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Calcific Aortic Valve Disease: Not Simply a Degenerative Process A Review and Agenda for Research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group

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INTRODUCTION

Calcific aortic valve disease (CAVD) encompasses the range of disease from initial alterations in the cell biology of the leaflets to end-stage calcification resulting in left ventricular outflow obstruction. The first detectable macroscopic changes in the leaflets, seen as calcification, or focal leaflet thickening with normal valve function, is termed aortic valve sclerosis, but it is likely that the initiating events in the disease process occur much earlier. Disease progression is characterized by a process of thickening of the valve leaflets and the formation of calcium nodules – often including the formation of actual bone – and new blood vessels, which are concentrated near the aortic surface. End stage disease, e.g. calcific aortic stenosis, is characterized pathologically by large nodular calcific masses within the aortic cusps that protrude through the outflow surfaces into the sinuses of Valsalva, interfering with opening of the cusps. For decades, this disease was thought to be a passive process in which the valve degenerates with age in association with calcium accumulation. Moreover, although calcific aortic valve disease is more common with age, it is not an inevitable consequence of aging. Instead, CAVD appears to be an actively regulated disease process that cannot be characterized exclusively as “senile” or “degenerative.”

The NHLBI convened a group of scientists from different fields of study, including cardiac imaging, molecular biology, cardiovascular pathology, epidemiology, cell biology, endocrinology, bioengineering, and clinical outcomes, to review the scientific studies from the past decade in the field of CAVD. The purpose was to develop a consensus statement on the current state of translational research related to CAVD. Herein, we summarize recent scientific studies and define future directions for research to diagnose, treat and potentially prevent this complex disease process.

NORMAL AORTIC VALVE ANATOMY AND FUNCTION

Key Structure-Function Correlations

Heart valves permit unobstructed, unidirectional forward flow through the circulation. Valve components must accomplish the second-to-second movements necessitated by the cardiac cycle and must maintain sufficient strength and durability to withstand repetitive and substantial mechanical stress and strain over many years. The functional requirements of the heart valves are accomplished by a specialized set of cells and heterogeneous extracellular matrix, arrayed in a spatially-specific and differentiated tissue structure which are temporally dynamic and highly responsive to the external biomechanical environment[1].

The aortic valve (AV) provides a paradigm for valvular structural specialization and tissue dynamics as viewed by echocardiography and bioreactor models (Figure 1, **Panel A**). The direction of flow during systole is allowing the valve cusps to open as the blood flows across the open aortic valve leaflets. The inflow surface is the located along the direction of flow as indicated in Figure 1, **Panel A.** The outflow surface is demonstrated in the diastole figure as the valves are closed and there is end diastolic pressure closing the valve leaflets along the outflow surface. Individual AV cusps attach to the aortic wall in a semilunar fashion, ascending to the commissures, and descending to the basal attachment of each cusp. In the closed phase, under the backpressure from the blood in the aorta, the AV cusps stretch and coapt and, thereby, occlude the orifice. Pulmonary valve structure is analogous to, that of the AV, consistent with the lower pressure environment. During diastole, the tissue of the cusps

is stretched via a backpressure; during systole, the cuspal tissue becomes relaxed and shortens owing to recoil of elastin, which was elongated and taut during diastole.

All four cardiac valves have a similar layered architectural pattern composed of cells, including the valvular endothelial cells (VECs) at the blood-contacting surfaces and the deep valvular interstitial cell (VICs), and valvular extracellular matrix (VECM), including collagen, elastin and amorphous ECM (predominantly glycosaminoglycans [GAGs]). The AV has a dense collagenous layer close to the outflow surface, and continuous with valvular supporting structures, which provides strength: the *fibrosa*; a central core of loose connective tissue: the *spongiosa* rich in glycosaminoglycans (GAGs); and a layer rich in elastin below the inflow surface: the *ventricularis* as shown in (Figure 1, **Panel B**). The GAGrich spongiosa facilitates the relative rearrangements of the collagenous and elastic layers during the cardiac cycle. Moreover, the diverse characteristics of the cell phenotype, such as smooth muscle alpha-actin, are associated with distinct locations within the valve leaflet in situ[2]. *In vitro*, this heterogeneity of phenotype is consistently demonstrated in primary cultures of VICs. Only recently, however, has it become possible to identify and characterize the behavior of discrete subpopulations of valvular cells using methods such as cloning[3], and subculturing based on differential adhesion[4]. These approaches have been used to demonstrate that discrete valvular cell subpopulations have unique morphological characteristics, synthesis of ECM, potential for calcification and ossification, and potential for promoting angiogenesis[5]. These latter two characteristics are particularly relevant to calcific valve disease, and hence these methods offer the potential for determining whether selected groups of cells within the entire population undergo specific pathologic changes that drive valve remodeling and mediate the progression of disease, which is the calcified valve leaflet as depicted in (Figure 1, **Panel C)**.

Cardiac Valve Cell Types: Valvular Interstitial Cells

VICs are abundant in all layers of the heart valves and are crucial to function. VICs synthesize VECM and express matrix degrading enzymes (including matrix metalloproteinases [MMPs] and their inhibitors [TIMPs]) that mediate and regulate remodeling of collagen and other matrix components. VICs comprise a diverse, dynamic, and highly plastic population of resident cells[6]. They modulate function among phenotypes in response to changes in stimulation by the mechanical environment or by certain chemicals, during valvular homeostasis, adaptation, and pathology. Adult heart valve VICs in-situ have characteristics of resting fibroblasts; they are quiescent, without synthetic or destructive activity for ECM. VICs are activated during intrauterine valvular maturation, by abrupt changes in the mechanical stress state of valves, and in disease states, and VICs continuously repair a low level of injury to the VECM that occurs during physiological functional remodeling of AV tissue[7]. Table 1[6] demonstrates the phenotypic transitions of the VIC cells, which are critical for normal development, homeostasis, and function of the aortic valve, and likely mediate the development of valve calcification. Once activated, VICs can differentiate into a variety of other cell types[3], including myofibroblasts and osteoblasts, although valve osteoblasts may respond to cellular signals differently than skeletal osteoblasts.

Valvular Endothelial Cells

VECs resemble endothelial cells elsewhere in the circulation in some respects. However, they are phenotypically different from vascular endothelial cells in the adjacent aorta and elsewhere in the circulation[8]. VECs likely interact with VICs to maintain the integrity of valve tissues and potentially mediate disease. Evidence indicates that different transcriptional profiles are expressed by VECs on the opposite (i.e., aortic and ventricular) faces of a normal adult pig aortic valve, and some investigators have hypothesized that these

differences may contribute to the typical localization of early pathologic aortic valve calcification predominantly near the outflow surface secondary to inhibitors along the inflow surface[9]. Studies indicate that abnormal hemodynamic forces (such as hypertension[10], elevated stretch[11], or shear stresses[11]) experienced by the valve leaflets can cause tissue remodeling and inflammation, which may lead to calcification, stenosis, and ultimate valve failure.

Normal Cardiac Valve Development

VIC and VEC phenotypes, critical for maintaining valve function, change throughout life in response to environmental stimuli as demonstrated in recent studies using quantitative histological assessment of human semilunar valves obtained from fetuses, neonates, children, and adults[7,12]. VECs express an activated phenotype throughout fetal development (e.g., VCAM-1, ICAM-1). Numerous signaling pathways have been proposed and tested in the critical pathways that promote endothelial-mesenchymal transition in the valves[12]. In addition, VIC density, proliferation, and apoptosis are significantly higher in fetal than adult valves. A tri-laminar architecture appears by 36 weeks of gestation, but remains rudimentary compared with that of adult valves. These data of the natural history of cell and matrix changes in valve development, extend the paradigm that cardiac valves can adapt to pathological conditions which suggests similar molecular mechanisms in physiological and pathological cell activation.

PATHOBIOLOGY OF CALCIFIC AORTIC VALVE DISEASE

Calcific aortic valve stenosis has characteristic pathological features[13]. The calcific process begins deep in the valvular tissue, near the margins of attachment. In advanced disease, the nodules extend through the outflow surfaces of the cusps and are nearly transmural. An early morphological stage of the calcification process is called aortic valve sclerosis. In the later stage, aortic valve stenosis, the functional valve area is decreased sufficiently to cause measurable obstruction to outflow and a significant gradient from left ventricle to aorta.

Lipids also play an important initiating role in the cell signaling of vascular and valvular calcification[14]. Surgical pathological studies have shown the presence of oxidized LDL in calcified valves[15, 16]. Patients with homozygous familial hypercholesterolemia (FH) provide an opportunity to test the hypothesis that lipids play a role in the development of calcific aortic stenosis because these patients have extremely elevated levels of low density lipoprotein cholesterol (LDL-c) without other traditional risk factors for coronary artery disease[17–20].

Renin-Angiotensin Signaling Pathway

Angiotensin converting enzyme (ACE) is expressed and colocalizes with LDL in calcified aortic valves[21]. In addition, an observational study showed slowing of progression of aortic valve disease in patients taking angiotensin converting enzyme inhibitors compared to those not on this therapy[22]. This first study is the first to demonstrate this novel signaling pathway in CAVD which is still preliminary and somewhat controversial, but promising for the future potential to target this pathway with ACE inhibitors and angiotensin receptor blockade in early stages of the disease.

Initiating Events: Oxidative Stress

In the presence of cardiovascular risk factors, similar to vascular atherosclerosis, an early event is abnormalities in oxidative stress. This has been demonstrated in abnormal endothelial nitric oxide synthase function, which decreases normal physiologic levels of nitric oxide along the valve endothelium[23, 24]. In atherosclerotic plaques, increased

oxidative stress seems to be due primarily to increases in NAD(P)H oxidase activity[25]. In calcified stenotic human[26] and murine aortic valves[27], levels of superoxide and hydrogen peroxide are markedly increased. In addition, uncoupling of nitric oxide synthase[23] may play an important role in generation of superoxide in calcified aortic valves.

Calcifying Phenotype: Myofibroblast Osteoblastogenesis

The initial confirmation of pathologic bone in the aortic valve was demonstrated by bone histomorphometry[28] and osteogenic gene expression[13] in diseased human valves. The likely sources of the myofibroblasts and osteoblasts that appear and persist in CAVD include native VICs, which contain mesenchymal progenitor-like cells that are highly plastic[3], and small numbers of circulating progenitors[29] and mesenchymal cells that transition from endothelial cells[30]. Possible triggers for VIC pathologic differentiation or dysfunction include abnormal biomechanical forces (such as hypertension[10], elevated stretch[11], altered shear stresses[11] or altered VECM stiffness[31]), reactive oxygen species, inflammatory cytokines[32–35] and growth factors [36], and the cellular environment caused by other disease states. Delivery of antagonists to pathologic stimuli or otherwise inhibiting VIC proliferation or apoptosis can significantly reduce calcification. *In vitro* administration of an ERK pathway inhibitor significantly decreased VIC proliferation, apoptosis, and nodule formation, enabling these cells to retain a quiescent phenotype[37]. Meanwhile, delivery of HMG-CoA reductase inhibitors (“statins”) in both in vitro and *in vivo* animal models decreased VIC proliferation, apoptosis, and calcification [38–41]. Statins represent a particularly intriguing avenue to pursue in regulating VIC function, as these drugs have demonstrated clinical but controversial slowing of the progression of CAVD[42].

Calcification-Bone Formation

Calcification is largely responsible for hemodynamic progression of aortic valve stenosis. Recent descriptive studies from patient specimens have demonstrated the cell changes associated with aortic valve calcification, including osteoblast expression, cell proliferation, and atherosclerosis[13, 28, 43, 44]. Furthermore, these studies have also shown that specific bone cell phenotypes present in calcifying valve tissue from human specimens[45, 46,47–49] demonstrate the potential for vascular cells to differentiate into calcifying phenotypes.

Recent observations in *ex vivo* human tissue suggest that rapid advancement in our understanding of the basic mechanisms involved in the initiation and progression of vascular and valvular calcification is now possible. If an osteoblast phenotype is present, then the factors important in the regulation of bone development and regeneration must be considered in the understanding of calcification of the aortic valve. It is well known that cardiovascular calcification is composed of hydroxyapatite deposited on a bone-like matrix of collagen, osteopontin (OP), and other minor bone matrix proteins[28, 50, 51], and regulation occurs via activation of specific transcription factors including MSX2[52], Runx2[45], and Sox9[45]. Calcified aortic valves removed from surgical valve replacement show bone formation (osseous metaplasia)[13, 28, 45]. Further characterization of this phenotype has proven, in calcified bicuspid aortic valves, that immunohistochemistry staining shows the expression of osteopontin[50]. In addition, osteopontin expression has been demonstrated in the mineralization zones of heavily calcified aortic valves obtained at autopsy and surgery[13, 28, 43, 45].

IN VIVO MODELS OF CALCIFIC AORTIC VALVE DISEASE

Studies in the field of vascular calcification have set the stage for the experimental studies in valvular heart disease. Elevated LDL and its oxidative modification represent one of the major factors of CAVD. Therefore, addressing the mechanisms of CAVD in

hypercholesterolemic animal models is a reasonable and essential approach. Development of CAVD has been shown in both apoE and LDL receptor deficient mice[27, 29, 53]. Aortic valves in hypercholesterolemic mice and rabbits[23, 41, 44], characterized by thickened leaflets with macrophage-rich subendothelial lesions in early stages and formation of calcific deposits on the aortic site of the valve in late stages, reproduce key pathologic features found in human valve disease. In addition, clinicopathological studies of stenotic aortic valves in humans identified lesions similar to those in inflamed atherosclerotic plaques[15, 16]. Cholesterol lowering in such models improves various features associated with atherogenesis and aortic valve disease[23, 41, 4454]. These animal models are important and need to be characterized further in regards to CAVD. However, these models also have limitations in that no one model recapitulates the human disease process completely, but each published model to date provides information provides incremental mechanistic insight into the human disease process. The evidence which compares the osteogenic process in the valve to the bone is the most compelling to dissect the molecular mechanism and to demonstrate the foundation for both of these cellular processes and the potential for medical therapy[49, 55–57]. Figure 2 demonstrates a current working model of the signaling events published in the field of calcific aortic valve disease.

CLINICAL STUDIES

Prevalence, Genetics and Cardiovascular Risk Factors

The presence or progression of CAVD has been associated with several clinical, genetic, and anatomic factors. Bicuspid aortic valve disease is the most common congenital heart abnormality. A congenitally bicuspid aortic valve is present in over 50% of adults undergoing valve replacement for severe CAVD and nearly all patients with a bicuspid valve will eventually need valve surgery, either for regurgitation in young adulthood or for stenosis in the 5th or 6th decade of life. Bicuspid valve disease appears to be inherited in an autosomal dominant pattern in some families, and a mutation in the NOTCH1 gene segregates with both bicuspid valve anatomy and premature valve calcification[58]. Calcification of trileaflet aortic valves also may be affected by genetic factors based on population studies and case control comparisons for specific polymorphisms, including the Vitamin D receptor[59], estrogen receptor[60], apolipoprotein E4[61] and interleukin 10 alleles[62]. Mild CAVD, called aortic sclerosis is present in about 25% of adults over 65 years of age and is associated with adverse cardiovascular outcomes with about a 50% increased risk of cardiovascular events over 5 years[63].

Similar to the cardiovascular risk factors defined by the Framingham study for vascular atherosclerosis, clinical factors associated with the presence of CAVD in the Cardiovascular Health Study included older age, male gender, serum Lp(a) and LDL levels, height, hypertension, metabolic syndrome and smoking as shown in Figure 3[63–79]. The association with elevated low-density lipoprotein (LDL) is relatively weak in those over 65 years old, the group at greatest risk of progressing to aortic stenosis.

Echocardiographic Imaging for CAVD

Due to its ability to detect and quantify valve-related hemodynamic obstruction, echocardiography long has been recognized as a useful clinical tool for monitoring aortic stenosis (AS), the later, obstructive stage of CAVD[80]. Echocardiography can reliably visualize aortic valve anatomy, although once severe calcification is present, distinguishing a bicuspid from a trileaflet valve can be difficult. Echocardiographic measures of AS severity have been well validated in numerous studies and now are the clinical standard for patient management. Guidelines recommend measurement of aortic jet velocity, mean pressure gradient, and continuity equation valve area. Although clinically robust, these

measures are subject to several sources of error, including physiologic changes, recording technique, and measurement variability[81]. In addition, there is marked variability between patients in the rate of hemodynamic progression and the degree of stenosis that results in clinical symptoms. Aortic sclerosis is defined on echocardiography as focal areas of leaflet thickening without significant obstruction to LV outflow, with an aortic velocity <2.6 m/s[82]. Echocardiographic measures of aortic jet velocity and leaflet calcification have been shown to be robust predictors of clinical outcome[82, 83]. In the design of clinical trials, we will need to consider the effects of variability of echocardiographic data on sample size calculations and defining the imaging standards and protocols for the use of this tool to quantify non-invasively the level of disease in the patients.

Computed Tomography in Quantifying Calcification in CAVD

The early stage of CAVD, aortic sclerosis, is characterized by aortic valve calcium (AVC) accumulation but not hemodynamic obstruction. Because echocardiography does not have the resolution for quantifying AVC, it is less useful for monitoring early-stage CAVD. In contrast, computed tomography (CT) is a relatively sensitive and precise tool for quantifying AVC[30,98]. Thus CT has emerged as a useful tool for studying aortic sclerosis, complementing the utility of echocardiography in studying AS[84]. Moreover, because the aortic valve leaflets lie in the same anatomic plane as the coronary arteries, AVC can be quantified by any CT scan obtained for the purpose of quantifying coronary artery calcium (CAC). Taking advantage of these issues, investigators have used CT to study traditional and novel risk-associations for AVC in the Multiethnic Study of Atherosclerosis (MESA), a 6,780 participant study of risk factors for subclinical coronary artery disease. In MESA, the metabolic syndrome (MetS) is a strong risk factor for prevalent[85] and early stage disease. Thus, MetS appears to be an adverse risk factor in all stages of CAVD.

Clinical Trials: HMG CoA-Reductase Pathway

The first randomized prospective study testing the effects of HMG CoA reductase inhibitors in aortic valve disease was published in 2005[86]. In this double-blind, placebo-controlled trial, patients with calcific aortic stenosis were randomly assigned to receive either 80 mg of atorvastatin daily or a matched placebo. Aortic-valve stenosis and calcification were assessed with the use of Doppler echocardiography and helical computed tomography, respectively. The SALTIRE investigators demonstrated a trend in slowing the progression of the aortic valve stenosis but not a statistically significant study for primary end-points. The SALTIRE investigators concluded that intensive lipid-lowering therapy does not halt the progression of calcific aortic stenosis or induce its regression[86], and the reason for this negative trial might be the timing of therapy[87]

In the RAAVE trial[88], performed a prospective trial of AS with Rosuvastatin targeting serum LDL, slowed progression of echo hemodynamic measurements, a providing the first clinical evidence for targeted therapy using an HMG CoA reducatase inhibitors in patients with asymptomatic moderate AS[88] in a hypothesis driven open label study. These results are small, controversial but test the lipid hypothesis. The next clinical trial, SEAS[89], examined intensive lipid lowering with Simvastatin and Ezetimibe in Aortic Stenosis. This trial was a randomized, double-blind trial involving 1873 patients with mild-to-moderate, asymptomatic aortic stenosis. Again, the investigators concluded that the medication did not reduce the composite outcome of combined aortic-valve events in patient with aortic stenosis including echo progression and vascular end-points. Finally, the most recent trial, Astronomer, randomized patients to Rosuvastatin versus placebo in patients with moderate aortic valve disease and bicuspid aortic valve disease. This study also did not demonstrate slowing of progression of this disease[90]. These four clinical trials have different results, which may be due a number of reasons including differences in trial designs, differences in

enrollment criteria, differences in statin medication, or timing of therapy[91]. Although, the three randomized trial did not demonstrate slowing of progression of aortic stenosis, the largest trial SEAS, did demonstrate improvement in primary end-points of ischemic vascular disease. The future of clinical valve trials may need further analysis of the trial design, the type of medications and the duration of the trials, but for now there is no primary indication for statin therapy in patients with valvular heart disease to slow progression of this disease. However, treatment of all cardiovascular patients with risk factors remains appropriate according to the guidelines as described by the American Heart Association and American College of Cardiology.

RECOMMENDATIONS

Based on this review of the current state of knowledge as summarized in this paper, the Working Group made the following recommendations for future research on CAVD. Recommendations are presented as being of equal weight, not in priority order.

1. Identify genetic, anatomic, and clinical risk factors for the distinct phases of initiation and progression of CAVD, to identify individuals at higher risk, to determine interactions between risk factors, and to determine whether the severity of aortic stenosis is a risk factor for surgical aortic valve replacement. These factors should encompass the unique contributions of atherosclerosis, metabolic syndrome, hypercholesterolemia, type II diabetes, and chronic kidney disease. New, larger epidemiological studies and existing epidemiological datasets in which CT scans, echocardiograms, or possibly magnetic resonance imaging scans have been obtained, could be used in this effort.
2. Develop high-resolution and high-sensitivity imaging modalities that can identify early and subclinical CAVD, including molecular imaging and other innovative imaging approaches. Continue research to define the state-of-the-art for detecting early calcification not identified by traditional echocardiographic imaging.
3. Understand the pathogenesis and pathophysiology of bicuspid aortic valve, especially to establish correlations between phenotype and genotype, and to clarify the key features of this disease process that potentiate calcification.
4. Understand the basic valve biology (e.g., early events, mechanisms and regulatory effects) of CAVD, including signaling pathways and the roles of valve interstitial and endothelial cells and the autocrine and paracrine signaling between them, the extracellular matrix and matrix stiffness, the role of age-related changes in both valve cells and extracellular matrix, the interacting mechanisms of cardiovascular calcification and physiologic bone mineralization, and micro-scale mechanotransduction and macro-scale hemodynamics.
5. Develop and validate suitable multi-scale in vitro, ex vivo, and animal models. Improved models are needed that realistically duplicate the conditions in which human CAVD develops. Metabolic studies are needed, from the cellular level through the patient level, to define those conditions.
6. Identify the relationship between calcification of the aortic valve and bone and the reciprocal regulation of these processes.
7. Encourage, promote, or establish tissue banks that make valve tissue from surgery, pathology, and autopsy unsuitable or unneeded for transplantation – with and without CAVD – available for research. Human valve cell lines should be derived including immortalized VICs.

8. Conduct clinical studies specific to CAVD to determine the feasibility of earlier pharmacological intervention in aortic valve sclerosis versus stenosis. Determine the correct design of the clinical trials to test the hypothesis whether it include measurement of calcification and further understanding of the biology of the aortic valve as measured by the continuity equation by Doppler Echocardiography
9. Determine the risk factors and optimal timing of surgical valve replacement in view of the current state of the data defining the biological mechanisms of CAVD.

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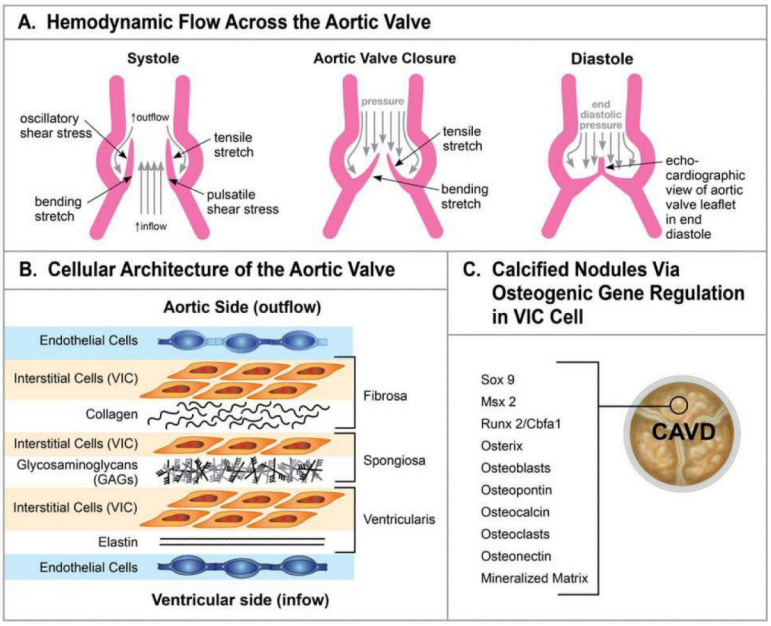

Figure 1.

Echocardiographic and Bioengineering and Hemodynamic Force Perspective of the diastole and systole in the aortic root affecting aortic valve leaflet cell and function **Panel A. Panel B,** demonstrates the cellular architecture of a normal aortic valve. **Panel C,** demonstrates the osteogenic phenotype of the calcified aortic valve.

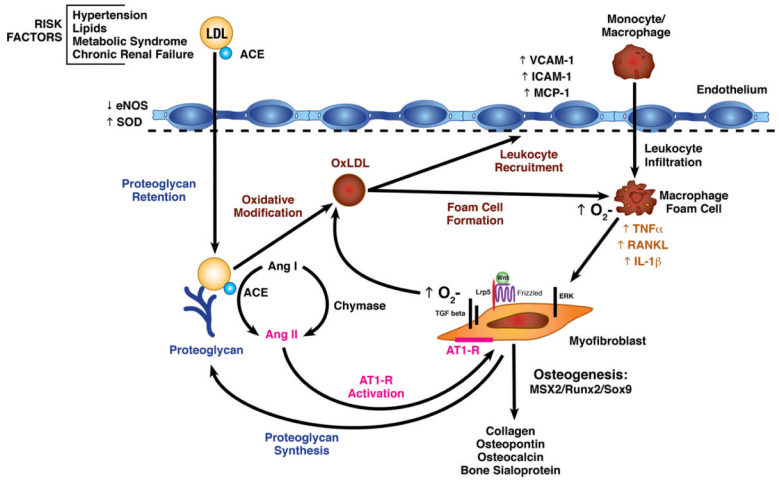

Figure 2.

Potential roles of Lipoprotein retention and signaling, oxidative stress, and reninangiotensin system activation in the pathogenesis of calcific aortic valve disease.

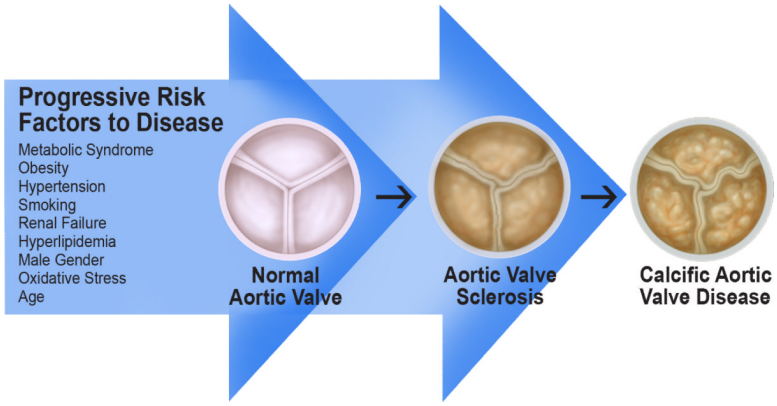

Figure 3.

The identified atherosclerotic cardiovascular risk factors involved in the development of aortic valve disease from sclerosis to calcific aortic valve disease

Table 1

In vitro Valvular interstitial cell phenotypes[6](Used with permission)

Cell type	Location	Function
Embryonic progenitor endothelial/ mesenchymal cells	Embryonic cardiac cushions	Give rise to resident qVICs, possibly through an activated stage. EMT can be detected by the loss of endothelial and the gain of mesenchymal markers
qVICs	Heart valve leaflet	Maintain physiologic valve structure and function and inhibit angiogenesis in the leaflets
pVICs	Bone marrow, circulation, and/or heart valve leaflet	Enter valve or are resident in valve to provide aVICs to repair the heart valve, may be CD34-, CD133-, and/or S100-positive
aVICs	Heart valve leaflet	α-SMA-containing VICs with activated cellular repair processes including proliferation, migration, and matrix remodeling. Respond to valve injury attributable to pathological conditions and abnormal hemodynamic/mechanical forces
obVICs	Heart valve leaflet	Calcification, chondrogenesis, and osteogenesis in the heart valve. Secrete alkaline phosphatase, osteocalcin, osteopontin, bone sialoprotein