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## Calcific Aortic Valve Stenosis: Methods, Models, and Mechanisms

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### Abstract

Calcific aortic valve stenosis (CAVS) is a major health problem facing aging societies. The identification of osteoblast-like and osteoclast-like cells in human tissue has led to a major paradigm shift in the field. CAVS was thought to be a passive, degenerative process, whereas now the progression of calcification in CAVS is considered to be actively regulated. Mechanistic studies examining the contributions of true ectopic osteogenesis, non-osseous calcification, and ectopic osteoblast-like cells (that appear to function differently from skeletal osteoblasts) to valvular dysfunction have been facilitated by the development of mouse models of CAVS. Recent studies also suggest that valvular fibrosis, as well as calcification, may play an important role in restricting cusp movement, and CAVS may be more appropriately viewed as a fibrocalcific disease. High resolution echocardiography and magnetic resonance imaging have emerged as useful tools for testing the efficacy of pharmacological and genetic interventions *in vivo*. Key studies in humans and animals are reviewed that have shaped current paradigms in the field of CAVS, and suggest promising future areas for research.

### Keywords

calcification; fibrosis; animal models; phenotyping; echocardiography

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Calcific aortic valve stenosis (CAVS) is an important clinical problem: 2.8% of adults over 75 years old have some degree of CAVS.<sup>1, 2</sup>, and as many as 25% of adults over 65 have valvular sclerosis<sup>3</sup>. Although risk factors and downstream mediators appear similar for CAVS and atherosclerosis (older age, male sex, hypertension, smoking, hypercholesterolemia, and diabetes<sup>4, 5</sup>, see Figure 1), as many as 50% of patients with CAVS do not have clinically significant atherosclerosis<sup>6, 7</sup>.

Studies of valves from humans and experimental animals have begun to clarify mechanisms that lead to CAVS<sup>8–10</sup>. A major obstacle to research in this area is that although several experimental models of CAVS develop valvular sclerosis, few develop hemodynamically significant stenosis. Two experimental models of CAVS have now been identified in mice, which consistently develop hemodynamically significant CAVS<sup>11–14</sup>. These models will

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allow studies of mechanisms contributing to valve calcification, the cardiac and systemic consequences of CAVS, and the efficacy of interventions.

In this review, we will summarize 1) methods to evaluate the normal and stenotic aortic valve in mice, by histology and imaging, 2) mechanisms that may contribute to valve calcification and fibrosis in humans and animal models of CAVS, and 3) mechanisms that be useful therapeutic targets to inhibit development and/or progression of CAVS. Finally, we will speculate about future directions of this area of research.

## Assessment of Aortic Valve Function in Mice

Imaging approaches have evolved from techniques that were introduced first in the clinical setting, and later scaled to studies in mice. Three approaches are summarized below to evaluate cardiac function in mice: echocardiography, magnetic resonance imaging, and invasive hemodynamic assessment.

### Echocardiography

Echocardiography is a mainstay of clinical evaluation of aortic valve disease<sup>2</sup>. Echocardiographic imaging techniques are non-injurious, which facilitates longitudinal studies. These techniques can readily be performed in minimally sedated mice, avoiding the risks and physiologic perturbations associated with general anesthesia.

Continuous- and pulse-wave Doppler evaluation of blood velocities are useful for estimation of transvalvular pressure gradients and valve areas (see Figure 2). This approach has been useful for quantitation of aortic valve function across a very broad range, from normal<sup>15</sup> to “sclerotic”<sup>16</sup> to severely stenotic<sup>11</sup>.

This approach also has some disadvantages. It is not always possible to register the line of Doppler interrogation parallel to the direction of blood flow, which can result in underestimation of valve gradients. The region of interrogation needs to be small, to avoid contamination of velocity profiles by adjacent tissue motion, especially during active respiration, which challenges the limits of commercially available Doppler equipment. The requirement for continuous-wave Doppler limits the choice of transducers to those that image at frequencies of < 12 MHz, when commercially available equipment is used, resulting in suboptimal visualization of valve structures. Doppler velocities can be affected by factors other than effective valve orifice area. Reduced left ventricular contractility, for example, can result in underestimation of the severity of aortic stenosis in humans<sup>2</sup>, and probably in mice. When alterations in valve tissue produce valvular regurgitation, the increased stroke volume and ventricular preload recruitment may increase transvalvular gradients, even in the absence of valve stenosis<sup>15</sup>(Figure 2). As in clinical studies, those findings invoke a note of caution when Doppler velocities alone are used as evidence of aortic valve stenosis.

The superior spatial and temporal resolution of M-mode echocardiography are useful for quantitative assessments of aortic valve function<sup>12-14</sup>, which correlate well with invasive hemodynamic measurements<sup>14</sup> (Figure 2).

M-mode echocardiography has several advantages for assessment of aortic valve function in mice. Images are readily obtained from parasternal short- and long-axis views, and do not require co-registration with a Doppler line of interrogation. Variance of valve orifice dimensions is relatively low, achieving statistical power in between-group comparisons, with manageable sample sizes<sup>12</sup>. M-mode derived valve orifice dimension, and by

extrapolation valve area, are not appreciably affected by left ventricular contractility or the presence of aortic regurgitation.

Disadvantages of M-mode echo techniques arise from reliance on a unidimensional measurement to portray valve function. Thus, in the presence of eccentric valve remodeling, e.g. partial or complete cusp fusion, M-mode methods are susceptible to both under- and overestimation of the severity of valve dysfunction.

2-D echocardiography using clinical equipment does not reliably provide sufficient spatial resolution for visualization of valve motion in normal mice. A newer generation of ultrasound devices, developed solely for use in small experimental animals, employs transducers capable of imaging at very high frequencies. These new devices hold promise for quantitative assessment of valve function in 2-D<sup>17</sup>.

2-D echocardiography is a powerful technique for characterization of the impact of aortic valve disease upon left ventricular structure and function<sup>14, 17</sup>. The presence and severity of left ventricular hypertrophy and systolic dysfunction can be ascertained rapidly and reproducibly in conscious minimally sedated mice, in longitudinal studies.

### Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) provides useful information about aortic valve function in patients<sup>18</sup>. In mice, MRI at field strengths  $\geq 4.7$ T provides sufficient spatial and temporal resolution to assess aortic valve function in two dimensions<sup>12</sup> (see Figure 2). Flow turbulence causes “dephasing” of the blood signal, which facilitates visualization of aortic regurgitation<sup>19</sup> (Figure 2). MRI affords the added benefit of precise quantitative assessment of the structure and function of both the right and left ventricles and, consequently, precise measurement of regurgitant volume in mice with aortic regurgitation<sup>20</sup>.

The advantages of MRI are balanced by distinct disadvantages, notably limited availability. Relatively long imaging times, on the order of 20 minutes per study, require deep sedation or general anesthesia, which places mice with severe aortic valve disease at a higher mortality risk<sup>21</sup>.

### Invasive hemodynamic techniques

In clinical studies of valvular and left ventricular function, invasive hemodynamic techniques have been employed as a “gold standard”<sup>2</sup>. In mice, microtransducer-tipped catheters provide high-fidelity assessments, by virtue of sampling rates on the order of 1000 Hz. The small caliber, e.g. 1.4Fr (Millar<sup>R</sup>, Houston, TX), allows retrograde introduction into the left ventricle via the carotid artery<sup>11, 14</sup> (see representative traces in Figure 2). Advantages of invasive techniques include precise ascertainment of transvalvular gradients, and left ventricular systolic and diastolic function<sup>22</sup>. As is the case in the clinical setting, invasive hemodynamic techniques in mice can serve as a validation standard for more convenient non-invasive methods<sup>11, 14</sup>.

Disadvantages of invasive methods include the need for arterial access, risking blood loss and rendering longitudinal studies very difficult. General anesthesia can result in cardiac depression, especially in mice with severe aortic valve disease – resulting in discordance of findings obtained by invasive studies and those acquired by echocardiography in minimally sedated mice<sup>11, 14</sup>. Catheter-induced valve trauma is also likely to introduce artefactual cellular and molecular changes in valve tissue.

**Summary of findings from studies of aortic valve function in mice**—In adult C57BL/6 mice, the systolic aortic valve dimension is approximately 1.2 mm<sup>14</sup>. Assuming

that the orifice is roughly circular, anatomic estimates of normal adult aortic valve area from various strains of mice are about 0.8 – 1.3 mm<sup>2</sup>. Estimates of normal aortic valve area are somewhat higher (~1.60 mm<sup>2</sup>), when Doppler methods are employed<sup>11</sup>.

Normal peak systolic velocity of blood flow across the aortic valve in mice is < 1.5 m/sec<sup>11, 15–17</sup>, predicting peak transvalvular gradients of < 10 mmHg, findings corroborated by invasive hemodynamic studies<sup>11, 14</sup>. Reduction of systolic aortic valve dimension by > 50%, corresponding to reduction of valve area by > 75%, is sufficient to induce hemodynamically important transvalvular pressure gradients of > 50 mmHg, a finding that recapitulates seminal findings in humans with aortic valve disease<sup>2</sup>. Hemodynamically significant aortic valve stenosis causes left ventricular hypertrophy and reduced systolic function in mice<sup>11, 14</sup>.

Aortic transvalvular systolic gradients are increased in mice with aortic valve regurgitation<sup>22</sup>. Thus, it is advantageous to evaluate valvular function with at least two imaging techniques (e.g., cusp separation distance by m-mode echocardiography and transvalvular velocity with Doppler), in addition to determining whether there is aortic valve regurgitation (e.g., with color Doppler imaging or MRI imaging). This is particularly important when evaluating the myocardial consequences of CAVS, as mice with moderate or severe aortic valve regurgitation develop left ventricular hypertrophy, bi-ventricular enlargement, and decreased systolic function of both ventricles, even in the absence of aortic stenosis<sup>20</sup>.

These findings were culled from studies in relatively few mice, and may not account for differences between “normal” mouse strains or differences between sexes. Thus, they may be useful for planning future studies, but do not supplant the need to address possible strain or sex differences in the development of CAVS.

Noninvasive imaging techniques are useful for longitudinal studies to characterize the evolution of aortic valve dysfunction, and responses to therapeutic interventions. Characterization of events at the cellular and molecular levels, however, generally require studies of tissue *ex vivo* or cells *in vitro*, which greatly increases the complexity of long term investigations. Development of multimodality imaging methods suitable for long-term, serial imaging studies of the aortic valve (similar to what has been accomplished in blood vessels, where movement artifact and sampling rate are less<sup>23</sup>) will undoubtedly provide significant insight into mechanisms contributing to the development of aortic valve stenosis and biological responses to therapeutic interventions.

## Assessment of histological, structural, and biological changes in mouse aortic valves

### Histological changes

Histological examination of the aortic valve is useful to quantify calcium deposition in sections of the valve. Staining with alizarin red is preferable to von Kossa, not only because of its specificity for calcium, but also because mice with a C57BL/6 background often have artefactual deposits of black pigment (perhaps lipofuscin) in the aortic valve that resemble the black stain of calcium with von Kossa<sup>24</sup>. Masson’s trichrome stain and picrosirius red staining are useful for detection of gross changes in collagen<sup>12, 25–27</sup>, and Movat’s pentachrome staining is useful for evaluation of changes in content of collagen, elastin, and proteoglycans<sup>28</sup>. Oil red O is commonly used for assessing lipid deposition in the valve<sup>12, 13, 24</sup>. It is important to evaluate histological changes not only in the cusps of the valve, but also at the attachment points of the valve cusps (where calcification often begins).

## Gene expression, protein levels, and enzyme activity

In studies of aortic valve from humans, the relatively large amount of tissue facilitates evaluation of DNA (e.g., genome sequencing), mRNA (e.g., using quantitative real-time RT-PCR), and protein (e.g., western blots, ChIP assays, etc.), often from the same patient or sample.

In mice, the amount of tissue in aortic valve from one mouse is sufficient for measurement of gene expression with quantitative real-time RT-PCR<sup>29–31</sup>. To examine changes in protein levels during various stages of valve disease, immunohistochemistry is useful<sup>12, 13, 15, 30</sup> but is limited because it is semi-quantitative. High levels of tissue autofluorescence in calcified tissue require careful correction for background fluorescence with adjacent sections.

Although valve tissue could be pooled from a cohort of animals to use in more quantitative assays (e.g., Western blotting), the amount of time required to generate animals with hemodynamically significant CAVS (9–12 months or longer) and number of animals required for pooling (>5) make it logistically and financially difficult to use such techniques.

Evaluation of enzymatic activity in mouse valve tissue is extremely challenging when isolated protein is required (for the sample size limitations listed above). Indirect assays of enzyme activity are frequently used in frozen histological sections. For example, we have used PEG-superoxide dismutase-inhibitable fractions of dihydroethidium to evaluate superoxide levels in mouse valves<sup>12, 13</sup>, and similar approaches could be used with enzymatic inhibitors (e.g., oxidase inhibitors, etc.). Recent development of high-sensitivity chemiluminescent compounds (e.g., L-012) have been used to measure superoxide levels in mouse basilar arteries<sup>32</sup>, providing hope for a more quantitative assay for use on micro-samples.

Finally, the emerging field of molecular imaging may be useful for valvular and vascular biology. Of particular interest are compounds that emit fluorescence after they are cleaved by specific enzymes. These molecules have been used to demonstrate that MMP activity<sup>19</sup>, cathepsin activity<sup>33</sup>, inflammatory cell infiltrate<sup>34</sup>, and osteoblast-like cell activity<sup>19, 33, 34</sup> are substantially increased in aortic valves from hypercholesterolemic mice. These compounds are available with different excitation/emission wavelengths, making them a powerful tool to understand valvular biology when they are combined with each other or with standard fluorescent immunohistochemical methods.

## Limitations and future directions

### Limitations

One major advantage of studying CAVS in mice is that they are the only species, other than humans, that have been shown to develop hemodynamically important stenosis<sup>11–14</sup>. Other advantages, like other studies in mice, are that genetic alterations are readily available, and new strains and colonies can be expanded rapidly. Perhaps more importantly, the relatively short lifespan of the mouse makes it an attractive model for the study of time and/or age-dependent diseases such as CAVS. There are, however, significant limitations associated with using mouse models of CAVS.

The major disadvantages of studying CAVS in mice relate largely to their size. The hemodynamic evaluation of severity of stenosis has been challenging. However, echocardiographic evaluation of severity of aortic valve stenosis in mice has been refined, and high resolution imaging systems are commercially available. Direct measurements of transvalvular pressures have been used to validate the use of echocardiographic

measurements of cusp separation distance, which correlate well with peak transvalvular blood velocity (in the absence of aortic valve regurgitation)<sup>11, 14</sup>.

Histological assessment of valvular structure and calcium deposition has inherent limitations. A great limitation lies in the methods required to reconstruct serial sections into a three-dimensional image that can be quantitated. Although advances in quantitative stereology and associated software packages have made some advances possible<sup>35, 36</sup>, it is still extremely challenging to accurately quantitate valvular collagen, cellular composition, and other variables. Accurate quantitation of valve structure from 3-dimensional confocal/multiphoton images from intact tissues is difficult due to image distortion, secondary to differing refractive indices<sup>37, 38</sup>, which also vary at different excitation/emission wavelengths (making image registration across wavelengths and imaging modalities challenging).

Quantitative analysis of protein levels in valves from mice is challenging. Semi-quantitative analyses of immunohistochemically stained tissues are the predominant tool to evaluate changes in protein levels and post-translational modifications. Fluorescent immunohistochemical techniques provide the distinct advantage of analyzing spatial distribution of changes in protein levels (e.g., base versus tip of valve) and double- or triple-staining methods allow for evaluation of co-expression/co-localization of specific molecules. Pooling of tissue from multiple animals for Western blotting is theoretically feasible for young animals/cohorts, but routine quantitative analysis of proteins in valves from mice with severe CAVS will require the refinement of microdissection, micropurification, and microanalysis techniques.

Perhaps the greatest challenge in using mice is examination of molecular mechanisms underlying CAVS. The small size of the valve cusps and base, and limited amount of tissue, makes isolation of pure tissue and examination of gene expression (by qRT-PCR) challenging. Yields of RNA are sufficient for use in such applications, however, when combined with high fidelity, high efficiency RT enzymes.

### Future directions

The resolution available with echocardiographic imaging of the aortic valve is likely to continue to improve, and provide greater accuracy and precision to analysis of aortic valve structure and function. Improvements in imaging systems, however, will further our understanding of CAVS only if measurements are taken under reasonably physiologically meaningful conditions (e.g., heart rate >500, EF > 75%). Furthermore, great care must be taken to evaluate not only the severity of aortic valve stenosis, but also the presence and severity of aortic valve regurgitation, which can alter the transvalvular systolic gradient.

Advances have been made in micro computed tomography imaging, which does not have distortion or registration issues associated with laser-/fluorescence-based, three-dimensional microscopy techniques. Thus, in complex structures such as the aortic valve, three-dimensional micro-CT imaging may prove to be useful for understanding the spatial distribution of calcium deposition during progression of CAVS.

Several echocardiographic and MRI systems can combine high resolution imaging with molecular probes targeting surface molecules of cells. Multimodality imaging methods suitable for long-term, serial imaging studies have already been applied to the study of atherosclerosis in mice<sup>23, 39-41</sup> and aortic valve sclerosis in rabbits<sup>42</sup>, and will undoubtedly provide significant insight into mechanisms contributing to the development of aortic valve stenosis and biological responses to therapeutic interventions.



## Pro-calcific and anti-calcific signaling during the progression of CAVS

Activation of pro-osteogenic signaling cascades is thought to be a central mechanism contributing to the initiation and progression of calcific aortic valve stenosis. Osteogenic signaling cascades, especially bone morphogenetic protein and Wnt/ $\beta$ -catenin signaling, are activated in calcifying valves. Because other papers in this series will focus on these signaling cascades in detail, we will discuss them only briefly.

### Bone morphogenetic protein (BMP) signaling

Increased levels of phospho-smad1/5/8, a hallmark of canonical BMP signaling, occur in stenotic valves<sup>43</sup>. Mechanisms that contribute to increased BMP elaboration are not clear, but recent data suggest that non-laminar flow patterns on the aortic side of the valve may be a key initiator of BMP2/4 secretion from the valvular endothelium<sup>44-46</sup>. In hypercholesterolemic mice, phospho-smad1/5/8 levels increase prior to reduction of valve opening, and increase further as valvular calcification progresses and valve function becomes impaired<sup>12, 13</sup>. Tonic suppression of BMP signaling by inhibitory Smads is important in preventing cardiovascular calcification, as Smad6-null mice develop cardiovascular calcification and have evidence of aortic ossification at only 2 weeks of age<sup>47</sup>.

### Wnt/ $\beta$ -catenin signaling

Increases in levels of low-density lipoprotein receptor-related protein 5 (Lrp5) and associated increases in nuclear accumulation of  $\beta$ -catenin have been reported in valves from humans with CAVS<sup>48</sup>.  $\beta$ -catenin immunofluorescence also increases in calcified valves from hypercholesterolemic rabbits<sup>49</sup> and in hypercholesterolemic mice with advanced CAVS<sup>13</sup>.

### TGF- $\beta$ signaling and calcification

The role of TGF- $\beta$  in the initiation and progression of aortic valve calcification is not clear. Data from valvular interstitial cells plated directly on plastic or glass culture dishes show convincingly that TGF- $\beta$ 1 induces cell apoptosis, cellular aggregation, and calcified nodule formation<sup>50-57</sup>, but administration of TGF- $\beta$ 1 to cells plated on a less stiff collagen matrix does not induce osteogenic differentiation and calcification<sup>58</sup>. Furthermore, there is a clear dissociation between TGF- $\beta$  signaling and osteogenic protein levels with lipid lowering *in vivo*<sup>13</sup>, which suggests that TGF- $\beta$  is not a primary inducer of pro-osteogenic signaling in hypercholesterolemic mice with advanced valve disease. Although some data suggest that TGF $\beta$  may actively suppress pro-osteogenic signaling in skeletal osteoblasts *in vivo*<sup>59</sup>, the role of TGF- $\beta$  in suppression or activation of osteogenic signaling *in vivo* is not clear.

### Identifying the origin of cells that redifferentiate to osteoblast-like cells

The conventional wisdom is that osteogenesis in stenotic valves results from activation of maladaptive signaling, which drives the redifferentiation of resident valvular interstitial cells to an osteoblast-like phenotype<sup>60</sup> (Figure 3). There are, however, several lines of evidence which suggest that cells other than the resident valvular interstitial cell can contribute to osteogenesis in the valve. First, a subset of valvular endothelial cells appears to undergo endothelial-mesenchymal transformation<sup>61, 62</sup>, which may provide a sub-population of cells with a propensity for activation and calcification (Figure 3). Second, circulating progenitor cells may contribute to vascular and valvular calcification by either redifferentiating to an osteoblast-like cell or by promoting interstitial cell calcification through paracrine signaling<sup>63-66</sup> (Figure 3). Studies from lethally-irradiated mice, which undergo green fluorescent protein marrow transplantation, suggest that ~15% or more of the valvular cell

population (endothelium,  $\alpha$ -smooth muscle actin positive cells, and inflammatory cell infiltrate) may be comprised of cells that originated from bone marrow<sup>15, 67</sup>.

## Endogenous mechanisms that may modulate pro-calcific signaling in CAVS

### Reactive oxygen species

Reactive oxygen species (ROS) appear to be a central pathophysiological component of a number of cardiovascular diseases, including atherosclerosis<sup>68–70</sup>, hypertension<sup>71–73</sup>, and thrombosis<sup>74–77</sup>, and can originate from a number of enzymatic sources<sup>68, 76</sup>. Superoxide and hydrogen peroxide are significantly increased in the calcified and peri-calcific regions of stenotic aortic valves<sup>78</sup>. Uncoupled nitric oxide synthase and reductions in antioxidant enzyme expression and activity appear to be major contributors to increased ROS in stenotic valves<sup>78</sup>. Although increases in global NAD(P)H oxidase activity do not appear to be major contributors to increased ROS in stenotic valves<sup>78</sup>, ROS derived from p47<sup>phox</sup>-dependent oxidases may be generated in peri-calcified microenvironments<sup>79</sup>.

There are several lines of evidence supporting the concept that ROS play an important role in progression of CAVS (Figure 4). First, ROS are increased prior to valve dysfunction in mice, which suggests that increased ROS are not merely the consequence of increased cusp stress associated with valve calcification<sup>12</sup>. Second, ROS have been implicated as a critical link in the transduction of pro-osteogenic and pro-fibrotic signaling cascades (see sections on TGF- $\beta$  signaling<sup>80</sup> and Figure 4). Third, addition of exogenous ROS accelerates calcification of VSMC's *in vitro*<sup>(81, 82)</sup>. Finally, administration of lipoic acid (which reduces superoxide and H<sub>2</sub>O<sub>2</sub>), but not tempol (which only reduces superoxide), attenuates calcification in rabbit model of valvular sclerosis<sup>79</sup>.

Although we have a general understanding of ROS that are increased and general enzymatic sources of ROS in stenotic valves, there are major gaps in our understanding of the role of ROS in valvular calcification. Specifically, it is not known which nitric oxide synthase isoforms and/or NAD(P)H oxidase isoforms contribute to increased generation of ROS, nor do we know the relative contributions of different antioxidant mechanisms (e.g., catalase, superoxide dismutases, and peroxidases) in their respective subcellular compartments. Genetically altered mice will allow elucidation of the role of specific ROS-related enzymes in the pathogenesis of CAVS.

### Nitric oxide bioavailability

Reduction of nitric oxide bioavailability is strongly associated with a number of cardiovascular diseases, and NO bioavailability is often inversely correlated with increases in ROS. Expression of endothelial nitric oxide synthase is increased on the aortic side of the valve in early stages of valve disease<sup>83</sup>, and increased eNOS immunofluorescence is evident in neovessels in advanced stages of valve disease<sup>84, 85</sup>. One might anticipate that increased expression of eNOS would protect against CAVS and suppress interstitial cell proliferation (Figure 4). Overexpression of eNOS in hypercholesterolemic mice, however, accelerates atherosclerosis due to NOS uncoupling<sup>86</sup>, and NOS uncoupling may contribute to increases in reactive oxygen species in human CAVS<sup>78</sup>.

Nevertheless, there are several observations which together suggest that increases in NO bioavailability may be a useful strategy to slow progression of aortic valve calcification (see Figure 4). First, endogenous inhibitors of nitric oxide synthase—such as asymmetric dimethylarginine—are significantly increased in patients with CAVS<sup>87, 88</sup>. Second, addition of exogenous NO slows calcium nodule formation in valve interstitial cells *in vitro*<sup>51</sup>. Third,



administration of statins to hypercholesterolemic rabbits is associated with robust increases in eNOS levels<sup>89</sup> and attenuation of valvular calcium deposition. Finally, administration of NOS cofactors slows progression of atherosclerosis in mice<sup>90</sup>.

### Renin-angiotensin system (RAS)

Several findings suggest that angiotensin II may lead to oxidative stress, inflammation, and accelerated development of CAVS. First, hypertension, which often involves the RAS, is a risk factor for vascular calcification<sup>91</sup> and CAVS<sup>1</sup>. Second, monocytes<sup>92</sup> and macrophages in atherosclerotic lesions<sup>93</sup> contain Ang II, and there are many macrophages in stenotic aortic valves.<sup>12, 94, 95</sup> Third, expression of angiotensin-converting enzyme (ACE) and angiotensin type I receptors (AT<sub>1</sub>R) is increased in stenotic valves, and co-localizes with macrophages and mast cells primarily in peri-calcific regions of stenotic valves<sup>95</sup>. Fourth, Ang II promotes oxidative stress and inflammation<sup>96-98</sup>, which are associated with CAVS.

In animal models of atherosclerosis and hyperlipidemia-induced valve disease, AT<sub>1</sub>R blockade prevented inflammatory cell infiltration and myofibroblast activation in early stages of valve disease<sup>99</sup>. A retrospective clinical study also suggested that an AT<sub>1</sub>R blocker, but not an ACE inhibitor, protected against progression of valve disease in early (but not late) stages of CAVS<sup>100</sup>. Other retrospective clinical studies, however, have yielded conflicting results with regards to the effects of ACE inhibitors on calcium and progression of CAVS<sup>101, 102</sup>. The efficacy of ACE inhibitors in reducing Ang II levels in patients with CAVS may be limited by high levels of chymase (which can convert Ang I to Ang II) in human valves<sup>95</sup>. Thus, although there is a strong biological rationale that implicates the RAS in progression of CAVS, the lack of prospective, experimental data (in studies in animals or humans) prevents a firm conclusion.

### RANK/RANKL/OPG

There is a complex interaction between receptor activator of NFκB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) in relation to oxidative stress and inflammation, through effects on NFκB<sup>103, 104</sup>. This interaction has important consequences for calcification of bone, arteries, and perhaps the aortic valve.

Increased RANK activation/RANKL levels may influence cardiovascular calcification through effects on both circulating cells and resident cells. RANKL elaborated from calcifying vascular smooth muscle cells is Runx2-dependent and sufficient to induce monocyte recruitment and osteoclast-differentiation in calcifying atherosclerotic lesions in mice<sup>105</sup>. This mechanism may modulate intimal plaque calcification. This RANKL-stimulated osteoclast differentiation may be mediated in part by ROS, and RANKL in turn induces further ROS generation<sup>106, 107</sup>. In contrast, RANKL increases calcification of vascular smooth muscle cells, perhaps through a BMP4 pathway<sup>108</sup>. Finally, OPG (which is an endogenous decoy receptor for RANKL, and inhibits inflammation and the pro-osteogenic pathway) inhibits aortic calcification in OPG<sup>-/-</sup> mice<sup>109</sup>.

Based on seminal studies in blood vessels<sup>110, 111</sup>, we speculate that OPG may inhibit calcification of the aortic valve. First, RANKL is greater in stenotic than normal aortic valves from humans<sup>112, 113</sup>, and promotes calcification of myofibroblasts *in vitro*<sup>112</sup>. Second, calcification of atherosclerotic lesions in innominate artery is accelerated in apoE-deficient mice that are OPG-deficient.<sup>110</sup> Third, injections of OPG prevented calcification of the aorta in *ldlr*<sup>-/-</sup> mice but had no impact on extent of total atherosclerosis. This finding suggests that intimal calcification can be dissociated from lipid deposition and tissue fibrosis in atherosclerosis<sup>111</sup>. It is not known whether similar phenomena occur in calcifying valves.

### **Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )**

PPAR $\gamma$  is a member of the nuclear hormone receptor superfamily of ligand-dependent transcription factors<sup>114, 115</sup>. Several findings imply that PPAR may protect against CAVS. First, increasing PPAR $\gamma$  impairs differentiation of progenitor cells and calcifying vascular cells into an osteoblast-like lineage in vitro<sup>116</sup>. Second, inhibition of PPAR $\gamma$  (pharmacologically or with siRNA) increases differentiation of embryonic stem cells to osteoblasts<sup>117</sup>. Third, PPAR $\gamma$  ligands promote antioxidant and anti-inflammatory gene expression profiles<sup>118</sup>.

The role of PPAR $\gamma$  in valve calcification is not known. Interestingly, PPAR $\gamma$ -related pathways, however, are increased in early stages of hypercholesterolemia-induced valve disease, which may contribute to protection of the endothelium<sup>83</sup>.

Thus, PPAR $\gamma$  regulates expression of genes that modulate expression of osteoblasts, are antioxidant and antiinflammatory, and may thereby protect against CAVS. Multiple signaling pathways appear to be important in the pathophysiology of CAVS. Activation of PPAR $\gamma$  is attractive as a potential treatment for CAVS because, instead of targeting a single mechanism, PPAR $\gamma$  affects a large clusters of genes<sup>119</sup>, and thus may protect the valve through multiple pathways.

### **Direct inhibitors of osteogenic signaling**

Downregulation of inhibitors of osteogenic signaling have been implicated in the progression of CAVS. Matrix Gla protein (MGP) binds bone morphogenetic proteins, rendering them inactive<sup>120</sup>, and enhances fetuin-dependent uptake of mineralizing matrix vesicles<sup>121</sup>. In the setting of cardiovascular calcification, however, it appears that MGP may be inactivated by undercarboxylation or physical interactions with inflammatory proteins (e.g., HSP70<sup>120</sup>). A retrospective clinical study suggested that warfarin use, which inhibits gamma-carboxylase (thereby impairing MGP), is a risk factor for progression of valve disease in patients with early CAVS<sup>100</sup>. MGP-deficient mice develop massive medial vascular calcification and aortic valve calcification early in life<sup>122</sup>, and hypercholesterlemic MGP-transgenic mice are protected against cardiovascular calcification<sup>123</sup>. Thus, inactivation of MGP may be a key permissive event in initiation of osteogenic signaling in cardiovascular tissue.

### **Notch signaling**

Loss-of-function polymorphisms in Notch1 are strongly associated with development and early calcification of bicuspid aortic valves in humans<sup>124</sup>. Mice that are haploinsufficient in Notch1 do not develop bicuspid aortic valves, but develop calcific aortic valve disease due to the derepression of BMP2 expression<sup>125, 126</sup>. The mice, however, do not develop significant abnormalities in valve cusp function/aortic valve stenosis<sup>125, 126</sup>. An intriguing possibility is that reductions in Notch1 may play a dual role in CAVS, being permissive for both BMP2/4 elaboration in the endothelium and for osteogenic differentiation in interstitial cells<sup>127</sup>. Interestingly, Notch1 activation induces Msx2-dependent osteogenic differentiation in vascular smooth muscle cells<sup>128</sup>, which implies that effects of Notch1 activation are highly context dependent.

### **Matrix metalloproteinases (MMP), cathepsins, and valvular calcification**

MMP-1<sup>129-131</sup>, MMP-2<sup>132</sup>, MMP-3<sup>133</sup>, MMP-9<sup>134</sup>, and cathepsins S<sup>135</sup>, K<sup>135</sup>, V<sup>135</sup>, and G<sup>136</sup> are increased in stenotic human valves. The functional significance of alterations in MMPs in valve calcification remains unclear, although matrix remodeling is likely to play an important role in permitting the expansion of calcified plaques and in the generation of pro-inflammatory collagen fragments<sup>137, 138</sup>. Elastin fragments produced by active

cathepsin S are a major contributor to valvular and vascular calcification in hypercholesterolemic mice with chronic renal failure<sup>33</sup>. Genetically altered mice will be useful in determining whether inhibitors of MMPs or cathepsins are a viable therapeutic target to slow the progression of CAVS.

### Pro-inflammatory cytokines

Inflammatory cell infiltrate and production of pro-inflammatory cytokines are markedly elevated in valves from both humans and mice with CAVS (Figure 1). Effects of pro- and anti-inflammatory cytokines on valve biology have not been thoroughly examined, but two lines of evidence suggest that TNF $\alpha$  may be a critical downstream mediator of inflammation-induced calcification. First, mice that are deficient in interleukin-1 receptor antagonist (IL-1rn) have pronounced valve thickening, calcification, and modest sclerosis/stenosis (peak velocity = ~2m/sec); this valvular phenotype is abrogated in IL-1rn/TNF $\alpha$  double knockout mice<sup>139</sup>. Second, TNF- $\alpha$  appears to be a critical intermediary in the induction of vascular calcification and MMP activation in diabetic mice, as administration of infliximab (a TNF- $\alpha$  neutralizing antibody) inhibits BMP2-Msx-Wnt signaling in aorta<sup>140</sup>. We speculate that isoform-specific receptor blockers may be useful when targeting TNF- $\alpha$  signaling, because activation of TNF- $\alpha$  receptor 1a is responsible for many of the deleterious effects of TNF- $\alpha$ , and activation of TNF- $\alpha$  receptor 1b may confer some beneficial/protective effects<sup>141</sup>.

Activation of receptors of advanced glycosylation end products (RAGE) can accelerate VSMC calcification both *in vitro*<sup>142–145</sup> and *in vivo*<sup>142, 146, 147</sup>. The contribution of this pathway to development of CAVS has not been tested experimentally, but several observations in patients suggest that RAGE activation may contribute to progression of CAVS. First, metabolic syndrome and diabetes are risk factors for development of CAVS<sup>148</sup>, and such patients have marked increases in plasma and tissue AGE levels<sup>149</sup>. Second, circulating soluble RAGEs, which prevent AGEs from binding to tissue RAGEs, are reduced in patients with CAVS<sup>150</sup>. Thus, reducing AGE levels and RAGE activation may prove to be useful in slowing progression of CAVS in some patients.

### Is valvular calcification always an osteogenic process?

Recent studies of mechanisms that contribute to CAVS have assumed that ectopic calcification is primarily or exclusively an active process resembling processes observed in bone. The relative importance of true “ectopic osteogenesis”, however, is not entirely clear in humans or in mice with CAVS. In our opinion, there are several potential mechanisms whereby calcium nodules may initiate or expand in CAVS (Figure 5).

First, valvular calcification may progress by a process that parallels bone. Approximately 15–20% of valve cusps from patients with CAVS have evidence of bone matrix, including osteoid cells, highly organized collagen scaffolds, multi-nucleated osteoclast-like cells, and marrow pockets<sup>151</sup>. To date, similar structures have not been described in murine CAVS.

A second possible mechanism of valvular calcium accumulation is accumulation of amorphous calcium deposits. Calcified nodules of this type typically have a crystalline ultrastructure, and lack live cells within the core of the calcified mass itself<sup>151, 152</sup>. Cellular necrosis and apoptosis are classical mechanisms of nodule formation and expansion of amorphous calcium<sup>152–154</sup>. *In vitro*, TGF $\beta$  induces caspase-dependent apoptosis and formation of calcified nodules, and TGF $\beta$  is markedly increased in valves from humans with CAVS<sup>52, 55</sup>. Although mechanisms of TGF $\beta$ -induced calcification are highly substrate/matrix sensitive *in vitro*<sup>57</sup>, these data support the concept that formation of osteoid cells is not always a primary event in initiation or expansion of calcified nodules. It is important to

note that, although accumulation of calcium may not occur via a process that resembles skeletal ossification, initiation (and perhaps progression) of calcium deposition via this mechanism may occur via: 1) tightly regulated “active” processes, such as caspase-dependent apoptosis, or 2) “passive” processes, via accumulation of calcium secondary to tissue necrosis. The relative contributions of both pathways to valve calcification remains poorly understood, and both may prove to be therapeutic targets for patients with CAVS.

A third mechanism may lead to valvular calcification in humans and mice. Active mineralization of valvular tissues may occur by cells that express a subset of osteogenic genes<sup>12, 13, 19, 155</sup>, but is regulated by processes that are fundamentally different from skeletal ossification. *In vitro*, calcifying cells of cardiovascular origin respond to several external stimuli in a manner that is fundamentally different from skeletal osteoblasts<sup>82</sup>. As mentioned above, cells expressing osteogenic markers frequently are found near calcified areas in both humans and mice<sup>12, 13, 19, 78, 151</sup>, although the functionality (and malleability of their function) has yet to be determined experimentally.

In summary, it is not clear which signaling cascades are responsible for initiation and progression of aortic valve calcification *in vivo*, or which mechanisms predominate in CAVS in humans or mice. These questions deserve attention, and will ultimately be addressed through careful histomorphometric studies which examine the cellularity, ultrastructure, composition, and molecular fingerprint of calcified nodules in human and murine CAVS.

### Role of fibrosis in CAVS

The conventional wisdom is that calcification is the major determinant of stenosis in CAVS. Acquired fusion of valve cusps also may contribute to stenosis, but commissural fusion is not common in CAVS, in contrast to rheumatic and congenital aortic stenosis. We suggest that fibrosis of the valve, as well as calcification, may contribute importantly to CAVS (see Figure 6).

There is extensive fibrosis of the aortic valve in humans<sup>30</sup> and mice<sup>12</sup> with CAVS. Extracellular matrix synthesis (ECM) can originate from activated myofibroblasts (i.e., alpha-smooth muscle actin positive cells in the valve). Myofibroblast activation occurs early in the development of aortic valve disease<sup>12</sup>, and myofibroblasts may actively secrete collagen<sup>156</sup>, hyaluronan<sup>157, 158</sup>, and other ECM components during development and progression of CAVS. ECM composition and stiffness may have a profound impact on the phenotype of valve interstitial cells, and ECM may contribute to differentiation of cells to an osteoblast-like phenotype<sup>57, 58, 159–161</sup>. If additional studies continue to implicate fibrosis in the pathogenesis of CAVS, it may be more accurate to use the term fibrocalcific aortic valve stenosis.

**TGF- $\beta$  and fibrosis**—TGF $\beta$  is an antiinflammatory and profibrotic cytokine<sup>162, 163</sup>. TGF $\beta$  plays a critical role in fibrosis of the myocardium after injury, and also may “stabilize” atherosclerotic plaques in arteries, by its antiinflammatory and profibrotic effects. TGF $\beta$  signaling and myofibroblast activation are markedly increased during development of CAVS in mice<sup>12, 13</sup> and in valves from patients with severe CAVS<sup>55</sup>, making it an attractive candidate as a primary driver of fibrosis in CAVS.

**Twist1**—Re-activation of developmental gene expression programs may occur in the stenotic valve. Specifically, increases in Twist1, which is essential for normal endocardial cushion development and remodeling, have been reported in the peri-calcific regions of stenotic aortic valves<sup>30</sup>. Overexpression of Twist1 in mice produces valvular

hypercellularity and excessive cusp fibrosis, which suggests that Twist1 may contribute to valvular fibrosis and interstitial cell proliferation in advanced CAVS<sup>30</sup>.

## **Aging, valvular calcification, and valvular fibrosis: failure of multiple regulatory mechanisms?**

Increasing age is one of the strongest predictors of cardiovascular calcification<sup>164</sup> and the development of aortic valve stenosis<sup>165</sup>. Several regulatory mechanisms may have a profound effect on lifespan, genomic stability, and age-related diseases. We will discuss a few areas of research in aging that hold promise for advancing our understanding of cardiovascular calcification during aging.

### **Progeric humans and mice**

Patients with progeroid syndromes (e.g., Hutchinson-Gilford syndrome or Werner's syndrome) have increased prevalence of severe aortic valve calcification and stenosis<sup>166–170</sup>. Non-progeroid humans with atherosclerotic lesions accumulate prelamin A (whose expression is increased in some forms of progeria) in areas close to senescent or calcifying smooth muscle cells<sup>171</sup>. Mice that overexpress progerin develop robust vascular calcification at a young age<sup>172</sup>. We speculate that characterizing changes in expression of these molecules in stenotic human valves, and the valvular phenotypes of progeric mouse models, will provide important insights mechanisms that contribute to CAVS.

Other mouse models of aging and progeria develop cardiovascular calcification. For example, *klotho*-deficient mice develop premature vascular calcification<sup>173</sup>. These mice, however, also develop calcification of the gut and other soft tissues, which are not typically associated with normal aging<sup>173, 174</sup>.

### **Post-transcriptional regulation of gene expression in CAVS**

An emerging field of study is the role of micro-RNA in the regulation of mRNA stability and translation. One microRNA may target many, perhaps hundreds, of mRNAs<sup>175</sup>, predominantly by destabilization of target mRNAs resulting in their subsequent degradation<sup>176</sup>. MicroRNA expression is dramatically altered in numerous tissues with aging<sup>177</sup>. A recent report describing microRNAs in cardiac valves suggested that several microRNAs are decreased in stenotic bicuspid aortic valves, compared to insufficient valves, and may modulate mRNA levels of several pro-calcific genes<sup>178</sup>. Downregulation of microRNAs modulates development of fibrosis in myocardium<sup>179</sup>, but the role of micro-RNAs in the regulation of cardiac valve fibrosis is not known.

### **Epigenetic modifications**

Changes in acetylation levels of transcription factors and histones are a critical determinant of availability and affinity of transcription factor binding sites, result from perturbations in the balance between acetyltransferase activity and deacetylase activity (class I–IV histone deacetylases), and are significantly altered by aging. Both class I deacetylases (such as histone deacetylase 3) and class III deacetylases (the sirtuins) influence several proteins involved in cardiovascular calcification: 1) HDAC3 suppresses Runx2 activity and prevents osteoblastic differentiation<sup>180</sup>, 2) reductions in Sirt1 increase vascular inflammation and endothelial cell activation<sup>181</sup>, 3) reductions in Sirt1 and Sirt6 increase histone acetylation, promote genomic instability, and are permissive for increases in NFκB binding in the nucleus<sup>182</sup>, and 4) the histone acetyltransferase GCN5 increases TGF-β binding efficiency and overall genomic instability<sup>183</sup>. Mice deficient in acetyltransferase or deacetylase enzymes have been generated, and will be useful in determining the role of histone and protein acetylation in the progression of CAVS.



DNA methylation also contributes to regulation of both global and specific gene expression, and aberrations in DNA methylation profiles are present in atherosclerosis, stroke, and cancer<sup>184</sup>. Interestingly, recent work has shown that CpG island methylation at the  $\alpha$ -smooth muscle actin promoter contributes to gene silencing in cultured smooth muscle cells, thereby facilitating re-differentiation of these cells to an osteoblast-like phenotype<sup>185</sup>. In contrast, hypermethylation of pluripotency-inducing transcription factors could potentially prevent cellular de-differentiation<sup>184</sup> and maintain resident valvular or vascular cells in a non-osteogenic lineage. While changes in DNA and histone methylation in stenotic aortic valves have not been examined at this time, we speculate that use of genetically altered mice and methyltransferase inhibitors will lend important insights into the role of epigenetic modifications in CAVS.

## **Histological and molecular changes with treatment of valve disease: is regression of CAVS possible?**

At present, surgical (i.e., valve replacement) or emerging interventional (i.e., percutaneous valve implantation) approaches are the only treatments for CAVS. When hypercholesterolemia is the primary driver of valve calcification, lipid lowering therapy may be a useful intervention in hypercholesterolemic patients with relatively early stages of CAVS<sup>186</sup>. Based on data from three large clinical trials (SEAS<sup>187</sup>, SALTIRE<sup>188</sup>, and ASTRONOMER<sup>189</sup>), however, lipid lowering is not likely to be beneficial for patients with severe CAVS. To determine molecular, histological, and functional changes with lipid lowering in early- and late-stage CAVS, we used a mouse model of CAVS in which lipids could be altered with a “genetic switch”, thereby avoiding confounding/pleiotropic effects of pharmacological interventions<sup>190–192</sup>.

### **Histological changes following lipid lowering**

Reduction of blood lipids in mice reduces valvular lipid content and inflammatory cell infiltrate in both early and late stages of hypercholesterolemia-induced CAVS<sup>12, 13</sup> (see Figure 6). Lipid lowering therapy also reduces BMP<sup>12</sup> and Wnt/ $\beta$ -catenin signaling<sup>13</sup> and, remarkably, reduces valvular calcium<sup>12, 13</sup>. This finding is markedly different from observations in advanced atherosclerotic lesions, where activity of osteoblast-like cells is markedly reduced by statins<sup>23</sup>, but calcium deposits are resistant to reduction and/or resorption<sup>193, 194</sup>. These contrasting effects in valves and arteries are somewhat surprising because osteoclast-like cells are present in both calcifying aortic valves<sup>79, 195–197</sup> and in arteries<sup>198, 199</sup>. Further work to examine differences in calcified plaque composition/ultrastructure, and differences in osteoblast-/osteoclast-like function in arteries and valves, will be important to understanding the susceptibility of calcified deposits to resorption in different cardiovascular tissues.

In contrast to valvular lipid and calcium, valvular fibrosis is remarkably refractory to lipid lowering (Figure 6). Fibrosis, extending into the spongiosa of the valve, persists even after 6 months of lipid lowering<sup>12</sup>. This finding is concordant with observations in atherosclerotic lesions during the first few months of reduction of blood lipids in hyperlipidemic monkeys, as decreases in lipid content of lesions are not accompanied by reduction of fibrosis<sup>200</sup>.

Although phospho-smad2/3 levels (indicative of TGF- $\beta$  signaling) and myofibroblast activation are reduced following lipid lowering in early stages of valve disease<sup>12</sup>, both remain elevated if lipid lowering is initiated in advanced valve disease<sup>13</sup>. We speculate that persistent myofibroblast activation may be due to epigenetic modifications resulting in sustained expression of TGF- $\beta$  and smad2<sup>201</sup>, or cell-matrix interactions that result in sustained activation of TGF- $\beta$ 1 signaling<sup>202</sup>. Thus, interventions that target both pro-calcific



and pro-fibrotic signaling may be required to slow progression of valvular dysfunction in end-stage CAVS. Similarly, improving valvular function/reversing histopathological changes in advanced CAVS may require interventions that effectively reduce valvular calcium content, connective tissue content, and their respective signaling cascades.

### Functional changes after lipid lowering

When initiated in early stages of valve disease (i.e., before reduction in aortic valve orifice area), lipid lowering in mice halts progression to severe aortic valve stenosis<sup>12</sup> (see Figure 6), although it is not clear whether lipid lowering confers similar benefits to patients with mild/moderate valve disease<sup>203</sup>, especially those with hyperlipidemia<sup>186</sup>. Similar to observations from large clinical trials in humans<sup>187–189</sup>, however, lipid lowering in advanced stages of aortic valve disease does not improve aortic valve function in hypercholesterolemic mice<sup>13</sup>. Taken in the context of the histological changes described above, it is clear that reducing valvular calcium *per se* is not sufficient to improve valvular function with lipid lowering. Thus, we speculate that reduction of valvular fibrosis may also be critical to improving valvular function.

### Interventions that may inhibit development and/or progression of CAVS in humans and mice

Based on previous studies, and discussion of endogenous mechanisms that may modulate CAVS (see above), we speculate that several interventions might potentially inhibit development and/or progression of calcification, fibrosis, and stenosis of the aortic valve (Figure 7).

First, lipid lowering therapy could slow progression of aortic valve disease under some conditions. Specifically, lipid lowering stops progression—but does not induce regression—of aortic valve stenosis in hypercholesterolemic mice<sup>12, 13</sup>. Thus, lipid lowering therapy may be a useful intervention in hypercholesterolemic patients in early stages of CAVS.

Second, we propose that altering oxidative stress and/or nitric oxide bioavailability may be useful in slowing progression of CAVS. High doses of antioxidants (e.g., vitamin E) rarely confer long-term therapeutic benefit<sup>204</sup>, and chronic treatment with tempol unexpectedly increased apoptosis and calcification in a rabbit model of CAVS<sup>79</sup>. Nevertheless, targeting antioxidants to different subcellular compartments may be more beneficial than those which affect redox state throughout the cell<sup>205</sup>. Reducing oxidative stress is also likely to increase nitric oxide bioavailability<sup>8, 68</sup>, which may be augmented by combining antioxidant therapy with treatments that reduce endogenous inhibitors of nitric oxide synthases (i.e., asymmetric dimethylarginine, which is increased in patients with CAVS<sup>87, 88</sup>).

Third, inhibition of the renin-angiotensin system may slow the development and/or progression of CAVS. Although retrospective studies suggest that ACE inhibitors do not slow the progression of CAVS<sup>100, 101</sup>, it is possible that AT1r inhibitors may be more effective at slowing the progression of CAVS<sup>100</sup> by allowing angiotensin II to exert beneficial effects via both the AT2 receptor<sup>206</sup> and through its cleavage product angiotensin-1-7<sup>207</sup>. Indeed, overexpression of AT2r reduces atherosclerosis in mice<sup>208</sup>, and administration of exogenous angiotensin 1–7 suppresses fibrosis in models of vascular injury<sup>209</sup>.

Fourth, inhibition of the RANK/RANKL pathway may prevent and/or slow progression of CAVS. Based on previous findings from models of intimal plaque calcification, it is likely that osteoprotegerin will slow progression of CAVS when initiated during early stages of disease<sup>111</sup>. Because RANKL may be a key mediator of monocyte recruitment and

osteoclastogenesis in vascular calcification, however, it is difficult to predict whether osteoprotegerin will induce regression of calcification in advanced vascular or valvular lesions.

Fifth, a PPAR $\gamma$  ligand may inhibit development of CAVS. Because PPAR $\gamma$  ligands prevent cells from differentiating to an osteoblast-like cell lineage, suppress inflammation, and increase antioxidant protein levels, it is possible that PPAR may slow the development of CAVS. However, the time at which treatment is initiated is likely to be of great importance. Thiazolidinediones appear to inhibit development of early atherosclerotic lesions<sup>210</sup>, but have little or no beneficial effect on advanced lesions in *ldlr*<sup>-/-</sup> mice, perhaps because they promote cell death in advanced lesions<sup>211</sup>.

Sixth, we speculate that manipulation of microRNA levels may be useful in prevention of fibrosis and calcification of the aortic valve, and inhibit development of CAVS. For example, antisense oligonucleotide-mediated (antimiR) knockdown and overexpression techniques are under development for prevention and/or treatment of cardiac fibrosis<sup>175, 179, 212</sup>. MicroRNAs that alter pro-calcific and pro-fibrotic signaling are dysregulated in CAVS<sup>178</sup>. Thus, altering expression of microRNAs may be effective in attenuating progression of valve disease, especially because microRNAs can modulate the functions of multiple genes in a signaling cascade (and may affect multiple signaling cascades), thereby conferring higher therapeutic efficacy.

Finally, pharmacological manipulation of epigenetic modifications and post-translational modifications of transcription factors may be useful in preventing calcification and fibrosis of the aortic valve. Acetylation levels of histones and proteins have a profound impact on genomic stability and transcription factor binding affinity, and the beneficial effects of acetyltransferase inhibitors and deacetylase activators on inflammation and atherosclerosis make them promising candidates for use in CAVS<sup>213</sup>. Small molecule methyltransferase inhibitors or demethylase activators may also further our understanding of the role of epigenetic modifications in CAVS, facilitate favorable changes in gene expression<sup>184</sup>, and perhaps slow progression of CAVS.

## Integration of findings from cardiovascular calcification and skeletal ossification

There is strong support for the concept that CAVS is an active process mediated by ectopic osteoblastogenesis, and that these osteoblast-like cells express markers similar to those found in skeletal osteoblasts<sup>12, 13, 214</sup>. Patients with CAVS generally are over the age of 65, and at risk of developing osteoporosis. Thus, therapies that are designed to slow the progression of CAVS via inhibition of osteoblastogenesis may not be useful, because they may augment osteoporosis. An example of this side effect is PPAR $\gamma$  agonists, which may slow progression of CAVS by diverting cells to an adipocyte-like lineage, but increase the prevalence of bone fractures in patients with type II diabetes via a similar mechanism<sup>215–217</sup>. An ideal pharmacological intervention would simultaneously suppress ectopic osteogenesis and improve skeletal osteogenesis. Two potential therapies may accomplish this challenging goal: antioxidants or suppression of RANKL signaling.

First, increasing oxidative stress promotes osteogenic differentiation of vascular smooth muscle cells, but attenuates mineralization of bone-derived osteoblasts *in vitro*. Antioxidants such as N-acetylcysteine attenuate bone loss in genetically altered mice<sup>218</sup>, but, direct evidence for a role of oxidative stress in initiation or progression of CAVS *in vivo* is limited. Antioxidant specificity<sup>79, 219</sup>, and perhaps subcellular compartmentalization/ targeting<sup>205</sup>, may be required for successful therapeutic interventions.

Second, modulation of the OPG/RANKL-axis attenuates calcification of atherosclerotic plaque, but preserves bone mineral density<sup>220, 221</sup>. Furthermore, estrogen appears to tonically inhibit aortic calcification through the suppression of RANKL signaling<sup>222</sup>. Thus, suppression of RANKL signaling with monoclonal antibodies or other strategies may be an efficacious treatment to preserve bone mineral density and attenuate progression of valvular calcification in men and post-menopausal women.

## Conclusions

Major advances have been made towards our understanding of mechanisms that contribute to aortic valve stenosis. Much of this insight has been gleaned from analysis of human tissue, because animal models that consistently develop hemodynamically significant CAVS have been available only for the past 5 years. These murine models are dependent upon hypercholesterolemia or background strain of the mice for the development of CAVS. The context dependence of cell signaling will always be an underlying issue with these models, which makes the development of additional models of CAVS important. These mouse models will provide an opportunity to examine mechanisms that lead to CAVS, and to test the efficacy of pharmacological interventions. We suggest that interventions outlined in this review hold promise for slowing the progression of CAVS and delaying the need for valve replacement surgery.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## NON-STANDARD ABBREVIATIONS

<b>CAVS</b>	calcific aortic valve stenosis
<b>RT-PCR</b>	reverse transcriptase-polymerase chain reaction
<b>BMP</b>	bone morphogenetic protein
<b>NOS</b>	nitric oxide synthase
<b>RANKL</b>	receptor activator of nuclear factor $\kappa$ B ligand
<b>OPG</b>	osteoprotegerin
<b>PPAR<math>\gamma</math></b>	peroxisome proliferator-activated receptor
<b>RAS</b>	renin-angiotensin system
<b>TGF-<math>\beta</math></b>	transforming growth factor- $\beta$ ROS, reactive oxygen species
<b>VSMC</b>	vascular smooth muscle cell
<b>RAGE</b>	receptor for advanced glycosylation end products
<b>MGP</b>	matrix gamma-carboxyglutamic (Gla) protein

<b>MMP</b>	matrix metalloproteinase
<b>TNF</b>	tumor necrosis factor
<b>HDAC</b>	histone deacetylase
<b>AT1r/AT2R</b>	angiotensin receptor 1/2
<b>CpG</b>	linear base sequence of cytosine and guanine in DNA

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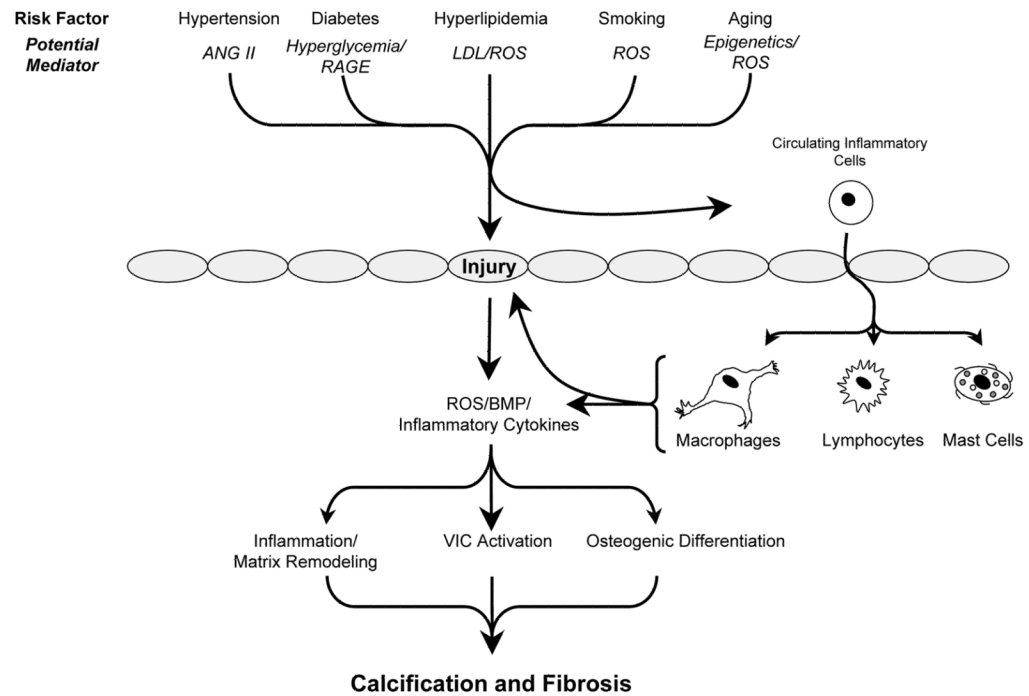
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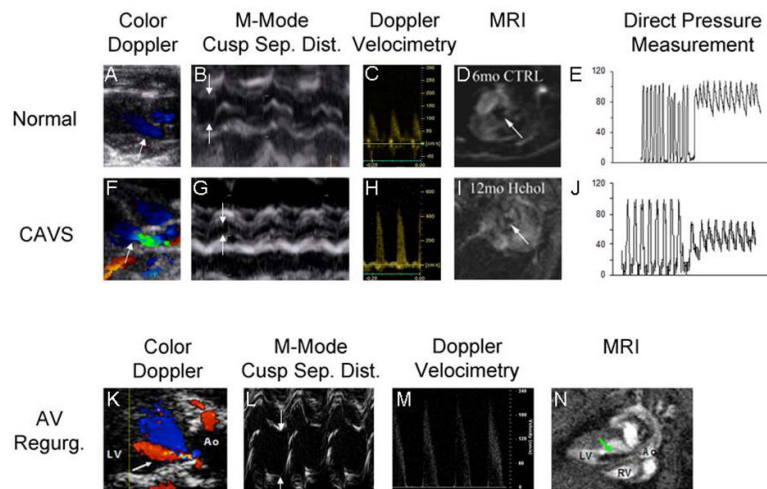
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**Figure 1.**

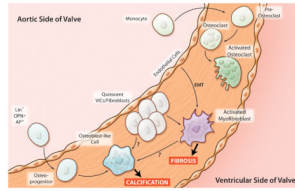
Overview of risk factors and potential mechanisms that contribute to calcification and fibrosis of the aortic valve. For clarity, effects of potential mediators on various cell types in the valve have been omitted. Abbreviations: ANG II = angiotensin II; RAGE = receptor for advanced glycosylation end products; LDL = low density lipoproteins; ROS = reactive oxygen species. Grey ovals depict endothelial cells.



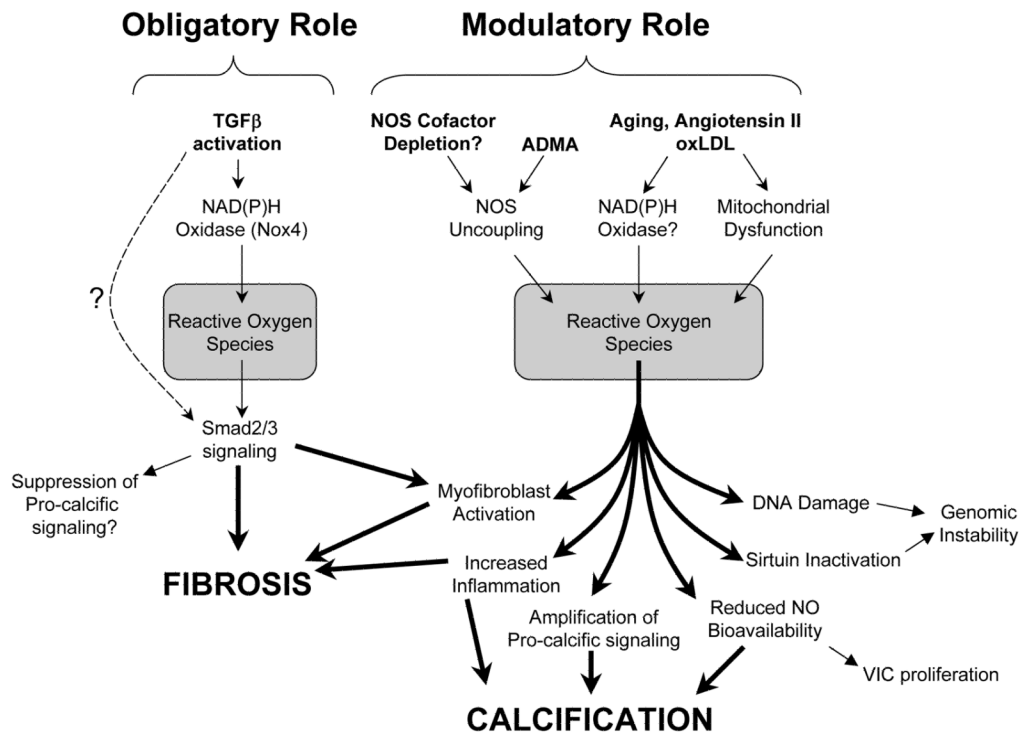


### Figure 2. Assessment of aortic valve function in mice

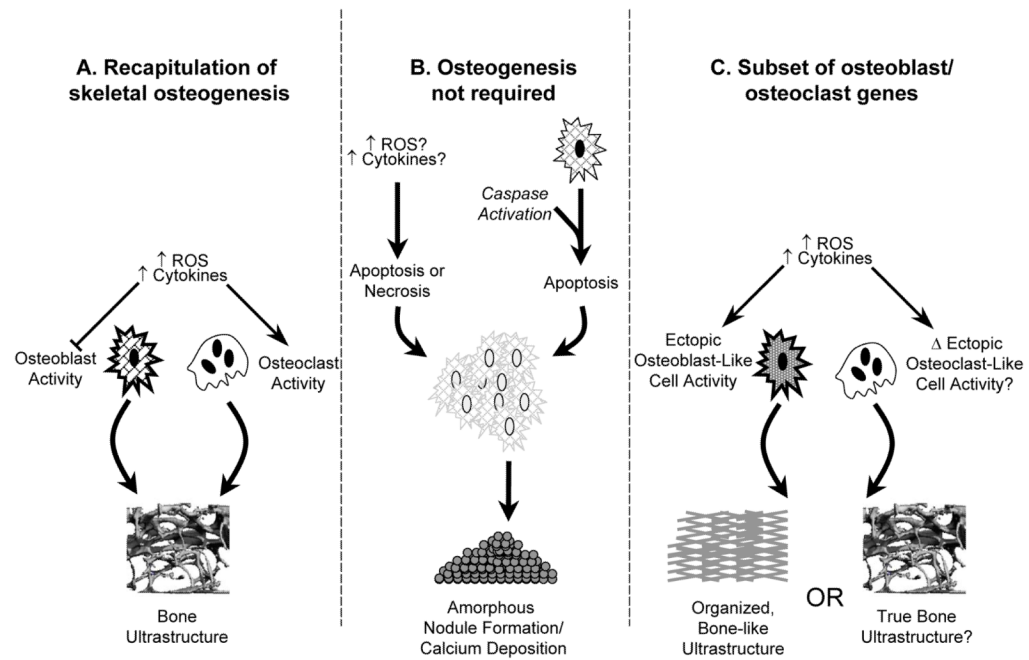
Two-dimensional color Doppler images (Panels A,F,K) are used to target M-mode imaging of the aortic valve and Doppler velocimetry, and to assess the presence (arrow, panel K) or absence of aortic regurgitation. Direction of blood flow is indicated by pseudocolors (red = blood flow towards probe, blue = blood flow away from probe). In CAVS, the irregular stenotic valve orifice causes flow acceleration and turbulence (green). The superior spatial and temporal resolution of M-mode echocardiography facilitates quantitation of systolic cusp separation (arrows, panels B,G,L). Doppler velocimetry (Panels C, H, M) allows for estimation of the transvalvular systolic pressure gradient via the Bernoulli Equation. Aortic regurgitation can cause a modest transvalvular systolic pressure gradient even in the absence of reductions in cusp separation (Panel L, green arrow points to regurgitant jet), by virtue of pre-load dependent increases in contractile force and stroke volume and subsequent increases in velocity (Panel M). Magnetic resonance imaging provides temporal and spatial resolution sufficient to portray the aortic valve orifice in two dimensions (arrows, Panels D,I), by virtue of magnetic dephasing of ejected blood. The same principle is employed to depict retrograde flow when aortic regurgitation is present (arrow, Panel N, cine images can be viewed in the Supplemental Movie). Direct pressure measurements can provide incontrovertible evidence of a transvalvular gradient (Panels E and J), but are influenced by the potential for cardiodepression and vasodilation caused by deep general anesthesia, especially in the presence of CAVS. **CAVS** calcific aortic stenosis; **AV Regurg.** aortic valve regurgitation; **Sep. Dist.** separation distance; **MRI** magnetic resonance imaging.



**Figure 3. Potential origins of cells that contribute to valvular calcification and fibrosis**  
Possible origin of osteoblast-like and osteoclast-like cells in aortic valves in human and murine CAVS. Activated myofibroblasts are likely to come from either quiescent valvular interstitial cells (VICs) or from a sub-population of endothelial cells that undergo endothelial to mesenchymal transformation (EMT). Osteoclast-like cells may originate from circulating monocytes. (**Illustration Credit: Cosmocyte/Ben Smith**).

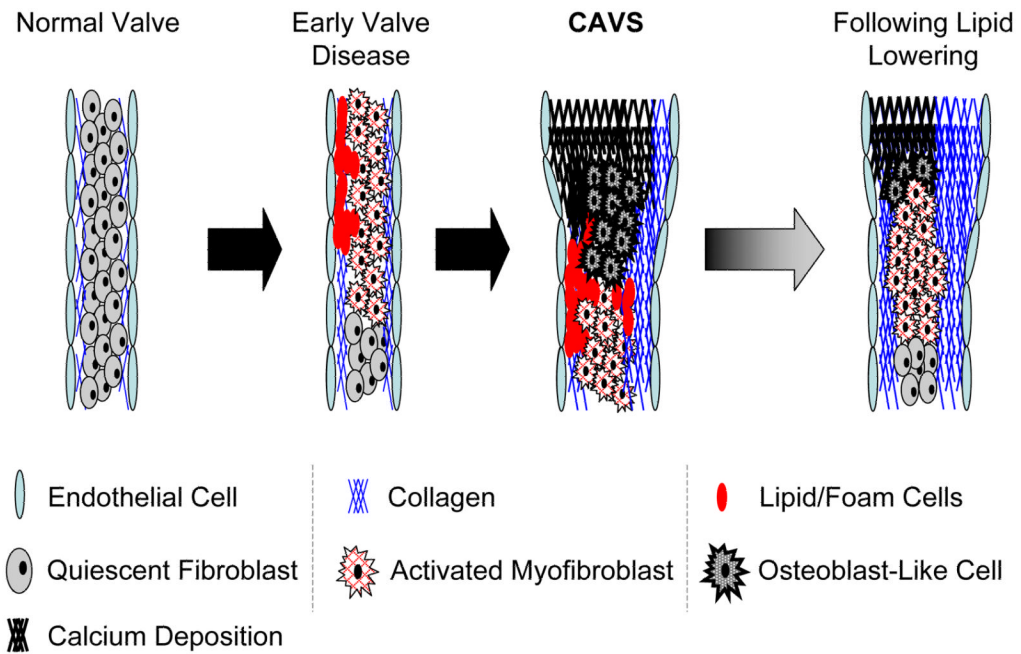


**Figure 4.** Mechanisms whereby reactive oxygen species (ROS) may modulate pro-calcific and pro-fibrotic signaling in CAVS. Nox4-derived ROS may play an obligatory role in TGF $\beta$  signaling and induction of fibrosis. In contrast, ROS may play a modulatory role in promoting aortic valve calcification.

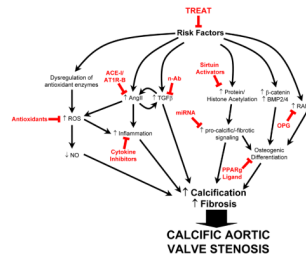


**Figure 5.**

Potential pathways contributing to calcified nodule formation in CAVS. A) recapitulation of classical skeletal osteogenesis, in which osteoblast and osteoclast cells respond to exogenous stressors (such as oxidative stress) in a manner similar to that found in bone-derived osteoblasts. B) formation of amorphous calcific nodules without a requirement for osteoblast-like cells, in which stressors initiate cellular aggregation, apoptosis or necrosis, and nodule formation. C) “pseudo-skeletal” ossification, in which cells expressing a subset of osteoblast or osteoclast genes are present in the aortic valve, but respond to exogenous stimuli in fundamentally different ways. For example, previous studies *in vitro* have shown that—unlike skeletal osteoblasts—cells from cardiovascular tissue typically increase their osteogenic potential in response to exogenous oxidative stress. Bone matrix, replete with marrow hematopoietic elements, has been identified in aortic valves of some patients with CAVS. It is not clear whether this requires processes identical to skeletal osteogenesis (panel A), or if similar structures can be formed by osteoblast-like cells (panel C).



**Figure 6.** Progression and “regression” of CAVS in “Reversa” mice<sup>12, 13</sup>. Early stages of CAVS in mice involve myofibroblast activation and lipid insudation/foam cell formation, and are followed by the appearance of osteoblast-like cells, valvular calcification, and substantial increases in valvular fibrosis. Following reduction of blood lipids (“regression” in right panel), there are substantial reductions in valvular lipid content and calcium content, but valvular fibrosis remains increased. Despite reduction of valvular lipid and calcium content, aortic valve function does not improve with substantial lipid lowering.



**Figure 7.** Potential targets and treatments to slow the progression of aortic valve stenosis. Risk factors (including hypercholesterolemia, hypertension, metabolic syndrome, and smoking) can be treated. Possible signaling cascades and treatments (in red), although supported by some experimental evidence, are speculative. See text for rationale. Abbreviations: ACE-I: angiotensin converting enzyme inhibitor; AT1R-B, angiotensin II receptor type I blocker; n-Ab, neutralizing antibody against TGFβ; miRNA, micro-RNA; ROS, reactive oxygen species; NO, nitric oxide; OPG, osteoprotegerin; PPARγ, peroxisome proliferator-activated receptor gamma.



**Table 1**

Echocardiographic and hemodynamic changes in animal models of aortic valve sclerosis and stenosis.

Species/Strain	Diet <sup>REF</sup>	Histopathological changes in aortic valve	Hemodynamically significant stenosis?
<b>Mice</b>			
C57BL/6	HF <sup>16</sup>	Lipid deposition Modest calcification	No
ApoE <sup>-/-</sup>	Chow <sup>15</sup>	Lipid deposition Calcification	<2%
	HF/HC <sup>19, 33</sup>	Monocyte/Inflammatory cell infiltration	<2%
	HF/HC <sup>16, 223</sup>	Lipid deposition Calcification Monocyte/Inflammatory cell infiltration	No
Ldlr <sup>-/-</sup>	Chow <sup>14</sup>	Lipid deposition Calcification Monocyte/Inflammatory cell infiltration	Yes, ~30% of mice
	HF/HC <sup>12, 13</sup>	Lipid deposition Calcification Fibrosis Monocyte/Inflammatory cell infiltration Myofibroblast activation	Yes, >50% of mice
EGFR <sup>Wa2/Wa2</sup>	Chow <sup>11</sup>	Fibrosis Calcification Inflammatory cell infiltration	Yes, but background strain dependent
eNOS <sup>-/-</sup>	Chow <sup>224</sup>	Bicuspid aortic valves in ~40% of mice	Not known
Notch1 <sup>+/-</sup>	HF/HC <sup>125, 126</sup>	Calcification	No
Perostin <sup>-/-</sup>	Chow <sup>225</sup>	Calcification Fibrosis	Not known
MGP <sup>-/-</sup>	HF/HC <sup>226</sup>	Reduced valve thickening and fibrosis	No
	Chow <sup>122</sup>	Calcification	Not known
Chm1 <sup>-/-</sup>	Chow <sup>227</sup>	Neoangiogenesis Lipid deposition Calcification	Not known
<b>Rabbits</b>			
New Zealand White	HF/HC <sup>49, 79, 89, 209, 228-238</sup>	Lipid deposition Calcification	<10%
			Mostly moderate sclerosis

Species/Strain	Diet <sup>REF</sup>	Histopathological changes in aortic valve	Hemodynamically significant stenosis?
		Inflammatory cell infiltration	
	Chow +	Fibrosis	<10%
	HTN <sup>229</sup>	Inflammation	
Watanabe	HF/HC <sup>49</sup>	Lipid deposition Fibrosis Calcification Inflammatory cell infiltration	No
<b>Pigs</b>			
Yorkshire	HF/HC <sup>83,239</sup>	Lipid deposition	No
Landrace			