In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial

Valentini Konstantinidou,[*,‡] Maria-Isabel Covas,[*,1] Daniel Muñoz-Aguayo,[*] Olha Khymenets,[†] Rafael de la Torre,[†] Guillermo Saez,[§] Maria del Carmen Tormos,[§] Estefania Toledo,[‖] Amelia Marti,[¶] Valentina Ruiz-Gutiérrez,[#] Maria Victoria Ruiz Mendez,[#] and Montserrat Fito[*]

[*]Cardiovascular Risk and Nutrition Research Group and [†]Human Pharmacology and Clinical Neurosciences Research Group, Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar), Centro de Investigación Biomédica Eu Ŗed (CIBER) de Fisiopatología de la Obesidad y Nutrición, Barcelona, Spain; [‡]PhD Program in Biomedicine, Departament de Ciencies Experimentals i de la Salut, Pompeu Fabra University, Barcelona, Spain; [§]Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain; [‖]Department of Preventive Medicine and Public Health and [¶]Department of Nutrition, Food Science, Physiology, and Toxicology, University of Navarra, Navarra, Spain; and [#]Instituto de Nutrition and Lipid Metabolism, Instituto de la Grasa, Seville, Spain

ABSTRACT **The aim of the study was to assess whether benefits associated with the traditional Mediterranean diet (TMD) and virgin olive oil (VOO) consumption could be mediated through changes in the expression of atherosclerosis-related genes. A randomized, parallel, controlled clinical trial in healthy volunteers (n=90) aged 20 to 50 yr was performed. Three-month intervention groups were as follows: *1*) TMD with VOO (TMD+VOO), *2*) TMD with washed virgin olive oil (TMD+WOO), and *3*) control with participants' habitual diet. WOO was similar to VOO, but with a lower polyphenol content (55 *vs.* 328 mg/kg, respectively). TMD consumption decreased plasma oxidative and inflammatory status and the gene expression related with both inflammation [INF-γ (*INF*γ), Rho GTPase-activating protein15 (*ARHGAP15*), and interleukin-7 receptor (*IL7R*)] and oxidative stress [adrenergic β_2-receptor (*ADRB2*) and polymerase (DNA-directed) κ (*POLK*)] in peripheral blood mononuclear cells. All effects, with the exception of the decrease in *POLK* expression, were particularly observed when VOO, rich in polyphenols, was present in the TMD dietary pattern. Our results indicate a significant role of olive oil polyphenols in the down-regulation of proatherogenic genes in the context of a TMD. In addition, the benefits associated with a TMD and olive oil polyphenol consumption on cardiovascular risk can be mediated through nutrigenomic effects.—Konstantinidou, V., Covas, M.-I., Muñoz-Aguayo, D., Khymenets, O., de la Torre, R., Saez, G., del Carmen Tormos, M., Toledo, E., Marti, A., Ruiz-Gutiérrez, V., Ruiz Mendez, M. V., Fito, M. *In vivo* nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *FASEB J.* 24, 2546–2557 (2010). www.fasebj.org**

Key Words: inflammation · oxidative stress · DNA damage · gene expression · IFNγ

In 1979, Keys *et al.* (1) provided ecological evidence of a reduced risk for coronary heart disease (CHD) associated with the Mediterranean diet despite its high monounsaturated fat content. This diet, when consumed in sufficient amounts, provides all of the known essential micronutrients (*i.e.*, vitamins and minerals), fiber, and other plant food substances to promote health (2). A high degree of adherence to the Mediterranean diet has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's disease and Alzheimer's disease (3, 4). The most impressive benefits of this diet are, however, related to cardiovascular morbidity and mortality (5).

Olive oil is the main source of fat in the Mediterranean diet. A large body of knowledge provides evidence of the benefits of the Mediterranean diet and the consumption of olive oil on risk factors for CHD, in particular, on the lipid profile, lipid and DNA oxidation, insulin resistance, and inflammation (6–9). In experimental studies, olive oil has also been shown to be able to influence stages of carcinogenesis, cell membrane composition, signal transduction pathways, transcription factors, and tumor suppressor genes (10). The beneficial effects of olive oil on cardiovascular risk factors are now recognized, but often are attributed

[1] Correspondence: Cardiovascular Risk and Nutrition Research Group, Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar), Parc de Recerca Biomèdica de Barcelona (PRBB), Carrer Dr. Aiguader, 88.08003, Barcelona, Spain. E-mail: mcovas@imim.es

doi: 10.1096/fj.09-148452

0892-6638/10/0024-2546 © FASEB

only to the high levels of monounsaturated fatty acids (MUFAs) present in olive oil (11). Olive oil, however, is a functional food that, besides a high content of MUFAs, contains other minor biologically active components (12). Among them, the best studied are the polyphenols. In human studies, olive oil polyphenols have been shown to reduce *in vivo* lipid oxidative damage (13), endothelial dysfunction (14), prothrombotic profile (15), and inflammatory status (16–18) in healthy volunteers and patients with stable CHD or hypercholesterolemia.

The exact mechanisms by which the Mediterranean diet and olive oil exert their health effects are not yet understood. Among these mechanisms, the gene-environment and/or gene-diet interaction could play an important role in the development and/or protection of chronic degenerative diseases. At present, few data exist on the *in vivo* effect of the Mediterranean diet on human gene expression (19, 20), particularly in healthy volunteers. Gene expression changes in human peripheral blood mononuclear cells (PBMNCs) after virgin olive oil consumption have been reported (21–23). However, no data exist concerning the *in vivo* nutrigenomic effects of olive oil polyphenols in humans. The aim of the present study was to evaluate whether a traditional Mediterranean diet (TMD) and the polyphenols present in olive oil promote changes in atherosclerosis-related genes in healthy volunteers.

MATERIALS AND METHODS

Study design

A randomized, parallel, controlled clinical trial with 3 dietary interventions was performed. From October 2007 to October 2008, 99 potential participants were recruited in primary care centers. Ninety eligible participants were community-dwelling men and women aged 20 to 50 yr. They were considered healthy on the basis of a physical examination and routine biochemical and hematological laboratory determinations. The institutional ethics committee [IMM Comitè Ètic d'Investigació Clínica–Institut Municipal d'Assistència Sanitària (CEIC-IMAS)] approved the protocol (2004/1827/I), and the volunteers gave written informed consent before initiation of the study. This trial was registered in Current Controlled Trials, London, with the International Standard Randomized Controlled Trial Number of ISRCTN53283428.

Volunteers were randomly assigned to 3 intervention groups (n=30/group), by means of a computer-generated random-number sequence. They received the following treatments during 3 mo: group 1, TMD with virgin olive oil (TMD+VOO); group 2, TMD with washed virgin olive oil (TMD+WOO); and group 3, control group with their habitual diet. Volunteers were advised by a dietitian to maintain their habitual lifestyle. Exclusion criteria were the following: intake of antioxidant supplements; intake of acetosalicylic acid or any other drug with established antioxidative properties; high levels of physical activity (>3000 kcal/wk in leisure-time physical activity); obesity [body mass index (BMI) >30 kg/m^2]; hypercholesterolemia (total cholesterol >8.0 mM or dyslipidemia therapy); diabetes (glucose >126 mg/dl or diabetes treatment); hypertension [systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg or antihypertensive treatment]; multiple allergies; celiac or other intestinal diseases; any condition that could limit the mobility of the subject, making study visits impossible; life-threatening illnesses or other diseases or conditions that could worsen adherence to the measurements or treatments; vegetarianism or a need for other special diets; and alcoholism or other drug addiction. Fasting blood and first morning spot urine samples were collected between 8 and 10 AM at study entry and after the 3-mo intervention.

Randomization and Mediterranean diet intervention

The baseline examination included the administration of a previously validated 137-item food frequency questionnaire (24); the Minnesota Leisure Time Physical Activity questionnaire, which has been validated for use in Spanish men and women (25, 26); and a 47-item general questionnaire assessing lifestyle, health conditions, sociodemographic variables, history of illness, and medication use. The same dietitian performed the interventions with the 3 randomized groups. On the basis of the assessment of an individual 14-point Mediterranean diet score (8), the dietitian gave personalized advice during a 30-min session to each participant, with recommendations on the desired frequency of intake of specific foods. Instructions were directed to upscaling the TMD score, including the use of olive oil for cooking and dressing; increased consumption of fruit, vegetables, and fish; consumption of white meat instead of red or processed meat; preparation of homemade sauce with tomato, garlic, onion, aromatic herbs, and olive oil to dress vegetables, pasta, rice, and other dishes; and, for alcohol drinkers, moderate consumption of red wine. At the end of the intervention (3 mo), all baseline procedures were repeated.

Olive oil characteristics

The WOO used in intervention group 2 was obtained from the VOO used in intervention group 1 in the Instituto de la Grasa (Sevilla, Spain). In brief, VOO was placed in a thermostatic reactor, washed twice with 10% water at 70°C, and shaken at 125 rpm. Temperature was maintained at 40°C for 20 min at 95 rpm. Oil-phase separation was performed by centrifugation, repeating the whole procedure 5 times. This WOO maintained the same characteristics as the VOO, with the exception of a lower content of polyphenols (55 and 328 mg/kg, respectively). Olive oils were provided to the subjects of both intervention groups 1 and 2 in a sufficient amount for the entire family (15 L/volunteer) during the intervention periods for both cooking and dressing purposes. The VOO used was of the Hojiblanca variety from Andalucía, Spain. The composition of the olive oils was as follows: MUFAs, 75%; polyunsaturated fatty acids (PUFAs), 18.6%; and saturated fatty acids, (SFAs), 6.4%. Minor components, other than polyphenols, were α-tocopherol (1.47 mg/kg), β-carotene (0.43 mg/kg), and sterols (15.6 mg/kg). The contents of squalene and terpenes were 4346 and 4026 mg/kg and 48.3 and 61.3 mg/kg for VOO and WOO, respectively. Both olive oils were stored to avoid exposure to air, light, and high temperature to prevent oxidation.

Oxidative damage and inflammation biomarkers

Serum glucose, total cholesterol, and triglyceride levels were measured using standard enzymatic methods, and HDL-cholesterol (HDL-C) was measured by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France), in a automated PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics). LDL-cholesterol (LDL-C) was calculated

by the Friedewald (27) formula whenever triglycerides were <300 mg/dl. Oxidized LDL (oxLDL) was determined in plasma by a sandwich ELISA procedure using the murine monoclonal antibody mAB-4E6 as a capture antibody and a peroxidase-conjugated antibody against oxidized apolipoprotein B bound to the solid phase (oxLDL; Mercodia AB, Uppsala, Sweden). Urine total F2α-isoprostanes were determined by an immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The amount of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) in urine was measured by HPLC with electrochemical detection. Values of isoprostanes and 8-oxo-dG in urine were normalized against creatinine concentration. High-sensitivity C-reactive protein (CRP) was measured by immunoturbidometry (ABX-Horiba Diagnostics). Plasma levels of IFN-γ, monocyte chemoattractant protein 1 (MCP-1), soluble P-selectin (s-P-selectin), and soluble CD40L (sCD40L) were measured by flow cytometry (Bender MedSystems Co. Ltd., San Diego, CA, USA). All analytical determinations were performed in the same batch.

Evaluation of the intervention

After 3 mo, all baseline procedures were repeated. Biological assessment of intervention compliance was performed in all participants. Tyrosol and hydroxytyrosol, the major polyphenols present in olive oil, were measured in urine by gas chromatography-mass spectrometry (28).

Gene expression analyses

The selection of candidate genes was performed on the basis of previous data from our group concerning atherosclerosis-related responsive genes in peripheral blood mononuclear cells (PBMNCs) of healthy volunteers after long-term (3 wk) (21) and short-term (23, 29) VOO consumption, and their biological plausibility as assessed by literature review (http://www.ncbi.nlm.nih.gov/pubmed/). Gene expression analyses were performed in a subsample of 56 participants (20, 16, and 20 in control, TMD+WOO, and TMD+VOO groups, respectively). A liquid-liquid method to isolate total RNA from PBMNCs was performed as described previously (21–23). The correct quality, quantity, and purity of total RNA were assessed. A total of 100 ng of tRNA in a 20-μl reaction was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. An array for gene expression analysis was performed in duplicate using 384-well Micro Fluidic cards (TaqMan Low Density Array by Design) for 48 genes (47+1 control) on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) gene was used as an endogenous control to correct the differences in the amount of total cDNA added to each reaction. Results from each run were analyzed separately using a software-defined baseline and a C_t threshold of 0.20. Changes in gene expression were calculated using the relative quantification (RQ) method and applying the $2^{-\Delta\Delta Ct}$ formula (30). Each gene expression was first normalized to the endogenous reference gene ($\Delta C_t = C_{t\ \mathrm{exp}} - C_{t\ \mathrm{ref}}$) and afterward to its untreated control (baseline) ($\Delta\Delta C_t$). Two genes, *NOX1* (NADPH oxidase 1) and *NOX2* (NADPH oxidase 2), did not amplify. Thus, they were excluded from the analyses. Data obtained were analyzed using SDS 2.1 software. We used the Functional Classification Tool of the DAVID Bioinformatics Database (31, 32) to generate a gene-to-gene similarity matrix.

Statistical analysis

The normality of continuous variables was assessed by normal probability plots and by means of the Shapiro-Wilk test. The relationship between continuous variables was measured by Spearman's rank correlation coefficient. Non-normally distributed variables were log-transformed before application of the *t* test or general linear modeling statistics. ANOVA was used for assessing differences between the control and the two TMD intervention groups at baseline. Comparisons of the 3-mo changes were performed by a covariance model with polynomial content, with age and sex as covariates. Statistical analyses were performed as 2-group analyses with the TMD global group (TMD+VOO and TMD+WOO) *vs.* the control group and as 3-group analyses, considering the 3 types of intervention separately (TMD+VOO, TMD+WOO, and control). An *a priori* defined value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS 12.3 software (SPSS Inc., Chicago, IL, USA) for Windows XP (Microsoft, Redmond, WA). Gene set enrichment analysis was applied to the Functional Classification Tool to determine whether an *a priori* defined set of genes showed statistically significant concordant differences between the two biological states (before and after the intervention). The enrichment score value was used to highlight the most overrepresented biological annotation out of thousands of linked terms and contents.

RESULTS

We excluded 9 of the 99 invited participants before randomization for various reasons, and 1 participant dropped out of the study after randomization (**Fig. 1**). **Table 1** shows the baseline characteristics of the 90 participants (26 men and 64 women) who entered the study. We observed lower levels of plasma IFN-γ in the TMD global and the TMD+WOO groups *vs.* control. We did not observe differences in general baseline characteristics among groups (Table 1). **Table 2** shows the changes in energy, nutrient intake, and key food items at the end of the intervention period. An increase in vegetable, legume, and fish consumption was observed in both TMD groups. Participants' compliance with the supplemented olive oil was good, as reflected by both the increase in VOO consumption and the decrease in olive oil (nonvirgin) in the TMD+VOO group, whereas the opposite effect was observed in the TMD+WOO group (Table 2), and the decrease in the urinary tyrosol and hydroxytyrosol concentrations in the TMD+WOO group and the increase in the TMD+VOO group ($P = 0.007$, for quadratic trend) (**Fig. 2**). In the two-group analyses (TMD global *vs.* control), plasma glucose levels, HDL-C, $F_{2\alpha}$-isoprostanes, IFN-γ, and CRP decreased after 3 mo of TMD intervention ($P < 0.05$;) (**Table 3**). In the 3-group analyses (Table 3), total cholesterol, HDL-C, and LDL-C decreased in the TMD+VOO group after 3 mo of intervention ($P < 0.05$), without changes in the total cholesterol/HDL-C or LDL-C/HDL-C ratios. The decrease in plasma IFN-γ, $F_{2\alpha}$-isoprostanes, and s-P-selectin was significant only after the TMD+VOO intervention ($P<0.05$) (Table 3). Similar trends and results

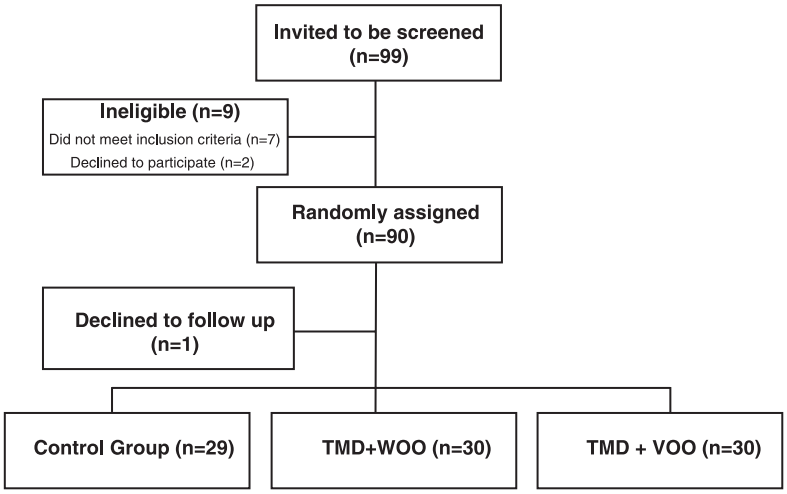

Figure 1. Study flow diagram. Gene expression analyses were performed in a subsample of 56 participants (20, 16, and 20 in control, TMD+WOO, and TMD+VOO groups, respectively).

were obtained when the subpopulation involved in gene expression analyses (n=56) was evaluated. When results were disclosed by sex, in females a decrease in IFN-γ in the control group and in CRP in the TMD global and TMD+WOO groups was observed. In addition, in the TMD+VOO group, we observed an increase in HDL-C in females and a decrease in LDL-C in males (Supplemental Table 1).

Intragroup comparisons showed no significant differences between pre- and posttreatment values in the evaluated gene expression in any intervention group. **Table 4** shows the intergroup comparisons of the gene expression changes in the 2-group analyses (TMD global *vs.* control) expressed as the $\log_2$ ratio of RQ between posttreatment and basal values. Five genes [adrenergic β_2-receptor (*ADRB2*), Rho GTPase-activating protein 15 (*ARHGAP15*), IFN-γ (*IFN*γ), IL-7 receptor (*IL7R*), and polymerase (DNA directed)-κ (*POLK*)] were down-regulated compared with the control group (P<0.05) (Table 4). When the 3-group analyses were performed, a decreasing linear trend from the control to the TMD+VOO group (P<0.05) was observed (**Fig. 3**) in *ADRB2, ARHGAP15, IL7R,* and *IFN*γ gene expression. The down-regulation was statistically significant in the TMD+VOO group *vs.* control group (P<0.05) for *ADRB2, ARHGAP15,* and *IFN*γ genes and had borderline significance (P=0.052) in the case of *IL7R* gene expression (Fig. 3). No differences in the expression of

TABLE 1. *Volunteer baseline characteristics*

Parameter	Control, n = 30	TMD global, n = 60	TMD+WOO, n = 30	TMD+VOO, n = 30
Age (yr)	43 ± 13	45 ± 10	44 ± 10	45 ± 10
Men (%)	34.5	25	27	23
Weight (kg)	66 ± 16	68 ± 13	69 ± 13	68 ± 14
BMI (kg/m^2)	25 ± 4	25 ± 4	26 ± 5	25 ± 4
SBP (mmHg)	117 ± 12	116 ± 15	117 ± 14	114 ± 16
DBP (mmHg)	69 ± 10	72 ± 10	72 ± 10	72 ± 10
Glucose (mg/dl)	85 ± 15	84 ± 12	84 ± 14	84 ± 9
Total cholesterol (mg/dl)	202 ± 54	207 ± 50	200 ± 47	214 ± 54
LDL-C (mg/dl)	127 ± 42	131 ± 44	124 ± 37	138 ± 50
HDL-C (mg/dl)	58 ± 13	60 ± 14	58 ± 13	61 ± 15
Total cholesterol/HDL-C	3.6 ± 1.0	3.6 ± 0.9	3.5 ± 0.6	3.6 ± 1.0
LDL-C/HDL-C	2.2 ± 0.7	2.3 ± 0.8	2.2 ± 0.5	2.3 ± 0.9
Triglycerides (mg/dl)	67 (52, 83)	70 (57, 103)	67 (58, 102)	70 (57, 105)
oxLDL (U/L)	66 ± 29	63 ± 21	62 ± 20	64 ± 22
$F_{2\alpha}$-isoprostanes in urine (pg/mmol of creatinine)	42 (39, 79)	67 (39, 83)	54 (41, 79)	72 (39, 85)
IFN-γ (ng/ml)	0.086 (0.009, 0.124)	0.018 (0.001, 0.073)*	0.001 (0, 0.068)*	0.027 (0, 0.086)
MCP-1 (pg/ml)	282 (203, 369)	217 (170, 307)	240 (195, 349)	174 (143, 243)
s-P-selectin (ng/ml)	935 ± 741	743 ± 493	768 ± 496	710 ± 498
s-CD40L (pg/ml)	937 (586, 2254)	1217 (602, 2354)	1389 (618, 2306)	1001 (558, 2449)
CRP (mg/dl)	0.02 (0.01, 0.09)	0.07 (0.03, 0.18)	0.11 (0.02, 0.25)	0.07 (0.03, 0.11)
8-Oxo-dG in urine (nmol/mmol creatinine)	10.09 ± 4.07	11.32 ± 4.01	11.10 ± 3.89	11.55 ± 4.19
EEPA (kcal/d)	129 (25, 269)	130 (47, 224)	113 (49, 183)	139 (32, 229)

Values are shown as means ± SD for normal variables and medians (25th, 75th percentiles) for nonparametric variables. Univariate ANOVA was used to assess differences between groups for the normal variables; Kruskal-Walls test was used for nonparametric variables. EEPA, energy expenditure in physical activity in leisure time. *P < 0.05 *vs.* control group.

TABLE 2. *Change in consumption of key foods and nutrients*

Variable	Change from baseline at 3 mo [mean (95% CI)]		
	Control (n = 29)	TMD + WOO (n = 30)	TMD + VOO (n = 30)
VOO (g/d)	−0.44 (−2.9 to 1.9)	−8.0 (−12.6 to −3.4)‡	22.2 (15.1 to 29.2)‡ †
Olive oil (g/d)[a]	−0.88 (−5.6 to 3.9)	10.3 (4.7 to 16.0)*,‡	−17.3 (−24.2 to −10.4)*,†,‡
Total olive oil (g/d)	−13.5 (−42.1 to 15.2)	22.9 (−11.5 to 57.5)	41.3 (11.9 to 70.7)‡
Fruits (g/d)	1.0 (0.1 to 1.2)	−0.76 (−2.84 to 1.33)	−1.31 (−5.06 to 2.44)
Vegetables (g/d)	1.72 (−2.44 to 5.89)	4.51 (−1.55 to 10.57)	10.31 (4.50 to 16.12)‡
Legumes (g/d)	−0.02 (−0.76 to 0.72)	1.36 (0.47 to 2.25)‡	2.25 (1.24 to 3.26)‡
Fish (g/d)	1.93 (−1.14 to 5.00)	3.87 (1.79 to 5.94)*	7.93 (2.89 to 12.98)‡
Nuts (g/d)	1.5 (−1.5 to −4.5)	1.2 (0.2−2.2)*	0.9 (−1.7 to 0)*
Dairy products (g/d)	6.28 (−6.59 to 19.15)	1.83 (−3.50 to 7.16)	−2.41 (−11.30 to 6.48)
Alcohol (g/d)	0.18 (−0.14 to 0.49)	0.17 (−0.08 to 0.5)	0.02 (−0.02 to 0.061)
Energy (kcal)	−20.08 (−53.66 to 13.51)	24.88 (−13.15 to 62.90)‡	51.01 (20.30 to 81.73)‡
Protein (%)	0.17 (−0.15 to 0.49)	−0.08 (−0.45 to 0.28)	0.033 (−0.25 to 0.32)
Carbohydrate (%)	0.04 (−0.76 to 0.83)	−0.57 (−1.38 to 0.24)	−0.78 (−1.27 to −0.29)*
Fat (%)	−0.28 (−1.35 to 0.79)	0.67 (−0.52 to 1.86)	0.80 (0.11 to 1.48)*
MUFAs (%)	−0.52 (−1.15 to 0.12)	0.47 (−0.47 to 1.41)	0.96 (0.06 to 1.86)*,‡
PUFAs (%)	0.10 (−0.09 to 0.30)	0.09 (−0.22 to 0.39)	−0.22 (−0.75 to 0.31)
SFAs (%)	0.38 (−0.24 to 1.00)	−0.43 (−1.0 to 0.13)‡	−0.77 (−1.24 to −0.29)*,‡
α-Linolenic acid (g/d)	0.01 (−0.06 to 0.09)	0.04 (0.01 to 0.07)*	0.05 (0.02 to 0.08)*
Marine n–3 fatty acids (g/d)	0.01 (−0.004 to 0.03)	0.03 (0.02 to 0.05)*	0.09 (0.02 to 0.17)*,‡

Univariate ANOVA was used to assess differences between groups. [a]Includes WOO. *$P < 0.05$ *vs.* baseline; †$P < 0.05$ *vs.* TMD + WOO; ‡$P < 0.05$ *vs.* control group.

other evaluated genes were observed, either between the TMD+VOO and TMD+WOO groups or between them and the control group. Gene expression changes were observed, particularly in the female groups (Supplemental Table 2). Correlation analyses showed that postintervention IL7R expression values (all volunteers) were inversely correlated with urinary tyrosol ($r=-0.273$, $P=0.044$) and hydroxytyrosol ($r=-0.284$, $P=0.035$) levels. In addition, changes in urinary levels of tyrosol after TMD+VOO intervention were inversely correlated with changes in the expression of *IFNγ* ($r=-0.390$, $P=0.006$). Functional annotation clustering of all 45 genes showed that 3 of the down-regulated genes, *IFNγ, IL7R*, and *ADRB2*, clustered to the same functional group (functional group 3, GO:0019219, regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process) (**Table 5**).

DISCUSSION

In the present study, we examined whether the adherence to a TMD modulates the expression of atherosclerosis-related genes and systemic oxidative stress and inflammation markers, focusing on the effect of olive oil polyphenols. Our results indicate that the TMD decreased the lipid oxidative and inflammatory status. The TMD also decreased the expression of genes

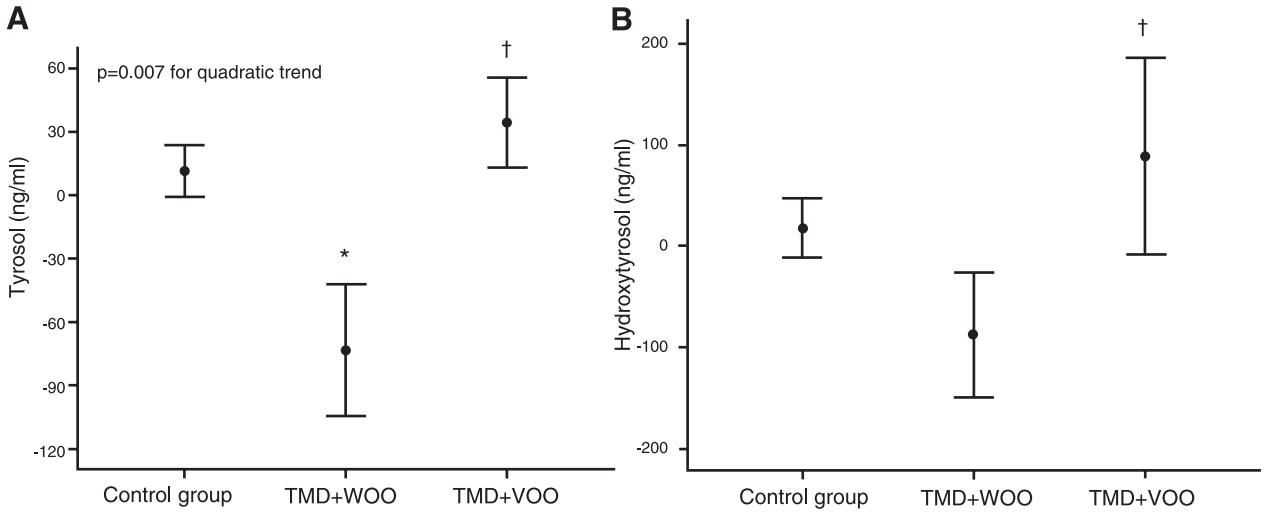

Figure 2. Changes in urinary tyrosol (*A*) and hydroxytyrosol (*B*) after the 3-mo interventions. *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* TMD+WOO.

TABLE 3. *Change in biomarkers after 3 mo of intervention*

Parameter	Control, $n = 29$		TMD global, $n = 60$	
	Postintervention	Change	Postintervention	Change
Weight (kg)	67 ± 16	0.19 (−0.59 to 0.97)	68 ± 14	−0.17 (−0.72 to 0.37)
BMI (kg/m^2)	25 ± 4	0.081 (−0.2 to 0.36)	25 ± 4	−0.068 (−0.26 to 0.13)
SBP (mmHg)	119 ± 15	1.40 (−2.60 to 5.40)	115 ± 15	−1.03 (−3.76 to 1.7)
DBP (mmHg)	71 ± 10	1.67 (−1.23 to 4.58)	72 ± 10	0.17 (−1.81 to 2.15)
Glucose (mg/dl)	82 ± 12	−2.55 (−5.4 to 0.31)	82 ± 10*	−2.1 (−4.09 to −0.09)
Cholesterol (mg/dl)	202 ± 57	−0.12 (−8.56 to 8.33)	202 ± 46	−4.85 (−10.75 to 1.04)
HDL-C (mg/dl)	57 ± 13	−1.82 (−4.34 to 0.70)	57 ± 13*	−2.0 (−3.75 to −0.29)
LDL-C (mg/dl)	129 ± 47	2.1 (−4.35 to 8.56)	128 ± 40	−2.80 (−7.22 to 1.63)
Cholesterol/HDL-C	3.6 ± 1.0	0.09 (−0.05 to 0.24)	3.6 ± 0.8	0.04 (−0.06 to 0.14)
LDL-C/HDL-C	2.3 ± 0.8	0.09 (−0.03 to 0.22)	2.3 ± 0.7	0.03 (−0.06 to 0.12)
Triglycerides (mg/dl)	62 (49, 98)	−2.5 (−17, 17.3)	71 (59, 99)	4 (−14, 19)
OxLDL (U/L)	70 ± 32	3.38 (−2.36 to 9.16)	64 ± 23	2.3 (−1.69 to 6.19)
Isoprostanes (pg/mmol of urine creatine)	39 (34, 65)	−2.8 (−14, 5.1)	49 (41, 66)*	−2.5 (−13.7, 6.6)
8-oxo-dG (nmol/mmol of urine creatine)	8.9 ± 3.8	−1.1 (−2.5 to 0.26)	10.4 ± 3.9	−0.95 (−1.89 to 0.003)
IFN-γ (pg/ml)	61 (0, 113)	−11 (−52, 5)	0 (0, 46)*	0 (−45, 11)
MCP-1 (pg/ml)	247 (211, 317)	−36 (−119, 27)	202 (176, 305)	0.14 (−37, 35)
s-P-selectin (ng/ml)	696 (493, 1063)	−78 (−286, 323)	578 (346, 808)*	−30 (−383, 122) †
s-CD40L (pg/ml)	1267 (498, 2013)	−228 (−1109, 789)	943 (587, 2437)	−77 (−1077, 804)
CPR (mg/dl)	0.04 (0.01, 0.14)	0 (−0.01, 0.06)	0.04 (0.02, 0.11)*	−0.02 (−0.07, 0)†
EEPA (kcal/d)	129 (52, 226)	6.8 (−23.7 to 37.2)	117 (32, 206)	−1.8 (−23 to 19)

Postintervention values are presented as means ± SD for normal variables and as medians (25th, 75th percentiles) for non-normal variables. Change values are presented as means (95% CI) and medians (25th, 75th percentiles) for non-normal variables. Univariate ANOVA, adjusted by sex and age, was used to assess differences between groups for the normal variables; Kruskal-Walls test was used for nonparametric variables. EEPA, energy expenditure in physical activity in leisure time. *$P < 0.05$ *vs.* baseline; †$P < 0.05$ *vs.* control; ‡$P < 0.05$ *vs.* TMD + WOO.

related to inflammation processes (*IFNγ*, *ARHGAP15,* and *IL7R*), oxidative stress (*ADRB2*), and DNA damage (*POLK*) in PBMNCs. All of the above-mentioned effects, with the exception of the decrease in *POLK* expression, were particularly observed when VOO, rich in polyphenols, was present in the TMD pattern. Our work provides, for the first time, evidence of the *in vivo* nutrigenomic effect of olive oil polyphenols down-regulating proatherogenic genes in humans. In addition and to the best of our knowledge, the *in vivo* human nutrigenomic effect of the Mediterranean diet in healthy individuals has not been reported previously.

When results were disclosed by sex, the gene expression changes were particularly lower in the female groups. In a previous work, we have reported gender differences in PBMNC gene expression, with higher expression of *SOD1* and *SOD2* in healthy males (31). In the present work, however, the low number of males in some groups could account for the gender differences observed. Gene expression can be considered as a quantitative trait that is highly heritable. We used the Functional Classification Tool of the DAVID Bioinformatics Database (32, 33) to generate a gene-to-gene similarity matrix. Grouping genes based on functional similarity can help to enhance the biological interpretation of large lists of genes derived from high-throughput studies. It has been shown that disease-related genes tend to interact (34, 35) and display significant functional clustering in the analyzed molecular network. In our results, after 3 mo of TMD+VOO intervention, 3 of the down-regulated genes (*IFNγ*, *IL7R,* and *ADRB2*) were clustered to the same functional group. In a previous exploratory approach concerning the human mononuclear cell transcriptome response after acute and sustained VOO consumption, we observed gene expression changes in PBMNCs of healthy volunteers (21–23). In this work (21, 23), the Gene Ontology analysis of the differentially expressed genes indicated that consumption of VOO could elicit changes in the regulation of transcription and translation activities of human PBMNCs.

The Mediterranean diet, in which the main source of fat is olive oil, is well known to be associated with a low prevalence of CVD (2), cancer (36), and inflammatory diseases (37, 38). Inflammation is heavily involved in the onset and development of atherosclerosis (39). Previous data from short- and long-term clinical trials have shown an anti-inflammatory effect of the Mediterranean-type diet in patients with risks for cardiovascular disease (8, 40). In these patients, a TMD enriched with VOO prevented the increase in cyclooxygenase-2 (*COX-2*) and LDL receptor-related protein (*LRP1*) gene expression and reduced monocyte chemoattractant protein (*MCP-1*), compared with a TMD enriched with nuts or with a low-fat diet (19). In experimental models, the anti-inflammatory effects of polyphenols and other olive oil minor components have been described (41). Some of the anti-inflammatory effects of olive oil polyphenols could be attributed to oleocan-

TABLE 3. *Continued*

TMD + WOO, $n = 30$		TMD + VOO, $n = 30$	
Postintervention	Change	Postintervention	Change
69 ± 14	−0.25 (−1.03 to 0.53)	67 ± 14	−0.1 (−0.86 to 0.67)
26 ± 5	−0.1 (−0.38 to 0.18)	25 ± 4	−0.04 (−0.31 to 0.24)
116 ± 14	−1.63 (−5.51 to 2.24)	114 ± 15	−0.4 (−4.31 to 3.45)
71 ± 9	−0.8 (−3.6 to 2.0)	73 ± 10	1.12 (−1.69 to 3.93)
82 ± 11	−1.76 (−4.58 to 1.06)	82 ± 9	−2.43 (−5.3 to 0.44)
200 ± 48	−0.2 (−8.1 to 7.7)	205 ± 45*	−10.5 (−19.1 to −1.84)
57 ± 12	−1.12 (−3.5 to 1.3)	58 ± 15*	−3.14 (−5.54 to −0.53)
126 ± 40	1.4 (−4.6 to 7.4)	131 ± 41*	−7.5 (−13.8 to −1.2)†‡
3.5 ± 0.7	0.06 (−0.08 to 0.20)	3.6 ± 0.8	0.02 (−0.13 to 0.17)
2.2 ± 0.6	0.06 (−0.06 to 0.18)	2.3 ± 0.8	−0.003 (−0.13 to 0.12)
73 (58, 100)	4.5 (−17.3, 18.5)	68 (60, 97)	4 (−10, 19)
65 ± 22	2.4 (−3.04 to 7.9)	63 ± 24	2.1 (−3.68 to 7.83)
52 (43, 66)	−1.6 (−10.5, 7.4)	47 (35, 75)*	−4.3 (−18.2, 6.6)
10.7 ± 3.5	−0.41 (−1.75 to 0.93)	10.1 ± 4.4*	−1.48 (−2.82 to −0.15)
16 (0, 51)	0 (−47, 33)	0 (0, 39)*	−2.5 (−47, 0)
253 (175, 328)	8 (−60, 49)	194 (176, 250)	−6 (−25, 29)
664 (368, 965)	19 (−375, 147)	549 (248, 634)*	−63 (−434, 75)
1256 (706, 2773)	123 (−875, 915)	923 (455, 2467)	−81 (−1435, 602)
0.0 (0.02, 0.12)*	−0.03 (−0.1, 0)	0.03 (0.02, 0.11)*	−0.02 (−0.06, 0)†
113 (61, 206)	6.7 (−23 to 37)	120 (23, 226)	−10 (−40 to 20)

thal, an olive oil polyphenol with ibuprofen-like activity in *in vitro* models (42). Besides its antioxidant and anti-inflammatory activity, recent data suggest that hydroxytyrosol, a major olive oil phenolic compound, may exert beneficial effects through stimulation of mitochondrial biogenesis (43). The *in vivo* anti-inflammatory role of olive oil polyphenols in humans is supported by several randomized controlled clinical trials (16, 17, 44).

The decrease in systemic inflammatory markers and in the expression of genes related with inflammatory processes observed in the present study is in agreement with the above described previous results concerning the protective effect of Mediterranean diet and olive oil phenolics on inflammation. The decrease in IFN-γ was observed both at phenotypic and gene expression levels. IFN-γ is considered to be a key inflammatory mediator for inducing IL-6, a prime regulator of CRP synthesis in the liver (45). We have previously reported a down-regulation of *IFN*γ expression in PBMNCs of healthy volunteers after a single dose of VOO (22). *ARHGAP15* encodes for a Rho GTPase-activating protein that regulates activity of GTPases (46). Ras superfamily GTPases have been identified as strategic molecular targets in statin-induced T-cell immunosuppression. Statins, besides being cholesterol-lowering drugs, also harbor strong anti-inflammatory properties (47). Members of the Rho GTPase family have been suggested to be mediators of cardiac hypertrophy (48); however to date little is known about their physiological roles (46).

The protein encoded by the *IL7R* gene is a receptor for IL-7, which has been related to inflammatory processes (49, 50). IL-7 has been shown to enhance the expression of chemokines in PBMNCs (51). A recent study has shown an up-regulation of stress-response genes, such as *IL7R* and *POLK*, in the case of induced carbon ion irradiation in murine tumor models (52). *POLK* is a DNA repair gene that copies undamaged DNA templates and is unique among human Y-family DNA polymerases (53). Somatic DNA mutations, promoted by DNA oxidation, are considered to be a crucial step in carcinogenesis as well as to be involved in atherosclerotic processes (16, 54). We did not observe changes in the levels of 8-oxo-dG after the global TMD interventions, although a decrease was observed after the TMD+VOO intervention. However, the results of the EUROLIVE study, an intervention study performed in 200 healthy males with 3 types of similar olive oils, but with differences in their phenolic content, showed that daily consumption of 25 ml of olive oil for 3 wk reduced DNA oxidation, irrespective of the olive oil polyphenol content (55). In agreement with the EUROLIVE results, the down-regulation of *POLK* gene expression observed in our study was associated with the TMD intervention, but not with the olive oil polyphenol content. All these data suggest a protective role for the MUFAs or other minor components of the olive oil on DNA oxidation and damage.

TABLE 4. *Change in expression of atherosclerosis-related genes after 3 mo of intervention*

Gene symbol	Gene name	Control, $n = 20$	TMD-global, $n = 36$	P value
Cholesterol, lipid transport, and metabolism				
ABCA1	ATP-binding cassette, subfamily A, member 1	0.320 ± 0.231	0.051 ± 0.159	0.334
ABCG1	ATP-binding cassette, subfamily G, member 1	0.146 ± 0.127	0.064 ± 0.092	0.608
ANXA1	Annexin A1	0.259 ± 0.229	−0.444 ± 0.161	0.160
ARHGAP15	Rho GTPase activating protein 15	0.448 ± 0.175	−0.040 ± 0.126	0.043
ARHGAP19	Rho GTPase activating protein 19	0.400 ± 0.151	0.134 ± 0.112	0.166
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	0.460 ± 0.144	0.157 ± 0.106	0.099
CD36	CD36 molecule (thrombospondin receptor)	0.197 ± 0.170	−0.009 ± 0.126	0.342
CETP	Cholesteryl ester transfer protein, plasma	−0.262 ± 0.331	−0.058 ± 0.257	0.631
MSR1	Macrophage scavenger receptor 1	0.542 ± 0.222	0.253 ± 0.157	0.301
PLA2G4B	Phospholipase A2, group IVB	0.148 ± 0.156	0.082 ± 0.109	0.735
SCARB1	Scavenger receptor class B, member 1	−0.025 ± 0.078	0.085 ± 0.056	0.261
Inflammation				
IFNg	Interferon, γ	1.048 ± 0.464	−0.109 ± 0.330	0.049
IL10	Interleukin 10	0.915 ± 0.360	0.609 ± 0.270	0.506
CHUK	Conserved helix-loop-helix ubiquitous kinase	0.325 ± 0.192	0.036 ± 0.140	0.236
ADAM17	ADAM metallopeptidase domain 17 (tumor necrosis factor, α, converting enzyme)	0.290 ± 0.153	0.008 ± 0.112	0.148
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.166 ± 0.208	−0.120 ± 0.150	0.277
IFNA1	Interferon, α 1	0.726 ± 0.356	0.001 ± 0.258	0.117
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	0.195 ± 0.219	−0.195 ± 0.156	0.157
TNFSF12_13	Tumor necrosis factor (ligand) superfamily, member 12-member 13	−0.021 ± 0.102	0.133 ± 0.075	0.235
IL6	Interleukin 6	−0.017 ± 0.588	0.356 ± 0.401	0.612
IL7R	Interleukin 7 receptor	0.580 ± 0.182	0.095 ± 0.132	0.037
USP48	Ubiquitin specific peptidase 48	0.380 ± 0.179	0.203 ± 0.131	0.431
MPO	Myeloperoxidase	−0.159 ± 0.121	−0.013 ± 0.090	0.343
RGS2	Regulator of G-protein signaling 2, 24 kDa	0.439 ± 0.268	0.289 ± 0.196	0.656
NFKB2	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2	−0.098 ± 0.082	0.008 ± 0.063	0.315
Nuclear receptors and fatty acids receptors				
NR1H2	Nuclear receptor subfamily 1, group H, member 2	−0.081 ± 0.070	−0.003 ± 0.050	0.369
NRIH3	Nuclear receptor subfamily 1, group H, member 3	0.166 ± 0.108	0.034 ± 0.077	0.331
PPARA	Peroxisome proliferator-activated receptor α	0.088 ± 0.123	0.068 ± 0.092	0.897
PPARBP	PPAR binding protein	0.341 ± 0.160	0.022 ± 0.105	0.084
PPARG	Peroxisome proliferator-activated receptor γ	0.002 ± 0.242	0.235 ± 0.175	0.463
PPARD	Peroxisome proliferator-activated receptor δ	0.066 ± 0.128	0.010 ± 0.096	0.732
Oxidative stress				
LIAS	Lipoic acid synthetase	0.228 ± 0.197	0.188 ± 0.148	0.874
PTGS1	Prostaglandin-endoperoxide synthase 1	−0.176 ± 0.171	−0.170 ± 0.117	0.978
PTGS2	Prostaglandin-endoperoxide synthase 2	0.170 ± 0.545	−0.231 ± 0.379	0.557
OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1	0.521 ± 0.948	0.113 ± 0.580	0.724
OSBP	Oxysterol binding protein	0.219 ± 0.130	0.035 ± 0.093	0.260
ADRB2	Adrenergic, β-2, receptor, surface	0.225 ± 0.135	−0.138 ± 0.098	0.036
OGT	*O*-linked *N*-acetylglucosamine (GlcNAc) transferase	0.373 ± 0.235	0.014 ± 0.162	0.218
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	−0.101 ± 0.187	−0.116 ± 0.135	0.949
DNA repair				
CCNG1	Cyclin G1	0.396 ± 0.192	0.004 ± 0.139	0.106
POLK	Polymerase (DNA directed) κ	0.595 ± 0.275	−0.115 ± 0.204	0.045
TP53	Tumor protein p53	−0.071 ± 0.077	−0.048 ± 0.056	0.812
DCLRE1C	DNA cross-link repair 1C	0.406 ± 0.169	0.052 ± 0.123	0.100
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5	0.401 ± 0.227	0.049 ± 0.169	0.221
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80 kDa)	0.267 ± 0.152	0.000 ± 0.111	0.166

Gene expression changes, adjusted by age and sex, are presented as means ± SE of the RQ $\log_2$ ratio (posttreatment *vs.* basal values).

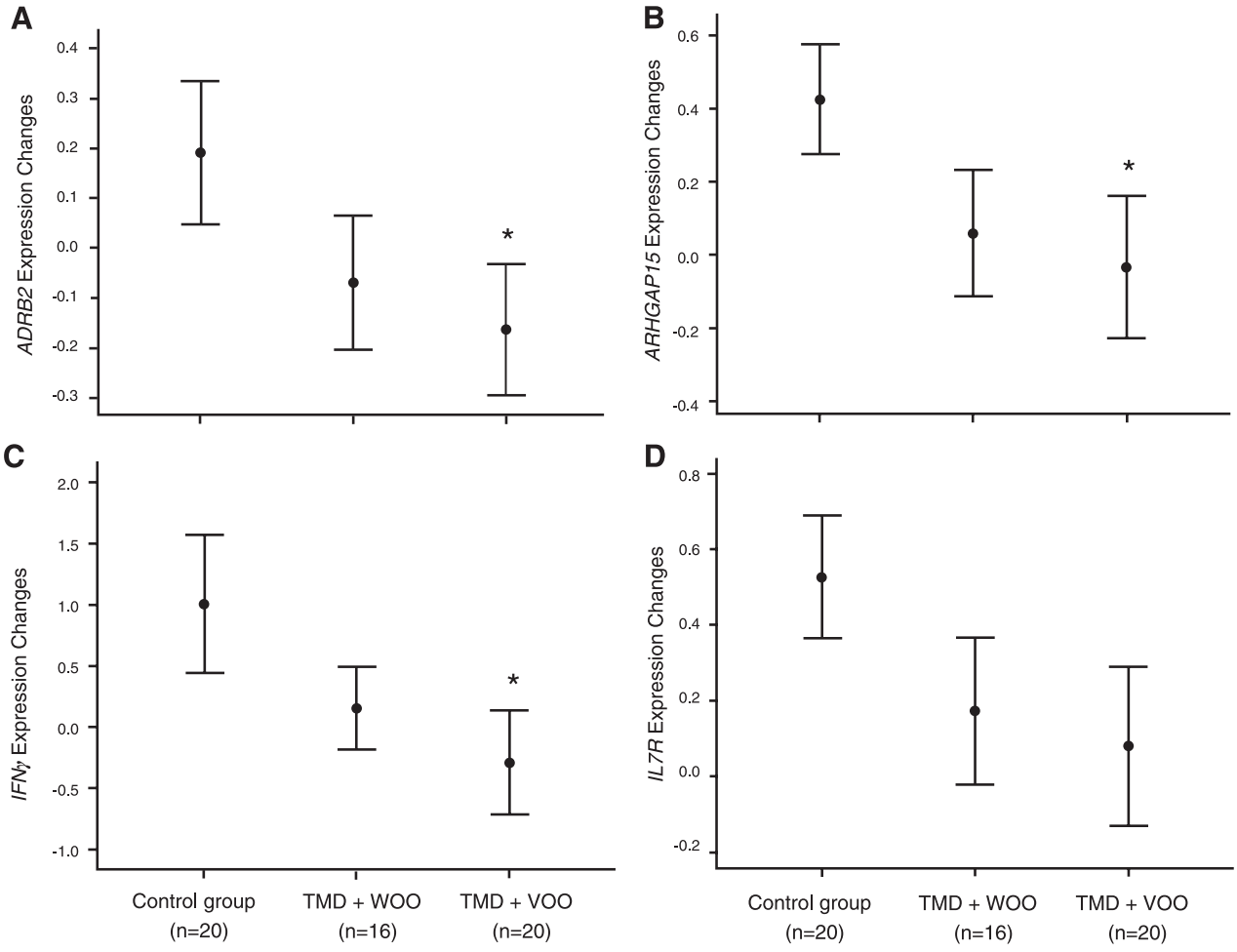

Figure 3. Gene expression changes in adrenergic β_2-receptor (*ADRB2; A*), Rho GTPase activating protein 15 (*ARHGAP15; B*), INF-γ (*IFNγ; C*), and IL-7 receptor (*IL7R; D*) genes after the 3-mo interventions. $P < 0.05$ for linear trend in all cases; $^*P < 0.05$ *vs.* control group.

The *ADRB2* gene was also down-regulated after 3 mo of TDM intervention, particularly in the TDM+VOO intervention group. A recent study has demonstrated that the *ADRB2* blockade reduces macrophage cytokine production and improves survival after traumatic injury (56). *ADRB2* agonists can affect glucose homeostasis through the modulation of insulin and glucagon secretion, hepatic glucose production, and glucose uptake into muscle (57). In this sense, we have previously reported up-regulation of the *ADRB2* expression in human PBMNCs at the postprandial state after ingestion of 50 ml of VOO (22). This olive oil ingestion promoted a postprandial peak of insulin, lipid oxidative damage, and triglycerides, and the *ADRB2* expression at 6 h postprandial was inversely correlated with plasma oxLDL and triglyceride concentrations (22). Oxidation of the lipids and apoproteins 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 (58). In functional studies the *ADRB2* receptor appears to be protective against oxidative stress (22, 59). In our present study, after 3 mo of TMD+VOO intervention, an improvement in the oxidative status of the volunteers was observed. These data are in agreement with those obtained in the EUROLIVE study, in which a dose-dependent decrease of the lipid oxidative damage was observed with the phenol content of the administered olive oil (13). One of our trial's strengths is that the study design is able to provide first-level scientific evidence (60), reflecting eating habits of community-dwelling individuals. Compliance of the volunteers was good as reflected in the changes of olive oil consumption patterns and urinary tyrosol and hydroxytyrosol. The lack of significance in the increase in urinary olive oil phenolics in the TMD+VOO group *vs.* the control group could be due to the fact that the control group participants followed their habitual diet, which in Mediterranean countries includes VOO, or the high interindividual variation in urinary phenolic values, particularly in the case of hydroxytyrosol (28). We worked with whole dietary patterns at real-life doses of food. Administration of isolated antioxidants (*i.e.*, hydroxytyrosol) at high doses has been shown to promote the atherosclerosis lesion, as well as an increase in oxidative damage, in apolipoprotein E-

TABLE 5. *Functional annotation clustering (biological processes level 5)*

Functional group	Enrichment	Gene Ontology	Gene symbol	Adjusted *P* value
1	3.86	GO:0008203; cholesterol metabolic process	*ABCG1, ABCA1, CETP, PPARD, SCARB1*	<0.001
		GO:0016125; sterol metabolic process	*ABCG1, ABCA1, CETP, PPARD, SCARB1*	<0.001
		GO:0008202; steroid metabolic process	*OSBP, ABCG1, ABCA1, CETP, PPARD, SCARB1*	<0.001
2	2.67	GO:0006915; apoptosis	*IL6, IFNG, MPO, ERCC5, TP53, IL10, TNFSF10, ARHGEF6, PPARD, ANXA1, SCARB1, ADRB2*	<0.001
		GO:0042981; regulation of apoptosis	*IL6, MPO, ERCC5, TP53, IL10, TNFSF10, ANXA1, ADRB2*	0.001
		GO:0043066; negative regulation of apoptosis	*IL6, MPO, ERCC5, IL10, ANXA1*	0.005
		GO:0043065; positive regulation of apoptosis	*TP53, TNFSF10, ADRB2*	0.181
3	2.19	GO:0031325; positive regulation of cellular metabolic process	*IL6, IFNG, MED1, TP53, IL10, PPARG, ABCA1, PPARA, ADRB2*	<0.001
		GO:0045935; ositive regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	*IL6, IFNG, MED1, TP53, PPARG, ABCA1, PPARA, ADRB2*	<0.001
		GO:0045893; positive regulation of transcription, DNA-dependent	*IL6, IFNG, MED1, TP53, PPARG, PPARA, ADRB2*	<0.001
		GO:0031324; negative regulation of cellular metabolic process	*IL6, TP53, IL10, PPARG, NR1H2, PPARD*	0.006
		GO:0006357; regulation of transcription from RNA polymerase II promoter	*MED1, TP53, PPARG,PPARA, PPARD, ADRB2*	0.014
		GO:0019219; regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	*IL6, MED1, TP53, PPARA, XRCC5, NR1H3, IL7R, IFNG, PPARG,NFkB2, ABCA1, PPARD, ADRB2*	0.047
4	1.64	GO:0006631; fatty acid metabolic process	*CD36, PTGS1, PTGS2, PPARA, PPARD*	0.002
		GO:0032787; monocarboxylic acid metabolic process	*CD36, PTGS1, PTGS2, PPARA, PPARD*	0.006
		GO:0008544; epidermis development	*PTGS2, PPARA, PPARD*	0.075
		GO:0009888; tissue development	*PTGS2, PPARA, PPARD, ADRB2*	0.084
5	1.58	GO:0048534; hemopoietic or lymphoid organ development	*IL6, IL7R, CHUK, IL10, NFkB2*	0.003
		GO:0002521; leukocyte differentiation	*IL6, CHUK, IL10*	0.045
		GO:0030097; hemopoiesis	*IL6, CHUK, IL10*	0.116

deficient mice (61). This finding points out the importance of the matrix and the dose of antioxidants. Changes in gene expression were modest, as was expected in real-life intervention conditions. The lack of a washout period at the beginning of the study could also be one factor responsible for the relative low gene response observed. We worked against our own hypothesis, by using the current Spanish dietary pattern in our control group, to maintain real-life conditions in all groups. A study limitation was the inability to assess potential interactions between the olive oil and other diet components that might affect the generalization of the results. However, the effects of food components are subtle and must be considered in the context of chronic exposure. Whether additional or different effects would have been observed over longer periods is unknown. A longer study, however, could have impaired the compliance of the participants.

In summary, a down-regulation in the expression of atherosclerosis-related genes occurs in human PBMNCs after 3 mo of TMD. Our results point out a significant role of olive oil polyphenols in the down-regulation of proatherogenic genes in the frame of the Mediterranean diet. Changes in gene expression were concomitant with decreases in lipid oxidative damage and systemic inflammation markers. Our results support the idea that the benefits associated

with a Mediterranean-type diet and olive oil polyphenol consumption on CHD risk can be mediated through changes in the expression of atherosclerosis-related genes. Data from this study provide further evidence to recommend the TMD and rich-polyphenol olive oils, such as VOO, as a useful tool for the prevention of atherosclerosis. FJ

The CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. This work was supported by Fóndo de Investigación Sanitaria–Fóndo Europeo de Desarrollo Regional (FIS-FEDER; PI041308), by Sistema National de Salud (SNS) contract Miguel Servet (CP06/00100) Instituto de Salud Carlos III, and by the Greek State Scholarship Foundation (Athens, Greece), and partially supported by the Generalitat of Catalunya (2005 SGR 00577). The authors declare no conflicting financial interests.

REFERENCES

1. Keys, A., Menotti, A., Karvonen, M. J., Aravanis, C., Blackburn, H., Buzina, R., Djordjevic, B. S., Dontas, A. S., Fidanza, F., and Keys, M. H. (1986) The diet and 15-year death rate in the seven countries study. *Am. J. Epidemiol.* **124,** 903–915
2. Trichopoulou, A., Bamia, C., and Trichopoulos, D. (2009) Anatomy of health effects of Mediterranean diet: EPIC prospective cohort study. *BMJ* **338,** b2337
3. Trichopoulou, A., Costacou, T., Bamia, C., and Trichopoulos, D. (2003) Adherence to a Mediterranean diet and survival in a Greek population. *N. Engl. J. Med.* **348,** 2599–2608
4. Sofi, F., Cesari, F., Abbate, R., Gensini, G. F., and Casini, A. (2008) Adherence to Mediterranean diet and health status: meta-analysis. *BMJ* **337,** a1344
5. Parikh, P., McDaniel, M. C., Ashen, M. D., Miller, J. I., Sorrentino, M., Chan, V., Blumenthal, R. S., and Sperling, L. S. (2005) Diets and cardiovascular disease: an evidence-based assessment. *J. Am. Coll. Cardiol.* **45,** 1379–1387
6. Covas, M. I. (2007) Olive oil and the cardiovascular system. *Pharmacol. Res.* **55,** 175–186
7. Pérez-Jimenez, F., Alvarez de Cienfuegos, G., Badimon, L., Barja, G., Battino, M., Blanco, A., Bonanome, A., Colomer, R., Corella-Piquer, D., Covas, I., Chamorro-Quiros, J., Escrich, E., Gaforio, J. J., Garcia Luna, P. P., Hidalgo, L., Kafatos, A., Kris-Etherton, P. M., Lairon, D., Lamuela-Raventos, R., Lopez-Miranda, J., Lopez-Segura, F., Martínez-González, M. A., Mata, P., Mataix, J., Ordovas, J., Osada, J., Pacheco-Reyes, R., Perucho, M., Pineda-Priego, M., Quiles, J. L., Ramírez-Tortosa, M. C., Ruíz-Gutiérrez, V., Sanchez-Rovira, P., Solfrizzi, V., Soriguer-Escofet, F., Torre-Fornell, R., Trichopoulos, A., Villalba-Montoro, J. M., Villar-Ortiz, J. R., and Visioli, F. (2005) International conference on the healthy effect of virgin olive oil. *Eur. J. Clin. Invest.* **35,** 421–424
8. Estruch, R., Martínez-González, M. A., Corella, D., Salas-Salvadó, J., Ruíz-Gutiérrez, V., Covas, M. I., Fiol, M., Gomez-Gracia, E., López-Sabater, M. C., Vinyoles, E., Aros, F., Conde, M., Lahoz, C., Lapetra, J., Saez, G., and Ros, E. (2006) Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann. Intern. Med.* **145,** 1–11
9. Salas-Salvado, J., Fernandez-Ballart, J., Ros, E., Martinez-Gonzalez, M. A., Fito, M., Estruch, R., Corella, D., Fiol, M., Gomez-Gracia, E., Aros, F., Flores, G., Lapetra, J., Lamuela-Raventos, R., Ruiz-Gutierrez, V., Bullo, M., Basora, J., and Covas, M. I. (2008) Effect of a Mediterranean diet supplemented with nuts on metabolic syndrome status: one-year results of the PREDIMED randomized trial. *Arch. Intern. Med.* **168,** 2449–2458
10. Menendez, J. A., Papadimitropoulou, A., Vellon, L., and Lupu, R. (2006) A genomic explanation connecting "Mediterranean diet," olive oil and cancer: oleic acid, the main monounsaturated fatty acid of olive oil, induces formation of inhibitory "PEA3 transcription factor-PEA3 DNA binding site" complexes at the Her-2/neu (erbB-2) oncogene promoter in breast, ovarian and stomach cancer cells. *Eur. J. Cancer* **42,** 2425–2432
11. U.S. Food and Drug Administration. Press Release P04-100. November 1, 2004. Retrieved March 30, 2009, from http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2004/ucm108368.htm
12. Covas, M. I., Ruiz-Gutierrez, V., De la Torre, R., Kafatos, A., Lamuela-Raventos, R. M., Osada, R. W., and Visioli, F. (2006) Minor components of olive oil: evidence to date of health benefits in humans. *Nutr. Rev.* **64,** 20–30
13. Covas, M. I., Nyyssonen, K., Poulsen, H. E., Kaikkonen, J., Zunft, H. J., Kiesewetter, H., Gaddi, A., de la, T. R., Mursu, J., Baumler, H., Nascetti, S., Salonen, J. T., Fito, M., Virtanen, J., Marrugat, J., and EUROLIVE Study Group. (2006) The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann. Intern. Med.* **145,** 333–341
14. Ruano, J., Lopez-Miranda, J., Fuentes, F., Moreno, J. A., Bellido, C., Perez-Martinez, P., Lozano, A., Gomez, P., Jimenez, Y., and Perez, J. F. (2005) Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients. *J. Am. Coll. Cardiol.* **46,** 1864–1868
15. Ruano, J., Lopez-Miranda, J., de la Torre, R., Delgado-Lista, J., Fernandez, J., Caballero, J., Covas, M. I., Jimenez, Y., Perez-Martinez, P., Marin, C., Fuentes, F., and Perez-Jimenez, F. (2007) Intake of phenol-rich virgin olive oil improves the postprandial prothrombotic profile in hypercholesterolemic patients. *Am. J. Clin. Nutr.* **86,** 341–346
16. Bogani, P., Galli, C., Villa, M., and Visioli, F. (2007) Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* **190,** 181–186
17. Fitó, M., Cladellas, M., de la Torre, R., Martí, J., Muñoz, D., Schröder, H., Alcántara, M., Pujadas-Bastardes, M., Marrugat, J., López-Sabater, M. C., Bruguera, J., Covas, M. I., and SOLOS Investigators. (2008) Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. *Eur. J. Clin. Nutr.* **62,** 570–574
18. Pacheco, Y. M., Bermudez, B., Lopez, S., Abia, R., Villar, J., and Muriana, F. J. (2007) Minor compounds of olive oil have postprandial anti-inflammatory effects. *Br. J. Nutr.* **98,** 260–263
19. Llorente-Cortés, V., Estruch, R., Mena, M. P., Ros, E., Martínez-González, M. A., Fitó, M., Lamuela-Raventós, R. M., and Badimon, L. (2010) Effect of Mediterranean diet on the expression of pro-atherogenic genes in a population at high cardiovascular risk. *Atherosclerosis* **208,** 442–450
20. Paniagua, J. A., Gallego de la Sacristana, A., Romero, I., Vidal-Puig, A., Latre, J. M., Sanchez, E., Perez-Martinez, P., Lopez-Miranda, J., and Perez-Jimenez, F. (2007) Monounsaturated fat-rich diet prevents central body fat distribution and decreases postprandial adiponectin expression induced by a carbohydrate-rich diet in insulin-resistant subjects. *Diabetes Care* **30,** 1717–1723
21. Khymenets, O., Fito, M., Covas, M. I., Farre, M., Pujadas, M. A., Munoz, D., Konstantinidou, V., and de la Torre, R. (2009) Mononuclear cell transcriptome response after sustained virgin olive oil consumption in humans: an exploratory nutrigenomics study. *OMICS* **13,** 7–19
22. Konstantinidou, V., Khymenets, O., Covas, M. I., de la Torre, R., Munoz-Aguayo, D., Anglada, R., Farre, M., and Fito, M. (2009) Time course of changes in the expression of insulin sensitivity-related genes after an acute load of virgin olive oil. *OMICS* **13,** 431–438
23. Konstantinidou, V., Khymenets, O., Fito, M., de la Torre, R., Anglada, R., Dopazo, A., and Covas, M. I. (2009) Characterization of human gene expression changes after olive oil ingestion: an exploratory approach. *Folia Biol. (Praha)* **55,** 85–91
24. Martin-Moreno, J. M., Boyle, P., Gorgojo, L., Maisonneuve, P., Fernandez-Rodriguez, J. C., Salvini, S., and Willett, W. C. (1993) Development and validation of a food frequency questionnaire in Spain. *Int. J. Epidemiol.* **22,** 512–519
25. Elosua, R., García, M., Aguilar, A., Molina, L., Covas, M. I., and Marrugat, J. (2000) Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish women. The MARATHOM Investigators. *Med. Sci. Sports Med.* **32,** 1431–1437
26. Elosua, R., Marrugat, J., Molina, L., Pons, S., and Pujol, E. (1994) Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. *Am. J. Epidemiol.* **139,** 1197–1209

27. Friedewald, W. T., Levy, R. I., and Fredrickson, D. S. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18,** 499–502
28. Miró-Casas, E., Farré-Albaladejo, M., Covas, M. I., Rodriguez, J. O., Menoyo-Colomer, E., Lamuela-Raventós, R. M., and de la Torre, R. (2001) Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. *Anal. Biochem.* **294,** 63–72
29. Khymenets, O., Ortuno, J., Fito, M., Covas, M. I., Farre, M., and de la Torre, R. (2005) Evaluation of RNA isolation procedures from human blood and its application for gene expression studies (Sod-1, Sod-2). *Anal. Biochem.* **347,** 156–158
30. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. *Methods* **25,** 402–408
31. Khymenets, O., Covas, M. I., Farré, M., Langohr, K., Fitó, M., and de la Torre, R. (2008) Role of sex and time of blood sampling in SOD1 and SOD2 expression variability. *Clin. Biochem.* **41,** 1348–1354
32. Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4,** 44–57
33. Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., and Lempicki, R. A. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* **4,** P3
34. Goh, K. I., Cusick, M. E., Valle, D., Childs, B., Vidal, M., and Barabasi, A. L. (2007) The human disease network. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 8685–8690
35. Feldman, I., Rzhetsky, A., and Vitkup, D. (2008) Network properties of genes harboring inherited disease mutations. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 4323–4328
36. Benetou, V., Trichopoulou, A., Orfanos, P., Naska, A., Lagiou, P., Boffetta, P., and Trichopoulos, D. (2008) Conformity to traditional Mediterranean diet and cancer incidence: the Greek EPIC cohort. *Br. J. Cancer* **99,** 191–195
37. Choi, H. K. (2005) Dietary risk factors for rheumatic diseases. *Curr. Opin. Rheumatol.* **17,** 141–146
38. Chrysohoou, C., Panagiotakos, D. B., Pitsavos, C., Das, U. N., and Stefanadis, C. (2004) Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: the ATTICA Study. *J. Am. Coll. Cardiol.* **44,** 152–158
39. Fuster, V., Badimon, L., Badimon, J. J., and Chesebro, J. H. (1992) The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N. Engl. J. Med.* **326,** 310–318
40. Esposito, K., Marfella, R., Ciotola, M., Di Palo, C., Giugliano, F., Giugliano, G., D'Armiento, M., D'Andrea, F., and Giugliano, D. (2004) Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *JAMA* **292,** 1440–1446
41. Martinez-Dominguez, E., de la Puerta, R., and Ruiz-Gutierrez, V. (2001) Protective effects upon experimental inflammation models of a polyphenol-supplemented virgin olive oil diet. *Inflamm. Res.* **50,** 102–106
42. Beauchamp, G. K., Keast, R. S., Morel, D., Lin, J., Pika, J., Han, Q., Lee, C. H., Smith, A. B., and Breslin, P. A. (2005) Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. *Nature* **437,** 45–46
43. Hao, J., Shen, W., Yu, G., Jia, H., Li, X., Feng, Z., Wang, Y., Weber, P., Wertz, K., Sharman, E., and Liu, J. (2009) Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3–L1 adipocytes. [E-pub ahead of print] *J. Nutr. Biochem.* 10.1016/j.jnutbio.2009.03.012
44. Visioli, F., Caruso, D., Grande, S., Bosisio, R., Villa, M., Galli, G., Sirtori, C., and Galli, C. (2005) Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. *Eur. J. Nutr.* **44,** 121–127
45. Coppack, S. W. (2001) Pro-inflammatory cytokines and adipose tissue. *Proc. Nutr. Soc.* **60,** 349–356
46. Seoh, M. L., Ng, C. H., Yong, J., Lim, L., and Leung, T. (2003) ArhGAP15, a novel human RacGAP protein with GTPase binding property. *FEBS Lett.* **539,** 131–137
47. Ghittoni, R., Patrussi, L., Pirozzi, K., Pellegrini, M., Lazzerini, P. E., Capecchi, P. L., Pasini, F. L., and Baldari, C. T. (2005) Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. *FASEB J.* **19,** 605–607
48. Laufs, U., Kilter, H., Konkol, C., Wassmann, S., Bohm, M., and Nickenig, G. (2002) Impact of HMG CoA reductase inhibition on small GTPases in the heart. *Cardiovasc. Res.* **53,** 911–920
49. Mobini, R., Andersson, B. A., Erjefalt, J., Hahn-Zoric, M., Langston, M. A., Perkins, A. D., Cardell, L. O., and Benson, M. (2009) A module-based analytical strategy to identify novel disease-associated genes shows an inhibitory role for interleukin 7 receptor in allergic inflammation. *BMC Syst. Biol.* **3,** 19
50. Gregory, S. G., Schmidt, S., Seth, P., Oksenberg, J. R., Hart, J., Prokop, A., Caillier, S. J., Ban, M., Goris, A., Barcellos, L. F., Lincoln, R., McCauley, J. L., Sawcer, S. J., Compston, D. A., Dubois, B., Hauser, S. L., Garcia-Blanco, M. A., Pericak-Vance, M. A., and Haines, J. L. (2007) Interleukin 7 receptor α chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat. Genet.* **39,** 1083–1091
51. Damas, J. K., Waehre, T., Yndestad, A., Otterdal, K., Hognestad, A., Solum, N. O., Gullestad, L., Froland, S. S., and Aukrust, P. (2003) Interleukin-7-mediated inflammation in unstable angina: possible role of chemokines and platelets. *Circulation* **107,** 2670–2676
52. Imadome, K., Iwakawa, M., Nojiri, K., Tamaki, T., Sakai, M., Nakawatari, M., Moritake, T., Yanagisawa, M., Nakamura, E., Tsujii, H., and Imai, T. (2008) Upregulation of stress-response genes with cell cycle arrest induced by carbon ion irradiation in multiple murine tumors models. *Cancer Biol. Ther.* **7,** 208–217
53. Kunkel, T. A., Pavlov, Y. I., and Bebenek, K. (2003) Functions of human DNA polymerases η, κ and ι suggested by their properties, including fidelity with undamaged DNA templates. *DNA Repair (Amst.)* **2,** 135–149
54. Cooke, M. S., Evans, M. D., Dizdaroglou, M., and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* **17,** 1195–1214
55. Machowetz, A., Poulsen, H. E., Gruendel, S., Weimann, A., Fito, M., Marrugat, J., de la Torre, R., Salonen, J. T., Nyyssonen, K., Mursu, J., Nascetti, S., Gaddi, A., Kiesewetter, H., Baumler, H., Selmi, H., Kaikkonen, J., Zunft, H. J., Covas, M. I., and Koebnick, C. (2007) Effect of olive oils on biomarkers of oxidative DNA stress in northern and southern Europeans. *FASEB J.* **21,** 45–52
56. Rough, J., Engdahl, R., Opperman, K., Yerrum, S., Monroy, M. A., and Daly, J. M. (2009) β_2 Adrenoreceptor blockade attenuates the hyperinflammatory response induced by traumatic injury. *Surgery* **145,** 235–242
57. Philipson, L. H. (2002) β-Agonists and metabolism. *J. Allergy Clin. Immunol.* **110,** S313–S317
58. Witztum, J. L. (1994) The oxidation hypothesis of atherosclerosis. *Lancet* **344,** 793–795
59. Takahata, Y., Takarada, T., Iemata, M., Yamamoto, T., Nakamura, Y., Kodama, A., and Yoneda, Y. (2008) Functional expression of β_2 adrenergic receptors responsible for protection against oxidative stress through promotion of glutathione synthesis after Nrf2 upregulation in undifferentiated mesenchymal C3H10T1/2 stem cells. *J. Cell. Physiol.* **218,** 268–275
60. Woolf, S. H., Battista, R. N., Anderson, G. M., Logan, A. G., and Wang, E. (1990) Assessing the clinical effectiveness of preventive maneuvers: analytic principles and systematic methods in reviewing evidence and developing clinical practice recommendations. A report by the Canadian Task Force on the Periodic Health Examination. *J. Clin. Epidemiol.* **43,** 891–890
61. Acín, S., Navarro, M. A., Arbonés-Manar, J. M., Guillén, N., Sarría, A. J., Carnicer, R., Surra, J. C., Oman, I., Segovia, J. C., de la Torre, R., Covas, M. I., Fernández-Bolaños, J. F., Ruiz-Gutierrez, V., and Osada, J. (2006) Hydroxytyrosol administration enhances atherosclerotic lesion development in ApoE deficient mice. *J. Biochem.* **140,** 383–391

Received for publication October 28, 2009.
Accepted for publication January 21, 2010.