

## Effect of Consumption of Phenols from Olives and Extra Virgin Olive Oil on LDL Oxidizability in Healthy Humans

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Accepted by Professor B. Halliwell

(Received 7 February 2001; In revised form 5 April 2001)

A high intake of olive oil has been proposed as an explanation for the low incidence of coronary heart disease in Mediterranean countries, but it is unclear whether olive oil offers specific benefits beyond a low content of saturated fat. Some types of extra virgin olive oil are rich in non-polar phenols, which might be taken up by plasma LDL particles and protect these from becoming atherogenic by oxidative modification. In a pilot study we found that consumption of 47 g fortified olive oil containing 31 mg phenols significantly increased the lag time of LDL oxidation from  $112 \pm 5$  min before to  $130 \pm 7$  min 2 h after the meal. However, this study was not controlled, and in the current study we therefore investigated whether olive oil phenols increase the lag time of LDL oxidation in postprandial samples when compared with a control group.

Twelve healthy men and women consumed four different olive oil supplements with a meal on four separate occasions: one similar to the supplement in the pilot study (positive control); one containing mainly non-polar olive oil phenols; one containing

mainly polar olive oil phenols; and one without phenols (placebo). Lag time significantly increased 2 h after the meals with the positive control ( $8 \pm 2$  min), the polar phenols ( $8 \pm 2$  min), and the placebo ( $8 \pm 2$  min), but not after the non-polar phenols ( $-0.4 \pm 3$  min). Increases were not statistically different between supplements.

These results indicate that the lag time of LDL-oxidation is increased after consumption of a meal. This increase is probably due to non-specific meal or time effects and not to phenols from olives or olive oil. Furthermore, these findings stress the need for adequate controlled studies to avoid misinterpretations of the data.

**Keywords:** Phenols; Tyrosol; Hydroxytyrosol; Oleuropein; Olive oil; Antioxidants; LDL Oxidation

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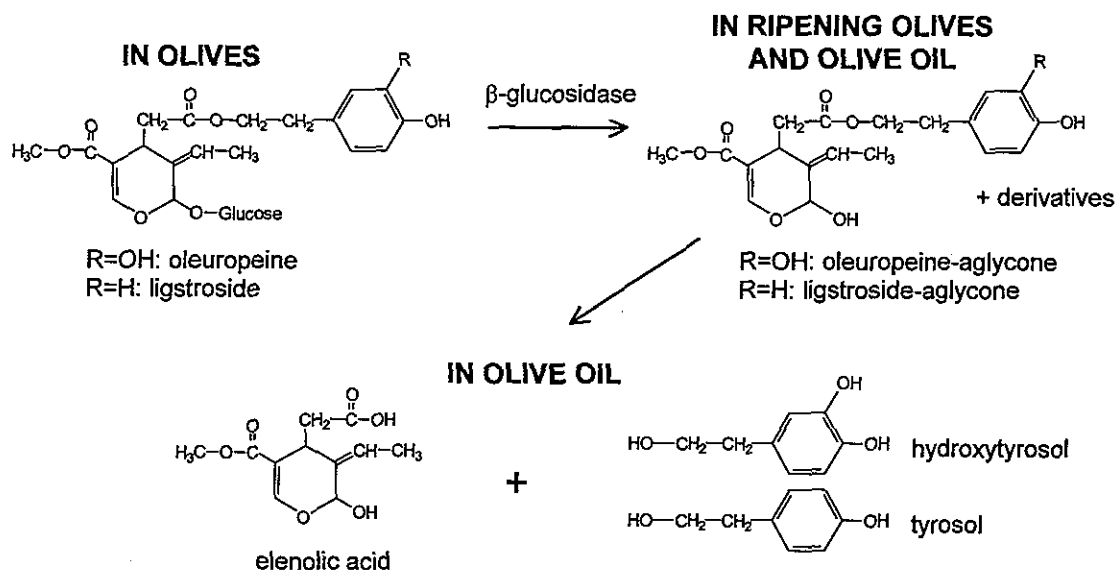


FIGURE 1 Structures of phenols present in olives and olive oil, their degradation into aglycones during ripening, and hydrolysis of aglycones into tyrosol and hydroxytyrosol.

## 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.<sup>[1]</sup> 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.<sup>[2]</sup> 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.<sup>[3-5]</sup> 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 aglycones formed in olives by removal of glucose from the parent compound oleuropein or ligstroside, and they are less-polar. End products of hydrolysis of ligstroside- and oleuropein-aglycones are tyrosol and hydroxytyrosol which are polar (Fig. 1).

Oxidation of LDL may be an important risk factor for atherosclerosis, the underlying cause of coronary heart disease, and olive oil phenols may be particularly effective in preventing oxidation of LDL. Due to their relatively lipophilic nature the olive oil phenols might directly dissolve into LDL particles where they can protect LDL from oxidation. Results of animal<sup>[6-8]</sup> and *in vitro* studies<sup>[9-12]</sup> do suggest a protective effect of phenols from olive oil on LDL-oxidation. Some human studies suggest that such an effect may also apply to the practical *in vivo* situation. Visioli and co-workers found that administration of phenol-rich oils resulted in a dose-dependent decrease in urinary excretion of F<sub>2</sub>-isoprostanes, which indicates less overall oxidation of arachidonic acid.<sup>[13]</sup> Results of other human studies, on the other hand, are inconsistent.<sup>[14-17]</sup> In a previous human study<sup>[14]</sup> we did not find an effect on LDL oxidizability in fasting blood after subjects had consumed extra virgin olive oil that was naturally high in phenols (21 mg/day) for three weeks. From that study we concluded that a dose of 21 mg/day of phenols was not high enough to affect markers of oxidation, or that we

missed an effect because we measured blood samples taken at least 12 h after the last meal. Because the metabolism of olive oil phenols might be fast, we investigated effects on LDL oxidation in plasma taken shortly after intake of olive oil phenols in a pilot study. In this pilot study, ten healthy men and women consumed in one meal 47 g of olive oil fortified with a phenol-rich extract from olives containing 32 mg phenols. The mean lag time of LDL oxidation increased from  $112 \pm 5$  min before to  $130 \pm 7$  min 2 h after the meal (95% CI of change, 5.3–30.8 min) (Fig. 2). However, this study did not include a concurrent control group, and the observed decrease in LDL oxidizability may have been due to non-specific meal or time effects. Therefore, we performed a controlled intervention study on whether olive oil phenols affect LDL oxidizability directly after intake.

## METHODS

### Subjects

The study protocol was approved by the Medical Ethical Committee of Wageningen University. The protocol was fully explained to the participants before they gave their written informed consent.

Twelve healthy subjects (eight females, four males) were recruited, with a mean age of 22 years (range 20–28 years), and with a mean body mass index of  $21.8 \text{ kg/m}^2$  (range  $18.3$ – $27.1 \text{ kg/m}^2$ ).

Subjects had no history of gastrointestinal, liver or kidney disease, did not use any drugs known to affect concentrations of serum lipids, and were not pregnant, lactating, or on a prescribed diet. Subjects were all healthy as judged by normal blood count, the absence of glucose and protein in urine, and a medical questionnaire, which was reviewed by an independent physician. Subjects had a serum

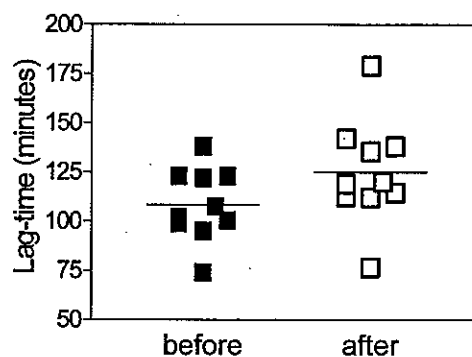


FIGURE 2 Individual lag times of LDL oxidation in samples taken before and 2 h after intake of the phenol-rich supplement in the pilot study ( $n = 9$ ). In this pilot study subjects started with a seven day run-in period in which they were not allowed to consume olives, olive oil, olive oil products. On day eight they consumed 47 g fortified olive oil containing 31 mg phenols with breakfast. The subjects consumed their breakfast within half an hour did not eat or drink anything else except water until three hours after supplement intake.

concentration of total cholesterol  $< 7.0 \text{ mmol/l}$ , and fasting triglycerides  $< 2.3 \text{ mmol/l}$ .

### Design and Treatment

Each subject consumed a single dose of four different supplements, in random order (cross-over). The study consisted of a four-week treatment period, during which subjects were not allowed to consume olives, olive oil, olive oil products, and products extremely rich in vitamin E, like peanut butter and nuts. At the end of each week, on days 8, 15, 22, and 29, we provided the subjects with a supplement between 8:00 and 9:30 h at our department. The supplement was consumed together with a standard breakfast, containing bread, tomato, cucumber, tuna, ham, cheese, milk or buttermilk, and tea or coffee with or without sugar and evaporated milk. Products were low in vitamin E to prevent any effect of vitamin E on LDL oxidation. The breakfast was not protein-free, but protein content was kept as low as possible to minimize the possible binding of phenols to proteins in the intestines. To this end, we instructed the subjects not to consume

more than one slice of ham or cheese and not to drink more than 150 ml milk or buttermilk. The breakfasts contained  $3.0 \pm 0.8$  MJ as energy, and  $26 \pm 7$  g protein. The breakfast with the positive control contained  $4.2 \pm 0.8$  MJ as energy because of the higher dose of olive oil in this supplement. The subjects consumed breakfast within half an hour. Each subject consumed exactly the same amount of breakfast on each of the four occasions. The only exception was that subjects were allowed to consume some more cucumber or salad with one of the supplements (positive control), in order to facilitate the intake of the higher amount of olive oil in this supplement than in the other three supplements. Tea, coffee or other foods and drinks were not allowed until the last blood sample had been taken. Blood samples were taken before, and 30 min and 2 h after the experimental breakfast. All subjects kept daily records of illness and deviations from the protocol.

### Supplements

The positive control supplement was similar to the supplement consumed in the pilot study in which we found a postprandial effect on the lag time. The supplement consisted of 65 g mayonnaise prepared with 47 g olive oil, water, egg yolk powder, vinegar, pizza herbs, mustard, salt

and pepper. This extra virgin olive oil was fortified with a phenol-rich olive fraction. The total phenol concentration in the fortified oil was 674 mg/kg. Thus, the supplement contained 31.1 mg of phenols (Table I).

The supplement with non-polar phenols consisted of 37 g mayonnaise prepared with 14.5 g phenol-enriched olive oil, sour cream and small amounts of water, egg yolk powder, vinegar, mustard, honey, pizza herbs, salt and pepper. The phenols were extracted from extra virgin olive oil by food-grade ethanol, which was evaporated in a later stage. The total phenol concentration of the olive oil used in this supplement was 6876 mg/kg, thus the supplement provided 100 mg of olive oil phenols (Table I).

The supplement with polar phenols was not a mayonnaise because the extract used was watery and not suitable to prepare a mayonnaise. The supplement was extracted from extra virgin olive oil by reversed osmosis. Subjects drank 8.7 g of this supplement containing a total phenol concentration of 11470 mg/kg. Thus, the supplement provided 100 mg of phenols (Table I). Subjects also consumed 37 g mayonnaise prepared with 14.5 g olive oil without phenols to equalize fat intake between breakfasts. The mayonnaise was similar to the placebo supplement.

The placebo supplement also consisted of 37 g mayonnaise prepared with 14.5 g olive oil with-

TABLE I The amount of the various phenols and vitamin E present in the supplements used in study 1 and 2, analysed by HPLC. The placebo did not contain any phenols or vitamin E

Type of phenol* (peak number)	Phenol intake		
	Positive control (mg/47 g oil)	Non-polar (mg/14.5 g oil)	Polar (mg/8.7 g extract)
Hydroxytyrosol (1)	3.7	1.4	36.2
Tyrosol (2)	1.2	16.9	22.9
Oleuropein-aglycone (peaks 3, 4, 5, 7, 8, 9, 10)*,†	19.0	19.0	34.1
Ligstroside-aglycone (peaks 6, 11, 12)*,†	7.2	62.8	6.9
Total phenols	31.1	100.1	100.1
Alpha-tocopherol	10	0.1	0

\*Ten derivatives of the aglycones were separated and the peaks identified (van Boom *et al.*, in preparation). The various derivatives differ mainly in their ring structure, which can be either open or closed in two different forms.

†The numbering of the various phenols in this table refer to the numbering of the peaks in Fig. 3.

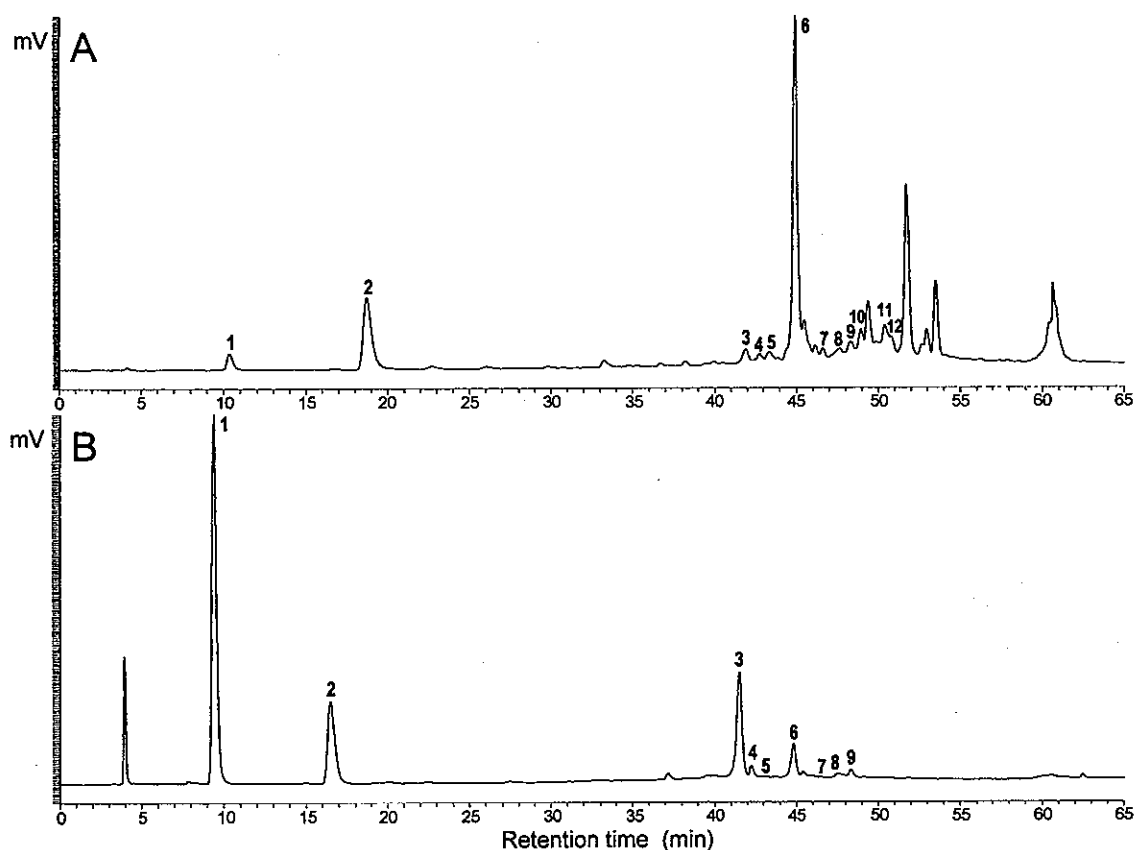


FIGURE 3 Chromatogram of various identified phenols in the non-polar (A) and the polar (B) supplement. The numbering of the peaks refer to the numbering of the various phenols in Table I; 1 = hydroxytyrosol; 2 = tyrosol; 3, 4, 5, 7, 8, 9, 10 = oleuropein-aglycones; 6, 11, 12 = ligstroside-aglycones.

out phenols and vitamin E, sour cream and little amounts of water, egg yolk powder, vinegar, mustard, honey, pizza herbs, salt and pepper. The olive oil without phenols and vitamin E was prepared by stripping it several times.

The phenol concentration of the fortified oil was measured with an HPLC method based on the method of Montedoro *et al.*<sup>[18]</sup> With this HPLC method hydroxytyrosol and tyrosol and 10 derivatives of the oleuropein- and ligstroside-aglycones were separated, and the peaks of the aglycones were identified using HPLC-MS-MS (Fig. 3, Table I). The 12 major peaks in the HPLC chromatogram were quantified using the

area/ $\mu\text{g}$  at  $\lambda = 280 \text{ nm}$  of the individual compounds hydroxytyrosol, tyrosol and oleuropein or of mixtures of aglycones. The aglycone reference mixture was obtained by enzymatic hydrolysis of oleuropein by  $\beta$ -glucosidase. The various derivatives differ mainly in their ring structure, which can either be open or closed in two different forms (van Boom *et al.*, in preparation). Because some peaks could not be identified, the concentrations of phenols in the experimental oils might be an underestimation of the total amount of phenolic substances from olive oil. Thus intake of phenols might have been even higher.

### Blood Sampling

Venous blood samples for determination of LDL oxidizability were taken using vacuum tubes containing EDTA. Blood was sampled before and 1/2 and 2 h after breakfast. Plasma was prepared within 30 min by centrifugation at 4°C for 10 min at 2500 g, and 10 µl of a 600 g/l sucrose solution was added per ml plasma.<sup>[19]</sup> Samples were stored at -80°C until analysis less than three months later.

### LDL Isolation and LDL Oxidation

Plasma lipoproteins were isolated by density gradient ultracentrifugation in an SW 41Ti rotor (Beckman Instruments, Palo Alto, USA) for 24 h at 10°C.<sup>[20]</sup> LDL was isolated in a density range of 1.019–1.063 g/ml. Density gradients solutions contained 0.1 mM Na<sub>2</sub>EDTA to inhibit metal ion catalysed LDL oxidation during the isolation procedure.

The susceptibility of LDL to copper-mediated oxidation was determined in duplicate by monitoring the formation of conjugated dienes, as described by Princen *et al.*<sup>[21]</sup> Determinations before, and 30 min and 2 h after supplement intake were done for two supplements of one subject in one single run. The duplicates of these determinations were done in another run.

### Statistical Analyses

The duplicate values obtained from each subject were averaged before data analysis.

LDL-oxidizability before, and 1/2 and 2 h after intake of the supplement were compared using Student's paired *t*-tests. To compare differences in LDL oxidizability between the various supplements we applied ANOVA using the general linear models (GLM) of the Statistical Analyses System.<sup>[22]</sup> Before analysis, the lag time and maximum rate of each individual subject before supplement intake were subtracted from the lag time and maximum rate at 30 min or 2 h

after intake. Then, these differences were compared among the various supplements. The positive control, the supplement also used in the pilot study, was statistically compared with the placebo supplement without phenols. The supplements containing polar or non-polar phenols were statistically compared with each other and with the placebo supplement only. The Tukey method was used to adjust for multiple comparisons and for calculation of 95% confidence limits of the differences between the supplements. Data are presented as mean ± SE (Table II).

## RESULTS

One subject did not consume the breakfast containing the non-polar phenol-supplement because of personal reasons not related to the study.

The mean lag time increased significantly both at 30 min and 2 h after the meals with the positive control ( $10 \pm 4$  min at 30 min and  $8 \pm 2$  min at 2 h), the polar phenols ( $4 \pm 1$  min at 30 min and  $8 \pm 2$  min at 2 h), and the placebo ( $7 \pm 3$  min at 30 min and  $8 \pm 2$  min at 2 h); but not after the non-polar phenols ( $4 \pm 2$  min at 30 min and  $-0.4 \pm 3$  min at 2 h) (Table II).

The maximum rate of diene formation was not affected by any of the four supplements (Table III).

The increase in lag time and maximum rate did not differ significantly between the positive control supplement and the placebo supplement, neither at 30 min nor at 2 h after intake (Table IV). Also, the increase did not significantly differ between the supplement with polar or non-polar phenols and the placebo supplement (Table IV).

TABLE II The lag time of LDL oxidation (minutes) before intake and 30 min and 2 h after intake of the four experimental supplements and differences in lag time 30 min and 2 h after compared to before intake supplements ( $n = 12$ ). Values are means  $\pm$ SE. All subjects (four men, eight women) were included in the analyses. Each subject consumed each supplement in random order

Supplement	Lag time of LDL oxidation			Differences in lag time compared to	
	Before supplement intake ( $t = 0$ )	30 min after supplement intake ( $t = 1/2$ )	2 h after supplement intake ( $t = 2$ )	( $t = 1/2$ )	( $t = 2$ )
Placebo	76 $\pm$ 2	82 $\pm$ 4*	84 $\pm$ 3	7 (0.4-14.2)	8 (3.5-11.9)
Polar phenols	74 $\pm$ 2	78 $\pm$ 2	82 $\pm$ 3	4 (1.7-6.8)	8 (2.9-13.3)
Non-polar phenols	79 $\pm$ 3*	83 $\pm$ 3*	79 $\pm$ 4*	4 (-0.3-8.3)	-0.4 (-6.9-6.1)
Positive control†	74 $\pm$ 3	83 $\pm$ 4	82 $\pm$ 4	10 (1.8-17.5)	8 (3.2-12.4)

\* $n = 11$ : one subject was not able to consume the non-polar phenol-supplement or there was not enough plasma of one subject to do the analyses (placebo supplement,  $t = 1/2$ ).  
 † Positive control is the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

TABLE III The maximum rate of LDL oxidation (nmol conjugated dienes/min/mg LDL protein) before intake and 30 min and 2 h after intake of the four experimental supplements and differences in maximum rate 30 min and 2 h after compared to before intake of the supplements ( $n = 12$ ). Values are means  $\pm$  SE. All subjects (four men, eight women) were included in the analyses. Each subject consumed each supplement in random order

Supplement	Maximum rate of LDL oxidation			Differences in maximum rate compared to	
	Before supplement intake ( $t = 0$ )	30 min after supplement intake ( $t = 1/2$ )	2 h after supplement intake ( $t = 2$ )	( $t = 1/2$ )	( $t = 2$ )
Placebo	23.4 $\pm$ 0.7	22.4 $\pm$ 0.9*	21.9 $\pm$ 0.8	-0.9 (-2.0-0.16)	-1.6 (-2.9-0.2)
Polar phenols	22.5 $\pm$ 0.7	21.5 $\pm$ 0.8	21.4 $\pm$ 0.4	-1.0 (-2.5-0.42)	-1.1 (-2.3-0.16)
Non-polar phenols	21.4 $\pm$ 0.7*	21.0 $\pm$ 0.6*	21.8 $\pm$ 0.9*	-0.4 (-1.4-0.6)	0.4 (-0.5-1.4)
Positive control†	22.9 $\pm$ 0.6	22.4 $\pm$ 0.7	22.6 $\pm$ 0.7	-0.5 (-1.8-0.9)	-0.3 (-1.4-0.9)

\* $n = 11$ : one subject was not able to consume the non-polar phenol-supplement or there was not enough plasma of one subject to do the analyses (placebo supplement,  $t = 1/2$ ).  
 † Positive control is the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

TABLE IV The difference in lag time and maximum rate of LDL oxidation between the supplements with non-polar phenols, polar phenols, and the placebo supplement and between the positive control and the placebo supplement: 30 min and 2 h after compared to before intake of the supplements ( $n = 12$ ). Values are means  $\pm$  SE. All subjects (four men, eight women) were included in the analysis. Each subject consumed each supplement in random order

Supplement	Difference in lag time compared to $t = 0$ (min)		Differences in maximum rate compared to $t = 0$ (nmol conjugated dienes/min/mg LDL protein)	
	$t = 1/2$ (95% CI)	$t = 2$ (95% CI)	$t = 1/2$ (95% CI)	$t = 2$ (95% CI)
	Polar phenols vs. placebo	-3.0 (-10.8-4.8)	0.4 (-7.5-8.4)	-0.1 (-2.1-2.0)
Non-polar phenols vs. placebo	-3.3 (-11.3-4.7)	-8.0 (-16.2-0.1)	0.5 (-1.5-2.6)	2.0 (-0.1-4.1)
Polar phenols vs. non-polar phenols	0.3 (-7.5-8.1)	8.4 (0.3-16.6)	-0.6 (-2.7-1.4)	-1.5 (-3.6-0.6)
Positive control* vs. placebo	2.3 (-8.8-13.5)	0.2 (-3.8-4.1)	0.5 (-1.3-2.2)	1.3 (0.0-2.5)

\*Positive control is the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

## DISCUSSION

Our study shows that consumption of a high dose of phenols from olives and extra virgin olive oil does not affect LDL oxidizability in postprandial blood samples. We found that the increases in lag time after intake of a meal with phenol-rich olive oil was not different from the increase after intake of a meal with placebo olive oil. Thus, the decrease in LDL oxidizability that we observed in the pilot study was not due to phenolic substances but probably to non-specific effects of the meal or to changes in LDL oxidizability over the day.

### Adequate Control Groups

Our findings stress the need for adequate control groups to avoid misinterpretations of the data. In the pilot study, we found that the phenol-rich breakfast decreased LDL oxidizability, which would have suggested a positive effect of the phenols on LDL oxidizability. However, if compared with a control group, the effect turned out to be a non-specific meal or time effect rather than an effect of the phenols. The importance of a control group should thus not be underestimated, and the results of studies should be interpreted very carefully when no control group is included. Thus, for a conclusive effect of

dietary components on antioxidative markers, including a control group is essential.

### Olive Oil Phenols and LDL Oxidation

Our findings are similar to those from the studies of Nicolaiew *et al.*<sup>[15]</sup> and Bonanome *et al.*<sup>[16]</sup> in which extra virgin olive oil also did not affect the lag time of the *ex vivo* susceptibility of LDL to oxidation, both in fasting and in postprandial plasma samples as compared to the control group. In contrast to what we found, those studies did not show an increase in lag time after eating a meal. A reason for that might be that blood samples were taken 6 h after the oral fat load in the study of Nicolaiew *et al.* instead of the 2 h in our study.<sup>[15]</sup> However, Bonanome *et al.* measured LDL oxidizability 30, 60, 120, 240 and 360 min after intake of 100 ml olive oil, and did not find an effect on lag time on all these time points.<sup>[16]</sup>

Bonanome *et al.* did find an increase of total plasma antioxidant capacity, another marker than lag time, in postprandial plasma samples 2 h after intake of a single dose of 100 ml extra virgin olive oil.<sup>[16]</sup> However, they did not include a control group and this effect might therefore, like in our study, also be a non-specific meal or time effect.



Other human studies addressed long-term effects of phenols from olive oil in fasting blood and not immediate effects in postprandial blood.<sup>[14-17]</sup> Ramirez-Tortosa *et al.*<sup>[17]</sup> showed a decreased LDL oxidizability after three months of consumption of extra virgin olive oil compared to refined olive oil. However, LDL oxidation was assessed as the amount of thiobarbituric acid-reactive substances (TBARS) formed after incubation for 24 h with various concentrations of copper ions instead of assessing LDL oxidation as the lag time as in the current study. The value of TBARS as a measure of *in vivo* oxidation is questionable,<sup>[23]</sup> but Ramirez-Tortosa *et al.*<sup>[17]</sup> also found that extra virgin olive oil reduced uptake by macrophages of oxidized LDL in fasting blood compared to refined olive oil. Other human studies did not find an effect on LDL oxidizability in fasting blood.<sup>[14-16]</sup> Taken together, results from human studies are thus inconsistent.

#### Increased Lag Time in Postprandial Samples

Few data are available on the diurnal variation of the lag time. Studies of LDL oxidation have mainly focussed on the fasting state. In the postprandial state triglyceride concentrations in the LDL fraction increase,<sup>[24,25]</sup> which is thought to increase the susceptibility of LDL to oxidation and thus, in contrast to what we found, decrease the lag time.<sup>[26]</sup> But the type of fatty acids in the supplement might also affect LDL oxidizability. Olive oil is rich in oleic acid, which reduces LDL oxidizability.<sup>[3]</sup> VLDL or chylomicrons might exchange lipid molecules with other lipoproteins,<sup>[27]</sup> like LDL, and thereby decrease the LDL oxidizability in postprandial plasma. Unfortunately we did not measure the fatty acids composition of the LDL fractions. However, other studies did not show an effect of boluses of up to 100 g olive oil on the lag time of LDL oxidation in postprandial samples.<sup>[15,16,28]</sup> Thus, an acute effect of an oleic acid bolus on LDL

composition and subsequent LDL oxidizability is not likely. Furthermore, fat intake was similar among all supplement groups and could not affect the results of the phenols on LDL oxidizability.

We did not measure vitamin E concentrations in plasma. However, we think it is not likely that vitamin E could have confounded the results. The amount of vitamin E that subjects consumed from the supplement was at maximum 10 mg in the positive control supplement. One dose-response study suggested that a daily ingestion for two weeks of at least 25 mg vitamin E was needed to reduce LDL susceptibility.<sup>[29]</sup> Another study even suggests that much higher daily doses are needed.<sup>[30]</sup> Furthermore, Dimitrov *et al.* demonstrated in a kinetic study that a single dose of as much as 440, 880, and 1320 mg vitamin E hardly elevated plasma vitamin E concentrations 2 h after intake, and that a peak is reached as late as 12–24 h after ingestion.<sup>[31]</sup> Thus, we think it is unlikely that the single dose of at most 10 mg of vitamin E materially influenced plasma vitamin E concentrations and LDL oxidizability. Furthermore, the amount of vitamin E in the background diet was  $2.3 \pm 1.0$  mg, which is probably too low to affect LDL oxidizability 2 h after intake.

The pizza herbs mainly consisted of oregano, which is known to have antioxidant properties *in vitro*.<sup>[32]</sup> However, subjects consumed only 0.37 g of the herbs, and each supplement contained exactly the same amount and type of the herbs, which thus could not have affected the differences in effects on LDL oxidizability between the supplements.

Thus, our results indicate that phenols from extra virgin olive oil do not affect LDL oxidizability, but a non-specific time or meal effect might be responsible for the decreased LDL oxidizability after the meal. Future research should more precisely determine the 24 h time course of LDL oxidizability, including effects of meals.

### Dose of Phenols

The plasma concentration of phenols needed to protect LDL from oxidation *in vivo* is unknown. *In vitro* dose-response studies by Visioli and co-workers demonstrated that pre-incubation of LDL with 10  $\mu\text{mol/l}$  oleuropein or 10  $\mu\text{mol/l}$  hydroxytyrosol protected vitamin E from oxidation, but that a concentration of 1  $\mu\text{mol/l}$  provided much less protection.<sup>[9,10]</sup> We do not know the plasma concentration of the various phenols that were reached in our studies, but a single dose of 100 mg (about 275  $\mu\text{mol}$ ) does probably not result in a plasma concentration higher than 1  $\mu\text{mol/l}$ .<sup>[33]</sup> Thus, it is likely that the plasma concentrations in our studies were too low to produce an effect on the *ex vivo* oxidation of LDL. Furthermore, estimations of the average daily intake of phenols from olive oil in the Mediterranean diet indicate that 10–20 mg/day of total phenols may be supplied by olive oil,<sup>[9]</sup> which is at least five times less than the amount given by us in a single dose. Therefore the phenol content of olive oil may be too low to produce a measurable effect on LDL oxidizability *in vivo*.

### Pharmacokinetics of Phenols from Olive Oil

In the pilot study we measured the LDL oxidizability 2 h after intake of the meals.<sup>[16]</sup> We found an intriguing increase in lag time and we therefore repeated measurement at this time point in the current study 2. In this study we also measured the effect at 30 min after intake because Hollman and co-workers found a plasma peak concentration of quercetin,<sup>[33]</sup> a compound that is comparable with the olive oil aglycones, 30 min after intake of a quercetin-rich breakfast. However, we cannot be sure that olive oil phenols were present in plasma or LDL 1/2 or 2 h after intake. Thus, more insight into the time course of plasma phenol concentrations after intake is required to conclude whether olive oil phenols can add a positive health effect in humans. Future research should focus more on the

kinetics and metabolism of phenols and other potential antioxidants in humans.

### Conclusion

We did not find an effect of a large single dose of olive oil phenols on plasma LDL oxidizability. More insight into the kinetics of phenol transport and metabolism in humans is required to study the potential health effects of olive oil phenols in humans.

### Acknowledgements

We thank the International Olive Oil Council and the Foundation for Nutrition and Health Research for financial support; Sheila Wiseman for her advise; Brenda Glas, Dieneke Kool, Saskia Meyboom, Els Siebelink, Lisette Wagenaar, and Caroline Wolderling for their assistance during the study; Joke Barendse, Stella van Boom, Jan van Buuren, Marjolein Hagemans, Ton van Immerzeel, Bep Knoop, Mirto Oduber, Eugenie Olde Riekerink, Eric Schuurbijs, and Anita Willebrand for their technical assistance; and the volunteers for their enthusiastic participation.

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