

# Development of Aortic Valve Sclerosis in a Rabbit Model of Atherosclerosis: An Immunohistochemical and Histological Study

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**Background and aim of the study:** It has been suggested that aortic valve sclerosis (AVS) is an atherosclerotic disease process that can proceed to aortic stenosis. The absence of reports studying an animal model of the early stages of this disease has precluded the development of preventive therapeutic strategies. A cholesterol-fed (0.25% cholesterol in chow) rabbit model of atherosclerosis that is characterized by a moderate level of hypercholesterolemia was studied to determine its efficacy as a model of early AVS. Cellular, structural and morphological changes in the aortic valves of these rabbits were studied.

**Methods:** Twenty rabbits were assigned randomly to four experimental groups: Group 1 received normal chow for 40 weeks; group 2 received 0.25% cholesterol-supplemented chow for 20 weeks; group 3 received 0.25% cholesterol-supplemented chow for 40 weeks; and group 4 received 0.25% cholesterol-supplemented chow for 20 weeks followed by nor-

mal chow for an additional 20 weeks. The aortas and aortic valves were analyzed using immunohistochemical and histological methods to detect cellular and structural components of the developing lesions. **Results:** All rabbits in groups 2, 3 and 4 developed atherosclerotic lesions in their aortas. Aortic valves from these animals demonstrated thickening, lipid deposition, a change in collagen content and organization, a reorganization of elastin, and the presence of both macrophage infiltrate and osteopontin.

**Conclusion:** These findings were consistent with the suggestion of a link between atherosclerosis and AVS. Results were also similar to changes reported in human sclerotic aortic valves, suggesting the suitability of this rabbit model of atherosclerosis as a model for AVS.

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Aortic valve sclerosis (AVS) is a common heart valve lesion that affects at least 25% of the population over the age of 65 years (1). The natural history of AVS is that of gradual progression, with the majority of patients showing detectable changes, by simple non-invasive means, within a three-year period (2). AVS can progress to calcific aortic stenosis, increasing in prevalence with advancing age, and affecting 2-3% of the population aged over 65 years (1). By the year 2030, almost one-quarter of the population will be aged over

65, making this disorder a potentially significant healthcare cost. Since the end-stage of this disease - symptomatic aortic stenosis - leads to death in less than five years, surgical valve replacement remains the primary management (2,3). Indeed, up to 90% of all aortic valve replacements in patients over the age of 75 years are performed for this disorder (4,5). At present, no preventive therapy exists for AVS.

The etiology of AVS remains poorly understood. Because of its close association with advanced age, most authors in the past have considered AVS to be a wear and tear phenomenon (6-9). However, considerable individual variation in the presence and severity of the lesion in patients aged over 65 has suggested a more complicated etiology (10). Several authors have noted a variety of clinical factors that appear to be associated with the disease. These include age, male gender, hypertension, smoking, and elevated levels of cholesterol, serum low-density lipoprotein (LDL) and serum lipoprotein a (LP(a)) (11-14). It is currently believed that a relationship exists between aortic scler-

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rosis and atherosclerosis (15-17). This association has been supported by the studies of several groups (13,14,18), and has been based on similarities in risk factors as well as histological and ultrastructural manifestations of each disease process (15). The concept that AVS may be an atherosclerotic disease process has therefore met increasing acceptance.

Animal models of AVS have been developed in the past, but have been dependent on surgical manipulation (19-21), or on extremely high plasma cholesterol levels (22-25). Such disease-inducing methods leave reasonable doubt as to the etiology of the disease, since the approaches used have required either physical manipulation or the possibility of developing a lipid storage disorder. In contrast, the present low cholesterol-fed rabbit model of atherosclerosis develops atherosclerotic lesions that resemble human atheroma (26,27). In the present study, this rabbit model was utilized to examine the potential link between atherosclerosis and AVS, and to examine the suitability of this model in the study of this disease.

## Materials and methods

### Animals

Twenty male New Zealand rabbits of body weight 1.6-1.8 kg were individually housed in a controlled environment maintained at 19°C, with water provided ad libitum. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care.

The rabbits were assigned randomly to four study groups, each consisting of five animals. Five control animals were fed standard rabbit chow (Prolab Laboratories, Agway Inc., Syracuse, NY, USA) for 40 weeks (Control), five were fed a 0.25% (w/w) cholesterol- (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) supplemented diet for 20 weeks (CH-20), five were fed a 0.25% cholesterol-supplemented diet for 40 weeks (CH-40), and the remaining five animals were fed a 0.25% cholesterol-supplemented diet for 20 weeks and then placed on a normal diet for an additional 20 weeks to determine if reducing plasma cholesterol influences lesion progression (RD = 'regression diet'). Total plasma cholesterol (TPC) levels were monitored periodically using standard methods (26,27).

### Tissue retrieval

Following administration of the diet regimen, animals were sacrificed and perfusion-fixed at 100 mmHg via the left ventricle, as described previously (27). The aortas and aortic valves were excised and cryoprotected sequentially for 1 hour each in 10%, 20% and 30% sucrose solutions. Thoracic descending aortas were rolled longitudinally with their luminal surfaces facing

inward, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, USA), snap-frozen in liquid nitrogen, and serially sectioned. Aortic valve leaflets were embedded in OCT horizontally, so that tissue sections would be cut in the radial direction.

### Aortic valve average thickness measurements

Aortic valve sections that represented the central portion of the leaflet between 1.5-5 mm from the commissure were analyzed. Aortic valve thickness was measured as the average thickness of six radial sections spanning the base, middle and free edge. Within each group, three leaflets of the aortic valve were analyzed for three rabbits from each group. Images were taken using a Carl Zeiss Axioskop microscope equipped with a Sony CCD camera. Area and length measurements were taken using Image Pro Plus 4.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). Average section thickness was expressed as a mean of the section area over the mean length in  $\mu\text{m}$ .

### Histology

Serial sections of aortic valve leaflets and aortas were stained with Oil Red O for neutral lipid deposition, with picrosirius red to enhance collagen visualization via polarized light microscopy, and with Alizarin Red and von Kossa's stain to detect mineralization (all stains from Sigma-Aldrich Canada, Oakville, ON, Canada).

Tissue sections stained with Oil Red O were first hydrated for 5 min, immersed in 100% propylene glycol for 5 min, and then stained with 0.5% Oil Red O in 100% propylene glycol, rehydrated and mounted in an aqueous mounting medium.

Sections stained with picrosirius red were first rehydrated with distilled water, washed an additional three times in distilled water, immersed in 0.1% Sirius Red F3BA in 90% saturated picric acid for 30 min, and then dehydrated with ascending concentrations of alcohol. Sections were then immersed in xylene for two 5-min intervals and mounted with DPX mounting medium.

Sections stained with Alizarin Red for  $\text{Ca}^{2+}$  localization were hydrated for 5 min in distilled water, and then stained in a 1% Alizarin Red solution. Next, sections were washed in water, differentiated in ethanol, dehydrated and mounted. Sections were also stained using Von Kossa's method to detect mineralization. Sections were rehydrated in water, treated with a 2% silver nitrate solution in a clear glass jar under a lamp for 1 h. They were then washed several times in water, treated with 2.5% sodium thiosulfate solution, washed again in water and counterstained in a 1% neutral red solution (Sigma). Slides were then dehydrated and mounted with a glass coverslip.

### Immunohistochemistry

Serial sections of aortic valve leaflets and aortas were surveyed with a number of antibodies directed against markers known to be prevalent in early AVS lesions and atherosclerosis. Sections were subjected to single labeling immunohistochemistry using the Dako envision HRP<sup>+</sup> system (Dakocytomation Inc., Canada, Mississauga, ON, Canada) according to the manufacturer's instructions and using the following antibodies; anti-macrophage RAM-11 (1:30) (Dakocytomation), anti-CD3 T-lymphocytes clone PC3/18A (1:50), anti-CD5 T-lymphocytes clone KEN5 (1:20) (Cedarlane Laboratories, Hornsby, On, Canada), anti-elastin clone BA4 (1:75) (Elastin Products Company, Inc., Owensville, MI, USA), and anti-osteopontin clone MPIIB10<sub>1</sub> (1:100) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IO, USA). For tissue sections to be stained with anti-elastin antibodies, a protease antigen retrieval method (28) was used by bathing sections in a protease solution consisting of 0.1% trypsin, 0.1% calcium chloride, 20 mM Tris (pH 7.8) for 20 min. Following staining, the slides were mounted using Aquatex (an aqueous mounting medium).

### Light microscopy and analysis

Digital images of stained tissue sections were captured using a Zeiss Axioskop microscope equipped with a Sony 3 chip CCD camera and Northern Eclipse imaging software (EMPIX, Inc., Mississauga, ON, Canada). Each image was then analyzed morphometrically for percentage of HRP or dye positive staining. Briefly, images were processed using Adobe Photoshop 5.5 to obtain two separate images, one of HRP/dye-specific positive staining and one of the entire tissue section. For picrosirius red-stained sections the collagen framework was visualized with polarized images, and positive pixels were isolated using Adobe Photoshop 5.5 and counted using Image

Pro Plus. Positive pixels for histology and immunohistochemistry were expressed as a percentage of the control group.

### Statistical analysis

Statistical analysis was performed using SPSS 10 software package for Windows (SPSS Inc., Chicago, IL, USA). Variables were analyzed using a one-way ANOVA, followed by a Scheffe's post hoc multiple contrast test. For all analyses, a p-value <0.05 was considered to be statistically significant. Transformations were performed on data where appropriate. All values were expressed as mean ± SEM.

## Results

### Total plasma cholesterol

During the first 20-week period, the control group TPC level was 41 ± 2 mg/dl, whereas rabbits in all cholesterol-fed groups had average TPC levels >500 mg/dl (Table I).

During the second 20-week period, the control group TPC level remained relatively unchanged (Table I), but that in the CH-40 group rose to >800 mg/dl. In contrast, TPC levels in the RD group fell to levels (121 ± 20 mg/dl) shown not to promote atherogenesis (unpublished observations).

### Development of atherosclerosis

The aortas of rabbits were analyzed using histological stains and immunohistochemistry. Previously, the development of diet-induced atherosclerotic lesions in the aortas of this rabbit model was found to range from fatty streaks to advanced atheromas (26,27). In the present study, increased lipid deposition, macrophage/foam cell infiltrate, osteopontin deposition, collagen and elastin deposition within the lesions of the vessel wall were observed, in addition to mineralization (Fig. 1).

Table I: Average total plasma cholesterol (TPC) levels of rabbits over time.

Group	Time-weighted average TPC level (mg/dl)	
	First 20-week period	Second 20-week period
Control	41 ± 2	30 ± 2
CH-20	588 ± 76*	-
CH-40	582 ± 39*	822 ± 61 <sup>+</sup>
RD	570 ± 57*	121 ± 20*

Values are mean ± SEM.

\*p <0.05 versus controls; <sup>+</sup>p <0.05 versus any other group.

Control: Rabbits fed normal chow for 40 weeks; CH-20: Rabbits fed cholesterol-supplemented chow for 20 weeks; CH-40: Rabbits fed cholesterol-supplemented chow for 40 weeks; RD: Rabbits fed cholesterol-supplemented chow for 20 weeks, then normal chow for an additional 20 weeks.

### Aortic valve investigations

#### Average thickness

There was no significant increase in valve thickness in the CH-20 and RD groups compared to controls (Fig. 2). In contrast, valves from the CH-40 group were significantly thicker, the majority of this change having occurred in the base and middle regions of the leaflet, primarily in the fibrosal layer (data not shown).

#### Histological analysis

Oil Red O staining was not seen in control group aortic valve leaflets (Fig. 3A), but was evident throughout the valves of all animals fed a cholesterol-supplemented diet (Fig. 3B-D). Most of the staining was found in the fibrosal layer. Valve leaflets from animals fed a cho-

lesterol-supplemented diet for 40 weeks (Fig. 3C) appeared to contain significantly more lipid than those of animals fed the diet for 20 weeks (Fig. 3B and D).

Collagen fiber disarray and deposition have been reported in human AVS (29-33), and aortic valve leaflets from the rabbit model showed characteristics of both these processes (Fig. 3F-H). The CH-20 group showed a reorganization in the collagen architecture, where collagen fibers seemed to accommodate a cellular infiltrate (Fig. 3F). However, there was no change in the percentage area of collagen when compared to controls (Fig. 4B). In contrast, the images revealed a similar (though significantly more dense) pattern in the CH-40 group when compared to CH-20 group (Fig. 3G and F, respectively). The morphometric analysis con-

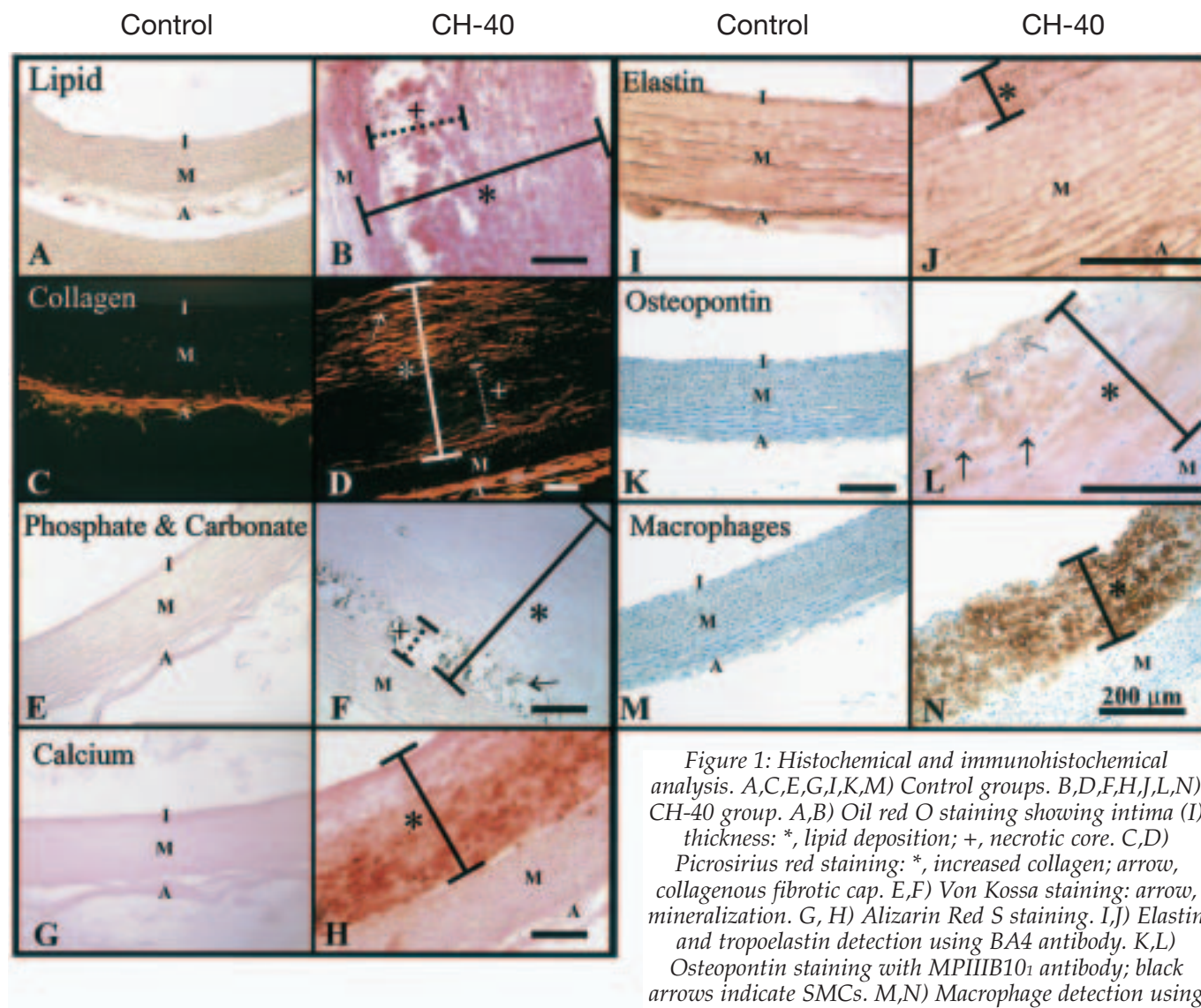


Figure 1: Histochemical and immunohistochemical analysis. A,C,E,G,I,K,M) Control groups. B,D,F,H,J,L,N) CH-40 group. A,B) Oil red O staining showing intima (I) thickness: \*, lipid deposition; +, necrotic core. C,D) Picrosirius red staining: \*, increased collagen; arrow, collagenous fibrotic cap. E,F) Von Kossa staining: arrow, mineralization. G, H) Alizarin Red S staining. I,J) Elastin and tropoelastin detection using BA4 antibody. K,L) Osteopontin staining with MPIIB10<sub>1</sub> antibody; black arrows indicate SMCs. M,N) Macrophage detection using RAM-11 antibody.

\* in all panels denotes the lesion, + in all panels denotes the necrotic core; I: in tissue, M: medical, A: adventitia.

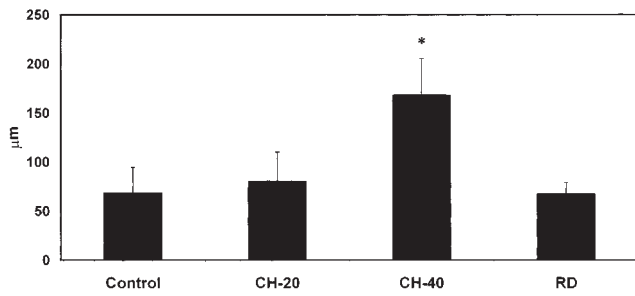


Figure 2: Aortic valve leaflet thickness in rabbits fed different atherogenic diets. \*, significant difference between groups ( $p < 0.05$ ).

firmed this finding (Fig. 4B), and revealed a significant increase in collagen area in CH-40 group valve leaflets compared to those of all other groups.

Although fine stippled mineralization is a characteristic of AVS in humans (32,34-37), Von Kossa and Alizarin Red staining were both negative in the rabbit aortic valves (data not shown). This was despite the fact that aortas of animals in the CH-40 group showed calcified areas within and at the base of the lesions (Fig. 1F and H).

#### Immunohistochemical analysis

In the control group, no macrophages/foam cells were detected in the aortic valve leaflets when sections were stained with RAM-11 (Fig. 3I). In contrast,

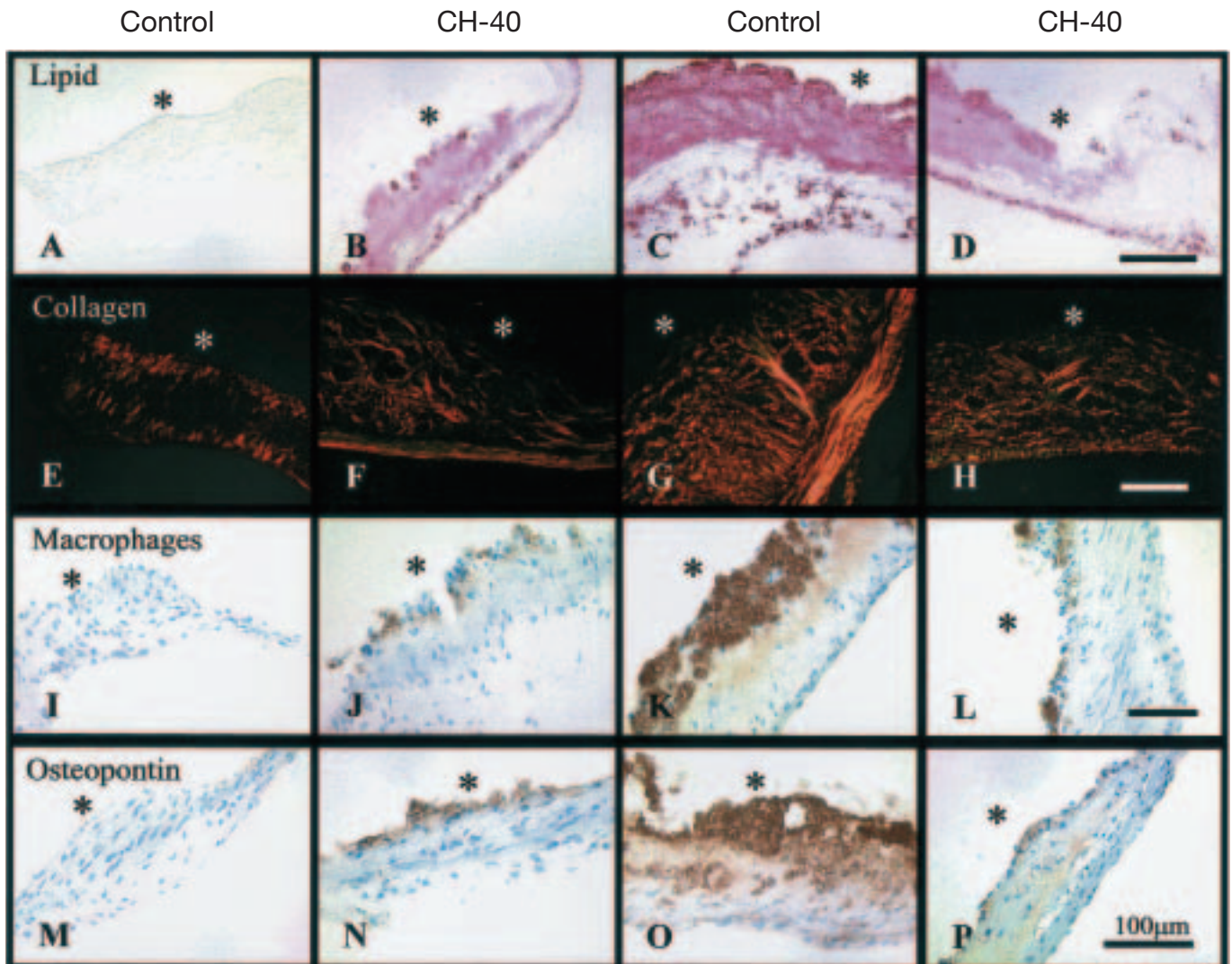


Figure 3: Histochemical and immunohistochemical staining of aortic valve leaflets of rabbits fed different diet regimens. A,E,I,M) Controls; B,F,J,N) CH-20 group; C,G,K,O) CH-40 group; D,H,L,P) RD group; \*, fibrosal side of the valve. A-D) Oil red O staining showing lipid deposition in the subendothelial area in the fibrosal layer. E,F) Picrosirius red staining showing collagen network. I-L) Macrophage detection using RAM-11 antibody. M-P) osteopontin detection in the MP111B1 antibody.

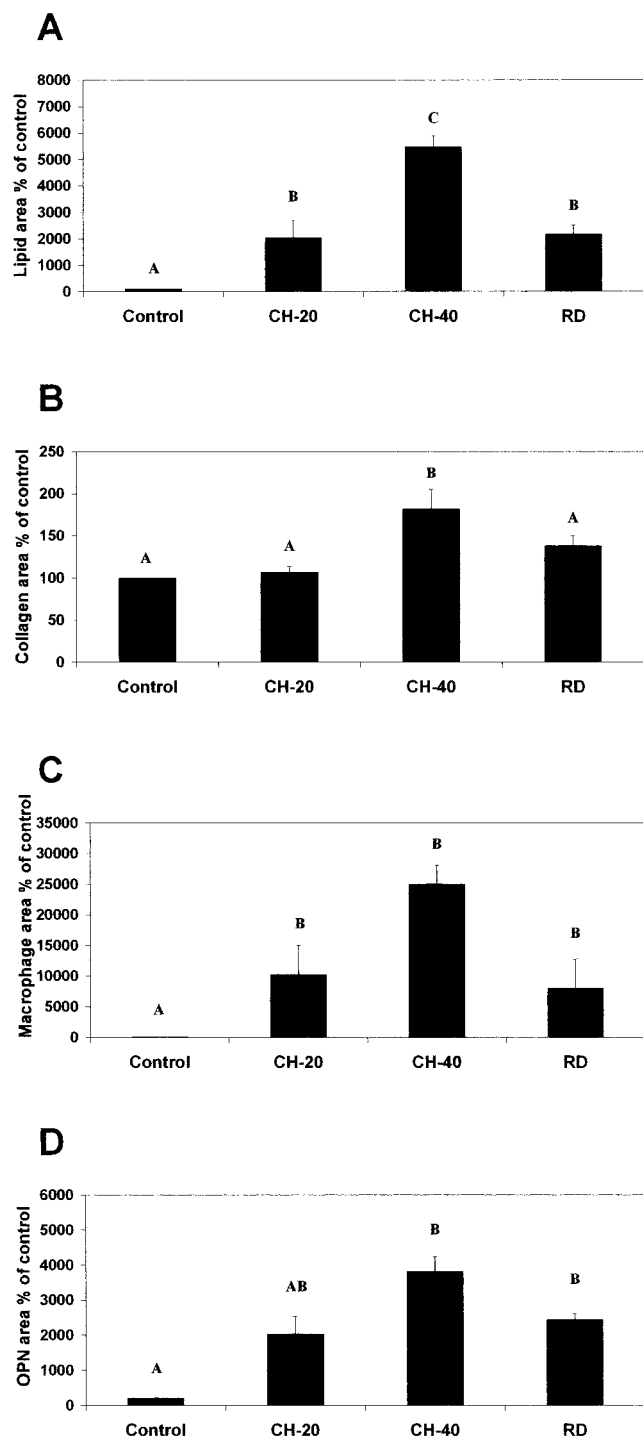


Figure 4: Lipid content (A), collagen area (B), macrophage/foam cell infiltration (C) and osteopontin (OPN) deposition (D) in aortic valve leaflets of rabbits fed different atherogenic diets. Different superscripts indicate significant inter-group differences ( $p < 0.05$ ).

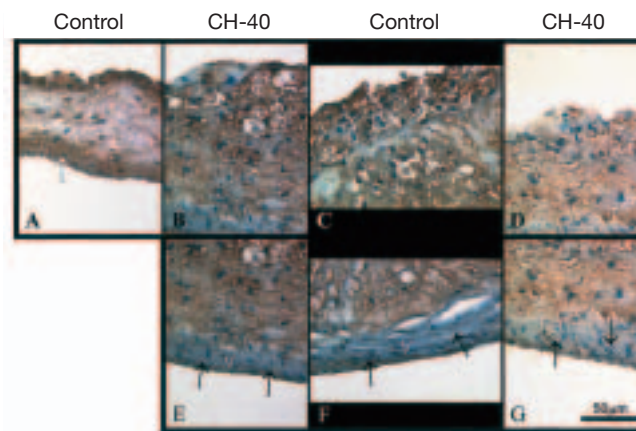


Figure 5: Elastin architecture in aortic valves of rabbits fed atherogenic diets, using BA4 antibody. A) Controls; elastin (white arrows) was concentrated mainly in the outer layers. In cholesterol-fed rabbits, elastin staining was increased in the fibrosa (F) and spongiosa (S) (B-D; white arrows), but reduced in the ventricularis (V) (E-G; black arrows).

macrophage/foam cells were detected in aortic valves of the CH-20, CH-40 and RD groups (Fig. 3J, K and L, respectively). Macrophage localization was similar to lipid deposition (Fig. 3B-D), suggesting that some of these cells might be macrophage-derived foam cells. Quantitative analysis of digital images revealed a significant increase in the presence of macrophage/foam cells in all groups fed a cholesterol-supplemented diet with respect to the control group (Fig. 4C); however, there was no significant difference among the cholesterol-fed groups of animals.

Osteopontin (OPN), like RAM-11, was found to concentrate within the fibrosa of the base and middle portions of the valve leaflets (Fig. 3M-P). The morphometric analysis revealed significant increases in OPN in the CH-40 and RD groups when compared to controls (Fig. 4D). The CH-20 group also appeared to show an increase in OPN expression, though compared to the control group this difference was not statistically significant.

The aortas of rabbits fed a 0.25% cholesterol diet for 40 weeks did not demonstrate the presence of CD-3- and CD-5-positive lymphocytes at the surface or within the lesions (data not shown). Like the aortas, T-lymphocytes were not found in the aortic valves of rabbits fed this diet. To confirm the validity of these observations, rabbit spleen tissue was used as a positive control for the immunohistochemical analysis, and anti-CD5 and anti-CD3 antibodies showed significant staining around periarterial lymphatic sheath within the splenic white pulp, consistent with known localization of T-lymphocyte proliferation and maturation (data not shown).

In the control group, staining of elastin within the aortic valves, using an anti-elastin antibody, revealed a focal localization to the surface layers of the valve, and sparse staining within the central spongiosa (Fig. 5A, white arrows). In valves from cholesterol-fed rabbits, elastin staining was prominent at the fibrosal surface and within the lipid-laden lesion (Fig. 5B-D, black arrows). In the spongiosa, elastin became more prominent, whilst in the fibrosal layer elastin distribution was maintained in a similar pattern to that in controls (Fig. 5E-G).

## Discussion

Aortic valve sclerosis has been correlated with a number of risk factors associated with atherosclerosis, and lesions found in both pathologies have been described as being similar (32). As a result, an increasing number of investigators believe that AVS, as a disease process, is closely related to atherosclerosis (13,15-17,38,39). AVS may be a modifiable disease process, and the development of animal models may provide a vehicle to test preventive and treatment strategies. Therefore, a rabbit model of atherosclerosis was used to survey the aortic valves of these animals for sclerotic changes characteristic of human-type lesions (26,27).

### Development of atherosclerosis

During the first 20 weeks, the cholesterol-supplemented diet led to a significant increase in TPC compared to normal chow-fed controls. During the second 20-week period, rabbits maintained on the cholesterol diet (CH-40) showed further increases in plasma cholesterol, while those switched to a normal chow diet (RD) had TPC that returned to near-control levels. The aortas of rabbits fed the cholesterol diet showed atherosclerosis-like changes, with evidence of lipid accumulation, macrophage/foam cell infiltration, elastin reorganization and/or deposition in the lesion, collagen reorganization and deposition, as well as calcification (see Fig. 1). These lesions resembled type II, III and IV lesions previously described in humans (26,27). The development of atherosclerosis with concomitant development of sclerotic lesions in the aortic valve leaflets, suggests an etiological link between these two diseases.

### Influence of atherogenic diet on valve morphology

Following 20 weeks of cholesterol feeding (CH-20), aortic valves were characterized by a build-up of Oil Red O-stained lipid and RAM-11-positive macrophages. Increases in other parameters studied, namely collagen production, OPN secretion and valve thickness, were not statistically significant until the animals had been maintained on the atherogenic diet

for 40 weeks (CH-40). Valve morphology in rabbits switched from the cholesterol-supplemented diet to normal chow (RD) was indistinguishable from that seen in rabbits analyzed following 20 weeks of cholesterol feeding (CH-20). The reorganization of the structural components (collagen, elastin) within the valve may account for the increase in macrophage infiltrate in the absence of any detectable increase in the thickness of the valves in the CH-20 and RD groups when compared to controls (see Fig. 2).

### Aortic valvular lesions in the rabbit model of atherosclerosis

In order to survey the aortic valves of the rabbits, morphological parameters were chosen that characteristically change in early AVS in humans. Early lesions in mild aortic stenosis are characterized by subendothelial thickening, which consists of an accumulation of lipid, protein, extracellular mineralization and cellular infiltration (32). Similarly, the aortic valves in the rabbit model exhibited increases in leaflet thickness and lipid deposition, remodeling and/or increases in collagen, changes in elastin distribution, along with increases in macrophage/foam cell infiltration and OPN deposition.

The structural components of the rabbit aortic valves were also investigated. Elastin was found to be concentrated primarily in the fibrosa and ventricularis layers of the leaflet in control animals, but in rabbits fed an atherogenic diet there was a clear reorganization and/or increase in elastin in the lesion forming in the subendothelial layer of the fibrosa, around the lipid-macrophage/foam cell infiltrate, and within the spongiosa. In contrast to valves from control animals, in which the ventricularis appeared to be enriched with elastin, the ventricularis of valves harvested from rabbits fed an atherogenic diet was relatively devoid of elastin staining. Fokin et al. (40) showed that reduced aortic root compliance, that can occur as a result of atherosclerosis, may promote the development of degenerative aortic valve disease. These authors showed that a rabbit model of reduced aortic root compliance created by applying 'SuperGlue' around the sinus of Valsalva, demonstrated a reduction in leaflet elastic fibers.

Collagen is the other major component of the aortic valve. Accordingly, Fokin et al. (40), also described a decrease in aortic valve type I collagen density, with no concomitant reduction in aortic valve thickness in rabbits subjected to stiffening of the aortic roots after 8-11 months. This would suggest that reorganization or collagen fiber disarray occurs, rendering leaflets vulnerable to an accumulation of lipid at areas of low shear stress and high mechanical strain, in turn creating a focal point for disease progression. The results of the

present study support these conclusions.

In the present analysis, collagen area was evaluated in picrosirius red-stained sections. Images of the collagen in the aortic valve leaflet of the CH-20 and RD groups revealed that a reorganization of collagen architecture occurred without a concomitant increase in the percentage area occupied by collagen. Authors of human studies of aortic stenosis have also described this as collagen fiber disarray (32,41). Since the present macrophage/foam cell morphometric analysis revealed a significant increase in cellular infiltrate in all groups fed an atherogenic diet when compared to controls, it is likely that the reorganization of elastin and collagen in the present experimental animals was due to accumulating macrophage/foam cells.

Lipid deposition is thought to be an early initiating factor in the development of AVS (32), and this is supported by the fact that both aortic sclerosis and stenosis have been repeatedly associated with elevated total cholesterol levels (42-46). In the present study, all groups fed an atherogenic diet displayed Oil Red O staining within the fibrosal surface, primarily in the base and middle portions of the leaflet. This staining was most prominent in those animals exposed to a full 40 weeks of the cholesterol diet.

In humans, it is well established that lipid deposition is associated with the presence of a macrophage infiltrate (32,37,47). Under atherogenic conditions, modified lipoproteins present in the tissue are ingested by macrophages, forming macrophage-derived foam cells (48). Along with lipid deposition, this is considered to be one of the earliest signs of lesion formation. In the present study, it was found that a macrophage/foam cell infiltrate existed within the fibrosal surface of leaflets of rabbit groups fed an atherogenic diet in a pattern that was clearly associated with the distribution of lipid.

Osteopontin has been shown to be involved in a wide range of physiological processes, including chemotaxis, cellular proliferation, inflammation and mineralization (49,50). Osteopontin is a glycoprotein produced by a wide variety of different cell types, including endothelial cells, smooth muscle cells and macrophages. In particular, it has been associated with areas of vascular (51,52) and valvular dystrophic calcification in both heavily and minimally calcified aortic valves in humans (35,37,47) and in animal models (25). Osteopontin is thought to act as a potent regulator of mineralization by binding to ionic calcium and hydroxyapatite crystals with high affinity (49). The distribution pattern of OPN was found to be similar to that of both lipid deposition and macrophage/foam cells - a finding which is consistent with other studies that have demonstrated OPN's association with inflammation (37,47). The presence of OPN and

macrophages/foam cells at similar sites may be explained by two possible mechanisms. Osteopontin is produced by the monocyte/macrophage, and is a known regulator of macrophage invasion, migration and phagocytosis (49,50). Although the significance of OPN deposition in the absence of mineralization is unclear, it has been shown previously that OPN expression in the vasculature may occur in non-calcified vessels (53) and is closely associated with neointima formation and vascular tissue remodeling (54).

Macrophages are not the only inflammatory cells seen in human AVS, as T-lymphocytes have also been reported to be associated with these lesions (32). In cholesterol-fed rabbits, T-lymphocytes were not detected in the lesions that formed in the aorta or in the aortic valves. Previous studies of T-cell expression in atherosclerotic lesions in rabbits have shown that their residence is transient (55), and that T-lymphocytes are primarily detected in the earliest stages of atherogenesis in this model. The lack of T-cells in the present tissue samples may be related to the advanced stage of lesion formation seen at 20 and 40 weeks of cholesterol feeding.

Two recent reports have also examined the development of AVS in rabbits. Rajamannan et al. (25) showed the development of lipid-filled lesions in the aortic valve of rabbits fed a 1% cholesterol diet for 8 weeks. Rabbits fed a diet containing 0.5-3.0% cholesterol have been shown in the past to develop lipid storage disease, and not true atherosclerosis (56). Rajamannan et al. (25) also reported that their animals developed a TPC level over 3000 mg/dl - a level that leads to the deposition of cholesterol in the reticuloendothelial system of these animals. Morphologically, the valvular lesions in these animals demonstrated collagen deposition, macrophage infiltration, and the expression of an osteoblast phenotype in the developing lesions, and provided evidence for the potential use of atorvastatin as an efficacious treatment in ameliorating mineralization (25). The results of the present study similarly demonstrated the presence of a macrophage/foam cell infiltrate and OPN deposition in the aortic valves with a change in collagen content which was quantified using a much lower level of dietary cholesterol. The present observations, based on findings from an animal model that develops human-type aortic lesions, may provide a complete and representative model of human AVS.

Drolet et al. (57) used a third rabbit model of atherosclerosis to study aortic valve stenosis. In their study, calcific aortic stenosis was induced with a 0.5% cholesterol-enriched diet supplemented with vitamin D2. Vitamin D2 has been used in the past to accelerate atherosclerosis in later stages in animal models, and is a known method of inducing calcification (58-62).

Exogenous vitamin D is not metabolized in the same manner as the endogenous source, and has a two-pronged pro-calcific effect (63). It interacts directly with LDL, and it may accumulate in the lesion area, possibly accelerating calcification (64). In addition, vitamin D is a known co-factor for the response elements in genes for the bone matrix proteins such as osteocalcin and OPN (65), and thus may influence calcification in vascular and valvular tissue. Drolet et al. (57) also confirmed mineralization with both Alizarin Red S and Von Kossa's staining of aortic valve tissue sections, which was not observed in the leaflets of the present rabbit model. This was the main reason why this model was categorized as one of AVS before mineralization. Nonetheless, in the present rabbit model, calcification was observed within the aortas, whilst OPN deposition was also observed in the aorta and aortic valves that may influence future mineralization events. Although a vitamin D-supplemented, high-cholesterol-fed rabbit model of AVS may represent a useful accelerated hemodynamic model, the results of management strategies may prove difficult to interpret.

*In conclusion*, the sclerotic changes documented in the aortic valves of this rabbit model of atherosclerosis suggest that it may be used to study early events in the genesis of AVS. If aortic stenosis is potentially a preventable disease, then an animal model of the early stages of this human pathology could be an invaluable tool for the development of intervention strategies (39). These findings support the view that AVS and atherosclerosis are related disease processes, and suggest that intervention strategies developed for the latter condition would be efficacious in the treatment of the former.

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