Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial

Eva Gimeno1, Karina de la Torre-Carbot1, Rosa M. Lamuela-Raventós1, Ana I. Castellote1, Montserrat Fitó2, Rafael de la Torre2, María-Isabel Covas2 and M. Carmen López-Sabater1*

1Department of Nutrition and Food Science, Reference Center in Food Technology, Faculty of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Spain
2Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d' Investigació Médica (IMIM-Hospital del Mar), C. Doctor Aiguader, 88, 0800, Barcelona, Spain

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Olive oil decreases the risk of CVD. This effect may be due to the fatty acid profile of the oil, but it may also be due to its antioxidant content which differs depending on the type of olive oil. In this study, the concentrations of oleic acid and antioxidants (phenolic compounds and vitamin E) in plasma and LDL were compared after consumption of three similar olive oils, but with differences in their phenolic content. Thirty healthy volunteers participated in a placebo-controlled, double-blind, crossover, randomized supplementation trial. Virgin, common, and refined olive oils were administered during three periods of 3 weeks separated by a 2-week washout period. Participants were requested to ingest a daily dose of 25 ml raw olive oil, distributed over the three meals of the day, during intervention periods. All three olive oils caused an increase in plasma and LDL oleic acid (P<0.05) content. Olive oils rich in phenolic compounds led to an increase in phenolic compounds in LDL (P<0.005). The concentration of phenolic compounds in LDL was directly correlated with the phenolic concentration in the olive oils. The increase in the phenolic content of LDL could account for the increase of the resistance of LDL to oxidation, and the decrease of the in vivo oxidized LDL, observed in the frame of this trial. Our results support the hypothesis that a daily intake of virgin olive oil promotes protective LDL changes ahead of its oxidation.

Olive oil: Oleic acid: Phenolic compounds: LDL: CVD risk

CVD is the main cause of death and disability in developed countries1–3. The type of fat consumed can modify the plasma and LDL lipid profile, which is directly related to the growth of atheroma plaque4,5. However, the antioxidant content of the diet is also crucial, as oxidized LDL seems to be involved in atherosclerotic plaque development6.

Olive oil, rich in MUFA and antioxidant minor components, is considered to be favourable for cardiovascular health7–12. Linoleic acid (C18: 2) accounts for 90 % of the PUFA present in LDL and is the main substrate for oxidation. However, diets rich in oleic acid, like those in Mediterranean countries, generate LDL particles which appear to be more resistant to oxidation13–17. Moreover, data from in vitro18,19 and in vivo studies20–23 show that the phenolic compounds of olive oil protect LDL from lipid peroxidation. Thus, olive oil phenolic compounds are good candidates to partially account for the prevention provided by diet against CVD. Due to this, studies directed at a better understanding of the protective mechanisms of olive oil on human health must be enhanced.

To date, few studies have analysed the effects of sustained olive oil consumption on human LDL composition. The few available data come from short-term studies20,24,25 or non-randomized trials where only virgin olive oil was used25. A double-blind, randomized, crossover, controlled trial was carried out to identify the effect of similar olive oils, but with a range of phenolic content, on the levels of plasma and LDL antioxidants and oleic acid in healthy subjects. Volunteers from a religious centre, a population with regular and similar lifestyles such as physical activity and dietary habits, were involved. In the frame of this trial we have previously reported21 a protective effect of an olive oil rich in phenolic compounds on LDL oxidation. From these results, our main hypothesis was that sustained real-life doses (25 ml/d) of raw rich phenolic olive oil could enhance the antioxidant load of the LDL, thus protecting the lipoproteins from oxidation. Here, we examined the fatty acid and antioxidant composition of the LDL after consumption of similar olive oils, but with differences in their phenolic content, in order to test our hypothesis.

Materials and methods

Study population

An in-person screening visit was conducted to ascertain eligibility and obtain baseline data. Forty-two subjects from a religious community were screened for inclusion. Nine of them were ineligible. Thus, thirty-three healthy volunteers, from 23 to 91 years old, with a regular lifestyle and dietary habits

Abbreviations: CAE, caffeic acid equivalents; FAME, fatty acid methyl esters.
* Corresponding author: Dr M. Carmen López Sabater, fax +34-93 403 59 31, email mclopez@ub.edu
were included. The volunteers gave their written consent prior to participating in the study. Subjects with any of the following conditions were excluded: smoking; intake of any drug or supplements with established antioxidative properties, either in the two weeks before the onset of the study or throughout the study; obesity (BMI > 30 kg/m²); diabetes; and any disease or condition that would impair compliance.

Diets were prepared and consumed in the religious centre. Subjects maintained their regular physical activity and lifestyle throughout the study. The local institutional Review Board approved the protocol according to the Helsinki Declaration of 1975.

### Olive oil composition

Three olive oils provided by the Olive Oil Cooperative Association of Catalonia were used. They were obtained from the same harvest which means that olive fruits were of the same cultivar, collection time, and soil. First, a virgin olive oil of 370 mol/kg (phenolic content of 0 mol/kg) and a common olive oil (phenolic content of 370 mol/kg) with similar fatty acid composition, α-tocopherol and β-carotene content, in order to match the virgin olive oil. Major and minor components of the three olive oils were examined in order to confirm their similar fatty acid and micronutrient profile, as well as the differences in their phenolic content. The characteristics of the oils are summarized in Table 1. The acidity value, the peroxide index, and the UV spectrophotometric index (K270) were determined following the analytical methods described in the European Union Commission Regulation CE/1989/2003. Fatty acids were transformed into methyl esters and analyzed by gas chromatography. α-Tocopherol was measured by HPLC, as previously described. Phenolic compounds were measured by the Folin-Ciocalteau method.

The sample size was calculated to provide a statistical power of 80%. In order to recognize as statistically significant a difference ≥10 min change in lag time, and a change of 4 nmol/mg apo B of phenolic compounds in LDL, ten and nine subjects were necessary in each order of olive oil administration respectively. It was assumed that standard deviations which have been validated for use on Spanish men.

### Study design

A placebo-controlled, double-blind, crossover, randomized, supplementation trial was conducted. A Latin square for the three treatments was used in the crossover trial, to randomize participants into three orders of olive oil administration: virgin–common–refined (order 1), common–refined–virgin (order 2), and refined–virgin–common (order 3). The three olive oils were administered over three periods of 3 weeks, each one preceded by 2-week washout periods (Fig. 1). Participants were requested to ingest a daily dose of 25 ml olive oil, distributed over the three meals of the day, during intervention periods. Refined olive oil was used as source of raw fat in washout periods. Other cooking fats were replaced by refined olive oil to maintain energy and oleic acid intake unchanged during the entire study.

Daily menus were recorded, as were extra food intakes between meals, and meals eaten outside the religious centre. Participants were requested to avoid a high intake of foods containing phenolic compounds such as fruit, vegetables, tea and red wine. Participants were managed by a trained physician who stayed at the religious centre throughout the study. Participants were instructed to return the 25 ml containers every morning when they collected their next daily dose, in order to register the amount of unconsumed oil. Treatment containers for the daily dose of olive oil to be ingested in the intervention periods were coded, concealed from participants, and distributed by investigators to the participants. Containers were opaque in order to conceal the olive oil and to avoid its degradation.

Diets were analyzed by a nutritionist and converted into nutrients using the software Medysystems (Conacyte S.A., Madrid, Spain). Anthropometric variables (i.e. height and weight) were recorded. Physical activity was assessed by the Minnesota Leisure Time Physical Activity Questionnaire, which has been validated for use on Spanish men.

### Sample size and power analyses

### Blood sampling and laboratory analyses

Laboratory measurements were carried out on samples from fasting subjects taken: before the first washout period (baseline); before administration of the three types of oil; and after olive oil administration. To prevent oxidation and aggregation of LDL, blood samples were drawn with EDTA tubes (1 g/l) and stored with saccharose (0.18 mM) at −80°C. Plasma was separated by centrifugation at 1000 × g at 4°C for 15 min. LDL isolation was performed by sequential flotation ultracentrifugation. All samples were stored at −80°C until analysis.
Total cholesterol, HDL-cholesterol and TAG levels were measured by standard enzymatic methods. The fatty acid composition of plasma and LDL was measured by the method described by Rodríguez-Palmero et al. Fatty acid methyl esters were prepared by alkaline hydrolysis with sodium methylate and esterification with boron trifluoride in methanol. Fatty acid methyl esters were extracted with hexane and injected into a gas chromatograph. The CV obtained ranged from 3·42 to 5·25 %. To determine α-tocopherol in plasma and LDL, an aliquot of the sample was deproteinized with ethanol. The analyte was then extracted with hexane and injected into an HPLC system. The CV was 4·27 %. Phenolic compounds in LDL were also determined by HPLC-Diode Array Detection, as previously described. Briefly, acidulated LDL was applied to a Waters Oasis™ HLB extraction cartridge (Milford, MA, USA) and washed with water and 5 % aqueous methanol. Phenolic compounds, measured only in LDL, were eluted with methanol, which was then evaporated under a stream of nitrogen. The residue was dissolved in acidulated water and injected into an HPLC system. The chromatogram was monitored at 280 nm and the areas of phenolic compounds were expressed as CAE. The analytical within-run precision was 5·25 %, and the between-run precision was 8·8 %.

Oxidized LDL was measured in plasma by ELISA (ox-LDL, Mercodia AB, Uppsala, Sweden). The LDL resistance to oxidation was determined by formation of conjugated dienes after copper (5 μM) oxidation of isolated LDL.

Results of LDL parameters were expressed according to apo B. Apo B concentrations were measured by immunoturbidimetry (Roche Diagnostics, Basel, Switzerland).

Dietary intake and adherence
The average of the main antioxidant (i.e. β-carotenoïd, vitamin C, α-tocopherol), or pro-oxidant (i.e. Fe) intake, energy, and any nutrient of interest were similar in the three groups during each type of olive oil intervention (Table 3). Participants’ alcohol intake was <30 g/d. Tyrosol and hydroxytyrosol in urine increased in a dose-dependent manner with the phenolic content of olive oil administered. Mean changes were 15 %, 147 %, and 190 % for tyrosol, and 12 %, 180 %, and 221 % for hydroxytyrosol, after refined, common, and virgin olive oil, respectively.

Plasma and LDL fatty acid and antioxidant content
The phenolic content of LDL was significantly higher after the virgin olive oil administration than at baseline (P<0·005) and
Table 2. Baseline characteristics by sub-groups of subjects depending on the order* of olive oil administration
(Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Order 1</th>
<th>Order 2</th>
<th>Order 3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>54.8 ± 21.4</td>
<td>61.0 ± 19.2</td>
<td>56.6 ± 19.3</td>
<td>0.80</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.2 ± 3.5</td>
<td>23.2 ± 3.3</td>
<td>23.6 ± 2.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.92 ± 0.05</td>
<td>0.89 ± 0.04</td>
<td>0.89 ± 0.05</td>
<td>0.40</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.4</td>
<td>1.0 ± 0.5</td>
<td>0.68</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.4 ± 1.1</td>
<td>5.7 ± 1.0</td>
<td>5.9 ± 1.2</td>
<td>0.25</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>0.66</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.4 ± 0.7</td>
<td>4.2 ± 0.5</td>
<td>4.6 ± 0.9</td>
<td>0.59</td>
</tr>
<tr>
<td>Plasma α-tocopherol (μmol/ml)</td>
<td>4.2 × 10⁻² ± 1.38 × 10⁻²</td>
<td>4.7 × 10⁻² ± 1.2 × 10⁻²</td>
<td>5.1 × 10⁻² ± 1.1 × 10⁻²</td>
<td>0.22</td>
</tr>
<tr>
<td>Plasma oleic acid (mmol/l)</td>
<td>2.1 ± 0.5</td>
<td>2.0 ± 0.9</td>
<td>2.0 ± 0.6</td>
<td>0.77</td>
</tr>
<tr>
<td>LDL α-tocopherol (μmol/mg apo B)</td>
<td>2.1 × 10⁻² ± 0.31 × 10⁻²</td>
<td>1.9 × 10⁻² ± 0.38 × 10⁻²</td>
<td>1.7 × 10⁻² ± 0.45 × 10⁻²</td>
<td>0.59</td>
</tr>
<tr>
<td>LDL phenolic compounds (nmol CAE/mg apo B)</td>
<td>8.7 ± 3.2</td>
<td>7.9 ± 2.3</td>
<td>7.8 ± 1.5</td>
<td>0.29</td>
</tr>
<tr>
<td>LDL oleic acid (μmol apo B)</td>
<td>0.31 ± 0.1</td>
<td>0.25 ± 0.05</td>
<td>0.31 ± 0.1</td>
<td>0.34</td>
</tr>
<tr>
<td>Physical activity (kJ/d)</td>
<td>1410 ± 966</td>
<td>1648 ± 879</td>
<td>1886 ± 1518</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Table 3. Daily intake of nutrients in each dietary period
(Mean values and standard deviations for thirty subjects)

<table>
<thead>
<tr>
<th>Olive oil administered</th>
<th>Refined (0 mg/μmol/kg CAE)</th>
<th>Common (370 μmol/kg CAE)</th>
<th>Virgin (825 μmol/kg CAE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>9567 ± 937</td>
<td>9639 ± 1079</td>
<td>9668 ± 966</td>
<td>0.84</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>20.4 ± 1.8</td>
<td>20.2 ± 1.8</td>
<td>20.3 ± 1.6</td>
<td>0.70</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>37.5 ± 4.1</td>
<td>37.7 ± 4.5</td>
<td>40.0 ± 4.8</td>
<td>0.60</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>41.9 ± 5.1</td>
<td>41.8 ± 4.9</td>
<td>41.6 ± 5.5</td>
<td>0.89</td>
</tr>
<tr>
<td>MUFAs (%)</td>
<td>20.2 ± 2.8</td>
<td>20.1 ± 2.9</td>
<td>20.2 ± 2.8</td>
<td>0.96</td>
</tr>
<tr>
<td>SFAs (%)</td>
<td>4.5 ± 0.5</td>
<td>4.4 ± 0.5</td>
<td>4.4 ± 0.4</td>
<td>0.55</td>
</tr>
<tr>
<td>α-Tocopherol (mg)*</td>
<td>8.4 ± 1.9</td>
<td>8.3 ± 2.4</td>
<td>8.6 ± 2.3</td>
<td>0.66</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>227 ± 99</td>
<td>228 ± 102</td>
<td>229 ± 97</td>
<td>0.75</td>
</tr>
<tr>
<td>Phenolic compounds (mg)*</td>
<td>14.9 ± 4.8</td>
<td>14.4 ± 5.3</td>
<td>14.7 ± 5.8</td>
<td>0.66</td>
</tr>
<tr>
<td>β-Carotene (μg)</td>
<td>2385 ± 354</td>
<td>2357 ± 361</td>
<td>2420 ± 332</td>
<td>0.74</td>
</tr>
</tbody>
</table>

CAE, phenolic content in caffeic acid equivalents.
* This amount excludes the phenolic compounds and α-tocopherol taken with the oils studied.
Virgin olive oil and phenolic content of LDL.

Table 4. Content in α-tocopherol, phenolic compounds and oleic acid at baseline and after each dietary period (Mean values and standard deviations for thirty subjects)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-consumption of refined olive oil</th>
<th>Post-consumption of common olive oil</th>
<th>Post-consumption of virgin olive oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol (μmol/ml)</td>
<td>4.8 x 10⁻² ± 1.1 x 10⁻²</td>
<td>4.6 x 10⁻² ± 1.2 x 10⁻²</td>
<td>4.4 x 10⁻² ± 1.5 x 10⁻²</td>
<td>4.4 x 10⁻² ± 1.1 x 10⁻²</td>
</tr>
<tr>
<td>Oleic acid (mmol/l)</td>
<td>2.02 ± 0.10</td>
<td>2.05 ± 0.07</td>
<td>2.12 ± 0.07</td>
<td>2.05 ± 0.10</td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol (μmol/mg apo B)</td>
<td>1.90 x 10⁻² ± 0.49 x 10⁻²</td>
<td>1.95 x 10⁻² ± 0.58 x 10⁻²</td>
<td>1.94 x 10⁻² ± 0.53 x 10⁻²</td>
<td>1.92 x 10⁻² ± 0.51</td>
</tr>
<tr>
<td>Phenolic compounds (nmol CAE/mg apo B)</td>
<td>7.88 ± 1.55</td>
<td>9.16 ± 3.7</td>
<td>9.55 ± 4.27</td>
<td>10.44± 4.0</td>
</tr>
<tr>
<td>Oleic acid (μmol/mg apo B)</td>
<td>0.31 ± 0.14</td>
<td>0.32 ± 0.12</td>
<td>0.39 ± 0.14</td>
<td>0.39 ± 0.14</td>
</tr>
</tbody>
</table>

* Mean values were significantly different from baseline values (P<0.005).
† P < 0.05 for linear trend from refined to common to virgin olive oil.

As is reflected in this study and others, olive oil phenolic compounds are absorbed in human subjects in a dose-dependent manner with the phenolic content of the olive oil20,21,42. Phenolic compounds from olive oil can bind the human LDL after virgin olive oil ingestion43,44. In a previous study, we observed that the postprandial LDL total phenolic content and LDL oxidation could be modulated by olive oil phenolic compounds in human subjects 20. Here, we report the same phenomenon after a sustained consumption of olive oil.

At baseline, subjects did not consume only olive oil as a source of fat. Instead, they consumed preferentially other vegetable oils for cooking, using olive oil for raw purposes. The increase in oleic acid in LDL observed throughout the study could be due to the consumption of all types of olive oil, given that the refined olive oil was consumed during the wash-out periods. From our results, olive oil consumption promoted an increase of MUFA in the LDL. MUFA are less susceptible to oxidation than PUFAs45. Due to this, the increase of MUFA in LDL could enhance the preservation of the phenolic compounds bound to LDL, given that they are not used to counteract the autocatalytic chain reaction of LDL fatty acid peroxidation46. This fact could also explain the non-significant increase in LDL phenolic compounds observed after refined olive oil intervention, compared with baseline values.

Phenolic compounds can protect LDL from oxidation20,21,44,47–50 through different mechanisms: (1) for the free radical scavenging properties (the capacity of hydrogen-donation and their ability to improve radical stability)51–53, (2) by means of the strong metal-chelation capacity54, (3) through the nitric oxide stimulation in endothelial cells55,56, (4) by stimulating antioxidant transcription and detoxification defence systems52,57,58, and (5) by modulating other enzymatic systems related with oxidation process (cyclooxygenases, lipoxygenases and NAD(P)H oxidase)53. In addition, olive oil phenolic compounds have been shown to be related with the prevention of platelet aggregation49,59, vasodilatation52, and anti-inflammation52,59–61. Thus, the protection provided by the olive oil phenolic compounds on CVD risk may be due to a combination of bioactive mechanisms.

Phenolic compounds of dietary origin have been shown to be involved in cholesterol and lipoprotein metabolism60.
In this study, we observed an increase in HDL cholesterol levels after virgin olive oil intervention. These results are in line with the recent results of the EUROLIVE study\textsuperscript{62}, a large inter-country intervention trial with three similar types of olive oils, but with differences in their phenolic content. Results of the EUROLIVE study showed a dose-dependent increase of the plasma HDL cholesterol levels with the phenolic content of the olive oil administered. Mechanisms by which phenolic compounds can enhance HDL cholesterol are at present unknown.

Phenolic compounds in olive oil may contribute to the health benefits\textsuperscript{63,64} and a Mediterranean diet, rich in virgin olive oil, improves protection against the major risk factors for CVD\textsuperscript{65,66}.

The design and conduct of the study had strengths and limitations. One strength was that the dose administered, 25 ml/d, closely reflects real-life consumption in Southern European Mediterranean countries. Another was the crossover design, which permitted the same participants to receive all olive oils, thereby minimizing interferences with confounding variables. Our design, however, did not allow modelling the first- and second-order possible carryover effects. Another limitation was the inability to assess potential interactions between olive oil and other diet components. Measurements of dietary intake relied on self-reporting and were, therefore, subjective. Another limitation is the short duration of the intervention periods. It is unknown whether additional or different effects would have been observed over longer periods. A longer duration of the study, however, could have impaired the compliance of the participants. Also, although the trial was blinded, some participants might have identified the refined olive oil by its taste and smell.

In summary, regular consumption of olive oil increases the MUFA content of the LDL lipoprotein. Regular consumption of olive oil rich in phenolic compounds increases the LDL total phenolic content in a dose-dependent manner with the phenolic content. The combined protective effect of the MUFA and phenolic content of the LDL could account for the decrease in LDL oxidation observed in the frame of this study.

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References


Virgin olive oil and phenolic content of LDL


