

## Dietary cholesterol suppresses the ability of olive oil to delay the development of atherosclerotic lesions in apolipoprotein E knockout mice

Sergio Acín<sup>a</sup>, María A. Navarro<sup>a</sup>, Ricardo Carnicer<sup>a</sup>, José M. Arbonés-Mainar<sup>a</sup>,  
Mario A. Guzmán<sup>a</sup>, Carmen Arnal<sup>b</sup>, Gabriel Beltrán<sup>c</sup>, Marino Uceda<sup>c</sup>,  
Nobuyo Maeda<sup>d</sup>, Jesús Osada<sup>a,\*</sup>

<sup>a</sup> Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain

<sup>b</sup> Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain

<sup>c</sup> Centro de Investigación y Formación Agraria "Venta del Llano", Mengibar, Spain

<sup>d</sup> Department of Pathology, University of North Carolina, Chapel Hill, USA

Received 29 September 2004; received in revised form 21 January 2005; accepted 27 January 2005

Available online 7 April 2005

### Abstract

To test the hypothesis that cholesterol might suppress the beneficial effect of olive oil in atherosclerosis, we fed apoE KO mice diets containing extra virgin olive oil, either with or without cholesterol, for 10 weeks and assessed the development of atherosclerosis. Within each sex, mice were assigned randomly to one of the following four experimental groups: (1) a standard chow diet, (2) a chow diet supplemented with 0.1% cholesterol (w/w) cholesterol, (3) a chow diet enriched with 20% (w/w) extra virgin olive oil and (4) a chow diet containing 0.1% cholesterol and 20% extra virgin olive oil. On the standard chow diet, average plasma cholesterol levels were higher in males than in females. Olive oil- and cholesterol-enriched diets, separately or in combination, induced hypercholesterolemia in both sexes, and abolished the difference between the sexes in plasma cholesterol levels. The addition of cholesterol to chow or olive oil diets decreased apolipoprotein A–I significantly in females and serum paraoxonase activities in males. The latter activity was higher in females than in males. In both sexes, the size of aortic atherosclerotic lesions was similar in olive oil- and chow-fed animals and smaller than in cholesterol-supplemented groups. Size of aortic lesions were positively correlated with circulating paraoxonase activity, particularly in males, and the relationship remained after adjusting for apolipoprotein A–I and HDL cholesterol levels. Our results demonstrate that the nutritional regulation of paraoxonase is an important determinant of atherosclerotic lesions dependent on sex. They also suggest that the mere inclusion of olive oil in Western diets is insufficient and the adoption of Mediterranean diet would be more effective in retarding the development of atherosclerotic lesions.

© 2005 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Olive oil; Cholesterol; Apolipoprotein; Atherosclerosis; Paraoxonase

**Abbreviations:** Apo, apolipoprotein; ApoE KO, apolipoprotein E-deficient mice; FPLC, fast performance liquid chromatography; HDL, high density lipoproteins; LDL, low density lipoproteins; MCP-1, monocyte chemoattractant protein-1; PON-1, paraoxonase 1; TG, triglycerides; VLDL, very low density lipoproteins

\* Corresponding author. Present address: Department of Biochemistry and Molecular Biology, Veterinary School, University of Zaragoza, Miguel Servet, 177, E-50013 Zaragoza, Spain. Tel.: +34 976 761644; fax: +34 976 761612.

E-mail address: Josada@unizar.es (J. Osada).

### 1. Introduction

ApoE KO mice develop spontaneous atherosclerosis that mimics most of the features that characterize the human pathology of atherosclerosis, such as fatty streaks, necrotic cores, fibrous caps [1,2] and plaque rupture [3,4]. Due to the rapid onset and accelerated development of atherosclerosis, apoE KO mice provide a unique animal model for studying the impact of environmental factors on the development of arteriosclerosis [5]. Among environmental factors, diet is essential because eating is essential for living. That

said—the definition of an appropriate diet for humans is far from clearly understood [6]. Moreover, recent findings of dietary responses as a function of genetic make-up make it impossible to make broad recommendations for all subjects [7].

There is considerable interest in understanding the reasons for the relatively low incidence of coronary heart diseases in Mediterranean populations, and the Mediterranean diet might be one of the factors involved in the phenomenon [8,9]. Indeed, interventions using a Mediterranean-like diet reduce cardiovascular-related mortality and exert a protective effect in patients, even after the intervention is concluded [10]. The Mediterranean diet is characterized by high fat, mainly from olive oil, complex carbohydrates in the form of cereals and legumes, high fiber in the form of fruit and vegetables and the limited consumption of animal proteins [11], representing a design tested by populations in the Mediterranean Basin for more than 2000 years.

Dietary fat is one of the most important environmental factors associated with the incidence of cardiovascular disease and, in this regard, considerable attention is being paid to the potential benefits of olive oil. Studies report that human consumption of olive oil decreases low density lipoproteins (LDL) cholesterol with maintenance of high density lipoproteins (HDL) that could generate a favorable lipid profile [12]. Improved endothelial function in humans consuming olive oil also has also been reported [13]. To improve our understanding of the beneficial properties and mechanisms of olive oil, analyses of specific components and interactions through examination of experimental models are required. Thus, the olive oil phenol, hydroxytyrosol, prevents smoking-generated oxidative stress in rats [14]. A virgin olive oil-enriched diet with low cholesterol content stops the progression of induced atherosclerosis in rabbits [15]. In a previous study, we showed that virgin olive oil in a low cholesterol diet decreased atherosclerosis in apoE-deficient female mice [16]; however, when apoE KO mice were fed a diet containing olive oil together with 0.2% cholesterol, atherosclerosis lesion did not change [17]. These findings suggested that cholesterol might suppress the beneficial effect of olive oil in retarding the development of atherosclerosis. To test the hypothesis, we fed apoE KO mice diets containing olive oil, either with or without cholesterol, for 10 weeks and assessed the extent of atherosclerosis. To determine the mechanisms involved, we performed a detailed analysis of lipid, lipoproteins, lesions and serum paraoxonase activity. The latter was included because genetic manipulations of paraoxonase in mice decreased atherosclerosis lesions when PON-1 was over-expressed [18] and increased atherosclerosis when the PON-1 gene was eliminated [19,20].

## 2. Material and methods

### 2.1. Animals

Homozygous apoE KO mice were bred in the Unidad Mixta de Investigación, Zaragoza, Spain. To estimate initial

plasma cholesterol and triglycerides, 43 male and 29 female two-month-old mice were fasted overnight, anesthetized with isoflurane and blood samples were obtained by retro-orbital bleeding. For each sex, four random groups of mice having similar plasma cholesterol and triglyceride levels were housed in sterile filter-top cages in groups of three or four animals per cage. Animals had ad libitum access to food and water. Food consumption, which was determined using metabolic cages (Biosys, Barcelona, Spain), and body weights were recorded weekly throughout the 10-week experiment. The protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

### 2.2. Diets

Within each sex, apoE KO mice were assigned randomly to one of the following four experimental groups: (1) a control group fed a standard chow diet, (2) a cholesterol group fed a diet supplemented with 0.1% (w/w) cholesterol, (3) an olive-oil group fed a diet enriched in (20%, w/w) extra virgin olive oil and (4) a cholesterol + olive oil group fed a diet containing 0.1% cholesterol and 20% extra virgin olive oil. Standard mouse chow diet was supplied by B & K Universal Ltd. (Humburside, UK) and the sources of protein and fat were vegetable grains. To avoid the potential confounding effects of variation between batches of chow, 25 kg from a single batch were reserved and used to prepare diets and feed experimental groups throughout the experiment. Weekly prepared diets were stored in N<sub>2</sub> at –20 °C and their composition analyzed, as previously described [16]. The chemical composition of the diets is shown in Table 1. The nutritional intervention lasted 10 weeks and was well tolerated by the subjects.

### 2.3. Blood determinations

After the 10-week experiment, overnight-fasted animals were sacrificed by suffocation in CO<sub>2</sub> and a sample of blood was drawn from the heart. Total plasma cholesterol and triglyceride concentrations were measured using commercial kits (Sigma Chemical Co., Madrid, Spain). HDL cholesterol concentrations were determined in a similar manner following phosphotungstic acid–MnCl<sub>2</sub> (Roche, Barcelona, Spain) precipitation of apoB-containing particles [21]. Total isoprostane 8-*iso*-PGF<sub>2α</sub> was measured by immunoassay (Cayman Chemical, Ann Arbor, MI). Paraoxonase was assayed as arylesterase activity [22]. Initial rates of phenylacetate hydrolysis at 37 °C were recorded spectrophotometrically at 270 nm. The reaction mixture contained 2 μL of sample in 800 μL of 1.0 mM phenylacetate, 0.9 mM CaCl<sub>2</sub> and 20 mM Tris–HCl, pH 8.0. Nonenzymatic hydrolysis of phenylacetate and variation in absorbance in the absence of substrate were subtracted from the total rate of hydrolysis. The value of the extinction coefficient at 270 nm was 1310 mol (L cm)<sup>–1</sup> and results are expressed as μmol phenylacetate hydrolyzed (min L)<sup>–1</sup> (IU L<sup>–1</sup>). Plasma

Table 1  
Composition of the experimental diets provided to apoE knockout mice

	Chow	Cholesterol	Olive oil	Olive oil + cholesterol
Energetic content (kJ/g)	13	13	18	18
Carbohydrate	57	57	46	46
Protein	16	16	13	13
Fat	3	3	22	22
Vitamin E (IU %)	13	13	12	12
Cholesterol (mg %)	31	125	29	125
Fatty acids				
Lauric (12:0)	0.1	0.1	0.7	0.7
Myristic (14:0)	1.5	1.5	0.5	0.5
Palmitic (16:0)	21.6	21.6	11	11
Margaric (17:0)	0.1	0.1	0.2	0.2
Stearic (18:0)	7.0	7.0	4.5	4.5
Arachidic (20:0)	2.5	2.5	0.2	0.2
Behenic (22:0)	0.9	0.9	0.3	0.3
Lignoceric (24:0)	0.1	0.1	0.1	0.1
Palmitoleic (16:1)	2.2	2.2	0.9	0.9
Margaroleic (17:1)	0.4	0.4	0.3	0.3
Oleic (18:1)	32.2	32.2	69.5	69.5
Gadoleic (20:1)	0.4	0.4	0.2	0.2
Linoleic (18:2 $n-6$ )	30.1	30.1	11.4	11.4
Linolenic (18:3 $n-3$ )	0.3	0.3	0.1	0.1
Saturated	33.8	33.8	17.5	17.5
Monounsaturated	35.2	35.2	70.9	70.9
P/S ratio	0.9	0.9	0.6	0.6

Dietary components are expressed as g % (w/w). Other components of the chow diet are crude fiber 4.5% and minerals 6.8%. A total dry matter of 87.5%. P/S ratio: polyunsaturated/saturated fatty acid ratio.

apoA-I concentrations were evaluated by immunoassay using immunopurified rabbit IgG as the primary antibody [23]. We determined plasma lipoprotein profiles in samples of 100  $\mu$ L from pooled plasma within each dietary group using fast protein liquid chromatography gel filtration (FPLC) in a Superose 6B column (Amersham Pharmacia, Barcelona, Spain), as described [16].

#### 2.4. RNA preparation and analysis

Immediately after animals were sacrificed, livers were removed and quickly frozen in liquid nitrogen. RNA was isolated using Trizol reagent following the manufacturer's protocol (MRC, Cincinnati, OH, USA). Total RNA was subjected to Northern blot analysis as described [24]. The mouse clone for paraoxonase-1 (4158951 IMAGE Clone) was obtained from The MGC Geneservice (Cambridge, UK). The *PON-1* probe was a 1397 bp *EcoRI/XbaI* fragment and the mouse *ApoA1* probe was a 180 bp *EcoRI/NheI* fragment corresponding to the fourth exon of the gene. To normalize the amount of RNA loaded onto the gel, we used a mouse 250 pb *KpnI/XbaI* fragment of  $\beta$ -actin as a probe. Probes were labeled using [ $\alpha$ - $^{32}$ P]-dCTP and Rediprime. Filters were exposed to Biomax film (Kodak, Amersham) and the intensity in film was quantified using a laser LKB 2202 densitometer (Amersham-Pharmacia).

#### 2.5. Evaluation of atherosclerotic lesions

The heart and arterial tree were perfused with phosphate-buffered saline and, later, with phosphate-buffered formalin (4%, pH 7.4, Panreac, Barcelona, Spain) under physiological pressure. Hearts and aortas were removed, cleaned and stored in neutral formaldehyde. For *en face* analyses, aortas were opened longitudinally and stained, as described by Tangirala et al. [25]. For cross-sectional analyses, the base of the heart and the aortic roots were embedded in paraffin. Serial 4  $\mu$ m sections were stained with orcein [26]. The average lesion sizes from four cross-sectional sections were used for morphometric evaluations using the method of Paigen et al. [27]. Images were captured and digitized using a Nikon microscope equipped with a Canon digital camera. Morphometric analyses were performed using NIH Image software.

#### 2.6. Statistical analysis

Mann–Whitney *U*-tests and one-way ANOVAs, followed by post hoc analyses, were used for comparisons. Differences were considered statistically significant when  $P \leq 0.05$ . Correlations between variables were tested using Spearman's rank-order correlation coefficient ( $r_s$ ). Tests were performed using Instat 3.02 for Windows (GraphPad, S. Diego, CA, USA). A two-way ANOVA to verify the variation of paraoxonase with HDL cholesterol and apoA-I as covariates was performed using StatView 5.0 (SAS Institute Inc. Cary, NC, USA).

### 3. Results

#### 3.1. Dietary characteristics

Table 1 summarizes the chemical composition of the diets used in this experiment. Vitamin E content was similar among diets. Chow and cholesterol-enriched diets were isocaloric, as were olive oil and olive oil + cholesterol diets. Olive oil-enriched diets were lower in carbohydrates, higher in percentage of fat and had slightly lower protein content. Chow and cholesterol-enriched diets had a monounsaturated fatty acid content of 35.2% of total fat and almost equal amounts of polyunsaturated and saturated fatty acids (P/S ratio 0.9). Olive oil-enriched diets were high in monounsaturated fatty acids and the P/S ratio was 0.6, which indicates an important decrease in polyunsaturated fatty acids.

#### 3.2. Changes in somatic and plasma parameters are sex-specific

The effects of the experimental diets on female mice are shown in Table 2. After the 10-week experiment, animals fed a chow diet gained, on average, 5 g of body weight. Although there was no significant variation in food consumption among groups of females ( $2.5 \pm 0.5$  g per day), all of the olive oil-fed

Table 2  
Effects of experimental diets on female apoE knockout mice

	Chow (n = 7)	Cholesterol (n = 7)	Olive oil (n = 7)	Olive oil + cholesterol (n = 8)
Body weight change (g)	+5 ± 1	+3 ± 2	+8 ± 3 <sup>†,§</sup>	+6 ± 2 <sup>#</sup>
Liver weight (g)	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.2 <sup>*,†,§</sup>
Plasma cholesterol (mmol L <sup>-1</sup> )	10 ± 4	32 ± 9 <sup>†</sup>	20 ± 5 <sup>‡,§</sup>	35 ± 4 <sup>*,§</sup>
HDL cholesterol (mmol L <sup>-1</sup> )	1.4 ± 0.4	1.0 ± 0.4	1.9 ± 0.4 <sup>§</sup>	1.3 ± 0.7 <sup>*</sup>
Plasma triglycerides (mmol L <sup>-1</sup> )	1.3 ± 0.3	1.5 ± 0.5	1.9 ± 0.3 <sup>†</sup>	1.6 ± 0.4
Plasma apolipoprotein A-I (mg dL <sup>-1</sup> )	31 ± 9	24 ± 6 <sup>‡</sup>	43 ± 4 <sup>‡,§</sup>	37 ± 12
8-iso-Prostaglandin F <sub>2α</sub> (pg mL <sup>-1</sup> ) <sup>a</sup>	394 ± 100	262 ± 42	383 ± 58	257 ± 30

Results are expressed as mean ± S.D. Mice were fed chow or experimental diets for 10 weeks and fasted overnight before blood was collected. Statistical analyses to evaluate dietary responses were done using nonparametric one-way ANOVA and Dunn Multiple Comparisons test as post hoc.

<sup>a</sup> For the estimation of this parameter, two pools of three to four animals per group were used.

\* *P* = 0.01 vs. olive.

# *P* = 0.05 vs. cholesterol.

† *P* = 0.01 vs. chow diet.

‡ *P* = 0.05 vs. chow diet.

§ *P* = 0.001 vs. chow diet.

§ *P* = 0.01 vs. cholesterol.

groups gained significantly more weight than did the standard chow-fed control group; however, only liver from females fed the olive oil + cholesterol diet were heavier.

In females, the intake of cholesterol significantly increased their plasma levels. Likewise, dietary supplementation with olive oil also increased plasma levels of cholesterol in females (Table 2). In females, the addition of cholesterol + olive oil to the diet induced levels similar increase to that of the cholesterol-only group, but higher than in animals in the olive oil-fed group. HDL cholesterol was lower in mice fed a cholesterol-enriched diet, and significantly increased in females fed an olive oil-enriched diet. In females, this effect was suppressed when cholesterol was added to the olive oil diet. Plasma triglycerides tended to be higher in animals fed olive oil-enriched diets, although there was considerable inter-individual variation and the differences were only statistically significant for the olive oil-fed group.

Diets supplemented with olive oil increased plasma apoA-I concentrations in females (Table 2). The addition of cholesterol to chow and olive oil diets decreased apoA-I concentrations, and this change was particularly significant

for the former group compared to the chow and olive oil-fed groups. In females, plasma levels of 8-iso-prostaglandin F<sub>2α</sub> did not vary significantly among dietary groups (Table 2).

The distribution of cholesterol among the plasma lipoproteins of females analyzed by FPLC is shown in Fig. 1. Dietary cholesterol increased very low density lipoproteins (VLDL) and LDL cholesterol (Fig. 1c), and olive oil mainly increased VLDL cholesterol (Fig. 1e). Females on the olive oil + cholesterol diet experienced an increase in LDL cholesterol (Fig. 1g). In agreement with the results obtained by a precipitation method, HDL cholesterol increased in females fed olive oil (Fig. 1e).

The effects of experimental diets on male mice are shown in Table 3. After the dietary intervention, males fed a chow diet gained on average 4 g of body weight. Despite no significant variation in food consumption among groups (3 ± 0.5 g), all of the olive oil-fed groups gained significantly more weight than did the standard chow-fed control group (Table 3). Accordingly, liver weight was significantly higher in males that were fed one of the high olive oil-enriched diets.

Table 3  
Effects of experimental diets on male apoE knockout mice

	Chow (n = 11)	Cholesterol (n = 10)	Olive oil (n = 11)	Olive oil + cholesterol (n = 11)
Body weight change (g)	+4 ± 1	+3 ± 1	+6 ± 1 <sup>†,§</sup>	+5 ± 2 <sup>#</sup>
Liver weight (g)	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.11 <sup>*,§</sup>	1.3 ± 0.2 <sup>*,§,¶</sup>
Plasma cholesterol (mmol L <sup>-1</sup> )	19 ± 4	34 ± 6 <sup>†</sup>	34 ± 6 <sup>‡</sup>	42 ± 3 <sup>*,§,¶</sup>
HDL cholesterol (mmol L <sup>-1</sup> )	1.8 ± 0.5	0.8 ± 0.7 <sup>†</sup>	1.7 ± 0.7 <sup>#</sup>	1.4 ± 0.7
Plasma triglycerides (mmol L <sup>-1</sup> )	1.7 ± 0.7	1.5 ± 0.3	2.0 ± 0.4	2.3 ± 0.6 <sup>*,†</sup>
Plasma apolipoprotein A-I (mg dL <sup>-1</sup> )	17 ± 2	18 ± 2	25 ± 5 <sup>‡,§</sup>	24 ± 2
8-iso-Prostaglandin F <sub>2α</sub> (pg mL <sup>-1</sup> ) <sup>a</sup>	490 ± 143	287 ± 26	419 ± 20	356 ± 13

Results are expressed as mean ± S.D. Mice were fed chow or experimental diets for 10 weeks and fasted overnight before blood was collected. Statistical analysis to evaluate dietary response was done using nonparametric one-way ANOVA and Dunn Multiple Comparisons test as post hoc.

<sup>a</sup> For the estimation of this parameter, two pools of three to four animals per group were used.

\* *P* = 0.01 vs. olive.

# *P* = 0.01 vs. cholesterol.

† *P* = 0.01 vs. chow diet.

‡ *P* = 0.05 vs. chow diet.

§ *P* = 0.001 vs. chow diet.

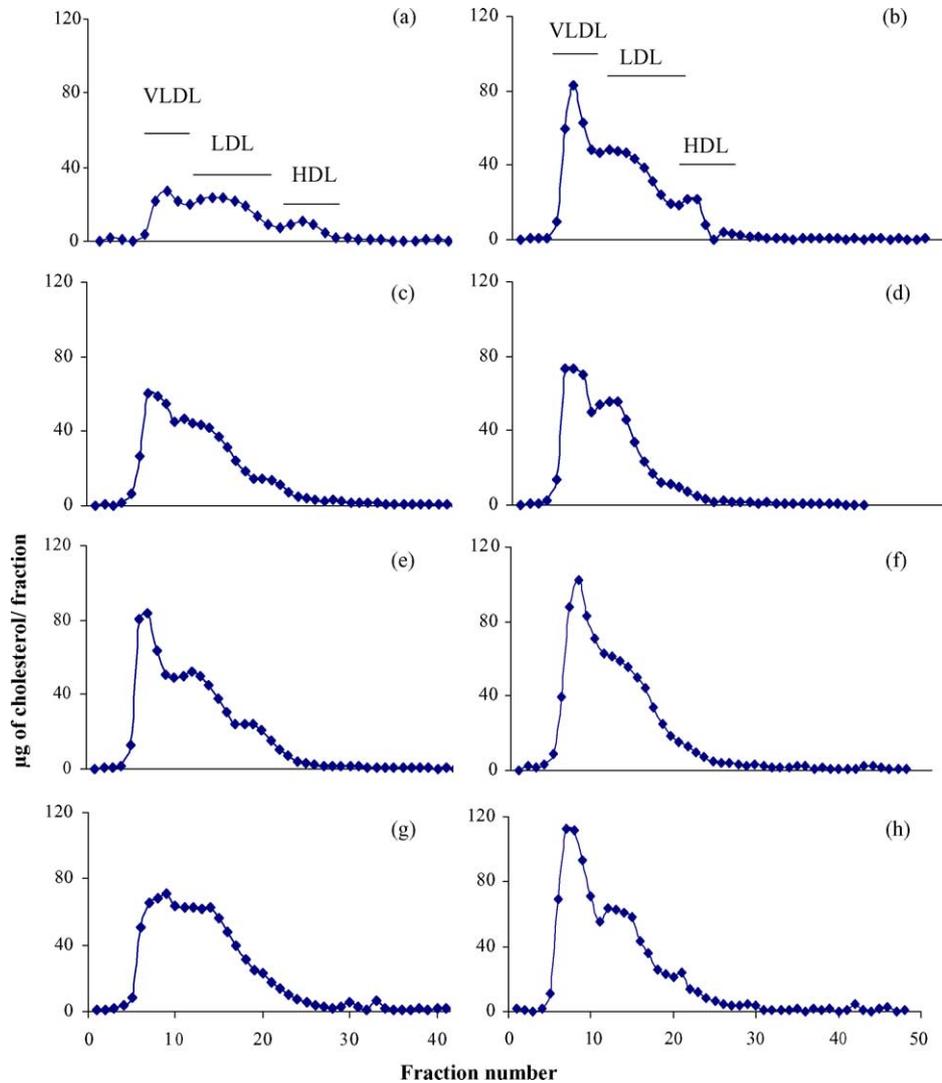


Fig. 1. FPLC plasma lipoprotein profile of apoE knockout mice following 10-week experimental diets. Results are presented as  $\mu\text{g}$  of cholesterol per fraction, (a) and (b) female and male fed a chow diet; (c) and (d) female and male fed a cholesterol-enriched diet; (e) and (f) female and male fed an olive-enriched diet; (g) and (h) female and male fed an olive + cholesterol-enriched diet.

Among mice fed the standard chow diet, plasma cholesterol levels were higher in males (Table 3) than in females (Table 2). The intake of cholesterol significantly increased plasma levels and abolished the difference between the sexes. In males, dietary supplementation with olive oil increased plasma levels of cholesterol and the addition of cholesterol + olive oil to the diet had an additive effect on plasma cholesterol (Table 3). HDL cholesterol was significantly lower in mice fed a cholesterol-enriched diet and tended to be lower when cholesterol was added to the olive oil diet. Plasma triglycerides tended to be higher in animals fed fat-enriched diets, and the differences were statistically significant following the dietary supplementation with cholesterol + olive oil.

Plasma apolipoprotein A-I concentrations were lower in males than in females (Tables 3 and 2). Diets supplemented with olive oil increased plasma apoA-I concentrations also in males, but the addition of cholesterol to chow or olive oil

diets did not result in a change in this parameter. In males, plasma levels of 8-*iso*-prostaglandin  $F_{2\alpha}$  tended to be lower in cholesterol-fed groups (Table 3).

The distribution of cholesterol among the plasma lipoproteins in males analyzed by FPLC is shown in Fig. 1. Dietary cholesterol increased VLDL and LDL cholesterol (Fig. 1d), and olive oil mainly increased VLDL cholesterol (Fig. 1f). Males on the olive oil + cholesterol diet experienced an increase in both VLDL and LDL cholesterol (Fig. 1h). As with the results obtained by a precipitation method, HDL cholesterol decreased in response to the administration of cholesterol in males (Fig. 1d).

### 3.3. Atherosclerotic lesion formation

Fig. 2 is a representative image of an aortic root atherosclerotic lesion in 18-week-old mice used in the present study. The characteristics of lesions are shown in Fig. 2a and b.

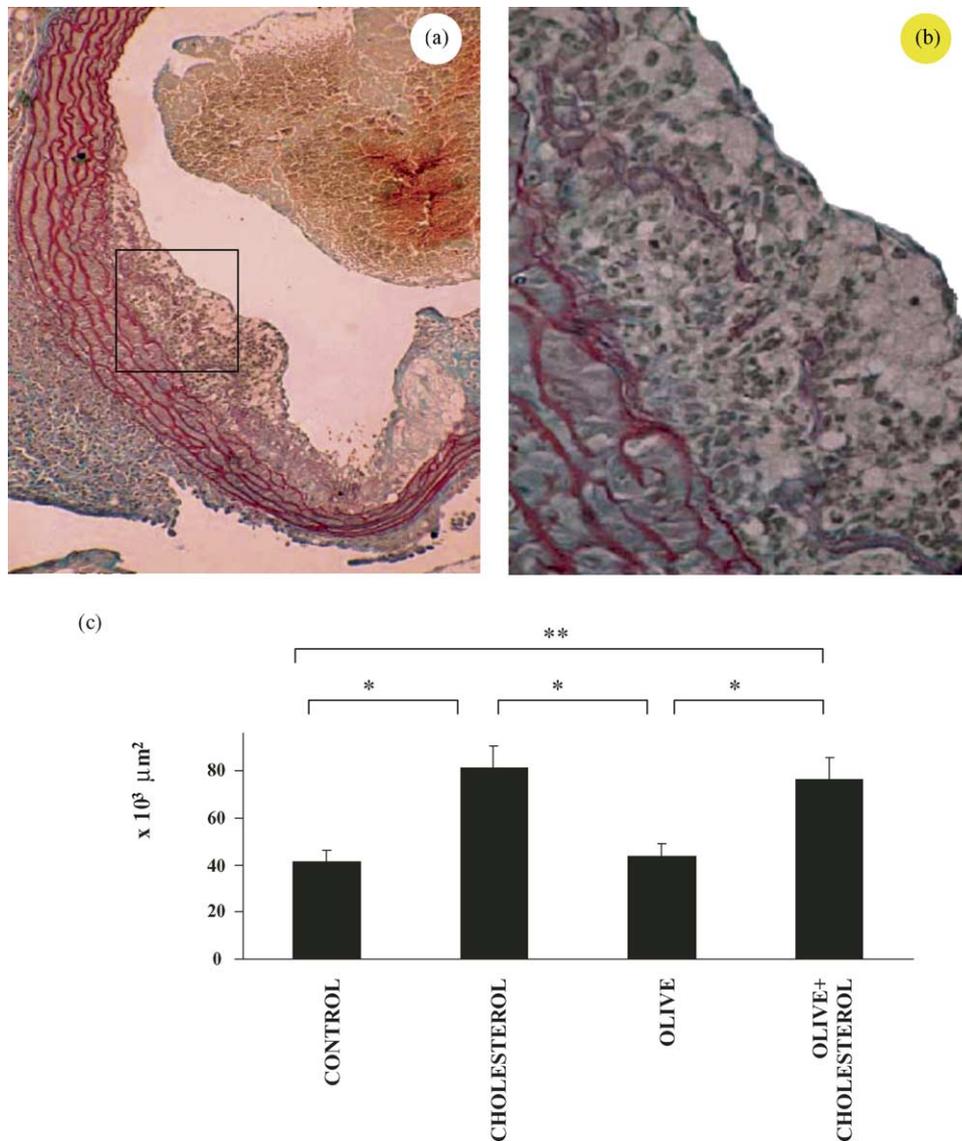


Fig. 2. Cross-sectional analysis of aortic lesions. Panels (a) and (b) correspond to representative lesions displayed by the animals and panel (c) summarizes lesion area in apoE knockout mice following different experimental diets. Data are mean  $\pm$  S.E.M. for each group. Statistical analyses are based on the Mann–Whitney *U*-test; (\*)  $P < 0.05$  and (\*\*)  $P < 0.02$ .

At the end of the 10-week experiment, the infiltration of macrophages and the transformation into foam cells are the characteristic features of the lesions. Quantitative analyses revealed no significant differences between males and females in any of the experimental groups; therefore, the results for both sexes are combined (Fig. 2c). A cholesterol-enriched diet induced a significant increase in the size of lesions relative to those found in mice fed the control diet, whereas mice fed an olive oil-enriched diet had lesions that were similar to those of mice in the control group. Note, however, animals fed the olive oil + cholesterol diet had lesions similar to those observed in animals of the cholesterol group.

Fig. 3 shows the results of the *en face* analysis. Dissection of an arterial tree (Fig. 3a) of a representative animal used

in the present study showed lesions at the aortic arch and the lumbar regions. In females, the amount of the aortic area covered with plaques was not significantly greater in mice fed cholesterol- or olive oil-enriched diets compared to the control group; however, female mice fed a cholesterol + olive oil-enriched diet showed significantly less plaques than the group that received only the diet enriched in cholesterol (Fig. 3b).

Response of plaque development to the different experimental diets appears to differ in males from females. Thus, the dietary supply of cholesterol, either with or without olive oil, significantly increased the amount of the aortic area covered by lesions (Fig. 3c). Lesions in males fed an olive oil-enriched diet were similar to those found in mice fed the carbohydrate-based control diet.

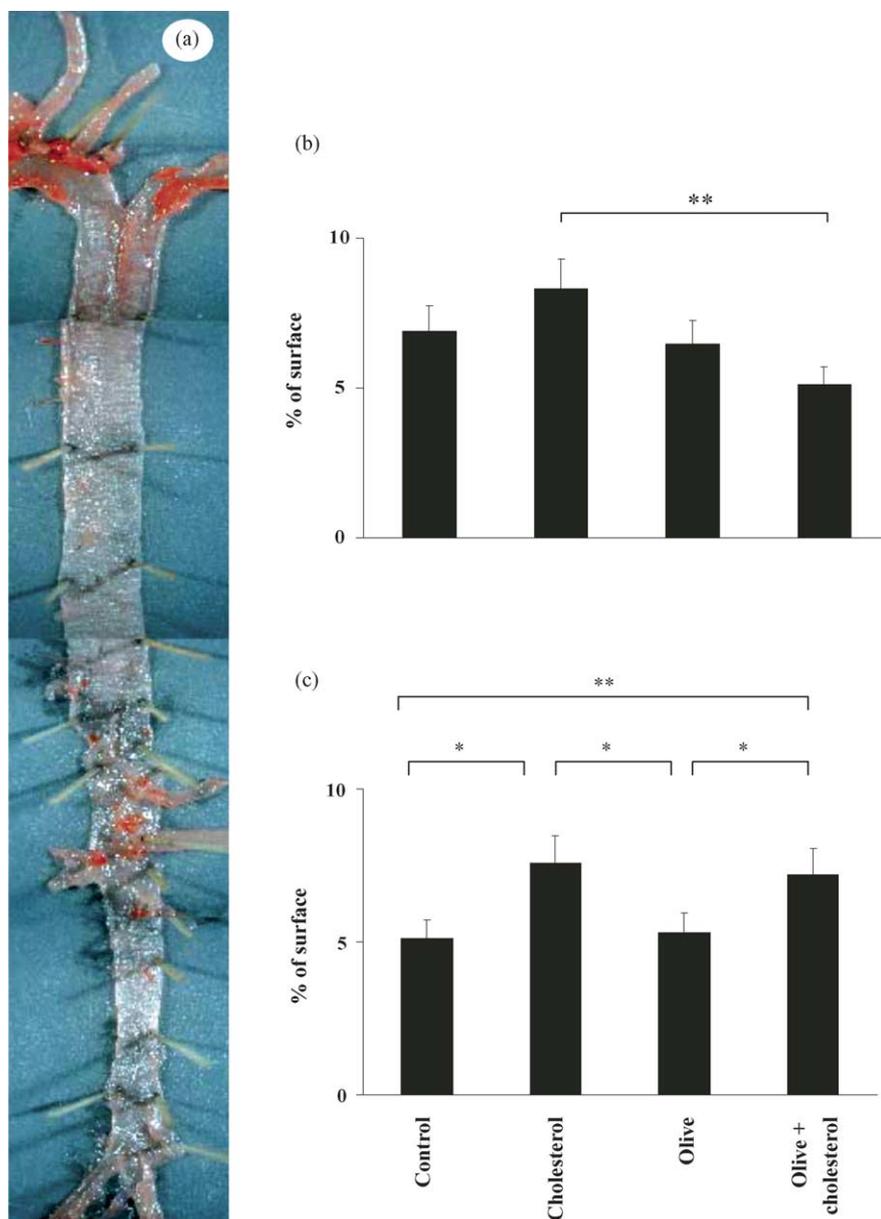


Fig. 3. Extent of atherosclerotic lesions in arterial trees from apoE KO mice fed different experimental diets. Panels show (a) the presence of atherosclerotic foci (b), quantification of area covered by lesions in females and (c) in males. Values are mean  $\pm$  S.E.M. for each group. Statistical analyses are based on the Mann–Whitney *U*-test; (\*)  $P < 0.05$  and (\*\*)  $P < 0.02$ .

### 3.4. Paraoxonase activity

Fig. 4a shows the arylesterase activity of paraoxonase. In females, the serum catalytic activity concentration of the enzyme was generally higher than in males and showed a resistance to being modified by the dietary interventions. Thus, only the female groups that received olive oil or cholesterol showed a significant variation in its levels.

In contrast, the activity of arylesterase in males was more likely to be influenced by differences in diet. Thus, the administration of cholesterol induced a significant decrease in the arylesterase activity. The administration of an olive oil-enriched diet caused a significant increase in

arylesterase activity compared to the cholesterol-enriched diet, but not the standard chow diet. The inclusion of cholesterol in the olive oil diet also significantly decreased arylesterase activity compared to both the olive oil or chow diets. PON activity and HDL cholesterol were positively correlated ( $r = 0.34$ ,  $P < 0.1$  and  $r = 0.34$ ,  $P < 0.05$  in females and males, respectively). A stronger positive correlation existed between PON activity and apoA–I concentration ( $r = 0.56$ ,  $P < 0.005$  and  $r = 0.63$ ,  $P < 0.001$  in females and males, respectively). Those data corroborate the relationship between PON and apoA–I, and emphasize that this relationship might exist in apoA–I HDL devoid of cholesterol.

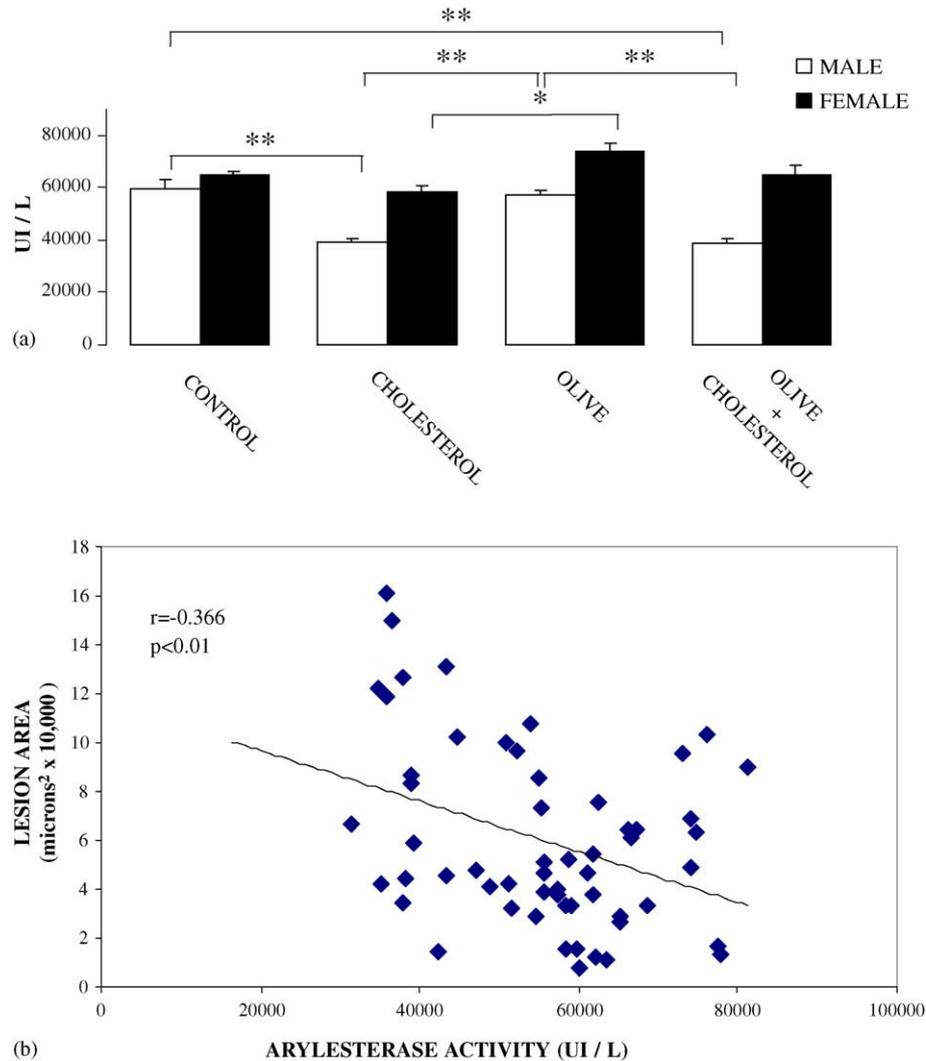


Fig. 4. Serum paraoxonase activity in apoE KO mice fed one of four diets over a 10-week period. (a) PON activity determined as described in Section 2. Values are mean  $\pm$  S.E.M. for each group; (\*)  $P < 0.01$  and (\*\*)  $P < 0.001$ . (b) Relationship between serum paraoxonase activity and cross-sectional aortic lesions in individual mice from all diet groups.

Globally, serum arylesterase activity concentrations and aortic atherosclerotic lesion areas were significantly and negatively correlated (Fig. 4b). However, in this regard, males showed a stronger relationship ( $r = -0.45$ ,  $P < 0.01$ ) than did females ( $r = -0.33$ ,  $P < 0.1$ ), which suggests that modulation of paraoxonase by the different diets might influence the growth of aortic lesions in apoE-deficient mice in a sex-dependent manner. A two-way ANOVA to verify the relationship between paraoxonase and aortic lesions, with HDL cholesterol as a covariate, indicated  $r = -0.4285$  ( $P < 0.013$ ) in males and  $r = -0.3346$  ( $P < 0.128$ ) in females. With the sexes combined,  $r = -0.352$  ( $P < 0.008$ ). A similar analysis to demonstrate the relationship between paraoxonase and aortic lesion area, with apoA-I as covariate indicated  $r = -0.4580$  ( $P < 0.014$ ) in males and  $r = -0.0866$  ( $P < 0.7$ ) in females. With the sexes combined,  $r = -0.261$  ( $P < 0.06$ ). Hence, the nutritional regulation of paraoxonase is an important determinant of atherosclerotic lesions, inde-

pendent of HDL and apoA-I concentrations, particularly in males.

### 3.5. RNA analysis

To determine whether hepatic mRNA was involved in changes in serum paraoxonase activity and apolipoprotein A-I concentrations, we determined apoA-I and PON-1 message levels in liver using Northern blot analyses (Fig. 5). In female and male mice fed a cholesterol-enriched diet, apoA-I and PON-1 mRNA expressions were significantly lower than in mice fed a chow diet. A similar effect was found in males fed an olive oil-enriched diet. Likewise, when both fats were combined in the diet, a further significant decrease was observed in both sexes. Collectively, those data suggest that cholesterol and olive oil act synergistically to decrease hepatic expression of both genes in male and female apoE KO mice.

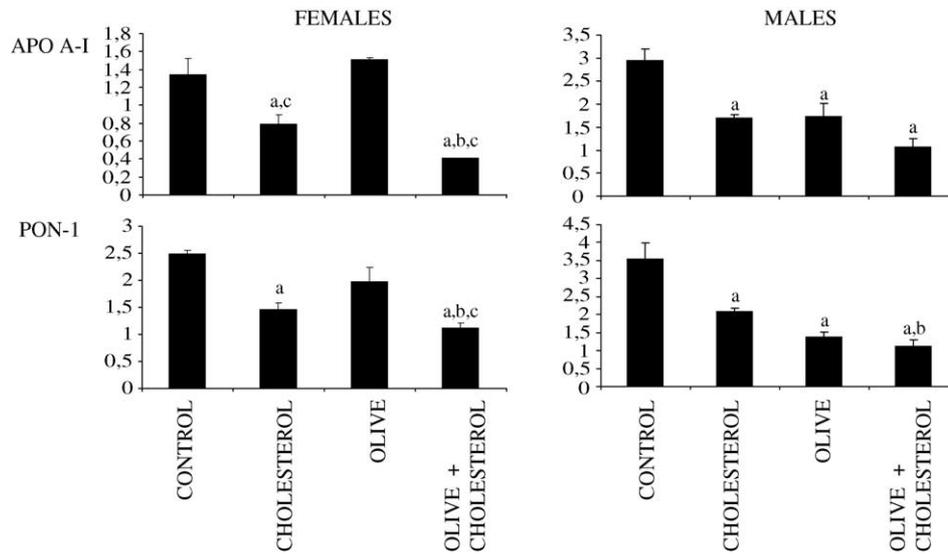


Fig. 5. Differential effects of experimental diets on hepatic apoA-I and PON-1 mRNA expressions. Results are expressed as mean  $\pm$  S.E.M. in arbitrary absorbance units normalized to the  $\beta$ -actin gene expression. Five micrograms of total RNA from animals in each group was subjected to Northern analysis. Statistical analyses are based on the Mann–Whitney *U*-test. Letters (a, b, c) denote  $P < 0.05$  versus control, cholesterol and olive oil diet group, respectively.

#### 4. Discussion

The Mediterranean diet is characterized by a high fat content, mainly derived from olive oil, and low cholesterol [11]. An extrapolation of this diet to our present condition supposes that the mere inclusion of olive oil in the human diet might provide the beneficial effect of lowering the incidence of coronary heart disease. To investigate this issue and to determine the mechanisms by which dietary fat regulates such parameters, we conducted a dietary experiment in which apoE knockout mice were given one of four diets for 10 weeks. We found that a dietary fat supplement of olive oil, in the absence of dietary cholesterol, administered at a level comparable to that of human energy intake did not result in an increase in the size of atherosclerotic lesions either in male or female apoE KO mice. The apparent protective effect of olive oil disappeared when cholesterol was added at levels consumed by Western human populations.

In an attempt to reproduce in mice the average intake of fat in humans of Western society, we provided animals with olive oil as 20% (w/w) of the diet. That oil supplement did not increase the growth of pre-existing plaques (Fig. 2a) to an extent greater than when mice consumed a low fat, standard chow diet. That result was surprising in the light of a previous study in which a 10% (w/w) virgin olive oil-enriched diet induced a significant decrease in atherosclerotic lesions in apoE KO female mice [16]. The absence of a response at higher levels of olive oil intake suggests a saturation effect. A similar interpretation is obtained when other parameters were examined. Thus, virgin olive oil administered at 10% (w/w) had no effect on body weight or plasma cholesterol levels, and even decreased triglycerides in apoE-deficient mice [16], whereas virgin olive oil administered at 20% (w/w) increased body weight, plasma cholesterol and triglycerides (Tables 2 and 3).

Those and our previous results suggest a “therapeutic window” for olive administration to apoE KO mice because 10% (w/w) olive oil content had a beneficial effect compared to the chow diet and 20% (w/w) olive oil-supplemented diets. Although a 20% olive oil supplement did not increase the size of atherosclerotic lesions, this level of oil intake clearly influences parameters such as body weight and plasma lipid levels. A similar phenomenon was reported by Asset et al. [28] who did not observe a hypotriglyceridemic effect of 20% (w/w) fish oil administered to apoE-deficient mice and suggested that apoE might be necessary for fish oil to lower plasma triglyceride concentrations. Indeed, hepatic apolipoprotein E is known to play an intracellular role in cholesterol excretion, and the absence of apoE contributes to the propensity for tissue cholesterol deposition [29]. In LDL receptor-deficient mice, an increase in cholesterol intake (higher than 0.15%, w/w) contributed to an increase in cholesterol deposition in liver [30]. Our results indicate that a combination of fat and an even lower cholesterol supply (0.125%, w/w) induces significant hepatomegaly (Tables 2 and 3). Collectively, those results indicate that the absence of apolipoprotein E or the LDL receptor in mice makes them highly susceptible to the effects of higher levels of dietary triglycerides and cholesterol, which might indicate a limitation of these models in testing the effects at high levels of fat intake or, a possible caution regarding the hepatic handling of lipids in dyslipidemic patients, who might be more sensitive to dietary changes than are healthy individuals.

The main finding of our study is that cholesterol supplementation of diets enriched with olive oil significantly increased the area of aortic lesions, independent of the sex of the mouse. Our data partly corroborate another study that reported no change in aortic lesions in apoE KO mice that consumed 20% olive oil or carbohydrates when both diets

were enriched in 0.2% cholesterol [17]. Our experimental design, however, allows us to establish that the inclusion of dietary cholesterol abolishes the ability of olive oil to retard atherosclerotic lesions. This might convey a reflexion regarding whether just an oil substitution in human nutrition without other components of a Mediterranean profile that reduce cholesterol sources might be more harmful than beneficial. Indeed, in the regions of Spain where the most olive oil and animal protein is consumed, the incidence of cardiovascular disease is higher than in other areas of Spain where olive oil consumption is accompanied by more vegetables and legumes and less animal protein [31].

Another important finding of our study is the sex-related differences in the development of aortic lesions and the presence or extent of atherosclerotic foci along the aortic tree, as estimated by the *en face* procedure. Thus, the pattern observed in males, in which the cross-sectional lesion analyses at aortic roots (Fig. 2c) is paralleled by the extent of lipid staining on the surface of aortic vessels (Fig. 3c), was not observed in females, where the addition of cholesterol to an olive oil-enriched diet does not increase extension or appearance of new foci (Fig. 3b). Consequently, our the overall correlation between both methods is lower ( $r=0.33$ ) than that obtained by Tangirala et al. [25] in apoE KO mice maintained on a chow diet ( $r=0.77$ ). When LDLr KO mice with different genetic backgrounds were fed different amounts of dietary cholesterol, the *en face* lesion was highly dependent on the genetic background at aortic arch region and the FVB females had lower aortic root cross-sectional lesions than did C57BL/6 females at low cholesterol intake [30]. In our study, the apparition of new atherosclerotic foci was efficiently blocked in females receiving olive oil + cholesterol diet (Fig. 3b) by a mechanism not effective to avoid growth of existing plaques and specific for this sex. Recently, the fractalkine knockout mice that show differences in the regional distribution of atherosclerotic foci have been described [32]. A hypothetical and plausible explanation for our findings might be that the dietary conditions used in our study modified the expression of this protein and its receptor. Collectively, the data indicate that in the initiation and development of atherosclerotic foci there are complex interactions among diet, sex and genetic make-up that can induce the positional expression of certain proteins that would favor macrophage recruitment.

Our study demonstrates a sex-related difference in the regulation of paraoxonase activity in cholesterol-fed mice. Thus, females are resistant to reduction of serum paraoxonase activity by diets enriched in cholesterol (Fig. 4a), despite a clear decrease in PON-1 steady-state levels of hepatic mRNA (Fig. 5). The dissociation of mRNA and enzyme activity was also observed in olive oil-fed mice. While PON-1 mRNA levels decreased in males fed this diet (Fig. 5) compared to mice in the chow diet group, there was no difference in circulating activities between the two groups (Fig. 4a). In contrast, when 20% (w/w) triolein was administered to male rats, Kudchodkar et al. [33] described an increase in plasma PON-1 activity.

Our results are partially in agreement with theirs, since olive oil contains triolein in a high percentage, but we did not provide only this triglyceride so the effect is not as dramatic as reported by them. In our study, when olive oil + cholesterol was provided to male apoE-deficient mice, the decrease in paraoxonase activity reflected a decrease in the expression of PON-1 mRNA (Fig. 5). Similarly, Shih et al. [34] showed that C57BL/6J mice fed a high saturated fat, cholesterol (1.25%, w/w), and cholate (0.5%, w/w) diet exhibited reduced serum PON-1 activities and hepatic PON-1 mRNA levels. In our study, mice were given low cholesterol (0.1%, w/w) in the absence of cholate. Our experimental dietary design using females and males allows us to conclude that cholesterol administration alone is able to reduce the circulating paraoxonase, but this effect is clearly dependent on sex. Likewise, association between apoA-I mRNA and plasma protein levels in olive fed animals is more pronounced for males than females. Therefore, the regulation of plasma PON1 enzyme and apolipoprotein A-I at several levels, including transcriptional, translational, association with certain HDL particles, kinetics and metabolic fate of these plasma particles, may be complex and influenced by both dietary components and sex.

Another remarkable finding of our study is the negative correlation between serum paraoxonase activity and the cross-sectional area of lesions in the aortic root (Fig. 4b), which is stronger in males than in females. The reduction in paraoxonase activity might lead to an increase in atherosclerotic lesions. In that regard, our results are consistent with those observed in mice genetically manipulated for the PON-1 gene [18–20] and emphasize that slight variations in serum paraoxonase activity induced by diet appear to be important factor for atherosclerosis particularly in males. A potential patho-physiological meaning of this association has to consider that PON-1 is expressed in liver, so the circulating level of this enzyme has hepatic origin and is associated with certain HDL particles. The stronger correlation observed between PON activity and apoA-I than that between PON activity and HDL cholesterol likely reflects that the enzyme is particularly vehicled in smaller size HDL particles containing apoA-I but devoid of cholesterol. Thus, the transference of PON carrying particles into subendothelial space would be increased and favor the inactivation of certain oxidized phospholipids species and, in turn could delay atherosclerosis progression [35]. In addition, a direct effect of plasma paraoxonase on endothelial cells to reduce MCP-1 production cannot be excluded [36]. Collectively, our results demonstrate that dietary factors interact with sex in determining the activity of paraoxonase and, therefore, the contribution of this activity in preventing the development of atherosclerosis.

In conclusion, the administration of cholesterol with olive oil abolishes the ability of olive oil to delay the progression of atherosclerosis in apoE KO mice. That is particularly important when implementing Mediterranean diets into the nutritional context of a 0.15% (w/w) cholesterol intake in Western societies. The classical Mediterranean diet was low in cholesterol intake, so the inclusion of olive oil in excess as a part of

the reappraisal to convey into this diet might be more harmful than beneficial based on the results of our study. Although our findings from using apoE knockout mice might not be directly applicable to humans, the results suggest the existence of a “therapeutic window” in which to obtain the benefits of olive oil consumption and this should be considered when modifying diets for humans. Furthermore, the extraordinary sensitivity to fat intake of apoE KO mice and the development of hepatic steatosis might indicate that dietary interventions in subjects with severe dyslipemias should be more closely monitored than in the general population.

## Acknowledgments

This research was supported by Grants FEGA-FEOGA (CAO99-014), CICYT (SAF2004-08173-C03-02), FISS (01/0202), Redes de investigación cooperativa C03-01 and G03-140 and by Fundación Española del Corazón. R.C., S.A., M.A.N. and M.A.G.G. were recipients of DGA, FEGA-FEOGA and PROMEP fellowships. We thank Drs. Surra and Villaroel-Robinson for their critical reading of this manuscript. We also thank Angel Beltran, Jesus Cazo, Jesus Navarro, Carmen Navarro and Clara Tapia from Unidad Mixta de Investigación for their invaluable help in maintaining animals.

## References

- [1] Reddick R, Zhang S, Maeda N. Atherosclerosis in mice lacking ApoE. *Arterioscler Thromb* 1994;14:141–7.
- [2] Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* 1994;14:133–40.
- [3] Rosenfeld ME, Polinsky P, Virmani R, Kausar K, Rubanyi G, Schwartz SM. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. *Arterioscler Thromb Vasc Biol* 2000;20:2587–92.
- [4] Johnson JL, Jackson CL. Atherosclerotic plaque rupture in the apolipoprotein E knockout mouse. *Atherosclerosis* 2001;154:399–406.
- [5] Osada J, Joven J, Maeda N. The value of apolipoprotein E knockout mice for studying the effects of dietary fat and cholesterol on atherogenesis. *Curr Opin Lipidol* 2000;11:25–9.
- [6] Willett WC. Diet and health: what should we eat? *Science* 1994;264:532–7.
- [7] Krauss RM, Eckel RH, Howard B, et al. AHA dietary guidelines: revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* 2000;102:2284–99.
- [8] Knuops KTB, de Groot LCPGM, Kromhout D, et al. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. *JAMA* 2004;292:1433–9.
- [9] Robertson RM, Smaha L. Can a Mediterranean-style diet reduce heart disease? *Circulation* 2001;103:1821–2.
- [10] de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors and the rate of cardiovascular complications after myocardial infarction. *Circulation* 1999;99:779–85.
- [11] Kris-Etherton P, Eckel RH, Howard BV, St Jeor S, Bazzarre TL. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style. National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. *Circulation* 2001;103:1823–5.
- [12] Mata P, Garrido JA, Ordovas JM, et al. Effect of dietary monounsaturated fatty acids on plasma lipoproteins and apolipoproteins in women. *Am J Clin Nutr* 1992;56:77–83.
- [13] Fuentes F, Lopez-Miranda J, Sanchez E, et al. Mediterranean and low-fat diets improve endothelial function in hypercholesterolemic men. *Ann Intern Med* 2001;134:1115–9.
- [14] Visioli F, Galli C, Plasmati E, et al. Olive phenol hydroxytyrosol prevents passive smoking-induced oxidative stress. *Circulation* 2000;102:2169–71.
- [15] Aguilera CM, Ramirez-Tortosa MC, Mesa MD, Ramirez-Tortosa CL, Gil A. Sunflower, virgin-olive and fish oils differentially affect the progression of aortic lesions in rabbits with experimental atherosclerosis. *Atherosclerosis* 2002;162:335–44.
- [16] Calleja L, Paris MA, Paul A, et al. Low-cholesterol and high-fat diets reduce atherosclerotic lesion development in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol* 1999;19:2368–75.
- [17] Merkel M, Velez-Carrasco W, Hudgins LC, Breslow JL. Compared with saturated fatty acids, dietary monounsaturated fatty acids and carbohydrates increase atherosclerosis and VLDL cholesterol levels in LDL receptor-deficient, but not apolipoprotein E-deficient, mice. *Proc Natl Acad Sci USA* 2001;98:13294–9.
- [18] Tward A, Xia YR, Wang XP, et al. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation* 2002;106:484–90.
- [19] Shih DM, Gu L, Xia YR, et al. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 1998;394:284–7.
- [20] Shih DM, Xia YR, Wang XP, et al. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000;275:17527–35.
- [21] Escola-Gil JC, Jorba O, Julve-Gil J, Gonzalez-Sastre F, Ordonez-Llanos J, Blanco-Vaca F. Pitfalls of direct HDL-cholesterol measurements in mouse models of hyperlipidemia and atherosclerosis. *Clin Chem* 1999;45:1567–9.
- [22] Aviram M, Dornfeld L, Rosenblat M, et al. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am J Clin Nutr* 2000;71:1062–76.
- [23] Calleja L, Trallero MC, Carrizosa C, Mendez MT, Palacios-Alaiz E, Osada J. Effects of dietary fat amount and saturation on the regulation of hepatic mRNA and plasma apolipoprotein A-I in rats. *Atherosclerosis* 2000;152:69–78.
- [24] Osada J, Maeda N. Preparation of knockout mice. *Methods Mol Biol* 1998;110:79–92.
- [25] Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res* 1995;36:2320–8.
- [26] de las Heras M. Estudio de los aspectos anatomopatológicos de la Angiostrongilosis canina, tras sensibilización con L<sub>1</sub> de *Angiostrongylus vasorum* (Baillet, 1886). Zaragoza: Zaragoza; 1987.
- [27] Paigen B, Morrow A, Holmes P, Mitchell D, Williams R. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 1987;68:231–40.
- [28] Asset G, Bauge E, Fruchart JC, Dallongeville J. Lack of triglyceride-lowering properties of fish oil in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2001;21:401–6.
- [29] Sehaye E, Shefer S, Nguyen LB, Ono JG, Merkel M, Breslow JL. Apolipoprotein E regulates dietary cholesterol absorption and biliary cholesterol excretion: studies in C57BL/6 apolipoprotein E knockout mice. *Proc Natl Acad Sci* 2000;97:3433–7.

- [30] Teupser D, Persky AD, Breslow JL. Induction of Atherosclerosis by low-fat, semisynthetic diets in LDL receptor-deficient C57BL/6J and FVB/NJ mice: comparison of lesions of the aortic root, brachiocephalic artery, and whole aorta (en face measurement). *Arterioscler Thromb Vasc Biol* 2003;23:1907–13.
- [31] Rodriguez-Artalejo F, Garces C, Gorgojo L, et al. Dietary patterns among children aged 6-7 y in four Spanish cities with widely differing cardiovascular mortality. *Eur J Clin Nutr* 2002;56:141–8.
- [32] Teupser D, Pavlides S, Tan M, Gutierrez-Ramos JC, Kolbeck R, Breslow JL. Major reduction of atherosclerosis in fractalkine (CX3CL1)-deficient mice is at the brachiocephalic artery, not the aortic root. *Proc Natl Acad Sci USA* 2004;101:17795–800.
- [33] Kudchodkar BJ, Lacko AG, Dory L, Fungwe TV. Dietary fat modulates serum paraoxonase 1 activity in rats. *J Nutr* 2000;130:2427–33.
- [34] Shih DM, Gu L, Hama S, et al. Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model. *J Clin Invest* 1996;97:1630–9.
- [35] Durrington PN, Mackness B, Mackness MI. The hunt for nutritional and pharmacological modulators of paraoxonase. *Arterioscler Thromb Vasc Biol* 2002;22:1248–50.
- [36] Mackness B, Hine D, Liu Y, Mastorikou M, Mackness M. Paraoxonase-1 inhibits oxidised LDL-induced MCP-1 production by endothelial cells. *Biochem Biophys Res Commun* 2004;318:680–3.