

Olive Oil Polyphenols Enhance High-Density Lipoprotein Function in Humans

A Randomized Controlled Trial

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Objective—Olive oil polyphenols have shown beneficial properties against cardiovascular risk factors. Their consumption has been associated with higher cholesterol content in high-density lipoproteins (HDL). However, data on polyphenol effects on HDL quality are scarce. We, therefore, assessed whether polyphenol-rich olive oil consumption could enhance the HDL main function, its cholesterol efflux capacity, and some of its quality-related properties, such HDL polyphenol content, size, and composition.

Approach and Results—A randomized, crossover, controlled trial with 47 healthy European male volunteers was performed. Participants ingested 25 mL/d of polyphenol-poor (2.7 mg/kg) or polyphenol-rich (366 mg/kg) raw olive oil in 3-week intervention periods, preceded by 2-week washout periods. HDL cholesterol efflux capacity significantly improved after polyphenol-rich intervention versus the polyphenol-poor one (+3.05% and −2.34%, respectively; $P=0.042$). Incorporation of olive oil polyphenol biological metabolites to HDL, as well as large HDL (HDL₂) levels, was higher after the polyphenol-rich olive oil intervention, compared with the polyphenol-poor one. Small HDL (HDL₃) levels decreased, the HDL core became triglyceride-poor, and HDL fluidity increased after the polyphenol-rich intervention.

Conclusions—Olive oil polyphenols promote the main HDL antiatherogenic function, its cholesterol efflux capacity. These polyphenols increased HDL size, promoted a greater HDL stability reflected as a triglyceride-poor core, and enhanced the HDL oxidative status, through an increase in the olive oil polyphenol metabolites content in the lipoprotein. Our results provide for the first time a first-level evidence of an enhancement in HDL function by polyphenol-rich olive oil. (*Arterioscler Thromb Vasc Biol.* 2014;34:2115-2119.)

Key Words: diet ■ high-density lipoproteins ■ olive oil

Olive oil consumption has proven to be protective against the development of cardiovascular pathologies¹ because of its monounsaturated fatty acid content² and to other bioactive compounds, such as polyphenols.^{3,4} Previous results from our group showed that olive oil polyphenols increased dose dependently high-density lipoprotein (HDL) cholesterol levels.³ Although it has been widely reported that low HDL cholesterol levels are strongly associated with high cardiovascular risk,⁵ recent data indicate that increased HDL cholesterol levels

do not imply a reduction in the risk of experiencing a myocardial infarction.⁶ A key objective in cardiovascular disease prevention strategies should, therefore, be not only to increase HDL cholesterol but also to enhance its biological function.

Within this context, our group recently reported that olive oil polyphenols are able to enhance the expression of genes related to HDL metabolism and function.⁷ However, no randomized controlled study in humans has been at present performed on the effects of olive oil polyphenols on HDL

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Nonstandard Abbreviations and Acronyms

CETP	cholesterol ester transfer protein
HDL	high-density lipoprotein
HPCOO	high-polyphenol content olive oil

functionality. Because of this, our aim was to elucidate whether long-term consumption of high-polyphenol content olive oil (HPCOO) would be able to enhance HDL activity and quality characteristics.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results**Participants Characteristics**

The crossover design of the study is described in Figure 1. As shown in Table I in the online-only Data Supplement, no significant differences in baseline parameters between our subsample and the whole EUROLIVE study population (The Effect of Olive Oil on Oxidative Damage in European Population; where our volunteers subsample was extracted from) were found. Energy expenditure in leisure time physical activity through the study and dietary patterns remained stable throughout the study. Volunteers' compliance was correct, as reflected in the biomarkers of compliance.³ Systemic lipid profile did not change after interventions, as described in Table II in the online-only Data Supplement.

In a subsample of 19 volunteers, a favorable but nonsignificant trend toward higher values of HDL particle count was observed after the HPCOO intervention. Raw values of all relevant HDL markers, before and after both interventions, are shown in Table III in the online-only Data Supplement.

Olive Oil Polyphenols Enhance HDL Cholesterol Efflux Capacity

As we see in Figure 2A, the cholesterol efflux capacity from THP-1 macrophages of the participants' HDL fraction was significantly higher after the HPCOO intervention, compared with the low-polyphenol content olive oil one ($P=0.043$).

Multiple linear regression analyses showed that basal values of cholesterol efflux capacity were significantly related to the HDL particle count. For each increase in 1 $\mu\text{mol/L}$ of baseline HDL particle count values, cholesterol efflux values increased by 0.06 points ($P<0.05$).

Olive Oil Polyphenols Bind to HDL in a Dose-Dependent Manner

As shown in Figure 2B, the main metabolites of olive oil polyphenols bound to HDL in a dose-dependent manner. This dose-dependent binding was especially relevant for hydroxytyrosol sulfate ($P<0.001$, when comparing the HPCOO intervention with both baseline and the low-polyphenol content olive oil one).

Increases of olive oil polyphenol metabolites in HDL were associated with enhancements of HDL function. Multiple linear regression analyses revealed that, after the HPCOO

intervention, cholesterol efflux capacity values increased significantly with the increment of olive oil polyphenol metabolites in HDL. For each increase in 1 ppb of hydroxytyrosol sulfate in HDL, there was an increase of 15.6% of cholesterol efflux capacity of the volunteers ($P<0.05$).

Olive Oil Polyphenols Induce the Formation of Larger HDLs

As described in Figure 2C, after the HPCOO intervention, levels of large HDL (HDL_2) in plasma increased significantly compared with baseline ($P=0.010$) or with the low-polyphenol content olive oil one ($P=0.050$). In parallel, levels of small HDL (HDL_3) were significantly lower compared with baseline ($P=0.039$).

Olive Oil Polyphenols Enhance Some HDL Biophysical Parameters

No changes were observed in HDL cholesterol, triglycerides, phospholipids, and apolipoproteins A1 and A2 after interventions (data not shown). However, olive oil polyphenols induced some changes in biophysical parameters related to the HDL fluidity and core quality (Table IV in the online-only Data Supplement).

When evaluating the triglyceride content in the HDL core, a significant decrease was observed after the HPCOO intervention compared with baseline ($P=0.049$), reaching a borderline significance ($P=0.057$) compared with the low-polyphenol content olive oil intervention. The fluidity of the HDL particle also changed after the consumption of olive oil polyphenols, increasing significantly compared with baseline ($P=0.033$).

Discussion

Our results show that a 3-week consumption of olive oil polyphenols induced a significant enhancement of the main HDL biological function, its cholesterol efflux capacity. Olive oil polyphenols also induced changes in the biochemical properties of the lipoprotein, which may have contributed to the HDL function enhancement. To our knowledge, this is the first time that first-level evidence about the *in vivo* health effects of polyphenols on HDL function is reported in healthy volunteers.

The main biological function of the HDL is extracting the excess of cholesterol from the peripheral cells and taking it to the liver to be metabolized and excreted. This cholesterol capture is broadly known as cholesterol efflux, and the main cell types involved in it are the macrophages under the endothelium in blood vessels.⁸ This HDL functional property has been broadly tested in several macrophage cell lines and has been inversely related to early atherosclerosis development and to high risk of experiencing a coronary event.⁹ In our study, the consumption of olive oil polyphenols enhanced the cholesterol efflux capacity of the volunteers' HDL in a physiological model of THP-1 monocyte-derived macrophages. A similar effect has been described after extravirgin olive oil consumption in a noncontrolled, linear study,¹⁰ after walnuts consumption¹¹ or a pioglitazone treatment.⁹

The HDL cholesterol efflux enhancement after the HPCOO intervention may be promoted by the observed increase in the

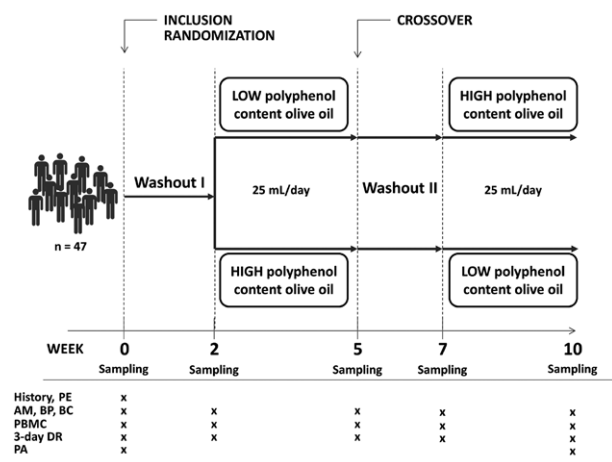


Figure 1. Study design (n=47). AM indicates anthropometric measurements; BC, blood/urine collection; BP, blood pressure measurements; DR, dietary record; PA, physical activity measurement by the Minnesota Leisure Time Physical Activity Questionnaire; PBMC, peripheral blood mononuclear cell collection for gene expression analyses; and PE, physical examination.

olive oil polyphenol metabolites bound to the lipoprotein. It has been previously described that an oxidized HDL is more rigid and presents a lower cholesterol efflux capacity.¹² Thus, a better antioxidative protection, conferred by a higher content of olive oil polyphenols in the HDL, may contribute to explain its functional enhancement. The olive oil polyphenol metabolites bound to the HDL after the HPCOO intervention have been shown to have antioxidant properties.¹³ Thus, a local antioxidant effect on the HDL is expected. In parallel, in the HPCOO intervention, a less rigid HDL was observed, and an increased HDL fluidity is considered an intermediate marker of enhanced HDL functionality.¹² Considering our evidences, we can hypothesize that the binding of olive oil polyphenols to HDL would increase the fluidity of the particle, thus enhancing the HDL capacity for promoting the cholesterol efflux from cells.

Olive oil polyphenols also induced changes in HDL size distribution, which has been closely related to HDL quality.¹⁴ Low levels of large HDL and high levels of small HDL are a pathological trait present in cardiovascular pathologies, as coronary heart disease,¹⁵ although they have not been described as associated with incident coronary events in some prospective studies.¹⁶ Thus, after the HPCOO intervention, large HDL (HDL₂) levels were significantly higher, whereas small HDL (HDL₃) levels were significantly lower. Our results agree with those reported after an olive oil-rich diet in healthy men¹⁷ or after a Mediterranean diet enriched in nuts.¹⁸

Up to the present, the only HDL functional parameter that has been correlated to incident coronary events is the HDL particle number.¹⁹ Thus, we assessed whether positive changes in this direction happened after the consumption of olive oil polyphenols. Although nonsignificant, we observed a trend toward higher HDL particle count values after the HPCOO intervention. Moreover, a direct relation between baseline values of this parameter and cholesterol efflux capacity has been found in the present work. However, as these results are not conclusive, and because few treatments

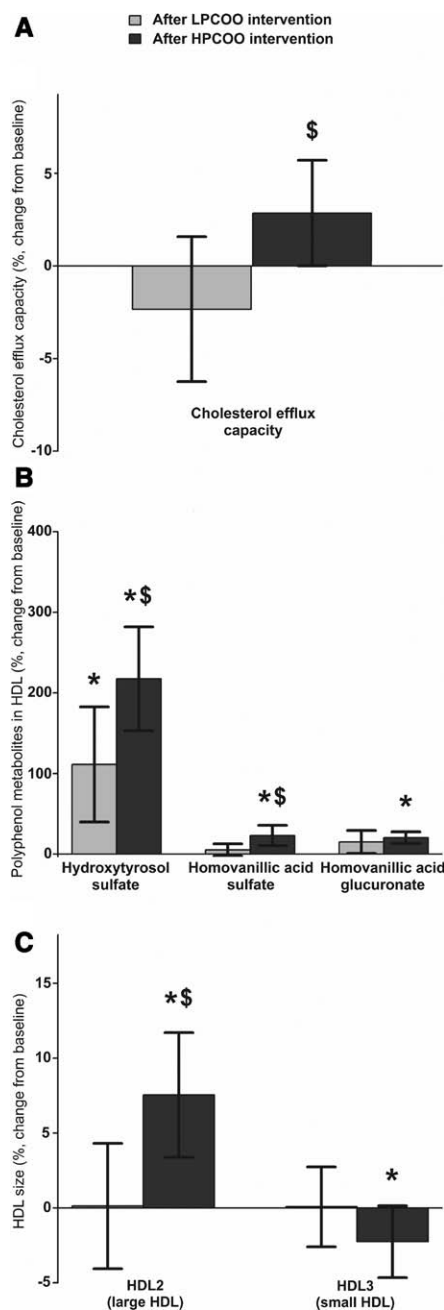


Figure 2. Main changes induced on high-density lipoproteins (HDL) by olive oil polyphenol consumption. **A**, Changes in the cholesterol efflux capacity of the HDL isolated fraction from THP-1 macrophages, expressed as the normalized ratio against the HDL control pool from each experiment. After the high-polyphenol content olive oil (HPCOO) intervention, the efflux is significantly higher, compared with the low-polyphenol content olive oil (LPCOO) one. **B**, Changes in the content of biological metabolites of olive oil polyphenols in HDL, after LPCOO and HPCOO interventions. Olive oil polyphenol metabolites bind to HDL in a dose-dependent manner. **C**, Changes in HDL size distribution show that large HDLs are significantly higher after the HPCOO intervention and that the small HDLs are moderately reduced. In all cases, changes in percentage compared with baseline values are shown, presented as mean±SEM. **P*<0.05, compared with baseline. \$*P*<0.05, compared with LPCOO intervention (mixed linear model).

have been able to induce an enhancement in HDL particle count values,²⁰ further studies in this line are priority to check this hypothesis.

Previous studies demonstrated that high triglyceride levels in the HDL core are present in several cardiovascular pathologies, such as coronary heart disease,²¹ and they are related to a less stable conformation of apolipoprotein-A1 in HDL surface (ie, a less stable lipoprotein structure).^{22,23} In our work, significantly lower triglyceride levels in the HDL core were observed. This decrease could be explained by a lower activity of the cholesterol ester transfer protein (CETP). CETP extracts esterified cholesterol from the HDL core to triglyceride-rich lipoproteins, returning triglycerides from triglyceride-rich lipoproteins to HDL.²⁴ We observed a trend toward a lower CETP activity after HPCOO intervention. This could have happened through a direct blockade of CETP enzyme, as it has been described for apple polyphenols,²⁵ or through a nutrigenomic inhibition of *CETP* gene expression, similar to the one reported after a Mediterranean diet consumption.²⁶ Further studies in this field are required to confirm these hypotheses.

One of the strengths of this study is its crossover design, which permitted the participants to ingest all olive oil types and also reduced possible interferences with confounding variables. As expected, changes after interventions were modest because real-life doses of a single food (which cannot be consumed in high quantities daily) were administered to healthy volunteers. A study limitation was its sample size, responsible for reduced statistical power in some biomarkers with high interindividual variability. It is also possible that an amount of polyphenols, similar to those provided by olive oil, could have come from other food types. A synergistic effect on HDL parameters between polyphenols and other olive oil biological metabolites is as yet unknown.

In conclusion, this is the first time that HDL cholesterol efflux enhancement by polyphenols has been reported in healthy, young population, in a randomized controlled trial. Apart from the enhancement in the main antiatherogenic HDL function, olive oil polyphenols induced a change in HDL size toward larger values. These, together with the enhancement of the stability and the oxidative status of the HDL particle, led to a better HDL functionality after the consumption of olive oil polyphenols. Our data are in line with the previous evidences supporting that the consumption of olive oil polyphenols helps to reduce cardiovascular risk.

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Disclosures

None.

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Significance

The cholesterol efflux capacity of high-density lipoproteins is considered their main antiatherogenic property, and, in our data, it improved after a long-term consumption of olive oil polyphenols. The dose-dependent binding of the biological metabolites of these polyphenols to the high-density lipoproteins could be responsible for the effect, through an enhanced oxidative status of the particles. Apart from this improvement, olive oil polyphenols induced in vivo a low-cardiovascular risk high-density lipoproteins profile in the volunteers of the study. This has been the first time that the previous effects have been associated to food polyphenols in healthy volunteers in a randomized, parallel, controlled trial with a robust design.

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MATERIAL & METHODS

Study design

The EUROLIVE study was a parallel, crossover, randomized controlled trial performed with 180 healthy European men, from six European cities, aged 20-60 years. From the 344 people who agreed to be screened, 200 were eligible and enrolled in the study from September 2002 to June 2003. Exclusion criteria were the following: smoking; use of antioxidant supplements, aspirin or any drug with known antioxidant properties; hyperlipidemia; diabetes mellitus; hypertension; intestinal disease; or any other physiological condition or disease that could impair adherence. Women were excluded to avoid possible interferences of estrogens, due to their potential antioxidant properties. In all cases, participants provided written informed consent to join the study, and local institutional ethics committees approved the protocol, whose details have been previously published (1). This protocol is registered with the International Standard Randomized Controlled Trial Number ISRCTN09220811 (www.controlled-trials.com).

HDL function and quality analyses were performed in a random subsample of 47 EUROLIVE participants (17 from Germany, 15 from Finland, and 15 from Spain). The samples studied were taken before and after interventions with high polyphenol content olive oil (HPCOO, a natural virgin olive oil with 366 mg/kg of polyphenols), and low polyphenol content olive oil (LPCOO, 2.7 mg/kg, a refined one). Since phenolic compounds are lost during the refinement process, the refined olive oil had a reduced phenolic content. Both olive oil compositions were identical, with the exception of their polyphenol content (1). In the crossover design (**Figure 1**), participants followed 3-week intervention periods, in which they ingested 25 mL/day of raw olive oil distributed among meals. Volunteers followed a brief training to learn to replace other dietary fats with olive oil in all meals. The intervention periods were preceded by 2-week washout periods, in which volunteers avoided olive oil and olive ingestion, as well as a high intake of antioxidants. According to the half-life of the main olive oil phenolic compounds in the body, a 2-week washout period was sufficient to guarantee a complete elimination of the antioxidants between interventions (2).

Dietary adherence and physical activity

24-hour urinary excretion of tyrosol and hydroxytyrosol (the two major phenolic compounds in olive oil, as simple forms or conjugates) were measured by gas chromatography and mass spectrometry (3), as biomarkers of adherence to the type of olive oil ingested. Taking this criterion into account, noncompliant volunteers were excluded from all the subsequent analyses.

To control their diet, participants were asked to keep a 3-day dietary record at the beginning of the study and after each intervention period, and to maintain their normal diet during the study. They also received nutritional education, learning to replace raw fats with olive oils.

Physical activity of the volunteers was measured at the beginning and the end of the study, by means of a validated Minnesota Leisure Time Physical Activity Questionnaire.

Systemic biomarkers

Plasma glucose, total and HDL cholesterol, and plasma triglycerides were measured using automatic enzymatic-colorimetric methods, in a Cobas-Mira Plus autoanalyzer (Roche, Basel, Switzerland), using specific reagents from Spinreact (Barcelona, Spain). LDL cholesterol was calculated with the Friedewald formula, whenever plasma triglycerides were below 300 mg/dL. Plasma oxidized LDL levels were measured by an ELISA (*Mercodia Oxidized LDL ELISA AB*, Mercodia, Uppsala, Sweden), in an INFINITE M200 reader (Tecan Group Ltd., Männedorf, Switzerland).

Lipoprotein extraction

Fasting human plasma from volunteers was collected in K₂-EDTA containing tubes. HDL lipoprotein fraction was extracted by ultracentrifugation, as previously described (4). HDL fraction was stored at -80°C in a buffer containing 2.5% sucrose until the moment of use.

Measurement of HDL cholesterol efflux capacity

THP-1 monocytes were grown in DMEM medium, supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin-streptomycin. Culture medium was refreshed each 72h. THP-1 monocytes were differentiated into macrophages incubating the cells in the presence of 200 nM of phosbol-myristate-acetate (Sigma, St. Louis, MO, USA) for 96h.

To test HDL cholesterol efflux capacity, these monocyte-derived macrophages were then incubated in fresh culture medium with 0.2 µCi/mL of [³H]-cholesterol for 24 hours. Afterward, macrophages were washed and incubated in DMEM supplemented with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for 24h for the equilibration process. Cells were then washed again and suspended in fresh DMEM medium, supplemented with 1% BSA, and with either 50 µg/mL of HDL from the volunteers or without HDL (control) for 16h. After this last incubation, the supernatant medium was extracted from the culture, the cells were lysed by freezing and the remains removed from culture plates.

The cholesterol efflux capacity of HDL fraction was determined by liquid scintillation counting. Radioactivity in both supernatant medium and cell lysate was measured with a beta scintillation counter Tri-Carb 2800TR (Perkin-Elmer, Waltham, MA, USA). Thus, the cholesterol efflux capacity was calculated using the following formula: (counts per minute in supernatant/counts per minute in supernatant + cells) x 100.

For all these determinations, samples were analyzed in triplicate, with a mean intra-plate coefficient of variation of 4.39% and a mean inter-plate coefficient of variation of 7.43%.

Determination of biological metabolites of olive oil polyphenols in HDL fraction

Determination and quantification of the biological metabolites of olive oil polyphenols in HDL were performed by means of High Performance Liquid Chromatography associated with mass spectrometry, following previously described methodology (5).

HDL size distribution

HDL size distribution was determined using the Lipoprint HDL System (Quantimetrix, Redondo Beach, CA, USA). The Lipoprint HDL System focuses on the size distribution of HDL lipoproteins and is able to detect nine size-dependent, HDL electrophoretic bands. The first band corresponds to the bigger HDLs present in the sample, the second band corresponds to slightly smaller HDLs, and so on until the ninth band. As has been previously published (6), the first three bands have been described as corresponding to the large HDL subclass (HDL₂) and, from 4th to 9th band, to the small HDL subclass (HDL₃).

HDL particle count analyses

HDL particle count, HDL average particle size and count of small, medium and large HDL particles were determined using NMR technology, in a Vantera Clinical Analyzer (LipoScience Inc., Raleigh, NC, USA), as previously described (7).

HDL composition and activities of HDL metabolic enzymes

In HDL lipoprotein fractions, total and free cholesterol, triglycerides and phospholipids were quantified by automatic enzymatic-colorimetric methods. Apolipoproteins A1 and A2 were also determined by automatic immunoturbidimetric methods. All these determinations were performed in a Cobas-Mira Plus autoanalyzer (Roche, Basel, Switzerland), using specific reagents from Spinreact (Barcelona, Spain) for both techniques. The triglyceride content of the HDL core was assessed as the ratio between HDL triglycerides and HDL esterified cholesterol. The activities of the main enzymes related to HDL metabolism were also analyzed. Cholesterol ester transfer protein (CETP) and lecithin:cholesterol acyl-transferase (LCAT) activities were measured, in serum and EDTA-plasma respectively, using specific commercial fluorimetric kits (*LCAT Assay Kit*, Calbiochem, Merck Millipore, Billerica, MA, USA; *CETP Assay Kit*, MBL International, Woburn, MA, USA) in an INFINITE M200 reader (Tecan Group Ltd., Männedorf, Switzerland).

HDL fluidity determination

The fluidity of the HDL particle was measured based on the determination of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described (8). In brief, HDL fractions were incubated with DPH 1 μM for 30 minutes at room temperature in constant agitation. After that, samples with the DPH probe were stimulated with a vertically polarized light at 360 nm.

Fluorescent emission intensities were detected at 430 nm in a Perkin-Elmer LS50B spectrofluorometer (Perkin Elmer, Waltham, MA, USA), through a polarizer orientated in parallel and perpendicular to the direction of polarization of the emitted beam. Subsequently, we were able to measure the intensities of the perpendicular polarized fluorescence produced by the probe (I_p), which could vary depending on the sample fluidity. The steady-state fluorescence anisotropy (r) was calculated with these I_p values, and with the grating correction factor of the monochromator (G), using the following formula:

$$r = (I_p - GI_p) / (I_p + 2GI_p)$$

The steady-state anisotropy refers to the rigidity of the sample. Therefore, the inverse value of this parameter ($1/r$) is the fluidity index.

Sample size calculation

A sample size of 45 participants allowed a $\geq 80\%$ power to detect a significant difference between olive oil interventions of 3 mg/dL of HDL cholesterol, considering a 2-sided type I error of 0.05 and a loss rate of 2%. Calculations were made from our previous data, taking into account the SD of HDL cholesterol levels in healthy volunteers (1).

Statistical analyses

Distribution of continuous variables was determined through normal probability plots and graphics and the Shapiro-Wilk test. Non-normally distributed variables were subsequently log transformed. A mixed linear model was used to determine the effect of each intervention compared to its baseline and the differences between treatments. The possible carry-over effect was determined by testing a period-by-treatment interaction. Pearson's correlation analyses were performed to determine relationships among variables. Multiple linear regression analyses were carried out, with the baseline values and changes after the HPCOO intervention, to explore whether HDL-associated variables could be related to cholesterol efflux capacity. To assess possible relations, age and those variables which presented a Pearson's correlation with cholesterol efflux (with a significance of $P < 0.1$) were introduced into the model. Then, a forward stepwise analysis was applied. Variables firstly introduced in the regression models were age, HDL cholesterol, apolipoprotein A1, HDL₂/HDL₃ ratio and HDL particle count for baseline values, and age, HDL₂/HDL₃ ratio and hydroxytyrosol sulfate in HDL for intervention changes. We considered any P value below 0.05 significant. All statistical analyses were performed with R Software, version 2.15.2 (R Development Core Team, 2013; www.R-project.org) and with SPSS Software, version 18.0 (IBM Corp).

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1 **SUPPLEMENTAL TABLE I**

2 Baseline characteristics of the participants

3

	Baseline value	
	Study subsample (n = 47)	EUROLIVE study whole population (n = 180)
Age (years)	33.5 (10.9) ¹	33.2 (11.0)
Weight (kg)	77.8 (10.9)	76.4 (10.5)
Height (m)	1.79 (0.07)	1.79 (0.07)
Total cholesterol (mg/dL)	170 (43)	174 (41)
LDL cholesterol (mg/dL)	96 (38)	98 (36)
HDL cholesterol (mg/dL)	52 (11)	53 (12)
Tryglycerides (mg/dL) ²	99 (70 to 129)	99 (68 to 134)

4

5 ¹: Mean (SD)6 ²: Median (1st to 3rd quartile in parentheses)

7

8 **SUPPLEMENTAL TABLE II**
 9 Changes in systemic parameters after olive oil interventions
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	Olive oil interventions				<i>P</i> between groups ²
	Low polyphenol content olive oil		High polyphenol content olive oil		
	Post-intervention	Change ¹	Post-intervention	Change ¹	
Systemic lipid profile					
Total cholesterol (mg/dL)	176 (47) ³	↑1.83% (12.2)	171 (45)	↓2.43% (10.9)	0.064
LDL cholesterol (mg/dL)	100 (42)	↑2.13% (20.5)	99 (41)	↓2.19% (16.2)	0.245
HDL cholesterol (mg/dL)	54 (12)	↑3.36% (13.2)	54 (13)	↓0.40% (11.4)	0.153
Triglycerides (mg/dL) ⁴	80.5 (55.1 to 123.4)	↓5.71% (-20.7 to 24.5)	78.8 (60.4 to 109.4)	↓7.03% (-23.3 to 9.38)	0.306
Oxidized LDL (U/L)	41.9 (20.3)	↑7.59% (38.5)	40.5 (16.2)	↓6.01% (22.0)*	0.023
HDL particle count analysis (n = 19)					
HDL particle count (μmol/L)	32.2 ± 3.80	↑0.30% (10.5)	32.0 ± 2.99	↑3.25% (9.79)	0.429
HDL average particle size (nm)	9.42 ± 0.53	↓0.16% (3.17)	9.36 ± 0.56	↑0.84% (3.34)	0.395
Small HDL particle count (μmol/L)	15.6 ± 5.25	↑15.8% (55.0)	15.8 ± 4.62	↑1.99% (22.6)	0.766
Medium+large HDL particle count (μmol/L)	16.5 ± 4.85	↑1.50% (32.7)	16.2 ± 5.24	↑5.07% (22.5)	0.444

11
 12 ¹: Change in percentage, compared to baseline, expressed as Mean (SD)
 13 ²: *P* for inter-group comparisons (mixed linear model)
 14 ³: Mean ± SD
 15 ⁴: Median (1st to 3rd quartile in parentheses)
 16 *: *P*<0.05 after intervention, compared to baseline (mixed linear model)

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18 **SUPPLEMENTAL TABLE III**

19 Raw measurements of main HDL-related parameters

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	Olive oil interventions						<i>P</i> between groups ³
	Low polyphenol content olive oil			High polyphenol content olive oil			
	Pre-intervention ¹	Post-intervention	Change ²	Pre-intervention	Post-intervention	Change ²	
HDL cholesterol (mg/dL)	53.0 (11.6)	54.0 (11.8)	↑3.36% (13.2)	54.5 (11.9)	54.2 (12.8)	↓0.40% (11.4)	0.153
Cholesterol efflux capacity ⁴	0.95 (0.18)	0.91 (0.17)	↓2.34% (13.5)	0.92 (0.20)	0.94 (0.19)	↑3.05% (9.98)	0.042
Hydroxytyrosol sulfate in HDL (ppb)	16.0 (12.2)	26.5 (18.6)	↑111% (224)*	17.2 (11.8)	46.8 (39.6)	↑215% (207)*	0.001
Homovanillic acid sulfate in HDL (ppb)	11.8 (3.76)	13.1 (4.46)	↑5.21% (12.5)	13.8 (6.13)	17.6 (9.96)	↑24.0% (22.7)*	0.028
Homovanillic acid glucuronate in HDL (ppb)	13.7 (3.64)	14.6 (5.33)	↑15.2% (30.8)	13.1 (3.12)	16.1 (5.13)	↑20.5% (15.8)*	0.163
HDL ₂ particles (%)	38.1 (9.80)	38.0 (10.5)	↓0.04% (14.2)	37.5 (9.13)	40.0 (8.03)	↑7.16% (13.7)*	0.050
HDL ₃ particles (%)	60.3 (9.01)	60.3 (9.82)	↑0.50% (8.57)	60.4 (8.62)	58.8 (8.12)	↓1.92% (7.79)*	0.146
HDL particle count (μmol/L)	31.6 (4.22)	32.2 (3.8)	↑0.30% (10.5)	31.1 (3.39)	32.0 (2.99)	↑3.25% (9.79)	0.429
Triglycerides in HDL core ⁵	0.36 (0.23)	0.38 (0.29)	↑22.1% (86.9)	0.37 (0.23)	0.32 (0.18)	↓9.29% (29.6)*	0.057
HDL fluidity	4.99 (0.21)	5.02 (0.21)	↑0.73% (3.54)	4.93 (0.18)	4.98 (0.22)	↑1.12% (3.61)*	0.555

21

22 ¹: Mean (SD)

23

23 ²: Change in percentage, compared to baseline, expressed as Mean (SD)

24

24 ³: *P* for inter-group comparisons (mixed linear model)

25

25 ⁴: Expressed as normalized values, respecting to control HDL

26

26 ⁵: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio

27

27 *: *P*<0.05 after intervention, compared to baseline (mixed linear model)

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28

29 **SUPPLEMENTAL TABLE IV**

30 Changes in HDL metabolic enzymes activities and biophysical parameters after olive oil interventions

31

	Olive oil interventions				<i>P</i> between groups ²
	Low polyphenol content olive oil		High polyphenol content olive oil		
	Post-intervention	Change ¹	Post-intervention	Change ¹	
HDL metabolism enzymes³					
CETP activity (U/L, <i>n</i> = 36)	496 ± 43.4 ⁴	↑1.52% (5.48)	489 ± 39.5	↓1.18% (7.78)	0.122
LCAT activity (U/L, <i>n</i> = 28)	0.63 ± 0.055	↑0.95% (7.03)	0.62 ± 0.051	↓0.68% (5.63)	0.390
HDL core-related parameters					
Triglycerides in HDL core ⁵	0.38 ± 0.29	↑22.1% (86.9)	0.32 ± 0.18	↓9.29% (29.6)*	0.057
HDL biophysical characteristics					
HDL fluidity	5.02 ± 0.21	↑0.73% (3.54)	4.98 ± 0.22	↑1.12% (3.61)*	0.555

32

33 ¹: Change in percentage, compared to baseline, expressed as Mean (SD)

34

34 ²: *P* for inter-group comparisons (mixed linear model)

35

35 ³: CETP, cholesterol ester transfer protein; LCAT, lecithin:cholesterol acyltransferase

36

36 ⁴: Mean ± SD

37

37 ⁵: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio

38

38 *: *P*<0.05 after intervention, compared to baseline (mixed linear model)

39

39

40 **SUPPLEMENTAL TABLE V**
 41 Pearson's correlation values for baseline measurements of HDL-related parameters
 42

	Age	HDL cholesterol	Cholesterol efflux capacity ¹	HDL2 (%)	HDL3 (%)	HDL particle count	HDL ApoA1	Triglycerides in HDL core ²	HDL fluidity
Age	1	0.292	-0.232	-0.241	0.264	0.322	0.020	-0.009	-0.013
HDL cholesterol		1	0.496**	0.622***	-0.655***	0.238	-0.072	-0.420*	-0.119
Cholesterol efflux capacity ¹			1	0.589***	-0.598***	0.562 (<i>P</i> =0.072)	0.053	-0.202	-0.305
HDL2 (%)				1	-0.964***	0.435	0.063	-0.346 (<i>P</i> =0.083)	-0.135
HDL3 (%)					1	-0.428	-0.099	0.410*	0.133
HDL particle count						1	0.147	0.179	0.578
HDL ApoA1							1	-0.327	-0.099
Triglycerides in HDL core ²								1	0.052
HDL fluidity									1

43
 44 ¹: Expressed as normalized values, respecting to control HDL
 45 ²: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio
 46 *: *P*<0.05. **: *P*<0.01. ***: *P*<0.001