a]

	Southern	Central	Northern	*P*
	(*n*=58)	(*n*=70)	(*n*=54)	
Age (years)	32 (31; 34)	33 (32; 34)	29 (28; 31)	0.112
Body mass index (kg/m²)	24.8 (24.5; 25.2)	23.7 (23.3; 24.0)	23.2 (22.9; 23.6)	0.009
Waist-to-hip ratio	0.96 (0.95; 0.96)	0.99 (0.98; 0.99)	0.97 (0.96; 0.98)	0.017
Nutrient intake:				
Energy (kcal)	2044 (1961; 2130)	2493 (2402; 2586)	2400 (2333; 2469)	< 0.001
Carbohydrates (energy-%)	44.2 (42.5; 45.9)	48.3 (47.4; 49.3)	48.0 (47.0; 49.0)	0.037
Protein (energy-%)	16.2 (15.6; 16.8)	14.9 (14.5; 15.2)	15.3 (14.9; 15.7)	0.091
Fat (energy-%)	31.9 (30.8; 32.9)	34.0 (33.2; 34.9)	31.0 (30.2; 31.9)	0.049
Saturated fatty acid (fat-%)	37.2 (36.1; 38.3)	45.9 (45.1; 46.8)	44.9 (43.9; 45.8)	< 0.001
Monounsaturated fatty acids (fat-%)	46.7 (45.9; 47.6)	37.4 (36.9; 37.9)	37.8 (37.2; 38.2)	< 0.001
Polyunsaturated fatty acids (fat-%)	13.9 (13.0; 14.8)	15.2 (14.6; 15.9)	16.2 (15.6; 16.9)	0.103
Vitamin E (mg)	5.84 (5.47; 6.23)	9.37 (8.85; 9.91)	7.40 (6.94; 7.88)	< 0.001
Phenolic compound excretion:				
Tyrosol (μg/24 h urine)	125 (109; 144)	59 (51; 67)	35 (30; 41)	< 0.001
Hydroxytyrosol (μg/24 h urine)	216 (186; 252)	76 (66; 84)	88 (75; 103)	< 0.001
O-Methylhydroxytyrosol (μg/24 h urine)	56 (46; 68)	15 (13; 17)	17 (12; 16)	< 0.001

[a] Data are shown as geometric mean and SE (lower; upper limit). *P* values estimated by using 1-way ANOVA.

energy intake ($P<0.001$), carbohydrate intake ($P=0.037$), saturated fatty acid intake ($P<0.001$), and a higher monounsaturated fatty acid intake ($P<0.001$) compared with Central and Northern Europe. The total fat intake was highest in Central and lowest in Northern Europe ($P=0.049$). In Southern Europe, monounsaturated fatty acids were the main component of total fat intake, whereas in Central and Northern Europe saturated fatty acids were the predominant component. Vitamin E intake was highest in subjects from Central Europe and lowest in Southern Europe ($P<0.001$).

Among markers of DNA and RNA oxidation, solely urinary excretion of 8oxoGuo and 8oxodG significantly differed between European areas at baseline. In both markers, excretion rates were higher in Northern Europe than in Central and Southern Europe (**Fig. 2**). There were no significant differences in the excretion of the nonoxidized nucleobases (data not given).

At baseline, urinary excretion of the phenolic markers tyrosol, hydroxytyrosol, and 3-*o*-methylhydroxytyrosol in Southern Europe was 2- to 4-fold higher than in Central and Northern European subjects. Urinary tyrosol excretion was lowest among Northern European subjects; in Central and Northern Europe, urinary hydroxytyrosol and 3-*o*-methylhydroxytyrosol excretions were similar.

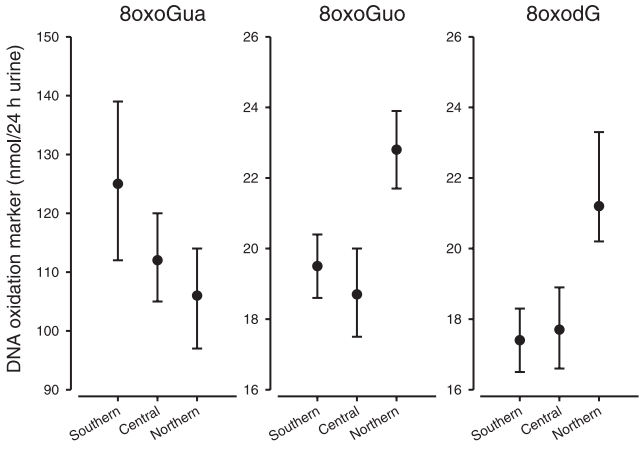

Figure 2. Baseline urinary nucleic acid oxidation markers of the study population in Northern, Central, and Southern Europe.

There were no differences in baseline characteristics such as age, body mass index, waist-to-hip ratio, blood lipids, and blood glucose (Glc), as well as nutrient intake in the randomized groups (Table 2).

Urinary excretion of phenolic compounds and subjects' compliance

During intervention, a dose-dependent increase in urinary excretion of phenolic markers was observed. After LPC consumption tyrosol, hydroxytyrosol, and 3-*o*-methylhydroxytyrosol concentrations remained unchanged. At the end of the MPC intervention period, urinary excretion of tyrosol showed a 1.2-fold, hydroxytyrosol a 2.1-fold, and 3-*o*-methylhydroxytyrosol a 1.5-fold increase. After the HPC intervention period, urinary excretion of the phenolic marker was 33-, 50-, and 44-fold (tyrosol, hydroxytyrosol, and 3-*o*-methylhydroxytyrosol, respectively) higher than at baseline ($P_{\text{Trend}}<0.001$). Thus, compliance of participants was considered sufficient (data not shown).

Effects of olive oil consumption on DNA and RNA oxidation markers

Energy intake of the study population in periods with and without olive oil consumption remained constant ($P=0.411$). Total fat intake increased significantly ~ 2.7–3.5 energy-% in each European area during olive oil intervention (Southern: $P=0.039$; Central: $P<0.001$;

Northern: $P=0.004$). Dietary intake of saturated fatty acids was significantly lower and monounsaturated fatty acids significantly higher during olive oil intervention compared with baseline ($P<0.001$). These effects were more pronounced in Central and Northern European countries compared with Southern Europe (olive oil×area interaction term; $P<0.001$). Dietary vitamin E intake significantly increased during olive oil intervention compared with baseline ($P<0.001$; data not shown).

Urinary 8oxodG decreased significantly after olive oil consumption by –13% ($P=0.008$) while urinary 8oxoGua, 8oxoGuo, and their corresponding nonoxidized forms as well as guanine were not affected by olive oil consumption (**Fig. 3**). No significant olive oil × area interaction term was found by ANOVA.

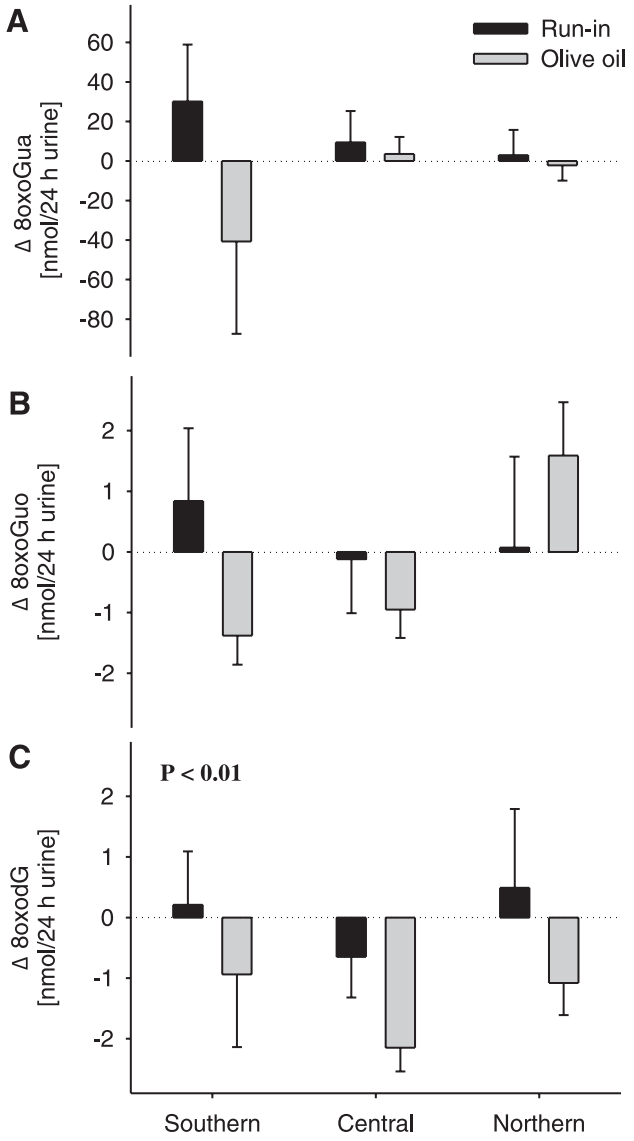

Figure 3. Changes in markers of oxidative damage. *A)* 8oxoGua, *B)* 8oxoGuo, *C)* 8oxodG in periods with and without olive oil consumption in Southern, Central, and Northern Europe. Data are shown as mean ± SEM. *P* values estimated using PROC MIXED, SAS. All models were adjusted for corresponding baseline values, administration sequence, age, difference in total fat intake from baseline, area, and treatment (no olive oil and olive oil).

Effects of phenolic compounds on DNA and RNA oxidation markers

Nutrient intake did not differ between LPC, MPC, and HPC intervention periods (data not shown). In the total study population, urinary excretion rates of guanine, guanosine, and deoxyguanosine and their corresponding oxidation products were not affected by the phenolic content of the olive oils administered (**Fig. 4**).

However, urinary excretions of 8oxoGuo and 8oxodG were higher in Northern European countries than in Southern and Central European countries before and after each olive oil intervention periods ($P=0.022$, $P<0.001$; respectively).

DISCUSSION

Most strikingly, we found that 25 ml of olive oil per day reduced DNA oxidation as measured by 24 h urinary excretion of 8oxodG, irrespective of the content of phenolic content. Since the three different oils used differed only with regard to their phenolic content, other components in the olive oil must be effective in reducing nucleic acid oxidation. The nature of these can only be speculated on, but further studies using the same biomarkers as those in the present study could be the starting point for identification. We also found pronounced cross-sectional differences in urinary excretion of 8oxoGuo and 8oxodG as biomarkers of DNA and RNA oxidation; their oxidation in Northern European countries was consistently higher than in Central and Southern European countries. This corresponds to a trend of increase from Northern to Southern Europe in the urinary excretion of olive oil phenolics and is consistent with the high consumption of olive oil, fruits, and vegetables in Mediterranean countries demonstrated earlier (22). The average intake of olive oil in Southern European countries is higher than in Central and Northern Europe (23), and may explain the higher excretion of olive oil phenolics in Southern European countries as observed in the present study.

The present beneficial effect of olive oil consumption on the urinary excretion of DNA oxidation markers with a considerable magnitude of 13% is comparable to the results of our earlier studies of smoking cessation (24). The olive oil intake led to a marked increase in monounsaturated fatty acid intake independent of the content of phenolic compounds in the olive oil. Again, this finding is supported by the observation that lifelong feeding of monounsaturated fatty acid-rich olive oil leads to a lower level of oxidative DNA damage compared with polyunsaturated fatty acid-rich sunflower oil (25). We also observed a higher vitamin E intake in the Central and Northern European regions. However, we have also shown that vitamin E administration does not change DNA oxidation (26), and thus

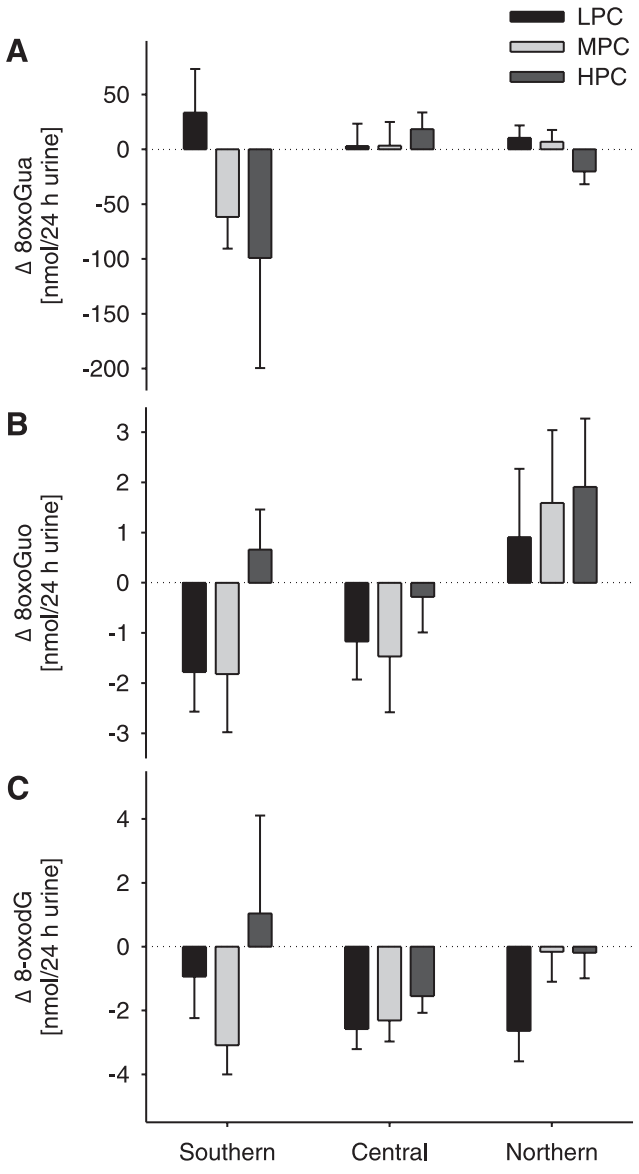

Figure 4. Changes in markers of oxidative damage. *A)* 8oxoGua, *B)* 8oxoGuo, *C)* 8oxodG after consumption of olive oil with low (LPC), moderate (MPC), or high (HPC) phenolic content in Southern, Central, and Northern Europe. Data are shown as mean ± SEM. *P* values estimated by using PROC MIXED, SAS. All models were adjusted for corresponding baseline values, administration sequence, age, difference in total fat intake from baseline, area, and phenolic content (LPC, MPC, and HPC)

do not find evidence that vitamin E has any effect on oxidative stress to nucleic acids.

The origins of 8oxodG, 8oxoGuo, and 8oxoGua are still not known in detail. As reviewed recently (27), 8oxoGua could result from the activity of the repair enzymes hOgg1, hOgg2, hNEH1, hNTH1. However, although 8oxodG could result from nucleotide excision repair and subsequent extracellular processing of the 24–32 oligomer excision product by an unidentified endonuclease, such activities have not been identified. An alternative origin of 8oxodG is degradation of 8oxdGPT in the nucleotide pool by the nucleotide sanitizing enzyme hMTH1. Nevertheless, 8oxodG is increased in smokers (28) and decreases after smoking cessation (24). Unpublished data from our laboratory do not reveal any differences in 8oxoGuo excreted into urine in smokers *vs.* nonsmokers. Therefore, we consider 8oxodG a better empirical measure of oxidative stress than 8oxoGuo. We did find that RNA oxidation, measured by 8oxoGuo, was influenced by olive oil. This is an intriguing observation, the significance of which is unclear at this time.

We were not able to demonstrate an effect of the phenolic compounds in olive oil on oxidative DNA damage, although phenolic compounds in olive oil have been described as powerful antioxidants (29). In some *in vitro* and *in vivo* studies, markers of lipid peroxidation decreased after virgin olive oil treatment (30, 31), but no beneficial effects of olive oil phenolics on DNA damage could be shown. We previously showed that none of the traditional antioxidants given as supplements are able to reduce oxidative stress to DNA (26). Taken together, these data support our interpretation that the beneficial effect of olive oil on oxidative stress to DNA relates to the monounsaturated fatty acid content.

From a design point of view, our finding of decreased oxidative stress to DNA after administration of monounsaturated fatty acid-containing oils was obtained by using historical controls since the analyses were done before and after oil administration, and we did not include a placebo-treated group. This fact gives a theoretical possibility of uncontrolled period effects. We do not, however, consider this an explanation for the effect of the oil because the sequence of the different oils was randomized to three different time periods. It is difficult to imagine a period effect able to bias our results consistently over three periods. Furthermore, a time period or other effects would have shown up in the other measures performed in the same assay, which was not the case. Therefore, we are confident in interpreting the findings as due to the administration of oil.

It could be argued that we should have used hard end points such as disease incidence or specific death rates. Although theoretically attractive, it has been empirically shown that controlled interventions with dietary factors are associated with extreme problems of almost all kinds besides being extremely costly and difficult to interpret. Epidemiological studies cannot provide evidence for single substances from an intake of complex foods, and we believe there is a dire need for evidence using validated biomarkers, according to the criteria given in Table 1, as the best means to gather reliable information.

DNA oxidation as measured by urinary 8oxodG is predictive for lung cancer in nonsmokers (32), and our previous studies (26, 33) with antioxidant intervention showed no effects on oxidative stress to DNA in accordance with the results of large interventions studies such as ATBC Study and Physicians Health Study.

DNA oxidation has been demonstrated to be a

promutagenic modification, and there seem to be hot spots in important genes like oncogens and tumor suppressor genes. Excretion of 8oxodG is stable in stable individuals over reasonably long periods (26), and identical results of urine measurements have been reported from laboratories all over the world. We have demonstrated that there is no change on storage (34), and since it only requires a urine sample the biomarker also fulfills the noninvasive criterion. The cost of analysis is not low compared with traditional hospital routine analysis; however, direct costs and time consumption do not prohibit analysis of trials of the present magnitude where seven measurements were performed on ~200 persons (*i.e.*, ~1500 samples). Provided funding, it would be possible to analyze even larger numbers. All criteria listed in Table 1 are thus fulfilled.

We conclude that olive oil consumption significantly decreases urinary excretion of 8oxodG, a marker of DNA oxidation. The effect is independent of the phenolic content of the olive oil and relates to the monounsaturated fatty acid content. This is also supported by the North-South gradients in oxidative stress to DNA, the North-South gradient in monounsaturated fatty acid intake, and the North-South gradient in cancer incidences. These data provide evidence that olive oil consumption explains some of the differences in cancer incidence between Northern and Southern Europe. FJ

The project has been carried out with financial support from the Commission of the European Communities, specific RTD program "Quality of Life and Management of Living Resources," QLK1–2001-00281, "EUROLIVE." The sponsor was not involved in the collection, analysis, or interpretation of data. The authors had full access to all the data in the study and final responsibility for the decision to submit for publication.

REFERENCES

1. Halliwell, B., and Whiteman, M. (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* **142,** 231–255
2. Poulsen, H. E., Prieme, H., and Loft, S. (1998) Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Cancer Prev.* **7,** 9–16
3. Whiteman, M., Hong, H. S., Jenner, A., and Halliwell, B. (2002) Loss of oxidized and chlorinated bases in DNA treated with reactive oxygen species: implications for assessment of oxidative damage in vivo. *Biochem. Biophys. Res. Commun.* **296,** 883–889
4. Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* **17,** 1195–1214
5. Evans, M. D., Dizdaroglu, M., and Cooke, M. S. (2004) Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.* **567,** 1–61
6. Kasai, H. (1997) Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res.* **387,** 147–163
7. Poulsen, H. E. (2005) Oxidative DNA modifications. *Exp. Toxicol. Pathol. 57 Suppl.* **1,** 161–169
8. Weimann, A., Belling, D., and Poulsen, H. E. (2002) Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Nucleic Acids Res.* **30,** E7
9. Lipworth, L., Martinez, M. E., Angell, J., Hsieh, C. C., and Trichopoulos, D. (1997) Olive oil and human cancer: an assessment of the evidence. *Prev. Med.* **26,** 181–190
10. La Vecchia, C., Negri, E., Franceschi, S., Decarli, A., Giacosa, A., and Lipworth, L. (1995) Olive oil, other dietary fats, and the risk of breast cancer (Italy). *Cancer Causes Control* **6,** 545–550
11. Braga, C., La Vecchia, C., Franceschi, S., Negri, E., Parpinel, M., Decarli, A., Giacosa, A., and Trichopoulos, D. (1998) Olive oil, other seasoning fats, and the risk of colorectal carcinoma. *Cancer* **82,** 448–453
12. Franceschi, S., Favero, A., Conti, E., Talamini, R., Volpe, R., Negri, E., Barzan, L., and La Vecchia, C. (1999) Food groups, oils and butter, and cancer of the oral cavity and pharynx. *Br. J. Cancer* **80,** 614–620
13. Bosetti, C., La Vecchia, C., Talamini, R., Simonato, L., Zambon, P., Negri, E., Trichopoulos, D., Lagiou, P., Bardini, R., and Franceschi, S. (2000) Food groups and risk of squamous cell esophageal cancer in northern Italy. *Int. J. Cancer* **87,** 289–294
14. Boskou, D. (2000) Olive oil. In *Mediterranean Diets* (Simopoulos, A. P., and Visiol, F., eds) pp. 56–77, Karger Press, World Rev. Nutr. Diet, Basel, Switzerland
15. Quiles, J. L., Farquharson, A. J., Simpson, D. K., Grant, I., and Wahle, K. W. (2002) Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. *Br. J. Nutr.* **88,** 225–234
16. Deiana, M., Aruoma, O. I., Bianchi, M. L., Spencer, J. P., Kaur, H., Halliwell, B., Aeschbach, R., Banni, S., Dessi, M. A., and Corongiu, F. P. (1999) Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radic. Biol. Med.* **26,** 762–769
17. Hoffmann, J. S., and Cazaux, C. (1998) DNA synthesis, mismatch repair and cancer (review). *Int. J. Oncol.* **12,** 377–382
18. Kunz, B. A., and Kohalmi, S. E. (1991) Modulation of mutagenesis by deoxyribonucleotide levels. *Annu. Rev. Genet.* **25,** 339–359
19. Kunz, B. A., Kohalmi, S. E., Kunkel, T. A., Mathews, C. K., McIntosh, E. M., and Reidy, J. A. (1994) International Commission for Protection Against Environmental Mutagens and Carcinogens. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutat. Res.* **318,** 1–64
20. Fidanza, F. (1991) *Nutritional Status Assessment,* Chapman & Hall, London
21. Miro-Casas, E., Farre Albaladejo, M., Covas, M. I., Rodriguez, J. O., Menoyo Colomer, E., Lamuela Raventos, R. M., and de la Torre, R. (2001) Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. *Anal. Biochem.* **294,** 63–72
22. Visioli, F., and Galli, C. (2001) The role of antioxidants in the Mediterranean diet. *Lipids* **36** Suppl., 49–52
23. Sanders, T. A. (2001) Olive oil and the Mediterranean diet. *Int. J. Vitam. Nutr. Res.* **71,** 179–184
24. Prieme, H., Loft, S., Klarlund, M., Gronbaek, K., Tonnesen, P., and Poulsen, H. E. (1998) Effect of smoking cessation on oxidative DNA modification estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion. *Carcinogenesis* **19,** 347–351
25. Quiles, J. L., Ochoa, J. J., Ramirez-Tortosa, C., Battino, M., Huertas, J. R., Martin, Y., and Mataix, J. (2004) Dietary fat type (virgin olive vs. sunflower oils) affects age-related changes in DNA double-strand-breaks, antioxidant capacity and blood lipids in rats. *Exp. Gerontol.* **39,** 1189–1198
26. Prieme, H., Loft, S., Nyyssonen, K., Salonen, J. T., and Poulsen, H. E. (1997) No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion in smokers. *Am. J. Clin. Nutr.* **65,** 503–507
27. Cooke, M. S., Lunec, J., and Evans, M. D. (2002) Progress in the analysis of urinary oxidative DNA damage. *Free Radic. Biol. Med.* **33,** 1601–1614
28. Loft, S., Vistisen, K., Ewertz, M., Tjonneland, A., Overvad, K., and Poulsen, H. E. (1992) Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* **13,** 2241–2247

29. Visioli, F., Poli, A., and Gall, C. (2002) Antioxidant and other biological activities of phenols from olives and olive oil. *Med. Res. Rev.* **22,** 65–75
30. Marrugat, J., Covas, M. I., Fito, M., Schroder, H., Miro-Casas, E., Gimeno, E., Lopez-Sabater, M. C., de la Torre, R., and Farre, M. (2004) Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation—a randomized controlled trial. *Eur. J. Nutr.* **43,** 140–147
31. Fito, M., Covas, M. I., Lamuela-Raventos, R. M., Vila, J., Torrents, L., de la Torre, C., and Marrugat, J. (2000) Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* **35,** 633–638
32. Loft, S., Svoboda, P., Kasai, H., Tjonneland, A., Vogel, U., Moller, P., Overvad, K., and Raaschou-Nielsen, O. (2005) Prospective study of 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion and the risk of lung cancer. *Carcinogenesis* **27,** 1245–1250
33. Van Poppel, G., Poulsen, H., Loft, S., and Verhagen, H. (1995) No influence of beta carotene on oxidative DNA damage in male smokers. *J. Natl. Cancer Inst.* **87,** 310–311
34. Loft, S., and Poulsen, H. E. (2000) Antioxidant intervention studies related to DNA damage, DNA repair and gene expression. *Free Radic. Res.* **33** Suppl., 67–83

Received for publication April 18, 2006.
Accepted for publication July 5, 2006.