Original Article

Sex as a Profound Modifier of Atherosclerotic Lesion Development in Apolipoprotein E-deficient Mice with Different Genetic Backgrounds

Joaquín C. Surra[1, 2], Natalia Guillén[1, 2], José M. Arbonés-Mainar[1], Cristina Barranquero[1, 2], María A. Navarro[1, 2], Carmen Arnal[2, 3], Israel Orman[4], José C. Segovia[4], and Jesús Osada[1, 2]

[1]Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Universidad de Zaragoza, Spain
[2]CIBER de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos Ⅲ , Spain
[3]Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Spain
[4]Unidad de Diferenciación y Citometría, Centro de Investigaciones Energé ticas, Medioambientales y Tecnoló gicas (CIEMAT) and Centro de Investigació n Biomé dica en Red de Enfermedades Raras (CIBER-ER), Madrid, Spain

***Aim*: Research suggests that sex may condition atherosclerosis development against different genetic backgrounds. This study addresses the hypothesis that this effect would be exerted by changes in the different apolipoproteins present in high-density lipoproteins.**
***Methods*: ApoE-deficient mice of both sexes with Ola 129 and C57BL/6J genetic backgrounds were fed a chow diet for 14 weeks. At the end of the dietary intervention, the development of atherosclerotic lesions, apolipoproteins, lipid metabolism, inflammation and paraoxonase were assessed.**
***Results*: Differences between atherosclerotic lesions in Ola 129 and C57BL/6J strains of apoE-deficient mice were sex-dependent and were only statistically significant in females. Plasma levels of HDL cholesterol and apolipoproteins related to these lipoparticles, such as apoA-Ⅰ, apoA-Ⅱ, apoA-Ⅳ, apoA-V and apoJ, were significantly different between these two strains and there were sex-related differences in some of these apolipoproteins. Hepatic steatosis was also related to the strain and was independent of sex. In females, changes in HDL cholesterol and apolipoproteins A-Ⅰ and A-Ⅱ were important determinants of atherosclerosis, while this was not the case in males.**
***Conclusions*: Our results demonstrate that atherosclerosis-related differences between Ola129 and C57BL/6J genetic backgrounds in apoE-deficient mice are sex-dependent and that this finding is explained by the differences in HDL cholesterol and its apolipoprotein components, mainly apoA-Ⅰ and A-Ⅱ. Overall, our findings highlight the importance of taking sex into account in the analysis of atherosclerosis and lipid metabolism in animal models.**

J Atheroscler Thromb, 2010; 17:712-721.

Key words; Sex, Apolipoprotein, Atherosclerosis, Chitotriosidase, Paraoxonase, Mouse strains

Cardiovascular diseases are the leading causes of mortality in Western societies and atherosclerotic processes are involved in their development. In addition, atherosclerosis is a complex disease, the outcome of which is determined by many genetic and environmental factors. One approach for dissecting the contribution of the two factors has been the use of laboratory mice with a spontaneous or induced mutation or a combination of both. Thus, a number of strains of mice with varying susceptibility to atherosclerosis have been characterized[1-3]; mice lacking apolipoprotein E (apoE) or low density lipoprotein receptor genes[4] have been created and the transfer of mutations into mice that have more prone (C57BL/6J) or more resistant (C3H/HeJ) atherosclerotic genetic backgrounds has been undertaken, and it has been observed that, whereas C57BL/6J develop large, human-like atherosclerotic lesions against the background of the apoE-

Address for correspondence: Dr. Jesús Osada, Department of Biochemistry and Molecular Biology, Veterinary School, University of Zaragoza, Miguel Servet, 177, E-50013 Zaragoza, Spain.
E-mail: Josada@unizar.es
Received: August 4, 2009
Accepted for publication: December 15, 2009

null mutation, C3H/HeJ mice develop almost no lesions unless fed a Western diet[5]. When genetic analysis was introduced to characterize potential genetic differences, a major locus contributing to atherosclerosis susceptibility was isolated on chromosome 9. In this region are located the genes coding for apolipoproteins ApoA1, ApoA4, ApoC3 and ApoA5[6]. When a more specific endeavor was undertaken to characterize genes associated with HDL cholesterol (HDL-C) using C57BL/6J and NZB/BlNJ mouse strains, hepatic expression of twelve genes was found to be significantly associated with this parameter (*Nr1i3, Apoa2, Sap, Tgfb2, Fgfbp1, Prom, Ppargc1, Tcf1, Ncor2, Srb1, App,* and *Ifnar*)[7]. In neither study were protein determinations carried out to verify whether the predicted involved gene products were translated accordingly. In our experience, hepatic *Srb1* gene expression did not correlate with protein levels[8]. Furthermore, few studies have addressed the characterization of the 129 strain, although female 129 mice showed less development of atherosclerosis when compared to C57BL/6J[9] and also had higher levels of HDL-C[10]. This difference in HDL levels has been associated with a more pronounced hypertensive phenotype of cystathione β-synthase and apolipoprotein A-I double deficiency in C57BL/6J mice[11]. Thus, HDL and their apolipoproteins represent an important source of variation in the phenotypic presentation of atherosclerosis and other cardiovascular diseases.

Sex-related differences in the manifestation of cardiovascular diseases are widely recognized in humans[12-14]. In this aspect, the mouse model is also providing some insights; for example, female apoE-deficient mice with the C57BL/6J genetic background developed larger atherosclerotic areas than males; however, this was not the case when apoE-deficient mice had the C3H/HeJ background[6]. Sex-related differences were also observed in HDL-C levels in mice as well in the potential genes responsible for those changes, suggesting that sex is an important determinant of the results[15]. Our group has also observed a different sex-related handling of hepatic fat in response to squalene administration[8] and a different, sex-specific contribution of paraoxonase to the atherosclerotic response in mice fed olive oil-enriched diets[16]. This study was undertaken to verify the influence of sex on the development of atherosclerosis against two genetic backgrounds (Ola129 and C57BL/6J) of apoE-deficient mice and to test the hypothesis that this effect might be exerted by changes in the different apolipoproteins present in HDL.

Materials and Methods

Animals

Three-month-old homozygous apoE-deficient mice with Ola129 ($n=13$) and C57BL/6J ($n=17$) genetic backgrounds, bred in the Unidad Mixta de Investigación, Zaragoza, were used. Four groups were established according to sex, background and their initial plasma cholesterol concentration, which had been quantified in samples taken from the retro-orbital plexus in isofluorane (Abbot, Barcelona) -anesthetized animals after an eight-hour fast. The mice were housed in sterile filter-top cages in a temperature-controlled facility, maintained on a 12-h light/12-h dark cycle, and had *ad libitum* access to food and water. The mice were fed the chow diet (2014 Global Rodent Maintenance; Harlan Tekland) for 14 weeks. The 2014 diet contained no cholesterol and provided 10.8% of energy as fat. The proportions of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids were 18%, 20% and 61% of total fat, respectively. The latter fraction of the control diet contained linoleic acid as the main fatty acid. To avoid the potential confounding effects of variations among batches of chow, 25 kg from a single batch was reserved and used throughout the experiment. Fresh food was provided daily. Food intake was recorded daily during the first two weeks of the experimental period using metabolic cages (Biosys, Barcelona, Spain). Body weights were recorded at the beginning and end of the experiment. The mice were handled while observing the criteria of the European Union for the care and use of laboratory animals in research, and the protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza, Spain.

Blood Cell Analysis of Surface Molecule Expression

After 13 weeks, the mice were fasted for eight hours, anesthetized using isofluorane, and blood samples were collected by retro-orbital bleeding. Approximately 1×10^6 white blood cells were resuspended in phosphate-buffered saline supplemented with 0.1% (w/v) BSA and 10 mmol/L sodium azide and analyzed for the expression of Mac-1 (Anti-CD11b; Becton-Dickinson, Madrid, Spain) and VLA-4 (Anti-VLA-4; Becton-Dickinson) using fluorescence-activated cell sorter analysis. The results are expressed as the proportion (%) of marker-positive cells recovered in the region corresponding to monocytes.

Biochemical Determinations

After the experimental period, animals were sac-

rificed by suffocation with CO_2 and blood was drawn from their hearts. Plasma nonesterified fatty acids (NEFA), total cholesterol (TC), and triglyceride (TG) concentrations were measured in a microtiter assay, using commercial kits from Wako and Thermo (Madrid, Spain). After phosphotungstic acid-$MnCl_2$ (Roche, Barcelona, Spain) precipitation of apoB-containing particles[17], HDL-C was determined in the supernatant using a commercial kit (Amplex Red; Molecular Probes, USA). Paraoxonase was assayed using arylesterase[16], paraoxonase or lactonase activities[18], and expressed as ($IU \cdot L^{-1} \times 1000$). The chitotriosidase enzyme assay was carried out in a microtiter plate as previously described[19] and expressed as nmol of substrate hydrolyzed per minute per litre of serum. Apolipoproteins A-I, A-II, A-IV, A-V and J were quantified by enzyme-linked immunosorbent assays with specific polyclonal antibodies (Biodesign, Saco, ME, sc-19036 and sc-66810; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described[20].

Lipoprotein Isolation

Plasma lipoprotein profiles were determined using 150 μL of two independent pools of plasma that contained samples from all animals within each experimental group, which were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6B column (Amersham Pharmacia, Barcelona, Spain), as previously described[21]. Cholesterol was assessed as described above.

HDL Capacity to Protect against Low-Density Lipoprotein Peroxidation

To assess the ability of HDL to inactivate peroxide formation in low-density lipoproteins (LDL), we used a modified version of the procedures followed by Navab *et al.*[22], which allowed us to monitor the presence of oxygen radicals in LDL by measuring the conversion of 2',7'-dichlorofluorescein diacetate (DCFH-DA) into highly fluorescent dichlorofluorescein (DCF). Cholesterol (0.2 μg) of FPLC-isolated HDL fractions (fractions 19 and 20) representing each experimental condition was incubated at 37 ℃, along with their LDL obtained from apoE-deficient mice of different sexes and backgrounds (5 μg cholesterol), 2 μg DCF, 25 μL of 0.1% sodium azide, and 100 μL phosphate-buffered saline in a total volume of 150 μL. After three hours, fluorescence was measured in a microplate reader (SPECTRAfluor Plus; TECAN) at 530 nm using an excitation wavelength of 485 nm.

Evaluation of Atherosclerotic Lesions

The heart and arterial tree were perfused with phosphate-buffered saline under physiological pressure. Hearts were dissected out and heart bases were placed in OCT (Bayer Diagnostic, Germany) on a cryostat chuck (Microm HM505E; Barcelona, Spain), covered with liquid OCT, frozen in liquid N_2-cooled isopentane and stored at −80℃ until analysis. Serial cryosections of the proximal aorta and aortic sinus were stained with Sudan IV (Sigma Chemical Company) and counterstained with hematoxylin (Sigma Chemical Company)[21]. Images were captured and digitized using a Nikon microscope equipped with a Nikon digital camera. Morphometric analyses were evaluated blindly using Scion Image software (Scion Corporation, Frederick, Maryland, USA). Average lesion sizes were used for morphometric evaluations based on the method of Paigen *et al.*[23].

Histological Analysis

Pieces of liver were stored in buffered formaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. Images were captured and digitized using a Nikon microscope equipped with a Nikon digital camera. Hepatic fat content was evaluated blindly 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.

Statistical Analysis

Results are expressed as the mean ± SEM. Unless otherwise stated, the nonparametric Mann-Whitney U-test for comparison between pairs was used for unpaired observations. Association between variables was assessed by the Spearman correlation test (p). All statistical tests were performed with SPSS version 15.0 (SPSS Inc., Chicago, IL), and $p \leq 0.05$ was considered significant.

Results

Somatic and Plasma Parameters of the Two Strains according to Sex

The data of female mice are shown in **Table 1**. After the 14-wk experiment, Ola129 mice gained significantly less body weight than C57BL/6J, although there was no significant variation in food consumption (3.7 ± 0.5 g per day). No significant change was noted in liver weight in females of either strain.

Plasma levels of initial and final cholesterol in females (**Table 1**) were significantly higher in Ola129 mice than in C57BL/6J. HDL-C, NEFA, TG and apoA-I were also significantly higher in the former strain than in the latter. In contrast, apoA-II, apoA-IV

Table 1. Initial cholesterol and final plasma lipid and apolipoprotein concentrations according to sex in apoE-deficient mice with different genetic backgrounds

	Female		Male	
	C57BL/6J ($n=10$)	Ola129 ($n=7$)	C57BL/6J ($n=7$)	Ola129 ($n=6$)
Body weight change (g)	7.5 ± 0.7	5.5 ± 0.6$^{\#}$	4.9 ± 1.1	4.0 ± 1.0
Liver weight (g)	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	0.9 ± 0.1$^{\#}$
Initial total cholesterol (mmol/L)	3.5 ± 0.3	5.0 ± 0.5*	5.2 ± 0.8	7.4 ± 1.1
Final total cholesterol (mmol/L)	9.4 ± 0.5	10.9 ± 0.5$^{\#}$	12.4 ± 1.9	19.8 ± 1.1$^{\#}$
VLDL + LDL cholesterol (mmol/L)	8.9 ± 0.6	9.6 ± 0.5	11.9 ± 1.9	18.5 ± 1.0$^{\#}$
HDL cholesterol (mmol/L)	0.5 ± 0.1	1.3 ± 0.1*	0.5 ± 0.1	1.3 ± 0.1*
NEFA (mmol/L)	0.2 ± 0.1	0.5 ± 0.1$^{\#}$	0.7 ± 0.1	0.4 ± 0.1$^{\#}$
Triglycerides (mmol/L)	0.6 ± 0.4	1.5 ± 0.2*	2.3 ± 0.4	1.7 ± 0.3
ApoA-I (AU/ L)	21 ± 3	185 ± 26*	46 ± 11	212 ± 14*
ApoA-II (AU/L)	24 ± 3	10 ± 1*	93 ± 15	18 ± 3†
ApoA-IV (AU/L)	16 ± 1	10 ± 2$^{\#}$	16 ± 2	7 ± 1†
ApoA-V (AU/L)	6.9 ± 0.2	6.2 ± 0.4*	6.4 ± 0.5	5.2 ± 0.2†
ApoJ (AU/L)	2.1 ± 0.2	2.0 ± 0.2	3.8 ± 0.8	1.9 ± 0.1*

Mice were fed chow diet for 14 weeks and fasted overnight before blood collection. Results are expressed as the means ± SEM. The Mann-Whitney U-test was used for pair-wise comparisons.
$^{*}p \le 0.001$ vs C57BL/6J $^{\dagger}p \le 0.004$ vs C57BL/6J $^{\#}p \le 0.05$ vs C57BL/6J
VLDL: very-low-density lipoproteins; LDL: low-density lipoproteins; HDL: high-density lipoproteins; NEFA: nonesterified fatty acids; Apo: apolipoprotein.

and apoA-V were elevated in C57BL/6J mice. No change was observed for apoJ. The FPLC profiles of cholesterol distribution into lipoproteins (**Fig. 1A**) corroborated the fact that the elevated cholesterol levels of the Ola129 strain were mainly due to increases in HDL and LDL cholesterol.

The results in male mice are also presented in **Table 1**. After the experimental period, males gained on average 4 g body weight with no significant difference between the two strains. No significant variation in food consumption was observed among the groups (3.7 ± 0.5 g), but the livers of Ola129 mice weighed significantly less than those of the C57BL/6J.

Final plasma cholesterol levels in males (**Table 1**) were significantly higher in Ola129 than in C57BL/6J mice, as were those of very-low-density (VLDL) + LDL cholesterol, HDL-C and apoA-I. No significant variation in TG was noted, despite the decrease in NEFA in Ola129 mice, and apoA-II, apoA-IV, apoA-V and apoJ concentrations were significantly increased in C57BL/6J compared to Ola129 mice. The FPLC profiles of cholesterol distribution into lipoproteins (**Fig. 1B**) corroborated the increased cholesterol levels of the Ola129 strain, particularly in VLDL and HDL fractions.

Oxidative and Inflammatory Parameters of the Two Strains according to Sex

The antioxidative mechanisms were tested through paraoxonase, an antioxidant enzyme present in HDL. The three activities reported for the enzyme, corresponding to arylesterase, lactonase and paraoxonase, were assayed and the results are presented in **Table 2**. In females, arylesterase and paraoxonase activities were significantly higher in Ola129 than in C57BL/6J mice. A similar finding was observed in males although, in this case, the significant differences corresponded to lactonase and paraoxonase activities. To verify whether these changes were due to changes in HDL-C, the ratio of all activities of paraoxonase was expressed in reference to HDL-C. As shown in **Table 2**, no matter which activity was studied, the ratio of paraoxonase to HDL-C was significantly higher in C57BL/6J than in Ola129 mice, irrespective of sex.

The antioxidant capacity of HDL particles against oxygen radicals of LDL was also tested. As shown in **Fig. 1C**, rather than reducing LDL oxidation, HDL from C57BL/6J mice of both sexes contributed to the further increase. HDL from female Ola129 mice was quite effective in inactivating oxygen radicals, while that from males was less effective.

Chitotriosidase activity has been found to be a

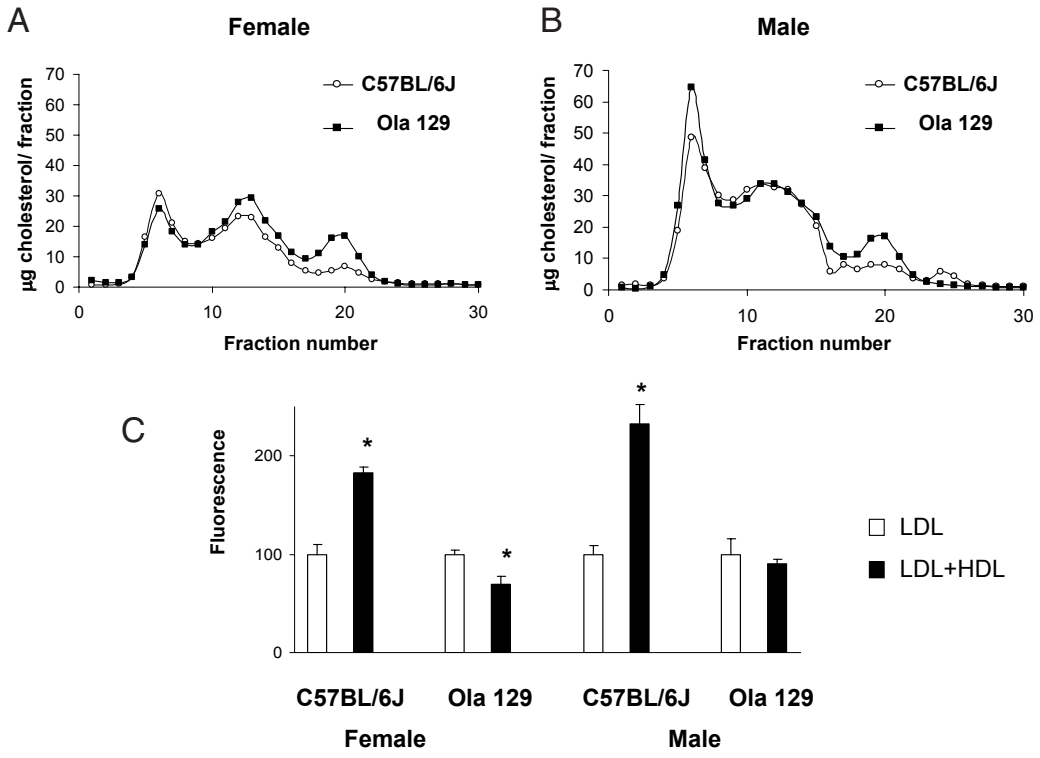

Fig. 1. Fast protein liquid chromatography (FPLC) plasma lipoprotein profiles of apoE-knockout mice from two genetic backgrounds, according to sex

Lipoproteins were fractionated by FPLC (two independent pools for each condition). Results are presented as µg of cholesterol per fraction. A and B, representative patterns from female and male mice, respectively. Isolated lipoproteins were: fraction numbers 4–8 corresponded to very-low-density lipoproteins (VLDL), 10–17 to low-density lipoproteins (LDL) and 18–23 to high-density lipoproteins (HDL). C; antioxidant capacity of HDL. The presence of oxygen radicals in LDL was estimated by fluorescence emission of mouse LDL incubated with HDL prepared from different experimental groups and assayed as described in methods. Data are the mean ± standard error of the mean (SEM) of three experiments. Statistical analyses were performed by the Mann-Whitney comparison between pairs. $^{*}p < 0.05$ vs LDL.

Table 2. Markers of oxidative stress and inflammation according to sex in apoE-deficient mice with different genetic backgrounds fed chow diet for 14 weeks

	Female		Male	
	C57BL/6J ($n = 10$)	Ola129 ($n = 7$)	C57BL/6J ($n = 7$)	Ola129 ($n = 6$)
Arylesterase activity (IU/L × 1000)	12 ± 1.6	18 ± 0.3$^{\#}$	17 ± 1.0	19 ± 0.7
Lactonase (IU/L × 1000)	3.2 ± 0.5	3.9 ± 0.5	2.9 ± 0.3	3.9 ± 0.3$^{\#}$
Paraoxonase (IU/L × 1000)	63 ± 10	89 ± 10$^{\#}$	56 ± 5	89 ± 8$^{\#}$
Arylesterase activity (IU × 1000/mmol HDL cholesterol)	32 ± 6.0	13 ± 1.3$^{\#}$	46 ± 5.3	15 ± 1.3*
Lactonase (IU × 1000/mmol HDL cholesterol)	8.6 ± 1.8	3.0 ± 0.5$^{\#}$	7.5 ± 0.7	3 ± 0.3*
Paraoxonase (IU × 1000/mmol HDL cholesterol)	166 ± 66	66 ± 9.0$^{\#}$	146 ± 14	68 ± 4*
Chitotriosidase (nmol/min/L)	1.9 ± 0.3	2.7 ± 0.5	2.1 ± 0.3	3.5 ± 0.2$^{\#}$
Mac-1 (% of cells)	17 ± 2	27 ± 3$^{\#}$	31 ± 7	28 ± 4
VLA-4 (% of cells)	69 ± 7	60 ± 6	55 ± 6	67 ± 6

Results are expressed as the means ± SEM. The Mann-Whitney U-test was used to pair-wise comparisons.
$^{*}p \leq 0.001$ vs C57BL/6J $^{\#}p \leq 0.05$ vs C57BL/6J
IU: international units; HDL: high-density lipoproteins.

biochemical marker of macrophage activation[19]. To test its relevance in this experimental setting, its activity was assayed and is presented in **Table 2**. The plasma concentration was increased in Ola129 mice compared to C57BL/6J, although only in males did the difference reach statistical significance. Mac-1 and VLA-4 are integrins present in circulating monocytes and involved in their vascular recruitment[24]. To verify whether there are differences in expression in the two strains, the expression in circulating monocytes was analyzed and is shown in **Table 2**. In females, circulating monocytes of Ola129 mice expressed a significantly higher level of Mac-1 than those of C57BL/6J females. No such difference was noted for males. Regarding VLA-4 expression, no significant differences were observed in either sex when the two strains were compared.

Atherosclerotic Lesion Area of the Two Strains According to Sex

Fig. 2 (**a-d**) shows representative atherosclerotic foci of experimental groups at the end of the experiment. Infiltration of the intima by foam cells and disorganization of the media were the main features of the lesions. A summary of the atherosclerotic lesion areas, expressed as cross-sectional aortic root lesion areas in mice at the end of the experimental period, is presented in **Fig. 2e**. In females, the lesion area was significantly smaller in Ola129 than in C57BL/6J mice. In contrast, in males no significant difference was observed between the two strains.

Histological Analysis of Livers of the Two Strains According to Sex

Fig. 3 shows representative histological images of livers from animals of both sexes from the two strains. Livers from female (**Fig. 3a**) as well as from male (**Fig. 3c**) C57BL/6J mice showed marked macrovesicular and microvesicular steatosis compared to those from Ola129 mice (**Fig. 3b**: females; **Fig. 3d**: males). Quantitative evaluation of the percentage of hepatic fat areas of all animals is shown in **Fig. 3e** and data showed a higher hepatic fat content in the C57BL/6J strain than in Ola129, irrespective of sex.

Association among Parameters in the Two Sexes

In females, HDL-C levels and ApoA-I were negatively associated with aortic atherosclerotic lesions, while the association with apoA-II was positive (**Table 3**). HDL-C was positively and negatively associated with ApoA-I and ApoA-II concentrations, respectively. An inverse correlation between the two apolipoproteins was also found. Plasma triglycerides were found to be associated with ApoA-I, ApoA-II, hepatic fat, HDL-C and liver weight.

In males, no significant association was observed between aortic atherosclerotic lesions and HDL-C, ApoA-I or ApoA-II (**Table 3**); however, HDL-C was positively associated with ApoA-I. ApoA-II was also positively associated with liver weight and hepatic fat (**Table 3**). Plasma TG was positively associated with plasma apoA-II and liver weight, in contrast to the situation in females and representing a trend toward a sex-related difference.

Discussion

From this study, focussed on the phenotypic characterization of apoE deficiency against Ola129 and C57BL/6J genetic backgrounds in both sexes, several findings have emerged. First, the differences in atherosclerotic lesions between Ola 129 and C57BL/6J strains of apoE-deficient mice are sex-dependent, being statistically significant only in females. Second, changes in HDL-C and apoA-I and apoA-II are important determinants in this variation. Third, the degree of hepatic steatosis is related to the strain but is independent of sex. Fourth, plasma apoA-II levels are associated with plasma TG in both sexes, although showing different trends. Finally, plasma TG is associated with liver weight and, again, the trend differs in the two sexes (**Table 3**).

Sex has emerged as an important determinant of the extent of atherosclerosis against these two genetic backgrounds, with differences between them in terms of penetrance. In this regard, C57BL/6J females have larger atherosclerotic lesions than males, while this is not so evident in Ola129. A similar result was observed by Wang *et al.*[6] when they compared apoE-deficient mice with C3H and C57BL/6J genetic backgrounds. These authors also described the lack of involvement of an immunological component, in line with the changes in chitotriosidase and markers of monocyte activation observed in this report, where the values were decreased in the strain in which aortic lesions were greater. Sexual dimorphisms among strains of mice have been evidenced by Svenson *et al.*[10] in plasma parameters from 43 strains fed high-fat diets. Using an SM/J by NZB/BlNJ intercross, Korstanje *et al.*[15] reported an important effect of sex on HDL-C. In the present study, we corroborated the latter finding and extended the results to the levels of apolipoproteins and paraoxonase, showing that HDL-C variation is accompanied by changes in plasma apolipoproteins A-I, A-II, A-IV and A-V, and paraoxonase load in HDL particles (**Table 1** and **2**). Furthermore,

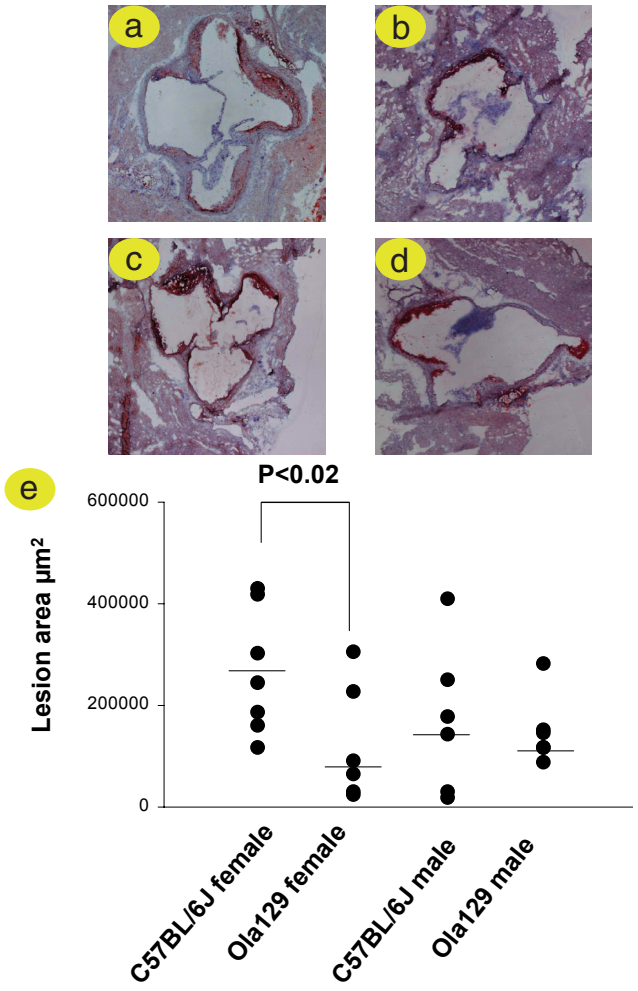

Fig. 2. Aortic root atherosclerotic lesions in female and male apoE-knockout mice with two genetic backgrounds

Representative micrographs at 40X magnification of aortic sections from female apoE-deficient mice with C57BL/6J (a) and Ola129 (b) genetic backgrounds. Images from male apoE-deficient mice with C57BL/6J (c) and Ola129 (d) genetic backgrounds. (e) Morphometric changes in aortic root lesions. Each symbol represents individual data of cross-sectional analysis of aortic lesion areas; bars represent means. Statistical analyses were performed according to the Mann-Whitney U-test and *p* vs C57BL/6J is shown.

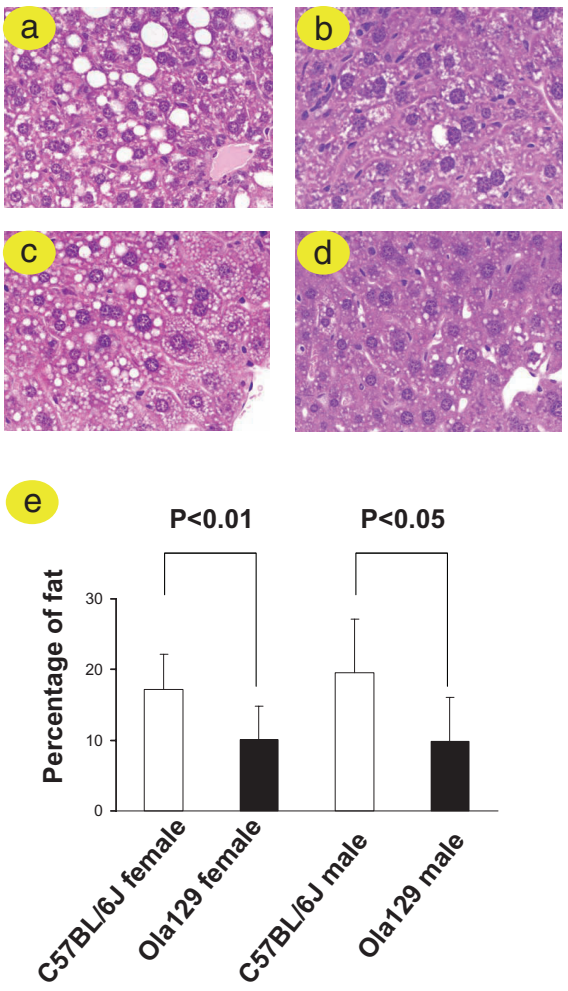

Fig. 3. Liver histology in female and male apoE-knockout mice with two genetic backgrounds

Representative liver micrographs at ×400 magnification from female apoE-deficient mice with C57BL/6J (a) and Ola129 (b) genetic backgrounds. Images from male apoE-deficient mice with C57BL/6J (c) and Ola129 (d) genetic backgrounds. Liver sections (4 µm) from each mouse were stained with hematoxylin and eosin, and evaluated blindly. (e) Morphometric changes in hepatic fat content. Data are the means ± standard error of the mean (SEM) for each group. Statistical analyses were performed according to the Mann-Whitney U-test.

the variation of apoA-I and apoA-II was only associated with the variation in atherosclerosis in females. It is interesting to note that, despite similar changes in males, these were not associated with atherosclerosis (**Table 3**). In this respect, several factors might be involved: the antioxidant capacity of HDL, as shown in **Fig. 1C**, was lower in male Ola129 mice than in females and, on the other hand, Ola129 males have a lipoprotein profile with a higher level of VLDL particles (**Fig. 1B**), which are considered less atherogenic[25].

Overall, these results point to a complicated network of interactions among apolipoproteins, HDL particles and their functionality, as well as the type of non-HDL lipoproteins that would result in atherosclerosis development in a sex-dependent manner.

An interaction between sex and genetic background likewise influences plasma levels of apoA-I and apoA-II and, to a lesser extent, apoA-IV and apoA-V levels. The former are the main protein components of HDL and are responsible for the differenc-

Table 3. Association of parameters in apoE-knockout mice according to sex

	Female				Male			
	Lesion size	ApoA-I	ApoA-II	Triglycerides	Lesion size	ApoA-I	ApoA-II	Triglycerides
ApoA-I	−0.64 $p<0.02$	----	-----	0.77 $p<0.000$	0.13 ns	-----	-----	−0.21 ns
ApoA-II	0.68 $p<0.01$	−0.83 $p<0.000$	-----	−0.70 $p<0.003$	0.18 ns	−0.48 ns	-----	0.63 $p<0.02$
Hepatic fat	0.33 ns	0.34 ns	0.43 ns	−0.61 $p<0.01$	0.33 ns	−0.47 ns	0.76 $p<0.002$	0.44 ns
HDL cholesterol	−0.63 $p<0.02$	0.75 $p<0.001$	−0.70 $p<0.003$	0.66 $p<0.004$	0.14 ns	0.92 $p<0.000$	−0.44 ns	−0.21 ns
Liver weight	0.34 ns	−0.37 ns	0.47 ns	−0.53 $p<0.02$	0.32 ns	−0.35 ns	0.83 $p<0.000$	0.54 $p<0.05$

Correlation analysis was performed according to Spearman's test. *p value* and its probability are shown.
Apo: apolipoprotein; HDL: high-density lipoprotein.

es in HDL-C concentrations between the two mouse strains. This would be partly in agreement with Wang *et al.*[7] although, through genetic analysis, using C57BL/6J and NZB/BlNJ mouse strains, these authors only predicted an association with hepatic expression of Apoa2. Using an SM/J by NZB/BlNJ intercross, Korstanje *et al.*[15] also described an important effect of sex on the involvement of the Apoa2 locus. These genetic analyses did not find the observed variation of apoA-I protein levels due to sex. Our data also provide further support for the finding that, in some strains, plasma apoA-I levels may be higher in males than in females, an observation previously reported by Korstanje *et al.*[15] at the level of HDL-C. Overall, the variation of HDL-C between sexes is typical of the variation in the genetic background, and genetic markers may not be enough to predict the involvement of proteins, as is the case of apoA-I.

Recently, Castellani *et al.*[26] proposed that apoA-II regulates the metabolism of TG-rich lipoproteins, HDL being a plasma reservoir of apoA-II that is transferred to TG-rich lipoproteins by a method similar to that by which they acquire most of their apoCs from HDL. In this line of evidence, our study of the association between apoA-II and TG would provide further *in vivo* support of this role of apoA-II (**Table 3**), although the different signs of the correlation coefficients point to the different relevance of the involvement according to sex. In fact, plasma apoA-II levels were higher in males irrespective of their genetic background (**Table 1**). Thus, in males, higher levels of this apolipoprotein would represent its important role, while the opposite might occur in females. Interestingly, liver weight and plasma TG were also significantly associated in both sexes (**Table 3**) and showed the same trend as apoA-II and TG, a fact that, considering that apoA-II is mainly biosynthesized in liver[27], would suggest that apoA-II is the intermediary involved in the latter associations. However, a significant association between this parameter and hepatic fat content has only been observed in males (**Table 3**), and the difference in hepatic fat content between the two strains is considerable but is not modified by sex (**Fig. 3**). Interestingly, the supply of NEFA to the liver was lower in Ola129 than in C57BL/6J males (**Table 1**), a circumstance that would contribute to explaining the difference in hepatic fat content between these two strains (**Fig. 3e**), and would also agree with recent results[28]. However, in females, the supply of NEFA was significantly higher in Ola129 than in C57BL/6J mice, in agreement with the increased plasma TG concentrations (**Table 1**). The latter finding would suggest an increased secretion of VLDL in the former strain; nonetheless, the chromatographic separation of plasma lipoproteins estimated as cholesterol content did not confirm this possibility (**Fig. 1A**), suggesting either a different composition of VLDL or secretion of a smaller particle in the range of LDL[29]. The latter possibility would agree with the observed Ola129 lipoprotein profile, but this is just a suggestion. Overall, these data indicate complex sorting of apolipoproteins and fat in the liver, with modulation according to strain and sex.

To summarize, we have found that differences in

atherosclerosis between Ola129 and C57BL/6J genetic backgrounds in apoE-deficient mice are sex-dependent and only statistically significant in females. This finding is mainly explained at the level of HDL-C and its apolipoprotein components, mainly apoA-I and apoA-II. The association between plasma apoA-II and TG levels differed in terms of the signs between sexes. Overall, our findings highlight the importance of taking sex into account in the analysis of atherosclerosis and lipid metabolism in animal models.

Acknowledgements

This research was supported by grants from FEDER-CICYT (SAF2007-60173 and SAF2008-1883), Gobierno de Aragón (PI025/08), Found de Investigaciones Sanitarias (RD06/0010/0015) and Redes DGA (B-69). CIBER is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. M.A.N. and N.G. were recipients of FEGA-FEOGA and Fundación Cuenca Villoro fellowships. We thank Angel Beltrán, Jesús Cazo, Jesús Navarro, Carmen Navarro and Clara Tapia from Unidad Mixta de Investigación for their invaluable help in maintaining the animals, and Rosario Puyó for her technical assistance. We thank Dr. M.D. Griswold for the generous gift of the antibody to mouse apoJ. We also thank Martha Messman for her assistance in manuscript editing.

References

1) Paigen B, Morrow A, Brandon C, Mitchell D, Holmes P: Variation in susceptibility to atherosclerosis among inbred strains of mice. Atherosclerosis, 1985; 57: 65-73
2) Nishina PM, Wang J, Toyofuku W, Kuypers FA, Ishida BY, Paigen B: Atherosclerosis and plasma and liver lipids in nine inbred strains of mice. Lipids, 1993; 28: 599-605
3) Allayee H, Ghazalpour A, Lusis AJ: Using mice to dissect genetic factors in atherosclerosis. Arterioscler Thromb Vasc Biol, 2003; 23: 1501-1519
4) Sarria AJ, Surra JC, Acín S, Carnicer R, Navarro MA, Arbonés-Mainar JM, et al: Understanding the role of dietary components on atherosclerosis using genetic engineered mouse models. Front Biosci, 2006; 11: 955-967
5) Shi W, Wang NJ, Shih DM, Sun VZ, Wang X, Lusis AJ: Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 mouse model: evidence for involvement of endothelial cells but not blood cells or cholesterol metabolism. Circ Res, 2000; 86: 1078-1084
6) Wang SS, Shi W, Wang X, Velky L, Greenlee S, Wang MT, et al: Mapping, genetic isolation, and characterization of genetic loci that determine resistance to atherosclerosis in C3H mice. Arterioscler Thromb Vasc Biol, 2007; 27: 2671-2676
7) Wang X, Le Roy I, Nicodeme E, Li R, Wagner R, Petros C, et al: Using advanced intercross lines for high-resolution mapping of HDL cholesterol quantitative trait loci. Genome Res, 2003; 13: 1654-1664
8) Guillén N, Acín S, Navarro MA, Perona JS, Arboné s-Mainar JM, Arnal C, et al: Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice. Atherosclerosis, 2008; 196: 558-564
9) Paigen B, Ishida BY, Verstuyft J, Winters RB, Albee D: Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. Arteriosclerosis, 1990; 10: 316-323
10) Svenson KL, Von Smith R, Magnani PA, Suetin HR, Paigen B, Naggert JK, et al: Multiple trait measurements in 43 inbred mouse strains capture the phenotypic diversity characteristic of human populations. J Appl Physiol, 2007; 102: 2369-2378
11) Carnicer R, Guzmán MA, Acín S, Surra JC, Navarro MA, Arbonés-Mainar JM, et al: Genetic background in apolipoprotein A-I and cystathionine β-synthase deficiency. Frontiers in Bioscience, 2008; 13: 5155-5162
12) Canto JG, Goldberg RJ, Hand MM, Bonow RO, Sopko G, Pepine CJ, et al: Symptom presentation of women with acute coronary syndromes: myth vs reality. Arch Intern Med, 2007; 167: 2405-2413
13) Shaw LJ, Bairey Merz CN, Pepine CJ, Reis SE, Bittner V, Kelsey SF, et al: Insights from the NHLBI-Sponsored Women's Ischemia Syndrome Evaluation (WISE) Study: Part I: gender differences in traditional and novel risk factors, symptom evaluation, and gender-optimized diagnostic strategies. J Am Coll Cardio, 2006; 47: S4-S20
14) Sheps DS, Kaufmann PG, Sheffield D, Light KC, McMahon RP, Bonsall R, et al: Sex differences in chest pain in patients with documented coronary artery disease and exercise-induced ischemia: Results from the PIMI study. Am Heart J, 2001; 142: 864-871
15) Korstanje R, Li R, Howard T, Kelmenson P, Marshall J, Paigen B, et al: Influence of sex and diet on quantitative trait loci for HDL cholesterol levels in an SM/J by NZB/BlNJ intercross population. J Lipid Res, 2004; 45: 881-888
16) Acín S, Navarro MA, Carnicer R, Arbonés JM, Guzmán MA, Arnal C, et al: Dietary cholesterol suppresses the ability of olive oil to delay the development of atherosclerotic lesions in apolipoprotein E knockout mice. Atherosclerosis, 2005; 182: 17-28
17) Escolà-Gil JC, Jorba O, Julve-Gil J, González-Sastre F, Ordóñez-Llanos J, Blanco-Vaca F: Pitfalls of direct HDL-cholesterol measurements in mouse models of hyperlipidemia and atherosclerosis. Clin Chem, 1999; 45: 1567-1569
18) Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, et al: Human serum paraoxonase (pon1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metab Dispos, 2000; 28: 1335-1342
19) Arbonés-Mainar JM, Navarro MA, Guzmán MA, Arnal C, Surra JC, Acín S, et al: Selective effect of conjugated linoleic acid isomers on atherosclerotic lesion development in apolipoprotein E knockout mice. Atherosclerosis, 2006; 189: 318-327
20) Navarro MA, Carpintero R, Acín S, Arbonés-Mainar JM, Calleja L, Carnicer R, et al: Immune-regulation of the apolipoprotein A-I/C-III/A-IV gene cluster in experimen-

tal inflammation. Cytokine, 2005; 31: 52-63
21) Calleja L, París MA, Paul A, Vilella E, Joven J, Jiménez A, et al: Low-cholesterol and high-fat diets reduce atherosclerotic lesion development in ApoE-knockout mice. Arterioscler Thromb Vasc Biol, 1999; 19: 2368-2375
22) Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST, Fogelman AM: A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. J Lipid Res, 2001; 42: 1308-1317
23) Paigen B, Morrow A, Holmes P, Mitchell D, Williams R: Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis, 1987; 68: 231-240
24) Libby P, Aikawa M: Stabilization of atherosclerotic plaques: New mechanisms and clinical targets. Nature Med, 2002; 8: 1257-1262
25) Veniant MM, Withycombe S, Young SG: Lipoprotein size and atherosclerosis susceptibility in Apoe(-/-) and Ldlr(-/-) mice. Arterioscler Thromb Vasc Biol, 2001; 21: 1567-1570
26) Castellani LW, Nguyen CN, Charugundla S, Weinstein MM, Doan CX, Blaner WS, et al: Apolipoprotein AII is a regulator of very low density lipoprotein metabolism and insulin resistance. J Biol Chem, 2008; 283: 11633-11644
27) Tailleux A, Duriez P, Fruchart JC, Clavey V: Apolipoprotein A-Ⅱ, HDL metabolism and atherosclerosis. Atherosclerosis, 2002; 164: 1-13
28) Guan HP, Goldstein JL, Brown MS, Liang G: Accelerated fatty acid oxidation in muscle averts fasting-induced hepatic steatosis in SJL/J mice. J Biol Chem, 2009; 284: 24644-24652
29) Stillemark-Billton P, Beck C, Boren J, Olofsson SO: Relation of the size and intracellular sorting of apoB to the formation of VLDL 1 and VLDL 2. J Lipid Res, 2005; 46: 104-114