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Effect of diet/atorvastatin on atherosclerotic lesions associated to nonalcoholic fatty liver disease in chickens

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Summary. Comparative histological examination of both liver and the supra-aortic arteries have not previously examined the consequences of atherosclerosis and nonalcoholic fatty liver disease (NAFLD), and their response to diet and atorvastatin therapy. This study evaluates the effects of diet alone or in combination with atorvastatin therapy on the progression/regression of atherosclerosis and its correlation with NAFLD. This research was performed on a cohort of chickens on standard (SD) or hyperlipidemic diets (HD), either with or without atorvastatin therapy. The development of atherosclerotic lesions was assessed by histology, immunohistochemistry and quantitative image analysis and correlated with liver histology. The lowest levels of atherosclerotic lesions were found in animals on the HD for 3 months, followed by 3 months of SD in combination with oral atorvastatin. There was a strong association between the histologic findings of atherosclerosis and those of NAFLD. These studies show that standard diet and atorvastatin therapy can positively affect both arterial and hepatic lesions, influencing the regression of the changes. These results support the hypothesis that NAFLD and atherosclerosis may be actually two aspects of a shared disease and suggest the possibility of regression of both disorders with dietary and pharmacologic manipulations.

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Introduction

Atherosclerosis and its consequences remain the major cause of death in humans in western countries (Sans et al., 1997; Kesteloot et al., 2012). Complex interrelationships exist between hyperlipidemia and the progression of atherosclerosis. The most important characteristic of atherosclerotic arteries is the accumulation of cholesterol within the vessel wall as a consequence of an unbalanced cholesterol influx-efflux (Badimon et al., 1990).

Atherosclerosis occurs in the aorta, the coronary, and carotid and other peripheral arteries (Duvall and Vorchheimer, 2004). Much of the clinical research on carotid atherosclerosis measures the intima-media thickness (IMT) of the carotid artery by non-invasive ultrasound techniques. In fact, an increased IMT has been shown to be a risk factor for myocardial infarction and stroke (O'Leary et al., 1999). In a review, 59% of studies presented the association of non-alcoholic fatty liver disease (NAFLD) and carotid intima-media thickness (Oni et al., 2013). In addition, patients with NAFLD show a cluster of risk factors for metabolic syndrome and advanced carotid atherosclerosis (Brea et al., 2005; Fargion et al., 2014). It was also found that there is an association of liver enzymes with biomarkers of subclinical myocardial damage (Lazo et al., 2014).

NAFLD appears to be a feature of metabolic syndrome, and its detection on abdominal ultrasound

could suggest the possibility of an increased cardiovascular risk (Brea et al., 2005; Oni et al., 2013; Fargion et al., 2014).

Diet-induced hypercholesterolemia and hypertriglyceridemia are associated with severe changes in liver histology (fat accumulation, inflammation and cell-ballooning), reproducing the histological features of human NAFLD in a chicken model (Ayala et al., 2009). Avian models of atherosclerosis helped pioneer the study of vascular biology, and they offer economic and technical advantages over mammalian models (Wang et al., 1999). The chicken is considered as a good animal model for the study of atherosclerosis (Gosling et al., 1969; Wong, 1975; Garcia-Perez et al., 2003; Ayala et al., 2005) and NAFLD (Ayala et al., 2009; Makovicky et al., 2011).

To our knowledge, no progression-regression studies, utilizing histologic comparison between atherosclerosis and NAFLD, and simultaneously investigating both arteries and the liver, have been reported in animals or human beings. Thus, the aim of this study was to evaluate the impact of high plasma cholesterol and triglyceride levels on the supra-aortic trunks of the NAFLD animal model, and the effects of atorvastatin on progression-regression of atherosclerotic lesions using histology, immunohistochemistry and image analysis. Moreover, we attempted to assess the potential histological relationship between the arterial atherogenic lesions and changes in NAFLD.

Materials and methods

Animals and diets

One hundred 3-week-old male White Leghorn chickens (Pollos Pujante, Murcia, Spain) were housed under controlled conditions (Martin Castillo et al., 2010). All experimental procedures were approved by the University of Murcia Institutional Animal Care Committee, following the EU Directive 2010/63/EU for animal experiments.

Water was given *ad libitum* and the chickens were randomly assigned to two types of diet as follows:

SD (standard diet): A standard growing mash (Broilers B-1, Piensos Meseguer, Murcia, Spain).

HD (hyperlipidemic diet): A standard growing mash with pure cholesterol (2% of the mixture) and 20% of the mixture of saturated oil (palm oil).

After a three-month induction period, the chickens fed on HD were randomly divided into four groups (B-E) and were kept for another three month period on different diets as follows:

Group A (n=16): SD for six months (healthy control group).

Group B (n=16): HD for six months (hyperlipidemic group).

Group C (n=16): HD for three months and SD during the next three months (spontaneous regression group).

Group D (n=16): HD for three months and SD during the next three months, at which stage they received oral atorvastatin at clinical doses (spontaneous and pharmacological regression group).

Group E (n=16): HD for the whole six months and oral atorvastatin at clinical doses during the last three months (pharmacological progression group).

Atorvastatin was administered orally at doses of 3 mg/kg/day. Medications were administered (force-fed) daily at 8 a.m.

Sampling

All animals were sacrificed by intraperitoneal administration of pentobarbital after six months of receiving both diet ± treatment. Blood samples were extracted and processed as previously described to obtain biochemical parameters (Martin Castillo et al., 2010). Supra-aortic trunks were removed for histological examination.

Histology and immunohistochemistry

Supra-aortic trunks (at the bifurcation site) were processed for histologic analysis as previously described (Martin Castillo et al., 2010). Other slides were used for immunohistochemistry, following the same procedure previously described (Adanez et al., 2008). Briefly, after being de-paraffinized and re-hydrated, slides were incubated for 30 min with 0.3% H_2O_2 in PBS to block endogenous peroxidase, washed with PBS, and blocked for 30 minutes at room temperature with 1:20 NRS (normal rabbit serum). Then, samples were incubated with mouse anti-α-actine (1:100, Dako, Barcelona, Spain) overnight at 4°C. After washing in PBS, sections were incubated with peroxidase conjugated rabbit anti-mouse Ig (1:100 Dako, Barcelona, Spain) for 1h and peroxidase was developed with 3,3-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide. After washing in tap water, sections were counterstained with haematoxylin. In the control, α-actine antibody was substituted for PBS.

H&E stained samples were analysed by light microscopy. A histological description of the different lesion levels of each sample was made, as to endothelial preservation, intima and media layer organization, cellularity, increase in smooth muscular fibers, and number and size of lipid deposits.

Semiquantitative analysis of lesion levels: Stary's modified classification

Atherosclerotic lesions in supra-aortic trunks were classified (0-3 points) based on histologic features analysed by H&E staining and light microscopy. A modification of the American Heart Association histological lesion type classification, as described by Stary (2000), was followed. Thus, the histological types were:

-Level I (normal, 0 points): normal intima size, organized, without vacuolized cells, scarce cellularity.

-Level II (mild, 1 point): slightly increased intima layer size with mildly increased cellularity and isolated subendothelial vacuolized cells.

-Level III (moderate, 2 points): moderately increased intima layer thickness with moderately increased cellularity, some isolated smooth muscular fibers and moderate lipid deposits.

-Level IV (severe, 3 points): highly significant increased intima layer thickness with lipidic core (atherogenic plaque), disorganization, dense cellularity, and smooth muscular fibers.

-Level V: fibroatheroma. This was not included in the semiquantitative classification as it is a more evolved stage of disease.

Once the score for each sample was calculated, the mean was obtained for each group and statistical analysis was performed.

Image analysis and inmunohistochemical quantification

Image analysis was performed to determine changes in morphometric parameters of supra-aortic trunks using MIP 4.5 software (CID, Barcelona, Spain). The following parameters were assessed: intima and media layer thickness, atherogenic plaque and sub-endothelial actin thickness, actin in the intima layer, and vessel characteristics. All histological and immunohistochemical quantifications were carried out by an expert pathologist blinded to the diet and/or treatment groups.

The following image analysis parameters for each experimental group were obtained: Media layer thickness (n=100); intima layer and atherosclerotic plaque thickness (n=100); actin in the intima layer as assessment of smooth muscular fibers by α-actin immunohistochemical techniques. These latter include three parameters: subendothelial actin thickness (n=40); volume density of actin which was estimated by the percentage of actin area in the reference area of intima layer (Aa=actin area/ reference area of intima layer, n= 20) and, finally, integrated density of actin (ID) which was estimated including volume density and intensity of α–actin immunoreactivity (ID=Aa x inverted grey mean, n=20).

Vessel characteristics (n=20): The analysed parameters were wall thickness, wall/lumen ratio (in order to assess vascular remodelling) and the level of vessel occlusion (intima area/intima area + lumen area).

Atherogenic score (AS) was estimated for each sample following a score system similar to the NAFLD activity score (NAS) used in liver disease (Kleiner et al., 2005). The AS was calculated by the addition of the scores of the parameters shown in Table 1, where each range of values is associated with a score. Mean and standard error were obtained for each experimental group.

Features of liver steatosis, cell-ballooning, and inflammation were scored, and single grades were added to obtain the NAS (NAFLD Score) ranging from 0 to 8 (Martin Castillo et al., 2010). Based on previously published data (Martin Castillo et al., 2010), related to the same experimental animals, liver and supra-aortic trunk lesions were compared (as measured by NAS and AS).

Statistical analysis

Results are expressed as mean ± standard error. Mann-Whitney and Kruskal Wallis non-parametric tests were used for semiquantitative analysis. Statistical significance for quantitative analysis was evaluated by ANOVA or Welch and the corresponding post-hoc test. Statistics were performed using SPSS v 15. A P-value <0.05 was considered statistically significant. NAS and AS were compared using Pearson's linear correlation analysis.

Results

Blood biochemical parameters

Values of the main lipids, enzymatic and hepatic proteins measured in the serum from animals of all different experimental groups were previously published (Martin Castillo et al., 2010).

Table 1. Parameters included for calculating the atherogenic score (AS).

Parameter	0 points	1 point	2 points	3 points	4 points	5 points	6 points
Stary grade (modified)	Level I	Level II	Level III	Level IV			
Plaque thickness (μm)	0-49	50-99	100-199	200-299	300-399	≥400	
Intima thickness (μm)	0-49	50-99	100-199	200-299	300-399	≥400	
Subendothelial actin thickness (μm)	0-19.9	20-39.9	40-59.9	60-79.9	80-99.9	100-199.9	≥200
α-actin volume density (%)	0-5	6-10	11-15	16-20	≥21		
α-actin integrated density	0-9.9	10-19.9	20-29.9	30-39.9	≥40		
Media layer thickness (μm)	≥500	400-499.9	300-399.9	200-299.9	100-199.9	0-99.9	
Wall thickness (μm)	1200-1399	1400-1599	1600-1799	1800-1999	2000-2199	≥2200	
Wall/lumen ratio	0.45-0.54	0.55-0.64	0.65-0.74	0.75-0.84	0.85-0.94	≥ 0.95	
Level of vessel occlusion	(%)	0-14	15-29	30-44	45-59	60-74	≥75

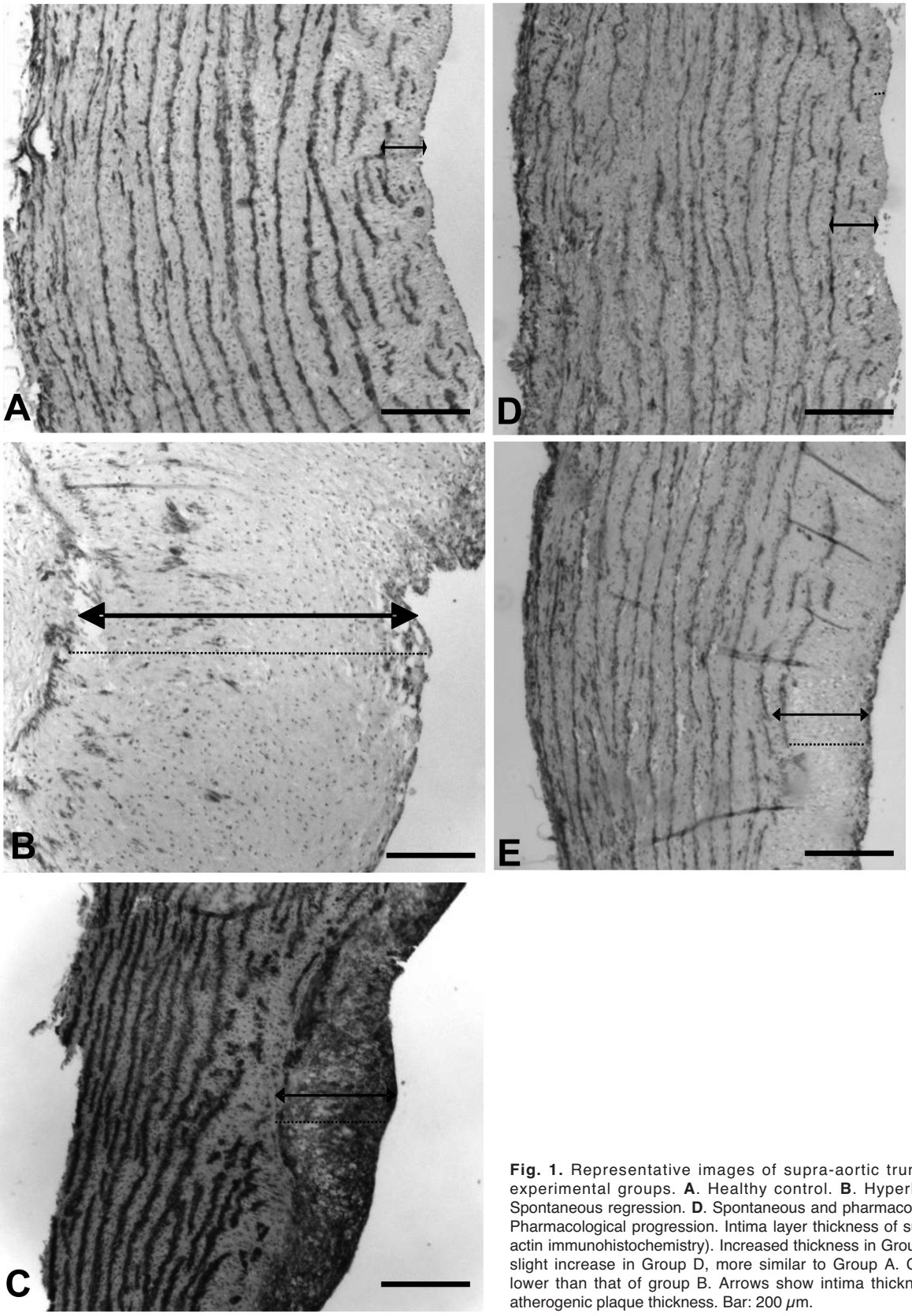

Fig. 1. Representative images of supra-aortic trunks in the different experimental groups. **A**. Healthy control. **B**. Hyperlipidemic group. **C**. Spontaneous regression. **D**. Spontaneous and pharmacological regression. **E**. Pharmacological progression. Intima layer thickness of supra-aortic trunks (α-actin immunohistochemistry). Increased thickness in Groups B and C and only slight increase in Group D, more similar to Group A. Group E thickness is lower than that of group B. Arrows show intima thickness. Dot line shows atherogenic plaque thickness. Bar: 200 μm.

Histology, inmunohistochemistry, semiquantitative and image analysis (Fig. 1)

Briefly, light microscopy evaluation showed the following histological characteristics:

Group A (healthy control). It showed a normal histology.

Group B (hyperlipidemic) showed increased thickening of intima layer, disorganization, numerous fat deposits in a large atherogenic plaque. Media layer with disorganized smooth muscular fibers and high cellularity.

Group C (spontaneous regression group): a conserved endothelium with an increased intima thickness, large atherogenic plaques, deposits of sub-endothelial smooth muscular fibers of significant thickness and depth. High laxity of media layer and moderate increase of cellularity.

Group D (spontaneous and pharmacological regression group): a conserved endothelium with a slightly increased intima thickness, some isolated fat deposits and a slight increase in muscular fibers and cellularity. Media layer appeared organized.

Group E (pharmacological progression group): a conserved endothelium with moderate increase of the intima layer and muscular fibers. Laxity of tissues because of fat vacuoles and atherogenic plaques, resulting in a disorganization of the intima layer.

Table 2 shows the different parameters studied from animals of all experimental groups.

With reference to supra-aortic trunks, the highest values of Stary modified lesion levels were found in groups B, C and E (hyperlipidemic, spontaneous regression, pharmacological progression groups). A significant decrease of lesion level was observed in group D (spontaneous and pharmacological regression group). Lesions up to level V were occasionally observed in group B (hyperlipidemic).

A significant decrease of media layer thickness and a significant increase of intima layer thickness were observed in all groups with respect to group A (healthy control).

Numerous fat deposits in a large atherogenic plaque occupying the whole intima layer were found in group B (hyperlipidemic). A significant decrease of atherogenic plaque thickness was found in groups C, D and E (spontaneous regression, spontaneous and pharmacological regression, pharmacological progression groups). Alpha actin content was used as a marker of smooth muscular fibers in neointima layer, evaluated by three different parameters (thickness, volume density and integrated density). A significant decrease of neointima layer was observed in group D (spontaneous and pharmacological regression) with respect to groups B, C and E (hyperlipidemic, spontaneous regression, pharmacological progression groups). The highest level of vessel occlusion was found in group B (hyperlipidemic) and the lowest ones were found in groups A and D (healthy control and spontaneous and pharmacological regression groups). No significant differences in the atherogenic score were observed between groups A and D (healthy control and spontaneous and pharmacological regression groups).

A complete description of liver histological analysis has previously been published (Ayala et al., 2009; Martin Castillo et al., 2010).

Correlation between AS (supra-aortic trunks) and NAS (liver)

We compared AS with NAS in the same experimental groups (Table 2). Data of NAS was previously published (Martin Castillo et al., 2010). A positive linear Pearson correlation (r=0.808; Sq r lineal=0.653) was found between the two scores, indicating that there exists an association between both

Table 2. Values of the different parameters studied from animals of all different experimental groups (mean ± standard error).

Experimental Groups	A	B	C	D	E
Stary (modified) lesion level (0-3)	0.00±0.00^{a}	2.90±0.10^{b}	2.78±0.15^{b}	1.50±0.34^{c}	2.33±0.87^{b,c}
Media thickness(μm)	585.19±9.06^{a}	345.45±18.21^{b}	342.26±18.38^{b}	359.42±19.02^{b}	371.44±18.18^{b}
Intima thickness (μm)	48.66±2.27^{a}	336.35±23.58^{b}	230.28±14.51^{c}	121.45±4.42^{d}	141.76±5.84^{e}
Atherogenic plaque thickness (μm)	0.00±0.00^{a}	394.51±34.71^{b}	110.84±11.45^{c}	38.25±1.82^{d}	69.64±6.33^{e}
Subendothelial α-actin thickness (μm)	0.00±0.00^{a}	43.28±3.18^{b,d}	81.87±14.99^{b}	13.24±2.73^{c}	35.42±4.33^{d}
α-actin volume density (%)	1.5±0.00^{a}	11±1.2^{b}	11.4±1.7^{b}	3.5±0.4^{c}	8.8±1.1^{b}
α-actin integrated density	3.11±0.39^{a}	16.01±1.78^{b}	17.99±2.42^{b}	6.46±0.69^{c}	16.72±2.13^{b}
Wall thickness (μm)	1507±45.21^{a}	1718±39.32^{b}	1715±40.69^{b}	1508±65.71^{a}	1649±87.28^{a,b}
Wall-lumen ratio	0.73±0.03^{a}	0.73±0.05^{a}	0.74±0.03^{a}	0.75±0.04^{a}	0.79±0.03^{a}
Level of vessel occlusion (%)	21±0.8^{a}	59±3.5^{b}	36±3.4^{c,d}	25±2.4^{a,d}	37±3^{c}
Atherogenic Score (AS)	5.07±0.26^{a}	24.02±0.63^{b}	15.62±0.84^{c}	6.37±0.43^{a}	12.90±0.56^{c}
NAFLD Activity Score (NAS)	0.00±0.00^{a}	7.13±0.12^{b}	2.90±0.14^{c}	1.83±0.11^{d}	4.58±0.16^{e}

By rows, different superscript letters indicate significant differences, same superscript letters indicate no significant differences between groups ($p<0.05$).

variables.

Discussion

Our study shows that chickens fed high cholesterol/high fat diets develop severe atherogenic lesions in the supra-aortic trunks in about 6 months, with pathological findings similar to those of humans. Additionally, there was a high positive correlation between the atherosclerotic lesions and hepatic steatosis.

One of the advantages of using chickens rather than other species is the short atherosclerosis regression time (Ayala et al., 2005). An emerging trend in NAFLD research is to utilize the mouse models traditionally targeted for studies of atherosclerosis (Bieghs et al., 2012), but in these, and most animal models, steatosis develops quite easily although progression to steatohepatitis is more difficult (Tous et al., 2005). In contrast, results from the present investigation show that the chicken model readily develops a significant steatohepatitis.

Levels IV and V atherogenic lesions (according to the Stary classification) that were observed in our study after hyperlipidemic diet feeding and atherogenic lesion regression through diet and/or therapy, have been found in several experimental animal models (Amstrong, 1976; Daoud et al., 1981; Badimon et al., 1990; Williams et al., 2008).

Semiquantitative analysis of lesion levels by the modified Stary's classification showed a statistically significant beneficial effect of the combination of diet and atorvastatin treatment (Group D). Neither the withdrawal of the HD diet (Group C) nor its continuation with the introduction of atorvastatin therapy (Group E) induced significant decreases in the levels. Our results are consistent with those showing no significant histological effects on the carotid arteries of withdrawal of a hyperlipidemic diet (Wilson et al., 1982).

All the groups showed a significant decrease in media layer thickness with respect to the healthy controls (Group A). These changes could be explained by endothelial dysfunction and consequent promotion of smooth muscle cell proliferation and migration (Cai and Harrison, 2000).

Statistically significant differences were observed for intima layer thickness between all the groups, from the lowest thickness in the healthy control A, followed by Group D (pharmacological regression) to the greatest thickness in Group B (hyperlipidemic group). Other studies in rabbits fed a low cholesterol atherogenic diet have shown arterial changes during the first 12 months consisting of smooth muscle cell and lipid accumulation in the intima (Wilson et al., 1982). Remarkably, in our study, Group E (pharmacological progression) showed a lower intima thickness than Group C (spontaneous regression), probably due to a higher HDL level in the former group. In this respect, HDL plasma fractions were able to induce regression of established aortic fatty streaks and lipid deposits in rabbits (Badimon et al., 1990). In humans, the REVERSAL trial found that the use of atorvastatin 80 mg daily for aggressive lowering of plasma LDL concentrations was associated with significant intima media thickness (IMT) regression due to atherosclerosis slowdown (Kastelein et al., 2004). Our data are also in agreement with experimental and clinical studies showing that statins reduce total atheroma volume (ASTEROID trial) (Nissen et al., 2006). Of particular interest is the fact that IMT and arterial lumen diameter, as measured by B-mode high-resolution ultrasonography and quantitative coronary angiography, respectively, are currently the only surrogate markers for progression of atherosclerotic disease recognized by regulatory authorities in the United States and Europe (Kastelein et al., 2004).

Neointima layer assessment by α-actin (smooth muscular fiber marker) showed that Groups A and D showed significant differences compared to the other groups: the deposits being very few in Groups A (control) and D (spontaneous and pharmacological regression). Similarly, a reduction in neointima formation has been shown to occur through decreased monocyte infiltration after atorvastatin therapy in rabbits (Hernandez-Presa et al., 1997).

The combined effect of diet (SD) and atorvastatin induced a significant reduction in vessel occlusion since we did not find statistically significant differences between Groups A (healthy control with only basal occlusion) and D (spontaneous and atorvastatin regression). However, the highest level of vessel occlusion, observed in Group B (hyperlipidemic diet), was quite low when considering the existing increased intima disorganization and large atherogenic plaques in this group. Glagov et al. (1987) studied histologic sections of the coronary artery in hearts obtained at autopsy and they concluded that human coronary arteries enlarge in relation to plaque area (compensatory enlargement to accommodate the plaque and maintain the lumen), avoiding lumen stenosis (positive remodeling). Furthermore, no statistically significant differences were found between the different groups studied for the wall/lumen relationship. This is in agreement with several experimental studies in animals, which report no changes in the vascular lumen after atherosclerosis progression/regression (Wilson et al., 1982; Ibáñez et al., 2008).

Atherogenic score assessment, which has not previously been described in the literature, showed that both spontaneous regression alone (using only a normal diet, Group C) and atorvastatin therapy alone (Group E) produced an improvement of atherosclerotic lesions in the supra-aortic trunks. When both a normal diet and atorvastatin therapy were administered in combination (Group D), the AS decreased significantly with values close to those of the control (Group A).

A highly positive linear correlation was found between AS and NAS, showing parallel changes in both parameters, with the highest scores for Group B (hyperlipidemic diet), intermediate values for Groups C (spontaneous regression) and E (hyperlipidemic diet and

atorvastatin) and the lowest scores for Groups A (healthy control) and D (normal diet and atorvastatin therapy). Other authors (Tous et al., 2005; Lohman et al., 2009) have shown histologic correlation between arterial and liver lesions when feeding ApoE $^{-/-}$ mice with a HD in progression studies. Recently, it was found that arterial stiffness in patients with NAFLD is related to liver fibrosis stage (Sunbul et al., 2014). The concept of concomitant development of atherosclerosis and fatty liver has been investigated for several years (Goldstein and Brown, 2008; Oni et al., 2013), which has been explained by attributing both processes to diet and abnormal activity of Sterol Regulatory Element Binding Proteins (SREBP) transcription factors, expressed in liver and vascular wall (Shimano et al., 1996). However, to our knowledge, a similar progression-regression study, with histologic comparison between atherosclerosis and NAFLD, by simultaneously investigating both the supra-aortic arteries and the liver in the same animals, has never been reported. The corresponding regression of atherosclerosis and NAFLD upon dietary or pharmacologic manipulation could be inferred in this study. Our finding of a positive correlation between AS and NAS, both in progression and regression of the lesions, contributes to the hypothesis that non-alcoholic steatohepatitis and atherosclerosis may actually be two aspects of a shared disease (Bieghs et al., 2012), but no conclusions on causal relationship can be drawn. Recently, it was found that mitochondrial aldehyde dehydrogenase activation inhibits atherosclerosis and attenuates hepatic steatosis in apolipoprotein e-knockout mice (Stachowicz et al., 2014).

In summary, our results suggest that hyperlipidemia in chickens induces atherosclerosis in their supra-aortic trunks and a combination of atorvastatin therapy and standard diet proved to be effective in promoting atherosclerosis regression. An association between these histologic findings of atherosclerosis in supra-aortic trunks and histologic evidence of NAFLD has been shown, as well as a similar positive impact of diet ± atorvastatin on both arterial and liver lesions in progression/regression groups. These findings support the hypothesis that NAFLD and atherosclerosis may actually be two aspects of a shared disease and suggest the possibility of regression of both disorders upon dietary or pharmacologic manipulation.

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