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Apolipoprotein E determines the hepatic transcriptional profile of dietary maslinic acid in mice

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Abstract

The hypothesis that the maslinic acid (MA) of olive oil (OO) dramatically influences hepatic gene expression was tested in mice. Two OOs only differing in the presence of MA were prepared. Using DNA microarrays, we analyzed hepatic gene expression in apolipoprotein E (apoE)-deficient mice with a C57BL/6J genetic background that were fed with isocaloric, isonitrogenous diets containing either 10% (w/w) OO or 10% MA-enriched OO. As an initial screening of potential candidate genes involved in a differential response, this study further considered only genes with remarkably modified expression (signal $\log_2$ ratio higher than1.5 or lower than −1.5). The nine genes fulfilling these prerequisites were confirmed by quantitative reverse transcriptase polymerase chain reaction and analyzed in C57BL/6J wild-type mice. Only *Cyp2b9*, *Cyp2b13* and *Dbp* expressions appeared significantly increased, and *Marco* was significantly decreased in apoE-deficient mice receiving the MA-enriched diet. *Dbp* was up-regulated to an extent depending on the genetic background of the mice and negatively associated with the expression of *Marco*, a gene strongly up-regulated by the absence of apoE. These expression changes could be used as markers of the intake of the MA-enriched OO and are influenced by genetic background generated by the absence or the presence of apoE. Overall, these results (a) indicate that MA in virgin OO is highly active in controlling hepatic gene expression and (b) highlight the important interaction between the response to MA and the presence of apoE. They also confirm that virgin OO cannot be simplistically classified as monounsaturated fatty-enriched oil without paying attention to its active minor components.

Keywords: Apolipoprotein E-deficient mice; Olive oil; Maslinic acid

1. Introduction

The "Seven Countries" study showed that the so-called Mediterranean diet is associated with a reduced risk for cardiovascular mortality despite its associated high intake of fat, mainly derived from olive oil (OO) [1]. OO, as a fruit juice, is a complex mixture where triglycerides (TGs) are combined with other biologically active substances, such as tocopherols, polyphenols and phytosterols, some of which have antioxidant and anti-inflammatory activities [2–4]. A whole panoply of experimental work had proven

Abbreviations: ALAT, alanine aminotransferase; ANOVA, analysis of variance; apoE, apolipoprotein E; ASAT, aspartate aminotransferase; BSA, bovine serum albumin; EVOO, extra virgin olive oil; IL-6, interleukin 6; MA, maslinic acid; MA-OO, maslinic acid-enriched olive oil; OO, olive oil; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ROS, reactive oxygen species; TG, triglyceride

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that these components might be responsible for the benefits of virgin OO [5–7]. Similar findings have also been described in animal studies dealing with the effects of OO on the vascular wall [8,9]. Thus, the unsaponifiable fraction of virgin OO improved the balance between the vasoprotective and prothrombotic factors released by endothelial cells [10], and two triterpenoids found in pomace OO, oleanolic acid and erythrodiol, showed vasorelaxant effects in rat aorta [11]. Profound changes in hepatic gene expression were observed in apolipoprotein E (apoE)-deficient mice consuming an unsaponifiable fraction-enriched OO. The slight hepatic steatosis of apoE-deficient mice was also corrected with OO diets [12]. Thus, the liver may undergo important metabolic changes under the influence of OO, and the steatotic-prone liver of apoE-deficient mice offers an excellent model in which to verify this. In OO of high unsaponifiable content, maslinic acid (MA) and waxes, phytosterols as well as vitamin E were the differential dietary compounds implicated in the observed changes.

MA is a pentacyclic triterpene present in high concentrations in pomace OO. This compound has previously demonstrated antioxidant properties against lipid peroxidation in vitro [13,14] and vasorelaxation in isolated aorta from spontaneously hypertensive rats [11]. In peritoneal murine macrophages, it significantly inhibited the enhanced production of nitric oxide induced by lypopolysaccharide, preventing oxidative stress [15]. This effect was a consequence of an action at the level of the inducible nitric oxide synthase gene expression. In this model, the secretion of the inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α from lypopolysaccharide-stimulated murine macrophages was also significantly reduced [15,16]. MA also reduced blood glucose, at least in part, through inhibiting hepatic glycogen degradation [17] and showed a potent inhibitory activity against HIV-1 protease [18]. MA has also been found to exert a significant antiproliferation effect in HT29 and Caco-2 colon cancer cell lines by inducing an apoptotic process characterized by caspase-3 activation by a p53-independent mechanism, which occurs via mitochondrial disturbances and cytochrome *c* release [19]. MA also induced cell differentiation in colon adenocarcinoma cells and was cytotoxic for other cell lines [19,20]. Likewise, in 1321N1 astrocytoma cells, MA induced apoptosis by activation of caspase 3 and increase of intracellular oxidative stress [21].

To test the hypotheses that the maslinic content of OO significantly influences hepatic gene expression and that the presence of apoE might condition the response, we fed apoE-deficient mice with diets supplemented with either 10% (w/w) OO or 10% MA-enriched OO; the dietary intervention was also carried out in wild-type mice. Gene expression was determined by microarray analysis and then confirmed by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

2. Material and methods

2.1. Animals

The experimental animals were twelve 2-month-old homozygous, apoE-deficient female mice with a C57BL/6J genetic background and 10 wild-type female mice also with a C57BL/6J genetic background. The founders of both colonies in C57BL/6J genetic background were obtained from Charles River Laboratories (Barcelona, Spain) and later bred at the Unidad Mixta de Investigación of the University of Zaragoza. To establish groups of each type of animals with similar initial plasma cholesterol and TG concentrations, we took blood samples (after overnight fasting) from the retro-orbital plexus after isofluorane anesthetization. All mice were housed in sterile filter-top cages in rooms maintained under a 12-h light/12-h dark cycle. They had ad libitum access to food and water. The study protocol was approved by the University of Zaragoza Ethics Committee for Animal Research.

2.2. Diets

Two study groups were established: the first group received a chow diet (Teklad Mouse/Rat Diet No. 2014, Harlan Teklad, Harlan Ibérica, Barcelona, Spain) supplemented with 10% (w/w) refined OO (OO diet; n=6 and n=5 for the homozygous apoE-deficient and wild-type mice, respectively); the other group received the same chow diet supplemented with 10% (w/w) refined OO enriched in MA (MA-OO diet; n=6 and n=5 for the homozygous apoE-deficient and wild-type mice, respectively). To avoid the potential confounding effects of variation among batches of chow, we reserved and used 25 kg from a single batch to prepare diets throughout the experiment. OO had been refined using nitrogen at low temperature, active carbon and low concentrations of phosphoric acid (to remove phospholipids). Those processes removed undesirable flavors, odors and colors, improved transparency and eliminated the influence of soluble phenol compounds as described by Alba-Mendoza et al. [22]. Chemically pure MA obtained as previously described [23] was added to the OO to reach the concentration found in pomace OO [12]. The OOs were analyzed in duplicate following the standard regulations of the European Union [24]. The distinctive feature between the oils was MA content (Table 1). All diets were prepared weekly and stored in an N_2 atmosphere at −20°C. Fresh food was provided daily. The animals were fed with the experimental diets for 4 weeks; both diets were well tolerated.

2.3. Plasma analyses

At the end of the experimental period and after an overnight fast, the animals were sacrificed by suffocation with CO_2 and blood was drawn from their hearts. Plasma total TG and cholesterol concentrations were determined by microtiter assay (Roche Diagnostics, Barcelona, Spain, and Infinity Cholesterol Reagent, Sigma Chemical, Madrid,

Table 1
Composition of the used OOs

Component	OO	MA-OO
Erythrodiol+uvaol	0.0017	0.0017
MA	0	0.011
Squalene	0.26	0.26
Total phytosterols	0.112	0.112
Total tocopherols	0.022	0.022
Waxes	0.012	0.012
Fatty acids		
Myristic (14:0)	0.02	0.02
Palmitic (16:0)	10.98	10.98
Stearic (18:0)	3.53	3.53
Arachidic (20:0)	0.42	0.42
Behenic (22:0)	0.12	0.12
Lignoceric (24:0)	0.05	0.05
Palmitoleic (16:1)	0.82	0.82
Oleic (18:1)	77.8	77.8
Gadoleic (20:1)	0.25	0.25
Linoleic (18:2*n*−6)	4.52	4.52
Linolenic (18:3*n*−3)	0.62	0.62
Saturated	15.14	15.14
Monounsaturated	78.87	78.87

Dietary components are expressed as g% (w/w).

Spain). Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) activities were assayed at 37°C using kits from Alfawassermann (Woerden, Holland) and expressed as IU L^{-1}. IL-6 was determined using a Lincoplex kit from Linco Research (Millipore, Madrid, Spain).

2.4. RNA isolation, Affymetrix oligonucleotide array hybridization and data analysis

At sacrifice, the livers were immediately removed and frozen in liquid nitrogen. RNA from each liver was isolated using Trigent reagent (MRC, Cincinnati, OH, USA). DNA contaminants were removed by TURBO DNase treatment using a DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was greater than 1.75). The integrity of the 28S ribosomal RNA and that of the 18S ribosomal RNA were verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining, and the 28S/18S ratio was greater than 2.

Twenty-microgram aliquots of total liver RNA from each apoE-deficient mouse of each group (n=6 for the OO group and n=6 for the MA-OO group) were pooled and purified using an RNeasy system (Quiagen, Barcelona, Spain). Eight micrograms of total liver RNA was then used for biotin labeling. Hybridization, washing, scanning and analysis with an Affymetrix GeneChip Murine Genome MOE430A array (Affymetrix, Santa Clara, CA) were performed according to the standard Affymetrix protocols at Progenika Biopharma (Derio, Spain). Fluorometric data were generated with the use of Affymetrix software, and the fluorimetric signal was adjusted so that all the probe sets provided intensities within a manageable range. Transcripts with signal intensities that were lower than the limit of detection estimated as background of the matrix ±3 S.D.'s [25] were not taken into account. The data obtained in the microarray hybridizations were processed with Microarray Suite 5.0 (Affymetrix) software. The identification of genes that were up- or down-regulated by the MA in OO was performed by comparing gene expressions in the livers of animals from the two diet groups (significance set at $P<.01$). Of these, we selected only those whose signal $\log_2$ ratio was higher than 1.5 (up-regulated genes) or lower than −1.5 (down-regulated genes). The signal $\log_2$ ratio is now recommended by Affymetrix software and several authors [26] because of the linear response observed in contrast to fold change. The complete data sets were deposited in the GEO database (accession number GSE7091).

2.5. Quantification of mRNA

The differences in mRNA expression observed with the microarrays were confirmed by real-time quantitative RT (qRT)-PCR analysis of individual samples. Equal amounts of DNA-free RNA from each sample of each animal were used in qRT-PCR analyses. First-strand cDNA synthesis and the PCRs were performed using a SuperScript II Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Madrid, Spain) according to the manufacturer's instructions and as previously described [27]. The primers used were designed by Primer Express (Applied Biosystems, Foster City, CA, USA) and checked by BLAST analysis (NCBI) to verify gene specificity. The following sets of primers were used: for *Apoe* — sense, 5′-AGG AAC AGA CCC AGC AAA TAC G-3′, and antisense, 5′-TCT TCC ACT ATT GGC TCG AAC C-3′; for *Cyp2b9* — sense, 5′-ACC AGA TCT ACT TCT TAG CCC GCT-3′, and antisense, 5′-GAG AAC AAC AGT AGA AGG AAG GGT G-3′; for *Cyp2b10* — sense, 5′-ATG TTT AGT GGA GGA ACT GCG G-3′, and antisense, 5′-ATA TTG GCC GTG ATG CAC TG-3′; for *Cyp2b13* — sense, 5′-CTC ATG CTG AGT CAC TTC CCT CTT-3′, and antisense, 5′-ACA GAC CAC AGA GTG TGA AGT TGG-3′; for *Dbp* — sense, 5′-GGA AAC AGC AAG CCC AAA GA-3′, and antisense, 5′-TTG CGC TCC TTT TCC TTC AG-3′; for *Hmox1* — sense, 5′-TCC AGA GAA GGC TTT AAG CTG G-3′, and antisense, 5′-AGG GCC GTG TAG ATA TGG TAC AAG-3′; for *Irg1* — sense, 5′-GGG ACG ATT AAT GCA CTT CTC C-3, and antisense, 5′-GGA GGG TGG AAT CTC TTT GGT A-3; for *Marco* — sense, 5′-AGC ACA GAA GAC AGA GCC GAT T-3, and antisense, 5′-CT TGG GCA CTG GAT CAT TGA-3; for *Orm2* — sense, 5′-TTG GAA GCT CAG AAC CCA GAA-3′, and antisense, 5′-TCG AAG CTC CAT CGT GTC ATT-3′; for *Saa2* — sense, 5′-CTG GCT GGA AAG ATG GAG AC-3′, and antisense, 5′-TGT CCT CGT GTC CTC TGC-3′; for *Xlr4b* — sense, 5′-TAC AGC TCT CAA ACG GAA GTGC-3′, and antisense, 5′-TTT GGT TGC TTG GGA GGT CT-3′; and for *cyclophilin B* — sense, 5′-GGA GAT GGC ACA GGA GGA A-3′, and antisense, 5′-GTA GTG CTT CAG CTT GAA GTT CTC AT-3′. The specificity of the PCR was confirmed by sequencing the products after

their electrophoretic separation in agarose gels. Real-time RT-PCRs were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and normalized to the invariant *cyclophilin B* mRNA expression.

2.6. Liver histology analysis

Aliquots of liver were stored in neutral formaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin–eosin and observed with a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as a percentage of total liver section [28].

2.7. Hepatic homogenate and microsomal preparation

One gram of liver was homogenized in 4 ml of homogenization buffer (10 mM sodium/potassium phosphate/1.14% KCl, pH 7.4) at 0–4°C. An aliquot was saved to estimate protein concentration and radical oxygen species. Microsomes were prepared by ultracentrifugation (105,000×*g* for 70 min) of the postmitochondrial supernatant (10,000×*g* for 10 min). The microsomal pellet was resuspended in homogenization buffer and again centrifuged for 70 min at 105,000×*g*. Washed microsomes were suspended in 1 ml of 50 mM sodium phosphate buffer and stored at −80°C until use. Protein concentration was determined by the BioRad dye binding assay (BioRad, Madrid, Spain).

2.8. Determination of reactive oxygen species

The presence of reactive oxygen species (ROS) was estimated by the 2′,7′-dichlorofluorescein diacetate assay where 20 μl of 40-μg/μl protein homogenates was incubated, at 37°C, with 2 μg of 2′,7′-dichlorofluorescein diacetate in a total volume of 150 μl and in the presence of 25 μl of 0.1% sodium azide and 80 μl of phosphate-buffered saline (PBS) [29]. Fluorescence, at 485-nm excitation and 535-nm emission, was measured after 24 h.

2.9. Western blot analysis

Ten micrograms of microsomal protein was loaded onto 12% SDS–polyacrylamide gel and electrophoresed for 45 min at 200 V in a BioRad Miniprotean cell (Hercules, CA, USA). Proteins were electrophoretically transferred to PVDF membranes (Millipore) using a BioRad Trans-Blot SD (semidry transfer cell) apparatus with 1.5 mA/cm^2 of membrane for 30 min. Membranes were blocked with PBS buffer (136 mM NaCl, 0.2 g/L of KCl, 1.44 g/L of Na_2HPO_4 and 0.24 g/L of KH_2PO_4, pH 7.4) containing 5% bovine serum albumin (BSA) and 2% Tween-20 for 1 h at room temperature and thereafter kept in blocking solution overnight (at 6°C). The primary antibody, diluted in PBS buffer containing 5% BSA and 1% Tween-20, was added, and the membranes were incubated at 30°C for 2 h. Cyp2b9 protein expression was evidenced by using a rabbit polyclonal antibody against human Cyp2B6 (Millipore) known to recognize this mouse protein [30]. Equal loadings were confirmed by using an anti-β-actin (Sigma Chemical). The membranes were washed with PBS containing 0.1% Tween-20 and then incubated with secondary antibody linked to horseradish peroxidase (Sigma Chemical) in PBS buffer containing 5% BSA and 1% Tween-20 (diluted 1/40,000) for 90 min at room temperature. Detection was carried out using an ECL detection kit (GE Healthcare, Madrid, Spain). Membranes were exposed to Hyperfilm-ECL (GE Healthcare) for several periods to achieve signal intensity within the dynamic range of quantitative detection, and films were scanned at a 600-dpi resolution. Intensity of bands for each condition, taken as volume of pixels per millimeter squared, was calculated using Quantity One software version 4.5.0 (BioRad) and normalized to that corresponding to the β-actin signal.

2.10. Statistical analysis

Microarray results were analyzed with the use of the algorithm of Microarray Suite 5.0 (Affymetrix) in Affymetrix GeneChip Operating Software version 1.4. The Mann–Whitney *U* test was used to compare the mean fluorimetric signal intensities of 11–20 perfect matches per probe set hybridized to liver RNA from the OO group with those obtained from the MA-OO group; in both cases, the intensities of a similar number of mismatched probes (background hybridization) were subtracted. Significance was set at $P<.01$. Using Instat 3.02 software for Windows (GraphPad, San Diego, CA, USA), we analyzed nonmicroarray results by the Kolmogorov–Smirnov test to check for normal distribution of the variable and by Bartlett's test to assess the homogeneity of variances. A one-way analysis of variance (ANOVA) was used, and post hoc tests were performed using Tukey–Kramer multiple comparisons. When the variables did not exhibit a Gaussian distribution, groups were compared using the Kruskal–Wallis test, and differences between pairs were tested using a Mann–Whitney *U* test. Unless otherwise stated, results are expressed as mean±S.D. Differences were considered significant when $P<.05$. Correlations between variables were tested by calculating Pearson's or Spearman's correlation coefficient.

3. Results

3.1. Plasma and hepatic parameters

Hepatic ROS were significantly increased in apoE-deficient mice compared with C57BL/6J control mice (Table 2), in agreement with previous reports [31,32]. No variation in hepatic ROS was observed by maslinic administration in apoE-deficient mice. In contrast, C57BL/6J mice receiving the MA-OO diet displayed increased hepatic ROS. The apoE-deficient mice also showed

Table 2
Effects of experimental diets on hepatic and plasma parameters

	C57BL/6J mice		apoE-deficient mice	
	OO (n=5)	MA-OO (n=5)	OO (n=6)	MA-OO (n=6)
Hepatic ROS (AU protein g^{-1})	16±1.0	20±1.4**	22±3.3**	23±2.7**
Plasma ALAT (IU L^{-1})	16±7	10±4*	37±15*,†	39±20*,†
Plasma ASAT (IU L^{-1})	96±28	64±27*	95±44	82±28
Plasma IL-6 (pg ml^{-1})[a]	1.8±0.5	0.4±0.2**	3.7±2†	3.6±2†
Plasma cholesterol (mmol L^{-1})	1.3±0.3	1.3±0.4	6.9±0.8**,†	6.9±0.9**,†
Plasma TGs (mmol L^{-1})	1.3±0.4	1.3±0.3	1.8±0.4*	2.0±0.4*

Results are expressed as mean±S.D. Mice were fed with experimental diets for 4 weeks and fasted overnight before blood collection. Statistical analysis to evaluate dietary response was done using one-way ANOVA and post hoc Tukey–Kramer multiple comparisons. AU, arbitrary units.

[a] Nonparametric Kruskal–Wallis one-way ANOVA test and post hoc Dunn's multiple-comparisons test.

* P=.05 vs. C57BL/6J mice fed with OO.

** P=.01 vs. C57BL/6J mice fed with OO.

† P=.01 vs. C57BL/6J mice fed with MA-OO.

significantly higher values of ALAT and IL-6 compared with the C57BL/6J mice and no variation by the presence of MA in the diet. However, C57BL/6J mice fed with the MA-containing diet had significantly lower values of ALAT, ASAT and IL-6 compared with their counterparts fed with the OO diet. No significant difference in plasma TGs or cholesterol due to the inclusion of MA in the diets in both genetic background animals was observed (Table 2). As expected, apoE-deficient mice showed significantly higher levels of cholesterol compared with C57BL/6J control mice. Similarly, plasma TGs were also significantly elevated in apoE-deficient mice.

3.2. Histological analysis of livers of mice fed with the different diets

Fig. 1 shows representative histological images of livers from animals of the two diet groups in both types of mice. C57BL/6J mice on OO (Fig. 1A) and MA-enriched OO (Fig. 1B) diets showed a slight accumulation of lipids. The apoE-deficient mice receiving both diets showed macrovesicular steatosis mainly in the hepatocytes of liver lobule zone 2 and microvesicular steatosis in hepatocytes in and outside this region (Fig. 1C), in agreement with the described accumulation of lipids in this model [33]. A further increased presence of hepatic lipids was observed in the liver of apoE-deficient mice consuming the MA-enriched OO diet (Fig. 1D). No compacted cell was detected in the liver of any animal, suggesting that the OO and MA-OO diets were well tolerated. Quantitative evaluation of the percentage of hepatic fat areas of all animals is shown in Fig. 1E and corroborates the abovementioned pattern. Therefore, an additive effect of the absence of apoE and maslinic intake is observed.

3.3. Gene expression in livers of Apoe-deficient mice fed with the different diets

To determine the changes in hepatic gene expression induced by the MA present in OO, we quantified the expression of 22,690 transcripts represented on the Affymetrix GeneChip Murine Genome MOE430A array in pooled liver samples of six animals that received the OO diet and another six that received the MA-enriched OO diet. The livers of OO animals expressed 13,634 transcripts, while those of the MA-OO animals expressed 13,786 (identified as "present" by Affymetrix software). Using the Mann–Whitney ranking feature of the Affymetrix software to determine significant differences in gene expression (P<.01) and a compromised limit of detection in variations of expressions lower than 55 (background matrix ±3 S.D.'s), we identified an increased expression of 177 sequences plus the reduced expression of 268 sequences in samples from the animals on the MA-OO diet compared with those on the OO diet when no multiple-test correction was applied. When the latter was taken into consideration by removing expressions with identical gene symbols, accession number and UNI-GENE number, the number of genes with increased expression and that with repressed expression were reduced to 166 and 256, respectively. To select the most relevant, we only took into account differentially regulated genes with a signal $\log_2$ ratio higher than 1.5 (for those genes up-regulated) or lower than −1.5 (for those repressed). Table 3 lists the genes whose mRNAs reflected these expressions. Six genes fulfilled the criterion of showing increased expression as a response to the MA content of OO. Three of these genes coded for electron transport (*Cyp2b9*, *Cyp2b10* and *Cyp2b13*), and three others did for proteins with miscellaneous functions [i.e., one was involved in lipid transport (*Apoe*), one was a member of the cognitive function (*Xlr4b*) and one was a transcription factor (*Dbp*)]. Three genes met the criterion of showing a reduced expression as a response to the presence of the MA content of OO (Table 3). Of these, two were involved in immunity (*Irg1* and *Marco*) and, finally, one was coding for an enzyme involved in heme metabolism (*Hmox1*).

To validate the results obtained with the microarray, we studied the expressions of nine genes (*Apoe*, *Cyp2b9*, *Cyp2b10*, *Cyp2b13*, *Dbp*, *Hmox1*, *Irg1*, *Marco* and *Xlr4b*) that were up- or down-regulated (signal $\log_2$ ratio higher than 1.5 or lower than −1.5) individually by specific qRT-PCR assays. *Cyclophilin B* was used to normalize the results that

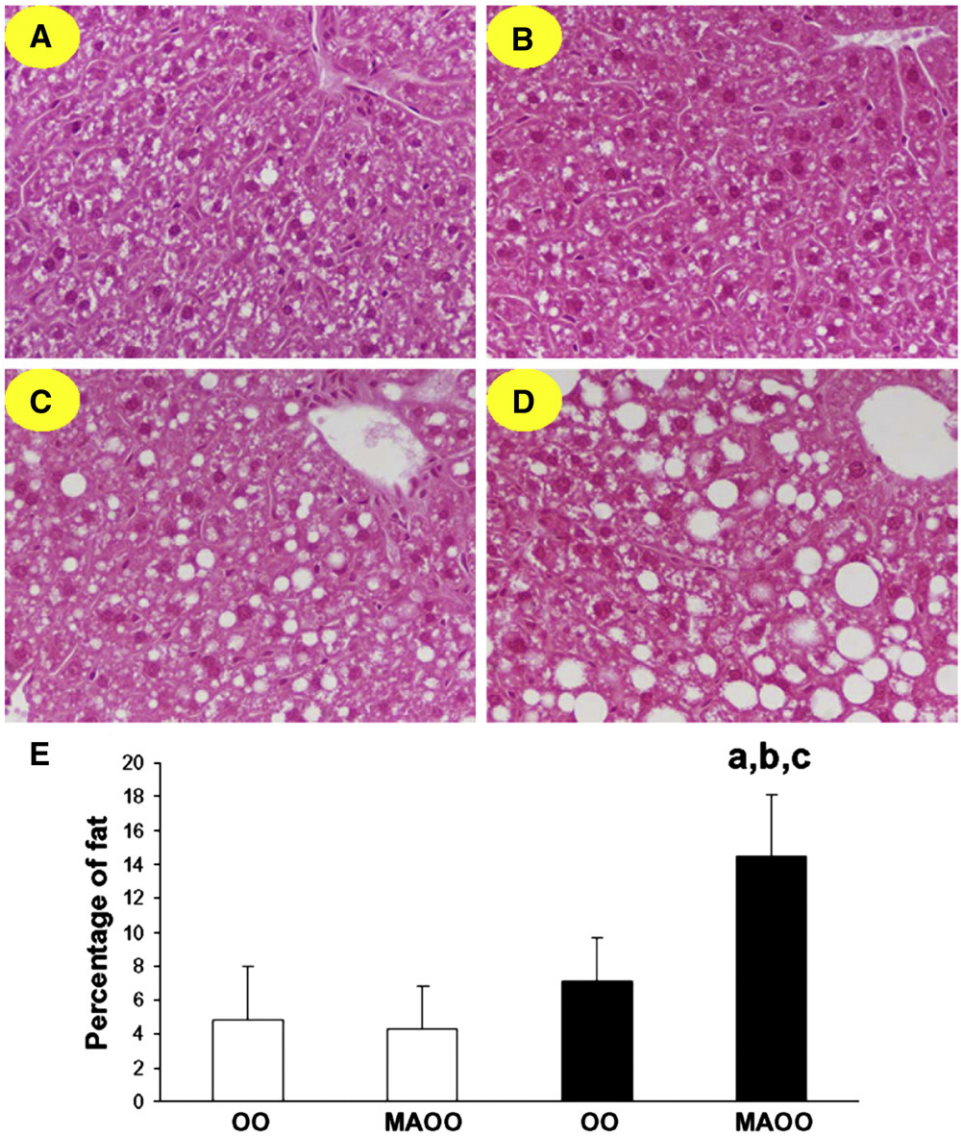

Fig. 1. Liver histology in mice fed with the different diets. (A) and (B) Representative liver micrographs at a magnification of 400× from C57BL/6J mice consuming (A) an OO diet (n=5) and (B) an MA-OO diet (n=5). (C) and (D) Images from apoE-deficient mice fed with (C) an OO diet (n=6) c) and (D) an MA-OO diet (n=6). Liver sections (4 μm) from each mouse were stained with hematoxylin–eosin and evaluated blindly. (E) Morphometric changes of hepatic fat content in mice consuming the different diets, where data are the mean±S.D. for each group. Open and black bars correspond to C57BL/6J and apoE-deficient mice, respectively. Statistical analyses were done according to ANOVA and Tukey–Kramer multiple comparisons as post hoc test. [a]P<.001 vs. C57BL/6J on OO. [b]P<.001 vs. C57BL/6J on MA-OO. [c]P<.01 vs. apoE-deficient on OO.

are shown in Table 4. Three of the six up-regulated genes included in the validation analysis (*Cyp2b9*, *Cyp2b13* and *Dbp*) appeared significantly increased in their expressions by the presence of the MA in OO. One of the three down-regulated genes selected (*Marco*) was significantly decreased in mice receiving the MA-enriched diet.

Fig. 2A shows the correlation between the mean values of signal $\log_2$ ratio for the abovementioned nine genes with the microarray assay performed with pooled samples of apoE-deficient mice (Table 3) and the mean of each group obtained after the analysis of samples from each animal in both experimental groups (Table 4). Two genes showing no expression change, *Orm2* and *Saa2* (data not shown), were also included in the analysis. Good agreement between these procedures was obtained (r=.76, P<.007), and all samples, except one, were correctly classified, although the magnitude of the response differed between the two methods. In an attempt to evaluate the accuracy of setting a cutoff point of a signal $\log_2$ ratio 1.5 in microarray analysis, we plotted the value of signal $\log_2$ ratio obtained in RT-PCR analysis using individual samples against the probability value obtained for comparison of individual expressions for each gene (Fig. 2B). A significant inverse relationship that fitted a logarithmic equation where a signal $\log_2$ ratio of 1.51 was the minimum value required to obtain a P<.05 was observed. These results indicate that pooled samples can be successfully used to

Table 3
Hepatic genes differentially regulated by the administration of MA-enriched OO at the level of signal $\log_2$ ratio higher than 1.5 or lower than −1.5 in apoE-deficient mice

Biological process	GenBank ID	Affymetrix ID	Name	Gene symbol	OO	MA-enriched OO	Signal $\log_2$ ratio
Up-regulated genes							
Electron transport	NM_007813	1449479_at	Cytochrome *P*450, 2b13	*Cyp2b13*	2	252	6.6
Electron transport	NM_010000	1419590_at	Cytochrome *P*450, 2b9	*Cyp2b9*	19	660	5.6
Lipid transport	AK019319	1432466_a_at	apoE	*Apoe*	408	7630	4.4
Electron transport	NM_009998	1422257_s_at	Cytochrome *P*450, 2b10	*Cyp2b10*	17	119	2.5
Transcription factor	BC018323	1418174_at	D site albumin promoter binding protein	*Dbp*	23	100	2.3
Cognitive function	NM_021365	1449347_a_at	X-linked lymphocyte-regulated 4	*Xlr4b*	39	102	1.5
Down-regulated genes							
Immunity	L38281	1427381_at	Immune-responsive gene 1	*Irg1*	43	5	−2.8
Oxidation of heme	NM_010442	1448239_at	Heme oxygenase	*Hmox1*	130	37	−2.3
Phosphate transport	NM_010766	1449498_at	Macrophage receptor	*Marco*	338	85	−1.9

Data represent intensity of signal for each condition with the Affymetrix chip.

provide an initial screening of gene expression, with the attending economic and time savings but with the limitation of no information on biological variability. In addition, the high biological variation of mRNA indicates a certain threshold of change to evaluate significant changes by nutritional components.

3.4. Gene expression in livers of C57BL/6J mice fed with the different diets

To examine whether the selected genes (*Apoe*, *Cyp2b9*, *Cyp2b10*, *Cyp2b13*, *Dbp*, *Hmox1*, *Irg1*, *Marco* and *Xlr4b*) were influenced in their response to dietary MA by the genetic background, we also assayed their expressions in C57BL/6J mice receiving both diets by qRT-PCR normalized to *cyclophilin B* (Table 5). Interestingly, *Apoe*, *Cyp2b9*, *Cyp2b10* and *Cyp2b13* mRNA expressions were significantly lower in mice receiving the MA-enriched diet. For the gene *Xlr4b*, a significant opposite response was seen. The decrease in the expression of *Cyp2b9* mRNA was not translated into a decrease of protein expression, estimated by Western blot analysis (Fig. 3A and B). In contrast, no influence of MA-OO diet was observed on the mRNA of the other four genes (*Dbp*, *Hmox1*, *Irg1* and *Marco*) in these mice (Table 5). These results, taken together with data from the previous section, indicate that response to MA-containing diets is modulated by the genetic background of mice.

Table 4
Effect of MA present in OO on the hepatic gene expression in apoE-deficient mice

	OO (n=6) (mean±S.D.)	MA-enriched OO (n=6) (mean±S.D.)	Fold change	Signal $\log_2$ ratio
Genes up-regulated				
Cyp2b13	0.3±0.3	2.1±1.4*	6	2.6
Cyp2b9	0.9±0.2	2.9±1.3*	3.2	1.7
Apoe	0.9±0.5	1.3±0.2	1.4	0.5
Cyp2b10	1.1±0.6	1.3±0.4	1.2	0.4
Dbp	1.8±1.9	9.9±12*	5.5	2.5
Xlr4b	0.8±0.4	1.0±0.6	1.2	0.3
Genes down-regulated				
Irg1	1.5±1.5	0.8±0.3	0.5	−0.9
Hmox1	1.1±0.4	1.2±0.5	1.1	0.1
Marco	1.3±1.3	0.4±0.3*	0.3	−1.7

Data represent arbitrary units normalized to the *cyclophilin B* expression for each condition with the qRT-PCR. Statistical analysis was carried out with the use of the Mann–Whitney *U* test.

* P=.05 vs. mice fed with OO.

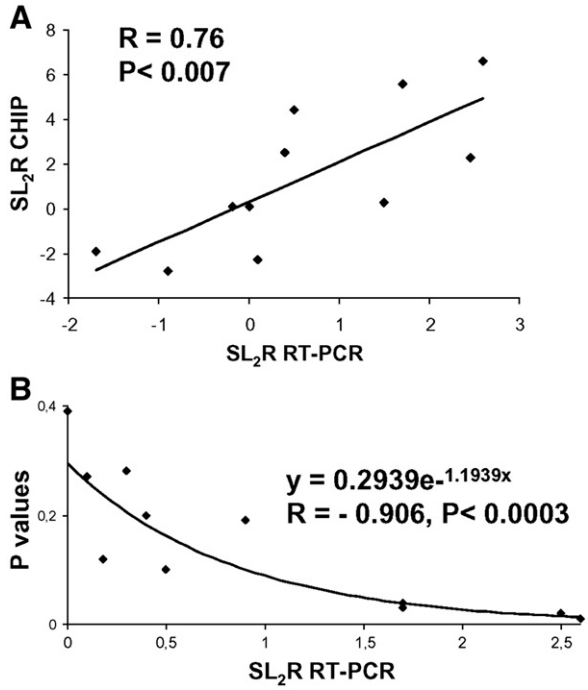

Fig. 2. Quality and biological meaning of microarray data. (A) Correlation analysis between microarray and qRT-PCR data. The expressions of 11 genes (*Apoe*, *Cyp2b9*, *Cyp2b10*, *Cyp2b13*, *Dbp*, *Hmox1*, *Irg1*, *Marco*, *Orm2*, *Saa2* and *Xlr4b*) were individually studied by qRT-PCR and normalized to the invariant *cyclophilin B* gene. The mean values obtained for signal $\log_2$ ratio from individual analyses (Table 4) were plotted against the microarray values that used pooled samples (Table 3). Good agreement between the procedures was seen (r=.76, P<.007). (B) Correlation analysis between signal $\log_2$ ratio of qRT-PCR data and P values obtained in individual comparisons using nonparametric Mann–Whitney *U* test. Signal $\log_2$ ratios are taking in absolute values.

Table 5
Hepatic genes regulated by the MA present in OO in C57BL/6J mice

	OO (n=5) (mean±S.D.)	MA-enriched OO (n=5) (mean±S.D.)	Fold change	Signal $\log_2$ ratio
Genes up-regulated				
Cyp2b13	1.0±0.4	0.6±0.3*	0.6	−0.7
Cyp2b9	1.0±0.2	0.5±0.3*	0.5	−1
Apoe	1.0±0.2	0.7±0.4*	0.7	−0.5
Cyp2b10	1.0±0.2	0.3±0.3*	0.3	−1.7
Dbp	1.3±0.8	1.6±3.0	1.2	0.3
Xlr4b	0.6±0.2	1.5±0.8*	2.5	1.3
Genes down-regulated				
Irg1	1.6±1.3	1.1±1.0	0.7	−0.5
Hmox1	1.0±0.6	1.1±0.6	1.1	0.1
Marco	1.0±0.4	1.0±0.6	1	0

Data represent arbitrary units normalized to the *cyclophilin B* expression for each condition with the qRT-PCR. Statistical analysis was carried out with the use of the Mann–Whitney *U* test.

* P=.05 vs. mice fed with OO.

Table 6
Effects of apoE genotype on gene expression in mice receiving the OO diet

	C57BL/6J mice (n=5)	apoE-deficient mice (n=6)
Cyp2b13	1.0±0.4	0.0±0.0*
Cyp2b9	1.0±0.2	0.0±0.0*
Apoe	1.0±0.1	0.0±0.0*
Cyp2b10	1.0±0.1	0.0±0.0*
Dbp	1.2±0.8	0.8±0.9
Xlr4b	0.6±0.3	0.9±0.4
Irg1	1.6±1.3	19±18*
Hmox1	1.1±0.6	1.4±0.4
Marco	1.5±1.4	4.3±3.8*

Results are arbitrary units and are expressed as mean±S.D. Mice were fed with the OO diet for 4 weeks and fasted overnight before tissue collection. Statistical analysis was carried out with the use of the Mann–Whitney *U* test.

* P=.01 vs. C57BL/6J mice.

3.5. Effects of Apoe deficiency on gene expression in mice fed with the enriched OO diet

To investigate which of the selected genes (*Apoe*, *Cyp2b9*, *Cyp2b10*, *Cyp2b13*, *Dbp*, *Hmox1*, *Irg1*, *Marco* and *Xlr4b*) were influenced by the presence of *Apoe*, we assayed their expressions in apoE-deficient (C57BL/6J genetic background) and C57BL/6J control mice receiving OO-containing diets by qRT-PCR (normalized to the invariant gene *cyclophilin B*). The results are shown in Table 6. As expected, the level of *Apoe* mRNA in apoE-deficient mice was significantly 10,000 times lower than that in control mice. Interestingly, *Cyp2b9*, *Cyp2b10* and *Cyp2b13* mRNA expressions were significantly lower in apoE-deficient mice. The striking decrease in the expression of *Cyp2b9* mRNA by the absence of *Apoe* was also corroborated at the protein level as evidenced by Western blot analysis (Fig. 3A). On the contrary, in the latter mice, *Irg1* and *Marco* mRNA expressions were significantly higher. *Dbp*, *Hmox1* and *Xlr4b* expressions were not influenced by the lack of apoE expression. An extraordinary significant inverse association was observed between *Marco* and *Dbp* expressions in all groups of animals (Fig. 4A). Equally significant negative associations were found between *Cyp2b9* or *Cyp2b10* and *Irg1* expressions in all groups of animals (Fig. 4B and C). These results illustrate that the genetic background generated by the absence of *Apoe* expression influences the expression of other genes and new connections between gene expressions.

4. Discussion

The nutrigenomic approach of this work clearly shows the important effect of MA at the level present in extra virgin OO (EVOO) on hepatic gene expression and the dramatic influence of apoE on those hepatic changes. In addition, it unveils nutritional regulation of genes with poorly known

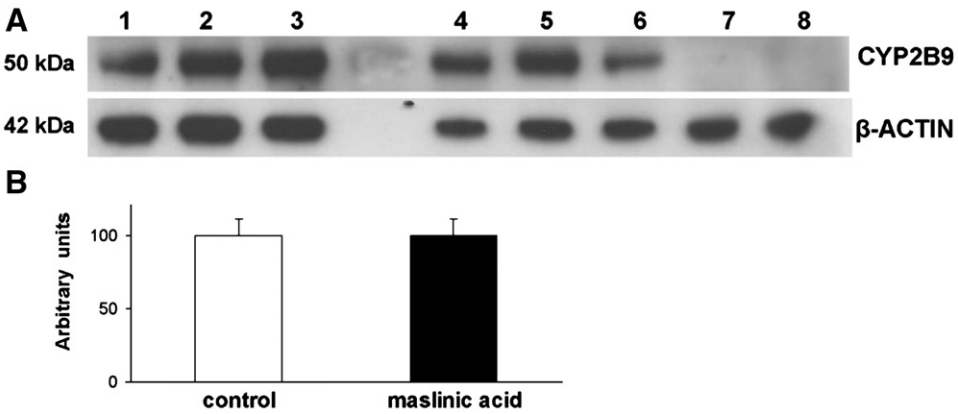

Fig. 3. Cyp2B9 protein expressions in mice fed with the different diets. (A) Representative blots. Lanes 1–3 correspond to C57BL/6J mice receiving the OO diet; lanes 4–6, to C57BL/6J mice receiving the MA-OO diet; and lanes 7 and 8, to apoE-deficient mice receiving the OO diet. (B) Quantification of Western blot analysis results in C57BL/6J mice. Data (mean±S.D.) expressed as arbitrary absorbance units normalized to the β-actin expression are given. Western blot analysis was carried out as described in Section 2. Statistical analysis was done according to the Mann–Whitney *U* test.

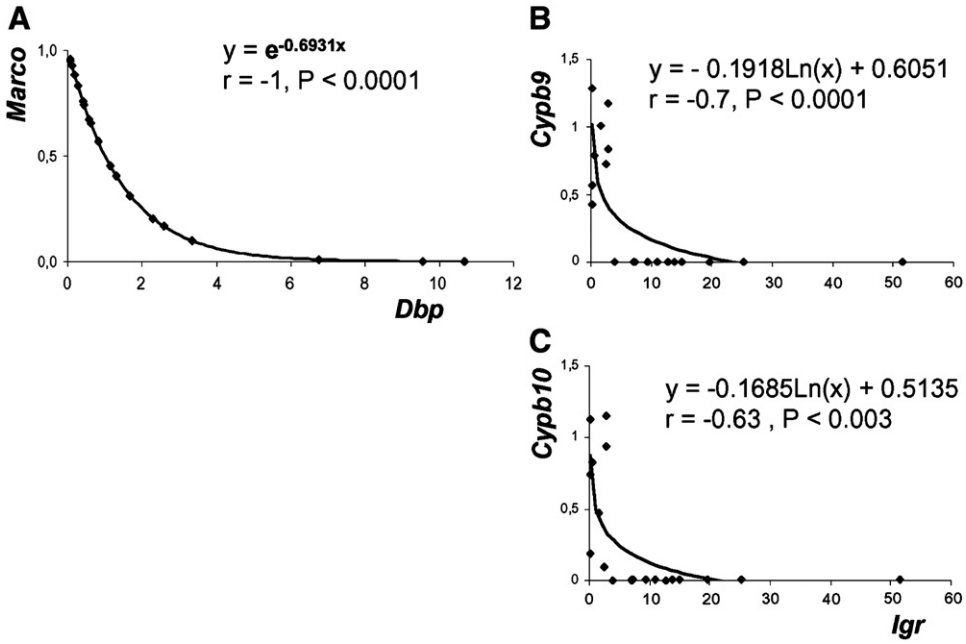

Fig. 4. Relationship among hepatic gene expressions in all groups of animals. A, *Marco* and *Dbp*; B, *Cypb9* and *Igr*, and C, *Cypb9* and *Igr* mRNA. Correlations were calculated according to Spearman's test.

function as well as the effectiveness of the algorithm adopted to obtain meaningful information.

In previous work, we have used a 10% (wt/wt) EVOO-enriched diet to prove that this functional food is able to delay atherosclerosis development in apoE-deficient mice, with females being the particular responder [8]. Using this animal model, our laboratory had also shown that diets containing 20% (wt/wt) EVOO are not as efficient as 10% and that the presence of dietary cholesterol suppresses the effect of EVOOs [34]. Based on this experience, we set up a nutrigenomic approach to test the effect of different OO components using 10% OO-containing diets and that of low cholesterol content [12]. With this approach and exploiting the latest advances in analysis and food technology, we proved that, when present in OO-containing diets, phytosterols, waxes, vitamin E and MA were responsible for modifying hepatic expression of an important number of genes and that this effect was dependent on the genetic background of mice [12]. The present report deals with the influence of MA at the level present in OO (110 mg/kg). With our experimental design, animals consuming the MA-enriched OO diet received an approximate dose of 1.5 mg/kg as functional food. This dose is much lower than the 25-mg/kg dose reported to induce DNA and hepatic hyperplasia in trout [35,36]. Due to the dose or the animal model, we have not observed any of the changes reported by these authors, such as cell packaging, decrease in sinusoidal spaces and cell size changes. Despite this fact, our results clearly show that the presence of MA in OO is quite active in determining gene expression changes in vivo, adding new evidence to previous findings of in vitro activity of this compound [13–15,20,21,37,38]. Independent of the mechanism involved, the present results reveal the additional effects of a minor component of EVOO with respect to the influence of OO and further support the idea that not all MUFA-containing oils behave in the same way [5–12]. Two main categories of MUFA-containing oils can be found, those that are fruit juices (OO and avocado) and those coming from seed extraction (oleic acid-enriched safflower, oleic acid-enriched sunflower and peanut oils). The first class, by its simpler obtaining procedure that simply requires physical pressure, is generally enriched in other compounds, such as squalene, polyphenols, phytosterols, waxes and terpenoids [6], that are not neutral.

In the present work, the huge amount of information provided by microarrays has been handled by selecting only the genes with the highest expression changes in agreement with our experience [12]. Considering that this approach provides meaningful and manageable data and is appropriate to find new candidate genes, we performed analysis by subjecting the microarray data to a restrictive step of selecting only those genes with a signal $\log_2$ ratio of ±1.5. With this criterion, only six genes were found to be remarkably up-regulated and three to be notably down-regulated. The modification of the expression of these genes was individually confirmed by qRT-PCR. Good agreement was observed between the Affymetrix chip and qRT-PCR data (Fig. 2A). In high-density microarrays, such as the Affymetrix MOE430A gene chip, several probes are used for a gene; therefore, the main concern regarding repeatability is the individual variability of mRNA expression. MIAME standards recommend using several chips for this reason (http://www.mged.org/index.html), but this option may be too stringent in terms of sample demands, time, cost and

manageability of information. The present data clearly show that pooling RNA from different animals and using this in microarray analysis are a reliable screening method for the search of biological effects in terms of saving samples as well as time and economic resources, as other authors have reported [39–41]. However, the main drawback of this approach is the lack of information on biological variability of individual samples [42], and this should be specially considered by the experimenter especially interested in finding responders and nonresponders. On the other hand, there is also an overestimate regarding the number of genes involved in a dietary response to a component such as that reported here. In fact, as our results in Fig. 2B indicate, when individually studied, the biological variation of mRNA is high enough that only genes with a certain threshold of change (signal $\log_2$ ratio ±1.5) would be candidates to experience significant biological response to an intervention. In this way, a significant reduction in the number of genes with biological variation is obtained. Accordingly, among the nine genes whose expression was strongly modified, only three of the six up-regulated genes included in the validation analysis (*Cyp2b9*, *Cyp2b13* and *Dbp*) appeared significantly increased and one of the three down-regulated genes selected (*Marco*) was significantly decreased in apoE-deficient mice receiving the MA-enriched diet. The dependency of the dietary response on the presence of apoE was evidenced using C57BL/6J mice consuming both diets. Interestingly, *Apoe*, *Cyp2b9*, *Cyp2b10* and *Cyp2b13* mRNA expressions were significantly decreased and *Xlr4b* significantly increased in C57BL/6J mice receiving the MA-enriched diet. These results clearly indicate that responses to MA-containing diets are different between different backgrounds of mice. In fact, the absence of apoE protein generated reduced expressions of *Cyp2b9* [at the mRNA (Table 6) and protein (Fig. 3A) levels] and of *Cyp2b13* at the mRNA level partly recovered in apoE-deficient mice consuming MA-containing diets (Table 4). Likewise, the induced *Marco* expression in apoE-deficient mice was significantly reduced by MA-containing diets (Table 4) and may be mediated by *Dbp* expression as the strong significant association between both parameters (Fig. 4A). This fact points out to an anti-inflammatory effect of MA, in agreement with the reduction of pro-inflammatory cytokine generation in macrophages [15] and liver of mice treated with some preparations of *Boswellia serrata*, an extract rich in particular derivatives of boswellic acid, triterpene-based compounds [43]. This selective action of triterpenes, together with the absence of change of another inflammatory gene, *Irg1*, and plasma ALAT and IL-6 levels in apoE-deficient mice, further reinforces the notion that the chemical structure of these compounds may be important for the anti-inflammatory action in liver, in line with the data obtained in other models [37]. The complexity of response of triterpenoids also arises when considering that MA induced transcription of phase 1 response (*Cyp2b9*, *Cyp2b13*) in apoE-deficient mice and no change of phase 2 response (*Hmox1*), in contrast with oleanolic acid and its triterpenoid analogs, which are phase 2 inducers [44]. The differential response in the presence or absence of apoE might be due to high oxidative stress present in apoE-deficient mice [31,32] and the fact that MA fails to counteract the ROS present in the apoE-deficient mice and promotes oxidative stress in C57BL/6J. The different mice strains are endowed with specific CYP450 isoforms [30] and therefore different possibilities to metabolize this compound, which could explain the discrepancy with the described antioxidant properties of MA in preventing lipid peroxidation [13]. Taken as a whole, these results suggest that the MA of OO plays an important role in controlling the expression of genes with roles in liver inflammation and that the effect is also modulated by genetic background.

An interesting observation of this work is the susceptibility of the apoE gene to the presence of MA in diet. In apoE-deficient mice, we detected a very low level of this mRNA as observed either by the microarray or by the RT-PCR (1024 times lower than in control mice) and confirmed by DNA sequencing (data not shown). This low abundant transcript (Table 6) could be a consequence of an alternative splicing as recently mentioned, GEO deposit GDS829, although it was not translated into an apoE protein as proved by Piedrahita et al. [45] and as the phenotype regarding plasma cholesterol levels (Table 1) and hepatic fat accumulation (Fig. 1) clearly reflect. An alternative splicing could explain the existence of this low abundant transcript since the sequence that represents the Affymetrix oligo used as a probe (1432466_a_at) corresponds to the 3′-end of the gene belonging to the fourth exon, where we designed the RT-PCR primers. An increase of this transcript has been observed in apoE-deficient mice transgenic for c-Jun GEO deposit GDS2776 (http://www.ncbi.nlm.nih.gov/geo/) with the use of the Affymetrix platform. Later on, when we confirmed it by RT-PCR, the increase did not reach statistical significance and was less pronounced. The discrepancy between these two procedures might be explained by the different experimental approaches: in the case of the chip analysis, a pool was used with an equal proportion of all animals; in RT-PCR, an individual analysis was carried out. In the former procedure, a high-expressing animal may skew the global result, while in the second procedure, the presence of this animal would contribute in lesser proportion to the mean value but would compromise the statistical significance by adding biological variation, as observed. This gene is a target for maslinic diet in control mice in which the expression was significantly decreased; a similar observation has been described in mice consuming a supplement of *B. serrata*, an extract rich in boswellic acid, a triterpene derivative compound [43].

In conclusion, this nutrigenomic approach clearly illustrates the important effects of MA at the level present in EVOO. Our approach using murine models also provides a simple strategy to advance in the field of finding target genes, nutritional components, pathology and their

interactions and consequently shows a new and strong relationship between nutrition and gene expression. The gene product *Dbp*, belonging to the basic region/leucine zipper protein family [46], was up-regulated to an extent depending on the genetic background of the mice and negatively associated with the expression of *Marco*, a gene strongly up-regulated by the absence of apoE. These modifications in expression could be used as markers of the intake of the MA-enriched OO and are influenced by genetic background generated by the absence or presence of apoE. The results further confirm that EVOO cannot simplistically be classified as a monounsaturated fatty-enriched oil without paying attention to its active minor components, which confuse the scientific community and consumers. The present results show the usefulness of Affymetrix chip technology for characterizing gene expression levels in response to nutritional components in intact animal systems.

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