Protection of LDL from oxidation by olive oil polyphenols is associated with a downregulation of CD40-ligand expression and its downstream products in vivo in humans[1–3]

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ABSTRACT

Background: Recently, the European Food Safety Authority approved a claim concerning the benefits of olive oil polyphenols for the protection of LDL from oxidation. Polyphenols could exert health benefits not only by scavenging free radicals but also by modulating gene expression.

Objective: We assessed whether olive oil polyphenols could modulate the human in vivo expressions of atherosclerosis-related genes in which LDL oxidation is involved.

Design: In a randomized, crossover, controlled trial, 18 healthy European volunteers daily received 25 mL olive oil with a low polyphenol content (LPC: 2.7 mg/kg) or a high polyphenol content (HPC: 366 mg/kg) in intervention periods of 3 wk separated by 2-wk washout periods.

Results: Systemic LDL oxidation and monocyte chemoattractant protein 1 and the expression of proatherogenic genes in peripheral blood mononuclear cells [ie, CD40 ligand (*CD40L*), IL-23α subunit p19 (*IL23A*), adrenergic β-2 receptor (*ADRB2*), oxidized LDL (lectin-like) receptor 1 (*OLR1*), and IL-8 receptor-α (*IL8RA*)] decreased after the HPC intervention compared with after the LPC intervention. Random-effects linear regression analyses showed *1*) a significant decrease in *CD40*, *ADRB2*, and *IL8RA* gene expression with the decrease of LDL oxidation and *2*) a significant decrease in intercellular adhesion molecule 1 and *OLR1* gene expression with increasing concentrations of tyrosol and hydroxytyrosol in urine.

Conclusions: In addition to reducing LDL oxidation, the intake of polyphenol-rich olive oil reduces *CD40L* gene expression, its downstream products, and related genes involved in atherogenic and inflammatory processes in vivo in humans. These findings provide evidence that polyphenol-rich olive oil can act through molecular mechanisms to provide cardiovascular health benefits. This trial was registered at www.controlled-trials.com as ISRCTN09220811. *Am J Clin Nutr* 2012;95:1238–44.

INTRODUCTION

A large body of knowledge supports the benefits of olive oil consumption for risk factors for chronic degenerative diseases and the aging process (1). In November 2004, the US Food and Drug Administration approved a health claim of olive oil consumption (23 g/d) on the basis of the MUFA content of the olive oil (2). However, olive oils, particularly virgin olive oil, contain bioactive polyphenols as minor components. Data from the European EUROLIVE study provided the final degree of evidence required to recommend polyphenol-rich olive oil to achieve additional benefits for both classical cardiovascular risk factors and novel ones such as the in vivo lipid oxidative damage including LDL oxidation (3). Recently, the European Food Safety Authority released a claim concerning the effectiveness of the ingestion of olive oil polyphenols (5 mg/d) on protecting LDL from oxidation (4).

Polyphenols can exert protective effects not only through the scavenging of free radicals but also by modulating signal transduction, cell signaling, gene expression, and cellular communication in various pathways (5). Some inflammatory genes have been reported to be modulated by phenolic-rich olive oil consumption (6–8). Increased amounts of oxidized LDL have been shown to correlate with an increase of *CD40* gene expression in hyperlipemic individuals (9). We have previously described that one of the mechanisms by which polyphenol-rich olive oil ingestion can reduce LDL oxidation is through the promotion of an increase in the antioxidant content of the LDL particle (10, 11). High plasma concentrations of soluble CD40 ligand (CD40L)[4] have been shown to be associated with reductions in the antioxidant content of the LDL (12). Therefore, we assessed the in vivo

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[4] Abbreviations used: CD40L, CD40 ligand; HPC, high polyphenol content; ICAM1, intercellular adhesion molecule 1; IFN-γ, interferon γ; LPC, low polyphenol content; MCP1, monocyte chemoattractant protein 1; PBMC, peripheral blood mononuclear cell.

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human peripheral blood mononuclear cell (PBMC) transcriptomic response, related with LDL oxidation, after sustained consumption of similar olive oils but with differences in their phenolic content, in healthy individuals.

SUBJECTS AND METHODS

Study design

The EUROLIVE study was a randomized, crossover, controlled study in which 180 nonsmoking, healthy men aged 20–60 y completed the study. Participants received 3 types of similar olive oils but with differences in their phenolic content. Exclusion criteria were was follows: the use of antioxidant supplements, aspirin, or drugs with known antioxidant properties, hyperlipidemia, obesity, diabetes, hypertension, intestinal disease, or any other disease or condition that could impair adherence. We excluded women to avoid the possible interference of estrogens, which are considered to be potential antioxidants.

All participants provided written informed consent, and the local institutional ethics committees approved the protocol. Details of the protocol have been published elsewhere (3). The protocol is registered with the International Standard Randomised Controlled Trial register (www.controlled-trials.com; ISRCTN09220811). Gene-expression analyses were performed in a random subsample of 18 participants (10%) (8 participants from Finland, 4 participants from Germany, and 6 participants from Spain) in samples taken before and after high polyphenol content (HPC; 366 mg/kg) and low polyphenol content (LPC; 2.7 mg/kg) olive oil interventions. In the crossover design (**Figure 1**), intervention periods were of 3 wk with a daily ingestion of 25 mL raw olive oil distributed among meals and preceded by 2-wk washout periods in which olives and olive oil were avoided.

Dietary adherence

We measured urinary tyrosol and hydroxytyrosol, which are the 2 major phenolic compounds in olive oil, as simple forms or conjugates as biomarkers of adherence to the type of olive oil ingested (13). We asked participants to keep a 3-d dietary record at the beginning of the study and after each intervention period and to maintain their habitual diet throughout the study. A nutritionist personally advised participants to replace all types of habitually consumed raw fats with the olive oils (eg, to spread the assigned olive oil instead of butter on bread).

Systemic biomarkers analyses

Serum glucose, total and HDL-cholesterol, and triglyceride concentrations were measured by using automated enzymatic methods. LDL cholesterol was calculated by using Friedewald's formula. Plasma oxidized LDL was determined by using an ELISA (Mercodia AB). Plasma concentrations of intercellular adhesion molecule 1 (ICAM1), monocyte chemoattractant protein 1 (MCP1), and soluble CD40L were measured by using flow cytometry (Bender Medsystems Co Ltd). High-sensitivity interferon γ (IFN-γ) was determined by using ELISA (Labclinics SA).

Gene-expression analyses

The selection of candidate genes was performed on the basis of their relation with LDL oxidation and its uptake via scavenger receptors. For messenger RNA–expression analyses, isolation of

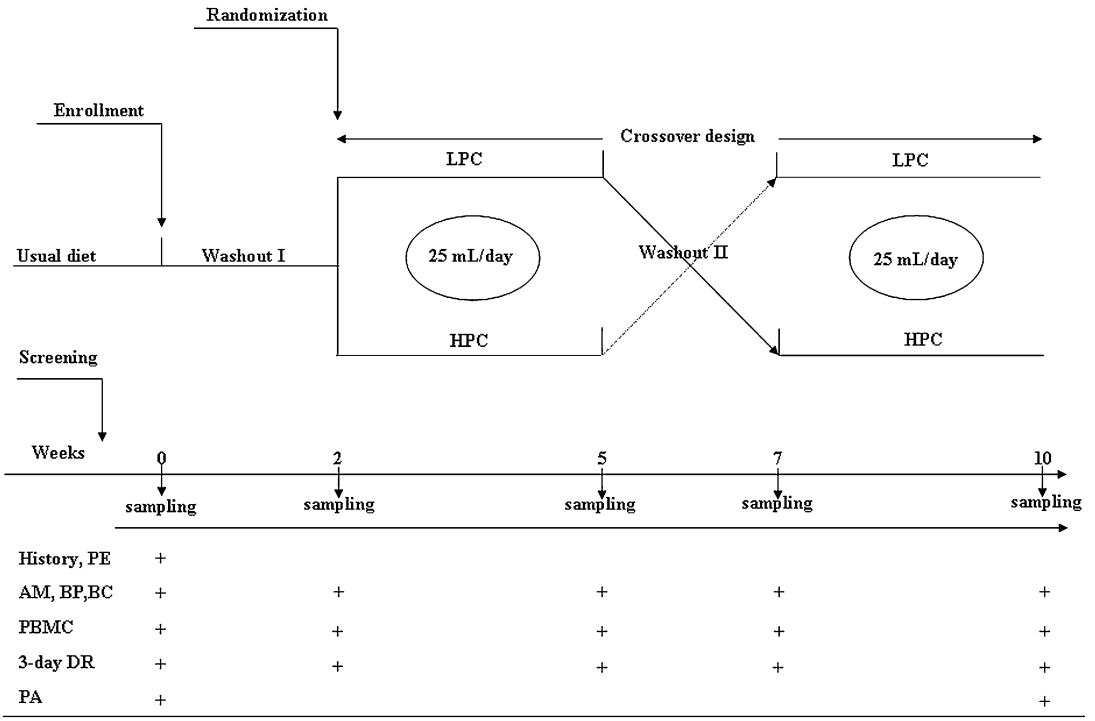

FIGURE 1. Study design (n = 18). AM, anthropometric measurements; BC, blood and urine collection; BP, systolic and diastolic blood pressure examinations; DR, dietary record; HPC, high polyphenol content; LPC, low polyphenol content; PA, physical activity assessment by the Minnesota Leisure Time Physical Activity Questionnaire; PBMC, peripheral blood mononuclear cell collection for gene-expression analyses; PE, physical examination.

total RNA from PBMCs was performed by using a liquid-liquid method. RNA purity and integrity were assessed. After complementary DNA conversion, duplicated by each sample, gene expression was measured by a real-time polymerase chain reaction with TaqMan Low Density Microfluidic (Applied Biosystems). Four replicates were performed for every RNA sample (2 polymerase chain reaction replicates per complementary DNA replicate) and analyzed with Sequence Detection System software (SDS 2.1; Applied Biosystems) according to the manufacturer's instructions. Changes in gene expressions were calculated by using the relative quantification method and by applying the $2^{-\Delta\Delta Ct}$ formula.

Sample size and power analyses

A total sample size of 18 participants allowed ≥80% power to detect a significant difference between olive oil groups of 0.5 units of $\log_2$ ratio relative quantification in the gene expression of IFN-γ measurement with consideration of a 2-sided type I error of 0.05. Calculations were made from our previous data concerning the SD of *IFNG* gene expression in healthy volunteers (8).

Statistical analyses

The normality of continuous variables was assessed by using normal probability plots and the Shapiro-Wilk test. Nonnormally distributed variables were log transformed. Pearson's correlation analyses were used to evaluate relations among variables. A paired *t* test was performed to assess the effect of each intervention compared with its baseline. Adjusted general linear mixed models were used to assess the effect of interventions. We tested for linear relations between oxidized LDL changes, or changes in tyrosol and hydroxytirosol urinary concentrations, and changes in gene expression. We used a random-effects linear regression model to account for within-person differences. The possible carryover effect was determined by testing a period-by-treatment interaction term in the general linear models. $P < 0.05$ was considered significant. Statistical analyses were performed with SPSS software version 13.0 (IBN Corp) and R software version 2.11.1 (R Development Core Team, 2011; www.R-project.org).

RESULTS

Participant characteristics and dietary adherence

The clinical characteristics of participants at the beginning of the study are shown in **Table 1**. No changes in daily energy expenditure in leisure-time physical activity were observed from the beginning to the end of the study. Throughout the study, no changes were observed in dietary patterns that were analyzed from data of the 3-d dietary records. Participants' compliance was good as reflected in the changes in urinary polyphenols after olive oil interventions (**Figure 2**).

Systemic biomarkers

Changes in systemic biomarkers after olive oil interventions are shown in **Table 2**. Diastolic blood pressure and BMI decreased after the HPC compared with after the LPC intervention. However, changes were nonclinically relevant. An improvement in the plasma lipid profile and a reduction in oxidized LDL (both adjusted and nonadjusted by LDL) and MCP1 was observed after the HPC intervention compared with after the LPC intervention ($P < 0.05$), and the reduction in soluble CD40L reached borderline significance.

TABLE 1
General characteristics of the participants ($n = 18$)

	Mean ± SD
Age (y)	38.2 ± 11.5
Weight (kg)	79.5 ± 11.4
Height (m)	1.79 ± 0.07
Waist-hip ratio	0.89 ± 0.07
BMI (kg/m^2)	24.7 ± 2.9
Systolic blood pressure (mm Hg)	129 ± 14
Diastolic blood pressure (mm Hg)	47 ± 10
Total cholesterol (mg/dL)	197 ± 45
HDL cholesterol (mg/dL)	47 ± 10
LDL cholesterol (mg/dL)	129 ± 44
Triglycerides (mg/dL)	110 ± 62
Glucose (mg/dL)	87 ± 14

Changes in gene expressions after olive oil interventions

We explored whether there was a carryover effect in all assessed outcomes. A significant carryover effect was observed for macrophage scavenger receptor 1 and *CD36* molecule (thrombospondin receptor) gene expression throughout treatments ($P < 0.01$). No significant differences were observed in the expression of these genes between before and after interventions (intragroup) when only each first period was analyzed. Therefore, these genes were excluded from the global statistical analyses. Changes in expressions of atherosclerosis-related genes after olive oil interventions are shown in **Figure 3**. The expressions of *CD40L*, *IL23A*, *IL7R*, *IL8RA*, *ADRB2*, and *OLR1* genes decreased significantly

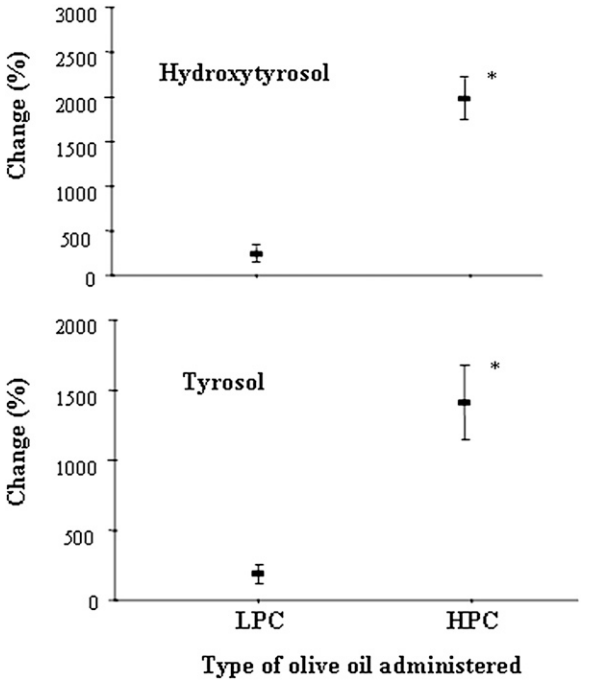

FIGURE 2. Mean (95% CI) percentage changes in urinary tyrosol and hydroxytyrosol ($n = 18$). Changes from baseline after LPC and HPC olive oil interventions. *$P < 0.01$ compared with LPC (general linear mixed model). HPC, high polyphenol content; LPC, low polyphenol content.

TABLE 2
Systemic changes after olive oil interventions[1]

	Olive oil interventions				
	Low-polyphenol olive oil (n = 18)		High-polyphenol olive oil (n = 18)		
	Postintervention	Change	Postintervention	Change	P between groups[2]
Systolic blood pressure (mm Hg)	125 ± 12[3]	0.88 ± 1.9	126 ± 12	−1.6 ± 2.3	0.361
Diastolic blood pressure (mm Hg)	79 ± 10	2.78 ± 1.7	79 ± 9.8	−1.22 ± 1.04	0.043
BMI (kg/m^2)	24.8 ± 2.8	0.13 ± 0.05	24.7 ± 2.9	−0.09 ± 0.08	0.033
Glucose (mg/dL)	87 ± 11	−1.0 ± 1.6	88 ± 11	1.3 ± 2.4	0.443
Cholesterol (mg/dL)	208 ± 50	8.2 ± 4.7	199 ± 46	−7.1 ± 4.2	0.016
LDL cholesterol (mg/dL)	135 ± 48	6.4 ± 4.8	129 ± 44	−6.3 ± 4.8	0.028
HDL cholesterol (mg/dL)	48.8 ± 9.6	1.8 ± 1.4	50.3 ± 12.2	1.4 ± 1.5	0.827
Triglycerides (mg/dL)	122 (84–160)[4]	2.72 (−11.5 to 10.8)	99 (74–124)	−10 (−84 to 57)	0.101
Oxidized LDL (U/L)	47 ± 21	6.4 ± 3.4	44 ± 17	−7.3 ± 3.4[5]	0.004
ICAM (ng/mL)	267 (235–299)	−1.45 (−78 to 25)	295 (266–324)	−8.0 (−81 to 35)	0.376
MCP1 (pg/mL)	716 (380–1052)	36 (−35 to 156)	659 (331–988)	−29 (−81 to 35)	0.022
sCD40L (pg/mL)	2.87 ± 0.26	0.01 ± 0.04	2.78 ± 0.40	−0.18 ± 0.07	0.063
IFN-γ (pg/mL)	4.0 (0.89–7.04)	0.04 (−0.18 to 0.93)	3.8 (1.07–6.89)	−0.13 (−0.42 to 0.84)	0.442

[1] ICAM, intercellular adhesion molecule; IFN-γ, interferon γ; MCP1, monocyte chemoattractant protein 1; sCD40L, soluble CD40 ligand.
[2] P for intergroup comparison.
[3] Mean ± SD (all such values).
[4] Median; 25th to 75th percentiles in parentheses (all such values).
[5] $P < 0.05$ after intervention compared with baseline (general linear mixed model).

after the HPC intervention compared with after the LPC intervention. The downregulation in *VEGFB* reached borderline significance ($P < 0.08$). *IFNG*, *IL7R*, *IL23A*, *CD40L*, *MCP1*, and *IL8RA* gene expressions decreased after HPC intervention ($P < 0.05$). A decrease in the expression of *ICAM1* (−27%) after HPC ingestion was observed, but significance was not achieved. The decrease of *MCP1* expression after LPC ingestion (−46%) reached significance ($P = 0.01$). No changes were observed in *ALOX5AP* or tumor necrosis factor (ligand) superfamily, member 10 (*TNFSF10*) gene expression.

Correlation analyses of gene-expression changes after HPC intervention showed cross-linked correlations in genes related with the CD40/CD40L cascade (**Table 3**). Changes in the expression of *CD40L* directly correlated with those of *IL23A*, *VEGFB*, *ADRB2*, *ICAM1*, *IL7R*, and *ALOX5AP* ($P < 0.05$). Direct correlations were also observed in changes in these genes ($P < 0.05$). The decrease in *ADRB2* directly correlated with the reduction observed in *OLR1*, *IL23A*, *VEGFB*, *IFNG*, *ICAM1*, *MCP1*, *IL8RA*, *IL7R*, and *ALOX5AP* ($P < 0.05$). A proposed integrated scheme for the in vivo reduced gene expression promoted by the ingestion of HPC instead of LPC is shown in **Figure 4**.

Linear relation between changes in plasma circulating oxidized LDL, olive oil phenolic compounds in urine, and the CD40L signaling pathway

Random-effects linear regression analyses showed a direct linear association between the decrease in oxidized LDL and those of *CD40*, *ADRB2*, and *IL8RA* gene expressions after HPC intervention. For each decrease in 1 U oxidized LDL/L, there was a 2.6, 3.1, and 2.4-fold significant decrease in expressions of *CD40L*, *ADRB2*, and *IL8RA*, respectively. Also, for each 10% increase in urinary tyrosol and hydroxytyrosol, there was a significant decrease of 2.8- and 2.6-fold in expressions of *ICAM1* and *OLR1*, respectively, after HPC intervention.

DISCUSSION

These outcomes showed that a randomized, crossover, controlled intervention with HPC olive oil reduced the gene expression of the *CD40* ligation and its downstream products, and this reduction was associated with a decrease in plasma LDL oxidation and an increase in urinary olive oil polyphenols. To our knowledge, this is the first time this result has been reported in vivo in humans. Our data also provided evidence that a decrease in proatherogenic and proinflammatory molecular mechanisms can be achieved with a polyphenol-rich olive oil intervention.

The CD40/CD40L system is considered to be proatherogenic and prothrombotic and links inflammation with atherothrombosis

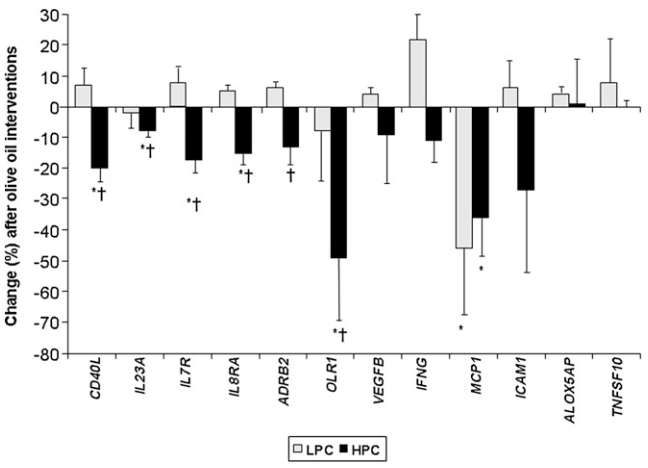

FIGURE 3. Mean (±SEM) percentage changes in gene expression (n = 18). Changes after HPC and LPC olive oil interventions (3-wk). Gene expression is referred to as the percentage of change (mean $\log_2$ ratio relative quantification) of post- compared with preintervention values. *$P < 0.05$ compared with baseline; †$P < 0.05$ compared with LPC intervention (general linear mixed model). HPC, high polyphenol content; LPC, low polyphenol content.

TABLE 3
Pearson's correlation analyses of gene-expression changes after HPC olive oil intervention ($n = 18$)[1]

	CD40L	*OLR1*	*IL23A*	*VEGFB*	*IFNG*	*ADRB2*	*ICAM1*	*MCP1*	*IL8RA*	*IL7R*	*TNFSF10*	*ALOX5AP*
CD40L	1											
OLR1	0.515	1										
IL23A	0.700[2]	0.259	1									
VEGFB	0.689[2]	0.619[3]	0.634[2]	1								
IFNG	0.319	0.420	0.537[3]	0.254	1							
ADRB2	0.702[2]	0.536[3]	0.667[2]	0.649[2]	0.527[3]	1						
ICAM1	0.527[2]	0.423	0.529[3]	0.673[2]	0.262	0.852[2]	1					
MCP1	0.473	0.569[3]	0.338	0.311	0.429	0.606[3]	0.609[3]	1				
IL8RA	0.405	0.001	0.641[3]	0.149	0.781[3]	0.554[2]	−0.143	−0.006	1			
IL7R	0.809[3]	0.158	0.599[2]	0.473	0.134	0.579[2]	0.278	−0.018	0.442	1		
TNFSF10	0.372	−0.003	0.304	0.082	−0.109	0.056	0.452	0.368	−0.080	0.367	1	
ALOX5AP	0.535[2]	0.240	0.561[2]	0.635[3]	0.245	0.727[3]	0.255	−0.240	0.429	0.665[3]	0.097	1

[1] All values are Pearson's correlation coefficient *r*. HPC, high polyphenol content.
[2] $P < 0.01$.
[3] $P < 0.05$.

(14). The activation of the *CD40* ligation can occur via several mechanisms. One of the mechanisms involves proinflammatory cytokines and IFN-γ, which have been reported to increase the surface amounts of CD40L in human vascular endothelial cells, smooth muscle cells, macrophages, and monocytes in experimental models (15, 16). The reduction in *IFNG* expression after polyphenol-rich olive oil could be linked with that in *CD40L* but also with the observed decrease in *IL23R* expression. The expression of proinflammatory cytokines is interlinked both among them and with that of *IFNG* (17). In experimental studies, IL23A increased the expression of human IFN-γ protein in mononuclear cells (17, 18). We also previously reported a reduction in IFN-γ plasma concentrations and messenger RNA expression associated with the consumption of virgin olive oil within the frame of the Mediterranean diet (8).

Another mechanism for CD40 activation involves a cross-linked interaction with *OLR1*. A stimulation of *OLR1* by oxidized LDL has been shown to induce the expression of *CD40*, and in turn, the stimulation of *CD40* by CD40L induced the expression of *OLR1* in endothelial cells (19). In our study, the decrease in oxidized LDL was directly associated with a decrease in *CD40L* expression, and the increase in urinary olive oil polyphenols was directly associated with the decrease in *OLR1* expression. Our results are also in line with results that reported a reduction in *CD40* and *CD40L* gene expression after intake of cocoa flavonoid or wine polyphenols (20, 21). *CD40L* enhances in vivo angiogenesis by directly upregulating the expression of vascular endothelial growth factor both in endothelial cells and monocytes (22). In this study, we observed, together with a reduced *CD40L* expression, a decrease in the expression of vascular endothelial growth factor in PBMCs after HPC compared with after the LPC intervention.

When CD40/CD40L system is internalized into cells, it binds to the tumor receptor associated factor and stimulates downstream signaling pathways (23). CD40 ligation has been reported to increase *MCP1*, *IL8* via the *IL8RA* receptor, and *ICAM1* expressions through the tumor receptor associated factor recruitment and mitogen-activated protein kinase activation (24, 25). Thus, the decrease in *ICAM1* expression after HPC intervention could be promoted by the reduction in both the *CD40L* and *IL8RA* expressions observed. After an acute intake of virgin olive oil, with HPC, a decrease in the gene expression of serine-threonine phosphatases, which downregulate effectors of the mitogen-activated protein kinase pathway, has been reported (6). Supplementation with olive oil, as well as with soy and cod-liver oils, has been shown to reduce ICAM and TNF-α plasma concentrations in humans (26). Olive oil phenolic extracts have also been shown to reduce *ICAM1* expression in cultured human umbilical vein endothelial cells (27). In the current study, increases in urinary olive oil polyphenols were associated with decreases in *ICAM* and *OLR1* gene expressions.

A key downstream product of the CD40/CD40L cascade is MCP1, which is a potent regulator of leukocyte trafficking. This cytokine is involved in the pathogenesis of diseases characterized by monocytic infiltrates, such as vascular diseases (28). Recently, an intervention study reported a reduction of the *MCP1* gene expression when the Mediterranean diet was supplemented with virgin olive oil (rich in polyphenols) in high–cardiovascular risk individuals (29). In our study, we observed a similar decrease (~40%) in *MCP1* expression after intakes of the 2 types of olive oil. However, the decrease in the MCP1 protein at the systemic level reached significance after the HPC intervention compared with after the LPC intervention. Our results suggest that olive oil polyphenols could act not only at pretranslational levels but also at posttranslational levels, decreasing the MCP1 protein.

Although the role of β-adrenergic receptors in heart disease remains controversial, overt activation of β-adrenergic receptors has been implicated in the progression of heart disease. Mice with transgenic *ADRB2* expression showed increased reactive oxygen expression (30). These data are in line with our current results that showed a decrease in *ADRB2* expression associated with lesser oxidative damage in LDL. We have previously reported a decrease in the *ADRB2* expression linked to the polyphenol content of olive oil within the frame of the traditional Mediterranean diet (8). In vivo studies have reported a significant decrease in MCP1 and IFN-γ after an ADRB2 blockade after an operative injury in rat models (31). Thus, a complementary mechanism for a mediated *IFNG CD40L* downregulation could be a decrease of the *ADRB2* expression mediated by olive oil polyphenols.

One strength of the current study is its crossover design that permitted the same participants to receive all treatments, which minimized interferences with possible confounding variables. Changes in outcomes were modest, as was expected from real-life

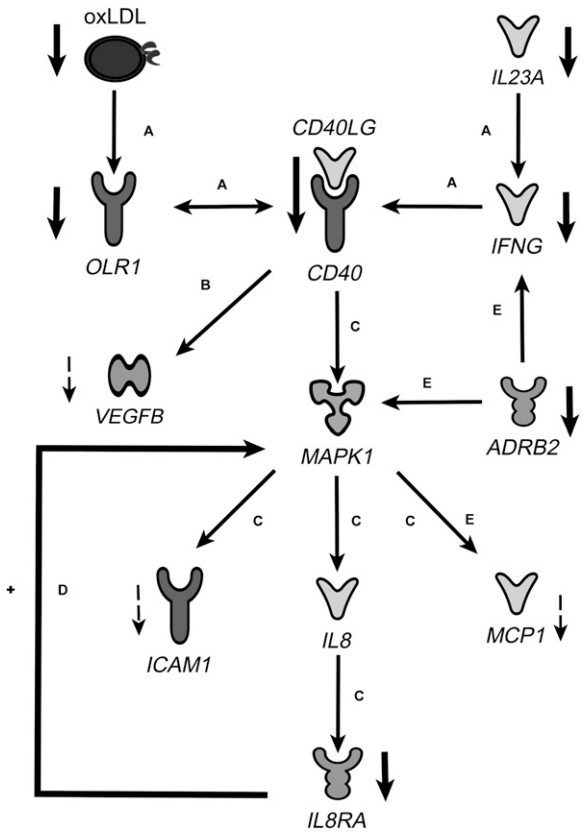

FIGURE 4. Proposed integrated scheme for the in vivo downregulation of the CD40/CD40L system and its downstream products promoted by olive oil polyphenols. A: Downregulation of *OLR1* expression, mediated by a decrease in oxidized LDL, or downregulation of *IFNG*, mediated by a reduced *IL23* expression, can reduce *CD40/CD40L* expression. This effect will lead to a reduction in the expression of its following downstream products: *VEGFB* (B) and *MCP1*, *IL8*, and *ICAM1* (C) via a decrease in *MAPK1* activation. D: The *CD40/CD40L*-mediated *ICAM1* downregulation could be amplified by IL-8 via the *IL8RA* receptor and through the *MAPK1* pathway by an independent mechanism. E: The reduction in the expression of *ADRB2* could contribute to the reduced expression of *MCP1* and *IFNG*. ⩛, cytokine; →, downregulation ($P < 0.05$); ·→, downregulation ($P < 0.08$); ⩛, G-protein coupled receptor; ⩛, growth factor; ⩛, kinase; ⩛, oxidized lipoprotein; ⩛, transmembrane receptor.

doses of natural olive oils and the healthy status of volunteers. A limitation of the study was the sample size, which could account for a lack of significance in changes in some plasma systemic biomarkers with high interindividual variability. It is not known whether additional or different effects in the analyzed gene expressions would have been observed over longer periods. Also, the same quantity of polyphenols present in a 25-mL daily dose of HPC olive oil could be provided by other types of foods. However, whether a synergic effect exists between MUFA and the polyphenol content of the olive oil on the gene expression of atherosclerosis-related genes is unknown.

In conclusion, reductions in LDL oxidation and increases in antioxidant polyphenols by a regular dietary intake of polyphenol-rich olive oil are associated with reductions in the expression of proatherogenic and proinflammatory genes related with the CD40/CD40L pathway. Our data support the concept that dietary polyphenols from olive oil, in addition to improving systemic cardiovascular risk factors, can modulate, in a protective mode, genes involved in chronic degenerative diseases such as cardiovascular and other inflammatory ones. Long-term intervention trials examining the effects of high–olive oil polyphenol diets on cardiovascular health are warranted.

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The authors' responsibilities were as follows—M-IC, MF, and RdlT: designed the research; OK, DM-A, VK, KN, H-FZ, MF, and RdlT: conducted the research; OC and JV: performed statistical analyses; and OC, MF, and M-IC: wrote the manuscript. The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. None of the authors declared a conflict of interest.

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