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Basic nutritional investigation

Dietary proteins modulate high-density lipoprotein characteristics in a sex-specific way in Apoe-deficient mice

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ABSTRACT

Objectives: The type and amount of dietary protein have become a topic of renewed interest, considering their involvement in several diseases. However, little attention has been devoted to the effect of avian proteins despite their wide human consumption. In a previous study, we saw that compared with soybean protein, the consumption of avian proteins, depending on sex, resulted in similar or lower atherosclerosis with a higher paraoxonase 1 activity, an antioxidant enzyme carried by high-density lipoproteins (HDL). This suggests that under these conditions, the HDL lipoproteins may undergo important changes. The aim of this research was to study the influence of soybean, chicken, and turkey proteins on the characteristics of HDL.
Methods: Male and female *Apoe*-deficient mice were fed purified Western diets based on the AIN-93 diet, differing only in the protein source, for 12 wk. After this period, blood and liver samples were taken for analysis of HDL composition and hepatic expression of genes related to HDL metabolism (*Abca1, Lcat, Pltp, Pon1,* and *Scarb1*). Depending on sex, these genes define a different network of interactions. Females consuming the turkey protein–containing diet showed decreased atherosclerotic foci, which can be due to larger very-low-density lipoproteins (VLDLs) calculated by molar ratio triacylglycerols/VLDL cholesterol and higher expression of *Lcat*. In contrast, in males, a higher ratio of paraoxonase1 to apolipoprotein A1 decreased the oxidative status of the different lipoproteins, and augmented *Abca1* expression was observed.
Conclusions: The source of protein has an effect on the development of atherosclerosis depending on sex by modifying HDL characteristics and the expression of genes involved in their properties.

Introduction

The amount and quality of ingested dietary protein is a crucial issue. US government dietary guidelines recommend a protein intake between 10% and 35% of the total caloric value (TCV) in adults depending on age and sex, for maintenance and growth; the current consumption of the population is around 16% of TCV [1]. It is important to note that not all the ingested protein is absorbed and that proteins are composed of 20 amino acids, 9 of which are

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essential in humans and other animals and must be obtained from the diet [2]. For this reason, a suitable proportion and amount of every essential amino acid is important for a well-balanced diet.

In 2013, The Food and Agriculture Organization (FAO) published a reference manual to evaluate nutritional protein quality [3], which incorporated the digestible indispensable amino acid score (DIAAS) to assess their bioavailability. In fact, soy protein isolate had a DIAAS of 0.84 [4], whereas chicken breast had a DIAAS of 1.08 [5] measured with crude protein throughout the whole digestive tract in pigs. In these experiments, the diets were prepared according to the recommended indications of protein sources to fulfill the different physiologic requirements for maintenance and/or growth and according to each species [6,7]. From this point of view, the bioavailability of essential amino acids could be lower in diets elaborated with proteins of vegetable origin compared with animal proteins because of the lower DIAAS value for the same amount of protein.

The influence of sources of proteins modulating the predisposition to certain pathologies related to metabolic diseases, atherosclerosis, and thrombosis has received little attention compared with other nutritional compounds, such as lipids or carbohydrates [8]. The fact that amino acids may require specific transporters and that some receptors are sensitive to these compounds are elements to hypothesize that they could contribute to the regulation of proteostasis and, through an imbalance of the latter, to the development of diseases [9].

The best way to evaluate the influence of the dietary amino acid composition of a certain protein, without changes in the rest of the nutrients, is achieved using purified diets, only differing on the type of protein [10]. The mouse lacking apolipoprotein E (APOE) has the peculiarity that develops spontaneous atherosclerosis and fatty liver in 10 to 12 wk of diet intervention, and it is considered a good animal model to test the influence of nutrients on these ailments [11,12]. Previous results showed that there were changes in the atherosclerotic lesion of mice lacking APOE depending on the source of purified protein: soy, turkey, and chicken, as depicted in Figure 1 [8]. This study showed that the consumption of avian proteins, depending on sex, resulted in similar or lower atherosclerosis development in females, and it was also associated with a higher paraoxonase 1 activity in males. Paraoxonase 1, an enzymatic glycoprotein synthesized mainly in the liver, is carried out by high-density lipoproteins (HDLs) and transferred to other tissues where its activity is required [13], thereby contributing to the cardioprotective properties of HDLs [14]. Paraoxonase 1 has an arylesterase activity and is a calcium-dependent enzyme, capable of hydrolyzing substrates such as organophosphates, aromatic carboxylic acid esters, N-acyl homoserine lactone, or lipo-lactones [15] and whose biological substrate remains unknown. A systematic review showed that few studies had addressed its regulation by dietary protein [16]. With this background, a detailed characterization of HDL of mice consuming different dietary protein sources is required. To continue this line of research, HDL properties, and some involved components are explored in Apoe-deficient mice in this experimental setting.

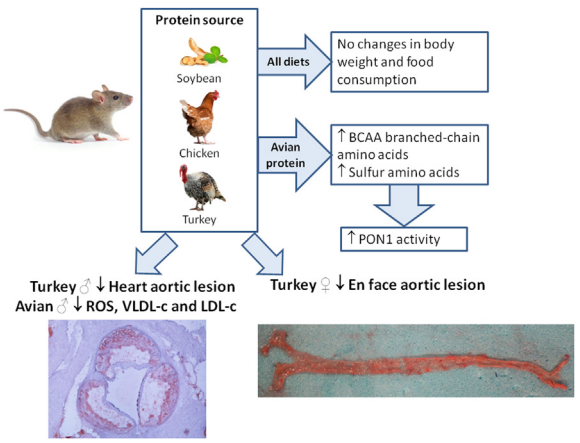

Fig. 1. Summary of previous findings (adapted from Martínez-Beamonte, et al. 2021 [8]). LDL-C, low-density lipoprotein cholesterol; ROS, reactive oxygen species; VLDL-C, very low-density lipoprotein cholesterol.

Material and methods

Animals and experimental procedure

We placed 2-mo-old Apoe-deficient mice of both sexes into homogeneous groups of similar body weight and plasma cholesterol at the beginning of the study. Male groups of 14 mice for soybean and chicken diets, 16 for turkey, and female groups of 10 mice for all groups were used. The mice were fed purified diets in which the only change was the source of protein, soy, chicken, or turkey proteins over 12 wk, the required time for this model to develop vascular injury consuming a Western diet [17,18]. The differences between the diets in the amino acid composition showed a lower amount of sulfured and branched-chain amino acids (BCAA) in the soybean diet (Fig. 1). After the dietary intervention, the mice fasted overnight, and serum and plasma samples were taken, as well as different tissues such as liver, heart, and aorta were taken. Animals were handled and sacrificed, observing guidelines from the European Union for the care and use of laboratory animals in research, and the Ethics Committee approved the study protocol for Animal Research of the University of Zaragoza.

Diets

To study only the effect of protein, purified diets were prepared differing only in the source of protein, soybean, chicken, and turkey, following the recommendations of the Nutrient Requirements of Laboratory Animals [7], as previously described [8]. After preparing the diets, they were frozen and lyophilized in vacuum bags, keeping them at −20°C until used. The intake was controlled weekly, and the weight was monitored every 2 wk [8].

Plasma parameters

Plasma determinations were performed using commercial kits: Infinity Reagent (Thermo Scientific, Madrid, Spain) for total cholesterol and triacylglycerides (Glucose ref. 11503; (Biosystems Reagents and Instruments Barcelona, Spain) for glucose and non-esterified fatty acids (NEFA) with a Fujifilm kit (Fujifilm Wako Chemicals Europe, Neuss, Germany). All kits were used according to the manufacturer's instructions. An in-house enzyme-linked immunosorbent assay was used to determine plasma APOA1 concentrations as previously described [19].

Paraoxonase 1 activity

Paraoxonase 1 activity was assayed as arylesterase activity in sera [20], according to a method previously described [21].

RNA extraction and quantification

Total RNA of individual liver samples was extracted using a Direct-zol RNA Miniprep TRIzol (Zymo Research, Irvine, CA, USA), using a DNAse I to eliminate the contaminating DNA traces, according to the manufacturer's instructions. The quantification of RNA samples was determined by absorbance at 260 nm, and purity was verified regarding the A260/280 ratio (>1.8) with SPECTROstar^{Nano} Spectrophotometer, using a LVis plate (BMG Labtech, Offenburg, Germany). Integrity of RNA samples was verified by 1% agarose gel electrophoresis containing ethidium bromide, observing the well-defined and without secondary band degradation of the 28S and 18S ribosomal RNAs.

We reverse-transcribed 500 ng of total RNA of each sample using the PrimeScript RT Reagent Kit (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. The quantitative reverse transcription polymerase chain reactions were done using PowerUp SYBR Green Master Mix reagents from Applied Biosystems (Bedford, MA, USA) according to the manufacturer's instructions. We evaluated the expression of the main genes related to reverse cholesterol transport, *Abca1* (ATP binding cassette sub-family A member 1), *Lcat* (lecithin-cholesterol acyltransferase), *Scarb1* (scavenger receptor class B type 1), *Pon1* (paraoxonase 1), and *Pltp* (phospholipid transfer protein). All reactions were determined by duplicating the $2^{-\Delta\Delta Ct}$ method and normalizing the results using the gene *Ppib* (peptidylprolyl isomerase B) [22,23]. The used primer concentrations, according to MIQE guidelines, are indicated in Supplementary Table 1.

Statistical analyses

Data are shown as means ± SD. Variables were tested for normal distribution (according to the Shapiro–Wilk test) and homology of variance among groups using Bartlett or Levene tests. Parameters fitting both criteria were compared using one-way analysis of variance (ANOVA), according to Tukey's multiple comparison test as post hoc analysis. Non-parametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons were used to compare the groups failing in any of the hypotheses. Differences were considered significant when $P < 0.05$. Correlations among all variables were analyzed using two-tail Spearman's correlation coefficient tests. SPSS version 25 (IBM, Armonk, NY, USA) or Prism 8 software for Windows (GraphPad, San Diego, CA, USA) was used for statistical analyses.

Results

Food consumption and body weight

During the dietary intervention, the experimental groups did not show statistical differences in body weight gains with independence of sex and food consumption (Fig. 1), with a daily food intake for males between 2.75 and 3.5 g/animal and between 2.25 and 2.75 for females in all groups and during the 12 wk of dietary intervention.

Molar ratio of TG/VLDL cholesterol

In this model, TGs are mainly vehicles in very low-density lipoprotein (VLDL) [24], so this ratio may be a subrogate of the size of these particles. The results showed significant differences in males among all the studied groups, being statistically significant between the soy and chicken groups and between turkey and chicken (Fig. 2A) since the smallest VLDL corresponded to the chicken group. In females, the VLDL size was larger than in males, with values of 0.25 TG mM/VLDL cholesterol (C) for the soybean and chicken groups and 0.37 TG mM/VLDL-C for the turkey group. The latter group showed the largest VLDL particles, being its results statistically significant compared with the soybean and chicken groups (Fig. 2B).

Ratio of paraoxonase 1 activity to APOA1

The ratio of paraoxonase 1 activity to APOA1 could be a measure of the antioxidant properties of the HDL particle. In males, the antioxidant properties of the HDL of the avian groups were higher than the soybean groups (Fig. 3A). In females, the values were slightly higher in the avian group but without statistical significance (Fig. 3B).

HDL-C/APOA1 ratio

The HDL-C to APOA1 ratio is a measure of the cholesterol load of the HDL [25,26]. Figure 3C depicts the male ratios, which did not show any statistical significance. In contrast, female HDL from the chicken group showed a significantly increased load in cholesterol compared with other groups.

Hepatic mRNA expressions and proposed networks

The expression of the main genes related with reverse cholesterol transport (RCT), one of the main functions of HDL [27], were studied in the liver. In males (Table 1), there were no statistical significances in *Lcat* and *Pltp* gene expressions. In contrast, *Pon1* was decreased in the turkey versus soybean group, *Abca1* was increased in the turkey group with respect to the soybean group, and *Scarb1* was decreased in both avian groups.

In contrast, in females (Table 1), statistical differences were observed in *Lcat*, increased in the turkey group with respect to the soybean group, and in *Pon1*, with higher expressions in the chicken group with respect to the others, and no changes were observed in *Pltp, Abca1*, and *Scarb1*.

Network correlations obtained from mRNA results revealed significant complex relationships among transcripts. Significant association of gene expression in males (Fig. 4A) evidenced two clusters: one formed by *Lcat, Scarb1*, and *Pon1* expressions and the other constituted by *Pltp* and *Abca1* transcript levels. The association between these two mRNAs was highly significant (Fig. 4B). Notably, *Pon1* expression in females was not connected to *Abca1, Lcat, Pltp*, or *Scarb1*, which showed one cluster (Fig. 4C). In this sex, the association between *Pltp* and *Abca1* transcripts was even stronger than in males (Fig. 4D).

Evaluation of aortic lesions

The complete evaluation of aortic lesions must be carried out with the en-face analysis, which analyzes the lesion surface

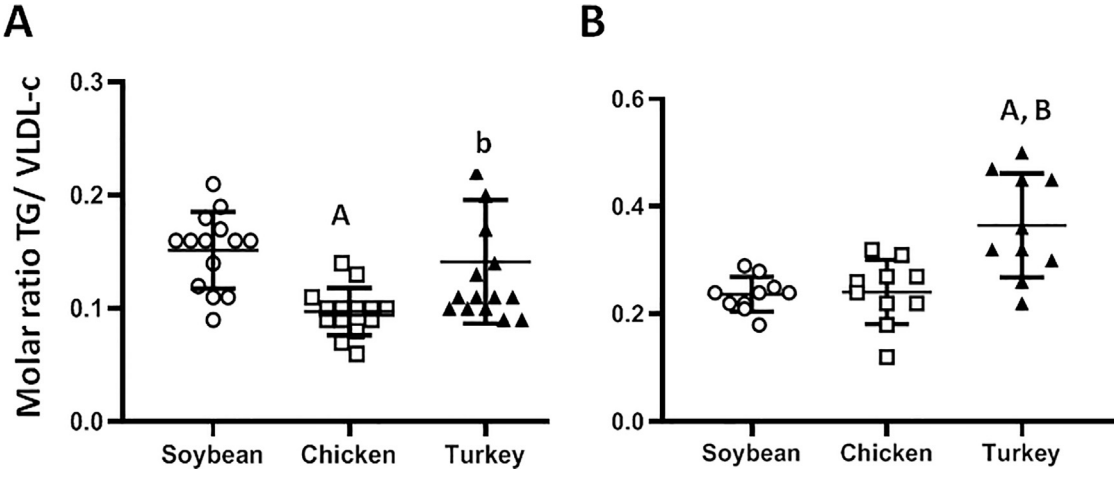

Fig. 2. Ratio of TG to cholesterol in VLDL. (**A**) Males and (**B**) females. Data are individual values with their means ± SD for each group. In males, n = 14 except for the turkey group (n = 16) and females (n = 10). Statistical analysis was carried out by one-way analysis of variance, followed by Tukey's post hoc test. $^{*}P < 0.001$ vs soybean and †,‡$P <$ 0.01 or 0.05, respectively vs chicken. TG, triacylglyceride; VLDL-C, very low-density lipoprotein cholesterol.

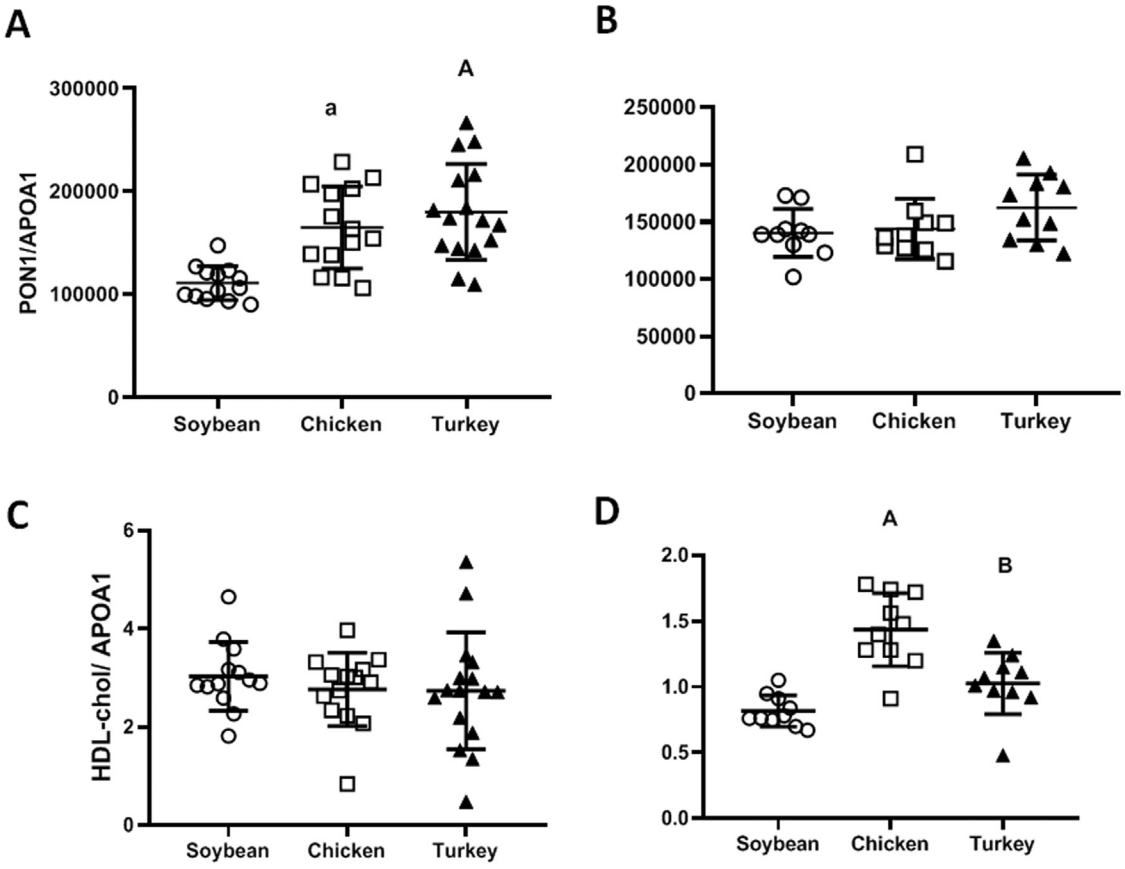

Fig. 3. Characteristics of HDL. (**A**) The ratio of PON1 activity to APOA1 corresponds to males and (**B**) to females. (**C**) The ratio of HDL-C to APOA1 corresponds to males and (**D**) to females. Scatter graph of individual samples with their means and SD. In males, n = 14 except for the turkey group (n = 16) and females (n = 10). Statistical analysis was carried out by one-way analysis of variance, with post hoc comparison using Tukey's test. $^{*}P \leq 0.05$ vs soybean. $^{\dagger}P \leq 0.01$ vs soybean. $^{\ddagger}P \leq 0.01$ vs chicken. HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol.

of the entire aorta, and the cross-sectional study, which evaluates the enlargement of the plaques at the level of the aortic valve. In males, the cross-sectional study revealed a decreased lesion in the turkey group (mean value of 324036 μm^2) compared with the soybean group (443098 μm^2). At the same time, in females, the en-face analysis showed a lower percentage of lesions in the turkey group (3%) versus 5% in the chicken group [8] as summarized in Figure 1.

Table 1
RT-qPCR results of gene expressions in males and females

Males	Soybean (n = 14)	Chicken (n = 14)	Turkey (n = 16)
Abca1	1 ± 0.3	1.7 ± 0.5	1.4 ± 0.4*
Lcat	1 ± 0.4	1.1 ± 0.4	1 ± 0.4
Pltp	1.1 ± 0.6	1.5 ± 1.2	1 ± 0.7
Pon1	1 ± 0.2	0.9 ± 0.3	0.8 ± 0.2*
Scarb1	1 ± 0.3	0.6 ± 0.1*	0.7 ± 0.2*
Females	Soybean (n = 10)	Chicken (n = 10)	Turkey (n = 10)
Abca1	1.4 ± 1.2	1.2 ± 0.4	1.9 ± 1.4
Lcat	1 ± 0.6	1.1 ± 0.4	2 ± 1.1*
Pltp	1.3 ± 1.0	1.2 ± 0.4	1.5 ± 0.9
Pon1	1.1 ± 0.4	1.7 ± 0.7*	1.0 ± 0.4†
Scarb1	1 ± 0.3	1.1 ± 0.3	1.3 ± 0.4

Results expressed as media ± SD
Statistical analysis carried out by Mann–Whitney based on their data distributions
$^{*}P \leq 0.05$ vs soybean.
$^{\dagger}P \leq 0.05$ vs chicken.

Discussion

The present investigation was carried out to study the influence of soybean, chicken, and turkey proteins on the characteristics of the HDL and the atherosclerotic lesion. The different purified diets were prepared in our laboratory according to the current recommendations [7]. To minimize the variables that could alter the different macro- and micronutrients, the unique variation among them was the source of protein. Using this approach, as reflected in a previous report of diet compositions [8] (Fig. 1), soy protein contained fewer BCAA and sulfur amino acids than proteins of animal origin. These differences in the amino acid compositions were accompanied by a lower DIAAS value for soybean protein isolates compared with chicken breast [28]. Despite these changes, the soybean group did not show significant changes in feed intake or body weight. However, more subtle effects might be observed in some biological functions such as the biosynthesis of proteins rich in BCAAs or sulfur-containing amino acids, mainly in males where protein requirements are larger than in females [1,8]. In fact, the male lipoprotein reactive oxygen species content was worse in the soybean group than in all studied lipoproteins, as previously reported [8] (summarized in Fig. 1). These changes could be due to the different amounts of BCAAs [29], as they were involved in endothelial dysfunction that was corrected using antioxidants [30]. The mentioned increase in oxidized lipoproteins of the soybean-fed mice was in concordance with the worse ratio of PON1 to APOA1 of the same group (Fig. 3A), despite the higher expression

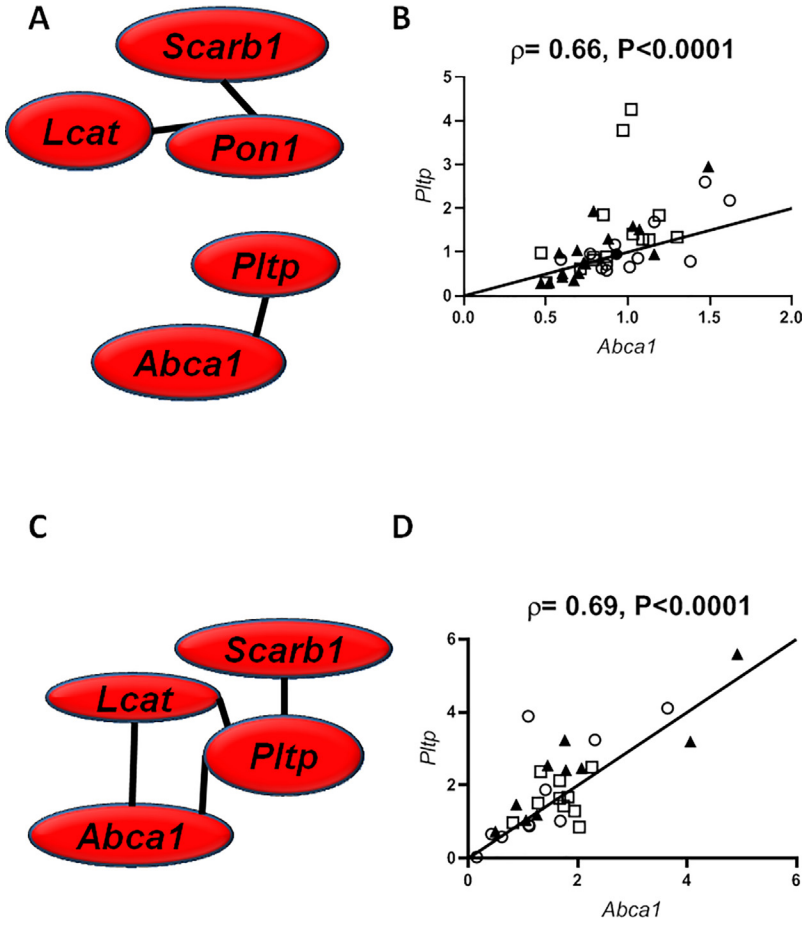

Fig. 4. Network of hepatic gene expressions codifying for proteins involved in high-density lipoprotein cholesterol metabolism. Significant association of gene expression in males (**A**) and in females (**C**) and detailed representation of individual values of *Pltp* and *Abca1* in males (**B**) and in females (**D**). Red denotes a positive significant association. Correlations were calculated according to Spearman's rho test.

of hepatic Pon1 mRNA compared with the turkey-fed group (Table 1). In contrast, a lower expression of hepatic Pon1 with a higher PON1/APOA1 ratio was observed in males in the turkey group with respect to the soybean group. Considering that the turkey group displayed a decreased cross-sectional atherosclerotic lesion [8], the characteristics of these lipoproteins could contribute to the observed pathologic changes. Moreover, the animals fed with the poultry protein had decreased *Scarb1* expression, and those consuming the turkey protein showed a higher *Abca1* expression with respect to the soybean group. Considering the roles of these two proteins in RCT [31,32], it could be hypothesized that consumption of turkey proteins might affect this pathway as well.

Our results of a higher ratio of PON1 to APOA1 (Fig. 3A) in males consuming the avian dietary protein raising values comparable to those observed in females pose a setting where dietary protein plays an important role (Fig. 3B). These results agree with decreased serum PON1 activity in male mice [33] and those of Bin et al., who described sex differences in serum PON1 activity between both sexes in Syrian hamsters [34]. Additionally, our results also indicate that the protein source influences the sex-differential effect, and the latter may represent a new way to favor this activity, particularly in males. Moreover, when gene networks were obtained using gene expressions (Fig. 4A), the clustering of *Pon1* was also sex-dependent. In males, it was associated with *Lcat* and *Scarb1* expressions, but this did not happen in females (Fig. 4C). It could be anticipated that hepatic regulation of *Pon1*, *Lcat*, and *Scarb1* expressions is shared in response to protein sources but differs between sexes. Moreover, the male increased ratio of PON1/ to APOA1 in both avian groups with respect to the soybean group (Fig. 3A), without changes in APOA1 [8], and the decreased hepatic Pon1 expression in the turkey group (Table 1) compared with the soybean group suggested other cellular mechanisms such as post-transcriptional or translational events may be involved and are also modulated by the source of dietary protein.

A strong association between hepatic *Pltp* and *Abca1* transcript levels independent of sex (Fig. 4B and D) was observed in this study. This would indicate a shared regulation of both genes, which would agree with the proposed function of PLTP, transporting cholesterol and phospholipids from cells to lipoprotein particles, by a process involving PLTP interactions with ATP-binding cassette transporter A1 (ABCA1) [35,36]. Our results, in this case, suggest that the source of protein has little effect on *Pltp* gene expression since no significant changes were observed.

Another important finding was the difference between both sexes in the molar ratio of TG to VLDL-C (Fig. 2), a subrogate of the size of these VLDL, with medium values of 0.12 in males and 0.30 in females, mainly due to the higher levels of total cholesterol in these male particles. Considering the relevance of lipoprotein sizes, since those <70 nm may easily penetrate the endothelial layer and remain in the subendothelial space [37], this would indicate that in males, the vascular lesions could be the consequence of these

smaller VLDLs. Groups receiving the turkey protein increased the size of these particles (Fig. 2), thereby making them less prone to enter the subendothelial space and contributing to explaining the observed atherosclerotic lesion (Fig. 1). This was particularly relevant in females consuming the turkey protein that showed the largest VLDL (Fig. 2B) and decreased en-face atherosclerosis lesion (Fig. 1). These results show that the source of protein may also induce changes in the size of lipoproteins and in this way contribute to the observed vascular lesion.

Our study provides evidence that the size of HDL particles (HDL-C per APO1) was also influenced by sex and diet. Females consuming the chicken protein showed the highest values compared with other groups (Fig. 3D). Thus, this group displayed a high load of cholesterol in these particles without modifying their PON1 activity/APOA1 (Fig. 3B). HDL-loaded cholesterol may be the consequence of activated cholesterol efflux by *ABCA1*, *Pltp*, and *Lcat* activities or decreased uptake by *Scarb1* [38]. When they were studied at the mRNA level, no change was observed in the liver of female mice consuming the chicken protein. Contrarily, females fed with the turkey protein showed an increased *Lcat* expression (Table 1), not reflected in the size of HDL-loaded cholesterol. Thus, a complex post-transcriptional or translational regulation of these genes is taking place by varying the source of protein, and sex has a crucial role.

Conclusion

The group fed with turkey protein displayed in both sexes the smallest lesions of the studied conditions. In females, the turkey group had less injury than the chicken group, probably due to a larger VLDL size, in addition to a higher expression of *Lcat* than the other groups. In males, other mechanisms were involved; the turkey group had lower lesion areas than the soybean group, thanks to a decreased oxidative status of the different lipoproteins, a better ratio of PON1 to APOA1, and augmented *Abca1* expression, able to increase the RCT. In both sexes, the turkey group presents a decreased atherosclerotic plaque area, reinforcing the relevance of the ratio of PON1 to APOA1 as antioxidant protection of HDL and TG/VLDL-C as an estimation of VLDL size in atherosclerosis.

Declaration of Competing Interest

David Botaya is an employee of the Aldelis Company.

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