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Inflammatory and Biomechanical Drivers of Endothelial-Interstitial Interactions in Calcific Aortic Valve Disease

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Abstract

Calcific aortic valve disease (CAVD) is dramatically increasing in global burden, yet no therapy exists outside of prosthetic replacement. The increasing proportion of younger and more active patients mandates alternative therapies. Studies suggest a window of opportunity for biologically based diagnostics and therapeutics to alleviate or delay CAVD progression. Advancement, however, has been hampered by limited understanding of the complex mechanisms driving CAVD initiation and progression towards clinically relevant interventions.

CAVD is a complex, multifaceted disease involving widespread inflammation and transdifferentiation of resident valvular cells in a mechanically active environment. Many early in-vitro studies on CAVD focused on 2D monocultures of valvular interstitial cells (VIC) and valvular endothelial cells (VEC) cells. However, these cells do not act in isolation but rather in concert with each other and inflammatory cells in the valve, notably in the case of NFκB signaling.

New experimental technologies that interrogate multiple valvular cells in 3D have yielded important insights into how they communicate with each other and their environment. Studies have demonstrated that VEC and VIC communicate using nitric oxide and cytokine signaling, and there is a large opportunity for discovery of additional communication mechanisms. Though it is known that inflammatory cells are present in diseased valves, they embody a protective and pathogenic role in valve disease through mechanisms that have yet to be elucidated. Mechanically-active experimental systems have demonstrated that VIC and VEC respond to altered mechanical stimuli with disease-like properties. This review synergizes understanding of these critical areas of research underpinning promise for the development of valve specific molecular diagnostics and biologically based therapeutics.

Subject Terms:

Cardiovascular Disease; Inflammation; Treatment; Vascular Disease

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Keywords

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I.   The aortic valve: Built to Last

Aortic valve structure and mechanical function in homeostasis

The human heart is responsible for delivering blood containing oxygen, nutrients, chemical messengers, cells, and much more to the organs of the body. It achieves this by pumping 3–5 liters of blood every ~60 seconds[1]. Thus, it must be strong, coordinated, and durable enough to last 80+ years over a human lifetime. Blood flows through the heart unidirectionally, such that all oxygenated blood is efficiently pumped to the body and does not backflow into the heart. In order to accomplish this feat, the body has developed four one-way valves that separate the chambers of the heart from each other, the pulmonary artery, and aorta. The aortic valve is the valve that separates the aorta from the left ventricle of the heart. This cardiac chamber is responsible for pumping oxygenated blood out of the heart to the rest of the body. Thus, its function is especially crucial to sustain life in humans (Figure 1a).

The unique structure of the aortic valve allows it to provide minimal resistance to blood flow in systole, when the ventricle pumps blood, and a leak-tight seal during diastole, when the ventricle relaxes. It has a tri-leaflet structure, and each leaflet is concave to the aorta side. When aortic pressure is equal to ventricular pressure, the valve begins to open[2]. As the ventricle pumps, blood flow forces the leaflets to fold back towards the walls of the aorta[2]. This results in a large opening to the aorta for blood flow. Vortexes that form behind each leaflet assist in valvular closure towards the end of systole[1].Once closed, during diastole, blood cannot flow back through the aorta to the left ventricular side, preventing mixing of the blood (Figure 1a, 1b).

In calcific aortic valve disease (CAVD), the aortic valve undergoes pathological matrix remodeling that thickens the valve and eventually exhibits calcified lesions that reduce its ability to open and close over the cardiac cycle (Figure 1c). As CAVD progresses, stiffening of the valve due to remodeling in addition to increased pressure/strain due to reduced valve mobility result in altered valvular mechanical stimuli. These mechanical stimuli include shear stress, cyclic strain, and stiffness. This hindered ability of the valve to open and close over the cardiac cycle also increases left ventricular pressure[3]. To compensate, the left ventricle increases muscle mass over time, called left ventricular hypertrophy (LVH), which can ultimately lead to heart failure[3,4]. Through CAVD was initially thought to be a degenerative process, seminal work in the field has discovered that the progression of CAVD is not merely a pathological occurrence of degredation but a mechanobiological manifesatation controlled by key cellular regulators of osteogenesis and inflammatory factors[5–8]

Failure of the Aortic Valve: CAVD Prevalence, Risk Factors, and Burden

12.6 million cases of CAVD have been reported globally in 2017, and an estimated 102,700 of these cases resulted in death[9]. There are no current therapeutic treatments for CAVD, and all patients seeking treatment must get surgical valve replacement. It is predicted that CAVD patients will undergo 80,000 surgical procedures related to valve disease per year by 2050[10].

Current surgical treatments include transcatheter aortic valve replacement (TAVR) or surgical aortic valve replacement (SAVR)[11]

In industrialized countries, the mean age of patients with heart valve related diseases is 55, compared to 20–25 for patients in low-and-middle-income countries[12]. In these countries, diseases such as Rheumatic Fever (RF) lead to aortic valve complications[13]. However, neither of the two available surgical methods are accessible in low-and-middle-income countries, especially those that lack a cardiac surgeon. In industrialized countries with more accessible healthcare, other limitations to surgical treatment include immunogenic responses and tissue rejection. Thus, cost effective and accessible alternatives to current treatments for aortic valve disease must be developed to meet the ever-increasing demands of the growing population and demographic discrepancies.

There are many risk factors for calcific aortic valve disease, including genetic mutation, smoking, hypertension/vascular stiffening, hyperlipidemia, diabetes, infection, sex, presence of kidney disease, and age, recently reviewed by Chen et al[14–16]. How these risk factors contribute to the disease is in many cases inferred by atherosclerotic literature and due to aberrant paracrine and autocrine nitric oxide signaling, inflammatory adhesion molecules, and production of pro-inflammatory destructive reactive oxygen species. These are summarized in Table 1.

CAVD Diagnosis and Prognosis

It has been known for many years that survival is poor after valve disease symptoms appear, yet logically patients often do not seek medical evaluation for AS until they experience symptoms[17]. Ross and Brunwald showed in their seminal graph of aortic disease timecourse that after onset of symptoms, survival declines rapidly due to angina, syncope and heart failure[18,19]. The absence of sensitive and specific diagnostic tools for early-stage AS prior to irreversible cardiac damage poses a challenge for the development of effective therapeutics, as many therapeutics may only be effective if administered at specific stages of disease. Thus, there is a need and great opportunity for the development of targeted-CAVD molecular diagnostics based on pathways specific to CAVD initiation and progression. Studies have previously been conducted to evaluate biomarkers for CAVD, reviewed by Beckmann et al. and more recently Small et al., and this remains an area of ongoing research[20,21]. Looking forward, novel bioinformatics technologies such as the use of cell-free DNA and proteomics to identify circulating factors associated with CAVD could provide a disease-specific method to identify CAVD in early stages, as evidenced in promising work published by Kossar et al.[22].

In addition to understanding pathways specific to CAVD initiation and progression, it is important to consider how these pathways diverge from the homeostatic valve for the

development of diagnostics and therapeutics. Features of the aortic valve in homeostasis are discussed in the following sections.

Aortic Valve Mechanics in Homeostasis

To facilitate folding and unfolding of the leaflet structure, the aortic valve leaflet has specialized extracellular matrix (ECM) structures on the fibrosa, or aortic side, of the valve and the ventricularis, or ventricle side, of the valve (Figure 2). The fibrosa side has collagen is oriented parallel to the bending axis (circumferential). It has a corrugated appearance when the leaflet folds, and a smooth appearance in diastole to support backpressure from the blood[1,41]., On the flip side, the ventricularis ECM consists mostly of disorganized collagen and elastin that is oriented radially along the leaflet[41]. Separated between these two layers is the spongiosa, which consists mostly of glycosaminoglycans (GAGs) to absorb shock in compression and provide lubrication from shear between the fibrosa and ventricularis[42]. Resident cells in the valve, called valvular interstitial cells, repair and maintain these ECM structures over the lifetime of the leaflet. Lining the valve are endothelial cells, which provide protection to the valve and can sense mechanics/hemodynamics on the leaflet. The mechanical properties of the valvular ECM structure is crucial to its function, as the valve must bend and extend approximately 3 billion times over its lifetime[43]. The rest of this section will focus on the mechanical forces experienced by the valve.

Shear stress: As the aortic valve opens and closes over a cardiac cycle, friction from the blood induces a shear stress on the fibrosa and ventricularis[44]. Yap et al. have conducted a series of in-vitro experiments using explanted porcine valves in a heart simulator to directly measure the shear stress experienced by the homeostatic valve. They found that the aortic leaflet approaches approximately 20dyn/cm^2 maximum shear stress and that the ventricularis shear stress peaks at $64\text{–}91 \text{dyn/cm}^2$. On the aortic side of the leaflet, they found that reducing stroke volume tended to reduce peak shear stress at the same heart rate, and that increasing heart rate also decreased peak shear stress[45,46]. Lower stroke volume could be attributed to a stenotic pathogenic state, but few studies have experimentally or computationally investigated how the shear profile on the valve is altered in CAVD. Additionally, the ventricularis side of the leaflet is exposed to a laminar shear stress, while the fibrosa side experiences an oscillatory one[47].Oscillatory shear stress as well as decreased magnitude of shear stress has been shown to promote atherosclerotic plaque formation in vessels, and this paradigm is also assumed to be the case in CAVD, as calcification typically presents on the fibrosa side of the valve[48,49].

One of the most well-known ways that valvular endothelial cells communicate to interstitial cells during homeostasis in response to shear is through nitric oxide (NO). Laminar shear stress in vessels is known to induce the production of NO, which promotes homeostasis of the vessels[50]. Studies have investigated endothelial NO production, in addition to the general valvular response to altered shear magnitudes and profiles, and are discussed below.

Cyclic Strain: As the valve opens and closes in response to blood pressure, it experiences a cyclic strain in both the radial and circumferential direction, and its associated stiffness accounts for this, as discussed below. The average strain has been shown to be 23%–40% in

the radial direction and 10% in the circumferential direction[1,44,51]. Unpublished work from the Yoganathan group indicated that cyclic stretch increases about 5% for every increase of 40mmHg in pressure[52]. Thus, many studies discussed below investigate 10% strain as physiologic, and 15–20% strain as pathologic strain induced by higher pressure in CAVD.

Stiffness: In the homeostatic porcine valve, both the ventricularis and fibrosa portions were found to be stiffer in the circumferential than the radial direction[53]. The ventricularis was found to have a stiffness of 7.41kPa in the circumferential direction and 3.68kPa in the radial direction[53]. Additionally, the fibrosa was found to have a stiffness of 13.02kPa in the radial and 4.65kPa in the circumferential direction[53]. In aortic valve sclerosis and aortic stenosis (AS), ECM remodeling causes valve fibrosis and changes in stiffness over time, affecting the valve's ability to open and close over the cardiac cycle. Santoro et al. found using atomic force microscopy (AFM) on an aortic valve leaflet that the local stiffness of the valve significantly increases near calcific lesions, indicating a spatial heterogeneity of matrix changes[54]. Recent studies have used computational models to aid in determining how this stiffness changes over disease progression. Maleki et al. were able to develop a model that determines a relative stiffness parameter in hopes of diagnosing disease severity in-vivo[55]. They found a relative increase in stiffness from 0.001 to 7.38MPa, and a 0.94 correlation between the valves tested in the study and electron beam computed tomography (EBCT) scores, which is a way to measure calcification in patients[56].

II.   Homeostatic mechanisms of resident valvular cells

Two primary cells inhabit the valve: valvular interstitial cells (VIC) which are embedded in the fibrosa, spongiosa, and ventricularis, and valvular endothelial cells (VEC) that line the outside of the valve leaflets (Figure 2). The valve interstitial cells aid in maintaining structural ECM components of the valve, through the secretion of structural proteins as well as proteinases. Endothelial cells provide a barrier between the valvular tissue and blood. These cells work together to sense their environment, respond to damage, and restore homeostasis in the valve. Studies have also discovered resident valvular immune cells, and their contributions to valvular homeostasis is beginning to be uncovered[57–59].

VEC Functions in Homeostasis

Barrier function/transport: Like the vascular endothelium, the valvular endothelium provides a barrier between the blood and interstitial tissue. It also directs transport of oxygen and nutrients to the inside of the valve, which is crucial because the valve is avascular, unlike large vessels that have the vasovasorum to supply nutrients. Despite these important functions, there have been limited studies investigating the homeostatic barrier function in valvular endothelial cells. One study investigating this feature has found that diffusion of LDL is much higher through the valvular endothelium in comparison to the arteries, and that the valve endothelium had higher permeability to LDL than the aorta[60]. In a follow up study, Zeng et al. modeled the valve leaflet as a non-continuous monolayer with leaky cells dispersed and a basement intimal lining. They found evidence of dispersed leaks in-vitro, and concluded that their model accounting for leakiness and parallel transport in the sub-endothelial space was able to explain the earlier results of Tompkins et al.[61,62]. These

unique transport and lipid diffusion properties demonstrate a distinct feature of valvular barrier function and sub-endothelial space.

Endothelial cells have also been shown to have vast immunomodulatory functions as barriers for tissues, with expression of MHCII on their surface, the ability to secrete pro- and anti-inflammatory cytokines, and express adhesion molecules that allow inflammatory cells to infiltrate the tissues they line[63]. Upon acute injury or infection, these mechanisms aid in restoring tissue to a homeostatic state.

Nitric Oxide Signaling: Nitric oxide production through the eNOS pathway in vascular endothelial cells provides protection to the vasculature by preventing platelet aggregation and adhesion, inhibiting monocyte and leukocyte adhesion, promoting quiescence of smooth muscle cells, and preventing apoptosis of the endothelial cells[64]. There have been many studies demonstrating that valvular endothelial cells can regulate the activity of valvular interstitial cells through nitric oxide signaling through the prevention of activation and calcification. Interestingly, there is more eNOS expression found in the ventricularis than the fibrosa endothelium, and this could in part explain why the ventricularis is protected from calcification, unlike the fibrosa[65]. Dysregulation of NO signaling through eNOS uncoupling has been implicated in CAVD and the role of NO in VIC-VEC interactions and eNOS uncoupling is discussed in detail in later sections.

Production of nitric oxide by endothelial cells occurs through nitric oxide synthase 3, also known as eNOS. In homeostasis, co-factors BH4 and NADPH are bound to eNOS[66]. NADPH is attached to the reductase domain of eNOS and BH4 is attached to the oxygenase domain[66]. In order to produce nitric oxide, NADPH donates an electron through BH4 to the oxygenase domain, which then is used to convert O_2 and L-arginine into nitric oxide and L-citrulline[66,67]. This nitric oxide then is able diffuse out of the endothelial cell and activate guanylate cyclase in a target cell, such as a valve interstitial cell or vascular smooth muscle cell[66]. Nitric oxide-activated guanylate cyclase then aids in transforming GTP to cyclic GMP in the target cell, which can interact with other signaling pathways[66,67] (Figure 3).

An early study demonstrating the influence of endothelial eNOS on aortic valve pathology was performed by T. C. Lee et al. in 2000[68]. They investigated the effects of eNOS-knockout on cardiovascular formation in mice, and found that there was a higher prevalence of bicuspid aortic valve in the knockout mice but no difference in aortic lumen diameter or morphology, indicating specificity to the valve[68]. Later studies performed in-vitro and in-vivo found that endothelial eNOS production provided a protective effect against valvular sclerosis and calcification. Co-culture of VIC with VEC has been shown to reduce pathologic alpha-smooth muscle actin (aSMA) and osteogenic expression in osteogenic-media (OGM) stimulated VIC in 3D, and has been shown to reduce myofibroblastic expression and expression of pSMAD2, a downstream target of the TGF-B pathway, in 2D culture[65,69].

This effect has not shown to be valve-endothelial cell specific, as Bosse et al. demonstrated that co-culture with both human umbilical vein endothelial cells (HUVEC) and lung

endothelial cells had an anti-calcific effect on porcine aortic valvular interstitial cells (PAVIC)[25]. Many gain and loss of function experiments have indicated that this protective effect of endothelial cell co-culture is largely due to the endothelial nitric-oxide signaling pathway outlined above[25,65,70,71]. Bosse et al. have further shown that in VIC cells, nitric oxide stimulus affects downstream Notch1 signaling in VIC, and this downstream Notch signaling is ultimately responsible for the anti-calcific effects of nitric oxide signaling[25]. Gould et al. have also investigated VIC and VEC co-culture in response to different stiffnesses, and found that NO signaling from VEC is able to reduce myofibroblastic activation of VIC on stiffer substrates[72]. They determined that similarly to atherosclerosis, valvular endothelial NO likely signaled to VICs through cGMP, and modulated anti-fibrotic effects through a rho-associated protein kinase (ROCK)-dependent mechanism[72]. They also found that addition of NO from VEC after VIC have already been activated could rescue VIC back to a quiescent state, pointing to paracrine NO as a potential therapeutic target[72].

Calcific inhibitors such as bone morphogenic protein (BMP) anagonists noggin, CV-2/BMPER, SMAD-6 levels, and C-type natriuretic peptide (CNP) in VEC also likely protect against disease and aid in homeostasis. This is evidenced by increased expression in the non-calcified ventricularis endothelium, and in the case of CNP the ventricularis interstitium, compared to the calcific-prone valvular fibrosa endothelium[73–75].

VIC Functions in Homeostasis

Sources of VIC Cells: Though VIC cells are generally considered a fibroblastic population, they have been shown to exhibit great plasticity and have much variation both in their phenotype and cellular origins. Li et al. report in their review on valvular injury that there are at least five types of VIC in the cardiac valve: progenitor mesenchymal/endothelial cells, VIC progenitors, activated (myofibroblast) VICs, quiescent VICs, and osteoblastic VICs[76]. Smooth muscle cells have also been identified in the aortic valve, and Chen et al. have demonstrated that there are VIC multipotent progenitors that have the potential to differentiate into myofibroblast, osteoblast, chondrocyte, and adipogenic phenotypes, further demonstrating the heterogeneity and plasticity of interstitial cells in the valve[77,78]. In development, endothelial cells in the cardiac jelly undergo a mesenchymal transition (EndMT), and invade the underlying matrix, becoming valvular interstitial cells[79]. EndMT in endothelial cells has been shown to depend on many pathways, including transforming growth factor-beta (TGFB), BMP, Notch1, and Wnt signaling[79]. In adult maintenance, Hadju et al. have also discovered that circulating bone-marrow derived cells can engraft into heart valves and differentiate into VIC, as evidenced by Hsp47 and Periostin staining[80]. Adult VEC EndMT has been postulated as another source of VIC turnover, however lineage tracing by our group has demonstrated that this likely only occurs in disease conditions[81].

Remodeling and maintenance of ECM in the valve: There have been few studies performed to directly investigate how VIC remodel and repair valvular tissue in homeostasis, and the studies that have investigated this have largely used the mitral valve[82–84]. In a mitral valve wound model, Lester et al. found that the interstitial cells seemed to be the main cell type driving repair[84].

In homeostasis, myocardial fibroblasts are known to deposit ECM, and also secrete MMP's to maintain the tissue structure of the cardiac tissue[85]. These fibroblasts proliferate upon injury, and become activated with aSMA expression and secretion of ECM proteins[85]. Aortic valvular fibroblasts are generally assumed exhibit a similar response to injury, as they also have been shown to differentiate to a myofibroblastic aSMA+ state that increases collagen production and MMP production upon activation[86]. However, in disease valvular fibroblasts also have a high propensity for calcification, which points to a divergent phenotype from cardiac fibroblasts. This in addition to the lack of studies in the aortic valve and more modern experimental technologies prompts further investigation into the role of VIC's in ECM regulation and turnover during homeostasis.

The Immune System in Valvular Homeostasis

Though much is still unknown about the contribution of the immune system to valvular homeostasis, a few studies have investigated the presence and identity of hematopoietic cells in the non-diseased adult aortic valve. Anstine et al. found that as mice age from postnatal to 16 months, the percentage of CD45+ hematopoietic cells increases with age from ~1.2 to ~10.9%[57]. In 6-week old mice, they did not find a substantial proportion of F40/80+ macrophages, indicating that these cells were largely undifferentiated. Further, in irradiated mice transplanted with reporter hematopoietic cells and depleted of macrophages, they did not see a substantial population of new F40/80+ macrophages in the valve[57]. Hulin et al. similarly found an increase in leukocyte density in aortic valves, rising from 5% of the valve at birth to 12% at eight weeks[58]. They conversely found that 60% of CD4+ cell in the valve were macrophages and 20% were dendritic cells, and that there was a higher percent recruitment of macrophages from one week to four weeks, indicating that further clarity into the role of macrophage engraftment in adult homeostasis is warranted[58]. Raddatz et al. also investigated the role of macrophages in perpetuating CAVD in WT and NOTCH–/+ mice[59]. They found a substantial number of F40/80+ macrophages at 10–12 weeks of age in WT and NOTCH+/– valves, and found that diseased valves likely home macrophages to the valve, and not the other way around. This indicates that in homeostatic conditions, macrophages are likely not recruited to the valve, but rather become pro-inflammatory upon interaction with a diseased valve. In a hyperlipemic disease model, Calin et al. found that depletion of macrophages perpetuated, rather than ameliorated lipid deposition and collagen secretion[87]. This indicates that macrophages homing to the valve may be attempting to serve a protective role against disease progression[87]. Further studies into the effects of macrophage depletion in homeostatic conditions are warranted to elucidate their role in valvular maintenance. Circulating bone-marrow progenitors have additionally been found to engraft into the valve and differentiate to valvular cells, as described above[80].

III.   VIC, VEC, and Cellular Interactions during Initiation and Progression of CAVD

CAVD has two clinical stages: sclerosis, which is valve thickening and some calcification that does not affect functional properties, and stenosis which is substantial obstruction by a heavily remodeled, stiff valve resulting in hemodynamic deficiency[88]. An exact cause of CAVD disease is unknown, however, there are many risk factors that have been discussed in

earlier sections and are presented in Table 1. In early stages of the disease, there are local areas of thickening, matrix disruption, and lipid deposition under the endothelial layer on the fibrosa side of the valve, accompanied by some micromineralization, usually found at the base of the valve[7]. These lesions typically have calcium and lipid deposits deeper in the leaflet with inflammatory cells on top[7]. Severe cases present with large calcific nodules on the fibrosa side of the valve that associate with inflammatory cells and can form mature bone structures and new blood vessels in the valve[89]. (Figure 4)

VIC contribute to valve thickening by undergoing myofibroblastic differentiation and depositing fibrotic matrix[90]. They also play a role in the calcification process through apoptosis, creating calcific nucleation sites. This apoptotic process is commonly referred to as dystrophic calcification.[90] VIC additionally undergo osteoblastic differentiation to directly modulate calcium deposition and bone formation[90].

It has been hypothesized that fibrotic differentiation of VIC may precede osteoblastic differentiation in a linear trajectory of disease. This hypothesis is supported by Schloter et al., who demonstrated with transcriptomics that VIC isolated from fibrotic areas of CAVD valves exhibit intermediate gene profiles between non-diseased and calcific regions[91]. Additionally, Hjortnaes et al. have demonstrated that myofibroblastic aSMA silencing prevents Runx2+ osteogenic differentiation and calcification in VICs[76] However, there is also evidence that VIC may directly undergo osteoblastic differentiation without a fibrotic intermediate[93]. Whether a fibrotic transition state is always necessary for osteoblastic VIC differentiation and why some myofibroblastic VIC further differentiate to an osteogenic state remains to be explored. Additionally, endothelial cells have been demonstrated to undergo transdifferentiation into myofibroblastic VIC, a process called EndMT, and subsequently invade the valve and promote remodeling[81,94–96].

Inflammation and Disease initiation: Risk Factors Promote NO-dysfunction in VEC

Though much is unknown about the initiation of CAVD, it is thought to start with early endothelial inflammation and dysfunction. This makes sense as the endothelium serves as a barrier and first defense protecting the valve tissue from the blood. Endothelial dysfunction allows for the extravasation of inflammatory cells into the valve tissue and allows for deposition of pro-inflammatory molecules and lipid deposition. Drawing off of the atherosclerotic literature, it is likely that smoking, diabetes, and in addition to hyperlipidemia initiate CAVD at least in part through an endothelial NO-dysfunction/ROS damage related mechanism[34,97]. However, studies directly investigating causality of these risk factors in CAVD are somewhat lacking.

In an early study assessing the role of eNOS in CAVD, Rajamannan et al. found that atorvastatin was able to upregulate eNOS and protect against calcification, suggesting a role for eNOS in VIC calcification prevention[98] Subsequently,, Rajamannan directly investigated the effects of adding an inflammatory lipid, low-density lipoprotein (LDL), on VEC eNOS activity. She found that addition of LDL significantly downregulated VEC eNOS enzyme activity, and increased nitrite and Caveolin I[99]. Caveolins are invaginations of the endothelial cell membrane that are involved in transcytosis of lipids and can directly inhibit eNOS, additionally reducing NO bioavailability[100]. It is also well known in the atherosclerotic

literature that shear stress can modulate VEC nitric oxide production, and this phenomenon in valve disease is discussed further below. NO dysfunction in endothelial cells can occur through a phenomenon called eNOS uncoupling[66,67,101]. This often happens when there is not enough BH4 or L-arginine present in the cell[66,67]. In this scenario, eNOS directly transfers electrons to the oxygen reactant creating superoxide, a damaging reactive oxygen species[66]. The superoxide can then react with nitric oxide to create peroxynitrate, another type of reactive oxygen species[66,67]. This peroxynitrate can oxidize BH4 and remove it from the system, creating a destructive positive feedback loop[67,102]. Our lab has demonstrated that endothelial eNOS uncoupling likely contributes to oxidative damage in valve disease, as addition of BH4 was able to reduce Tumor Necrosis Factor-alpha (TNFα) induced reactive oxygen species in endothelial cells-vitro[71]. Interestingly, this effect of BH4 was only observed in the fibrosa, and not ventricularis side of the valves[71] (See Figure 3). It follows that eNOS uncoupling reduces bio-available NO for VIC, abrogating the protective role VEC have been found exert on VIC and perpetuating disease (Figure 3).

Endothelial Sources of VIC Pathogenesis

There are many cytokine pathways that have been found to play a role in the fibrotic and calcific remodeling of CAVD, however, their cellular source and whether they act in an autocrine, juxtacrine, or paracrine manner largely remains to be elucidated.

Specifically, the signaling pathways BMP, Notch, Wnt, and TGFB have been found to play a role in chondrogenic and osteogenic processes of CAVD, and could be modalities through which dysfunctional VEC promote osteogenic differentiation in VIC in CAVD, although studies directly investigating these interactions are limited.. At the transcriptional level, many of these pathways appear to target Runx2 and/or Sox9 nuclear activity. Sox9 promotes chondrogenic differentiation of mesenchymal cells[103]. Runx2 inhibits chondrogenic differentiation and differentiates cells into an immature osteoblastic lineage[103–105]. In limb development, Sox9 acts upstream of Runx2 expression and osteoblastic differentiation, as knockdown of Sox9 prevents both cartilage and bone formation[106]. In endochondral ossification, where cartilage is gradually replaced by bone in limb development, Runx2 was required for ossification but its knock-out did not affect Sox9 expression, indicating a time-dependence for these two transcription factors in osteoblastic differentiation[107]. Furthermore, continued Sox9 expression can inhibit Runx2, indicating its upregulation and subsequent downregulation is necessary for bone formation through endochondral ossification[104,108].

Pathways generally on the pro-osteogenic spectrum of VIC differentiation include BMP and Wnt signaling, while TGFB and Notch are generally considered anti-calcific, with caveats. BMP stands for bone morphogenic protein, and signals through down-stream SMAD 1/5/8 in the cell[109]. It is mainly found localized to the endothelium and sub-endothelial area of the valve as BMP2/4 and also evidenced by SMAD 1/5/8 expression[75,110]. BMP2/4 has also been noted in myofibroblasts/osteoblasts near inflammatory infiltrate and bone formation, and BMP-4 was found in the fibrosa and spongiosa in response to shear stress[6,110]. In VIC cells, BMP pathways are known to promote osteoblastic differentiation, through downstream expression of ALP, Runx2, and osteopontin[111–113]. It is possible that this

endothelial-specific expression/activation of the endothelial BMP pathway could promote osteoblastic differentiation in VIC, and could be a promising area for future study.

TGFB is in the same superfamily as BMP, and signals downstream through SMAD2/3 in the cell[114,115]. It is known to induce Sox9 nuclear localization and aSMA+ fibrotic differentiation in VIC, and is also found to be upregulated in CAVD valves[116–118]. In response to shear stress, its expression is localized to the valvular endothelium, like BMP[110]. Huk et al. discovered that in co-culture and in mice, TGFB from VEC induces Sox9 nuclear localization in VIC which prevents VIC calcification[117]. However, many studies investigating calcification in VIC have also paradoxically shown that TGFB supplementation is necessary for nodule formation. This has been seen in-vitro culture and leaflet explants, where cells form nodules in a spatial pattern with necrosis and apoptosis, especially in a cyclic-strained environment[28,118–120]. One possible explanation is that TGFB supplementation initially upregulates Sox9, which inhibits nodule formation especially in the absence of a mechanical stimulus, but this initial upregulation of Sox9 primes the cells for a subsequent Runx2 upregulation terminal osteoblastic differentiation in a time-dependent endochondral ossification like process[121,122]. Further investigation is warranted to elucidate this mechanism.

The Wnt pathway is largely considered to induce calcification in the aortic valve. Usually B-catenin, a downstream signal of the Wnt pathway is continuously phosphorylated and degraded. However, upon Wnt binding B-catenin is not phosphorylated and translocates to the nucleus[123]. Upon activation, the Wnt/b-catenin pathway generally increases calcification and osteoblastic differentiation through upregulation through upregulation of Runx2[124]. Jenke et al. have demonstrated that TGFB, which has been found to be secreted by VEC to effect VIC, can inhibit both Wnt/B-catenin and BMP signaling[116,123]. They present a paradigm where TGFB induces myofibroblastic differentiation of VIC, while Wnt and BMP induce osteogenic/calcific differentiation, with TGFB an inhibitor of Wnt/BMP[116].

Notch1 signaling usually requires cell-cell contact, and is activated when the protein is cleaved and the intracellular domain is translocated to the nucleus. From there, it upregulates Hairy repressors which are classically thought to induce an anti-calcific effect through repression of Runx2 and BMP[15,125–128]. The Notch1 pathway one of the first pathways whose mutation was directly shown to induce CAVD[15]. However, since this seminal paper, there has been debate on whether Notch-1 itself has pro- or anti- calcific effects on VIC cells. A follow up study by Nigam and Srivastava corroborated Garg et al.'s findings in-vitro and in-vivo, finding that Notch1 prevented calcification through inhibition of BMP-2[128]. Additionally, Acharya et al. have found that Notch1 inhibits calcification by upregulating Sox9 activity[129]. They also interestingly found increased total Notch1 expression in calcified valves, with reduced Notch1 locally in areas of calcification[129]. Furthermore, a study conducted by Hadji et. al. found increased expression of long non-coding RNA H19 from DNA hypomethylation to be inductive of osteogenic cell fate through the down-regulation of Notch1[130]. This indicates a role for not just genetics, but also epigenetics in modulating disease.

Conversely, Zeng et al. found in response to lipopolysaccharide (LPS) stimulus, Notch1 inhibition with DAPT actually decreased osteogenic BMP and alkaline phosphatase (ALP) levels in VIC, possibly through an inflammatory ERK1/2 / NFKB pathway[131]. Wang et al. further discovered that intercellular adhesion molecule (ICAM) and lymphocyte-function associated antigen 1 (LFA) signaling is also involved in this phenomenon, and another group found that Notch signaling in VEC promotes apoptosis in VIC[132,133]. Differences in the results of these papers may be due to the differences in osteogenic stimulus used. It is important to note that even though Notch1 is a cell-cell contact pathway, few studies have investigated whether VIC and VEC communicate using this pathway and possible cross-talk effects on calcification. Future studies investigating Notch1 signaling between VIC and VEC in a direct-contact co-culture system are warranted.

Reactivation of EndMT by VEC in CAVD

Perhaps one of the largest direct interactions between valvular endothelial cells and interstitial cells in CAVD could occur during valvular endothelial to mesenchymal transition. For an in-depth review of VEC EndMT in CAVD, the reader is encouraged to reference Ma et al.[94]. Briefly, endothelial to mesenchymal transition (EndMT) is thought to be a re-activation of endothelial differentiation to an interstitial-cell phenotype and invasion into the valvular interstitial space. These transformed endothelial cells have been shown to have a myofibroblastic phenotype that can induce remodeling and could interact directly interact with valvular interstitial cells in the surrounding area[95,96]. Additionally, it can be imagined that widespread invasion of transformed endothelial cells into the interstitial space would compromise endothelial barrier function, allowing disease-promoting lipids, toxins cytokines, and inflammatory cells from the blood into the interstitial space.

Over the twenty years since the ability of aortic endothelial cells to undergo EndMT was discovered by Paranya et al., there has been much debate over the presence of EndMT in adult valves in both homeostasis and disease[134]. Our group has discovered through lineage tracing that adult aortic endothelial cells are able to undergo invasion into the interstitial space and that these cells are localized near calcific lesions in a CAVD mouse model but not in non-CAVD mice[81]. This indicates that this process occurs in adult disease but likely not homeostasis[81]. However, a study by Kim et al. came to the opposite conclusion. In their Tie2-CreER marfan-syndrome mice, they did not see aSMA+ CD31+ mice, and found that the lineage tracing they performed using g-gal+ Tie2 was actually marking CD45+ lymphocytes[135]. It is important to note that these two mouse models differed and the study by Kim et al. investigated the mitral valve, not aortic valve, which may reconcile some of these differences.

Hjortnaes et al. investigated the role of VIC paracrine signaling on VEC EndMT in-vitro using a transwell co-culture system[96]. They used two modes of promoting EndMT: TGFB media and osteogenic media. VIC were able to suppress aSMA, matrix metallo-proteinase (MMP)-2, and Slug expression in TGFB+ treated VEC, as well as osteocalcin, aSMA, osteopontin, Runx2, and calcium deposition in osteogenic-media treated VEC. This points to a protective paracrine role of VIC against EndMT in VEC cells, although the mechanism through which this signaling occurred was not investigated in this study. Interestingly,

Hjortnaes et al. did not observe a reciprocal protective paracrine effect of VEC co-culture with VIC, and instead actually saw increased mineralization in the co-culture condition in osteogenic media vs. VIC alone[96]. This is in direct contrast to a large body of work indicating a protective role for VEC on VIC calcification[25,65,69,117,136]. Our group, as well as Hjortnaes et al. found evidence of increased remodeling ability of EndMT VEC, through upregulated MMP's, and a notable propensity of EndMT transformed VIC to remodel collagen fibers[96,137]. Our group was able to attribute the EndMT process in response to TNFα to an AKT/NFKB pathway[137]. There has also been evidence of EndMT VEC present in calcified human explanted aortic valves, but not healthy control valves[96,137]. Gendron et al. have recently the ability of diseased VIC to undergo the reverse process back to endothelial cells. When challenged with vascular endothelial growth factor B (VEGF-B) and after 10 days of in-vivo implantation in matrigel, there was no evidence that VIC from diseased patients were able to differentiate to an endothelial phenotype, indicating that VEC EndMT plasticity is likely not reversible[138].

IV. Inflammation in the Diseased Valve

Risk Factors Promote Adhesion Molecule Expression in VEC

Inflamed VEC express adhesion molecules and exert immunomodulatory functions, to interact with the immune system. Some of these markers include P/E selectins, ICAM, vascular adhesion protein 1(VCAM), and activated leukocyte adhesion molecule (ALCAM). Selectins facilitate rolling of leukocytes and attachment, and ICAM/VCAM bind leukocytes and assist with extravasation[139]. Interestingly, Guerraty et al. found that a unique adhesion molecule ALCAM was the only differentially expressed endothelial adhesion molecule between the fibrosa and ventricularis, indicating a potential role in CAVD and warranting further investigation[140]. Vandana et al. and Ciortan et al. investigated the effects of hyperglycemia on VIC/VEC co-cultures in a diabetes model[35]. They found that high glucose resulted in direct upregulation of the adhesion molecules VCAM-1 and E-selectin in VEC at 7 and 14 days[35]. Mechanical stimuli have also been found to directly cause increased inflammatory adhesion markers in VEC, and are discussed below. It is important to note that VIC have also been shown also express inflammatory adhesion molecules like VCAM, ICAM, and E-selectin, however the expression of adhesion molecules to facilitate leukocyte infiltration is usually physiologically attributed to endothelial cells[36,141].

Inflamed VIC/VEC Recruit Inflammatory Cells to the Valve

As discussed above, pro-inflammatory VIC and VEC express adhesion molecules that allow inflammatory cells to the enter the valve. These inflammatory cells are generally thought to wreak more havoc on the valve, though destructive proteinases, and the release of additional pro-inflammatory cytokines, which promote pro-fibrotic and calcific phenotypes of resident valvular cells. Paradoxically, these infiltrating inflammatory cells may also serve a protective role against disease. Resident valvular inflammatory cells may also contribute to this process, but specifics regarding their role in disease has yet to be elucidated.

Initiation of Inflammatory cell infiltrate:

In CAVD, inflammation appears before valvular calcification. This has been demonstrated in-vivo by Abdelbaky et al., who used cancer surveillance fluorodeoxyglucose positron emission tomography (FDG-PET) images from patients without active cancer. They were able to demonstrate in images taken approximately two years apart that inflammation identified by FDG-PET independently predicted subsequent valvular calcification[142]. Though inflammation likely plays a role in the initiation of CAVD, there have been varying reports of its extent in late-stage disease. Coté et al. noted that only 28.4% of the 285 explanted human CAVD valves that they investigated contained inflammatory infiltrate, while Natorska et al. reported 95% inflammation in their CAVD specimens[89,143]. However, Coté et al. also noted that there was a correlation between leukocyte density and stenosis progression, and inflammatory cells tended to localize around calcific nodules and cluster in areas of bone deposition and angiogenesis, indicating an active role in disease progression[89]. Additionally, Hjortnaes et al. found that a macrophage-targeting fluorescent nanoparticles, as an indicator of inflammation, were positively correlated with valvular calcification, indicating that inflammation increases with disease severity[144]. This diverging evidence for the extent and presence of inflammation in late stage valve disease demonstrates the inherent variability in pathogenesis from patient to patient and prompts a nuanced understanding of inflammation in a wide range of CAVD disease etiologies.

Immune cells in Calcific Lesions

Pathophysiologically, inflammation in early CAVD is thought to be in many cases a response to lipid deposition through an ill-functioning endothelial barrier, similarly to atherosclerosis[145]. In diseased valves and in-vitro models, vascular endothelium has been reported to upregulate Lox1 which is involved in endocytosis of oxidized low-density lipoprotein (OxLDL), in addition to caveolins (discussed above), and CD73, although much remains unknown about this aspect of VEC barrier function[99,146–148]. A distinct cellular source for the conversion of LDL to OxLDL remains unclear, however it could be any ROS-producing cell[149]. ROS are discussed further in the section entitled "Reactive Oxygen Species". However, key differences have been noted between the lipid deposition, lesions, and inflammatory infiltrate in CAVD and atherosclerosis. An early study noted that these lesions had lipid deposition and were primarily under the basement membrane of endothelial cells with some extension to the fibrosa[7]. Olsson et al. further reported that the lipids remained in the fibrosa subendothelium and did not move deeper, unlike in atherosclerotic plaques[150]. Further investigation into these lipids concluded that only small, dense LDL's correlated with aortic stenosis progression, and not high-density lipoprotein-c (HDL-c), LDL-c, or triglycerides[151]. The presence specifically of oxidized low-density lipoprotein (OxLDL) in aortic valves correlated with T-cell, leukocyte, and macrophage presence as well as increased TNFα and tissue remodeling[151]. Otto et al. noted that lesions tend to accumulate these inflammatory cells including macrophages and T-cells on their surface, and Olsson et al. noted that unlike atherosclerotic lesions, valve lesions did not form necrotic cores with a high density of foam cells, although some foam cells were identified[7,150]. Widespread micromineralization has been noted in the aortic valve, and is also seen in atherosclerosis[7,152]. Olsson et al. postulated that deposited valvular OxLDL may aid in calcific deposit nucleation instead of inducing necrosis, and that differences in

OxLDL turnover in the valve vs. vasculature may be in part responsible for the differing calcific pathology in CAVD vs. atherosclerosis, which typically exhibits plaque rupture and necrosis[150]. Studies have found that small LDL particles and lipoprotein (a) (Lp(a)) presence has been associated with oxLDL in CAVD, and a mutation in Lp(a) has been found to be associated with aortic valve stenosis and calcification[24,126,151]. However, the role of LDL-C in CAVD is still unclear[14]. This is in contrast to atherosclerosis, where LDLc is implicated in lesion development[153] It is important to consider these differences between CAVD and atherosclerosis, as drugs such as statins that have shown incredible success in treating atherosclerosis have failed in the treatment of CAVD[154]. The role of lipid deposition in general CAVD pathogenesis has been reviewed extensively elsewhere[154,155], and the remainder of this section will focus on inflammatory cellular mediators and pathways in CAVD.

Many inflammatory cell types have been discovered in the aortic valve, and for a more extensive review of these cell types the reader should refer to Raddaz et al.[156]. Briefly, cell types include macrophages, T-cells, B-cells, mast cells, dendritic cells, and platelets. These inflammatory cellular mediators have been shown to be sources of pro-inflammatory cytokines and matrix-remodeling proteases in CAVD. Macrophages are perhaps the most studied of the inflammatory cells in CAVD, largely because their polarization state is known to induce homeostatic and pathogenic inflammatory remodeling or resolution. Notably, Li et al. discovered that M1 macrophages are increased and M2 macrophages are decreased in calcified valves vs. controls, indicating a more pro-inflammatory state[157]. Macrophages have colocalized or associated with matrix-remodeling MMP's EMMPRIN, MT1-MMP, MMP2/9, and Cathepsin B[158,159]. Macrophages have also been shown to express pro-inflammatory cytokine TNFα and leukocytes express IL-1B in stenotic valves[160,161]. It is assumed that macrophages would additionally express pro-inflammatory IL-6 in CAVD valves as they do in-vitro[157]. VIC cultured with conditioned media from undifferentiated macrophages resulted in an increase in osteogenic markers, and this was additionally increased by M1 differentiation[157].

Despite this incriminating evidence against leukocytes and macrophages in the pathogenesis of CAVD, complete depletion of macrophages in hyperlipidemic hamsters resulted in larger valvular lesions with increased lipid and collagen deposition, suggesting that macrophages also serve a protective role against CAVD[87]. Mast cells, an inflammatory cell type infamous for its role in allergic reactions, have additionally been found in the aortic valve near microvessels and regions of calcification[162,163]. Interestingly, Milutinovic et al. found that mast cells may also provide protection against calcification in CAVD, as stenotic valves with mast cells had lower calcification than valves without them[164]. These findings indicate that inflammatory cells at least in part serve a protective role in disease, in addition to potentiating it.

Interestingly, the platelet to lymphocyte ratio has been found to be increased in severe aortic stenosis when compared to patients with mild/moderate stenosis[165]. Additionally, areas of thrombin, fibrin, and tissue factor have been found in calcified aortic valves implicating a role for coagulation in the disease[166–168]. Valves that stenosed had significantly higher tissue factor-positive areas compared to aortic insufficiency, and additionally patients with aortic

stenosis had fewer large von-Willebrand factor (vWF) proteins, and lower rates of platelet retention indicating abnormal coagulation[166,169]. Recently, platelets have been implicated as potential sources of TGFB in valve disease, a pro-calcific and EndMT cytokine that is discussed earlier sections[170]. Investigation into valvular endothelium interactions with the blood is still somewhat lacking, and could provide important insights into disease initiation and progression.

Reactive oxygen species:

Reactive oxygen species (ROS) in many cases are produced to cause damage to invading pathogens, and can wreak havoc on native tissue[171]. The presence of reactive oxygen species and ROS-producing intermediates have been found around calcific nodules in human valves[172,173]. Though superoxide has been found in macrophages of calcified valves, this was more pronounced in areas of non-macrophage cells around the calcification[172]. These areas were found to contain osteoclastic and osteoblastic cells, and indicate that unlike atherosclerotic lesions, macrophages may not be the primary contributor of ROS in valves[172]. Miller et al. found that eNOS uncoupling likely plays a role in superoxide production, and this has been further validated in VEC, as discussed[71,173]. In-vitro studies by Branchetti et al. and Liu et al. have additionally investigated the role of ROS and intermediates in VIC[174,175]. Liu et al. found that Nox2, an enzyme that produces ROS, is elevated in diseased valves and osteogenic-media cultured VIC[175]. Inhibition of Nox2/ROS production with viral transduction and a drug called Celastrol was able to reduce calcific effects in-vitro and in-vivo through a GSK3B/B-catenin pathway[175]. Furthermore, Branchetti et al. discovered that treatment of VIC with H_2O_2 induced DNA damage repair proteins more so in sclerotic and stenotic valves than control valves[174]. Additionally, they found that H_2O_2 increased Runx2 signaling through an AKT pathway, and that catalase and SOD were able to mitigate some of the H_2O_2-driven DNA damage and osteogenic differentiation effects[174]. Given that both VIC and VEC appear to produce and react to ROS, and that studies have indicated that macrophages may not be the main cellular source of ROS in CAVD, it is imperative to test the response of these two cell types in co-culture to more clearly understand how ROS in disease valves initiates and manifests.

Neovascularization

One feature of diseased aortic valves is the presence of neovascularization in the normally avascular valvular tissue[176]. Steiner et al. postulated that one source of this neovascularization could come from the valvular endothelium[176]. A study by Arevalos et al. investigated the ability of VIC and VEC cells in co-culture to undergo pericyte differentiation and angiogenic sprouting on an angiogenic Matrigel scaffold[177]. VIC and VEC in co-culture were able to form angiogenic sprout networks which interestingly collapsed into VEC-VIC spheroids over prolonged culture. The VIC seemed to exhibit some pericyte-like behaviors, including wrapping around VEC tubules and invasive stalks. Cells from these spheroids notably invaded the matrix, with VIC cells paving the way for VEC cell migration, and this was found to be modulated by Ang/Tie and ROCK pathways. This pericytic differentiation of VIC has been further confirmed by Gendron et al., who discovered that conditioned media from VIC isolated from diseased patients were able to

promote significantly more proliferation, migration, and sprouting in cord blood endothelial progenitor cells[138].

NFκB Signaling- Conserved Mode of VIC/VEC Inflammation inducing CAVD

Pro-inflammatory cytokines have been shown to contribute to CAVD, including TNFα, IL-1B, IL-6, etc[178,179]. Many of these cytokines, including TNFα, IL1B, and IL-6 converge in downstream activation of the pro-inflammatory nuclear factor kappa B (NFκB) pathway, which has been associated with atherosclerosis and CAVD[137,180,181]. Homeostatically, NFκB is inactivated through binding to inhibitory kappa B (IκB). Upon activation, a complex called IκB kinase (IKK) complex, which contains a subunit IKKβ (not to be confused with IκB), phosphorylates IκB, which releases NFκB and allows for translocation to the nucleus[180]. There, it induces inflammatory cytokines/chemokines, matrix degrading enzymes, and adhesion molecules, including VCAM-1, ICAM, Selectins, MMP's, TNF, and IL1/6[180]. In innate immunity, toll-like receptors (TLR's), and NOD-like receptors present in the cells of resident tissues, which sense molecular patterns associated with pathogens, can also activate NF-kB[182]. A large body of work has demonstrated that NFκB can be activated in VICs in response to TLR signaling, and that TLR signaling is associated with CAVD[37]. This has been reviewed extensively by Garcia-Rodriguez et al., and thus will not be discussed in depth here[37].

Our group along with others have begun to study the role NFκB plays in CAVD. We have found that in the fibrosa of calcified valves, there is more NFκB nuclear signaling than the fibrosa of sclerotic and non-calcified valves[81]. As discussed above, Zeng et al. and Wang et al. have found that stimulation with an inflammatory LPS stimulus induces osteogenic differentiation of VIC through a downstream NFκB pathway, indicating an active role of NFκB in driving CAVD[131,132]. They found this pathway was activated through TLR receptor activation, Notch-1 and adhesion molecule ICAM-1 signaling. In VEC, our group found that the inflammatory cytokines TNFα and IL-6 can induce EndMT in adult endothelial cells through a downstream NFκB pathway[137]. In a follow up study, we additionally found that NFκB signaling can induce a calcific response in VEC in the presence of an osteogenic stimulus, evidenced by nodule formation, alizarin red S (ARS) deposition, and Runx2/osteocalcin (OCN) expression[81]. Knock out of NFκB in the entire valve of LDLR−/− mice had a protective role against CAVD, exhibited by decreased aortic peak velocity, peak gradient, mean gradient, leaflet thickness, and increased ejection fraction compared to LDLR−/− controls[81]. Further, knock out of NFκB exclusively in valvular endothelial cells was able to rescue hemodynamic function in LDLR−/− high-lipidemia mice, but not ejection fraction, indicating that NFκB-induced signaling from VEC to resident valvular cells plays a substantial but not all-encompassing role in the modulation of CAVD[81]. Notch signaling in VEC has been shown to promote apoptosis in VIC likely through a TNFα related pathway[133]. Thus, it would be interesting to investigate whether Notch 1 signaling is also involved with VEC NFκB signaling, and whether this signaling in VEC modulates VIC propensity towards calcification (Figure 5). The compounds EMD, curcumin, andrographolide, hydrogel sulfide, and caffeic acid have been discovered to have an anti-calcific effect on VIC by modulating at least in part the NFκB pathway, highlighting not only its importance in CAVD but also its promise for therapeutic targets[183–185,186,187].

IV. Altered mechanics in CAVD perpetuate disease

Risk factors such as age as well as inflammation result in progressive stiffening of the aortic valve as resident cells differentiate and remodel the matrix, and this stiffness can be sensed by resident cells, modulating their phenotypes and contributing to disease in a positive feedback loop. As the valve stiffens, its mechanical properties alter, preventing its ability to efficiently open and close over the cardiac cycle. Thus, hemodynamic shear stress and cyclic strain in the valve is altered, and these mechanical stimuli can induce a pro-disease and pro-inflammatory state in valvular cells. Hypertension could also induce altered shear stresses on the valve, initiating and/or perpetuating disease. The following section describes current knowledge of VIC and VEC cellular response to these mechanical stimuli, in isolated mono-culture and dynamic co-culture studies.

VIC and VEC Responses to stiffness

The VIC response to stiffness under an activating stimulus has been studied extensively under many conditions generally ranges from a myofibroblastic and apoptotic state in stiff substrates to osteogenic in softer substrate[54,188–190,191,192]. Yip et al. investigated the formation of calcific nodules under stiff and soft substrates, and found interestingly that stiffer substrates tended to form aggregates that were apoptotic, while softer substrates formed more nodules overall that were osteoblastic and not apoptotic[193]. They attributed the formation stiffer apoptotic nodules to AKT signaling, and this was corroborated later by Wang et al., who found that inhibiting PI3K (upstream of AKT) was able to block nodule formation on stiff substrates[191]. Chen et al. found additionally that an increase in aSMA on increasingly stiff substrates was due to B-catenin signaling, while Santoro et al. found a role for YAP/TAZ signaling in this process[54,190]. It is likely that these pathways are acting either separately or in concert to modulate the VIC response to stiff substrates. Investigation into pathways involved in VIC nodule formation in softer substrates have found a role for RhoA in osteoblastic differentiation through demonstration that addition of Rock inhibitor decreased Runx2 and OCN at 1kPa[188]. Contrary to the studies mentioned above, in a 3D model Duan et al. reported that there was more aSMA activation of embedded VIC in softer vs. stiffermethacrylated hyaluronic acid/oxidized hyaluronic substrates[194]. However, high circularity indicative of poor cell spreading due to the methacrylated hyaluronic acid material may have affected cellular expression, as this trend was not seen in methacrylated hyaluronic acid/ gelatin combination gels[194]. It is important to consider 2D vs. 3D culture when interpreting stiffness results, as some stiffer substrates investigated in 2D may elicit different responses when embedded in a more physiologically relevant 3D model.

The role of matrix stiffness and composition has also been evaluated in endothelial cells[195,196]. Endothelial cells tend to increase EndMT evidenced by aSMA expression and matrix invasion with increasing stiffness of the substrate[195,196]. Dahal et al. found that this VEC transdifferentiation occurs through an ERK pathway, while Zhong et al. found a role for B-catenin, similarly to Chen et al's VIC study[190,195,196].

Gould et al. have investigated the role of stiffness on VIC activation in a VIC+VEC co-culture model[136]. They found similarly to previous reports that increased stiffness resulted in increased myofibroblastic activation and significantly more nodule formation.

However, co-culture with VEC seeded on one end and VIC seeded on either side of a polyethelyne glycol (PEG) hydrogel was able to reduce the number of myofibroblastic VIC and nodule formation. As discussed above, VEC modulated this myofibroblastic and osteogenic activation in VIC through NO signaling, which likely modulates VIC ROCK activity[136]. As apoptotic VIC nodule formation through PI3K/AKT signaling has been found to dominate on substantially stiffer substrates, it would be interesting to see if VEC co-culture is also able to modulate these pathways through PI3K/AKT signaling. Additionally, as VEC have been shown to undergo increased EndMT in response to substrate stiffness, the effects of VIC-VEC co-culture on substrates of varying stiffnesses with characterization of VEC invasion could yield important insights in future studies.

VIC and VEC Responses to Pathologic Shear Stress

As the endothelium is the only cell type directly exposed to fluid flow from the valve, many studies have focused on the role it plays in mechanosensing and responding to shear in homeostasis and disease. Butcher et al. investigated transcriptional readouts of porcine aortic valvular endothelial cells (PAVEC) in comparison to porcine aortic endothelial cells (PAEC) under 20 dyne/cm^2 shear[197]. They found that both cell types down regulated BMP-4 with shear, but specifically cadherin-11 (CAD-11) was downregulated with shear in PAVEC, and that baseline levels of CAD-11 were much higher in PAVEC vs. PAEC[197]. This study indicates that though there are some similarities between the valvular and aortic endothelial response to shear, there are also marked differences. Our lab also investigated shear stress of different magnitudes on aortic endothelial cell transformation. We found that low laminar shear stress and low to moderate oscillatory shear stress induced EndMT markers aSMA, Snail, TGFB, NfKB, and ICAM-1 in endothelial cells[198]. These inflammatory markers and cytokines, notably TGFB, have been associated with VIC differentiation to a myofibroblastic and calcific phenotype. Holliday et al. interestingly found differences in mRNA due to applied shear stress but no significant differences in mRNA due to side origin of human aortic valvular endothelial cells[199]. This indicates that applied shear stimulus may supersede biological origin in driving shear responses of valvular endothelial cells. Holliday et al. also found that laminar shear reduces BMP4, and increases eNOS and IkB signaling. These eNOS results are corroborated by Richards et al.[65]. As discussed above, NO directly communicates with VIC, and IkB prevents the inflammatory transcription factor NFκB, which when activated could assist in recruitment of inflammatory cells and inflammatory activation of neighbors through cytokine expression[200].

Studies have also investigated the role of shear stress on expression in the fibrosa and ventricularis endothelium in porcine leaflet explants[110,201–203]. In aortic explanted leaflets, elevated pulsatile shear stress resulted in increased VCAM-1, ICAM-1, BMP-4 and TGFB signaling on the aortic side of the leaflet[110]. A combination of BMP and TGFB-1 inhibitors was able to reduce VCAM-1, BMP-4, and TGFB-1 signaling compared to media control in supraphysiologic shear conditions on the aortic side of the leaflet[201]. It was concluded that there may be a potential synergistic role for BMP and TGFB signaling in promoting pro-inflammatory activation of endothelial cells under pathologic shear stress[201]. Sun et al. used same model to study the effects of both magnitude and frequency of shear[203]. They found that high magnitude normal frequency shear increased BMP-4 and TGFB (as

expected) and also MMP's 2/9 and Cathepsin L/S, and abnormal frequency at normal shear magnitude also increased degrative enzymes but not BMP's/TGFB-1. Former studies in addition to this one noted that there was usually TGFB and BMP signaling localized along the endothelium and sub-endothelially[110,203], while this study found MMP's and cathepsins more uniformly spread throughout the tissue[203]. The effects of side-specific shear stress was also investigated in a similar model system, and it was found that inducing aortic shear flow pattern on the aortic side of the leaflet increased collagen and GAG content, while under ventricular flow increased elastin content compared to aortic flow[202]. This could indicate that shear stress type sensed by aortic or ventricular endothelium can influence ECM deposition type in VIC cells.

Butcher and Nerem used a more simplified collagen gel co-culture system to study VIC alone in comparison to VIC+VEC co-culture under shear conditions[204]. They found that ECM remodeling and overall protein deposition and proliferation responses differed in response to shear in VIC only vs. VIC+VEC co-culture. Additionally, VIC+VEC had more GAG content than VIC alone under 20dynes shear at 4 days, less proliferation under shear at 4 days, and more protein content with shear[204]. In contrast, Wang et al. tested VIC alone in a PDMS microfluidic chip and found that above $0.78 dyn/cm^2$ the VICs were elongated and aligned parallel to flow and that they had increased TGFB expression above $0.49 dyn/cm^2$ [205]. This differs from the results found by Butcher et al., which did not largely find VIC alignment[204]. The 2D vs. 3D culture may play a role in differing results between these two studies. Wang et al. mentioned addition of VEC co-culture in their future work, and this will yield important information about VIC+VEC interactions under shear that could not be captured in their study[204,205]. For a timecourse summary of shear stress studies, please refer to figure 6.

VIC and VEC responses to Pathologic Static or Cyclic Strain

Studies investigating VEC only in response to homeostatic and pathologic cyclic strain have found that the least amount of inflammatory adhesion molecules ICAM-1, VCAM-1, and E-selectin are present at 10% strain in comparison to 20%[206]. Further investigation looking at differences between fibrosa and ventricularis VEC found that fibrosa VEC upregulate B1 integrin in comparison to the ventricularis, and ventricularis VEC alternatively upregulate VE-cadherin in comparison to the fibrosa at 20% strain[207]. These studies demonstrate that VEC alone are able to sense and respond to altered cyclic strain, specifically with changes in adhesion molecules that could promote inflammation. McIntosh and Warnock's results also demonstrate that location matters when it comes to the response: i.e. VEC from the fibrosa will respond differently than VEC in the ventricularis to a cyclic-strain stimulus.

In contrast, many studies investigating VIC only and VIC+VEC concerted efforts on valve remodeling and calcification have focused on 14–15% strain. In VIC only, 15% strain yields the lowest expression of inflammatory markers, reduced IKKB, and increased pSMAD2/3[141]. A cyclic strain at 14–15% has also been reported to be pro-calcific and pro-remodeling in both VIC only[120,208,209] and valve explant systems[28,52,210]. These results point to a complex role of inflammation on VIC remodeling and calcification, as the strain that induced the least number of inflammatory markers also exhibited notable calcific

spheroids with hydroxyapatite (HA) deposits[120,141,208]. Our lab has investigated the role of VIC only culture in a 3D cyclically strained collagen gel. We have found that when VIC are cultured in 3D static-strained monoculture in ostogenic conditions, they exhibit a spike in RhoA signaling at day 1 that resolves at later timepoints, and that the addition of dominant negative (DN)-RhoA reduced the number of nodules at 14 days. Bouchareb et al. also found that 30 minutes after cyclic strain ROCK activity increased, returned back to baseline levels after one hour, and that addition of Rock inhibitor reduced calcification in a 2D VIC cyclic stretch model[208]. It appears that rapid RhoA/Rock signaling exerts a large influence over calcification and nodule formation in these VIC- only systems[208,211]. Interestingly, Gould et al. have discovered that endothelial NO signaling likely exerts an anti- myofibroblastic and calcific protective effect on VIC through ROCK signaling[136]. Investigation into the temporal regulation of calcification through RhoA/ROCK signaling in a co-culture cyclic-strain model could yield important insights into how these cells regulate each other in homeostatic and pathologic conditions.

Current methods to study VIC and VEC under a mechanical stimulus together in vitro include valvular explants mounted in a bioreactor, and mechanically-constrained gel co-culture. A series of studies by the Yoganathan group and others have tested explanted porcine aortic valves under cyclic stretch[28,52,212]. One notable finding was that the addition of cyclic stretch resulted in the upregulation of BMP2 specifically in the fibrosa side of leaflet explants, and that this co-localized with VWF, an endothelial marker[28]. Addition of noggin reduced the total calcification observed under mechanical stretch[28]. This indicates that the endothelial BMP side-specific response to cyclic stretch may be crucial to overall valve calcification, as BMP was co-localized with the endothelium and noggin, a BMP inhibitor, was able to reduce calcification[17].

Our lab has also employed a collagen gel co-culture model containing aortic valvular endothelial cells seeded on top of interstitial cells to investigate the effects of co-culture on the cellular response to static mechanical constraint[65]. We discovered that addition of PAVEC to PAVIC prevents calcification and compaction in OGM conditions, and reduces aSMA, OCN, and Runx2 expression. With loss and gain of function experiments, we were able to attribute these effects to NO signaling from the valvular endothelial cells, indicating that NO signaling plays a role in modulating VIC phenotype not just under shear but also in static mechanically-constrained conditions. For a timecourse summary of the results discussed in this section, please refer to figure 7.

V.   Future Directions for CAVD Research

Temporal phases of CAVD progression

As discussed in the introduction, CAVD is usually not diagnosed in patients until they already have hemodynamic abnormalities due to widespread calcification and valve remodeling and left ventricular hypertrophy. In comparison, many in-vitro models test therapeutics on their ability to prevent valvular calcification. It is still unclear when a model investigating the prevention of calcification corresponds to in-vivo disease progression. Looking forward, as the field uncovers more therapeutics that show great promise in-vitro,

it is crucial to gain a better understanding of when the effects of these therapeutics might provide the most benefit in-vivo.

For example, studies investigating the efficacy of preventative strategies against CAVD development have found that a combination of exercise and high cholesterol diet was able to prevent aortic valve disease in comparison to high cholesterol only. The endothelial lining was more intact in the exercise+cholesterol group than cholesterol alone, indicating the importance of maintaining the endothelial barrier in preventing CAVD[213]. However, this same group found in a follow up study that removing the high cholesterol diet or beginning exercise 16 weeks after mice were placed on a high cholesterol diet was not able to prevent valvular disease[214]. Additionally, in this study there was no longer preservation of the endothelial lining[214]. This indicates that there is likely a window for treatments that attempt to reduce the initiating factors, specifically endothelial damage, in CAVD. When that window begins and ends in humans and other animal models remains unclear.

Additionally, as discussed above, many studies investigating the interaction of mechanics and calcification have found that there is a time-dependent acute activation and subsequent return to baseline of the RhoA/Rock pathway[208,211]. This initial upregulation of the RhoA pathway had widespread effects on later calcification, although it was no longer upregulated during later timepoints. Though the Rho/Rock pathway played a significant role in calcification in these studies, targeting the pathway at later timepoints would not likely show efficacy. Thus, not only is it important to understand macroscopically when in-vitro models correspond to disease progression but it is also important to define when certain pathways are temporally modulated over disease progression.

Work from our lab has attempted to model later-stage calcification in-vitro by introducing hydroxyapatite nanoparticles to our 3D cultures[215]. We have found that smaller and less-crystalline calcium deposits tend to promote more calcific-disease responses in VIC-only and VIC+VEC co-cultures than more crystalline structures. Additionally, there was no longer a protective effect against myofibroblastic/osteogenic differentiation and calcification from adding VEC in co-culture with VIC in the presence of HA nanoparticles. Interestingly, there was still an upregulation of eNOS in these cultures indicating that its effectiveness in promoting quiescence in VIC may be diminished when there is already calcification present. Further work modeling late-stage disease is warranted to identify VIC-VEC interactions that may offer protection in early disease progression but are no longer effective in later-stage disease.

Risk Factors in CAVD Progression

Table 1 presents many risk factors for CAVD, mechanisms for how they could cause the disease, as well as current experimental models to investigate them. Porras et al. has pointed out that unlike atherosclerosis, it appears that the risk factor for causing valve disease can directly influence how the disease progresses, as evidenced by sex-specific disease etiologies in CAVD[216]. Though CAVD patients in many cases present with many of these risk factors, understanding how each may play a role in the disease in-vitro could assist in identifying which populations promising therapeutic targets would benefit most. This could be accomplished by first developing representative models of each risk factor and then

testing potential therapeutic targets across multiple models to determine whether there is a preferential efficacy in certain systems. For instance, a therapeutic that shows potential in preventing calcification in a diabetes-specific model with only male cells may not show the same effect with female cells, or in aged vs. young cells, or in a hyperlipidemia model. With the advent of personalized medicine and the invention of ever-increasing high-throughput experimental systems, the feasibility of performing multiplexed therapeutic experiments has shifted from dream to reality. Information gleamed from these types of experiments could help to explain failed clinical trials and prompt more targeted future clinical trials to bring therapeutics from bench to bedside.

3D Environmental Context of CAVD Progression

Although many in-vitro studies investigating the valve have focused on 2D culture systems, the unique 3D-structure of the valve positions VIC and VEC such that they may regularly communicate with each other during homeostasis and disease. This communication can influence calcific disease pathways, as evidenced by many of the articles discussed above. Specifically, it appears that VEC are able to exert a homeostatic influence on VIC, and serve as the gateway for infiltrating immune cells, lipids, cytokines, and other disease-initiating stimuli. Therapeutics that are able to rescue endothelial barrier function, therefore maintaining the semi-permeable barrier and restoring homeostasis, could prove fruitful.

It is also important to note that calcific lesions are not uniform over a diseased valve leaflet, but are often highly-organized structures spread heterogeneously over the diseased leaflet. This indicates that in addition to considering purely VIC and VEC interactions in CAVD, investigations into the spatial heterogeneity and differentiation spectrum in their relation to lesion formation could provide clues for how the disease progresses, and highlight nuanced complexity needed for therapeutic targets.

Though the interactions between VIC and VEC are critical to understand in the progression of CAVD, they are difficult to resolve using classical biological tools. Single cell gene sequencing and spatially-resolved proteomics are two novel techniques that will likely yield important information at a cell-resolved level to aid in the understanding of differentiation states and interactions between different cell types in CAVD, and how these might be exploited for future therapeutics. A novel study by Xu et al. is the first to employ single cell sequencing on calcified and healthy human aortic valves[217]. They were able to identify seven cellular sub-populations that were significantly upregulated in CAVD valves vs. healthy valves. They noted upon staining that these populations tended to be in the interstitial layer, and identified them as VDSC's. However, there was still much unanswered about the identity and activity of these cell types.

Xu et al. additionally performed a differentiation trajectory, and determined that these VDSC's likely originated from VIC, and a cellular cluster that likely was from a VEC-transitioning EndMT state[217]. Though this study yielded important insights at the single-cell level, there is much cellular heterogeneity from patient to patient, which may overshadow genes associated with disease progression. Thus, use of this technique in a more controlled experimental environment could yield more information about differentiation trajectories and expression at the single cell level.

Schlotter et al. also performed transcriptomics and proteomics on healthy, fibrotic, and calcific valve leaflets[91]. They were able to identify unique proteins and genes to each stage of disease progression. In studying pathways using network analysis of CAVD proteomic information, Schlotter et al. found MAPK signaling, lipid metabolism, coagulation, inflammation and ECM were the top subnetworks identified, further highlighting the important role of inflammation in disease progression. Additionally, they were able to find proteins specific to each layer of the valve in healthy and diseased valves, indicating that there is a baseline heterogeneity in these populations. They were able to identify a unique subpopulation of VIC that are GFAP+ and localize to the spongiosa, further highlighting the inherent heterogeneity of cells in the aortic valve based on location.

Additionally, it is important to remember that the valve is not a free-floating tissue, but rather is mechanically anchored and constantly exposed to mechanical stimuli. Abnormal mechanical stimuli appear to drive pro-inflammatory expression and promote CAVD myofibroblastic/osteogenic disease progression. Understanding how CAVD progresses under different mechanical stimuli can aid in elucidating risk factors as well as gain an understanding of which therapeutics might be most effective in which mechanical contexts.

Spatially, inflammation has been shown to be associated with calcific deposits, and is an important predictive factor for disease progression and severity. Modulating inflammatory cells themselves, specifically macrophages, has gained increasing interest in a wide range of diseases[218]. However, this proves to be a difficult strategy because the complexity through which the inflammatory system drives homeostasis and disease not only through the valve but in the body generally is still not fully understood. Modulating the macrophage response through blanketed removal was shown to promote, and not prevent disease, indicating that their presence in the valve is somewhat necessary[87]. Raddatz et al. found that is likely an already inflammatory valve that brings inflammatory infiltrate into the tissue in disease, and not the other way around, indicating that systems investigating isolated VIC and VEC interactions are likely a good model for disease initiation[59]. Information gathered using novel single-cell transcriptomics and spatially-resolved proteomics, as discussed above, could provide more detailed clues about the spatial distribution and identity of inflammatory cells that populate more advanced-stage CAVD valves.

Conclusions

In conclusion, as the aging population grows, incidence of calcific aortic valve disease will continue to rise. Though valve disease due to infection has been largely eradicated in western countries, worldwide this remains a major cause of heart disease and this should be considered when designing therapeutic strategies. As surgical treatment is not a long term solution for many or inaccessible due to resources and/or costs, there is a great opportunity to help patients globally through the design and implementation of medicinal therapeutics. Improved diagnostic tools that are biologically based could shed light to the elusive window of therapy where symptoms are not present, bolstering the odds of successful translation of therapeutics that modulate time-dependent CAVD processes. We have gained understanding of intricacies regarding the interplay of inflammation, VIC-VEC interactions, and mechanics in valve disease, which can pave the way for the development of effective biologically-based

and targeted therapeutics. New proteomics and sequencing tools provide an opportunity for investigation of more nuanced biological processes, and will allow for further uncovering of unanswered questions that still remain regarding CAVD.

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Abbreviations

CAVD	Calcific Aortic Valve Disease
LVH	Left Ventricular Hypertrophy
ECM	Extracellular Matrix
GAG	Glycosaminoglycans
NO	Nitric Oxide
EBCT	Electron Beam Computed Tomography
AS	Aortic Stenosis
AFM	Atomic Force Microscopy
RF	Rheumatic Fever
RHD	Rheumatic Heart Disease
TAVR	Transcatheter Aortic Valve Replacement
SAVR	Surgical Aortic Valve Replacement
LV	Left Ventricle
VIC	Valvular Interstitial Cell
VEC	Valvular Endothelial Cell
eNOS	Nitric Oxide Synthase 3
aSMA	alpha-Smooth Muscle Actin
OGM	Osteogenic Media
PAVIC	Porcine Aortic Valvular Interstitial Cells
HUVEC	Human Umbilical Vein Endothelial Cell
ROCK	Rho-Associated Protein Kinase

BMP	Bone Morphogenic Protein
CNP	C-type Natriuretic Peptide
EndMT	Endothelial to Mesenchymal Transition
LPS	Lipopolysaccharide
ALP	Alkaline Phosphatase
TGFB	Transforming growth factor beta
LDL	Low-density lipoprotein
TNFα	Tumor-Necrosis Factor Alpha
ICAM	intercellular adhesion molecule-1
LFA	Lymphocyte function-associated antigen 1
VEGF	Vascular endothelial growth factor
VCAM	Vascular cell adhesion protein 1
ALCAM	Activated Leukocyte Adhesion Molecule
FDG-PET	Fluorodeoxyglucose positron emission tomography
HDL	High-density lipoprotein
OxLDL	Oxidized low-density lipoprotein
MMP	Matrix Metallo-Proteinase
Lp(a)	Lipoprotein(a)
vWF	Von-Willebrand Factor
NFκB	Nuclear Factor Kappa B
TLR	Toll-like Receptor
ROS	Reactive Oxygen Species
IkB	Inhibitory Kappa B
IKK complex	IkB Kinase Complex
IKKB	IKK-complex Beta subunit
ARS	Alizarin Red S
OCN	Osteocalcin
EMD	Anthraquinone Emodin
PEG	Polyethylene Glycol

PAVEC	Porcine Aortic Valvular Endothelial Cells
PAEC	Porcine Aortic Endothelial Cells
CAD-11	Cadherin 11
HA	Hydroxyapatite
DN	Dominant-Negative

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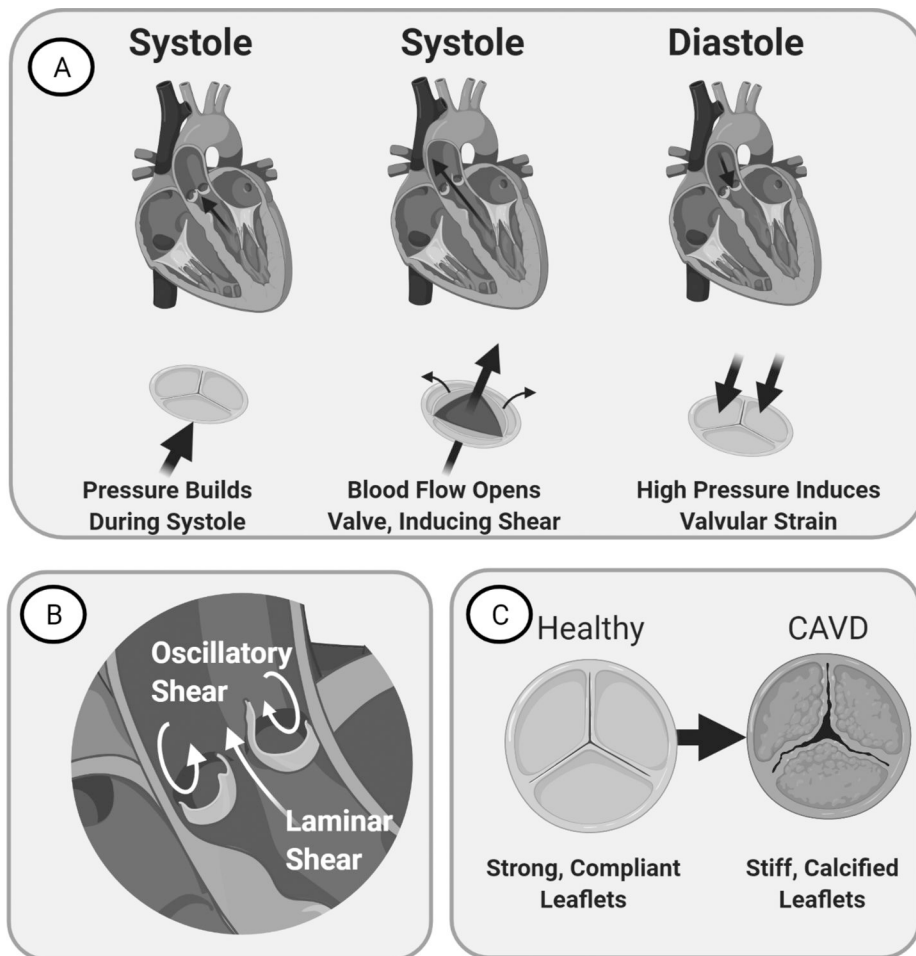
Figure 1. Anatomy and Physiology of the Aortic Valve in homeostasis and disease.
(A) Left: Valve just before systole. Blood flow assists opens the valve. Middle: Valve during Systole. Leaflets ends bend to create an opening for blood flow. Right: Valve during Diastole. Valve provides seal that resists back-flow pressure, inducing strain. (B) Image of the valve demonstrating laminar flow on the ventricularis side and oscillatory flow on the fibrosa side. (C) CAVD manifests as valvular thickening with stiffening and calcification that reduces leaflet mobility over time.
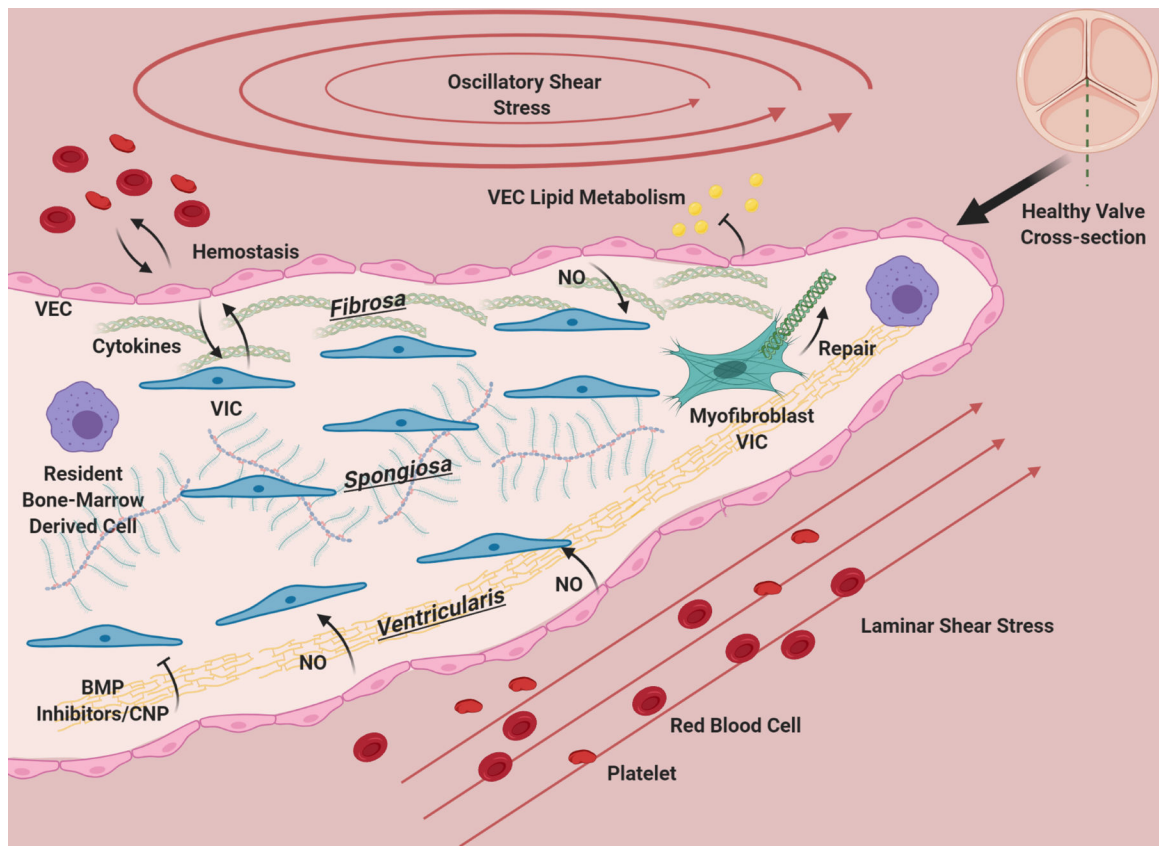
Figure 2. The aortic valve during homeostasis (cross-section).

The aortic valve has three layers: the fibrosa, consisting primarily of circumferentially oriented collagen, the spongiosa, consisting primarily of GAGs, and the ventricularis containing radially-aligned elastin. A diverse population of interstitial cells broadly defined as VIC inhabit these layers in the interstitium of the valve (blue) and become activated to remodel the ECM of the valve as needed (green). VEC line the valve and provide a barrier from the blood, preventing clotting, mediating infiltration of lipids, nutrients, and modulating extravasation of inflammatory cells. VEC (pink) promote quiescence in VIC through nitric oxide signaling, and VIC and VEC also communicate with cytokines. Osteogenic inhibitors such as BMP inhibitors and CNP from the ventricularis endothelium likely maintain homeostasis by preventing VIC activation. The homeostatic valve also contains resident immune cells (purple), whose functions are just beginning to be uncovered. The ventricularis of the valve experiences laminar shear, while the fibrosa has oscillatory flow. The valve is constantly under tension/compression as the valve opens and closes, and resists pressure. The ECM composition of the valve and cellular maintenance ensures that it remains flexible, strong and compliant so that it is durable over a human lifespan.
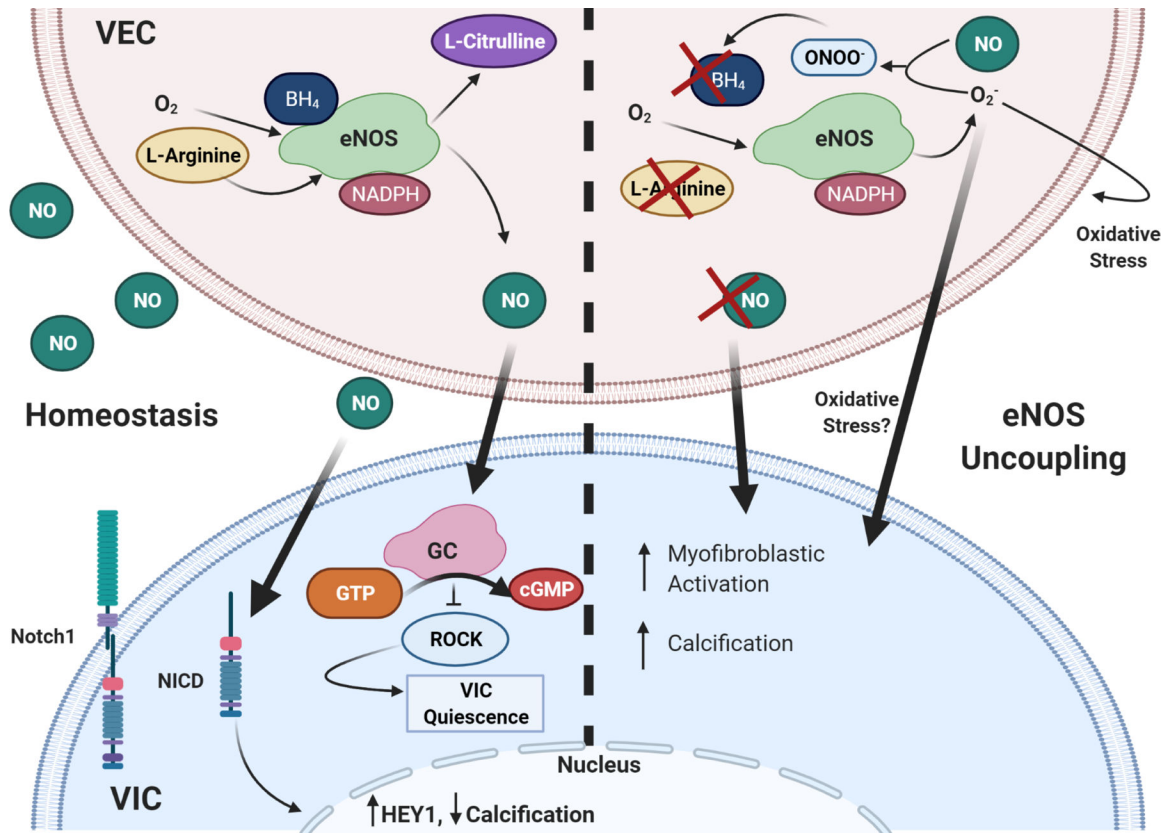
Figure 3. Role of NO and eNOS uncoupling in CAVD.

NO signaling from VEC stimulates VIC quiescence through activation of GC/cGMP pathway and subsequent downregulation of RhoA pathway. It also prevents calcification through assisting with Notch1 translocation to the nucleus. eNOS uncoupling causes endothelial dysfunction, such that there is little to no NO signaling to VIC, increasing myofibroblastic activation and calcification. ROS is known to induce cell damage, Superoxide can further cause oxidative stress in VEC and VIC. Thick arrows are VIC-VEC signaling, thin are intracellular or autocrine VEC effects.
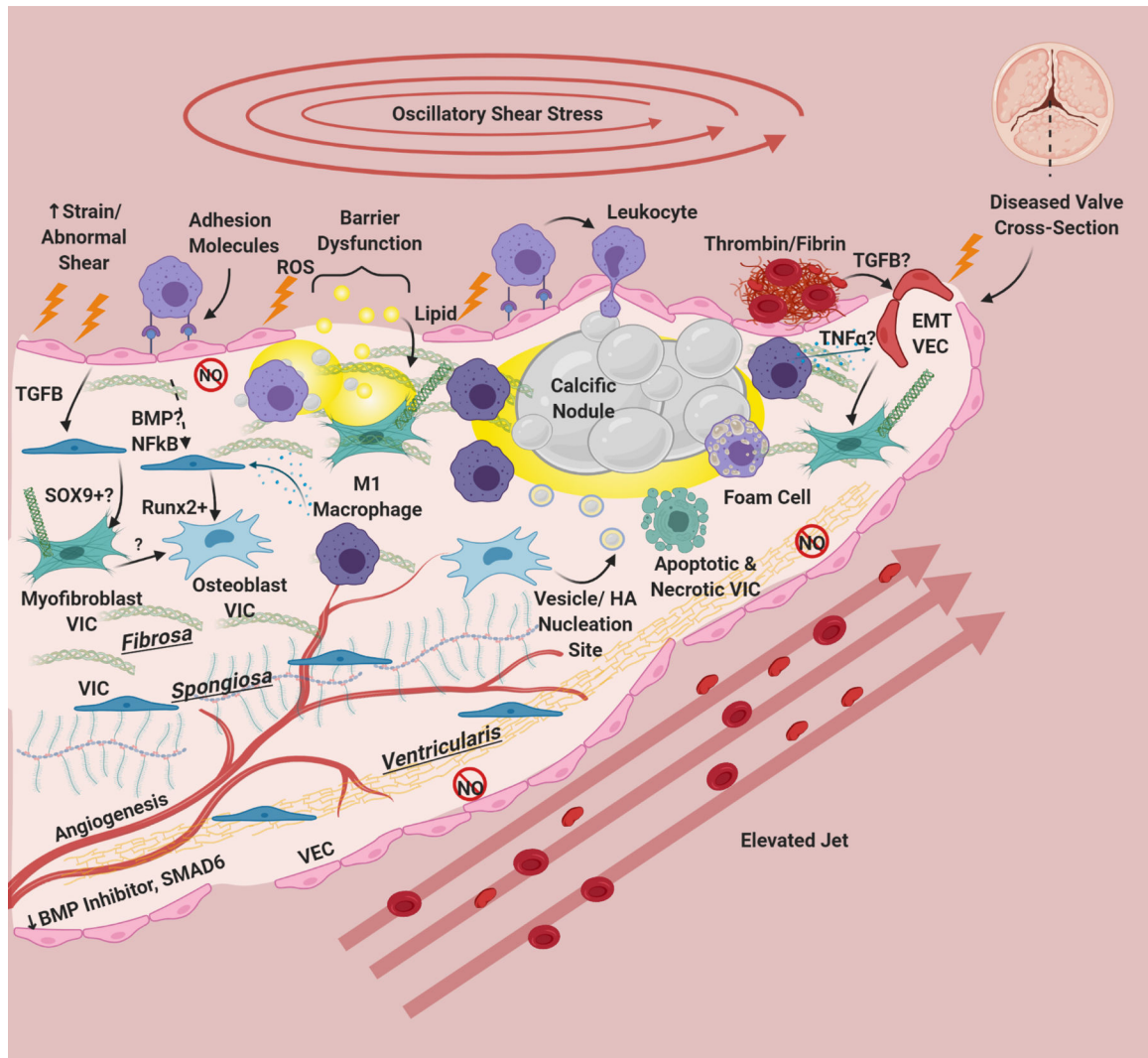
Figure 4. The Aortic Valve in CAVD.
Risk factors cause damage, inflammatory adhesion expression, barrier disruption, and lipid deposition in/underneath the endothelium. Deposited lipids, specifically oxidized lipids can cause inflammation and damage in resident cells. Endothelial dysfunction results in eNOS down-regulation and dysfunction, abrogating protective eNOS signaling to VIC and in hemostasis. This is especially apparent on the ventricularis side of the valve. There is also a reduction in protective BMP inhibitors and SMAD6 on the ventricularis side of the valve. Leukocytes bind to VEC adhesion molecules and undergo extravasation into the tissue. Clotting factors including thrombin, tissue factor, and fibrin have been associated with calcified valves, and platelets recently have been implicated as sources of TGFB in valve disease. TGFB from VEC has been shown to induce Sox9 expression and to produce activated myofibroblast-like VIC. Paracrine signals from macrophages, specifically M1 macrophages has been shown to induce osteogenic differentiation of VIC. NFκB and BMP (possibly) from VEC can also modulate VIC osteoblastic differentiation. Osteoblastic VIC may excrete vesicles or undergo apoptosis/necrosis that deposit calcium and create HA nucleation sites, which grow over time to form large calcific nodules. Inflammatory infiltrate

localizes around nodules, and areas of angiogenesis. Foam cells are observed in CAVD, but do not form dense necrotic cores. Inflammatory cytokines like TGFB and TNFα can cause VEC to undergo EndMT, creating fibrotic activated VIC-like cells. Thickening of the valve happens not only through lipid deposition/calcification but also fibrotic remodeling from activated VIC. CAVD induces angiogenesis in the normally avascular leaflet. For reasons unknown but hypothesized to be due to laminar shear stress, the ventricularis of the CAVD valve remains largely unaffected
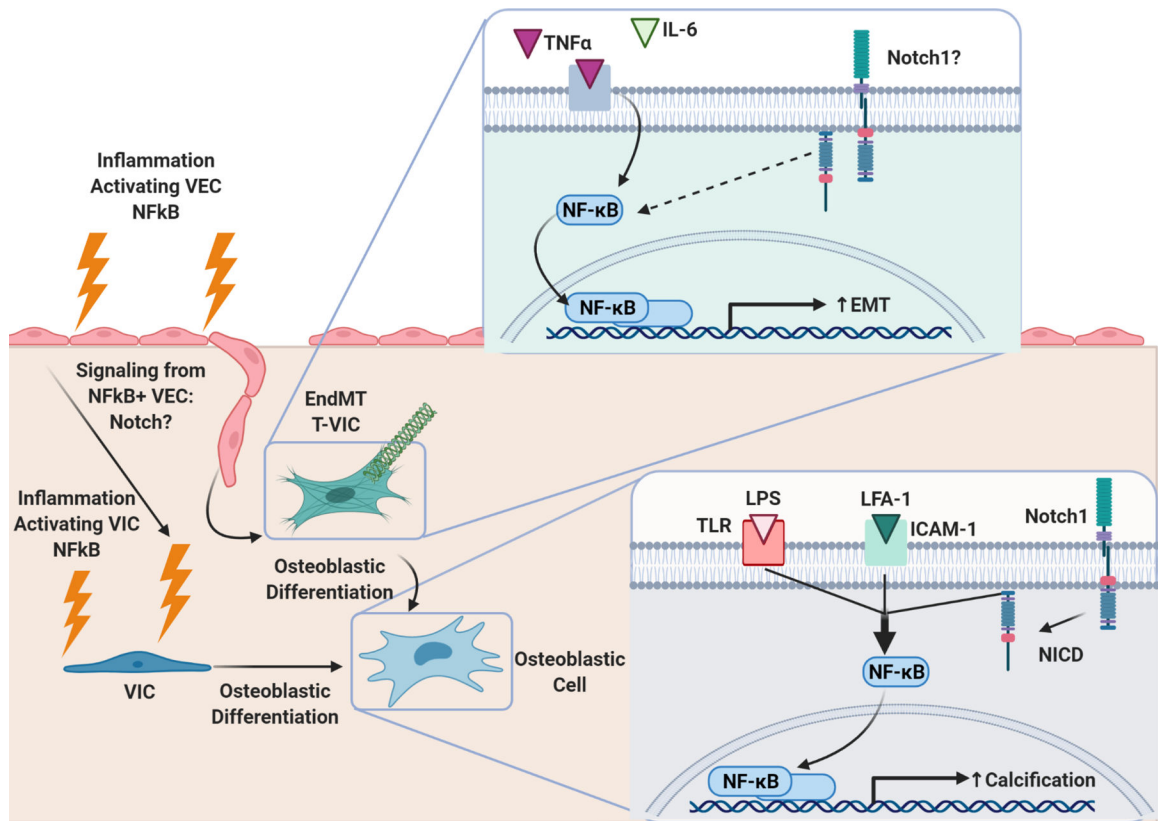
Figure 5. Inflammatory pathway NFκB Modulates CAVD intracellularly and through cell-cell interactions.

Bottom Right: NFκB activation in VIC through concerted efforts of inflammatory LPS/TLR signaling, LFA-1/ICAM-1 signaling, and Notch-1 signaling induces VIC osteoblastic differentiation and calcification. Left: Inflammation induces NFκB activation in VEC. NFκB+ VEC undergo EndMT, transforming into VIC (T-VIC). NFκB+ VEC have also been shown to exhibit evidence of osteoblastic differentiation. Knock-out of valve-specific NFκB in CAVD models prevents calcification and adverse valvular remodeling, consistent with the paradigm that NFκB activation in VEC promotes pathologic differentiation of VIC. Top right: Inflammatory cytokines TNFα and IL-6 activate NFκB signaling in VEC, which induces EndMT. It is unknown if Notch1 also plays a role in NFκB signaling in VEC. If so, Notch pathway could be a method through which VIC and VEC communicate in response to inflammation.

Mechanical Stimulus: Shear Stress Timepoint Observations

Key:
3D culture above line, 2D below line
■ *VIC (italicized)*
■ VEC (standard)
■ **Co-culture/Explant (bold)**

48 hours:
Cells in 3D collagen type 1 gels were exposed to constant shear. Alignment perpendicular to laminar shear was observed. Oscillatory shear led to upregulation of EndMT- (ACTA2, Snail, TGFB1) and inflammation-(ICAM1, NFKB1) related gene expression, matrix invasion, EndMT-related (αSMA) protein expression[214].

14 days:
Greater cGMP production was observed on the fibrosa side of the shear-stress patterns. On both sides, OGM media ↑ α-SMA expression. In co-culture with OGM, BGLAP and RUNX2 expression ↑. Hemodynamics regulate endothelial NO and calcification of valve leaflet. No signaling is side specific and regulated by shear. In static and flow patterned shear, cGMP expression was increased.[82]

48 hours:
↑ in BMP-4, TGF-β1, ICAM-1, and VCAM-1 expression were observed[123].

14 days:
On the fibrosa side, shear ↑BMP-dependent inflammation[11].

Immediate 0-48hrs.	Intermediate 3-7 days	Advanced 14 days

48 hours:
CAD11 was highly expressed. Shear resulted in downregulation of CAD11, CAV-1 protein, and BMP-4[213].

48 hours:
Cells expressed α-SMA and calponin, SMC markers. Cells aligned in the direction of laminar shear (LS). LS ↑ eNOS expression in static and OS conditions. LS ↓BMP-4, ↑IkB expression. LS ↑ phosphorylated eNOS. eNOS and Klf2 were at the mRNA level[216].

48 hours:
Minimal alignment with, α-SMA increase. BMP-4, , CAD11, and CAV-1 were downregulated while CYP1A1 was upregulated[224].

Figure 6. Mechanical Stimulus: Shear Stress Time Point Observations.
The findings of various studies observing the effects of shear stress over the course of the experiments. Based on the time points of experimental findings, the observations are classified as Immediate (0–48 hrs.), Intermediate (3–7 days), and Advanced (14 days).

Mechanical Stimulus: Cyclic Stretch/ Constant Tension Timepoint Observations

Key:
3D culture above line, 2D below line
- VIC (italicized)
- VEC (standard)
- Co-culture/Explant (bold)

24 hours:
Osteoblast-like VIC (OVIC) showed f-actin fibers aligned with tension. ↑ expression of αSMA. OVIC had ↑of RhoA mRNA than control VIC at day 1, pointing to a quick mechanotransducive response[6].

Day 5
↑ disorganization of f-actin fibers in direction of tension and ↑ expression of aSMA. ALP dominantly upregulated over Runx2. Cell and pro-calcific response in OGM. OGM inhibited ↑ in internal stress, change in VIC ability to regulate leaflet stiffness [6].

7 days:
Fibrosa side showed ↑ Ca2+ (calcification) compared to ventricularis side under 15% stretch. In OGM media, Runx2 expression ↑. Stretch & OGM w/ ↑ phosphate and TGF-β1 levels required for Ca2+[11].

14 days:
(15%) strain ↑ Ca2+ 5x compared to (10%). Noggin blocked stretch induced Ca formation. Rapid ↑ RhoA/Rock in static 3D culture[11].

14 days:
Stress free VIC, no Ca2+ in OGM. Ca observed in 3D stressed gels in OGM. No Ca2+ in control. VIC showed Ca2+ while VEC had no Ca2+ with mechanical strain, statically anchored[34].

14 days:
Cells ~2.5x more contractile w/ ~ 3x type I collagen compared to the control. With cyclic tension, ↑ TGF-β1 resulted in ↑ in contractile and biosynthetic proteins[211].

14 days:
Nodules cells + for αSMA, collagen disruption observed near the nodules. Compared to the control, OVIC showed ↑ in pro-Ca2+ cytokine TGF-β1 initially[6].
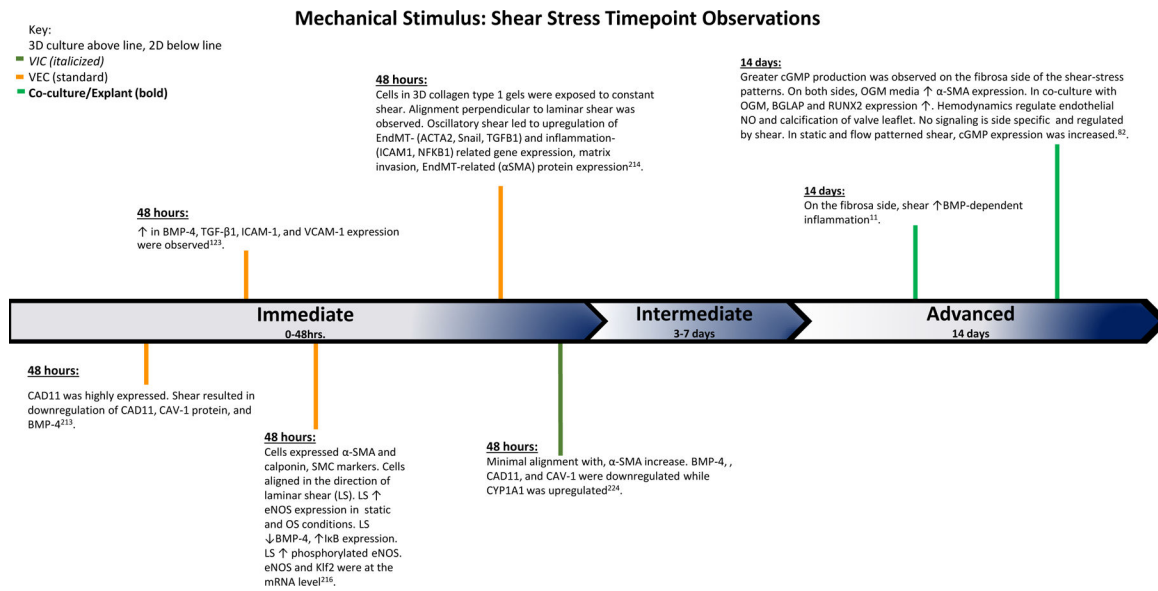
| Immediate 0-48hrs. | Intermediate 3-7 days | Advanced 14 days |

2 hours:
Cyclic strain ↓ MCP-1, VCAM, and GM-CSF mRNA[157].

24 hours:
Pro-inflammatory molecules at 5% and 20% strain. 10% strain had no inflammatory response. At 5% and 20%, up-regulation of adhesion molecules VCAM-1, ICAM-1 and E-selectin were ↑ compared to the 10% strain[207].

24 hours:
In Fibrosa , beta-1-integrin was ↑ at the 20% strain. Cells from the ventricularis were more reactive to cyclic strain[208].

4 days:
Cell orientation and matrix fiber alignment was driven by cyclic biaxial strain, leading to extracellular matrix remodeling[220].

30 minutes:
Rho kinase activity ↑ and returned to baseline after 60 min. w/ 15% stretch. ROCK activity was ↑ 1.6x (Bouchareb et. al. 2014). Cells swelled and formed a circular shape. RhoA hypothesized to play role in mineralization of VIC in response to strain. Inhibition of ROCK downstream of RhoA with HA1100 ↓ strain-induced mineralization [209].

24 hours:
120x increase in mineralization w/ 15% strain. Mechanical strain + mineralizing medium ↑ enzyme activity 12.5x at 15% .(MVICs) ↑ expression of osteoblastic genes was up-regulated following strain (Fig. 1C). ENPP activity ↑ 12.5x w/ mineralizing media + stretch. ENPP1 promoted apoptosis when overexpressed[209].

24 hours:
VCAM-1 expression ↑ at 5 and 10% strain vs. 15 and 20% . GM-CSF expression was ↑at 5 and 20% strain . Cyclic strain ↓ VCAM-1, MCP-1 and GM-CSF mRNA in VICs. 15% strain ↑ pSMAD 2/3. IkB ↑ inhibiting NFκB w/ strain[157].

48 hours:
Biaxial strain anisotropy ↑ cell proliferation and apoptosis. Cyclic stretch↑ f-actin. ACTA2 gene downregulated[220].

48 hours:
Cells under 15% strain showed a necrotic core surrounded by an apoptotic ring. TGF-β1 mediated remodeling of the cell monolayer. αSMA expression was ↑ in the monolayer in the presence of TGF-β1. TGF-β1 treated cells ↑ αSMA expression leading to ↑r tension[121].
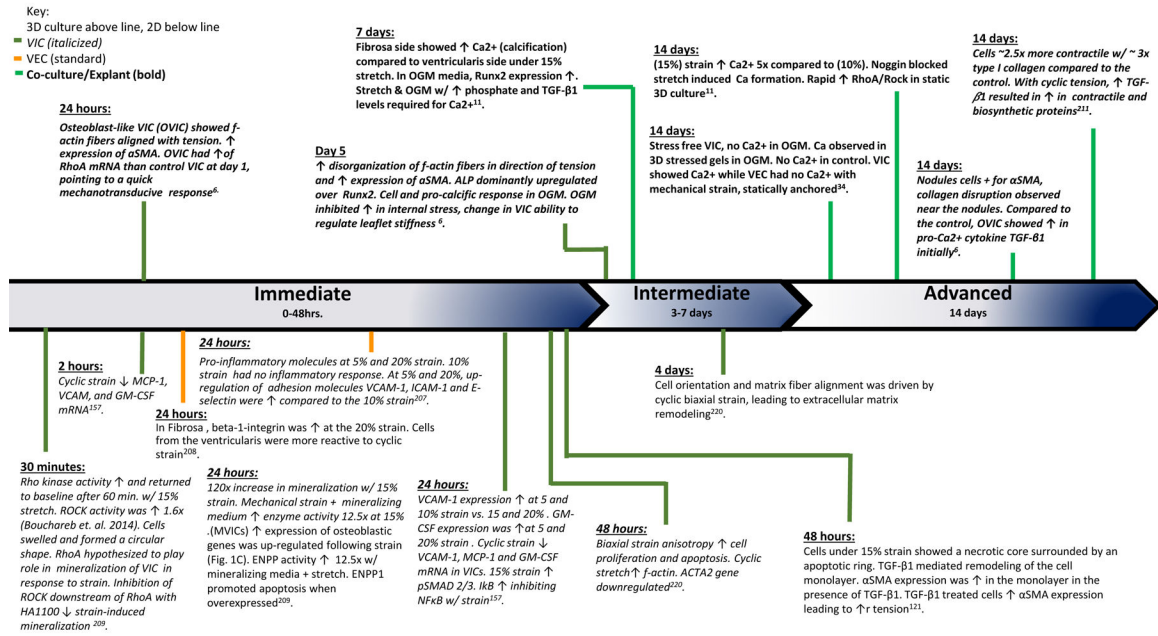
Figure 7. Mechanical Stimulus: Cyclic Stretch and Constant Tension Time Point Observations:
The findings of various studies observing the effects of cyclic stretch and constant tension over the course of the experiments. Based on the time points of experimental findings, the observations are classified as Immediate (0–48 hrs.), Intermediate (3–7 days), and Advanced (14 days).

Table 1.

Risk factors linked to valve deterioration and disease.

Per risk factor, in-vivo, in-vitro, and human observations are presented from various studies.

Risk Factor	How it could cause CAVD	*In-Vivo*	*In-Vitro*	Human
Genetic MutationsNotch1, LPA gene, PALMD, TEX41	-Valve malformation in development could lead to abnormal biomechanics. -NOTCH1 mutations cause developmental defects in the aortic valve, de-repression of the composition of calcium leads to AVD[23]. -LPA, PAMLD and TEX41 variants associated with valve defects lead to malformation[23,24].	-In mice, Notch1 repressed Runx2 activity, a transcriptional regulator of osteoblast cell fate[15].	-In VIC, endothelial NO stimulus plays a role in downstream Notch1 signaling leading to anti- calcific effects[25].	-Genome wide association study (GWAS) of two families with genetic histories of valve disease revealed Notch1 mutations in family members affected[15]. -Multi-ethnic study found variation in the LPA locus is associated with AVD and calcification[24]. -GWAS identified PALMD and TEX41 variants ↑ risk of CAVD by 20–28% and 15%, respectively, per copy of allele[23].
Smoking	-Mechanism unclear[26].	Potential area for further study.	Potential area for further study.	Strong association between Degenerative Aortic Valve Disease in a study of 756 healthy male workers[26].
Hypertension (HTN) /Increased Valve Resistance	-Altered shear profiles on endothelium. -Altered cyclic strain magnitude/profile. -Changes in local stiffness sensed by cells. -Stage I/II HTN associated with Aortic Valve Calcium (AVC)[27].	-*Ex-vivo* models of porcine valves showed nodule formation on the aortic side under 15% stretch HTN conditions in OGM media with TGF- β1[28].	Potential area for further study.	-HTN group ↑ progression of stenosis. Angiostatin converting enzyme inhibitors (ACEIs) group ↓ progression, patients on angiotensin receptor blockers (ARBs) ↑ progression than ACEIs but ↓ than patients with HTN[27].Patients <65 with ↑ systolic and pulse pressure were prevalent in AVC[29] .
Hyperlipidemia	-Infiltration of ApoB containing lipoproteins is a key initiator of an inflammatory response promoting atherosclerosis[30]. -Local ROS causes lipids to oxidize, which ↑ inflammation, prevents phagocytosis, and can be calcium nucleation sites[31,32].	-In SMC-specific PTEN deletion mice, AKT upregulated Runx2 which promoted VSMC calcification[31]. -WT, LDLR–/–, LDLR–/– Apob100, ApoE–/– mice +/– High fat diet. >50% ↓ in valve diameter, ↑ systolic pressure,	-Oxidized cholesterol stimulates calcification in VICs[33].	-ApoB (LDL, triglyceride rich remnants of VLDL, IDL, chylomicron remnants, and Lp(a) are present in all atherogenic lipoproteins[24].

Risk Factor	How it could cause CAVD	*In-Vivo*	*In-Vitro*	Human
		left ventricular hylertrphy[32].		
Diabetes/ Hyperglycemia	-Atherosclerosis causes ↓ NO and ↑ ROS due to insulin levels[34]. -Recent studies have associated high glucose with inflammation and pro-calcific gene expression in VIC/VEC model[35,36].	Potential area for further study.	-VIC actively remodeled when in hydrogels, ↑ expression of ECM proteins and MMPs. Exposure to TGF- β1 led to upregulation of BMP-2/4, SMAD, Runx2 and ↑ calcium deposition[35].	-L-arginine transport ↑ hCAT-1 activity leading to accumulation of ROS, which ↓ NO[34.].
Infection 16,3717,3817,38	-Rheumatic fever (RF) driven by an autoimmune response leading to valve damage, if untreated[16.] -Periodontal bacterial infection[37].	-Upon ligand binding, TLR dimerization activates pro-inflammatory molecules through the activation of NF-κB[37].	Potential area for further study.	-If untreated RF can cause cardiac inflammation, eventually leading to Acute RF, leading to valve damage[16].
Sex	-Male valves calcify where female valves fibrose. Many known disease pathways exclusive to each sex[38].	Potential area for further study.	Potential area for further study.	-Higher fibrosis scores observed in AS valves in the female sex. In male sex, calcification correlated with fibrosis. BAVs and TAVs can be correlated to calcific aortic stenosis. Post-operatively, remodeling of the left ventricle more prominent in females. Females displayed a 50% ↓rate of stroke, coronary bypass surgery, and 31% ↓ total mortality rate in a 4 year follow up period[38].
Kidney Disease	Abnormal phosphate, calcium, and vitamin D levels, which ↑ formation of calcific deposits[39].	Potential area for further study.	Potential area for further study.	- ↑ progression in patients with end stage kidney disease compared to the general population. Mineral imbalances such as Ca and Vitamin D could ↑ bone formation[39].
Age	The valve thickens and stiffens with increasing age[40].	Potential area for further study.	Potential area for further study.	-The fibrosa thickened with age, collagen ↑ in the valve layers, especially in the

Risk Factor	How it could cause CAVD	*In-Vivo*	*In-Vitro*	Human
				fibrosa/ ventricularis layers[40].