

Fibrotic Aortic Valve Stenosis in Hypercholesterolemic/Hypertensive Mice

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Objective—Hypercholesterolemia and hypertension are associated with aortic valve stenosis (AVS) in humans. We have examined aortic valve function, structure, and gene expression in hypercholesterolemic/hypertensive mice.

Approach and Results—Control, hypertensive, hypercholesterolemic (*ApoE*^{−/−}), and hypercholesterolemic/hypertensive mice were studied. Severe aortic stenosis (echocardiography) occurred only in hypercholesterolemic/hypertensive mice. There was minimal calcification of the aortic valve. Several structural changes were identified at the base of the valve. The intercuspid raphe (or seam between leaflets) was longer in hypercholesterolemic/hypertensive mice than in other mice, and collagen fibers at the base of the leaflets were reoriented to form a mesh. In hypercholesterolemic/hypertensive mice, the cusps were asymmetrical, which may contribute to changes that produce AVS. RNA sequencing was used to identify molecular targets during the developmental phase of stenosis. Genes related to the structure of the valve were identified, which differentially expressed before fibrotic AVS developed. Both RNA and protein of a profibrotic molecule, plasminogen activator inhibitor 1, were increased greatly in hypercholesterolemic/hypertensive mice.

Conclusions—Hypercholesterolemic/hypertensive mice are the first model of fibrotic AVS. Hypercholesterolemic/hypertensive mice develop severe AVS in the absence of significant calcification, a feature that resembles AVS in children and some adults. Structural changes at the base of the valve leaflets include lengthening of the raphe, remodeling of collagen, and asymmetry of the leaflets. Genes were identified that may contribute to the development of fibrotic AVS. (*Arterioscler Thromb Vasc Biol.* 2016;36:466–474. DOI: 10.1161/ATVBAHA.115.306912.)

Key Words: aortic valves ■ constriction, pathologic ■ hypercholesterolemia
■ hypertension ■ plasminogen activator inhibitor 1

Aortic stenosis in humans is associated with multiple risk factors, including hypercholesterolemia and hypertension.^{1–4} Single risk factors have failed to consistently produce hemodynamically significant calcific aortic valve stenosis (CAVS) in mice. In the first model of CAVS, hypercholesterolemia was produced by deficiency of low-density lipoprotein receptors (*Ldlr*^{−/−}) together with homozygous expression of apo B₁₀₀ (LA mice).⁵ On the basis of the idea that 2 hits may be needed to produce aortic stenosis, we have studied the effects of chronic hypertension, produced by activation of the renin–angiotensin system,⁶ combined with hypercholesterolemia (in apoE-deficient mice). Combining these 2 risk factors has led to discovery of the second experimental model that consistently develops severe aortic stenosis but in sharp contrast to the first model,^{5,7} with minimal calcification of the valve.

In hypercholesterolemic mice, the amount of calcium in the valve is associated with changes in function of the valve

in some,^{8,9} but not all,⁷ interventions. In humans, in some,^{10,11} but not all, studies,^{12,13} the amount of calcification in the aortic valve is a predictor of valve function. Thus, this is a new model of fibrotic aortic valve stenosis (FAVS) without significant valve calcification, findings that resemble children and some adults with aortic stenosis.^{12,13}

Structural changes that account for aortic stenosis are not clear. In this model of FAVS, we found little change in total collagen in the valve. We now propose that collagen remodels at the base of the valve leaflets to produce a mesh, which may contribute to development of stenosis. We also propose that lengthening of the raphe of the valve leaflets may limit the opening of the valve. A method was developed to measure the length of the raphe (or seam) at the base of the valve leaflets. Another method was developed to measure the size of the valve leaflets. It has been proposed that asymmetry of leaflets in humans may lead to processes that produce aortic stenosis.^{14,15}

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Nonstandard Abbreviations and Acronyms	
ACS	aortic cusp separation
AVS	aortic valve stenosis
CAVS	calcific aortic valve stenosis
FAVS	fibrotic aortic valve stenosis
PAI-1	plasminogen activator inhibitor 1
RNA-Seq	RNA sequencing
SEM	scanning electron micrograph

Medical treatments for aortic stenosis in humans have failed.¹⁶ To identify new targets for treatment of aortic stenosis, we have performed whole transcriptome profiling using RNA sequencing (RNA-Seq) of the aortic valve to identify differentially expressed genes in this new model of FAVS. Gene expression was examined before the development of aortic stenosis to avoid confounding effects of advanced disease. No other study of the aortic valve, in humans or in mice, has used RNA-Seq to compare gene expression with that of normal (control) tissue. Because this model of FAVS is characterized by only minimal calcification, one of our goals was to look with RNA-Seq for genes that promote or limit calcification and fibrosis in blood vessels.^{17–19}

Materials and Methods

Materials and Methods are available in online-only Data Supplement.

Results

Using echocardiography to examine opening of the aortic valve during systole, we measured aortic cusp separation (ACS). ACS was similar in control and hypertensive mice (Figure 1). In hypercholesterolemic mice, there was a small decrease in ACS ($P<0.05$; Figure 1E), which was not hemodynamically significant, based on our previous comparison of ACS and transvalvular gradient.⁵ In hypercholesterolemic/hypertensive mice, there was a significant decrease in ACS, with severe aortic stenosis (AVS, <0.66 mm) in about half of the mice (Figure 1E). Thus, severe aortic stenosis was identified only in hypercholesterolemic/hypertensive mice. Significant aortic regurgitation was not observed in any mice.

When mice died before we planned to euthanize them at 12 months of age, the cause of death usually was not clear because deaths were not observed and were not preceded by premonitory signs. Regional wall motion abnormalities of the left ventricle (suggestive of myocardial infarction) were not detected by echocardiography. Gross examination at autopsy did not reveal myocardial infarction or blood in the thorax or abdomen, which would have suggested the rupture of aortic aneurysms.

Histological Measurements

We used morphometry to examine structural components of the aortic valve. There was minimal calcification in the aortic valve of control mice (Figure 2A). Calcification in the valve was modest and similar in hypertensive, hypercholesterolemic, and hypercholesterolemic/hypertensive mice. There was substantial calcification in the atheroma around the base

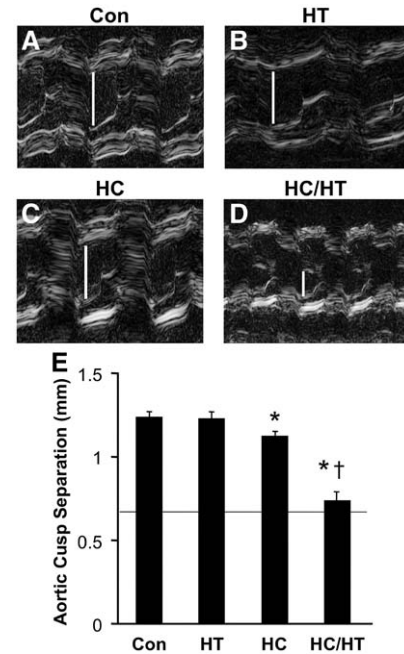


Figure 1. Echocardiographic evaluation of aortic valve function. M-mode echocardiograms (with aortic cusp separation [ACS], white line) in control (Con; **A**), hypertensive (HT; **B**), hypercholesterolemia (HC; **C**), and HC/HT (**D**) mice. Group data for ACS (**E**) were obtained from M-mode echocardiograms. Horizontal line (at 0.66 mm) indicates severe aortic stenosis,⁹ based on previous hemodynamic validation. Values are mean \pm SE; * $P<0.05$ vs Con and † $P<0.05$ vs HC.

of the valve, which indicates that the Alizarin red stain was able to detect calcification when it is present (Figure III in the online-only Data Supplement). Thus, calcification of the aortic valve was minimal and not greater in hypercholesterolemic/hypertensive mice that are the group that develops AVS.

Total collagen and elastic fibers were not changed in hypertensive, hypercholesterolemic, and hypercholesterolemic/hypertensive mice compared with control mice (Figure 2B and 2C). Lipids in the aortic valve were increased in hypercholesterolemic and hypercholesterolemic/hypertensive mice (Figure 2D). Because lipids in the valve were not greater in hypercholesterolemic/hypertensive mice than in hypercholesterolemic mice, they cannot account for aortic stenosis.

Base and Cusps of the Aortic Valve

We next focused on other structural changes that might account for AVS. A new method was developed to examine the raphe, which is the seam between cusps (Figure 3). The length of the raphe (from annulus to separation of the cusps) tended to increase (not significant) in hypertensive and hypercholesterolemic mice and increased >2 -fold ($P<0.05$) in hypercholesterolemic/hypertensive mice (Figure 3E). Thus, the raphe was significantly longer in the group (hypercholesterolemic/hypertensive mice) that developed aortic stenosis.

Area of all 3 cusps tended to increase in hypertensive mice (not significant; Figure 4A). Area of the right and left, but not noncoronary, cusps increased in hypercholesterolemic/hypertensive mice (Figure 4A). Asymmetry of the cusps was expressed as the ratio of the average areas of the right and

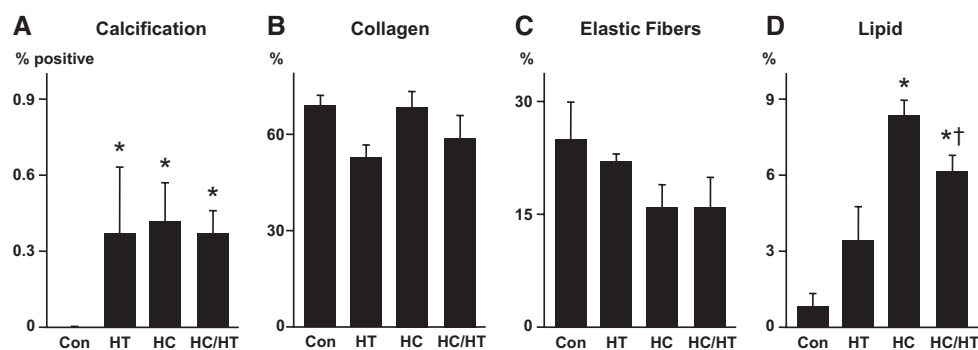


Figure 2. Morphometric evaluation failed to demonstrate a structural basis for aortic valve stenosis in hypercholesterolemia (HC)/hypertensive (HT) mice. Calcification (**A**; Alizarin red), collagen (**B**; picrosirius red), elastic fibers (Movat pentachrome; **C**), and lipid (**D**; oil red-O) in aortic valve. Values are mean \pm SE; n=4–8. * P <0.05 vs control (Con) and † P <0.05 vs HC.

left cusps to noncoronary cusp. Aortic valve cusps were asymmetrical in hypercholesterolemic/hypertensive mice but not in other groups (Figure 4B). In addition, in scanning electron micrographs (SEMs) of hypercholesterolemic/hypertensive mice, the noncoronary cusp was smaller in hypercholesterolemic/hypertensive mice than in other groups (Figure 4C). Thus, with echo measurements, and in SEMs, there is asymmetry of the valve cusps in hypercholesterolemic/hypertensive mice. We have described aortic stenosis in *Ldlr*^{-/-} *Apob*^{100/100} (LA) mice previously⁵ and now provide SEMs for comparison with hypercholesterolemic/hypertensive mice (Figure 4D). In SEMs, there also seems to be asymmetry of the valve in LA mice, with a small noncoronary cusp.

Although total collagen did not change in the aortic valve of hypercholesterolemic/hypertensive mice, we examined the possibility that collagen is remodeled, especially at the raphe (Figure 5). In control, hypertensive, and hypercholesterolemic mice, collagen at the base of the cusps was oriented primarily parallel or perpendicular to the long axis of the attachment site (Figure 5C). In hypercholesterolemic/hypertensive mice, there was a mesh of collagen at the base of the valve cusps, oriented around a 45° angle to the attachment of the cusps, which tethered adjacent cusps to one another (Figure 5B). Thus, although total collagen in the valve was not increased in hypercholesterolemic/hypertensive mice (Figure 2B), collagen is remodeled at the base of the valve in hypercholesterolemic/hypertensive

mice to form a mesh that spans adjacent cusps, which may restrict opening of the valve.

We also used SEMs to examine the structure of the aortic valve (Figure 6). SEMs show enlargement of the base of the valve in both hypercholesterolemic and hypercholesterolemic/hypertensive mice (Figure 6C and 6D). In 1 hypercholesterolemic/hypertensive mouse (Figure 6D; with ACS=0.5 mm), there was a band of tissue between 2 cusps. In hypercholesterolemic/hypertensive mice, there was minimal atheroma/annulus abutting the base of the valve (Figure 4C). In contrast, in LA mice, there was a large amount of atheroma/annulus abutting the base of the valve (Figure 4D).

RNA-Seq

For RNA-Seq analysis, differential expression is defined as >2-fold increase or 50% decrease with P <0.01. We found statistically significant differential expression of 483 genes in hypercholesterolemic/hypertensive mice compared with control mice. Of these, only 150 genes were identified that were differentially expressed in hypercholesterolemic/hypertensive compared with hypercholesterolemic (Table II in the online-only Data Supplement). Some genes of potential interest (Table) include those related to structural organization of the valve (mineralization and collagen organization), endothelium (or endothelial dysfunction), growth factors, and inflammation. Of particular interest, expression of anticalcific (as well

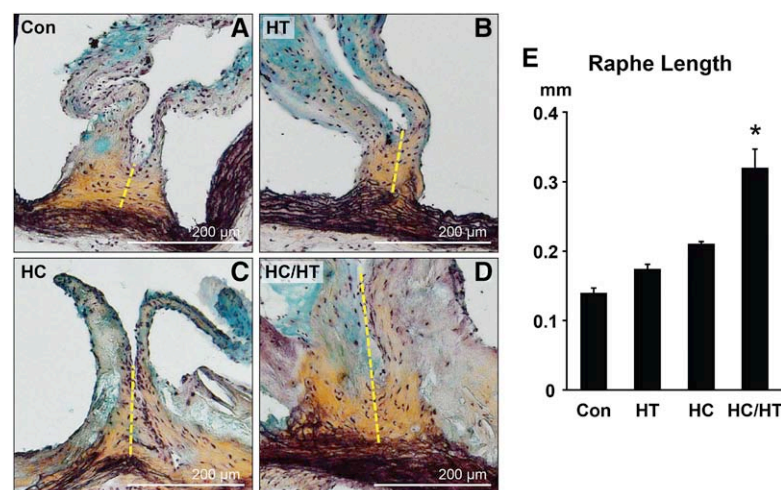


Figure 3. Novel method to examine the raphe (or seam) between valve leaflets. Raphe length (dashed lines; pentachrome stain) was greater in hypercholesterolemia (HC)/hypertensive (HT; **D**) than in control (Con; **A**), HT (**B**), or HC (**C**) mice. Values (**E**) are mean \pm SE, n=7. * P <0.05 vs Con.

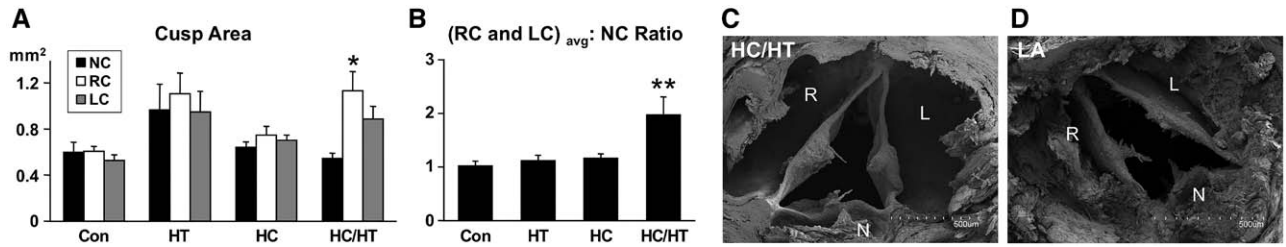


Figure 4. Echocardiographic measurements of size of valve leaflets demonstrated asymmetry of aortic valve leaflets in hypercholesterolemia (HC)/hypertensive (HT) mice. On the basis of 2-dimensional echocardiograms and areas of the noncoronary cusp (NC), right coronary cusp (RC), and left coronary cusp (LC; **A**), areas of RC and LC were averaged and compared with NC area (**B**). * $P < 0.05$ vs NC and ** $P < 0.05$ vs other 3 groups. Scanning electron micrographs (SEMs; **C** and **D**) of aortic valve from an HC/HT mouse with aortic cusp separation of 0.5 mm (**C**) and an *Ldlr*^{-/-}*Apob*^{100/100} (LA) mouse⁵ (**D**). The SEMs are left-right inverted to correspond with the echoes in Figure II in the online-only Data Supplement. There was asymmetry of the cusps, as the NC cusp (bottom) was smaller than other cusps. The SEM of the LA mouse (which is not described elsewhere in the article) is provided for comparison with HC/HT mice. The noncoronary cusps (N in **C** and **D**) are wrinkled and not fully extended. Although plasma cholesterol is similar in the 2 strains of mice, tissue deposition in and around the aortic valve is greater in LA than HC/HT mouse.

as procalcific) genes was increased in hypercholesterolemic/hypertensive mice, consistent with the finding that calcification was minimal in the valve.

To begin to determine whether protein, as well as mRNA, is increased in aortic valves from hypercholesterolemic/hypertensive mice, we quantified plasminogen activator inhibitor 1 (PAI-1) by immunofluorescence (Figure 7). PAI-1 protein levels were increased ≈ 3 -fold in hypercholesterolemic/hypertensive mice (Figure 7E).

Examples of changes in gene expression for molecular signaling networks are shown in Figure V in the online-only Data Supplement. There was differential expression of many peroxisome proliferator-activated receptor- γ -linked genes in

hypercholesterolemic/hypertensive mice versus control mice. When the set of genes was limited to those with differential expression in hypercholesterolemic/hypertensive mice, but not in hypertensive or hypercholesterolemic mice, which do not develop significant aortic stenosis, only 5 peroxisome proliferator-activated receptor- γ -linked genes were identified in the network.

Discussion

There are several major new findings in this study. First, we have discovered a second experimental model that develops severe aortic stenosis. In contrast to the first model, which is CAVS (with 10- to 100-fold greater calcification^{7,9}), this

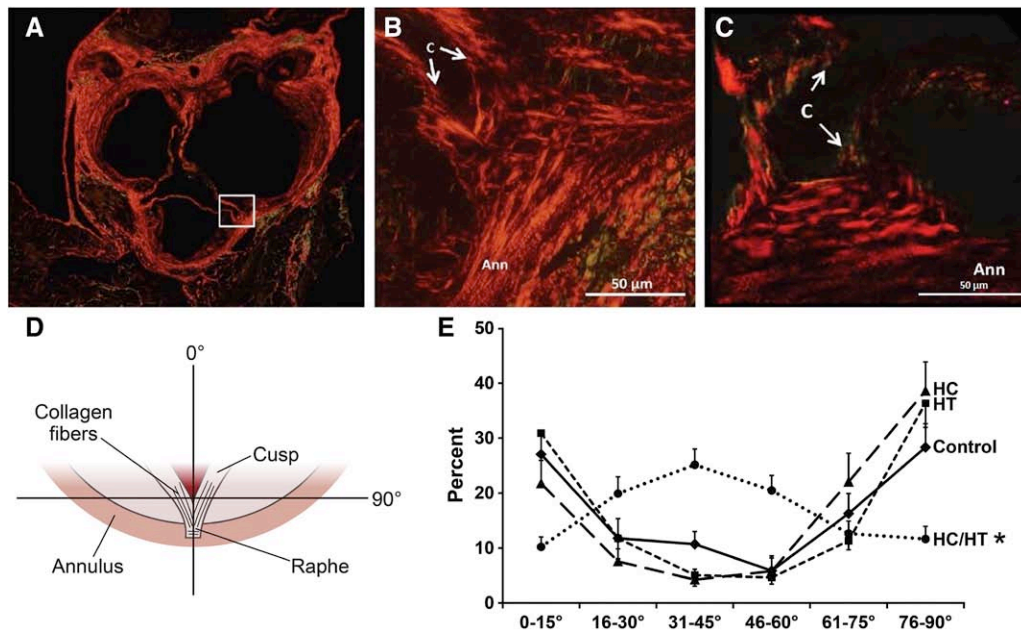


Figure 5. Orientation of collagen fibers at the base of the aortic valve, with remodeling of the fibers in hypercholesterolemia (HC)/hypertensive (HT) mice. Aortic valves from HC/HT mouse (**A**, low power; **B**, high power) and control mouse (**C**, high power), stained with picrosirius red and visualized with polarized light. Red, fibrous collagen; c, valve cusps; and Ann, valve annulus. The bases of the cusps of HC/HT mice have cross fibers of collagen at about a 45° angle. In the control mouse, the collagen fibers are oriented primarily in the direction of the cusps or perpendicular. Orientation of collagen fibers was calculated (**D**) using the intercusp raphe as the 0° reference line. The absolute value of the angle of deviation from the reference line is measured and recorded as a value between 0° and 90°. In the schematic, collagen fibers are oriented $\approx 15^\circ$ and 90° from the reference line. Orientation of collagen fibers (**E**) in control, HT, HC, and HC/HT groups is summarized. * $P < 0.05$ vs other groups.

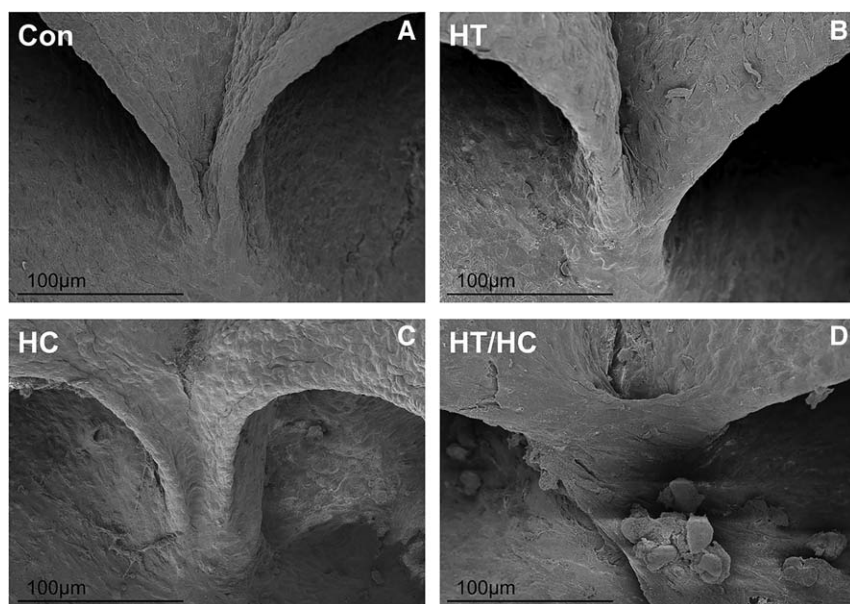


Figure 6. Scanning electron micrographs (SEMs) suggest enlargement of the base of the aortic valve in hypercholesterolemia (HC) and hypertensive (HT)/HC mice. SEMs of the aortic valves from control (A), HT (B), HC (C), and HC/HT (D) mice (representative of SEMs from 3–7 mice in each group). The perivalvular region at the base of the valve appeared enlarged in each of the HC and HC/HT mice. In this HC/HT valve, there was a band across the raphe.

new model has minimal calcification. Thus, this is the first model of FAVS. Second, although there is no change in the total amount of collagen in the valve in FAVS, a change in the arrangement of collagen at the base of the valve may knit the leaflets together and restrict opening of the valve (Figure 8). Also, the raphe at the base of the valve is longer in the group of mice with FAVS than in the other groups. In addition, there is marked asymmetry of the size of valve leaflets in the group of mice with aortic stenosis (Figure 8), a finding that is associated with valve dysfunction in humans.^{15,16} Third, RNA-Seq identified 150 genes that are differentially expressed in stenosis-prone valves in hypercholesterolemic/hypertensive mice; some of which are related to fibrosis.

Experimental Model of FAVS

Prolonged hypertension produced by the renin–angiotensin system, like hypercholesterolemia alone, does not produce FAVS in mice by 12 months of age. In contrast, the combination of hypertension and hypercholesterolemia produced moderate to severe FAVS. Thus, >1 risk factor was necessary to induce FAVS in mice. The observation is concordant with the apparent need for multiple risk factors to produce aortic stenosis in humans.^{1,2} No previous study has examined the role of chronic hypertension in the development of AVS in mice.

At least 30 previous studies have examined the aortic valve in apoE-deficient mice, usually in mice that were fed a high-fat diet. Many studies demonstrated aortic valve disease, but none has reported hemodynamically significant stenosis (ie, a large decrease in pressure across the aortic valve). Although severe aortic stenosis has been observed with echocardiography in apoE-deficient mice, the prevalence is low (1 of 64 mice with Doppler velocity of >4.0 m/s).²⁰ We observed statistically significant reduction in ACS in hypercholesterolemic mice, but the physiological effect of that reduction would be expected to be minimal, based on previous hemodynamic correlation studies in mice.⁵

We and others have assumed that calcification or fibrosis mediate hemodynamically significant FCAVS²¹ and that global measurements of calcification and fibrosis in the valve would predict hemodynamically significant aortic stenosis. We have not found consistent evidence to support this assumption. During development of CAVS, calcification increases in the valve.^{21,22} Reduction of calcium in the valve by 70% during treatment of hypercholesterolemia, however, failed to reduce stenosis.⁷ Furthermore, in children¹³ and adults,¹² aortic stenosis often occurs in the absence of gross calcification. Thus, associations between calcification of the aortic valve and valve function are highly variable, in humans as well as mice, reflecting heterogeneity of underlying disease processes.

In this study, calcification of the aortic valve was minimal with hypertension or hypercholesterolemia alone and did not produce hemodynamically significant aortic stenosis. Furthermore, the amount of calcification of the valve was not greater in hypercholesterolemic/hypertensive mice with stenosis. Thus, calcification of the valve does not account for stenosis in this experimental model of severe aortic stenosis.

Calcification and collagen content in the valve do not account for AVS in this model. It is theoretically possible that neighboring annulus atheromata could restrict valve function. On the basis of SEMs (Figure 4C and 4D), however, atheromatous tissue abutting the valve is minimal in hypercholesterolemic/hypertensive mice, which differs from CAVS in LA mice. Thus, generalized encroachment of atheroma/annulus on cusp excursion is unlikely as an explanation for valve stenosis. Second, although total collagen in the valve did not increase in hypercholesterolemic/hypertensive mice, there was reorganization of collagen in regions that critically influence cusp mobility.

The experimental model that we described previously⁵ are *LDLr^{-/-} Apob^{100/100}* mice. The mice develop CAVS. The current model is *Apoe^{-/-} REN⁺ AGT⁺*, and the mice develop FAVS. Mechanisms that account for the strikingly different phenotype are not clear, but one might speculate that activation of

Table. Change in Selected Genes by Category

	Gene	Name or Function	Fold Change vs Control			Fold Change HC/HT vs HC
			HT	HC	HC/HT	
Mineralization	SPP1	Osteopontin	4.3	95	30	...
	BMP2	BMP	3.2	4.8
	GDF10	BMP3	0.39
	ASPN	Asporin (tissue mineralization)	0.3	0.32
	LRRC17	Osteoclast differentiation	0.37	...	0.17	0.24
Collagen organization	SERPINE1	PAI-1	5.5	3.7
	OGN	Osteoglycin	0.12	0.15
	LGALS3	Galectin 3: myofibroblast proliferation	...	10.0	7.0	...
	COL8A1	Collagen 8	3.2	...	6.5	3.2
	FLNB	Filamin-β	2.4	2.6
	EFEMP1	EGF-containing fibulin-like	0.46	...	0.29	0.33
	ADAMTS8	Disintegrin-like and MMP	2.8	...	5.2	3.1
	ADAMTS5	Disintegrin-like and MMP	0.4
	MFAP4	Microfibrillar associated	0.34	...	0.36	0.4
	TNC	Tenascin	...	5	...	0.35
	GUCY1A3	Guanylate cyclase	0.26	0.28
	PDGFB	Platelet-derived growth factor	3.8	2.9	9.6	3.4
	FGF14	Fibroblast growth factor 14	0.42	0.47
Inflammation	CFH	Modulates C3	0.46	0.46
	IL33	Interleukin 33	0.44	0.46
	IL11RA	Interleukin 11r α-subunit	0.41	0.39
	C1S	Complement	0.33
	C3	Complement	...	5.9	...	0.27
Ion channels	CYTL1	Cartilage destruction	0.24	0.4
	CACNG7	Calcium channel, voltage dependent	2.82
	TRPV4	Transient receptor potential cation channel	3.13	2.23
	ATP1A2	ATPase, Na ⁺ /K ⁺ transporting	0.45	...	0.23	0.41
	KCNJ8	Potassium inwardly rectifying channel	0.36
	GPM6A	(Ca ⁺⁺ transport) glycoprotein	0.26
	RYR2	Ryanodine receptor 2, cardiac	0.11	0.15

P < 0.01 for all numeric values. BMP indicates bone morphogenetic protein; EGF, epidermal growth factor; HC, hypercholesterolemia, HT, hypertension; MMP, matrix metalloproteinase; and PAI-1, plasminogen activator inhibitor 1.

the renin–angiotensin system in REN⁺ AGT⁺ mice may contribute to fibrosis observed in *Apoe*^{−/−}REN⁺ AGT⁺ mice.

One likely explanation for the failure of pharmacological treatments to slow the progression of AVS in humans is that underlying mechanisms may be heterogenous. This possibility is supported by findings in humans^{10–13} and now in mice that the amount (and perhaps role) of calcification differs in human adults and children and in 2 experimental models.

Base and Cusp of Aortic Valve

Two lines of evidence indicate that changes at the base of valve leaflets occur in hypercholesterolemic/hypertensive mice, which may account for FAVS. First, in hypercholesterolemic/hypertensive mice, the length of the raphe more than doubles. Second, with polarized light, there is a mesh of cross fibers at the base of leaflets in hypercholesterolemic/hypertensive but not in control, hypertensive, or hypercholesteremic mice. On

the basis of change in orientation of collagen fibers in hypercholesterolemic/hypertensive mice, we conclude that there is reorganization of collagen fibers and structural remodeling. If fibrosis is viewed as a process where extracellular matrix remodeling leads to reduced tissue distensibility or mobility, then our novel findings are consistent with the concept of FAVS. Our finding of severe aortic stenosis in the absence of gross increases in valve collagen is analogous to findings in some patients with aortic stenosis. Côté et al²³ found at least 25-fold variability in valve collagen among patients with severe aortic stenosis and also found that ≈20% of explanted valves demonstrated minimal valve collagen. This new finding, taken together with studies in CAVS mice, demonstrates that mechanisms leading to severe aortic stenosis can be diverse in mice, as is the case in humans.

The stimulus for remodeling, with formation of a mesh between leaflets in hypercholesterolemic/hypertensive mice,

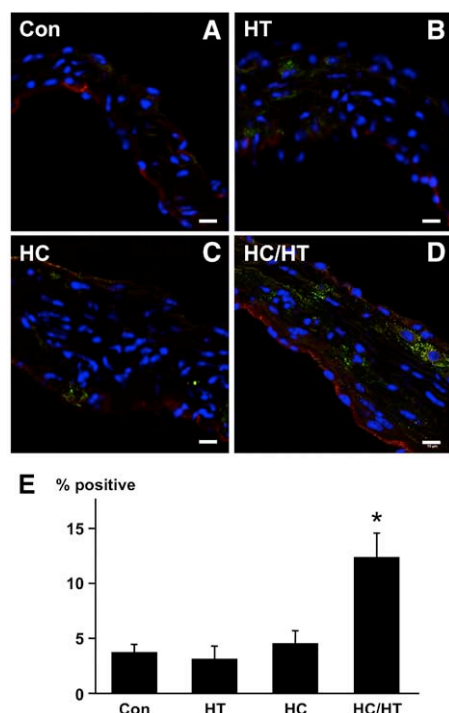


Figure 7. Increased expression of plasminogen activator inhibitor 1 (PAI-1) protein in aortic valve of hypercholesterolemia (HC)/hypertensive (HT) mice. Immunofluorescence of PAI-1 (green), CD31 (red), and DAPI (4',6-diamidino-2-phenylindole; blue) in aortic valve of control (Con; **A**), HT (**B**), HC (**C**), and HC/HT mice (**D**). Expression of PAI-1 (y axis is the percentage of area of PAI-1 staining; **E**) increased about 3-fold in HC/HT mice (n=4 for each group; * $P<0.05$).

is not clear. Remodeling may be driven by cells within the leaflets or possibly because of remodeling or changes in stress of the aortic wall.

Changes in collagen²⁴ and proteoglycans²⁵ have been observed in aortic stenosis, which seem to be related to calcific nodules in valve cusps. Because the observations were made in valves removed from humans, the base of the valves was not examined. We have previously measured collagen in valves from mice²⁶ but did not examine the base of the valves specifically.

RNA-Seq

A unique aspect of our RNA-Seq approach is that expression is compared in an experimental model that is documented to develop severe aortic stenosis versus disease models that do not consistently develop severe AVS. No other study, in humans or mice, has used RNA-Seq to compare valves with aortic stenosis or are diseased but not yet stenotic versus controls. In addition, we examined expression before development of aortic stenosis to avoid reporting epiphenomena associated with end-stage disease.

An advantage of RNA-Seq compared with arrays is the greater potential for gene discovery. Whereas arrays examine genes that are selected and limited, RNA-Seq covers the entire transcriptome. This approach could result in identification of genes that have not been fully annotated.

A recent study²⁷ used RNA-Seq to compare tricuspid and bicuspid valves from humans. The study illustrates a

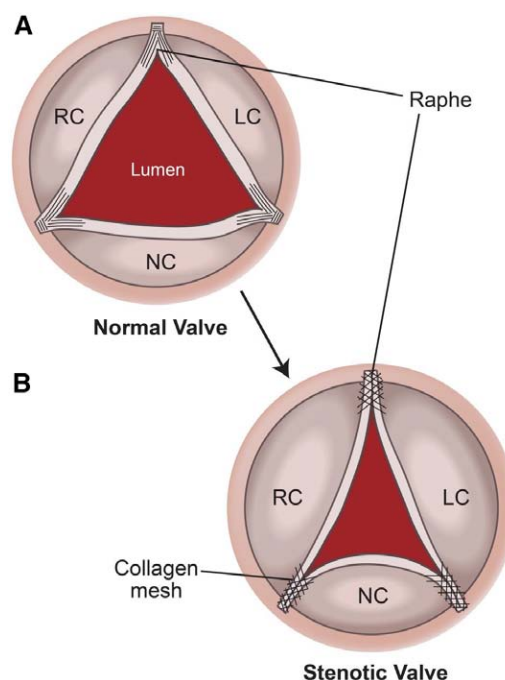


Figure 8. Some mechanisms that may contribute to transition from normal (**A**) to stenotic aortic valve (**B**) in fibrotic aortic valve stenosis (FAVS). A collagen mesh forms at the base of the leaflets that may knit the leaflets together and extend the raphe. In addition, the noncoronary cusp (NC) is smaller than the right coronary cusps (RC) and left coronary cusps (LC) in mice with FAVS. Asymmetry of valve leaflets is associated with impairment of opening of the valve.

productive approach to examine the aortic valve. Seven of 10 genes that differed in the 2 groups produce or degrade extracellular matrix,²⁷ which implies an important role of fibrosis.

Many genes were differentially expressed in the aortic valve of hypercholesterolemic/hypertensive mice (summarized in the Table). Expression of bone morphogenetic protein 2 (which stimulates bone formation) was greater in hypercholesterolemic/hypertensive mice, but this difference may be offset by enormous increases in osteopontin (which protects against calcification), decreases in bone morphogenetic protein 3 (which negatively regulates bone density), and decreased expression of asporin (which may induce collagen mineralization). The balance of these changes may contribute to the finding that the mice do not develop the calcific type of aortic stenosis (CAVS). Expression of some other genes (LDLR-related protein 6 and TNFRSF11B [which encodes osteoprotegerin])^{18,19} that inhibit calcification in blood vessels is not increased in the aortic valve of hypercholesterolemic/hypertensive mice.

In the aortic valve of hypercholesterolemic/hypertensive mice, there are prominent changes in genes associated with collagen organization. Expression of osteoglycin, which contributes to collagen organization (and also induces ectopic bone formation), was reduced >60% in hypercholesterolemic/hypertensive versus hypercholesterolemic mice. Differential expression of galectin 3, which is profibrotic, increased. There were large changes in matrix metalloproteinases.

RNA-Seq, in addition to providing information about genes related to calcification and collagen organization, also

demonstrated differential regulation of genes that modulate endothelial dysfunction and ion channels (Table). Expression of guanylate cyclase, an effector of endothelial function,¹ is decreased in hypercholesterolemic/hypertensive mice. In valves from hypercholesterolemic/hypertensive mice, there is differential expression of several genes that are associated with ion channels, including TRPV4. In vascular endothelium, TRPV4 is activated by laminar shear and regulates endothelial permeability.²⁸

After RNA-Seq, we chose to examine PAI-1 protein based on 2 criteria. First, expression of the gene is increased in valves from hypercholesterolemic/hypertensive mice and not in the 3 other groups. Second, PAI-1 is profibrotic and procalcific in other organs and is highly upregulated by inflammation.²⁹

Expression of PAI-1 also correlates with calcification in human aortic valves.³⁰ Thus, increased expression of PAI-1, demonstrated with RNA-Seq and protein, supports the hypothesis that PAI-1 contributes to development of FAVS in hypercholesterolemic/hypertensive mice. The findings provide evidence for the potential utility of this approach for identification of new therapeutic targets.

Limitations

The degree to which some novel findings in hypercholesterolemic/hypertensive mice, for example, raphe length, collagen fiber orientation, and gene expression patterns, can be extrapolated to humans with aortic stenosis is not yet known.

Several mice (primarily hypercholesterolemic/hypertensive mice) died before the time at which we planned to euthanize the groups. Although immediate cause of death was usually not known, it is likely that the mice that died were more severely affected and that we underestimated the synergy of hypertension and hypercholesterolemia in induction of FCAVS.

RNA-Seq revealed differential expression of a number of genes that are known to be operative in other settings in the processes of endothelial dysfunction, calcification, fibrosis, and inflammation. We confirmed increased levels of 1 gene product, PAI-1. Determination of the pathophysiologic effect of many newly identified candidate gene products is beyond scope of this initial report of a new model of FAVS.

In summary, we used novel approaches to examine structural changes in the aortic valve and their relationship to valve function. First, the length of the intercusp raphe nearly doubled in the group (hypercholesterolemic/hypertensive) that developed stenosis. A small increase in length of the intercusp raphe would be expected to profoundly restrict opening of the valve. Second, a collagen mesh was observed at the base of the valve in hypercholesterolemic/hypertensive mice, which may lengthen the raphe. In addition, there was asymmetry of the cusps, which may contribute to initiation of processes that produce dysfunction of the aortic valve. Finally, RNA-Seq identified novel candidates, including PAI-1, during the prestenotic disease phase in hypercholesterolemic/hypertensive mice.

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Disclosures

None.

References

- Freeman RV, Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. *Circulation*. 2005;111:3316–3326. doi: 10.1161/CIRCULATIONAHA.104.486738.
- Messika-Zeitoun D, Bielak LF, Peyser PA, Sheedy PF, Turner ST, Nkomo VT, Breen JF, Maalouf J, Scott C, Tajik AJ, Enriquez-Sarano M. Aortic valve calcification: determinants and progression in the population. *Arterioscler Thromb Vasc Biol*. 2007;27:642–648. doi: 10.1161/01.ATV.0000255952.47980.c2.
- Boon A, Cheriex E, Lodder J, Kessels F. Cardiac valve calcification: characteristics of patients with calcification of the mitral annulus or aortic valve. *Heart*. 1997;78:472–474.
- Nkomo VT, Gardin JM, Skelton TN, Gottdiener JS, Scott CG, Enriquez-Sarano M. Burden of valvular heart diseases: a population-based study. *Lancet*. 2006;368:1005–1011. doi: 10.1016/S0140-6736(06)69208-8.
- Weiss RM, Ohashi M, Miller JD, Young SG, Heistad DD. Calcific aortic valve stenosis in old hypercholesterolemic mice. *Circulation*. 2006;114:2065–2069. doi: 10.1161/CIRCULATIONAHA.106.634139.
- Merrill DC, Thompson MW, Carney CL, Granwehr BP, Schlager G, Robillard JE, Sigmund CD. Chronic hypertension and altered baroreflex responses in transgenic mice containing the human renin and human angiotensinogen genes. *J Clin Invest*. 1996;97:1047–1055. doi: 10.1172/JCI118497.
- Miller JD, Weiss RM, Serrano KM, Castaneda LE, Brooks RM, Zimmerman K, Heistad DD. Evidence for active regulation of pro-osteogenic signaling in advanced aortic valve disease. *Arterioscler Thromb Vasc Biol*. 2010;30:2482–2486. doi: 10.1161/ATVBAHA.110.211029.
- Chu Y, Lund DD, Weiss RM, Brooks RM, Doshi H, Hajj GP, Sigmund CD, Heistad DD. Pioglitazone attenuates valvular calcification induced by hypercholesterolemia. *Arterioscler Thromb Vasc Biol*. 2013;33:523–532. doi: 10.1161/ATVBAHA.112.300794.
- Miller JD, Weiss RM, Serrano KM, Brooks RM 2nd, Berry CJ, Zimmerman K, Young SG, Heistad DD. Lowering plasma cholesterol levels halts progression of aortic valve disease in mice. *Circulation*. 2009;119:2693–2701. doi: 10.1161/CIRCULATIONAHA.108.834614.
- Messika-Zeitoun D, Aubry MC, Detaint D, Bielak LF, Peyser PA, Sheedy PF, Turner ST, Breen JF, Scott C, Tajik AJ, Enriquez-Sarano M. Evaluation and clinical implications of aortic valve calcification measured by electron-beam computed tomography. *Circulation*. 2004;110:356–362. doi: 10.1161/01.CIR.0000135469.82545.D0.
- Clavel MA, Messika-Zeitoun D, Pibarot P, Aggarwal SR, Malouf J, Araoz PA, Michelena HI, Cuffe C, Larose E, Capoulade R, Vahanian A, Enriquez-Sarano M. The complex nature of discordant severe calcified aortic valve disease grading: new insights from combined Doppler echocardiographic and computed tomographic study. *J Am Coll Cardiol*. 2013;62:2329–2338. doi: 10.1016/j.jacc.2013.08.1621.
- Mohler ER 3rd, Medenilla E, Wang H, Scott C. Aortic valve calcium content does not predict aortic valve area. *J Heart Valve Dis*. 2006;15:322–328.
- Hinton RB Jr, Lincoln J, Deutsch GH, Osinska H, Manning PB, Benson DW, Yutzey KE. Extracellular matrix remodeling and organization in developing and diseased aortic valves. *Circ Res*. 2006;98:1431–1438. doi: 10.1161/01.RES.0000224114.65109.4e.
- Aicher D, Bewarner M, Kindermann M, Abdul-Khalique H, Schäfers HJ. Aortic valve function after bicuspidization of the unicuspid aortic valve. *Ann Thorac Surg*. 2013;95:1545–1550. doi: 10.1016/j.athoracsurg.2013.02.030.
- Joseph L, Krishnaswamy A, Tuzcu EM, Sonny A, Ozkan A, Svensson LG, Griffin BP, Thomas J, Kapadia SR. Relation of cuspal asymmetry to development of aortic stenosis in adults with tricuspid aortic valves. *J Heart Valve Dis*. 2014;23:395–405.

16. Borer JS, Sharma A. Drug therapy for heart valve diseases. *Circulation*. 2015;132:1038–1045. doi: 10.1161/CIRCULATIONAHA.115.016006.
17. Demer LL, Tintut Y. Inflammatory, metabolic, and genetic mechanisms of vascular calcification. *Arterioscler Thromb Vasc Biol*. 2014;34:715–723. doi: 10.1161/ATVBAHA.113.302070.
18. Morony S, Tintut Y, Zhang Z, Cattley RC, Van G, Dwyer D, Stolina M, Kostenuik PJ, Demer LL. Osteoprotegerin inhibits vascular calcification without affecting atherosclerosis in *ldlr*($-/-$) mice. *Circulation*. 2008;117:411–420. doi: 10.1161/CIRCULATIONAHA.107.707380.
19. Cheng SL, Ramachandran B, Behrmann A, Shao JS, Mead M, Smith C, Krcchma K, Bello Arredondo Y, Kovacs A, Kapoor K, Brill LM, Perera R, Williams BO, Towler DA. Vascular smooth muscle LRP6 limits arteriosclerotic calcification in diabetic *LDLR* $-/-$ mice by restraining noncanonical Wnt signals. *Circ Res*. 2015;117:142–156. doi: 10.1161/CIRCRESAHA.117.306712.
20. Tanaka K, Sata M, Fukuda D, Suematsu Y, Motomura N, Takamoto S, Hirata Y, Nagai R. Age-associated aortic stenosis in apolipoprotein E-deficient mice. *J Am Coll Cardiol*. 2005;46:134–141. doi: 10.1016/j.jacc.2005.03.058.
21. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. *Circ Res*. 2011;108:1392–1412. doi: 10.1161/CIRCRESAHA.110.234138.
22. Weiss RM, Miller JD, Heistad DD. Fibrocalcific aortic valve disease: opportunity to understand disease mechanisms using mouse models. *Circ Res*. 2013;113:209–222. doi: 10.1161/CIRCRESAHA.113.300153.
23. Côté N MA, Fournier D, Boulanger MC, Couture C, Després JP, Trahan S, Bossé Y, Pagé S, Pibarot P, Mathieu P. Angiotensin receptor blockers are associated with reduced fibrosis and interleukin-6 expression in calcific aortic valve disease. *Pathobiology*. 2014;81:15–24. doi: 10.1159/000350896.
24. Eriksen HA, Satta J, Risteli J, Veijola M, Väre P, Soini Y. Type I and type III collagen synthesis and composition in the valve matrix in aortic valve stenosis. *Atherosclerosis*. 2006;189:91–98. doi: 10.1016/j.atherosclerosis.2005.11.034.
25. Stephens EH, Saltarrelli JG, Baggett LS, Nandi I, Kuo JJ, Davis AR, Olmsted-Davis EA, Reardon MJ, Morrisett JD, Grande-Allen KJ. Differential proteoglycan and hyaluronan distribution in calcified aortic valves. *Cardiovasc Pathol*. 2011;20:334–342. doi: 10.1016/j.carpath.2010.10.002.
26. El Accaoui RN, Gould ST, Hajj GP, Chu Y, Davis MK, Kraft DC, Lund DD, Brooks RM, Doshi H, Zimmerman KA, Kutschke W, Anseth KS, Heistad DD, Weiss RM. Aortic valve sclerosis in mice deficient in endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol*. 2014;306:H1302–H1313. doi: 10.1152/ajpheart.00392.2013.
27. Padang R, Bagnall RD, Tsoutsman T, Bannon PG, Semsarian C. Comparative transcriptome profiling in human bicuspid aortic valve disease using RNA sequencing. *Physiol Genomics*. 2015;47:75–87. doi: 10.1152/physiolgenomics.00115.2014.
28. Monaghan K, McNaughten J, McGahon MK, Kelly C, Kyle D, Yong PH, McGeown JG, Curtis TM. Hyperglycemia and diabetes downregulate the functional expression of TRPV4 channels in retinal microvascular endothelium. *PLoS One*. 2015;10:e0128359. doi: 10.1371/journal.pone.0128359.
29. Kruihof EK. Regulation of plasminogen activator inhibitor type 1 gene expression by inflammatory mediators and statins. *Thromb Haemost*. 2008;100:969–975.
30. Kochtebane N, Alzahrani AM, Bartegi A. Expression of uPA, tPA, and PAI-1 in calcified aortic valves. *Biochem Res Int*. 2014;2014:658643. doi: 10.1155/2014/658643.

Significance

This study describes a new experimental model of aortic valve stenosis. In striking contrast to the first model, calcification is minimal, a feature that resembles children and some adults with aortic stenosis. Remodeling of collagen at the base of the valve leaflets may restrict opening of the valve. RNA sequencing identified molecular targets during the developmental phase of stenosis.