Fibrocalcific aortic valve disease (FCAVD) is a growing health problem in the developed world.\textsuperscript{1,2} The incidence of overt FCAVD rises exponentially with age, with a steep increase after 65 years of age.\textsuperscript{3} As developed societies age, therefore, the burden of disease is also expected to increase. Recognition of the emerging importance of FCAVD, together with seminal studies pointing to a complex orchestrated sequence of processes which culminate in a lethal phenotype, has spurred interest in elucidating the nature of the disease and in identifying effective preventive interventions.

In a recent series, mechanisms of cardiovascular calcification were reviewed, including emphasis on events in the aortic valve.\textsuperscript{4-9} In this compendium, we will review the rationale for studies in mice, the growing complexity of assessing aortic valve function in vivo, emerging mechanistic themes relating to the pathophysiology of FCAVD, and studies designed to ameliorate the course of FCAVD in mice.

**Abstract:** Studies in vitro and in vivo continue to identify complex-regulated mechanisms leading to overt fibrocalcific aortic valve disease (FCAVD). Assessment of the functional impact of those processes requires careful studies of models of FCAVD in vivo. Although the genetic basis for FCAVD is unknown for most patients with FCAVD, several disease-associated genes have been identified in humans and mice. Some gene products which regulate valve development in utero also protect against fibrocalcific disease during postnatal aging. Valve calcification can occur via processes that resemble bone formation. But valve calcification can also occur by nonosteogenic mechanisms, such as formation of calcific apoptotic nodules. Anticalcific interventions might preferentially target either osteogenic or nonosteogenic calcification. Although FCAVD and atherosclerosis share several risk factors and mechanisms, there are fundamental differences between arteries and the aortic valve, with respect to disease mechanisms and responses to therapeutic interventions. Both innate and acquired immunity are likely to contribute to FCAVD. Angiogenesis is a feature of inflammation, but may also contribute independently to progression of FCAVD, possibly by actions of pericytes that are associated with new blood vessels. Several therapeutic interventions seem to be effective in attenuating the development of FCAVD in mice. Therapies which are effective early in the course of FCAVD, however, are not necessarily effective in established disease. (\textit{Circ Res.} 2013;113:209-222.)

**Key Words:** aortic valve \& aortic valve stenosis \& aortic valve calcification

\textbf{Fibrocalcific Aortic Valve Disease}

\textbf{Opportunity to Understand Disease Mechanisms Using Mouse Models}

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\textit{Circulation Research} is available at http://circres.ahajournals.org

\textbf{DOI: 10.1161/CIRCRESAHA.113.300153}
Studies in Whole Animals

Genetic factors that predispose to FCAVD have been identified. Overt FCAVD, however, usually appears after a long latency, with incomplete penetrance. Mechanisms by which a given gene (product) affects the course of FCAVD cannot be ascertained fully in vitro. A clinical risk factor for FCAVD, such as hypertension, itself typically has long disease latency. Cellular and molecular influences of a specific mediator of hypertension, for example, angiotensin II, can be evaluated in vitro. But recapitulation of the complex pro-FCAVD milieu of hypertension cannot be replicated in vitro. In addition, it can be difficult to determine whether changes that are observed in vitro are important in the pathophysiology of a disease, or whether they represent a compensatory protective response to disease processes. Studies in whole animals allow integration of biochemical, humoral, neural, and inflammatory mechanisms into a broader mechanistic understanding of the pathophysiology of FCAVD. Longitudinal studies in experimental animals may serve as a platform for testing of translational hypotheses in humans at risk for the development of overt FCAVD.

Functional Impact of FCAVD

Foremost among the advantages of studies in whole animals is the ability to assess the impact of risk factors on aortic valve function and biventricular responses to valve disease. Commonly used tools for assessment of aortic valve function in humans can be applied in mice to place experimental findings in a clinically relevant context.

Echocardiography is the mainstay of quantitative assessment of aortic valve function in mice. A comprehensive report describes morphological and functional responses of the aortic valve to aging, from fetal development to senescence. Aortic valve cusp motion can be observed using 2-dimension(al 2D) echocardiography, but the measurement is challenging at physiological heart rates, even with newer high-resolution equipment. M-mode echocardiography provides greater spatial and temporal resolution than 2D echocardiography, is feasible in almost all mice, and normal values have been defined. M-mode assessment of stenosis severity has been validated against hemodynamic measurements in mice, in the absence of aortic regurgitation.

Technical Considerations in Mice

Some technical challenges that apply to studies in mice are summarized in Table 1. Accurate measurement of transvalvular pressure gradient obviously can be useful for the assessment of aortic stenosis severity. In humans and mice, intraventricular flow acceleration can produce a significant subvalvular gradient, which is optimally measured in the left ventricular outflow tract, using depth-gated (pulse wave) Doppler velocimetry. In hyperdynamic states, such as hypertrophic obstructive cardiomyopathy or aortic regurgitation, a very significant intraventricular cavity (subvalvular) pressure gradient can occur. Thus, accurate assessment of the severity of aortic valve stenosis requires subtraction of the intraventricular cavity gradient from the total left ventricle-to-aorta pressure gradient. When aortic regurgitation is known or suspected to be present, reliance solely on assessment of left ventricle-to-aorta pressure gradient will be prone to overestimation of aortic stenosis severity, even when invasive methods are used to measure the gradient (Figure 1).

Late in the course of valvular heart disease caused by aortic stenosis, left ventricular systolic function can become significantly impaired. Decreased stroke volume and reduced rate of ejection of blood from the left ventricle can result in a modest transvalvular gradient, even when severe aortic stenosis is present. In humans, that combination of findings, known as low flow low gradient stenosis, is associated with a particularly dire prognosis. The phenomenon has been observed anecdotally in mice, but comprehensive studies have not yet been published.

Continuous wave Doppler is the technique of choice for noninvasive assessment of transvalvular gradients in humans. Continuous wave Doppler capability, however, is not currently offered on high-resolution echo imagers designed for studies in mice. Use of continuous wave Doppler in mice thus requires equipment designed for use in humans, which provides lower spatial and temporal resolution.

When the direction of blood flow deviates from the Doppler line, angle correction must be used to report accurate blood velocities. Angle correction is prone to magnification of errors when the angle approaches 90°, especially with disturbed blood flow (Figure 2). These potential problems can be minimized by optimizing the angle of exit between transvalvular blood flow and the Doppler line. If color Doppler screening reveals no significant aortic regurgitation, and 2D B-mode images confirm the presence of normal left ventricular systolic function, then Doppler velocimetry can produce a reasonable measurement of severity of aortic stenosis (Figure 1D). However, we are not aware of a published, experimentally validated, maximum acceptable angle of exit, below which
accurate assessments of stenosis severity can be achieved with confidence, using Doppler velocimetry in mice.

Severity of stenosis can also be ascertained by using the excellent spatial resolution provided by high-field MRI. MRI carries the added advantage of allowing precise measurements of stroke volumes of both ventricles, which facilitates measurement of the severity of aortic regurgitation. Invasive hemodynamic measurements of left ventricle-to-aorta pressure gradients are of great value and avoid some of the technical drawbacks of Doppler echocardiography. Nevertheless, invasive measurements are still prone to over- or underestimation of stenosis severity in the presence of aortic regurgitation or left ventricular systolic dysfunction (Figure 1). When severe aortic stenosis is present, invasive techniques are technically challenging, require deep general anesthesia, and may result in tissue injury.

A summary of the attributes of methods used for assessment of valvular heart disease in mice in vivo is provided in Table 2.

Disadvantages of Studies in Mice
Although there are anatomic and pathophysiological similarities between murine aortic valves and human aortic valves, there are also important differences. The aortic and ventricular sides of the valve are exposed to different hemodynamic stimuli and, in humans, the thickness of the valve cusps allows a gradient of responses by valve interstitial cells (VICS). The gossamer-like structure of murine aortic valve cusps may not allow a gradient of responses by VICS.

As in humans, there is significant heterogeneity in the severity and rate of progression of FCAVD among individual mice, even in carefully controlled studies of syngeneic mice. In fact there can be significant heterogeneity of histopathologic changes within the valve of a single mouse. Postmortem histological and gene-expression studies offer only a single snapshot characterization of disease-associated events. Thus, it can be challenging to identify the cellular and molecular mediators that govern the initiation and propagation of FCAVD, when whole animals are used for study. Newer imaging methods may help clarify the temporal relationship between processes, such as inflammation, valve calcification, and valve dysfunction.

Mechanistic Themes
Mechanical Factors May Influence the Course of FCAVD
In vascular endothelial cells (ECs), longitudinal shear increases the activity and expression of endothelial nitric oxide synthase (eNOS) and attenuates inflammation. Mechanistic similarities between vascular disease and valve disease suggest that longitudinal shear stress could be an important regulator of valve homeostasis.

Congenitally bicuspid aortic valves are more prone than trileaflet valves to develop clinically important FCAVD. FCAD occurs at a much younger age in humans with bicuspid aortic valves than in those with trileaflet valves. Bicuspid aortic valves often develop predominant aortic stenosis, but predominant aortic regurgitation is also common in patients with bicuspid aortic valves. Although not rigorously tested, the prevailing explanation for susceptibility to FCAVD in patients with bicuspid aortic valves relates to disruption of flow across the abnormal valve, resulting in diminished longitudinal shear along the valve surface. The concept is supported by mathematical modeling of regional stresses in bicuspid and trileaflet aortic valves and by ex vivo experimental models that simulate congenital bicuspid aortic valves.

Asymmetry of Aortic Valve Endothelium and Shear Stress
In humans, FCAVD usually begins on the aortic side of valve cusps, with relative sparing of the ventricular side, which is exposed to high shear induced by ejection of ventricular blood. Recently, several investigators have sought to identify
paracrine signals that originate from endothelium.\textsuperscript{23} Targeted approaches and high-throughput molecular screening have provided key insights into molecular mechanisms, which regulate paracrine signals from both sides of the valve.\textsuperscript{23} After careful isolation of endothelium from both sides of the valve,\textsuperscript{23} targeted approaches and high-throughput molecular screening have provided key insights into molecular mechanisms, which regulate paracrine signals from both sides of the valve.\textsuperscript{23} Interrogation. In the illustration, the angle of exit of presumed blood flow is 75°. Thus, measured velocity would be divided by the cosine of 75° (\(\cos\theta = 0.26\)), to compute actual flow velocity. When transvalvular blood flow is disturbed, a portion of the flow jet will be directed more parallel to the Doppler line and will be detected as a higher velocity. In this case, correction for a presumed angle of exit of 75° will overestimate true blood velocity by a factor of \(\approx 4\). \textbf{B}, Doppler velocimetry from a normal mouse, with neither aortic stenosis nor regurgitation. The angle between the Doppler cursor and the presumed direction of transvalvular flow was 75° (image not shown). Peak velocity is reported as 3.6 m/s, erroneously predicting a peak valve gradient of 52 mm Hg. \textbf{C}, M-mode echocardiogram of the aortic valve in the same mouse. White bar indicates aortic cusp separation of 1.3 mm, which is normal. Color Doppler and MRI both confirmed the absence of aortic regurgitation (images not shown). LV indicates left ventricular.

Expression of antioxidant enzymes is relatively high in ECs isolated from the aortic side of the normal valve.\textsuperscript{24} In contrast, expression of proinflammatory molecules does not differ between the aortic and ventricular sides of the valve.\textsuperscript{24} The findings are surprising because disturbed flow on the aortic-side endothelium would be expected to induce both inflammation and increase oxidative stress and suggest that increased antioxidant enzyme levels may serve as an adaptive mechanism to protect the valve in this region.

Expression of inhibitors of osteogenic signaling is significantly reduced in endothelium from the aortic side of the valve.\textsuperscript{24,25} Inhibitors of bone morphogenetic protein (BMP) signaling, such as BMP-binding endothelial regulator and noggin, are reduced in endothelium from the aortic side of the valve, even in healthy/unstressed aortic valves.\textsuperscript{25} Molecular mechanisms underlying these changes are not clear. Given the important role of osteogenic morphogens in the initiation and progression of FCAVD, identification of novel interventions to increase expression of BMP antagonists on the aortic side of the valve may prove to be useful in preventing initiation of aortic valve calcification.

Studies to address the importance of EC asymmetry have usually been performed using valves from large mammals. It is possible that the proximity of the 2 EC layers to one another in murine aortic valves diminishes or eliminates some of the endothelial asymmetry observed in valves from larger mammals. We note, however, a study which reported that marrow-derived progenitor cells home preferentially to the ventricular side of the uninjured aortic valve in wild-type mice, indicating the plausibility of asymmetrical responses in murine aortic valves.\textsuperscript{26}

**Valve Homeostasis Is Regulated by Epigenetic Events**

Epigenetic modifications are emerging as major regulators of gene expression in health and disease.\textsuperscript{27–29} Alterations in histone acetylation, histone methylation, and DNA methylation can have a profound impact on transcriptional responses to multiple stimuli, but the interaction between these regulatory mechanisms is not understood well.

Knockdown of SIRT1 (a nicotinamide adenine dinucleotide-dependent histone deacetylase) accelerates smooth muscle cell calcification in vitro.\textsuperscript{30} Furthermore, loss-of-function mutations in SIRT1 are associated with coronary artery calcification.\textsuperscript{31} Depletion of endothelial SIRT1 increases vascular inflammation.\textsuperscript{32} In thoracic aortic aneurysms associated with bicuspid aortic valves, levels of the injury-response element pSmad2, and its translocation to the nucleus, are increased in smooth muscle cells.\textsuperscript{33} In addition, hyperacetylation of histone 3K9/14 and hypermethylation of H3K4 (both of which can be permissive for transcription factor binding) seem to contribute to the constitutive expression of Smad2.\textsuperscript{31} Little is known, however, about the role of histone deacetylases, histone acetyltransferases, and histone methyltransferases in regulation of aortic valve calcification or fibrosis. Studies which examine structural and functional consequences of altering levels of

| Table 2. Assessment of Valvular Heart Disease in Mice In Vivo |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | M-Mode Echo     | 2D Echo         | Doppler Echo    | MRI Catheterization |
| Cost            | +++             | +++             | +++             | ++++            | +               |
| Depth of sedation| +               | +               | +               | ++              | +++             |
| Valves morphology| ++              | ++              | –               | +++             | –               |
| Severity of stenosis| +++ +       | +++*            | +++             | ++++*           | –               |
| Severity of regurgitation| –          | –               | ++              | +++             | –               |
| LV anatomy | ++              | +++             | –               | +++             | –               |
| LV function | ++              | +++             | +++             | +++             | +++             |
| RV function | +               | ++              | +++             | +++             | –               |
| Production of tissue injury | –         | –               | –               | –               | –               |

2D indicates 2-dimensional; LV, left ventricular; and RV, right ventricular. *When aortic regurgitation is known to be absent.
histone-modifying enzymes in vivo will be critical in determining whether these processes are viable therapeutic targets in FCAVD.

Although there are extensive data implicating changes in DNA methylation in altered transcriptional patterns reported in cancer, stroke, and atherosclerosis, data examining the role of DNA methylation in FCAVD are only beginning to emerge. For example, expression of the proinflammatory enzyme 5-lipoxygenase is inhibited by methylation of its promoter, and hypomethylation of the 5-lipoxygenase promoter is associated with consequent increases in mRNA levels in inflammatory cells. This phenomenon is not likely to be restricted to inflammatory cells, as inhibition of DNA methylation results in increased 5-lipoxygenase expression in cultured VICs.

There are accumulating data from other tissues suggesting that DNA methylation may play an important role in transcriptional regulation of other genes implicated in FCAVD. First, expression of matrix Gla protein, a potent inhibitor of BMP signaling, can be markedly reduced by promoter hypermethylation. Second, expression of alkaline phosphatase is dramatically increased in osteogenic cells after treatment with compounds that induce DNA hypomethylation, which suggests that tonic suppression of expression of alkaline phosphatase may protect against initiation of aortic valve calcification.

MicroRNAs contribute to epigenetic control of gene expression. Using high-throughput, unbiased screening methods, 30 shear-sensitive, and 3 side-sensitive (ie, ventricular- versus aortic-side endothelium) microRNAs have been identified in aortic valve ECs. Identifying mechanisms whereby these microRNAs exert their deleterious or protective effects is challenging because a single microRNA can alter expression of hundreds of target genes. Prospectively, the combination of experimentally altering microRNA levels with high-throughput molecular screening will be critical to determine whether pharmacological or genetic manipulation of microRNAs can orchestrate a coordinated, protective phenotype and ultimately become an attractive therapeutic target in patients with FCAVD.

**Mechanical Properties of the Aortic Valve**

Cardiovascular tissue, in general, responds to increased radial stress by increasing its thickness, which acts to buffer or neutralize mechanical stress. However, mechanisms by which mechanical forces invoke cellular and molecular responses within the aortic valve are not yet well understood. The eccentric shape (Figure 3), heterogeneous deformation of the valve during the cardiac cycle, and oscillating direction of pressure-stress all render mathematical modeling problematic. Clinical translation will, therefore, require meticulous experimental validation of mathematically modeling of aortic valve mechanics.

Studies in cell culture are clarifying ways in which valve cells respond to changes in their physical environment. VICs that are grown in standard growth media, on a stiff synthetic matrix, rapidly transdifferentiate to myofibroblasts, assemble in nodules, undergo apoptosis, and calcify. The processes are attenuated when VICs are cultured on a soft matrix. Reduction of matrix stiffness, using photo-responsive material, can actually reverse the processes that lead to the formation of calcific nodules. Contractile proteins play a critical role in the formation of calcific nodules, which are produced by VICs in vitro after transdifferentiation of fibroblasts to myofibroblasts. Expression and polymerization of α-smooth muscle actin are critical to the formation of calcific nodules.

In a different setting, where VICs are grown on a collagen matrix with variable stiffness, supplementation of culture medium with pro-osteogenic factors (ascorbate, β-glycerophosphate, and dexamethasone) induces VICs to differentiate into osteoblast-like cells, which produce calcification of the extracellular matrix. Osteogenic calcification, in apparent contrast to apoptosis-related calcification, is decreased by a stiffer matrix composition. The relative importance of the 2 mechanisms (myofibroblast differentiation with formation of apoptotic calcific nodules versus osteogenic transdifferentiation leading to bone-like calcification), in vivo, is not known.

Translation of findings on the impact of matrix stiffness on VIC behavior from cell culture to studies in native aortic valves from FCAVD-prone mice could provide significant new insight about mechanisms that are operative in the development of FCAVD. In normal valves, matrix elements, such as collagen and elastin, are organized and stratified. In diseased valves, matrix elements are disorganized, and composition differs from that found in normal valves. The impact of matrix remodeling in the aortic valve has been addressed using atomic force microscopy in cryosectioned murine aortic valves, in which increased cusp stiffness correlates with increased abundance of extracellular matrix in several FCAVD-prone mouse strains.

Studies using micropipette aspiration of excised whole valves from normal mice demonstrate a gradient of decreasing mechanical stiffness from cusp base to cusp tip. Surprisingly, perhaps, mechanical stiffness of the valve annulus and valve cusps seems to decrease with age in normal mice, findings which correlate with replacement of collagen by proteoglycan at the annular site of cusp attachment.

Individual layers of the valve respond differently to applied mechanical stress. Valve tissue nearest the left ventricle has a lower elastic modulus than tissue nearest the aortic side of the valve. The findings provide a mechanical context for the cellular and molecular asymmetries described above. Evolution of methods for assessment of the material properties of the aortic valve will be of great importance for elucidating mechanisms by which mechanical forces are transduced into cellular and molecular events, and vice versa.

**Bicuspid Aortic Valves**

As discussed above, perturbation of blood flow occurs in patients with bicuspid aortic valves and has been proposed as an explanation for the increased incidence and severity of aortic stenosis in patients with bicuspid aortic valves. That long-standing belief, however, has not been rigorously tested in humans or in experimental animals. There is ongoing debate on the relative importance of cusp number versus underlying genotype, in humans with bicuspid aortic valve. In patients with bicuspid aortic valves, prediction for the development of aortic root aneurysm follows a strong familial pattern. The risk for the development of clinically significant aortic valve...
dysfunction, however, is less predictable, even within single families with high prevalence of bicuspid aortic valve.55 Thus, environmental and epigenetic factors influence the risk for overt valve disease, perhaps more so than the risk for development of aortic aneurysms, in patients with bicuspid aortic valve (Figure 4).

The discovery of mouse strains with increased prevalence of bicuspid aortic valves offers the opportunity to elucidate mechanisms of disease in syngeneic kindreds with either bicuspid or trileaflet aortic valves. About 30% of mice that are deficient in eNOS−/− are born with bicuspid aortic valves.56,57 When fed a high-fat diet, eNOS−/− mice with bicuspid valves, but not trileaflet valves, develop calcific aortic stenosis by 6 months of age.58 Similarly, preliminary reports suggest that experimentally altering endogenous inhibitors of nitric oxide synthases (eg, asymmetrical dimethylarginine) in mice with experimentally altering endogenous inhibitors of nitric oxide synthases (eg, asymmetrical dimethylarginine) in mice with bicuspid aortic valves does not alter the early initiation phases of CAVD.59 The combination of prolonged hypercholesterolemia and elevated asymmetrical dimethylarginine levels for 6 months, however, does seem to accelerate progression of CAVD in mice with trileaflet valves.59 Those findings suggest that deficiency of endothelium-derived NO alone is not sufficient to cause physiologically important aortic valve calcification in both bicuspid and trileaflet aortic valves, but that NO deficiency augments valve calcification in the presence of hypercholesterolemia.

A preliminary report suggests, however, that eNOS−/− mice with bicuspid valves are prone to develop aortic valve fibrosis and calcification, even in the absence of hypercholesterolemia.50 Interestingly, fibrosis, but not calcification, was increased in both bicuspid and trileaflet aortic valves, which suggests a gene effect, independent of cusp number.

Other genetically engineered mice that are prone to develop bicuspid aortic valves offer the opportunity to understand further the relative role of genotype (or gene dose) versus aortic cusp number in the development of overt FCAVD. Bicuspid valve occurs in ≤11% of mice that are haploinsufficient for the transcription factor Nkx2.5, and the prevalence is influenced by background strain.49 Although loss-of-function mutations in Nkx2.5 are associated with other congenital heart malformations, it is not yet clear whether bicuspid aortic valve is a manifestation of Nkx2.5 deficiency in humans.

Humans that are heterozygous for some decrease-of-function mutations in Notch1, a regulator of cell fate and intercellular communication, are prone to develop bicuspid aortic valves.60 Notch signaling represses osteogenic signaling in the aortic valve postnatally.61,62 Human carriers of some decrease-of-function Notch mutations develop overt FCAVD, even in trileaflet aortic valves.62 In mice, suppression of embryonic Notch1 signaling by knockdown of the gene encoding peristin causes cusp fusion and upregulation of osteogenic signaling, leading to FCAVD in postnatal life.63 Surprisingly, however, fibrotic valve changes caused by a high-fat diet are actually attenuated by knockdown of the gene encoding peristin, a paradox possibly related to compartmentalization of peristin expression in the normal aortic valve.64

The transcription factor GATA-5 regulates both eNOS signaling and Notch1 signaling during embryonic development. Deficiency of GATA-5 in mice results in increased incidence of bicuspid aortic valves, in association with hypoplastic heart syndrome, in mice.53 Bicuspid aortic valves with varying degrees of dysfunction have been reported in humans with rare GATA-5 mutations.65 Thus, factors such as eNOS, Notch1, and peristin all regulate the critical phase of cardiac morphogenesis known as epithelial-to-mesenchymal transformation. Deficiency of any one of those factors increases the likelihood of congenital aortic valve anomaly and also accelerates osteogenic valve calcification during adult life. It is not yet known whether bicuspid aortic valves that develop because of deficiency of Nkx2.5 or GATA-5, which also regulate epithelial-to-mesenchymal transformation, are prone to develop overt valve calcification.

Are Valve Calcification and Valve Dysfunction Mechanistically Linked?

One might expect that early stages of aortic valve dysfunction, with alterations in fluid dynamics and mechanical stresses, would promote fibrosis and calcification. At least 1 study, however, has demonstrated significant aortic valve dysfunction during postnatal life in mice, without evidence of subsequent valve calcification. Knockdown of the gene encoding activin receptor-like kinase-2 (Alk2, also known as activin receptor type I, or Acvr1), which is embryonically activated in the developing heart after epithelial-to-mesenchymal transformation, results in functionally bicuspid aortic valves with a high embryonic penetrance66 (Figure 4). Alk2 knockout mice that survive to adulthood develop increased systolic pressure gradients across the abnormal aortic valves, and also increased left ventricular stroke volume, which suggests aortic regurgitation or mixed...
valve dysfunction. The knockdown mice also demonstrate some elements of an osteogenic gene-expression profile, including increased osteopontin and reduced Sox9. However, expression of the committed osteoblast marker, Runx2, was decreased in Alk2 knockdown mice, and valve calcification was not observed, even during mature adult life.69 Resistance to valve calcification can follow a familial pattern in humans with bicuspid aortic valves, a finding corroborated by the absence of Notch1 mutations in subjects with bicuspid aortic valve, dilation of the ascending aorta, but absence of detectable aortic valve calcification.70 Those findings in mice and humans suggest that perturbation of flow associated with a bicuspid aortic valve alone is not sufficient to produce valve calcification, and that additional genetic or environmental stimuli may be necessary for the development of overt calcific aortic valve disease. It also seems that the specific genetic basis for the formation of a bicuspid valve can influence the predilection for valve calcification during postnatal life (Figure 4).

Mice that are haploinsufficient for elastin (Eln+/−) have normal aortic valve function at birth, and later develop aortic regurgitation.71 The composition and organization of the extracellular matrix are abnormal, and VICs are activated in aortic valves from Eln+/− mice. Neither apoptosis nor valve calcification was reported in that study. Thus, hemodynamically significant valve dysfunction does not inevitably initiate valve calcification, and aortic valve dysfunction is not invariably dependent on valve calcification.

**Nonosteogenic Calcification**

For many years, it was assumed that calcification of the aorta and aortic valve is secondary to cell death, with calcification of cell debris.72,73 A simplistic (not necessarily inaccurate) view was that, as cells die in arteries, they may calcify, and wall off inflammation.

It seems likely that apoptotic cell death does in fact contribute to calcification of the aorta and aortic valves.74 When smooth muscle cells undergo apoptosis, the cells release matrix vesicles.75 Apoptotic vesicles are present in calcified aortic valves,73 as well as aorta, and may contribute to calcification. Transforming growth factor-β stimulates, and a caspase inhibitor attenuates, apoptosis and calcification of VICs in vitro.76 Recently, we found that pioglitazone, a peroxisome proliferator–associated receptor γ (PPARγ) ligand, preserves aortic valve function in hypercholesterolemic mice, an effect best explained by attenuation of apoptosis-related calcification in the valve.77

Matrix Gla protein is expressed in vascular muscle and chondrocytes and is a potent inhibitor of mineralization.78 Severe arterial calcification develops in matrix Gla protein–deficient mice, and the mice usually die within 8 weeks of age.
from aortic rupture. Perhaps paradoxically, apoptotic vesicles that are released by smooth muscle cells contain inhibitors of calcification, including matrix Gla protein, as well as pro-apoptotic factors. Thus, apoptotic vesicles modulate vascular calcification, but because they contain factors that may both promote and inhibit calcification, their role is not clearly understood.

On the basis of changes in the signaling pathways that mediate osteogenesis, it is clear that active calcification is important in the aorta and aortic valve. It is now generally assumed that this mechanism is the predominant mechanism of vascular calcification. We wish to emphasize, however, that the relative importance in the development of FCAVD of apoptosis versus an active osteogenic pathways is not clear.

Aortic Valve Differs From Blood Vessels
Aortic stenosis is often associated with dilatation of the ascending aorta. Dilatation of the aorta in patients with aortic stenosis has been attributed to a jet of blood striking the aortic wall, with passive enlargement of the aorta. In our view, an important role for this mechanism is unlikely. A complementary explanation for the association between bicuspid aortic valve and aortic root dilatation is that gene mutations that alter connective tissue in the aortic valve may also alter connective tissue in the aorta.

Several inherited diseases affect both the aortic valve and the aorta. Marfan syndrome, Ehlers-Danlos syndrome, Loeys-Dietz disease, annuloaortic ectasia, and bicuspid aortic valves are associated with aortic regurgitation and enlargement of the aorta. Enlargement of the aorta may precede aortic valve disease and presumably is the result primarily of aortic disease, rather than valvular disease.

Risk factors for atherosclerosis and aortic valve stenosis are generally similar, but the relative importance of the risk factors differs in relation to association with aortic disease versus aortic valvular disease. Similarly, the rate and severity of calcification seem to differ in aorta and aortic valve.

Hypotheses in relation to mechanisms of calcification of the aorta valve often are based on observations in the aorta, with the assumption that mechanisms are similar in the aortic valve and aorta. There are, however, major differences in structure and cellular composition of aortic valve and aorta. Normal aortic valve consists of ECs and valvular interstitial cells. In contrast, the aorta is composed predominantly of smooth muscle cells and connective tissue, with relatively far less endothelium.

Recently, we have reported fundamental differences in responses in mice of the aortic valve and aorta to pioglitazone, a PPARγ ligand. Pioglitazone strongly attenuated lipid deposition and calcification of the valve, but had no effect on fibrocalcific events of the aorta. There also were striking differences in gene expression between the aortic valve and aorta during hypercholesterolemia and treatment with pioglitazone. The findings imply that one may not be able to predict mechanisms that lead to aortic valve disease based on studies of the aorta.

Hypertension is a strong risk factor for aortic stenosis in humans. It is surprising, perhaps, that the impact of hypertension on the aortic valve has not been extensively studied in mouse models, and that blood pressure itself has not been consistently reported in studies of mouse models of aortic valve disease.

Redox State Regulates Aortic Valve Homeostasis
Oxidative stress is increased in valves from patients with end-stage aortic valve stenosis. Several mechanisms may contribute to increased oxidative stress in the valve, with evidence implicating uncoupled nitric oxide synthase and reduced antioxidant enzyme activity, and conflicting evidence on the role of increased nicotinamide adenine dinucleotide phosphate oxidase activity.

Three experimental observations have provided insight into the role of oxidative stress in the pathogenesis of aortic valve calcification. First, increases in reactive oxygen species precede aortic valve dysfunction in a mouse model of FCAVD, which suggests that oxidative stress is not merely an epiphenomenon associated with valve calcification and dysfunction. Second, experimentally increasing oxidative stress amplifies osteogenic signaling and accelerates calcification of aortic VICs in vitro. Third, reducing blood lipids in mice with advanced FCAVD reduces osteogenic signaling in the aortic valve, but does not reduce oxidative stress in these same regions.

Collectively, these data suggest that, under some circumstances, oxidative stress can amplify osteogenic signaling in the aortic valve, but that elevated oxidative stress does not always initiate or independently propagate calcification in the aortic valve. Future studies aimed at understanding the role of oxidative stress in different cell types (eg, endothelium versus VICs) and different subcellular compartments (eg, mitochondria, cytosol, or peroxisomes) will be instrumental in determining the therapeutic usefulness of targeting oxidative stress in CAVD.

Inflammation
Many studies indicate that inflammation is associated with aortic valve disease. There are major gaps, however, in our understanding of mechanisms that produce inflammation in the valve and the role of inflammation in the pathophysiology of aortic valve disease.

Dendritic Cells
It is intriguing that dendritic cells are not only present in the aortic valve, but are also especially dense in the valve base and aortic sinuses (Figure 5). Ralph Steinman, MD, who received the Nobel Prize for his discovery of dendritic cells, demonstrated that dendritic cells, which are primarily sub-intimal, have processes that extend into the lumen of blood vessels and play a critical role in capture of pathogens and presentation to T cells.

Dendritic cell biology is complex and evolving, and it is now known that there are several types of dendritic cells. We will briefly summarize some aspects of dendritic cells, with potential relevance to the aortic valve.

Cardiovascular dendritic cells localize mainly in areas with disturbed flow, including the aortic valve, aortic sinuses, and origin of arterial branches (which are prone to the development of atherosclerosis). Normal flow patterns may attenuate, and disturbed flow may facilitate, capture of pathogens by dendritic cells. Studies of dendritic cells in valves were performed in murine valves, and the authors could not localize dendritic cells to the ventricular or aortic surface of the valves.
Considering the predisposition to disturbed blood flow on the aortic side of the valve, we speculate that dendritic cells may predominate on the aortic side and perhaps contribute to predisposition of aortic valve disease to begin on the aortic side and base of the valve.

The role of dendritic cells, especially in relation to macrophages, in the development of atherosclerosis is unsettled. Classical dendritic cells (which are strongly immunostimulatory) protect against the development of atherosclerosis in the aorta of Ldlr−/− mice. The implication is that the immune response may protect against atherosclerosis. It is not clear whether those findings in the aorta, and our speculation, are applicable to the aortic valve.

A second type of dendritic cells, in addition to classical dendritic cells, are macrophage-colony stimulating factor (M-CSF)–dependent, monocyte-derived dendritic cells. During atherogenesis, the number of monocyte-derived dendritic cells, which also strongly stimulate T cells, increases even more than classical dendritic cells. M-CSF is proatherogenic, and it is not known whether the proatherogenic effect is mediated through monocyte/macrophages or M-CSF–dependent dendritic cells.

M-CSF, which is one of the links between inflammation and cardiovascular disease, is also critical for formation of osteoclasts. M-CSF, together with receptor activator of nuclear factor-κB and receptor activator of nuclear factor-κB ligand, is required for fusion of pro-osteoclasts into multinucleated osteoclasts, which critically regulate tissue mineralization in bone. It is not known, however, whether M-CSF regulates osteoclast function and tissue mineralization in the aortic valve.

**T Cells and Macrophages**

Inflammation occurs early in the development of aortic stenosis and is associated with calcification. T cells and B cells are present in valves, adjacent to myofibroblasts and preosteoblasts. Expression of BMP2 and BMP4 occurs adjacent to the inflammatory infiltrate, which implies that inflammation may contribute to calcification of the valve.

Mice deficient in interleukin-1 receptor antagonist (IL-1Ra−/−) develop thickening and fibrosis of the aortic valve, with myofibroblast transdifferentiation. Transvalvular blood velocity increases, which (to our estimate) predicts a gradient of about 15 mm Hg. Thus, unopposed inflammation alone (without other risk factors) produces mild aortic valve dysfunction. Valve disease can be produced by transplantation of T cells (only) that are deficient in the IL-1 receptor antagonist, which indicates a powerful role for T cells in FCAVD. Tumor necrosis factor-α deficiency prevents FCAVD produced by IL-1 receptor deficiency.

Calcified aortic valves from humans contain many expanded T-cell clones, which are also expanded in circulating blood. The authors suggest that there is an ongoing systemic adaptive immune response in patients withicuspid and triicuspid FCAVD, which involves activation of circulating CD8 T cells and clonal expansion, with trafficking of T cells between blood and the aortic valve.

Atherosclerosis is an inflammatory disease, perhaps triggered by an autoimmune response to low-density lipoprotein. To suppress the inflammatory response, dendritic cells treated with apolipoprotein B100 and IL-10 were injected in hypercholesterolemic mice. Injection of tolerogenic dendritic cells reduced the autoimmune response and greatly decreased atherosclerosis.

Two trials, using methotrexate or an antibody against IL-1B, have been initiated to determine whether inhibition of inflammation prevents atherosclerotic disease. The rationale for targeting vascular inflammation as a means to reduce atherosclerosis is reasonable, and it is possible that a similar approach that targets inflammation would protect against the development of FCAVD. We speculate, however, that immunomodulatory therapies may (like reduction of lipids) be most effective when initiated before the development of overt FCAVD.

**Figure 5. Speculative and simplified diagram of disease pathways in cusps of the aortic valve.** Left. Valvular interstitial cells (VICs), when activated, transdifferentiate to myofibroblasts or osteoblasts, which produce collagen and calcification. Pericytes, near new blood vessels in the valve, also may transdifferentiate to myofibroblasts. Vascular endothelial growth factor-A (VEGF-A) may stimulate differentiation of myofibroblasts to osteoblasts. Right. When mature dendritic cells, primarily at the base of the valve, bind to naïve T cells, the T cells are activated. Interleukin (IL)-1, IL-17, interferon-γ, and other proinflammatory cytokines produce inflammation and may contribute to calcification. Tumor necrosis factor (TNF)-α, released by monocyte/macrophages and activated T cells, also plays a key role in inflammation and calcification in the valve (illustration credit: Ben Smith).
Monocyte/macrophages infiltrate the aortic valve and almost certainly play a key role in the development of FCAVD. In a series of studies, the Demer group demonstrated that several cytokines released by macrophages stimulate calcification of vascular muscle cells. This calcific mechanism presumably can be extrapolated to arteries in vivo, and to smooth muscle cells which are present at the base of the aortic valve. Imaging studies also link macrophages with osteogenesis in the development of aortic valve disease.

Macrophages can internalize basic calcium phosphate crystals in vitro and trigger release of high concentrations of several inflammatory cytokines, including tumor necrosis factor-α. Tumor necrosis factor-α promotes osteogenic differentiation and vascular calcification. The findings imply positive feedback, with calcification begetting inflammation and then calcification. With advanced calcification of tissues, however, inflammation may decrease.

**Vitamin D**

Deficiency of vitamin D₃ seems to be a risk factor for cardiovascular disease. Administration of vitamin D₃ decreases macrophages and CD4⁺ T cells in the aortic sinus, decreases dendritic cells and maturation, and attenuates development of atherosclerosis in apoE⁻/⁻ mice. However, administration of high doses of vitamin D produces aortic valve disease (but with minimal pressure gradient across the valve) in rabbits. Vitamin D also produces aortic sclerosis in mice with decreased signaling by a downstream effector of Notch1. Thus, effects on the aortic valve of vitamin D deficiency and therapeutic levels of vitamin D are not clear.

**Angiogenesis**

Normal heart valves are avascular. Inflammation of a heart valve, for example, from bacterial infection or autoimmune disease, is associated with neovascularization of valves. Hypercholesterolemia, which can be viewed as a systemic inflammatory disease, is associated with neovascularization of the aortic valve in apoE⁻/⁻ mice. In humans, neovascularization occurs in stenotic aortic valves, but not in valves without significant calcification. In humans, intraleafllet hemorrhage colocalizes with neovascularization and is associated with rapid decline of aortic valve function. In organ culture, abundant EC migration and tubule formation occur in explanted human stenotic aortic valves, but are minimal in cultured nonstenotic valves. The observation that normal articular cartilage is avascular led to isolation of chondromodulin-1, a powerful antian- giogenic protein that is also present in normal cornea. Deficiency of chondromodulin-1 produces aortic valve sclerosis and neovascularization in mice. Chondromodulin-1 is expressed in normal human aortic valves, but its expression is decreased in stenotic human valves.

Those findings suggest that neovascularization of the aortic valve is not only a marker for disease, but may also play a role in initiating or propagating the disease, perhaps in part by facilitating entry of cellular and humoral mediators of inflammation into valve interstitium. However, in valve tissue, as in other tissues, angiogenesis is associated with matrix remodeling and expression of growth factors, such as vascular endothelial growth factor, which may propagate FCAVD in ways that do not depend on formation of new blood vessels. In bone, vascular endothelial growth factor-A directly mediates osteoblast differentiation via intracrine Runx2 and PPARγ signaling. It is possible that similar processes occur in diseased aortic valves.

Although neovascularization of the aortic valve is associated with FCAVD, it is not clear whether angiogenesis contributes, or is secondary, to the disease. For example, thickening of the valve may exceed the diffusion distance of oxygen, and thereby stimulate angiogenesis. A similar mechanism has been described in the aorta during development of atherosclerosis.

Myofibroblasts are present in stenotic aortic valves from humans and play an important role in calcification of the valve. An intriguing observation is that pericytes are present near neovessels in stenotic valves (Figure 5). Pericytes may transform to myofibroblasts. We speculate that neovascularization of aortic valves may be accompanied by growth of pericytes, which transform to myofibroblasts, and thereby contribute to calcification and fibrosis of the valve. It has been known for many years that vascular pericytes (from retina) have osteogenic potential.

Thus, pro-FCAVD features of pericytes may provide a mechanistic link between neovascularization and progression of FCAVD. Confirmation of valve angiogenesis per se as a viable therapeutic target for FCAVD will require studies that isolate the process of neovascularization from the global inflammatory and procalcific milieu of FCAVD.

**Therapeutic Interventions for FCAVD**

FCAVD results from diverse complex-regulated processes, and it seems likely that specific targets may be identified for therapeutic interventions designed to attenuate FCAVD. Studies in FCAVD-prone mouse offer the ability to assess the efficacy of clinically relevant therapeutic interventions within a workable time frame, while controlling for important genetic and environmental covariables. A few interventions have shown promise in mice that are prone to develop severe aortic stenosis.

**Amelioration of the Underlying Cause of FCAVD**

Ldlr⁻/⁻ApoB⁰⁰/¹⁰₀ mice are hypercholesterolemic and are prone to develop severe aortic stenosis in old age. A high-fat diet accelerates the disease processes in Ldlr⁻/⁻ApoB⁰⁰/¹⁰₀ mice. Early in the course of FCAVD, reversal of hypercholesterolemia by means of a genetic switch normalizes aortic valve superoxide levels, decreases myofibroblast activation, reduces valcular calcium burden, suppresses pro-osteogenic signaling cascades, and prevents further impairment of aortic valve function, in Ldlr⁻/⁻ApoB⁰⁰/¹⁰₀/Mttp⁰⁰/Mx1Cre⁺/+ mice. When moderate aortic valve stenosis is present, reversal of hypercholesterolemia significantly reduces pro-osteogenic signaling and valve calcification, in Ldlr⁻/⁻ApoB⁰⁰/¹⁰₀/Mttp⁰⁰/Mx1Cre⁺/+ mice. Probiofic signaling in the aortic valve remains elevated, however, after reversal of hypercholesterolemia. The net effect of cholesterol lowering on aortic valve function is negligible in Reversa mice with advanced FCAVD, despite a 70% reduction in valve calcification. The findings...
recapitulate findings in studies of lipid lowering in patients with moderate or severe FCAV.

Two themes emerge from studies of cholesterol lowering in FCAV-prone mice. First, therapeutic efficacy during the early stages of disease does not necessarily predict efficacy for advanced disease. The findings suggest that processes that initiate FCAV may differ mechanistically from processes that propagate FCAV. Second, a potential therapeutic intervention may result in differential effects on individual FCAV processes, for example, calcification versus fibrosis, even when the therapy is targeted at the underlying cause of FCAV.

**Exercise**

Regular aerobic exercise reduces oxidant stress and improves endothelial function in patients with atherosclerosis, effects which could also be beneficial for patients with FCAV. In very young Ldlr−/− mice fed a high-cholesterol diet, regular exercise preserves endothelial integrity, reduces profibrotic and procalcific signaling, and protects against the development of aortic valve sclerosis. Beneficial effects of exercise occur, although plasma cholesterol levels are unaffected by exercise. When the exercise intervention is delayed until 5 months of age, however, after development of aortic valve sclerosis, regular exercise has no demonstrable effect on endothelial integrity, profibrotic signaling, procalcific signaling, or valve morphology in cholesterol-fed Ldlr−/− mice. The age dependence of the efficacy of exercise for attenuation of FCAV reinforces the concept that FCAV initiation can occur via mechanisms that are different from FCAV progression, and that efficacy of a therapeutic intervention may depend on the ambient phase of FCAV.

**Targeting a Pro-FCAV Gene Cluster**

PPARγ is an attractive therapeutic target because it affects a cluster of genes and modulates several mechanisms that may play an important role in the pathophysiology of FCAV. PPARγ protects against the development of atherosclerosis, inhibits differentiation of progenitor cells to osteoblasts, and is anti-inflammatory. We and others have observed that pioglitazone, a PPARγ ligand, attenuates the development of FCAV. Perhaps, a PPARγ would be useful in patients at high risk for FCAV, such as patients with a bicuspid aortic valve, in prevention of stenosis.

**Prospects for Clinical Translation**

A critical and challenging step will be to apply findings in mice to therapy in patients. One approach will be retrospective analysis of drugs (e.g., PPARγ ligands or receptor activator of nuclear factor-κB ligand inhibitors), used for other diseases to determine whether they slow the development or progression of FCAV. It is unlikely, however, that sensitive methods for evaluation of aortic valve function (echo, MRI) will be available in retrospective studies of treatments prescribed for other indications. Second, although long-term prospective studies will be most valuable, it is not likely that large, long-term studies will be feasible. Studies of a few years duration, as have been performed with statins, may be feasible. Third, imaging methods, including molecular imaging, which target inflammation, calcification, and other mechanisms will be valuable. They are surrogate end points, however, and do not provide functional end points.

A key question is whether there is positive feedback which, once established, can be interrupted to slow or reverse FCAV. A concern is that matrix stiffness can influence the propensity for VICs to produce calcification. It is encouraging that, in an experimental model with calcification, VICs in vitro can dedifferentiate away from myofibroblasts when matrix is less stiff. It is unknown, however, whether stiffness of the valve can be reduced, and what the net effect on valve calcification would be.

The future holds great promise for further elucidation of mechanisms by which FCAV develops and progresses, and for the development of effective medical therapy for FCAV. Studies in mice will be critical to assess the functional impact of systemic and local cellular and molecular processes, and effects of therapeutic interventions, on aortic valve function. Translation of findings from in vitro studies, and from studies in mice, to effective therapies in humans will be challenging, but necessary.

**Summary**

Studies of mouse models of FCAV in vivo provide an opportunity to integrate complex mechanical, cellular, and molecular processes that preserve or disrupt valve homeostasis. FCAV begins and progresses in a setting of complex interactions between mechanical forces and an ever-changing host tissue milieu. Some mouse models of FCAV recapitulate important features of clinical FCAV, including overt syndromic calcific aortic stenosis, but there are important differences relating to anatomic scale. Evaluation of aortic valve function in vivo allows examination of cellular and molecular processes and their physiological consequences—hemodynamically significant valve stenosis and regurgitation.

ECs and VICs are the predominant tissue in normal and minimally diseased valves. During evolution of FCAV, immune cells exert injury-response influences and also regulate the interaction between VICs and ECs. Some genes regulate valvulogenesis and also regulate postnatal valve homeostasis. Genetic control of processes in the aortic valve is itself regulated by epigenetic, posttranscriptional, and posttranslational processes.

The rapidly expanding body of knowledge on regulation of valve homeostasis holds great promise for identifying viable therapeutic targets for clinical application. A few therapeutic interventions have shown promise in murine models of FCAV. Murine models of FCAV will continue to provide new basic and translational insights in this clinically important disease.

**Acknowledgments**

We thank Dr Z. Ballas for advice about mechanisms of inflammation, H. Wang for advice about studies of valvular interstitial cell in vitro, and T. Ruggle for assistance with preparation of figures. We wish to acknowledge E. Oehler, K. Zimmerman, and M. Davis, whose technical expertise was critical for acquisition, analysis, and presentation of echocardiographic data.
Sources of Funding

Original studies by the authors were supported by grants from the National Institutes of Health (HL62984, RR026293, HL08610, HL092235, and HL111121).

Disclosures

R.M. Weiss and D.D. Heistad have received research funding from Amgen Corp. J. D. Miller reports no conflicts.

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doi: 10.1161/CIRCRESAHA.113.300153

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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