Original Research

Changes in LDL Fatty Acid Composition as a Response to Olive Oil Treatment Are Inversely Related to Lipid Oxidative Damage: The EUROLIVE Study

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Objective: The aim of our study was to assess the changes in the fatty acid composition of low density lipoproteins (LDL) after sustained consumption of olive oil at real-life doses (25 mL/day) and their relationship with lipid oxidative damage.

Methods: A multi-center randomized, cross-over, clinical trial with 3 similar types of olive oils, but with differences in the phenolic content, was conducted on 200 healthy European subjects. Intervention periods were of 3 weeks separated by 2-week washout periods. The LDL fatty acid content was measured in samples drawn at baseline and after the last intervention period.

Results: After olive oil ingestion oleic acid concentration in LDL increased (1.9%; $p < 0.001$) and those of linoleic (1.1%; $p < 0.002$) and arachidonic acid (0.5%; $p < 0.001$) decreased. Monounsaturated/polyunsaturated fatty acid and oleic/linoleic acid ratios in LDL increased after olive oil consumption. An inverse relationship between the oleic/linoleic acid ratio and biomarkers of oxidative stress was observed. One unit increase in the oleic/linoleic acid ratio was associated with a decrease of 4.2 μg/L in plasma isoprostanes.

Conclusion: Consumption of olive oil at real-life doses improved the fatty acid profile in LDL, the changes being associated with a reduction of the oxidative damage to lipids.

INTRODUCTION

The Mediterranean diet is considered to be a protective factor in the primary and secondary prevention of coronary heart disease (CHD) [1,2], and against oxidative stress associated processes [3,4]. This protection has been related to the relatively high content in this diet of non hydrogenated, monounsaturated fatty acids (MUFA) [5,6], since olive oil is the main source of fat in the Mediterranean diet [1]. The predominant fatty acid in olive oil is the MUFA oleic acid (18:1, n-9) with percentages ranging from 56% to 84%, while the polyunsaturated fatty acid (PUFA) linoleic acid (18:2, n-6) is usually found at percentages between 3% and 21% [7]. However, olive oil also contains several minor components with potentially healthy biological properties [7]. Several mechanisms have been proposed to explain the preventive effects of olive oil on atherosclerosis development. Among them, the reduction of the low-density lipoprotein (LDL) susceptibility to oxidation has been one of the main

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mechanisms addressed. Consequences of this reduction are a protection on cellular oxidative stress, thrombogenicity, and atheroma plaque formation [8,9].

In the frame of the EUROLIVE study we have previously reported an increase in plasma fatty acids after olive oil consumption [10]. However, contradictory data have been reported about the effects of olive oil (and its main components) on lipoprotein metabolism: some authors report that olive oil supplementation did not modify LDL fatty acid composition [11,12]. In contrast, an increase in the oleic acid incorporation to LDL after olive oil consumption has been reported in humans [13,14] and in animal studies [15–17]. In some of these studies [15] a decrease in the LDL oxidability was observed together with an increase in oleic acid in LDL. Data from these studies are difficult to interpret because, in most of them, olive oil was ingested as a dietary supplement, nor in replacement of other fats [18–20]. To the best of our knowledge, there are no studies with a large sample size in which the effect of olive oil ingestion on fatty acid LDL composition has been examined. In this context, the aim of the present study, based on data from the Eurolive Study (5th EU-Framework Program) [21], is to assess the effect of olive oil on the LDL fatty acid content and its relationship with plasma oxidation markers in a large sample of healthy European male adults.

Table 1. Characteristics of the Olive Oils Administered

	Type of olive oil		
	LPC	MPC	HPC
Quality parameters			
Free acidity (% oleic acid)	0.03	0.08	0.18
Peroxide value (mEq O_2/kg)	4.12	5.89	11.28
Fatty acids (%)			
C14:0	0.01	0.01	0.01
C16:0	10.63	10.50	10.63
C16:1	0.88	0.86	0.88
C17:0	0.05	0.05	0.04
C17:1	0.09	0.09	0.09
C18:0	3.27	3.13	2.84
C18:1	79.08	79.80	80.60
C18:2	4.64	4.21	3.35
C20:0	0.39	0.39	0.35
C18:3	0.58	0.58	0.58
C20:1	0.26	0.25	0.25
C22:0	0.11	0.10	0.10
C24:0	0.01	0.02	0.02
α-Tocopherol (ppm)	229	228	228
Phenolic compounds (ppm)	2.7	164	366
Squalene (mg/g)	3.0	3.2	3.4
β-sitosterol (mg/g)	1.4	1.5	1.5

MATERIALS AND METHODS

Study Design and Study Population

The EUROLIVE study [21] was a randomized, crossover trial with three intervention periods of three weeks and two wash-out periods of 2 weeks. Three types of olive oils with high (HPC, 366 mg/Kg), medium (MPC, 164 mg/Kg), and low (LPC, 2.7 mg/Kg) phenolic content were used. Olive oils were specially prepared for the trial from an extra virgin olive oil (produced from Picual olives, Spain) as it follows: a virgin olive oil with a high natural phenolic content (366 mg/kg) was selected. Fatty acid and vitamin E composition were measured. Harvests of this type of virgin olive oil, from the same cultivar and soil, which were submitted to refinement, were tested in order to choose one with a similar fatty acid and micronutrient profile to that of the virgin olive oil selected. An adjustment of vitamin E to similar values of that present in the virgin olive oil selected was performed. Phenolic compounds are lost in the refination process, due to this, the refined olive oil had a low phenolic content (2.7 mg/kg). By mixing virgin and refined olive oil, an olive oil with an intermediate phenolic content (164 mg/kg) was obtained. Finally, the three oils had similar fat and micronutrient (i.e. vitamin E, triterpenes, sitosterols) composition, but with relevant differences only in their phenolic content (Table 1) [14]. Fatty acid composition was determined by gas chromatography [22].

We enrolled 200 healthy European males (mean age: 33.1 ± 10.6 years) recruited from September 2002 through June 2003 in 6 Centers of 5 European Countries (Denmark, Finland, Germany, Italy, and Spain). Eligibility criteria were a willingness to provide written, informed consent and to agree to adhere to the protocol. Exclusion criteria were: smoking, intake of antioxidant supplements, aspirin, or drugs with established antioxidant properties, hyperlipidemia, obesity (body mass index >30 kg/m^2), diabetes, hypertension, celiac or other intestinal disease, any condition limiting mobility, life-threatening diseases, or any other disease or condition that could impair compliance. Subjects were considered healthy on the basis of physical examination and routine biochemical and hematological laboratory determinations. The protocol was approved by the Ethics Committee of each Clinical Trial Center involved.

Olive oils were sequentially administered over three periods of 3 weeks preceded by two-week wash-out periods in which participants were requested to avoid olive oil and olive consumption. In intervention periods, subjects were provided with 25 mL/day of olive oil, administered among meals. Participants were requested to avoid a high intake of foods listed as containing antioxidants. Participants recorded their habitual diet on diet records during three consecutive days at baseline and the end of the study period. Participants were personally advised by a nutritionist on how to record food consumption and follow the above mentioned dietary recommendations. Food consumption was converted into the corresponding nutrient intake by means of a validated nutrition software from each country. Physical activity was recorded at baseline and at the end of the study [23]. The full protocol has been previously and fully described elsewhere [21].

For the present study we used data of the baseline and the endpoint (last intervention). Serum glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triacylglycerols (TG) were determined by standardized enzymatic methods. LDL cholesterol was calculated by the Friedewald formula. Plasma circulating oxidized LDL (oxLDL) was measured by enzimoimmunoassay. Plasma total $F_{2\alpha}$-isoprostanes were determined using high performance liquid chromatography and stable isotope dilution mass spectrometry [24]. Serum LDL uninduced conjugated dienes (CD) were measured by spectrophotometry at 234 nm and 300 nm. CD concentration was adjusted for the cholesterol concentration in LDL. LDL isolation was performed by sequential flotation ultracentrifugation from plasma EDTA samples [21]. The fatty acid composition of the isolated LDL was determined following the method described by Bondía et al. [25] in which fatty acids are transformed into methyl esters and analyzed by gas chromatography. Apolipoprotein B in LDL was measured by immunoturbidimetry. Fatty acids were expressed as mg/g of LDL-apolipoprotein B100. All determinations were centralized in reference laboratories.

Statistical Analyses

Baseline data are shown as mean ± SD and 5% trimmed mean values. Kolmogorov Smirnov test and normal probability plots were used to assess normal distribution. One-factor ANOVA and Kruskal-Wallis test were used to determine differences in basal characteristics and nutrient intake among the three olive oil interventions. A Student's t test for paired samples was used to compare LDL fatty acid composition at baseline and at the end of the intervention.

Multiple regression models were fitted in order to evaluate the association between oxidative markers and fatty acids in LDL. These models used the oxidative markers postintervention values, adjusted by basal values, as dependent variable and the difference in the oleic/linoleic ratio in LDL as independent variable. For the plasma isoprostanes model, arachidonic acid in LDL was also included as an adjusting variable. Because there was no interaction with the olive oil administration order, it was not included in the models. Statistical significance was defined as $p < 0.05$ for a two-sided test. All tests were performed using the SPSS System for Windows release 11.0.

RESULTS

Eighteen participants (9%) did not complete the study. No one relevant side effect was registered during the research. At the beginning of the study, 193 (96.5%) participants submitted food records. Mean total energy intake was 2261 calories daily, with 48.6%, 33.8%, and 15.8% of calories derived from carbohydrate, fat, and protein, respectively. Table 2 shows the mean nutrient intake, at the beginning and at the end of the study. Mean total energy intake was unchanged. There was a significant increase in fat intake from the beginning to the end of the study (mean values, 86.4 g/day versus 95.1 g/day, $p < 0.005$), mainly linked to the increase of MUFA (mean values, 30.9 g/day versus 39.8 g/day, $p < 0.001$) as oleic acid (mean values, 27.5 g/day versus 34.8 g/day). Polyunsaturated (PUFA) and saturated fat (SFA) intake remained constant. Consumption of carbohydrates decreased (3.3% as average, $p < 0.001$), while alcohol and protein intake did not change.

Plasma total and LDL cholesterol did not change. HDL cholesterol ($p < 0.001$) and glucose ($p < 0.05$) increased, whereas $F_{2\alpha}$-isoprostanes decreased ($p < 0.01$). Lipid values and oxidative stress markers before and after interventions are shown in Table 3.

The fatty acid composition of LDL expressed as an absolute value in mg/g of LDL-apolipoprotein B100 and as a percentage of fatty acids is shown in Table 4. The Kolmogorov-Smirnov test demonstrates that the main LDL fatty acids have a baseline distribution that is not normal: the Q-Q plots between expected and observed values show that the overlapping area between the expected "normal" distribution and that observed is very high, but in two tails some values are markedly higher or lower than expected. In the hypothesis that these outliers represent subjects with peculiar characteristics, we also repeated comparative analyses with 5% trimmed means.

A significant enrichment of oleic acid (C18:1 n-9) in LDL was observed ($p < 0.001$). MUFA/PUFA and oleic/linoleic acid ratios in LDL increased. When considering the LDL percent content of fatty acids, oleic acid increased ($p < 0.001$), and linoleic and arachidonic acid decreased ($p < 0.005$). No changes were observed in the percentage of palmitic and stearic acids. When paired comparisons in absolute values obtained at the end of the study were examined, olive oil administration increased significantly oleic acid in LDL ($p < 0.001$). When differences were adjusted for energy intake and order of olive oil administration, the comparisons described above remained significant. The significance of the association was maintained when stratifying by center.

Isoprostanes were inversely correlated with the difference in the oleic/linoleic ratio: for every increase of 1 mg/g in the oleic/linoleic ratio, $F_{2\alpha}$-isoprostanes decreased by 4.2 μg/L. There was no relationship between adjusted isoprostanes and difference in arachidonic acid between interventions. $F_{2\alpha}$-isoprostanes, adjusted by baseline levels, were, however, directly related with levels of arachidonic acid after olive oil consumption ($B = 0.024$, 95% CI 0.002–0.046; $p = 0.030$). The overall model, with difference in oleic/linoleic ratio age and arachidonic acid in LDL simultaneously as independent variables, explains a 7.6% of the isoprostane variation ($r = 0.276$, $p = 0.004$). The difference in the oleic/linoleic ratio is being the variable with the strongest relationship to isoprostanes (Table 5). Further adjustments by other covariates (such as. energy intake, change in fat intake from baseline, and olive oil administration sequence) did not modify the trend. No relationship was observed with the other variables, including uninduced dienes.

Table 2. Dietary Intake Characteristics, as Mean Nutrient Intake and Differences, at the Beginning and at the End of the Study

Component (g)		Mean	SD	Absolute change	p value
Carbohydrates	baseline	275.8	91.1		
	end	256.9	84.4	−18.8	<0.001
Protein	baseline	88.7	27.5	−3.1	0.137
	end	85.5	26.8	−3.1	0.137
Total fat	baseline	86.4	30.6		
	end	95.1	34.6	+8.6	0.002
SFA	baseline	33.7	15.4		
	end	32.6	14.6	−1.1	0.360
MUFA	baseline	30.9	12.6		
	end	39.8	16.1	+8.9	<0.001
PUFA	baseline	12.1	6.2		
	end	12.3	6.0	+0.2	0.630
Cholesterol (mg)	baseline	329.7	149.5		
	end	310.9	151.6	−13.5	0.243
Oleic acid	baseline	27.5	11.3		
	end	34.8	15.1	+7.3	<0.001
Alcohol	baseline	81.0	191.0		
	end	74.3	191.0	−6.7	0.292
% Kcal					
Energy (Kcal)	baseline	2275.3	654.3		
	end	2366.3	1258.8	+91.0	0.302
Carbohydrates	baseline	48.5	8.2		
	end	45.2	9.4	−3.3	<0.001
Protein	baseline	16.0	3.9		
	end	15.2	3.6	−0.8	0.020
Total fat	baseline	34.1	6.9		
	end	37.5	10.3	+3.4	<0.001
SFA	baseline	13.0	3.7		
	end	12.6	3.5	−0.4	0.207
MUFA	baseline	12.3	4.0		
	end	15.9	5.9	+3.6	<0.001
PUFA	baseline	4.8	2.1		
	end	4.8	1.9	0.0	0.902

Saturated fat = SFA, monounsaturated fat = MUFA, polyunsaturated fat = PUFA.

Table 3. Lipid Values and Oxidative Stress Markers at the Beginning and at the End of the Study

	Base line		End		p
	Mean	DS	Mean	DS	
Cholesterol (mg/dL)	182.7	40.2	184.4	42.1	ns
Triacylglycerols (mg/dL)	95.6	49.0	91.4	44.5	ns
HDL cholesterol (mg/dL)	47.3	11.1	50.5	12.6	<0.001
LDL cholesterol (mg/dL)	116.5	36.7	115.7	38.2	ns
Glucose (mg/dL)	85.7	9.7	87.3	10.8	0.035
Oxidized LDL (U/L)	49.4	22.8	47.2	22.4	ns
Serum-LDL uninduced conjugated dienes (umol/mmol cholesterol)	11.8	3.5	11.7	3.8	ns
Plasma-$F_{2\alpha}$-isoprostanes (ng/L)	29.2	6.7	28.0	6.9	<0.01

DISCUSSION

In this study, sustained consumption of olive oil, in replacement of other fats, at a real life dose of 25 mL per day, increased the oleic acid content of the LDL as well as the MUFA/PUFA and oleic/linoleic acid ratios in LDL. Changes in the LDL fatty acid composition were inversely related with oxidative lipid and LDL damage. These results were independent of the type of olive oil consumed.

Higher MUFA intake has been associated with a more favorable cardiovascular risk profile [26]. In our study, carbohydrate consumption decreased with the increase in olive oil consumption. Low carbohydrate/high fat diets typically increase HDL cholesterol levels versus high carbohydrate/low fat diets [27–29]. In agreement with this, and with our previous results [10,21,30], we observed an increase in serum HDL-cholesterol after olive oil ingestion. Benefits of olive oil consumption on the lipid profile were also reflected in the reduction of total/HDL cholesterol and

Table 4. Baseline and Final Fatty Acid Content of LDL in the Studied Subjects; Absolute Values Are Given as mg/g of LDL-Apolipoprotein B100

Fatty acids		Baseline			End		
		Mean	SD	5% trimmed mean	Mean	SD	5% trimmed mean
Oleic	mg/gApo B 100	147.30	74.82	140.61	166.66**	80.12	160.48
	%	21.12	2.66	21.09	22.99**	2.81	22.96
Linoleic	mg/gApo B 100	303.29	150.82	293.01	312.62	156.19	302.50
	%	43.24	4.90	43.33	42.22*	4.41	42.36
Palmitic	mg/gApo B 100	144.72	75.24	137.86	150.80	74.39	144.70
	%	20.78	2.70	20.83	20.56	2.18	20.47
Stearic	mg/gApo B 100	48.07	23.27	46.11	49.71	24.80	47.73
	%	7.00	1.46	6.85	6.83	1.63	6.64
Arachidonic	mg/gApo B 100	54.65	31.14	51.68	53.85	27.42	51.85
	%	7.86	1.18	7.80	7.39**	1.52	7.33
Oleic/linoleic ratio		0.50	0.14		0.55**	0.12	

* $p < 0.01$, ** $p < 0.001$.

Table 5. Linear Regression Coefficients (Standard Error) of the Relations between $F_{2\alpha}$-Isoprostanes with Difference in Oleic/Linoleic Ratio, Adjusted for Age and Arachidonic Acid in LDL

	F2α-isoprostanes (μg/L)
Change inoleic/linoleic (units)	−4.208 (1.897)*
Age(year)	−0.0520 (0.026)*
Arachidonic Acid in LDL (mg/g Apo B)	0.0241 (0.011)*
Constant	28.563 (0.908)**
	R = 0.276
	R^2 = 0.076

Change in oleic/linoleic ratio = difference in the oleic/linoleic ratio in LDL between baseline and after olive oil consumption, Ox-LDL = oxidized LDL, $F_{2\alpha}$-isoprostanes = plasma $F_{2\alpha}$-isoprostanes after olive oil ingestion.
* $p < 0.05$, ** $p < 0.001$.

LDL/HDL cholesterol ratios. The total/HDL cholesterol ratio is an established and efficient indicator of lipid atherogenesis, reflecting the balance of cholesterol transport in and out of the arterial intima [31]. In a recent report with data from the 20-year follow up Framingham Offspring Study, total/HDL cholesterol and LDL/HDL cholesterol ratios have been reported to be the most efficient lipid parameters for predicting CHD [32].

We also observed a decrease in F_2-isoprostanes from the beginning to the end of the study. In a previous work [21] we did not observe statistically significant differences in this oxidative biomarker when comparing values before and after each one of the three olive oil intervention periods. The fact that significant changes in F_2-isoprostanes were observed after several periods of olive oil consumption suggests, despite the fact that no carryover was detected in the washout periods [21], that a long-term consumption of olive oil is required to observe changes in F2-isoprostane plasma concentrations.

Dietary fat can modulate the susceptibility of LDL to oxidative modification. Most studies comparing the effect of a MUFA-rich diet with that of a PUFA-rich one concluded that MUFA-rich diets reduced the susceptibility of LDL to oxidation [33]. Thus, oleate rich LDL appeared to be more resistant to oxidation than linoleate-enriched LDL. Oxidation of the lipids and lipoproteins present in LDL leads to a change in the lipoprotein conformation by which LDL is better able to enter the monocyte/macrophage system of the arterial wall, and promote the atherosclerotic process [34]. After olive oil consumption the susceptibility of LDL to oxidation, however, depends not only on the change in the LDL fatty acid content promoted by olive oil, but also on the phenolic content bound to the LDL. Recent results of the EUROLIVE study [21] have provided evidence of the protective role of phenolic compounds from olive oil on the in vivo LDL oxidation in humans. Olive oil phenolic compounds have also been shown to modulate the LDL phenolic content and the in vivo LDL oxidation at postprandial state in humans [35]. In the present study, we observed a reduction of the in vivo LDL oxidative damage associated with an enrichment of oleic acid in LDL after sustained olive oil consumption. However, the decrease in the degree of the in vivo LDL oxidation from the baseline to the end of the study did not reach significance. The differences among the olive oil phenolic content ingested in the last intervention period could account for this fact.

In our study, we also observed an inverse relationship between the oleic/linoleic acid ratio in LDL and the plasma concentration of isoprostans. Plasma F2-isoprostanes are considered to be a systemic marker of oxidative stress [36] and high levels of circulating F2-isoprostanes have been shown to be predictors of cardiac events in CHD patients [36]. The increase in the oleic/linoleic acid ratio in LDL has been shown to promote favorable changes in inflammatory markers. Tsimikas et al [37] observed that an increase in the oleic/linoleic acid ratio in LDL induced less monocyte chemotaxis and adhesion when exposed to oxidative stress.

In summary, real-life daily doses of olive oil (25 mL/day) increased oleic acid and the oleic/linoleic acid ratio in LDL and improved the cardiovascular risk lipid profile. This increase in

the oleic/linoleic acid ratio was inversely related with the degree of lipid and LDL oxidation. Our study adds further evidence to recommend the use of olive oil as a source of fat in order to achieve benefits against classical and novel risk factors of cardiovascular disease.

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