

Spatial Patterns of Matrix Protein Expression in Dilated Ascending Aorta with Aortic Regurgitation: Congenital Bicuspid Valve versus Marfan's Syndrome

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Background and aim of the study: Aortic wall stress has been shown to increase locally at the convex aspect of the ascending tract when axial root motion is increased, as occurs in aortic valve regurgitation. The study aim was to assess the expression of extracellular matrix (ECM) proteins involved in stress-induced vascular remodeling in the convexity and the concavity of dilated ascending aortas with aortic valve regurgitation.

Methods: Aortic wall specimens, harvested at the convexity and concavity of eight dilated ascending aortas with severe aortic valve regurgitation underwent morphometry, Western blot, RT-PCR and confocal immunohistochemistry. Five patients (group A) had congenital bicuspid aortic valve (BAV), and three (group B) had Marfan's syndrome. Specimens from the aorta of three multi-organ donors served as controls.

Results: At morphometry, medial degeneration was more severe in the convexity than in the concavity, especially in group A. Western blot, RT-PCR and immunohistochemistry disclosed an asymmetric pattern in the expression of some ECM proteins

(laminin, tenascin, fibronectin). Fibronectin was increased in the convexity of both groups compared to controls at Western blot. Immunohistochemistry confirmed this pattern only in BAV. Higher levels of tenascin were found in the convexity in group A. The laminin content was greater in the concavity than in the convexity of both groups, but in group B the type of laminin was different, with the $\beta 2$ chain particularly expressed, and almost absent in non-Marfan patients. Type I and type III collagens were more markedly reduced in the convexity than in the concavity in BAV. In group B, type I collagen was decreased and type III increased, but without any significant difference between the two aspects of the aorta.

Conclusion: A tissue remodeling response to valve disease-related wall stress may underlie aortic dilatation with BAV regurgitation. Although morphometry showed similar changes in Marfan aortas, molecular investigations differentiated this condition, qualitatively, from BAV.

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Bicuspid aortic valve (BAV) is the most common congenital cardiovascular malformation, with a population prevalence of 1-2%, associated with congenital (coarctation) and acquired (aneurysm, dissection) diseases of the aorta (1). Marfan's syndrome is a genetic disorder which affects one to two out of 10,000 subjects in the general population. The condition is caused by *FBNI* gene mutations, and is characterized by systemic

elastic tissue abnormalities with a high incidence of aortic aneurysms and dissections (2). BAV-associated aortic dilatations and Marfan aneurysms share the same histopathological picture of medial degeneration, with elastic fiber degradation and loss of smooth muscle cells (SMCs). Since in BAV patients aortic aneurysms usually develop at a younger age than in patients with a non-malformed aortic valve, the condition is often assimilated, from a clinicomorphological perspective, to Marfan's syndrome (3,4), and aggressive indications for surgery have been recommended (5,6).

Controversy relating to the pathogenesis of BAV-related ascending aorta aneurysm has recently gained renewed interest (7,8). Some authors have advocated a congenital defect of neural crest cells (involved in both valve and media embryogenesis) (9) as the culprit for

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aortic wall degeneration in BAV. However, the fact that no clear inheritability has ever been demonstrated, and that BAV is not associated with systemic arterial disorders, may contradict the hypothesis of a genetic defect of the arterial wall components. Others have maintained that the aortic wall pathology could be mainly secondary to abnormal post-valvular hemodynamics (10). Against this second theory, it has been observed that aortic dilatation also occurs in the absence of valve dysfunction at echocardiography (11), and that aortic diameters are, on average, larger than in tricuspid aortic valve patients matched for degree of aortic valve stenosis or regurgitation (12). However, by means of finite element models, the eccentric geometry of the BAV has recently been shown to produce, even without clinical stenosis, a turbulent flow (13). Uneven wall stress distribution is derived, with maximal burden occurring at the right anterolateral aspect (convexity) of the ascending tract (13). This abnormal stress pattern could play an important role in determining aortic wall changes that promote dilatation: flow characteristics and mechanical forces are known to influence the microstructure of the vessel wall, according to the so-called process of flow-induced vascular remodeling (14,15). On the basis of these findings, the aim of the present study was to assess the spatial patterns of medial changes in the dilated ascending aorta of BAV patients and of Marfan patients with severe aortic regurgitation, and to compare these data with those obtained from the normal aortic wall. The convexity and concavity of the aorta were studied separately, focusing on certain extracellular matrix (ECM) proteins involved in the processes of vascular remodeling that underlie arterial enlargement.

Materials and methods

Tissue sampling

Between January 2003 and December 2004, among 85 patients undergoing surgery for ascending aorta aneurysm, five with a severely regurgitant congenital BAV (group A; mean age 49.4 ± 11.3 years; mean ascending aorta diameter 5.5 ± 0.6 cm) and three with Marfan syndrome and severe aortic regurgitation (group B; mean age 38 ± 3 years; mean ascending aorta diameter 6.1 ± 0.7 cm) were selected for the present study. In order to reduce the heterogeneity of local hemodynamic conditions, patients with hypertension and those with mixed valve disease (combined stenosis and regurgitation) were excluded. During surgery, two large samples of aortic wall were retrieved from each patient at the aortic convexity (right anterolateral aspect) and concavity (opposite wall) respectively, 2 cm beyond the sinotubular junction. Samples were sectioned into four specimens each: one specimen was

used for morphometry, one for Western blot analysis, one for reverse transcription-polymerase chain reaction (RT-PCR), and the final specimen was paraffin-embedded for immunohistochemistry. Aortic wall specimens were also retrieved from three normotensive heart donors (mean age 40.3 ± 12.2 years) who had neither aneurysmal or atherosclerotic disease of the aorta nor BAV, during multiorgan harvesting. The sites of retrieval in control subjects were as described above.

The study was approved by the ethics committee of the authors' institution.

Morphometry

Specimens were prepared with hematoxylin and eosin, periodic acid-Schiff (PAS), Weigert-van Gieson's stain, Alcian-PAS, Alcian-Weigert stain for elastic fibers, and von Kossa's stain. Morphometric analysis was performed using two programs (RM 2100 or RM 5200) of a computer-assisted image analysis system (VIDAS Kontron Elektronik; Zeiss). For each specimen, 10 microscopic fields (original magnification $\times 20$) were randomly selected, and the number of elastic fibers, total SMCs and normal SMCs alone (excluding those with signs of cell degeneration, including cytoplasmic vacuolization, loss of fusiform shape and/or nuclear dysmorphisms) was counted and mean values were computed. The length of elastic fibers (taking into consideration their angulation) and their density per field were also measured.

Western blot

Samples were washed in phosphate-buffered saline (PBS) and then lysed with lysis buffer (1% Triton X-100 in PBS), containing protease inhibitors, for 30 min at 4°C . Lysates were centrifuged at 10,000 r.p.m. for 10 min at 4°C ; protein concentrations were determined using a protein assay (BioRad, Richmond, CO, USA). For each sample, the same amount of total proteins was added with Laemmli sample buffer and separated by electrophoresis in a 30% sodium dodecyl sulfate-polyacrylamide gel. The gels were then electroblotted on polyvinylidene difluoride filters (Millipore, Bedford, MA, USA); membranes were blocked with 6% fat-free dry milk for 1 h at room temperature. After three passes in washing solution, the membranes were incubated overnight at 4°C with antibodies against laminin, tenascin, type I and III collagens (Sigma, St. Louis, MO, USA) or with anti-fibronectin (Chemicon Int., Temecula, CA, USA). After washing, the membranes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated goat secondary antibodies (BioRad). After further washing, the membranes were developed in Luminol substrate and exposed to film (ECL for laminin and fibronectin, ECLplus for tenascin; Amersham Biosciences, Little

Chalfont, Bucks, UK). Computer-acquired images were quantified using ImageQuant software (Amersham Biosciences).

Immunohistochemistry

Specimens were fixed in buffered 10% formalin, embedded in paraffin and sectioned. Serial 4 µm-thick sections of aortic specimens were deparaffinized, covered with primary monoclonal antibodies against type I collagen, tenascin and laminin or polyclonal antibody for fibronectin (Sigma) and type III collagen (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and incubated in a moist chamber for 1 h at 37°C. After several washings in PBS, the specimens were covered with fluoresceinated secondary antibodies (Sigma) and submitted to the same treatment. Nuclei were stained with propidium iodide, after which sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed with a Leica DMLB fluorescence microscope (Leica Microsystems, Inc., Bannockburn, IL, USA), and with a Zeiss SM510 confocal microscope (Karl Zeiss International, Jena, Germany). Samples were evaluated by three independent observers, using a four-point scale to quantify staining, from none (-) to greatest (+++).

RT-PCR

Total RNA was isolated by lysing the frozen aortic vessel tissue samples (150-300 mg) in Trizol solution (Gibco BRL, Life Technologies, Rockville, MD, USA) according to the supplier's protocol. RNA was precipitated and quantified by spectroscopy. A 2-µg portion of total RNA from each sample was reverse-transcribed using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the manufacturer's protocol. The random hexamer primers provided in the kit were used. The same cDNA product obtained from each sample was used for subsequent PCR amplification with the primer sets prepared for the target gene and *GAPDH* housekeeping gene. Primer sequences were designed using the software Primer 3 (developed by

Steve Rozen and Helen J. Skaletsky; available on-line at <http://www-genome.wi.mit.edu>); the mRNA for fibronectin, laminin, collagen I and III were addressed. The optimal annealing temperature for all primer pairs was 55°C. Amplification of the *GAPDH* gene was used as a double internal control. The ratio between the samples and the housekeeping gene was calculated to normalize for initial variations in sample concentration, and as a control for reaction efficiency. The amplified products (12 µl of each sample) were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of ECM protein mRNA were estimated by densitometric scanning and normalized against *GAPDH* loading controls. Densitometric analyses of the PCR products were performed using ImageJ v1.29 software (developed by Wayne Rasband; available on-line at <http://rsb.info.nih.gov/ij/>). All PCR products were purified using the QIAquick PCR purification kit (Qiagen, Santa Clarita, CA, USA), and their identities verified by automated DNA forward and reverse sequencing using a dideoxy terminator reaction chemistry for sequence analysis (model 373A DNA sequencer; Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Data were presented as mean ± SD. Comparisons between control versus BAV and control versus Marfan syndrome were made using a standard Student's *t*-test. Comparisons between convexity and concavity within each study group were made using a paired *t*-test. A *p*-value <0.005 was considered to be statistically significant.

Results

Morphometry

Morphometric findings are listed in Table I. In the two study groups, both total and normal SMC numbers were reduced in the convexity and the concavity. Elastin fragmentation into shorter fibers resulted in an

Table I: Results of morphometric analyses. Values are mean number per field.

Parameter	BAV		p-value*	Marfan		p-value*
	Convexity	Concavity		Convexity	Concavity	
Total SMCs	364.4±36.2	476±83.2	0.012	454.0±56.3	522.7±87.3	0.47
Normal SMCs	242±121.3	514.8±159.7	0.015	246.7±100.2	370±45.8	0.06
EF number	825±195.1	464.2±179.5	0.008	910.6±118.2	586.7±276.8	0.07
Max EF length (µm)	98±41	134±14	0.05	63±7	114±50	0.22
Min EF length (µm)	4±1	15±2	0.003	4±1	15±6	0.07

*Concavity versus convexity.

EF: Elastic fibers; SMCs: Smooth muscle cells.

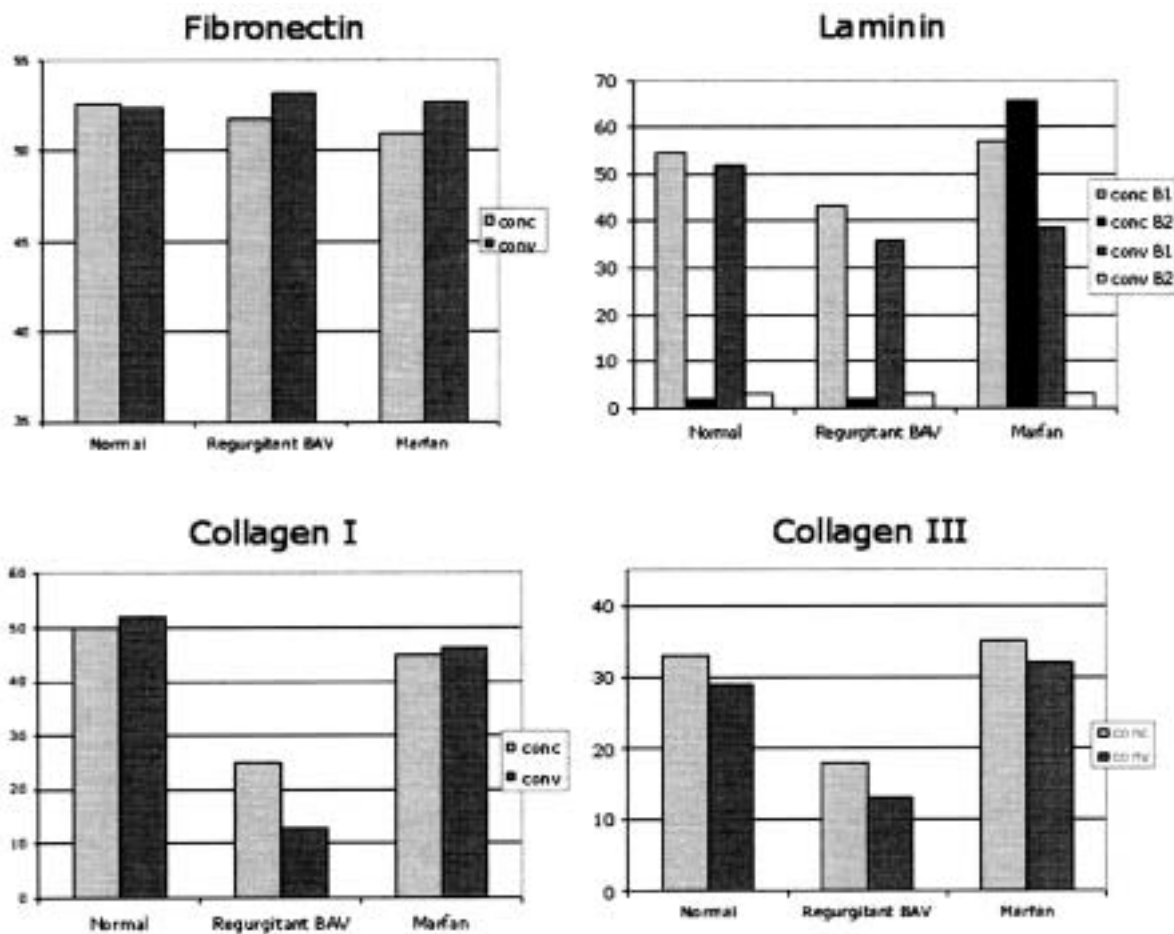


Figure 1: Western blot results for fibronectin, laminin, type I and type III collagen (protein content expressed in nanomoles). B1 and B2 indicate the two different isoforms of laminin.

increased number of them being counted by the computer. The difference in the number of SMCs and elastic fibers between the convexity and the concavity was significant only in group A.

Western blot

Western blot results are shown in Figure 1. The fibronectin content was found to be slightly increased ($p = \text{NS}$) in the convexity of the dilated aortas when compared to specimens from normal convexity. At the concavity, the fibronectin content tended to be reduced, more evidently in group B ($p = 0.16$) than in group A ($p = 0.78$). The laminin content was also slightly greater at the concavity than at the convexity in control aortas: the $\beta 1$ chain was mostly expressed, while the $\beta 2$ chain was almost absent. The laminin ($\beta 1$ chain) content was decreased at the convexity in groups A ($p < 0.01$) and B ($p = 0.03$); however, the $\beta 2$ chain content was significantly ($p < 0.01$) increased at the concavity in Marfan patients. Tenascin C was almost undetectable

in normal aortas, while its three subunits (190, 200 and 220 kDa) were found in all dilated aortas, though in much lower quantities than the other proteins studied. Tenascin was, on average, uniformly represented at the two aspects of the aorta in Marfan patients, but its distribution was asymmetric in BAV patients (a higher content at the convexity than the concavity; $p < 0.05$). A decreased content of type I collagen was found in dilated aortas ($p < 0.05$ only in group A) compared to controls, and a significant difference between convexity (less collagen) and concavity was observed in group A ($p < 0.01$), with no asymmetry in group B. The collagen III content was always decreased in group A, but was selectively increased at the convexity in some Marfan patients.

Immunohistochemistry

Immunohistochemistry results are detailed in Table II and Figure 2. In control subjects, the expression and distribution of ECM proteins was uniform between the

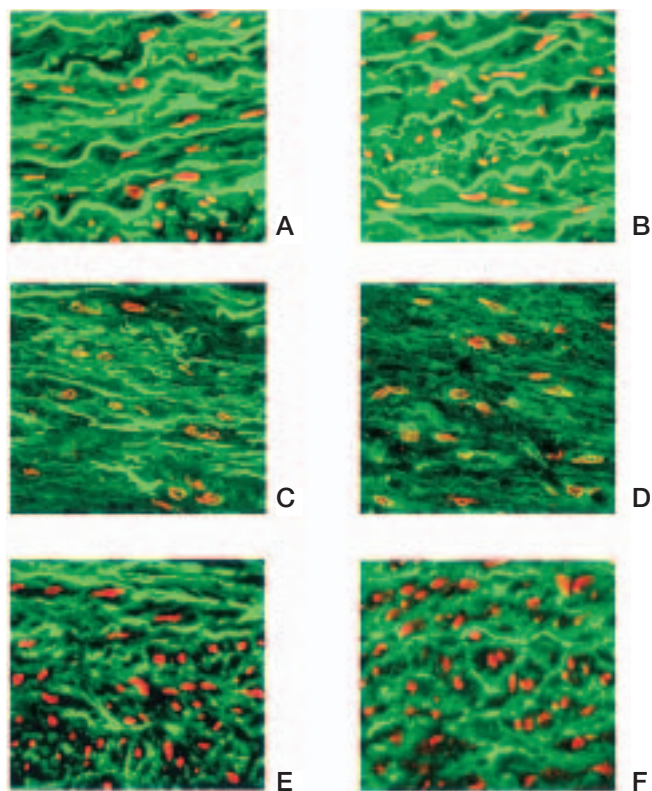


Figure 2: Examples of confocal immunohistochemistry preparations for type I collagen (in green). Smooth muscle cells appear stained in red. Details are shown from: A) Control specimen, concavity; B) control specimen, convexity; C) BAV specimen, concavity; D) BAV specimen, convexity; E) Marfan specimen, concavity; F) Marfan specimen, convexity. Collagen appeared reduced in C,D,E,F when compared to A and B. It was more uniformly distributed between convexity and concavity level in Marfan patients than in BAV patients, in whom it was more evidently reduced at the convexity (D).

convexity and concavity; tenascin was virtually absent. The increase in amounts of fibronectin at the convexity observed previously with Western blot was more evident with immunohistochemistry. With regard to type I and III collagens, an asymmetric expression was found in group A, with lower positivity in the convexity. Frequently, proteins that were reduced were also disarrayed and distributed less homogeneously along the thickness of the wall, with scattered layers of comparative hyperexpression.

RT-PCR

mRNA for fibronectin was reduced in group A compared to normal, with a tendency towards higher levels at the convexity; conversely, mRNA was uniformly increased in the aorta of Marfan patients. The mRNA for laminin was also decreased in group A and increased in group B: in regurgitant BAVs its expression was significantly lower at the convexity than at the concavity. The oligonucleotide sequence for tenascin C was identified, but it was not possible to measure its mRNA. The mRNA for type I collagen was reduced in all dilated aortas compared to control specimens; no significant differences were observed between the convexity and concavity in both groups. In regurgitant BAVs slightly higher levels of mRNA for type III collagen were found compared to normal, whereas in Marfan patients they were much higher, without differences between the sites of retrieval.

Discussion

Although classic morphometric methods cannot differentiate qualitatively the medial lesions underlying aortic dilatation in BAV patients from those in Marfan subjects, the results of the present study revealed remarkable differences between these two conditions in terms of type, grade and, above all, spatial pattern of ECM changes. The idea of assessing ECM changes separately in the convexity and concavity of each dilated

Table II: Results of immunohistochemistry.

Tissue	Fibronectin	Tenascin	Laminin	Collagen I	Collagen III
Normal					
Convexity	+	+/-	+ / ++	++	+
Concavity	+	+/-	++	++	+
BAV					
Convexity	+++	+	+	+/-	+/-
Concavity	+	+/-	+ / ++	+	+
Marfan					
Convexity	++	+	+	+	+ / ++
Concavity	++	+	++	+	+ / ++

BAV: Bicuspid aortic valve.

aorta was derived from the frequent surgical observation by others (16,17) and by the present authors (18), of BAV-associated asymmetric dilatations of the ascending aorta, which appear to maximally involve the right anterolateral aspect (convexity), while the opposite wall (concavity) is relatively unaffected. The genetically determined defect of the aortic wall in BAV patients that has been postulated by some authors (7,9,11) should be expected to involve uniformly the whole circumference of the vessel, as in Marfan's syndrome. In contrast, the pathogenetic factor of wall stress, as advocated by others (10), was shown recently to be distributed asymmetrically in space (13). Flow and stress patterns in the ascending aorta change substantially with different aortic valve morphology and function; therefore, the hypothesis of a pathogenetic role of wall stress can be tested only in well-selected and rheologically homogeneous patient populations. This explains the strict criteria of BAV patient enrolment in the present study. In computational fluid dynamics studies (13), aortic wall stress in BAV was found to be abnormal even in the presence of a non-stenotic valve, as its asymmetric orifice induces turbulence per se, with vortices directed towards the right and beyond the sinotubular junction, instead of being confined within the sinuses of Valsalva, as in the trileaflet valve. Moreover, others (19) have found that amplification of the normal aortic root motion (downward displacement in systole) that occurs with aortic valve regurgitation causes an increase in wall stress at the right anterolateral aspect (convexity) of the ascending aorta. The asymmetric pattern of ECM changes observed in regurgitant BAV-associated dilatations of the aorta in the present study coincides, both qualitatively and quantitatively, with previously reported patterns of wall stress.

The phenomena of vascular tissue remodeling are currently considered to be involved in clinically important processes, such as atherogenesis, restenosis after balloon angioplasty, and aneurysm development (20). At present, a key role appears to be played in all of these situations by medial SMC changes, from the contractile phenotype to a synthetic one. Bunton et al. (21), in a mouse model of Marfan's syndrome, found that the loss of SMC attachment to the elastic laminae, normally mediated by fibrillin-rich microfibrils, induces SMC conversion to the synthetic phenotype and over-production of ECM components, among which fibronectin and tenascin (21,22) and proteolytic enzymes including matrix metalloproteinases, which are responsible for subsequent massive elastolysis and collagenolysis (21,23). However, vascular SMC activation and matrix degradation have been found also to be triggered by acquired conditions, especially mechanical stimuli, such as increased transmural pres-

sure, high flow, shear stress and cyclical strain (24-26). In the present study, in the aortas of BAV patients, fibronectin and tenascin were expressed more at the convexity - which is considered to be an area of stress concentration (13,19) - than in the concavity. Fibronectin is known to further promote the modulation of SMCs from contractile to synthetic phenotype, whereas laminin retains the cells in a contractile phenotype (27). SMCs in the synthetic state have been shown to produce tenascin both in vitro and in vivo (22,28). Tenascin was scarcely represented in aortic wall specimens from normal subjects in the present study, and this was consistent with previous findings in normotensive animals (28). Conversely, its presence in specimens from BAV patients suggests SMC transition to the synthetic phenotype, prevailing at the convexity probably due to stress distribution patterns. Such a role of wall stress could be overwhelmed by that of the congenital defect of cell-matrix connections in Marfan disease (21), and this would explain the uniform distribution of proteins between the convexity and concavity in group B. The reduction in collagens and laminin found in the present study could be derived, at least in part, from the over-expression of collagenases and stromelysins (23). The loss of laminin is expected to enhance SMC activation (27), while the decrease in fibrillar collagens could reduce wall resistance to traction forces (29).

All of the observed changes in ECM components should be considered as the result of a fine balance between two opposing phenomena of abnormal synthesis and increased degradation of the same proteins. According to the present results, the first phenomenon may prevail for proteins such as fibronectin and tenascin, and the second phenomenon for collagens and laminin, though further studies are required to confirm this interpretation. In both protein and mRNA evaluations, Marfan aortic tissue generally showed a more pronounced synthetic and proliferative tendency than BAV aorta. However, the relationships between ECM composition and SMC activity are surely more complex, and feedback inhibition of protein synthesis/transcription could also be implicated, perhaps with different effects between BAV and Marfan patients. This proposal is in part supported by the discordance found between some protein changes and the respective quantities of mRNA.

Study limitations

The present study was unable to differentiate between early and late changes in ECM composition (21). When speculating on the possible pathogenesis of BAV-related aneurysms, it should be borne in mind that a cascade of events is expected to occur, and that both the primary stage and precise sequence are still

unknown. The aortic wall stress, for example, could play a triggering role, but in turn it is increased by dilatation itself, according to Laplace's law. In order to distinguish between causative factors, maintaining conditions and epi-phenomena, it might be useful to assess spatial patterns of ECM changes in early versus mature lesions (i.e., BAV without versus with aortic dilation, or BAV with progressive degrees of aortic enlargement). Moreover, no evaluation of aortic wall stress was possible in the present patients; consequently, the results could not be matched with actual stress patterns, but only compared with those reported by others in mathematical models (13,19).

In conclusion, the changes in aortic ECM composition found in BAV-associated and Marfan-related aortic aneurysms were mostly qualitatively similar; they represent hallmarks of the vascular remodeling process, known to underlie arterial enlargement (15,20-28). The different spatial distribution of ECM proteins, with asymmetric patterns in BAV, and symmetric in Marfan, might depend on different causative mechanisms, which argues against an exclusively genetic pathogenesis of BAV-associated forms. Whether the uneven wall stress distribution demonstrated by others (13,19) is the main factor responsible for aortic dilatation in BAV disease, or whether it simply plays an important cofactor role, must be assessed in further studies.

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Meeting discussion

DR. AJIT YOGANATHAN (Atlanta, Georgia, USA): Your patient population had bicuspid valves with aortic regurgitation, but have you included any patients who had bicuspid valves but no aortic regurgitation?

DR. ALESSANDRO DELLA CORTE (Naples, Italy): We have not yet done that, but we have begun to evaluate patients with bicuspid aortic valves with stenosis and compared them to patients with regurgitation. In these cases there is still asymmetric expression of changes in extracellular matrix proteins, but sometimes they are qualitatively different. For example, bicuspid patients with a stenotic valve have a greater tendency to overproduce tenascin and fibronectin. So the smooth muscle cells may respond differently to different types of stress loading in stenotic and regurgitant settings.

DR. YOGANATHAN: I would expect that, in the stenotic setting, there would be a far more turbulent flow downstream of the valve influencing the stress levels of the wall. The pressure fluctuations might even be larger than in patients who only have regurgitation.

DR. FREDERICK SCHOEN (Boston, Massachusetts, USA): You have shown there to be abnormalities in all of these aortas, and they are all slightly different. How, then, can you distinguish between abnormalities which precipitate the problem and those that are a response to correct the problem? What methods could be used to solve that problem in human material?

DR. DELLA CORTE: Perhaps a similar analysis should be performed in bicuspid patients without aortic dilatation and bicuspid patients with aortic dilatation but the same type of valve dysfunction. In that way we could determine which were the first steps of the mechanism and which were mature lesions, and whether there was an anomaly of the vascular remodeling process.