

# Morphological Changes of the Aortic Valve Leaflets in Non-Compliant Aortic Roots: In-Vivo Experiments

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**Background and aim of the study:** Age-related loss of elasticity of the naturally compliant aortic root disrupts the coordinated function of the valve leaflets. Morphological changes that developed over time in the aortic valve leaflets of non-compliant aortic roots were studied.

**Methods:** Stiffening of the aortic roots was achieved in vivo by applying Super Glue around the sinus of Valsalva in 27 New Zealand White rabbits. In nine animals, glue was applied only partially, and eight untreated rabbits served as controls. Histological evaluation of the aortic valves was performed at 8-11 months after surgery, and included immunohistochemistry and confocal microscopy with quantitative tissue assessment. Levels of collagen I, as a main component of fibrosis, and matrix metalloproteinases (MMP) -1 and MMP-9 and angiotensin-converting enzyme (ACE), as regulators of fibrosis, were analyzed. The morphological structure of the aortic valve leaflets was studied, and the length, thickness and area of leaflets were measured.

*"A Parisian tailor, not yet old, having dined and left his house had walked hardly 40 paces when he suddenly fell to the ground and expired. His body was opened and no disease was found except that the three semilunar cusps leading to the aorta were bony". (Bonet, 1679) (1)*

Second to coronary atherosclerosis, degenerative aortic valve disease is the most common indication for cardiac surgery. Despite its frequent occurrence, however, the cause of this condition remains uncertain. Many factors have been reported to contribute to the development of degenerative disease of the trileaflet aortic valve, including aging, hypertension and hyperlipidemia (2,3). It has been shown that systolic-dias-

**Results:** Leaflets in all groups were found to be composed of a continuous layer of collagen fibers at the mural side, and loose connective tissue containing fibroblasts and few capillaries on the aortic luminal aspect. In stiffened aortic roots, the length and area of the leaflets were increased. The area occupied by collagen was elevated in non-compliant aortic root leaflets, but collagen fluorescence intensity was decreased, indicating less densely packed collagen fibers. Degradation and synthesis of collagen as reflected by MMP-1, MMP-9 and ACE levels was up-regulated.

**Conclusion:** Loss of compliance in aortic roots leads to elongation of the leaflets which, combined with a decrease in collagen density, may render leaflets more susceptible to mechanical stress. In time, this may promote the development of degenerative changes in the aortic valve.

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tolic caliber changes of the compliant human aortic root exert a 'pull-and-release' action upon the commissures, which in turn assures well-coordinated, synchronous and low-stress function of the aortic valve leaflets (4). On the other hand, artificially created stiffening of the aortic wall induced abnormal function and significant stress-overload upon the aortic valve apparatus (5). A similar loss of elasticity of the aortic wall, which occurs as the result of atherosclerosis or age-related fibrosis may have similar effects, and in turn may promote the development of degenerative aortic valve disease (4-7). The aim of the present study was to examine the morphological changes that occurred over time in the aortic valve leaflets of non-compliant aortic roots in a chronic animal model.

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## Materials and methods

### Animal studies

An in-vivo model of the non-compliant aortic root was designed to study the effect of lost elasticity on the leaflets of the aortic valve. The experiments were performed in male New Zealand White rabbits of body weight 2.8-4.2 kg. The animals were housed and cared for in accordance with the guidelines provided in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996). Preoperatively, the rabbits were given an intramuscular injection of ketamine (20 mg/kg) and acepromazine (3 mg), while atropine (1 mg) was given intravenously. A 24-gauge intravenous catheter was placed in a vein of one ear, and hydration was maintained at 50 ml/h with lactated Ringer's solution. A 24-gauge arterial catheter was placed in the artery of the other ear for blood pressure measurements using a pressure monitor (Model 11-4183-09; Gould, Valley View, OH, USA). The rabbits were intubated with a 3-mm tracheal tube and ventilated in volume control mode using a ventilator (SV900C; Siemens, Sweden). A deep anesthetic plane was maintained with 1.5-2% isoflurane. Body temperature was maintained and observed using a Tropi-Cool Hyper/Hypothermia monitor (Seabrook, IL, USA). End-tidal CO<sub>2</sub>, saturated O<sub>2</sub>, and isoflurane levels were monitored with an anesthetic gas analyzer (Rascal II; Ohmeda, Salt Lake City, UT, USA).

### Surgical procedure

A right anterior thoracotomy in the fourth intercostal space was performed under sterile conditions. The ascending aorta, including the sinotubular junction and sinuses of Valsalva, were exposed and dissected from the surrounding tissues. In group A (n = 27), Super Glue (Pacer Technology, CA, USA) was applied so that it encircled the aortic root and formed a rigid ring about 10 mm wide. In group B (n = 9) the glue was applied in a 'spotty' fashion, so as not to restrict the aortic root.

The glue adhered immediately after application to the surface of the aortic wall and within seconds had created a rigid ring around the aorta, thus rendering the aortic root stiff and non-compliant. This was intended to simulate the loss of elastic properties of the aortic wall. The chest was then closed layer-by-layer. Any remaining air was evacuated through a 14-gauge catheter inserted through the chest wall. The rabbits, on average, received about 100 ml of fluid during surgery.

Group C (n = 8) consisted of normal, untreated animals, and served as a control.

The animals were given nalbuphine hydrochloride (Nubain®; 1 mg/kg) subcutaneously every 4 h as anal-

gesia during the first postoperative day. An antibiotic (enrofloxacin; Baytril®; 5.5 mg/kg, given once daily) was administered for up to five days postoperatively. The animals were sacrificed at 8-11 months after the surgical procedure. An autopsy was performed, during which the status of the glue-ring was evaluated and the leaflets of the aortic valves were harvested for assessment and measurements.

### Histology

Upon removal of the aortic roots, the ring of solidified glue and the sinus of Valsalva were carefully opened at the commissural line in order to avoid damage to the leaflets. Prior to freezing in liquid nitrogen, the tissues were secured on cork with needles, and mounted in Tissue-Tec to achieve an appropriate and reproducible orientation for sectioning. Tissues were stored at -80°C. Small samples of the valvular tissue were immediately fixed in glutaraldehyde and embedded for electron microscopy.

### Immunohistochemistry and confocal analysis

Cryosections (8 µm thick) were prepared by cutting each leaflet in the direction of its axis (i.e. from the aortic root to the free edge). The orientation of all leaflets was confirmed by light microscopy of sections stained with 1% toluidine blue. Elastic fibers and collagen were stained using elastica-van Gieson stain in Bouin-fixed sections and evaluated by light microscopy.

Cryosections were fixed either in 4% paraformaldehyde (PFA) for monoclonal anti-MMP-1 and anti-MMP-9 antibodies (matrix metalloproteinases; both Biotrend), and for PECAM (CD31, endothelial marker),  $\alpha$ -smooth muscle actin, vimentin and fibronectin (all Sigma), in acetone-methanol (1:1) for monoclonal anti-ACE (angiotensin-converting enzyme) antibody (Chemicon), or in acetone for monoclonal anti-collagen I antibody (Sigma). All primary antibodies were incubated for 12 h at 4°C. Biotinylated anti-mouse or anti-rabbit IgG (Immunoglobulin G; Dianova) were used as secondary antibodies, and Cy 2-conjugated streptavidin (Rockland) was utilized as the detection system. F-actin was fluorescently stained with TRITC (tetramethylrhodamine isothiocyanate)-conjugated phalloidin (Sigma). Repeated washes with phosphate-buffered saline were carried out after each step of incubation. Sections from the middle part of the leaflets were taken for analysis.

### Confocal microscopic studies and quantitative tissue analysis

All samples were examined with a laser scanning confocal microscope (Leica TCS NT) using Leica Neofluar  $\times 40/1.0$  or  $\times 25/1.0$  objectives. Qualitative changes in experimental valves were determined and

compared with normal and sham-operated animals. The total valve area and area occupied by collagen (or 'collagen area') were delineated and directly measured in the confocal microscope, along with the length and thickness of the valve sections. The length of the section was defined as the distance from the aortic root to the natural rim (one to two measurements from three sections per valve), and the thickness as the distance between the cusp and the aortic luminal side (at least six and up to 12 measurements from three sections).

Following a standardized labeling protocol, collagen I fluorescence intensity (FI) was quantified using the appropriate software (Leica TCS NT version 1.6.587) and presented as collagen I density (calculated by dividing total intensity per leaflet by the total leaflet area). The samples were analyzed with identical confocal settings such as laser power, photomultiplier (PMT), gain (detector voltage), offset or pinhole. During all measurements of FI, intensity levels were kept in a range of 0 to 255.

Collagen I was examined because it is the main component of fibrotic tissues. MMP-1, MMP-9 and ACE were analyzed as they are considered to be regulators of collagen and therefore of fibrosis, with the MMPs being involved in degradation, and ACE in synthesis (8,9).

### Statistical analysis

Values were reported as mean  $\pm$  SE. For statistical analysis, a paired *t*-test and ANOVA were carried out, and a *p*-value  $<0.05$  was considered to be statistically significant.

## Results

### Histology and immunohistochemistry

Both normal and experimental cusp tissues were of similar macroscopic appearance, with the tissue appearing white and almost transparent. Longitudinal sections through valves stained with toluidine blue revealed that the tissue consisted of fine elongations alternating with thickened structures. The same was evident in collagen I-stained sections (Fig. 1). At the cusp side, the tissue contained a collagen layer of varying thickness. Loose connective tissue was present on the aortic luminal side.

### Normal cusp tissue

All leaflet tissues were delineated by endothelium, which was thickened locally, especially on the aortic luminal side. Occasionally, PECAM-positive capillary endothelial cells were found in thickened areas of the cusps. Collagen, as evidenced by collagen I antibody, was clearly localized at the cusp side of the valve, thus confirming the light-microscopic findings. Throughout

the sections, numerous smooth muscle cells and fibroblasts, identified by staining with  $\alpha$ -smooth muscle actin and vimentin, respectively, were evident. These cells were more numerous in the collagen free layer of loose connective tissue. This was also confirmed by electron microscopy.

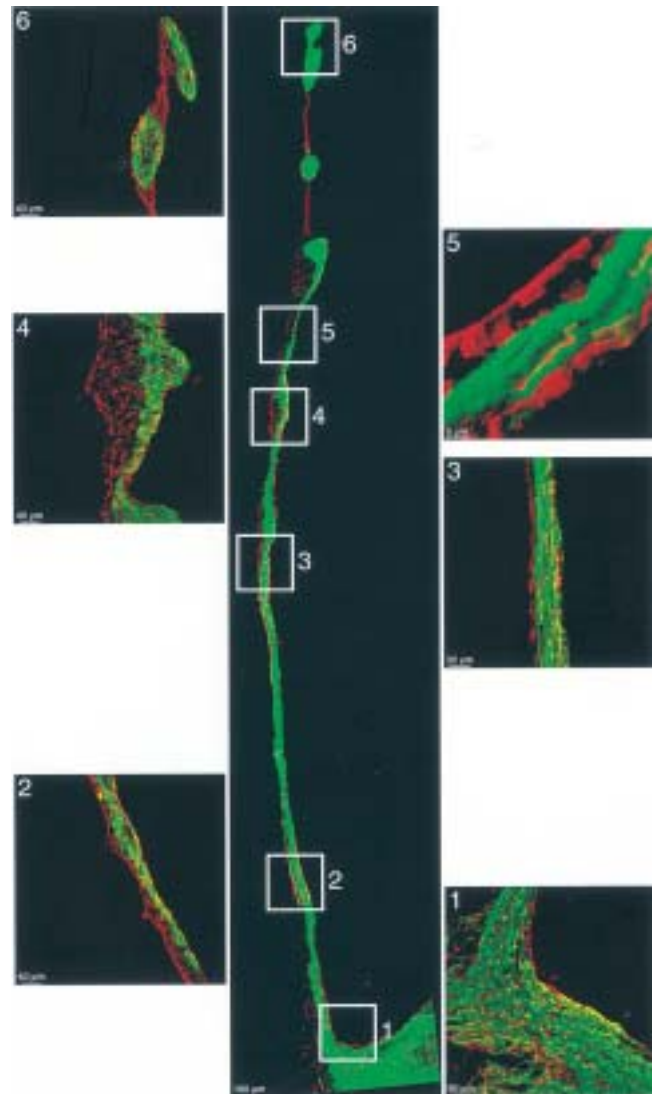


Figure 1: Typical appearance in the confocal microscope of aortic valve leaflets. Green indicates collagen, and red are nuclei from the connective tissue (fibroblasts, smooth muscle cells or endothelial cells). Different sections of cusp tissue along the leaflet indicate: 1. The leaflet near the aortic root is collagen-rich; 2. A section of tissue containing loose connective tissue and collagen in equal parts, the tissue is thin; 3. Thin section of the leaflet, containing mostly collagen; 4. Locally thickened section, containing more loose connective tissue than collagen; 5. Collagen embedded in connective tissue; 6. The free edge of the leaflet with irregular distribution of collagen as small nodules.

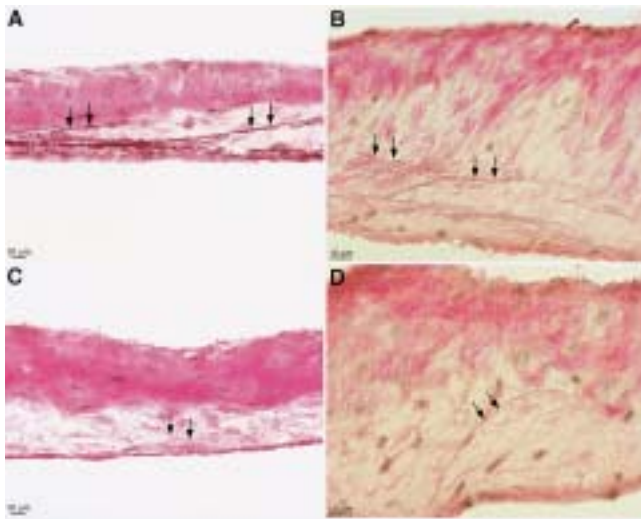


Figure 2: Elastic fibers (indicated by arrows) in valve tissue. Light microscopy; hematoxylin and eosin staining. A, B) Normal valve; note the abundant dark elastic fibers. C, D) Stiffened valve; note the loss of elastic material.

#### Experimental tissue

The general structure of the aortic cusps was similar to that observed in normal tissue. Sections showed the typical varying thickness with focal nodular structures. The number of smooth muscle cells was reduced throughout the cusp, while the number of fibroblasts was unchanged. While elastic fibers were abundant in normal valves, these were reduced in long-term stiffened aortic roots (Fig. 2). Macrophages were found in the experimental valves, but not in control tissue (Fig. 3).

#### Quantitative measurements

##### Leaflet size

There was a statistically significant increase in the length of the leaflet sections in group A, as compared

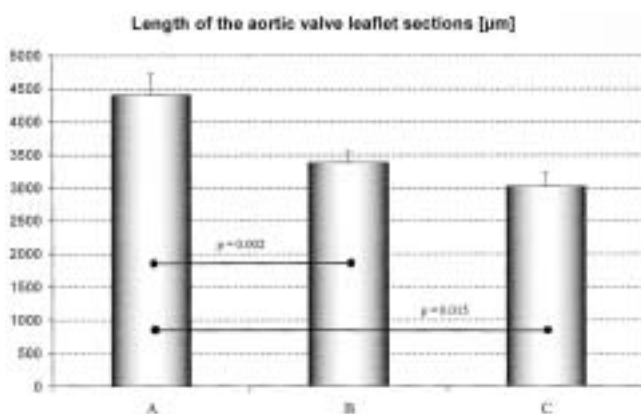


Figure 4: Length of the aortic valve leaflet sections (µm).

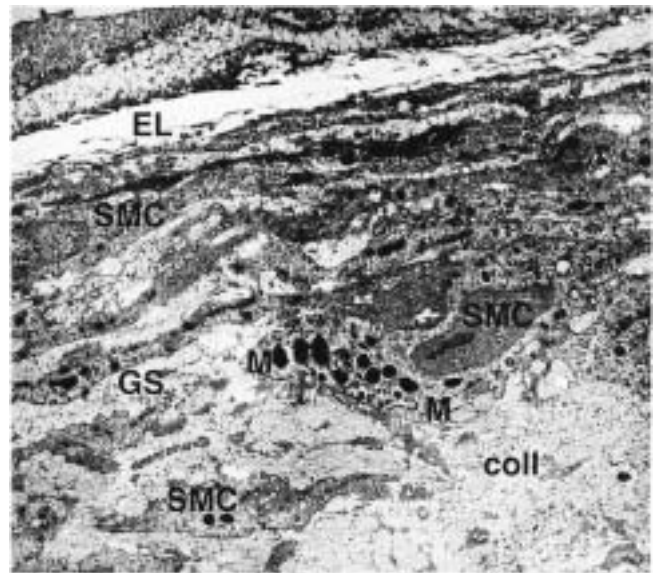


Figure 3: Electron microscopic ultrastructural appearance of experimental valve tissue exhibiting an elastic lamina (EL) and a macrophage (M) in addition to smooth muscle cells (SMC), ground substance (GS) and collagen (coll). Scale indicated by bar.

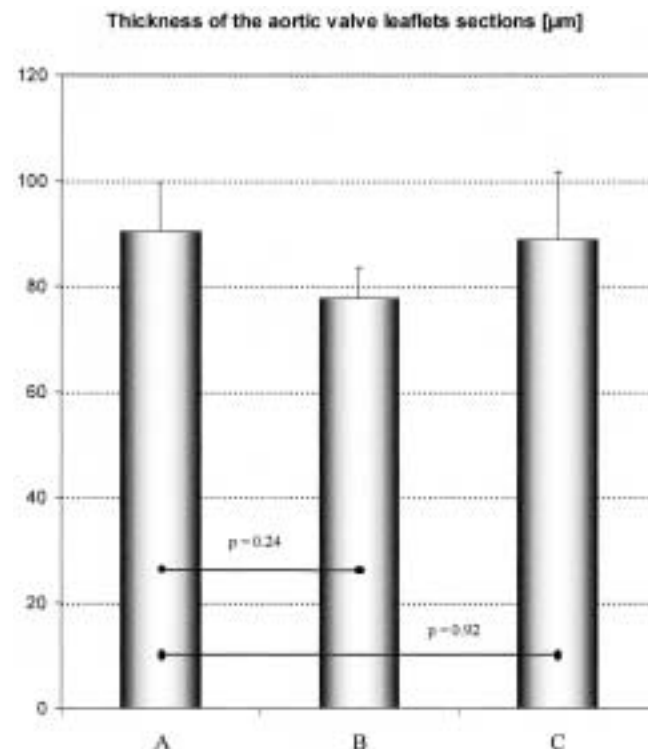


Figure 5: Thickness of the aortic valve leaflet sections (µm).

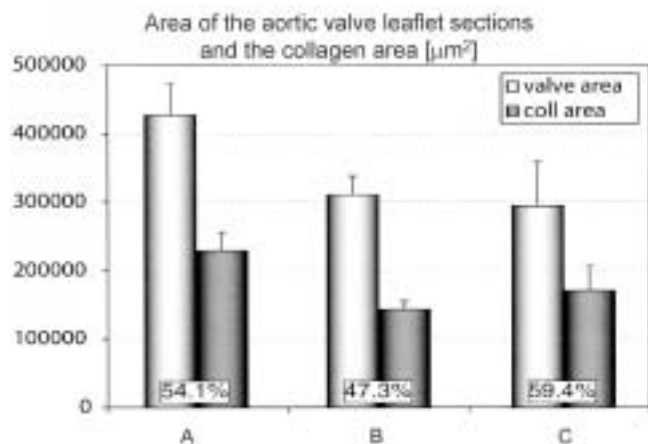


Figure 6: Area of the aortic valve leaflet sections and the collagen area ( $\mu\text{m}^2$ ). The ratio shows that despite the increase of both areas in experimental animals, the relationship between collagen and loose connective tissue remains similar. A) Experimental group; complete glue ring; B) Partial glue application; C) Control; normal untreated valves.

to groups B and C ( $p = 0.002$  and  $p = 0.015$ , respectively) (Fig. 4), but the thickness was unaffected (Fig. 5). The area of the leaflet sections was larger in experimental group A by 30% (mean  $4.25 \times 10^5 \mu\text{m}^2$ ) as compared to group B (mean  $3.0 \times 10^5 \mu\text{m}^2$ ), but the difference did not reach statistical significance due to a high standard deviation ( $p = 0.16$ ) (Fig. 6).

### Collagen

The area occupied by collagen was larger in group A than in the partially glued and control animals (Fig. 6). The ratio of collagen area:total valve area was similar in experimental valves when compared with the other groups (Fig. 6). This was interpreted as an increase of the collagen area proportional to an increase in total valve area. Measurements of FI, however, showed that the enlarged collagen area in the experimental valve

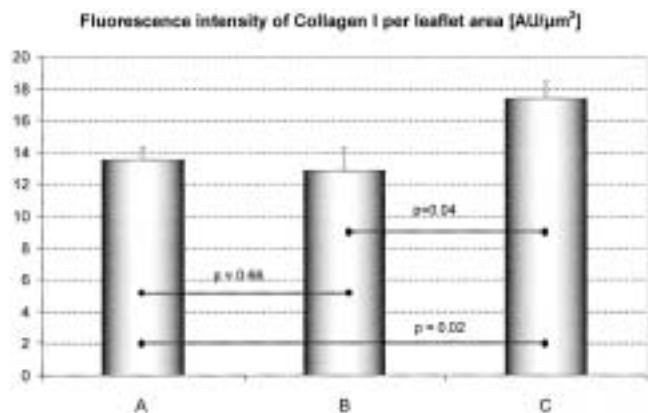


Figure 7: Fluorescence intensity of collagen I per leaflet area [arbitrary units (AU)/ $\mu\text{m}^2$ ].

tissue contained significantly less collagen per unit area, indicating less densely packed collagen ( $p < 0.05$ ) (Fig. 7).

### MMP and ACE

To test for possible mechanisms responsible for altered collagen distribution, a study was made of the protein-degrading system of the MMPs and the profibrotic enzyme, ACE.

MMP-1 and MMP-9 were localized in fibroblasts of the collagen area, as well as in smooth muscle cells, but were also seen to be stored in the extracellular matrix. Quantitative measurements of FI were attempted, but were not representative due to the focal accumulation of both MMPs. Zymography would be a more appropriate method for MMP assessment, though this was not feasible due to the limited amount of tissue available from these small valves. Based on histological

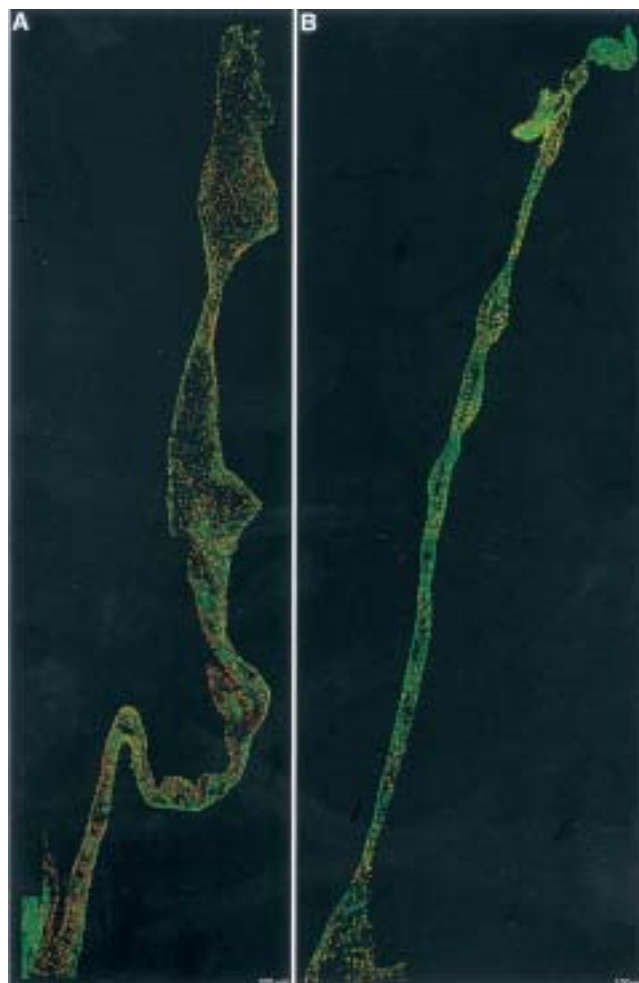


Figure 8: Irregular distribution of matrix metalloproteinase (MMP) -1 over the entire length of the valve section (MMP shown in green, nuclei in red). MMPs are more abundant in experimental tissue. A) Normal valve; B) Experimental valve.

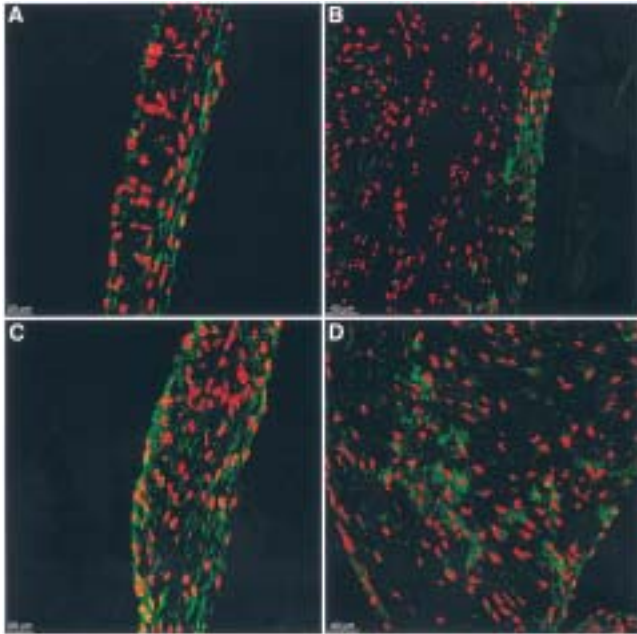


Figure 9: Occurrence of angiotensin-converting enzyme (ACE) in aortic valve leaflets. (ACE shown in green, nuclei in red). Increased fluorescence of ACE in experimental valves. A, B) Normal valves; C, D) Experimental valves.

findings it can be stated that, in experimental tissue, the amount of MMPs was increased as compared to sham or control animals (Fig. 8A and B).

The same observation was made regarding the presence of ACE. In control animals, ACE was present mostly on the cusp side (Fig. 9A and B), but in experimental animals it was seen to occur throughout the valve (Fig. 9C and D).

## Discussion

The morphological findings presented here indicate that, in stiffened aortic roots, the length and subsequently the area of the leaflet sections were increased. Of most importance was the fact that, although the area occupied by collagen at the cusp side was proportionally enlarged in experimental valves, the density of collagen was reduced. The increases in MMP-1, MMP-9 and ACE suggests up-regulated collagen degradation, synthesis and, most probably, turnover. This signifies that in the non-compliant roots, leaflets became longer, with less densely packed collagen.

During the course of previous investigations to identify factors that might contribute to degenerative diseases of the trileaflet aortic valve, normal human aortic roots were mounted into a left heart simulator and their function and configuration was studied using

high-speed (500 frames/s) cinematography, intravascular ultrasound and digitized computer modeling (4-7). The function of these normal aortic valves was characterized by normal hemodynamics and morphology. After application of a synthetic adhesive to the exterior of the mounted human aortic roots, thereby simulating age-related aortic wall stiffening, pressure-flow measurements remained unchanged; however, the function of the valve leaflets, as observed by cinematography (500 frames/s) was altered dramatically. The previously smooth leaflet opening and closure became irregular and asynchronous, and the leaflet motion showed whipping and curling. Correspondingly, the leaflet surface developed multiple wrinkles and creases. This change from the smooth and coordinated leaflet function of the naturally compliant aortic root to an irregular leaflet function in the stiffened root occurred in every specimen and was caused evidently by the loss of compliance.

The above-mentioned functional and macroscopic data correspond with the current findings observed using immunofluorescence microscopy. In contrast to the results that were expected - namely increased thickness and occurrence of fibrosis - the leaflets from the stiffened aortic valves were as thin as in controls, but longer - that is, they extended in the longitudinal direction. However, the collagen density was reduced, which might explain the ability of the leaflets to bulge and to show whipping and curling. It is theorized therefore that, in the situation of aortic root stiffening, the extension of the leaflets is combined with an inadequate increase in collagen that renders the leaflets even more susceptible to mechanical stress. In addition, the reduced amount of elastic fibers might contribute to this process. It cannot be excluded that, over time with abnormal turbulence in the aortic valve cusps, fibrosis may occur. The latter may require time to become detectable, as it was reported that the results of valve-sparing operations are encouraging (10). Further in-depth morphological and ultrastructural studies of human aortic valves should clarify this issue.

It is interesting to note that levels of both MMPs and of ACE were increased in the experimental valves. MMPs are responsible for extracellular protein degradation, whereas ACE promotes the formation of fibrosis (8,9). As both systems were up-regulated, it might be assumed that a constant dynamic turnover of extracellular matrix material occurs in these stiffened valves. As long as the balance between proteolysis and fibrosis formation is kept constant, the connective tissue components will stay unchanged. However, when the balance shifts and proteolysis prevails - which most probably is occurring in the experimental valves at this stage - the collagen density decreases and the

valves become more vulnerable to injury by hemodynamic forces - in other words, they bulge.

In studies of function-related leaflet stresses determined by finite element analysis, a multifold increase in leaflet stresses following the loss of aortic root compliance was found (7). Similar finite element studies of mitral valve leaflets also revealed elevated leaflet stress under conditions of increased tissue stiffness (11).

It has been shown that with an increase in pressure from 80 to 120 mmHg, the diameter of the intact aortic roots mounted in the simulator usually increased at the commissural level by an average of 9% in each cardiac cycle. Thus, it appears that after stiffening the root, the loss of compliance and corresponding restrictions on the expansion of the root were responsible for changes in the leaflet dynamics (4-6).

The synthesis of results from the above-mentioned experiments, supplemented with those presented herein, indicate that the loss of sinus compliance does indeed lead to cessation of the 'pull-and-release' function of the aortic root, places undue stress upon the leaflets, and contributes to changes in their microstructure (2-4,6). It is possible that a similar process commonly occurs in humans and, given time, may promote the development of degenerative aortic valve disease.

Based on clinical experience, it is known that cases involving a totally calcified aorta ('porcelain aorta') are relatively rare, though situations in which the aorta is only partially calcified are common in aged patients and may present additional technical difficulties during surgery (12). Consequently, the detection of the degree and depth of reduced elastic properties of the aorta is important clinically, and clearly has some prognostic value in that it may lead to changes in the surgical approach employed. However, quantification of any correlation between the degree of calcification (extent of encompassment of the circumference and the severity and depth of stiffening of the aortic root) and functional impairment of the aortic valve may require additional studies to be conducted.

### Study limitations

The limitations of this experimental model were based on the assumption that even partial glue application may cause a decrease in aortic root compliance, thus influencing the corresponding changes in the leaflets and reducing the chances of finding a more pronounced difference between groups. In fact, irregular distribution of the collagen was seen in all three groups, which explained the large SD values. In addition, the limited duration of the experiment may not have allowed sufficient time for more severe morphological and functional changes to develop in the exper-

imental group. The sudden introduction of non-compliant properties in the aortic wall also differs from the gradual stiffening that occurs in an aging population, though these conditions were very similar to those that developed as a result of valve-sparing operations, in which the aortic valve was sutured inside a rigid Dacron graft (10,13).

**In conclusion**, the present series of experiments demonstrated in vivo that artificial stiffening of the aortic root alters the morphology of the aortic cusps, inducing elongation and bulging of the leaflets of the valves in non-compliant aortic roots. The affected leaflets also had reduced collagen density in comparison to normal valves. Although these changes were observed during a limited time span, were moderate, and did not reach the level of full-blown aortic valve disease (which usually takes decades to develop), they are similar to changes that might be observed in the initial stages of the condition. There is a possibility that, given time, these changes may further advance into a condition that resembles human degenerative aortic valve disease.

### Acknowledgements

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### References

1. Bonet T. *Sepulchretum sive anatomica practica*, Geneva, Cramer and Perachon, 1, 891, 1700, 1st ed., 1679
2. Stewart BF, Siscovick D, Lind BK, et al. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. *J Am Coll Cardiol* 1997;29:630-634
3. O'Brien KD, Reichenbach DD, Marcovina SM, Kuusisto J, Alpers CE, Otto CM. Apolipoproteins B, (a), and E accumulate in the morphologically early lesion of 'degenerative' valvular aortic stenosis. *Arterioscler Thromb Vasc Biol* 1996;16:523-532
4. Robicsek F, Thubrikar MJ, Fokin AA. Cause of degenerative disease of the trileaflet aortic valve. Review of the subject and presentation of a new theory. *Ann Thorac Surg* 2002;73:1346-54
5. Robicsek F, Thubrikar MJ. Role of sinus wall compliance in aortic leaflet function. *Am J Cardiol* 1999;84:444-449
6. Robicsek F, Thubrikar MJ. Etiology of degenerative disease of the tri-leaflet aortic valve. A simple explanation for a complex problem. *Z Kardiol* 2001;90:VI, 35-38
7. Beck A, Thubrikar MJ, Robicsek F. Stress analysis of the aortic valve with and without the sinuses of Valsalva. *J Heart Valve Dis* 2001;10:1-11

8. Spinale FG. Matrix metalloproteinases: Regulation and dysregulation in the failing heart. *Circ Res* 2002;90:520-530
9. Sun Y, Weber KT. RAS and connective tissue in the heart. *Int J Biochem Cell Biol* 2003;35:919-931
10. David TE, Armstrong S, Ivanov J, Feindel CM, Omran A, Webb G. Results of aortic valve sparing operations. *J Thorac Cardiovasc Surg* 2001;122:39-46
11. Kunzelman KS, Quick DW, Cochran RP. Altered collagen concentration in mitral valve leaflets: biochemical and finite elemental analysis. *Ann Thorac Surg* 1998;66(6 Suppl.):S198-S205
12. Fokin A, Robicsek F. Direct anastomosis of the saphenous vein to the unclamped aorta. *J Cardiovasc Surg* 1998;39:311-312
13. David TE. Aortic valve sparing operations. *Ann Thorac Surg* 2002;73:1029-1030