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European Journal of Clinical Nutrition (2001) 55, 334–341
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Original Communication
Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers

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Objective: We studied whether consumption of phenol-rich extra virgin olive oil affects the susceptibility of low density lipoproteins (LDL) to oxidation and other markers of oxidation in humans.
Design: Randomized cross-over intervention trial, stratified according to sex, age and energy intake.
Setting: Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands.
Subjects: Forty-six healthy men and women completed the study.
Intervention: Subjects consumed two diets supplying 69 g per day of extra virgin olive oil either rich or poor in phenols for 3 weeks each. The mean difference in phenol intake between the treatments was 18 mg per day. Vitamin E intake was low during the whole study. Fasting blood samples were taken twice at the end of each period.
Results: Resistance of LDL and high density lipoprotein (HDL) to oxidation was not affected by treatment. The mean lag time of copper-induced formation of conjugated dienes was 1.6 min shorter in LDL and 0.4 min longer in HDL after the high phenol diet. Other markers of antioxidant capacity in plasma were also not affected: mean lipid hydroperoxides were 0.07 μmol/l higher, mean malondialdehydes were 0.001 μmol/l higher, mean protein carbonyls were 0.001 nmol/mg protein lower, and the mean ferric reducing ability of plasma (FRAP) was 0.006 mmol/l higher after the high phenol diet. All 95% confidence intervals enclosed zero. Serum cholesterol concentrations were not affected by the treatment.
Conclusion: Consumption of 18 mg per day of phenols from extra virgin olive oil for 3 weeks did not affect LDL or HDL oxidation or other markers of antioxidant capacity in fasting plasma samples.
Sponsorship: Supported by the International Olive Oil Council and the Foundation for Nutrition and Health Research.
Descriptors: phenols; tyrosol; hydroxytyrosol; oleuropein; olive oil; antioxidants; LDL-oxidation; HDL-oxidation; lipid hydroperoxides; malondialdehyde; protein carbonyls; FRAP
European Journal of Clinical Nutrition (2001) **55,** 334–341

Introduction

The Mediterranean diet, with olive oil as the major fat source, has been shown in epidemiological studies to be associated with a reduced incidence of coronary heart disease (Keys *et al*, 1986). The replacement of dietary saturated fatty acids with monounsaturated oleic acid from olive oil decreases plasma LDL concentrations, which presumably contributes to the low incidence of coronary heart diseases (Katan *et al*, 1995). It has also been suggested that a high-monounsaturated fat diet lowers the risk of coronary heart disease by producing oleic acid-enriched LDL particles, which are more resistant to oxidative modification (Bonanome *et al*, 1992; Reaven *et al*, 1991; Aviram & Eias, 1993). Oleic acid, however, may not be the only component of olive oil protecting LDL from oxidation; some types of extra virgin olive oil contain phenols with antioxidative properties. These phenols are formed during ripening of olives by hydrolysis of the parent compound oleuropein (Figure 1), and they are, in contrast to phenols from, for example tea and wine, partly lipid-soluble.

The oxidative modification hypothesis of atherosclerosis suggests that LDL particles are oxidatively modified and then taken up by macrophages inside the arterial wall. Such cholesterol-laden macrophages form the start of athero-

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Contributors: All authors were involved in designing the study. MV prepared and co-ordinated the field work, analysed the data, and wrote the paper. PZ, SW and SM co-operated in the preparation and organisation of the field work. PZ and MK contributed to the writing of the paper.
Received 15 June 2000; revised 23 November 2000; accepted 24 November 2000

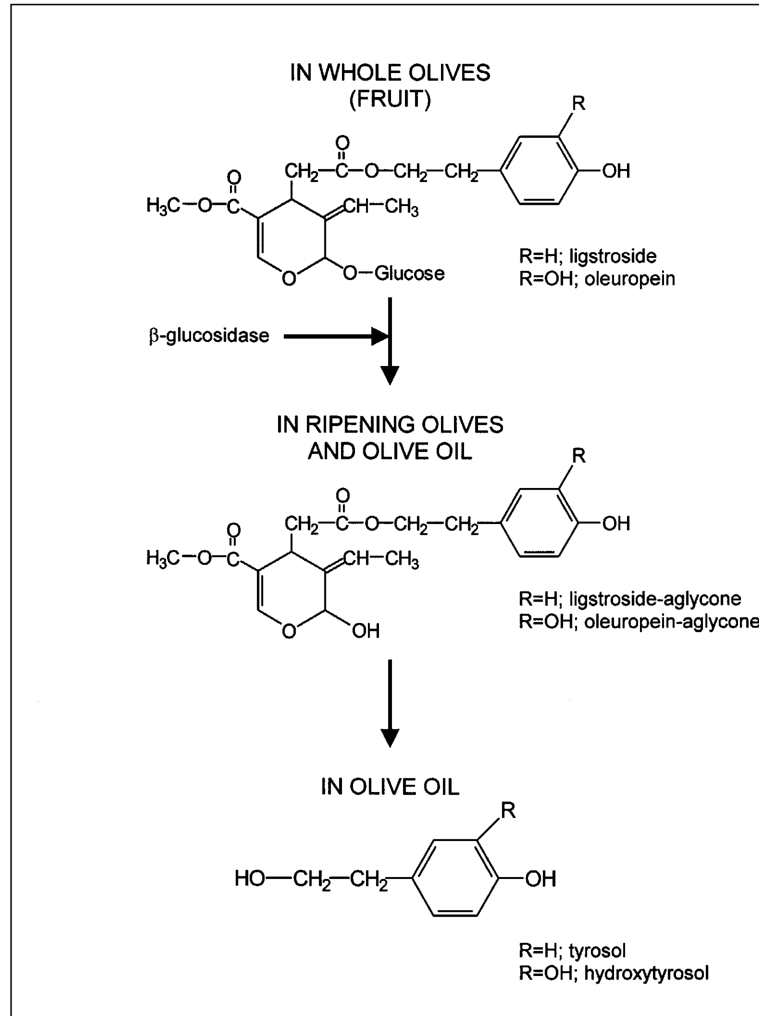

Figure 1 Structures of various phenols and their degradation into tyrosol and hydroxytyrosol in olives and extra virgin olive oil.

sclerotic plaques. Dietary antioxidants may therefore inhibit atherogenesis by inhibiting oxidation of LDL and accumulation of LDL in macrophages (Witztum & Steinberg, 1991). When olive oil is ingested, the lipid-soluble phenols are possibly taken up by LDL particles in plasma. Thus, these phenols may protect LDL particles from becoming atherogenic by oxidative modification. The aim of this study was to determine whether consumption of phenols from olive oil affects the susceptibility of LDL to oxidation and other markers of oxidation in normolipemic humans.

Subjects and methods

Subjects
Subjects were recruited via publicity in local newspapers and posters in university buildings and student apartments. We carefully explained the study protocol before subjects gave their written informed consent. The study protocol was approved by the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University.

Subjects were eligible if they were older than 17 y, did not use any drugs known to affect concentrations of serum lipids, were not pregnant, not lactating, and not on a prescribed diet. Volunteers filled out a medical questionnaire that was reviewed by an independent physician. Persons with a history of gastrointestinal, liver or kidney disease were excluded, as were those with glucosuria, proteinuria, anaemia, a serum concentration of total cholesterol >7.0 mmol/l, or fasting triglycerides >2.3 mmol/l, and serum values of liver enzymes >30 U/l for alanine aminotransferase (ALT), or >30 U/l for aspartate aminotransferase (AST). Thirty-two women and 17 men, 18–58 y, were enrolled in the study. Three subjects withdrew during the study; one because of illness unrelated to the treatment, and two because of personal reasons.

Forty-six subjects completed the study. None of the subjects used medications that could have affected the results.

Design and treatment
The study consisted of two 3-week treatment periods, during which each subject consumed two diets, one with phenol-rich extra virgin olive oil and one with phenol-poor extra virgin olive oil, in random order (cross-over). Before the study subjects were stratified according to sex, age and energy intake and then randomly allocated to one of the two sequence groups. During the 2 weeks before the study (run-in) and 2 weeks in-between the treatment periods (wash-out) subjects consumed diets without olives, olive oil and olive oil products.

The high phenol olive oil was prepared from Tsunati olives with a high content of phenols. The low phenol olive oil was prepared from Koroneiki olives; most of the phenols were removed by washing with hot water. Vitamin E was added to the high phenol olive oil to obtain the same vitamin E content in both oils. The two olive oils had a similar fatty acid composition (data not shown). The phenol concentration in the experimental oils was determined as described by Montedoro *et al* (1993). The high phenol olive oil contained 308 mg/kg of phenols, of which 2% was tyrosol, 1% was hydroxytyrosol, 72% were oleuropein-aglycones, and 25% were ligstroside-aglycones. The low phenol olive oil contained 43 mg/kg of phenols, of which 16% was tyrosol, 2% was hydroxytyrosol, 13% were oleuropein-aglycones, and 69% were ligstroside-aglycones. The mean difference in phenol intake between the high and low phenol diet was 18 mg/day.

Before the trial, energy intake of individual subjects was estimated by a food frequency questionnaire (Feunekes *et al*, 1993). Each subject received the amount of olive oil that fulfilled energy needs. Energy intakes were subdivided into four levels, ranging from 7.5 to 17.5 MJ per day. The amount of olive oil per day varied from 55 to 102 g, with a mean intake of 69 g. Forty percent of the daily oil was incorporated into a mayonnaise (78 w/w% olive oil), 30% was incorporated into sauces for hot meals, and 30% in cookies and raisin rolls baked by a local bakery. We recorded body weights twice weekly and adjusted the intake of olive oil when necessary so as to maintain a stable weight. Over the duration of the trial average body weight increased by 0.3 ± 0.9 kg (range $-1.2-2.8$ kg). Body weight did not differ between treatments; it was 68.0 ± 9.6 kg on the high phenol diet, and 67.8 ± 9.5 kg on the low phenol diet.

Each study diet was assigned a colour code that was used for labelling all foods supplied during the trial. In this way, subjects were blinded to the type and the sequence of the olive oils. On weekdays at noon, hot meals were served and eaten at the department in the presence of the researchers. These meals supplied at least 50% of the experimental olive oil. Foods that contained the remaining part of the olive oil were consumed at home. Foods containing the experimental olive oil were weighed or counted for each subject. During the whole study subjects followed instructions for a background diet low in vitamin E. A margarine with a low vitamin E content was supplied (Van den Bergh Foods, UK). Apart from this, subjects were asked to maintain their usual diet. During the study subjects were not allowed to take vitamin supplements or aspirin, which is a radical scavenger (Kuhn *et al*, 1995; Hermann *et al*, 1999). Therefore they were provided with acetaminophen (paracetamol). Subjects were asked to maintain their usual pattern of physical activity and not to change their smoking habits, consumption of coffee, or use of oral contraceptives. All subjects kept daily records of illness and deviations from the protocol. Diaries and anonymous questionnaires administered after the trial showed that consumption of experimental olive oil was 90% of the scheduled amount for the low phenol diet and 87% for the high phenol diet. To check adherence, we added 6.8 μmol/g of lithium chloride to the mayonnaise. The daily intake of lithium was $190-360$ μmol/day, depending on the level of energy intake and consequently the amount of olive oil consumed per day. Mean plasma lithium concentrations after olive oil consumption was 4.7 ± 1.3 μmol/l, which was about five times the concentration of 0.9 ± 0.3 μmol/l in similar subjects that did not consume added lithium chloride (De Roos *et al*, 2001). This confirmed adherence of the subjects to the protocol.

Blood sampling
Venous blood samples were taken twice after a 12-h fast at the end of each 3-week period (days 17 and 21). Plasma or serum was immediately obtained by low speed centrifugation and stored at -80°C. For the determination of the concentration of serum lipids, the liver enzymes, uric acid and the ability of plasma to reduce iron (III) (FRAP), the two samples of each subject at the end of each period were analysed separately and the results were averaged before statistical analysis. For the determination of the susceptibility of LDL and HDL to oxidation, the two samples obtained at the end of each period were pooled before analytical analyses. For all other analyses, only the last samples obtained at the end of each period were used.

Lipoprotein isolation
Plasma lipoproteins were isolated by density gradients ultracentrifugation in a SW 41Ti rotor (Beckman Instruments, Palo Alto, USA) for 24 h at 10°C (Redgrave *et al*, 1975). LDL was isolated in a density range of $1.019-1.063$ g/ml, and HDL in a density range of $1.063-1.210$ g/ml. Density gradients solutions contained 0.1 mM Na_2EDTA to inhibit metal ion catalysed LDL and HDL oxidation during the isolation procedure.

Markers of oxidizability
The susceptibility of LDL and HDL to copper-mediated oxidation was determined by monitoring the formation of conjugated dienes, essentially as described by Princen *et al* (1992). Malondialdehyde in plasma was determined as described by Wong *et al* (1987), except that the HPLC eluent was monitored using fluorescence detection. The

excitation wavelength was 537 nm and the emission wavelength was 554 nm. Lipid hydroperoxides in plasma were determined by the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA USA). Protein carbonyls in plasma were determined by an ELISA method as described by Buss *et al* (1997). The ability of plasma to reduce iron (III) (FRAP) was determined by the method of Benzie and Strain that measures the reduction of ferric to ferrous iron in the presence of antioxidants (Benzie & Strain 1996).

Vitamin and uric acid concentrations in serum

The concentrations of lycopene, β-carotene, retinol and α-tocopherol in serum samples were determined by high performance liquid chromatography (HPLC, Waters Instruments, Milfort, USA). Serum was deproteinised with ethanol-internal standard solution (1:1 v/v) and extracted with hexane. A part of the hexane layer was evaporated after centrifugation and reconstituted in the mobile phase. Samples to determine retinol and α-tocopherol were injected into a Resolve C_{18}-5 μm column (30 cm×3.9 mm; Waters Instruments, Milfort, USA) with methanol/dichloromethane/acetonitrile (10:20:70, v/v) as the mobile phase, with detection at 325 and 292 nm, respectively. Samples to determine lycopene and β-carotene were injected into a Sperisorb 5 μm ODS-2 column (25 cm×4.6 mm; Waters Instruments, Milfort, USA) with methanol/dichloromethane/acetonitrile (30:20:50, v/v) as the mobile phase, with detection at 472 and 450 nm, respectively. Calibration was performed by a single standard solution on the same way as a sample. Serum uric acid was determined by UA plus kit (Boehringer, Mannheim, Germany).

Serum lipids and liver enzymes

Serum lipids were analysed enzymatically (Siedel *et al*, 1983; Warnick *et al*, 1982; Fossati & Prencipe, 1982). Mean bias for control samples provided by the Centers of Disease Control in Atlanta was −1% for total and high density lipoprotein cholesterol and 10% for triglycerides. The coefficient of variation within runs ranged from 0.5 to 1.1%. Low density lipoprotein cholesterol concentrations were calculated (Friedewald *et al*, 1972). Alanine and aspartate aminotransferase were measured at 37°C using Abbott Spectrum reagents (Bergmeyer *et al*, 1978). The mean bias for 'Monitrol' control sera (Baxter Dade, Switzerland) ranged from 0 to 2%. The coefficient of variation within runs ranged from 2 to 8%.

Statistical analyses

The data were analysed by ANOVA using the General Linear Models (GLM) of the Statistical Analyses System (SAS Institute Inc., 1989). The Tukey method was used for calculation of 95% confidence limits of the differences between the two diets.

Results

Markers of oxidizability

Resistance of LDL and HDL to copper-mediated oxidation and other markers of antioxidant capacity or oxidative processes were not affected by treatment (Table 1). The lag time of copper-induced formation of conjugated dienes was 1.6 min shorter in LDL and 0.4 min longer in HDL after the high phenol diet. Maximum rate of diene formation in LDL and HDL were similar after both treatments (Table 1).

Other oxidation markers were also not affected. Plasma lipid hydroperoxides and malondialdehydes, both markers of lipid peroxidation, were respectively 0.07 and 0.001 μmol/l higher after the high phenol diet. Protein carbonyls, a marker of protein oxidation in plasma, were 0.001 nmol/mg protein lower after the high phenol diet. The ferric reducing ability of plasma (FRAP), a marker for the antioxidant capacity of plasma, was 0.006 mmol/l higher after the high phenol diet. All 95% confidence intervals enclosed zero (Table 1).

Table 1 Oxidizability of LDL and HDL, and other oxidation markers at the end of the high and the low phenol diet (*n* = 46)

	Low phenol diet	*High phenol diet*	*Differences between high and low phenol diet (95% CI)*
LDL oxidizability[a]			
Lag time (min)	110.2 ± 25.0	108.6 ± 20.4	−1.6 (−8.2−5.0)
Maximum rate (nmol dienes/min/mg LDL protein)	12.0 ± 2.6	11.8 ± 2.3	−0.1 (−0.6−0.4)
HDL oxidizability			
Lag time (min)	69.3 ± 47.5	69.7 ± 50.5	0.4 (−12.8−13.5)
Maximum rate (nmol dienes/min/mg HDL protein)	4.6 ± 2.2	4.4 ± 2.2	−0.1 (−0.3−0.03)
Malondialdehyde (μmol/l)	0.68 ± 0.15	0.69 ± 0.13	0.001 (−0.03−0.03)
Lipid hydroperoxides (μmol/l)	0.36 ± 0.52	0.44 ± 0.54	0.07 (−0.07−0.21)
Protein carbonyls (nmol/mg protein)	0.23 ± 0.12	0.23 ± 0.12	−0.001 (−0.02−0.02)
Ferric reducing ability of plasma (mmol/l)	1.05 ± 0.18	1.06 ± 0.18	0.006 (−0.01−0.02)

Values are means ± s.d. Participants (15 men and 31 women) consumed both diets in random order for 3 weeks each.
[a] *n* = 44.

Vitamin and uric acid concentrations in serum
Serum uric acid, lycopene, retinol, β-carotene or α-tocopherol was not affected by the treatment (Table 2). The concentration of α-tocopherol in our subjects was 19.4 ± 5.0 μmol/l, which is relatively low compared to normal plasma vitamin E concentrations from 11 to 37 μmol/l (Cohn, 1997). This can be explained by the low vitamin E content in the diet. This suggested adherence of the subjects to the protocol.

Serum lipids and liver enzymes
Mean total cholesterol was 0.06 mmol/l lower (not significant) on the high phenol diet than on the low phenol diet. Concentrations of LDL, HDL, triglycerides and the liver enzymes alanine and aspartate aminotransferase also did not differ between the high and low phenol diet (Table 3).

Discussion

Our data show that consumption of 18 mg per day of phenols from olive oil for 3 weeks does not affect *in vitro* susceptibility of LDL to oxidation or other markers of oxidation in fasting blood of healthy volunteers.

Our results are in line with two other human studies that addressed the effect of minor components in olive oil on the susceptibility of LDL to oxidation in fasting blood (Nicolaïew *et al*, 1998; Bonanome *et al*, 2000). In these studies, extra virgin olive oil *vs* high oleic acid sunflower oil or refined olive oil also did not affect the *in vitro* susceptibility of LDL to oxidation in fasting plasma samples. Other human studies on the effect of olive oil on LDL oxidation addressed the effects of oleic acid rather than olive oil phenols *per se* (Reaven *et al*, 1991; Bonanome *et al*, 1992; Tsimikas *et al*, 1999). *In vitro*, the olive oil phenols hydroxytyrosol and oleuropein strongly inhibited LDL oxidation (Visioli *et al*, 1995; Visioli & Galli, 1994). Also, tyrosol, oleuropein and extracts of minor components from extra virgin olive oil decreased the oxidation of LDL as assessed by oxysterol formation (Berra *et al*, 1995; Caruso *et al*, 1999). We realise that LDL oxidation *ex vivo* is different from LDL oxidation *in vitro*. Phenols or antioxidants might get lost during isolation of LDL from plasma by centrifugation, a process that is absent when doing experiments *in vitro*. However, in rabbits and rats, non-tocopherol antioxidants from olive oil showed a favourable effect on the susceptibility of LDL to oxidation (Wiseman *et al*, 1996; Coni *et al*, 2000; Scaccini *et al*, 1992). This suggests that during isolation phenols might stay in or attached to lipoprotein particles where they might affect the resistance of LDL to oxidation. Thus, results of animal and *in vitro* studies suggest a protective effect of phenols from olive oil on LDL oxidation, but such an effect was not observed in fasting blood samples of humans in the

Table 2 Serum concentration of uric acid, lycopene, β-carotene, retinol and α-tocopherol at the end of the high and the low phenol diet ($n = 46$)

	Low phenol diet	*High phenol diet*	*Differences between high and low phenol diet (95% CI)*
Uric acid (μmol/l)	260.6 ± 56.1	264.4 ± 58.0	3.8 (−2.2−9.8)
Lycopene (μmol/l)[a]	0.59 ± 0.20	0.61 ± 0.19	0.01 (−0.05−0.08)
β-Carotene (μmol/l)[a]	0.54 ± 0.23	0.52 ± 0.24	−0.02 (−0.08−0.04)
Retinol (μmol/l)[a]	2.51 ± 0.55	2.47 ± 0.70	−0.04 (−0.2−0.1)
α-tocopherol (μmol/l)	19.77 ± 4.58	18.97 ± 5.37	−0.8 (−2.1−0.5)

Values are means ± s.d. Participants (15 men and 31 women) consumed both diets in random order for 3 weeks each.
[a]$n = 40$.

Table 3 Serum lipid and lipoprotein cholesterol concentrations at the end of the high and the low phenol diet ($n = 46$)

	Low phenol diet	*High phenol diet*	*Differences between high and low phenol diet (95% CI)*
Lipoproteins (mmol/l)			
Total cholesterol[a]	4.25 ± 0.83	4.19 ± 0.76	−0.06 (−0.15−0.04)
HDL cholesterol[a]	1.54 ± 0.36	1.52 ± 0.37	−0.01 (−0.06−0.04)
LDL cholesterol[a]	2.29 ± 0.65	2.26 ± 0.59	−0.04 (−0.12−0.05)
Triglycerides[b]	0.92 ± 0.35	0.90 ± 0.32	−0.02 (−0.08−0.04)
Liver enzymes (U/l)			
Alanine aminotransferase	20.3 ± 7.0	20.7 ± 7.7	0.3 (−1.0−1.6)
Aspartate aminotransferase	21.7 ± 4.7	21.8 ± 5.1	0.1 (−1.0−1.2)

Values are means ± s.d. Participants (15 men and 31 women) consumed both diets in random order for 3 weeks each.
[a]To convert to mg/dl, multiply by 38.67.
[b]To convert to mg/dl, multiply by 88.54.

present and other studies (Nicolaïew *et al*, 1998; Bonanome *et al*, 2000).

One explanation might be that our study did not address postprandial effects. If phenol clearance is fast, phenol concentrations might be elevated in the first hours after a meal and during that time protect LDL from oxidation, but not after 12 h of fasting as in our study. Visioli *et al* actually found that the phenols tyrosol and hydroxytyrosol were mostly excreted within 24 h after intake of 50 ml extra virgin olive oil, which indicates that clearance of phenols from plasma is fast (Visioli *et al*, 2000). Furthermore, Bonanome *et al* found a significant postprandial effect 2 h after intake of 100 g of extra virgin olive oil on plasma antioxidant capacity, but they did not include a control group (Bonanome *et al*, 2000). Nicolaïew *et al*, on the other hand, did not find a significant effect on the lag time or maximum rate of LDL oxidation of extra virgin olive oil 6 h after intake (Nicolaïew *et al*, 1998). The proper study of postprandial effects requires more insight into the kinetics of phenol transport and metabolism in man and more studies are needed on this aspect.

We also did not find an effect of phenols from extra virgin olive oil on the susceptibility of HDL to oxidation. Like modified LDL, oxidatively modified HDL is suggested to increase intracellular cholesterol accumulation (Nagano *et al*, 1991; Gesquiere *et al*, 1997; Bonnefont-Rousselot *et al*, 1995). The physiological role of HDL oxidation is still unclear, but in our study 18 mg of phenols per day for 3 weeks did not influence the susceptibility of HDL to oxidation in fasting plasma samples.

Furthermore, we did not find an effect of olive oil phenols on other plasma markers of lipid and protein oxidation (lipid hydroperoxides, malondialdehyde, protein carbonyls and FRAP). The average plasma values of the oxidation markers in our study were in line with those found in other studies. Lag time of LDL oxidation was about 110 min, which is relatively high (Esterbauer & Jürgens, 1993). This can be explained by methodological differences and the high dose of oleic acid that increases the resistance of LDL to oxidative modification (Bonanome *et al*, 1992; Reaven *et al*, 1991; Tsimikas *et al*, 1999). Mean plasma lipid hydroperoxide and malondialdehyde concentrations and FRAP were similar to values found by others (Wong *et al*, 1987; Benzie & Strain, 1996). Protein carbonyls were slightly higher in our subjects than in healthy subjects in the study by Buss *et al*, but much lower than in 23 critically ill subjects in the same study (Buss *et al*, 1997). Thus, plasma values of the markers of oxidation were within previously reported ranges.

The phenol oleuropein-aglycone and some of its derivatives (Figure 1) from extra virgin olive oil are lipid-soluble, while oleuropein, tyrosol and hydroxytyrosol are water-soluble (Unilever Research Vlaardingen, unpublished data). We therefore hypothesised that the lipid-soluble aglycones, which are the main phenols in olive oil, may accumulate in LDL and HDL particles and provide better protection of LDL against oxidation than other, more water-soluble, dietary compounds like phenols from tea and wine, for example. The amount of phenols needed to protect LDL and HDL from oxidation is unknown. Also, data about the amounts of other antioxidants needed to protect LDL from oxidation are scarce. Vitamin E is the most important lipid-soluble antioxidant in the body (Princen *et al*, 1995; Jialal *et al*, 1995). In one dose–response study Princen *et al* suggested that intake of 25 mg/day of vitamin E for 2 weeks was sufficient to reduce the susceptibility of LDL to oxidation (Princen *et al*,1995). In another dose–response study, Jialal *et al* investigated the effects of vitamin E intake in doses of 60, 200, 400, 800 and 1200 mg/day. They found that at least 400 mg/day of vitamin E was needed to reduce the susceptibility of LDL to oxidation (Jialal *et al*, 1995). Thus, the minimum dose of vitamin E needed to reduce the susceptibility of LDL to oxidation is as yet unclear. However, if 25 mg/day of vitamin E is the minimum effective amount and if olive oil phenols and vitamin E would be equally effective, then the amount of 18 mg/day in our study might have been too low to detect an effect on LDL oxidizability. Moreover, vitamin E might partition better into LDL particles and thus be more effective in reducing the susceptibility of LDL to oxidation then phenols from olive oil. This implies that even more dietary phenols would be needed to detect an effect. Thus, the amount of 18 mg/day of olive oil phenols in our study, a very high intake in terms of practical realistic diets, might have been too low to affect LDL oxidizability.

More is known about the *in vitro* than about the *in vivo* antioxidant activity of olive oil phenols. *In vitro* dose–response studies by Visioli and co-workers demonstrated that pre-incubation of LDL with 10 μmol/l oleuropein or 10 μmol/l hydroxytyrosol inhibited the decrease of vitamin E in LDL during copper-mediated oxidation and delayed the formation of conjugated diene formation, lipid peroxides and thiobarbituric acid-reacting substances (Visioli *et al*, 1995; Visioli & Galli, 1994). These studies showed that the protection of vitamin E from oxidation was correlated with the concentration of oleuropein or hydroxytyrosol, and that a concentration of 1 μmol/l provided much less protection than a concentration of 10 μmol/l (Visioli *et al*, 1995; Visioli & Galli, 1994). Visioli *et al* suggested that olive oil phenols retard the oxidation of the natural vitamin E pool in LDL and in this way indirectly protect LDL from oxidation. We do not know the plasma concentration of the various phenols that were reached in our study, but it is unlikely that it reached 1 μmol/l after a whole-body dose of 18 mg (about 50 μmol) per day for 3 weeks. Thus, on the basis of *in vitro* data the concentration of phenols in LDL was probably too low to produce detectable effects on LDL oxidizability.

It remains possible that amounts of olive oil phenols higher than the 18 mg/day fed in our study can affect LDL oxidizability. However, we supplied the maximum dose that is achievable with practical every-day diets. Olives were specially selected to obtain a large difference in phenol concentration between the experimental oils, and the subjects consumed large amounts of olive oil

(69 g/day). The average daily intake of phenols from olive oil is not exactly known, but estimations from the Mediterranean diet indicate that 10–20 mg/day of total phenols may be supplied by olive oil (Visioli *et al*, 1995). According to our data, this is probably not enough to affect markers of antioxidant status in fasting plasma samples.

It is conceivable that the background diet of our subjects contained too high an amount of antioxidants to allow additional antioxidant effects of the phenols of extra virgin olive oil. Our subjects consumed a background diet low in vitamin E, which was confirmed by the low plasma vitamin E concentrations. Furthermore, subjects were not allowed to take vitamin supplements. However, it is possible that tissue stores of vitamin C and vitamin E for example, after the run-in period were still considerable, and it may require depletion periods to show an effect. Thus, olive oil might theoretically still affect oxidizability of fasting LDL in subjects who are depleted in dietary antioxidants.

The oxidative modification hypothesis of atherosclerosis is an attractive one, but it is still not proven that dietary antioxidants prevent coronary heart disease (Zock & Katan, 1998). Only a few studies have reported the relation between markers of oxidation and coronary heart disease, and the results of these studies are inconsistent (Van de Vijver *et al*, 1998; de Rijke *et al*, 1995; Cominacini *et al*, 1993; Croft *et al*, 1992). Thus, none of the oxidation markers have yet been validated as risk factors for atherosclerosis, although it may be that markers more representative of *in vivo* changes, like oxidised LDL antibodies, may be more sensitive to changes in LDL antioxidant content. Much work remains to be done here. In addition, randomised clinical trials with hard endpoints are needed to prove that dietary antioxidants, such as the phenols from olive oils, may offer protection against coronary heart disease.

The serum concentrations of total and LDL cholesterol did not differ between the two diets. It has been suggested that minor components, such as phenols, may be responsible for a cholesterol-lowering effect (Matsumoto *et al*, 1998). We therefore studied the effects of olive oil phenols on serum cholesterol concentrations. Our results are in line with those of Nicolaïew *et al*, who found that a diet with extra virgin olive oil and a diet with oleic-acid-rich sunflower oil produced the same cholesterol concentrations (Nicolaïew *et al*, 1998). Thus, the cholesterol-lowering effect of olive oil is probably not due to its phenolic compounds.

We also measured the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Phenols from extra virgin olive oil are responsible for the bitter taste of extra virgin olive oil. This bitter taste might be a warning from nature for possible adverse or toxic effects. We therefore measured ALT and AST as indicators of such effects. In our study the serum concentrations of ALT and AST did not differ between the two diets. This suggests that phenols from olive oil have no adverse affects on liver integrity.

In conclusion, we did not find an effect of a high intake of phenol-rich olive oil on the susceptibility of LDL and HDL to oxidation and other markers of oxidation in fasting plasma. The natural concentration of phenols in olive oil might be too low or their clearance from plasma too fast to produce an effect on markers of oxidation in the post-absorptive phase.

Acknowledgements—We thank the International Olive Oil Council and the Foundation for Nutrition and Health Research for financial support; Dr Karel van Putte for his advice; Professor Anthony Kafatos and co-workers for analyses of vitamins; Els Siebelink, Wilma van de Geer and Ariënne Stehouwer for their assistance during the study; Jan Harryvan, Joke Barendse and the laboratory staff of the Division of Human Nutrition of Wageningen University and Unilever Research Vlaardingen for their technical assistance; and the volunteers for their enthusiastic participation.

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