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Chapter 7

Genetics of Bicuspid Aortic Valve and Calcific Aortic Valve Disease

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1. The clinical taxonomy: Malformation vs. disease

Aortic valve malformation is a spectrum including Bicuspid Aortic Valve. Aortic valve malformation has been appreciated since the Renaissance when artists advanced our understanding of anatomy and specifically, Leonardo da Vinci illustrated and described variants of aortic valve morphology [1]. Aortic valve malformation is the most common cardiovascular malformation (CVM), and bicuspid aortic valve (BAV, MIM#109730) is the most common type of aortic valve malformation. BAV is present at birth and is characterized by two rather than three cusps. The incidence of BAV is 1-2% in the general population and affects an estimated 3 million people [2,3]. BAV itself is subclinical and the valve is typically functional, making BAV an endophenotype. Two patterns of BAV morphology are commonly observed: ~70% of isolated cases have fusion of the right and left (RL) coronary cusps with the remainder consisting almost entirely of those with fusion of the right and non (RN) coronary cusps [4,5]. Rarely, cases have shown fusion of the left and non (LN) coronary cusps. In addition to BAV subtypes, there is a spectrum of aortic valve malformation (Figure 1), ranging from various types of unicuspid to quadricuspid aortic valves with the three BAV morphology patterns and a thickened tricommissural aortic valve representing intermediate phenotypes [7]. Presently, it remains unclear to what degree these variations of malformation represent true differences.

Calcific Aortic Valve Disease is a growing public health problem. Aortic valve disease is defined by abnormal valve function. Valve disease may manifest as *stenosis*, an obstruction to normal forward blood flow, or *insufficiency*, a defective closure resulting in backward blood flow. Valve disease tends to progress. Ultimately, ventricular function can be compromised. Aortic valve stenosis is the most common manifestation of CAVD and classically presents as angina, syncope and heart failure. The diagnosis can be made clinically and confirmed by echocardiography, which quantifies the severity, and, over time, the progression of disease [8].

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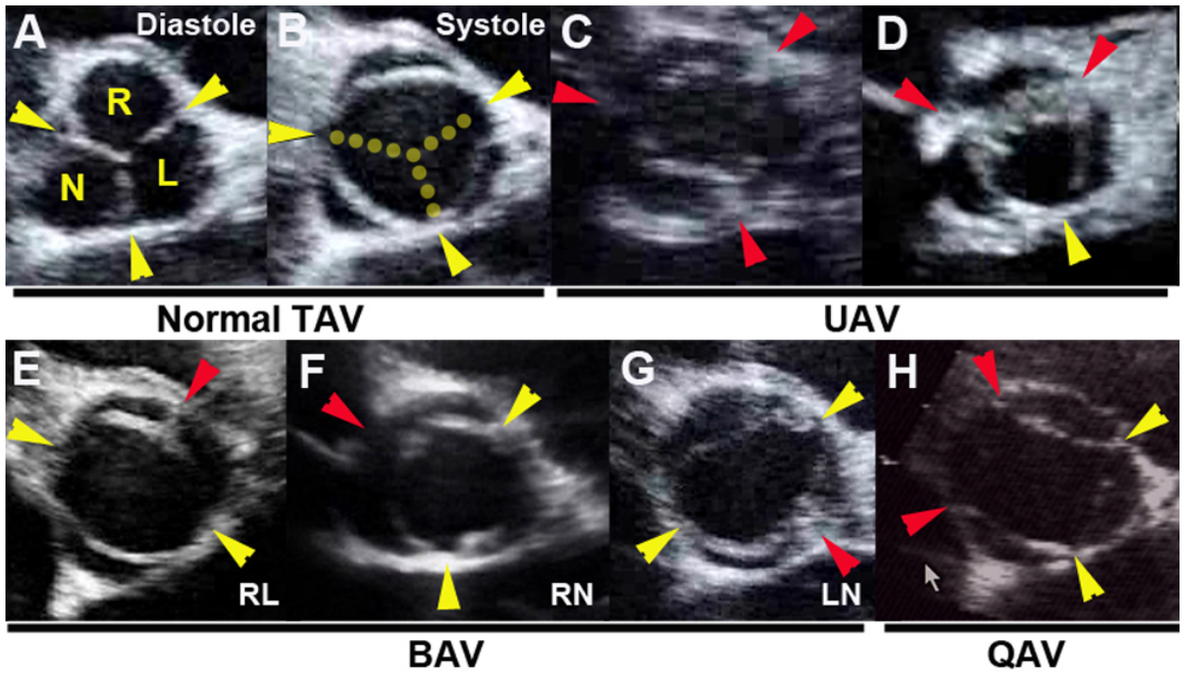


Figure 1. Phenotype definition: spectrum of aortic valve malformation. Aortic valve malformation Parasternal short axis echocardiographic views at the base of the heart showing the aortic valve en face (A-H). Normal tricommissural aortic valve (TAV) morphology is demonstrated in diastole (A) and systole (B). Distinct morphologies are based on fusion patterns of the commissures (dotted lines, B) as they relate to the right (R), left (L) and non (N) coronary sinuses of Valsalva (A). Aortic valve malformation ranges from unicuspid (UAV) to bicuspid (BAV) to a thickened tricuspid (not shown) to quadricuspid (QAV) morphology. Three normal commissures are demonstrated in panel A, and normal opening of the commissures results in complete cusp separation to the wall of the aorta at the sinotubular junction (yellow arrowheads). UAV manifests as either partial fusion of all three commissures (red arrowheads, C) or complete fusion of both the RN and RL commissures (D). Bicuspid aortic valve (BAV) may manifest as fusion of the RL (E), RN (F), and rarely LN (G) commissures. Rarely, a quadricuspid aortic valve (QAV, H) is identified. Adapted from [6].

Histopathology from diseased valves explanted at the time of surgery from patients with CAVD demonstrates large nodules of overt calcification, in addition to cell-matrix abnormalities (Figure 2). Research efforts have focused on the valve cusp, and as a result the valve annulus has been largely overlooked [4,7,9]. Human studies investigating valve disease have suggested that the base of the valve cusp and valve annulus regions is the origin of disease processes, including both sclerosis and calcification [10,11]. Greater than 2.5% of the population has AVD, causing more than 25,000 deaths annually in the US [12,13]. The actual direct cost for valve disease in the US alone has been estimated at 1 billion dollars per year [14]. Taken together, the public health impact and burden to society of CAVD is significant and underappreciated. The majority of valve disease at any age has an underlying valve malformation suggesting a genetic basis [15]. Aging is an independent risk factor for CAVD, resulting in a higher prevalence of disease as the population achieves greater longevity [16,17]. Aortic valve sclerosis, a marker of cardiovascular risk, and to a lesser extent valve disease, is present in more than 25% of the aged [18]. Therapy for CAVD remains primarily surgical and is restricted to late stage disease. Aortic valve replacement is the second most frequent cardiovascular surgical procedure [3,9], and the need for re-intervention is common [19]. Bioprosthetic replacement approaches are effective, but not durable [20,21]. Because there is a lack of

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pharmacologic treatments for CAVD, the indications for surgical intervention dominate the clinical landscape. Early disease processes and progression remain poorly understood, and there are presently no pharmacologic based treatment options for CAVD.

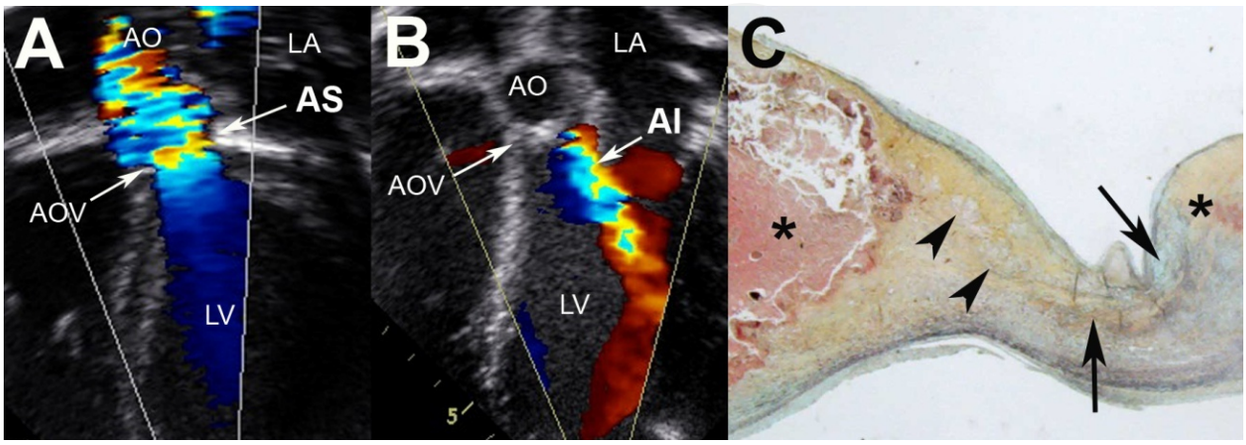


Figure 2. Phenotype definition: types of aortic valve disease. Color Doppler echocardiographic apical four chamber images demonstrate the two basic types of aortic valve disease. Aortic valve disease is characterized by a dysfunctional valve and is classified as stenosis (obstruction, A) and/or insufficiency (incompetence, B). Aortic stenosis (AS) and aortic insufficiency (AI) result in hemodynamic perturbations that lead to clinical disease states. Advanced calcific aortic valve disease is typically characterized by stenosis, and histopathology identifies gross calcific nodules in the fibrosa layer of the cusp (asterisks, C), clusters of cartilage like interstitial cells (arrowheads, C), and marked heterogeneity of extracellular matrix abnormalities (arrows, C). AO aorta; AOV aortic valve; LA left atrium; LV left ventricle.

Bicuspid Aortic Valve is an independent risk factor for Calcific Aortic Valve Disease. BAV is an established risk factor for CAVD [3,7,13]. The majority of CAVD cases at any age have an underlying BAV, and longitudinal studies in young adults with BAV have shown that >20% ultimately require surgical intervention [15,22,23]. In addition, those CAVD patients with an underlying BAV tend to develop calcification a decade earlier than those with normal aortic valve morphology [24]. Recently, a National Heart Lung and Blood Institute Executive Statement on CAVD identified a critical need to identify "clinical risk factors for the distinct phases of initiation and progression of AVD" [25], where standard cardiac risk factors including sclerosis have not yet been applicable. There has been avid interest and conflicting reports regarding the potential use of BAV morphology as a specific predictor of CAVD. Fernandes et al identified an association between RN BAV and AVD in a pediatric population, while Tzemos et al found no association in an adult population [5,22]. Exploring AVD in a pediatric population allows for examination of the disease process free from the confounding effects of cardiovascular comorbidities. Risk factors for AVD in children are poorly understood [23], but recently Calloway et al. reported that children with RN BAV and adults with RL BAV were more likely to develop AVD, suggesting BAV morphology may have predictive value for the time course of AVD [26]. It is unclear if AVD in children, which is not characterized by calcification, represents a different genetic type of disease or one end of a spectrum of the same disease.

Careful clinical phenotyping is critical for research, especially genetic discovery. Phenotype definition and stratification are necessary to advance our understanding of CAVD, especially in the context of genetic discovery. In addition to distinguishing malformation from disease, CAVD phenotyping needs to be detailed and comprehensive using all aspects of the clinical taxonomy, even those currently considered clinically inconsequential. The first step of any human genetic research study is to clearly and precisely define the phenotype. Studies that use too broad or too narrow a phenotype definition may fail to find association with an existing genetic variant or identify a pathologic one. Thus, identification of the phenotype most aligned with the underlying genetic etiology is essential for successful identification of associated genetic variants, a concept recently described as "deep phenotyping" [27]. Cardiovascular risk factors have been established for a variety of cardiovascular diseases, including substantial overlap for CAVD and coronary artery disease (CAD) or atherosclerosis [16,28,29]. While these disease processes often co-occur, as evidenced by the high frequency of concurrent coronary artery bypass grafting and aortic valve replacement surgery, only a small proportion of CAVD patients have CAD [30]. Likewise, there is an increased incidence of CAVD in patients with other cardiovascular disease, including systemic hypertension and chronic kidney disease [31,32]. Substantial investigation has established the adverse effects of common comorbid cardiovascular diseases on the progression of AVD; however, increasing attention on the underlying genetic and developmental processes will identify early mechanisms that incite disease processes. Emerging evidence suggests that both specific genetic factors and clinical cardiac risks may be necessary for disease initiation and progression.

Phenotype definition must expand to include non-clinical paradigms. Like many diseases, especially cardiovascular diseases, the clinical taxonomy of CAVD is based on anatomy and physiology. Classification schemes are organized with clinical standard of care, particularly surgical intervention, in mind [33,34]. The gold standard for diagnosis of cardiovascular diseases is imaging, such as echocardiography or magnetic resonance, modalities that define anatomy and physiology. While these approaches have been clinically useful, there is substantial phenotypic heterogeneity of unclear significance, including for example, the distinction between malformation and disease. Expanding phenotype to include an improved understanding of embryologic patterns underlying malformation will provide insight into pathogenesis [35-37]. Increasingly, combinations of phenotypes long held to be independent from a clinical perspective are now understood to be related from an etiologic perspective, challenging classic notions of disease classification. Molecular insights may inform new pharmacologic treatments the same way imaging informs surgical decision-making. Ultimately, identifying the genetic causes of disease will require reconciling clinical and molecular taxonomies of disease.

2. The genetic basis of BAV and CAVD

BAV has a strong genetic basis, but the precise causes remain unknown. Heritability estimates the proportion of a disease attributable to genetics. BAV heritability estimates are high, ranging from 75 to 89%, indicating that major genetic factors contribute to the develop-

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ment of BAV [38]. Pedigree and segregation analyses have consistently identified autosomal dominant inheritance with reduced penetrance and complex inheritance underlying BAV [38-41], acknowledging that BAV is subclinical and therefore may be underestimated. Interestingly, while BAV is highly heritable, AVD is not, suggesting the phenotypic variability of CAVD is determined largely by non-genetic factors [26]. Consistent with these human observations, an established hamster model of BAV also shows the same characteristics of complex inheritance [42,43]. An additional quantitative measure of familial risk is recurrence risk. The recurrence risk of a disease measures the proportion of relatives who have the disease. BAV recurrence risk in siblings has been estimated to be approximately 9% [44], identifying further evidence of a genetic basis. Linkage analysis determines whether susceptibility variant segregates with disease in families. Previous studies have supported a strong underlying genetic basis for isolated nonsyndromic BAV, including family-based studies that have identified numerous loci [44-46]. Combined, these loci harbor hundreds of genes that may contribute to BAV. Multiple loci identify BAV as a genetically heterogeneous trait. Missense mutations in *NOTCH1* have been identified in a small proportion of nonsyndromic CAVD patients with BAV [47,48]. NOTCH1 is an intriguing biological candidate gene. In animal systems, Notch loss of function recapitulates the AVD phenotype, and actively regulates the maladaptive development of associated calcification, further supporting a mechanistic role [49-51]. In addition, a recent report described copy number variants (CNVs) in 10% of left-sided CVM cases, including BAV and aortic stenosis, potentially identifying new causes and/or modifiers of CAVD [52]. Association studies have not been used for BAV due to the large number of cases required to perform analyses (typically at least 1000), but combined linkage-association may be an excellent approach for discovery to leverage the strengths of each method. It is unclear how whole exome sequencing will impact discovery, but combining the various new tools for discovery promises to yield increasing insight into the genetic basis of BAV and CAVD.

BAV is a congenital malformation, a defect in cardiac development. Malformations present at birth often have strong genetic causes, if not monogenic etiology. Primary cardiac development occurs in humans from 2-8 weeks gestation, and semilunar valve (including the aortic valve) formation occurs in the seventh and eighth weeks. The heart is the first organ to form and continued survival of the organism is dependent on the circulation. The primitive heart tube is composed of a myocardial cell layer surrounding an endothelial cell layer. The formation of endocardial cushions is the first event of valve development. Endocardial cushion formation is accomplished by an early epithelial to mesenchymal transition (EMT) that generates a progenitor cell population embedded in a loosely organized extracellular matrix (ECM), followed by a late ECM remodeling stage that results in mature cusp organization (ventricularis, spongiosa, fibrosa) and valve interstitial cells [35-37]. Early defects in this process result in embryonic lethality, but late defects result in viable malformation and disease [53], hypothetically making the mechanisms of late developmental defects more applicable to human disease. It remains unknown why there are uneven frequencies of the different BAV types, but several developmental hypotheses have been proposed including a neural crest contribution that is not necessary but when present results in fusion of the right and left coronary cusps [42]. Further, the relatively rare unicuspid morphology underlies the majority

of cases of critical aortic stenosis in the newborn and is associated with hypoplastic left heart syndrome (HLHS), suggesting genetic ("severe" malformation) and environmental (flow perturbations) factors combine to result in disease manifestation [15,54,55]. Elucidation of the genetic basis of both BAV and CAVD will result in a reconciled classification system that integrates the molecular basis of cardiac development with the pathologic basis of disease in a clinically meaningful manner.

Genetic factors contributing to CAVD are numerous and relatively small. Common complex traits are generally the result of numerous factors, each with a small additive effect and none necessary or sufficient to cause disease [56]. Coronary artery disease (CAD) and systemic hypertension (HTN) are well-described examples of this type of trait. While there is unequivocal evidence that BAV with CAVD is a complex trait, it is not nearly as common as CAD or HTN, and is more strongly linked to developmental processes, therefore it is likely that BAV/CAVD is an "intermediate" phenotype between the "rare single-gene" and "common complex" diseases. Importantly, this suggests that it is more likely to discover clinically useful patterns of variants associated with CAVD. Clinically, CAVD, CAD and HTN are considered discrete disease states, but there is a preponderance of epidemiologic and molecular evidence suggesting some pathogenesis is shared. Therefore, variants that have been identified studying individuals with CAD and HTN may inform risk assessment in patients with CAVD. Just as some clinical cardiovascular risk factors are common to all cardiovascular disease states, some genetic variants may pertain to predisposition of any cardiovascular disease depending on the aggregate risk (Figure 3). For example, the 10q24 locus has been identified in probands from BAV, CAD, HTN, thoracic aortic aneurysm (TAA) and intracranial aneurysm families [44, 57-60], suggesting the gene(s) in this region plays a role in each of these related cardiovascular phenotypes and therefore may be a general cardiovascular risk variant. It remains unclear whether a specified number of general cardiovascular risk variants are sufficient to cause any one disease, or more intuitively both *specific* and *general* disease variants are necessary.

CAVD is a latent phenotype, an injury or defect in valve maintenance. Typically, aortic valve disease does not manifest until the fourth or fifth decade of life and often does not progress to require surgical intervention until a decade later. How can developmental defects be functional for so long, only to fail in adulthood? The prevailing view is that individuals with a genetic predisposition for CAVD require an additional "second" insult to trigger disease initiation and progression that otherwise would not have occurred. While many of the genes that have been implicated in CAVD effect valve development [61,62], they may have additional distinct roles in valve maintenance [63], that is, how the tissue responds over time to the hemodynamic demands of constant motion and changing physiology. Similarly, there are genes that do not have a role in valve development but may be necessary for valve homeostasis [35,63]. Indeed, CAVD has been labeled a "degenerative" condition for decades, and age-related "wear and tear" contributes to valve failure. For example, elastic fiber degradation occurs with advanced age and predisposes the individual to inflammation, which may contribute to CAVD acceleration in later life [64,65]. Equally important, however, are comorbid conditions such as CAD that may serve to be an injury, or second hit, in vulnerable aortic valve tissue. For example, in an individual with genetic variants predisposing specifically for CAVD, the presence of CAD

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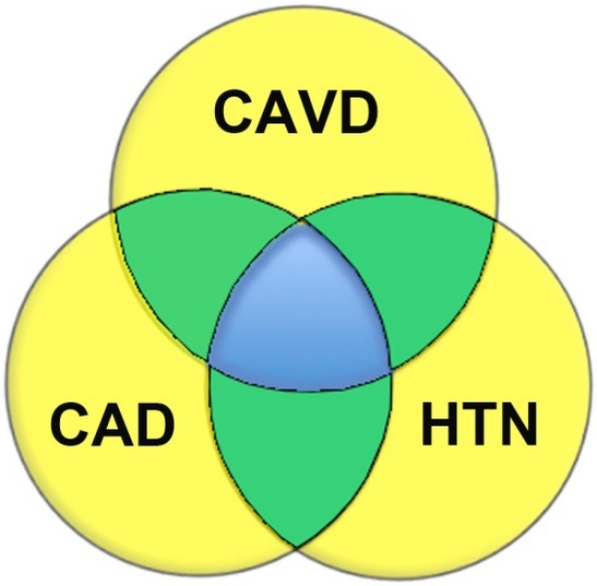


Figure 3. Shared predisposing genetic risk variants in common cardiovascular diseases. Cardiovascular diseases characterized by complex inheritance may have genetic variants specific to the clinical disease state, e.g., CAVD, CAD, HTN (yellow), as well as nonspecific genetic variants that may contribute to two (green) or three (blue) different cardiovascular diseases.

may initiate additional disease processes that incite CAVD (e.g. endothelial dysfunction). Taken together, a nonspecific cardiovascular insult in the context of a specific genetic predisposition for BAV may be necessary and sufficient for the manifestation of CAVD. As the genetic and developmental basis of valve malformation and disease is elucidated, opportunities for novel medical therapies will emerge and potentially preclude or delay the need for surgery. Defining regulation of valve tissue maintenance and homeostasis will provide exciting opportunities for cell-based or molecular therapies for valve disease.

Complex inheritance is characterized by a liability threshold. Polygenic conditions are characterized by a fixed number of susceptibility genes and a liability threshold, whereby a variety of combinations of predisposing variants may reach a specified level (e.g., 3 risk variants) to cause in combination the phenotype. In general, the importance of genetic modifiers and epigenetics is rapidly emerging, but little is known about these factors in the context of BAV/CAVD. Different BAV morphologies may reflect different combinations of shared genetic variants that carry different clinical risks, e.g. CAVD, thoracic aortic aneurysm and dissection, or associated CVM. It has been shown for example that RN BAV morphology is associated with a higher risk of developing valve disease and experiencing a cardiac event [5,22]. Together, patterns of predisposing genetic variants, which may be reflected in part by anatomical subtleties such as BAV morphology, may translate to variations in clinical disease states, suggesting major modifiers play a significant role in phenotype definition. Identifying these patterns may impact care, for example by facilitating the ability to consistently predict natural history [66,67].

3. The molecular taxonomy: Genes, pathways, and proteins

Genetic syndromes provide important biologic insights. Turner syndrome is associated with BAV and aorta abnormalities, and is the only monosomy compatible with life despite the fact that the vast majority of cases result in early spontaneous abortion. Turner syndrome can occur for a variety of reasons, including nondisjunction and mosaicism, and the exact genetic abnormality correlates with the severity of the malformations with 45,X more likely mosaicism less likely to have associated CVM. While there have been some studies examining possible maternal effects in nonsyndromic CVM [68], similar studies in Turner syndrome have not identified genomic imprinting in general or specifically with regard to BAV [69]. Interestingly, BAV morphology was RL in over 95% of cases, nearly uniform and significantly more disproportionate than the general ratio [70], suggesting a genotype-phenotype relationship of potential clinical significance. This is consistent with the observation that RL BAV is more commonly associated with aortic coarctation [5]. Little is known about long-term outcomes, e.g. the prevalence of CAVD requiring surgical aortic valve replacement or associated thoracic aneurysm that dissects, and there is not a mouse model to date that recapitulates the cardiac phenotype, but involvement of one of the sex chromosomes provides novel ways to explore specific genetic factors contributing to BAV.

The classic connective tissue disorders, Marfan and Ehlers-Danlos syndromes, caused by mutations in the FIBRILLIN-1 and COLLAGEN Type 3 genes respectively, are well-known to effect the aortic valve. While there is clearly reduced penetrance for BAV in these groups, there is a significantly increased incidence for BAV in both conditions of 10-30% [71,72]. Additional genetic syndromes that affect the connective tissue include Williams syndrome and osteogenesis imperfecta, caused by mutations in the ELASTIN and COLLAGEN Type 1 genes respectively, which also have an increased incidence of valve malformation and disease [73,74]. In addition, there are a number of genetic syndromes that are associated with BAV, often in the context of complex CVM. These include aneuploidies such as deletion 4p, deletion 10p, deletion 11q (Jacobsen syndrome), trisomy 18 (Edwards syndrome), deletion 20p12 (Alagille syndrome), as well as other genetic syndromes, including Adams-Oliver syndrome and Kabuki syndrome [75,76]. Trisomy 18 is a particularly interesting entity that is associated with polyvalvular disease, an unusual type of valve disease that is characterized by malformation, including BAV, and dysplasia of the valves, a poorly understood process that does not have a clear association with CAVD but challenges the malformation-disease distinction [77]. In addition, BAV is often one of multiple CVMs in the same individual and the patterns of co-occurrence can inform cause [78]. Taken together, there is a multitude of ways that valve tissue can be affected, and a molecular understanding of these conditions will inform CAVD.

Developmental signaling pathways identify basic regulatory factors in valvulogenesis. From a cardiac development perspective, there are three transcription factors that are considered the master regulators of basic heart development, NKX2.5, GATA4 and TBX5. Loss of function mutations in each of these genes has been associated with various forms of CVM [79-83]. While none of these genes has been associated with BAV, the Nkx2.5 mutant mouse is characterized by a variety of CVMs including BAV [84], suggesting like NOTCH1, NKX2.5

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may account for a very small proportion of cases of BAV and therefore may contribute to the pathogenesis underlying CAVD. As the focus has shifted from early to late (post endothelial-mesenchymal transition) regulatory factors, the role of additional factors, such as Notch and Wnt have been studied in the context of ECM stratification in the mature cups [53,63,85]. The progression of CAVD includes activation of osteogenic gene regulatory pathways and calcification, generally localized to the fibrosa layer [25,86-88]. Atherosclerotic mechanisms have been implicated in valve calcification, and there are overlapping risk factors for CAVD and CAD as described above, suggesting endothelial injury and inflammation play a key role in disease progression [17,87,89]. However, it remains unclear if these are inciting causal factors or exacerbating factors. TGFB signaling dysregulation has been associated with CAVD and cardiovascular disease progression, especially as it pertains to fibrosis and inflammation [90-92]. During human aortic valve calcification, expression of several genes associated with osteogenesis, including *Runx2*, *osteocalcin*, *osteopontin*, *alkaline phophatase*, and *bone sialoprotein,* is induced [93-97]. There is increasing evidence that CAVD recapitulates gene regulatory interactions characteristic of osteogenesis.

The molecular basis of aberrant calcification is poorly understood. While physiologic mineralization in the context of bone development and maintenance has been used successfully as a paradigm to study aberrant calcification in CAVD [86,98], less is known about the genetic basis of disease phenotypes characterized by aberrant calcification. Vascular calcification and the calcification that can occur in advanced CAD has been studied extensively and forms some of the basis for the prevailing view that CAD and CAVD are related disease states. Using a rare genetic disease, alkaptonuria, Hannoush et al identified a metabolic link between vascular calcification and advanced CAVD in a cohort of nearly one hundred patients [99]. Importantly, CAVD in this population was present and advanced, often requiring surgery, independent of standard cardiac risk factors, suggesting a primary link in pathogenesis not related to common comorbidities. In vitro, studies have focused on vascular smooth muscle's role in calcification, especially in the context of clinical comorbidities of CAVD such as CAD and HTN, as well as the context of pathways regulating the associated inflammation and the renin-angiotensin system [92,100]. While vascular smooth muscle cells are not present in valve tissue, there are subsets of VICs that have smooth muscle cell-like properties [101,102] and the expression of smooth muscle actin is considered a marker of activated VICs, the cells implicated in CAVD progression [103].

Understanding valve tissue homeostasis or maintenance will require proteomics. Focusing on valve injury or defects in valve homeostasis or maintenance requires increasing attention to processes downstream from the transcriptional regulation that dominates cardiac development paradigms of CAVD. Proteomics is one emerging field that provides a compelling strategy to address the challenges of dynamic post-translational biology in valve tissue and will undoubtedly have significant impact on our understanding of healthy valve maintenance and CAVD pathogenesis [104]. Proteomics involves a sophisticated technical approach that requires in vitro validation and substantial bioinformatics support. Angel et al. have demonstrated a number of seminal observations by defining the semilunar valve proteome in the adult mouse using MALDI mass spectrometry [105,106]. Specifically, this rigorous and

unbiased approach has yielded the identification and characterization of global protein expression and protein-protein networking provides a specific cell-matrix definition of valve maintenance that can be used further to explore the impact of aging, physiologic hemodynamic stresses due to constant motion, and systemic pathologic insults on specific signaling and metabolic dynamics. Importantly, this study provides proof of concept in mouse that will allow the approach to leverage the power of targeted mutagenesis [107]. Despite the difficulty of obtaining healthy controls, early observations have been made in human valve disease specimens, that when compared with control tissue, demonstrate misexpression of critical matrix proteins, including specific lipoproteins, inflammatory proteins, and proteases [108]. One study has focused this approach on VICs exposed to pro-calcific stimuli and has shown that specific chaperone proteins alter transport and cytoskeletal organization, providing insight into both valve homeostasis and CAVD [109]. Taken together, proteomics promises to generate novel insight into disease progression as well as potentially develop a new clinical tool that uses novel global proteomic analyses in plasma as a noninvasive comprehensive biomarker panel.

Dysregulation of structural proteins and remodeling enzymes is a common pathway. Normal valve function requires coordinated movement of complex structures. Gross and Kugel proposed nomenclature for valve tissue organization in 1931 that is now well established [110]. The mature valve structure is made up of highly organized ECM that is compartmentalized into three layers, the fibrosa, spongiosa, and ventricularis [9,53,111]. The annulus, composed primarily of fibrous collagens, provides a buttress for dispersion of forces, and tethering of the cusp in a crown-shape for tissue stabilization [112,113]. Studies examining ECM in valve tissue have focused by convention on structural properties, specifically durability (collagens) and flexibility (proteoglycans and elastic fibers). However, several studies have shown that ECM components reciprocally regulate growth factors and signaling pathways, in addition to causing architectural abnormalities, suggesting a primary rather than secondary role in pathogenesis [Reviewed in 53]. Studies in mouse models lacking ECM components critical for the mature aortic valve structure, including proteoglycans, collagens and elastic fiber components, demonstrate that the expression and organization of diverse ECM components are essential to the formation and structural integrity of the valves during development and after birth [114-123]. Further, mouse studies have shown that age and dietary manipulation can lead to ECM changes and CAVD [124-126].

During valve remodeling, the VICs regulate expression and organization of the valve ECM [127,128]. Additional ECM remodeling enzymes such as matrix metalloproteases (MMPs) and cathepsins also are expressed during valve maturation [128,129]. VICs from developing valves are highly synthetic, and extensive remodeling is required to achieve the mature organization [127,130]. In normal adult valves, the VICs are largely quiescent with little or no cell proliferation and maintain baseline levels of ECM gene expression necessary for valve homeostasis [103]. ECM enzyme dysregulation is established in the valve disease literature [131-135]. The elastin insufficient mouse demonstrates cartilage-like nodules in the valve annulus reminiscent of calcific nodules [119,136]. MMP misexpression malformation and more disease, suggesting malformation processes are due in part to remodeling defects and malformation

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and disease processes are shared [136]. Similar nodules are seen in the aortic valve annulus of the Adamts9 null mouse [137], confirming the importance of ECM remodeling enzymes. Elastolysis and associated elastic fiber fragments have been implicated as a trigger for myofibroblast mediated calcification [138,139]. Loss of balance between elastases and elastase inhibitors has been identified as one fundamental cause of elastolysis [140]. Interestingly, previous studies have shown that different elastic fiber fragments have different biologic functions, for example, some fragments induce calcification while others are chemo-attractants for endothelial cells [141,142].

The extracellular matrix is an interface between genetics and the environment. The heart valves function essentially to maintain unobstructed unidirectional blood flow. Valve structure-function relationships provide important insight in understanding mechanisms of valve homeostasis as well as developmental and disease processes. Valve ECM composition and biomechanics reflect underlying hemodynamics. There are three basic loading states that affect valve tissue during the cardiac cycle: flexure, shear and tension. Flexure occurs when the valve is actively opening or closing, shear occurs when blood is passing through the open valve, and tension occurs when the valve is closed [143]. Shear, compressive, and longitudinal stresses contribute to valve deformation, or displacement of the valve tissue during the constant motion of the cardiac cycle [144]. Valve tissue has exceptionally high strain because the tissue cycles to a completely unloaded state with each heart beat [145]. The heart beats more than 100,000 times per day handling approximately 5 liters of blood per minute. Over the average lifetime, there are greater than 3 billion heartbeats, or cardiac cycles. The long held appreciation of age-related degeneration and latent valve disease may in fact represent subtle defects in valve tissue maintenance.

CAVD is characterized by VIC activation, which in turn results in increased ECM and increased remodeling enzyme gene expression [103,127,128], and hemodynamic factors may activate VICs and therefore contribute to pathology. VIC activation is apparent by induction of myofibroblast markers, such as vimentin, smooth muscle actin, and embryonic non-muscle myosin heavy chain [129]. Some VICs have been shown to be dynamic and play an active role in ECM maintenance, as well as potentially regeneration and repair, and these VICs are progenitor cells with smooth muscle like properties [101,102,103,123,146,147]. Recently, two studies have demonstrated the complex interaction between developmental programs that predispose tissue to disease and shear stresses that trigger inflammation [148,149], providing examples of how these factors when combined may cause AVD. Research efforts are beginning to reconcile developmental and biomechanical considerations in an effort to more closely examine CAVD in vivo. A better understanding of hemodynamic-induced cell-matrix perturbations may inform the search for durable valve bioprostheses [150].

4. National Heart Lung and Blood Institute's research agenda for CAVD

New research agenda emphasizes genetics and development. Recently, the National Heart Lung and Blood Institute Aortic Stenosis Working Group defined a comprehensive research

agenda for CAVD [25]. There are nine research priorities outlined in the statement that are summarized in Table 1. These priorities emphasize the identification of genetic factors that inform etiology, risk, and pharmacologic response, pointing to the clinical impact of these efforts being new diagnostic tests, biomarkers that may improve surveillance, and panels that may inform response to specific drugs. In addition, there is an emphasis on identifying genotype-phenotype relationships focusing on BAV. Improved understanding of valve biology, especially as it pertains to genetic predispositions for CAVD, is critical and will facilitate the identification of specific mechanisms involved in disease initiation and progression. The identification of molecular developmental processes and animal models of CAVD in vivo are needed to establish early pathogenesis and the effectiveness of new pharmacologic treatments for disease. In addition, genetic information will be increasingly important in the assessment of clinical studies that aim to refine clinical risk factors and identify new diagnostic and risk stratification tests.

1. Identify genetic, anatomic, and clinical risk factors for the distinct phases of initiation and progression of CAVD to identify individuals at higher risk, to determine interactions between risk factors, and to determine whether the severity of AS is a risk factor for surgical AV replacement.
2. Develop high-resolution and high-sensitivity imaging modalities that can identify early and subclinical CAVD, including molecular imaging and other innovative imaging approaches.
3. Understand the pathogenesis and pathophysiology of BAV, especially to establish correlations between phenotype and genotype, and to clarify the key features of this disease process that potentiate calcification.
4. Understand the basic valve biology (e.g., early events, mechanisms, and regulatory effects) of CAVD, including signaling pathways and the roles of valve interstitial and endothelial cells and the autocrine and paracrine signaling between them, the extracellular matrix and matrix stiffness, the role of age-related changes in both valve cells and extracellular matrix, the interacting mechanisms of cardiovascular calcification and physiological bone mineralization, and micro-scale mechanotransduction and macro-scale hemodynamics.
5. Develop and validate suitable multi-scale in vitro, ex vivo, and animal models. Improved models are needed that realistically duplicate the conditions in which human CAVD develops.
6. Identify the relationship between calcification of the AV and bone and the reciprocal regulation of these processes.
7. Encourage, promote, or establish tissue banks that make valve tissue from surgery, pathology, and autopsy unsuitable or unneeded for transplantation, with and without CAVD, available for research.
8. Conduct clinical studies specific to CAVD to determine the feasibility of earlier pharmacological intervention in aortic AV sclerosis versus stenosis.
9. Determine the risk factors and optimal timing of surgical valve replacement in view of the current state of the data defining the biological mechanisms of CAVD.

Table 1. Current NHLBI Research Agenda for CAVD. Reproduced from [25].

There is an increasing need for networks and biorepositories. The current paradigm in translational human genetics research involves discovery (the identification of sequence variation associated with disease), mechanistic investigation (definition of pathogenesis), and finally development of new clinical approaches (application). Findings from human genetic studies are being taken into the laboratory where increasingly sophisticated animal models are providing the basis to define pathogenesis in a variety of diseases. The elucidation of pathogenesis subsequently results in the development of new diagnostic and therapeutic strategies, which can then be taken back to the patient. Taken together, this is referred to as the "bedside to bench to bedside" approach to disease and has led to numerous initiatives aiming to realize "translational" research goals, e.g., the NHLBI's Bench to Bassinet Program supporting excellence in pediatric cardiovascular translational research (http://www.bench-tobassinet.com), including CVMs such as BAV. Given the incidence of BAV and the sample size required to use new genetic discovery tools, it is necessary to combine cohorts. Genetic information is also impacting the understanding of pharmacology as it relates to drug indications and drug responses further facilitating improved care. Taken together, genetic information provides an impetus to shift the focus of medicine from treatment of end stage disease to strategies emphasizing primary prevention and early intervention.

Given some of the specific research priorities, for example the need to immortalize valve interstitial cell (VIC) lines, it will be important both to design biorepositories that are specifically built for cardiovascular disease needs and to organize virtual biobanks that can leverage combined resources from multiple centers. In effect, this will maximize translational impact and return on investment. The organization of biorepositories has advanced considerably in recent years, and significant strides have been made by international groups to coordinate resources. For example, the mission of the International Society for Biological and Environmental Repositories (ISBER) is to address technical, legal, ethical and managerial issues relevant to the governance of wide ranging biorepositories (http://www.isber.org) [151]. Several institutions have initiated biorepositories that include blood and tissue from CAVD patients. Virtual repositories, or multiple repositories that coordinate efforts to leverage sample size considerations, are becoming operational and the current funding climate is accelerating development of special rules to optimize tissue utility [152]. Funding bodies at the government and foundation levels need to recognize valve disease as a significant public health problem and establish valve specific funding opportunities. Further, valve biology and CAVD specific symposia are needed at large conferences, such as the American Heart Association.

5. Comprehensive counseling and genetic testing increasingly impact clinical care

A detailed family history remains a powerful tool and genetic testing will advance its impact. A detailed family history refers to questioning multiple individuals within a family and requires specific demographic information (e.g., age at disease onset) and documentation of disease and other pertinent health issues by medical record review [153]. The results of a

detailed family history may warrant referral to a cardiovascular genetics service. A detailed family history is a powerful tool and can help establish a diagnosis and initiate comprehensive care in a timely fashion [154-158]. There are significant barriers to the optimal use of family history information, primarily a lack of awareness on the family's part and considerable time restrictions on the health care professional's part. Studies have shown that a majority of people do not know their family history and do not appreciate its relevance in medical management, and consequently the potential impact of family history information is diminished [159]. In an effort to increase family history awareness, tools have been developed and are available to the general public to generate and maintain a detailed family history. For example, the Health and Human Services Family History Initiative has designed a publicly available, web-based program providing a means to generate and maintain a detailed family history [160]. Genetics has transformed the use of family history information and has led to the reemergence of the detailed genetic family history. Detailed family history information is necessary for the optimal use of genetic screening and testing and this translates to the essential need of genetic counselors embedded in cardiology clinics.

Genetic testing is anticipated for BAV and CAVD. As the etiology of BAV is defined and the complex genetics of CAVD is elucidated, a variety of variants associated with BAV and CAVD will be identified, including variants associated with etiology as well as variants associated with specific types of subsequent risk. All variants pertaining to CAVD will have to be organized based on utility. Once a significant proportion of cases can be diagnosed using genetic testing, clinical testing may be warranted. Presently, there are no CLIA (Clinical Laboratory Improvement Amendments) approved tests for the diagnosis or stratification of BAV or CAVD. NOTCH1 screening is of too little yield to justify testing (<2%), but may be included in larger panels of tests at a future time. Presently, there is no diagnostic utility for genetic testing for BAV or CAVD, but given the rate of discovery and the various technological advances being made, it would appear that this will occur in the near term. It is imperative that cardiologists understand the indications and limitations of clinical genetic testing [161,162]. However, genetic testing is being used for various clinical management reasons, and several of these uses have cardiovascular applications. For example, sequence variants in CYP2C9 and VKORC1 are associated with an increased bleeding risk and drug resistance, respectively, in patients taking warfarin [163,164]. Because CAVD patients often require valve replacement, and mechanical prostheses require anticoagulation, this particular example may be directly useful for CAVD patients. Ultimately, diagnostic panels of genetic variants that identify cause, and may provide insight regarding natural history, and additional management panels that identify disease-specific risks may inform clinical decision-making. Taken together, panels of genetic variants may be used in a manner similar to newborn screening, becoming an important part of the working information for every patient.

The opportunities and challenges of genotype definition in the clinic. Genotype definition will empower individuals and families to further control their health, extending the paradigm shift that occurred when the medical field embraced preventive medicine [165]. Increasing genetic information in the clinic creates new opportunities to improve cardiovascular health. However, this development also creates new challenges, including ethical and legal issues that

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challenge the existing regulatory landscape and directly impact application in the clinic [166]. For example, the meaning of a negative test often will not be clear, in addition to the ambiguity variants of unknown significance present. Despite the passage of the Genetic Information Nondiscrimination Act (GINA), a law that protects the public from insurance companies using genetic information for underwriting purposes, there are increasing concerns about privacy issues. Public education, including physician awareness, will be critical to facilitate the anticipated clinical uses of genetic information. Genetic testing will play an increasing role in the clinical management of BAV and CAVD patients. Ultimately, genotype definition may be able to identify those patients with BAV that are at risk (or not at risk) of developing CAVD or other associated problems, impacting clinical management decisions. As more is learned about the genetic basis of BAV and CAVD, the yield of clinical genetic testing will be sufficient to warrant routine diagnostic testing. As the genotypes associated with BAV and CAVD are defined, there will be a need to expand Consensus Guidelines for BAV to include full consideration of genetic information, especially overlapping silent and/or latent disease processes. Clinical applications of genetic variant panels will potentially include refined diagnosis, risk stratification (early intervention, timing of surgery), pharmacogenomics (which drug, what dose, risk of adverse effects), and screening strategies for relatives.

The clinical implications of genotype definition: examples. Because CAVD remains essentially a surgical problem, early clinical impact may be realized first in surgical considerations. For example, the pulmonary artery dimension is increased in BAV patients [167,168], consistent with previously reported histopathologic abnormalities in the pulmonary artery of BAV patients [169]. This may be clinically relevant in BAV patients who require aortic valve replacement and may be candidates for the Ross procedure (autologous pulmonary valve placed in the aortic position). Some patients with apparently isolated CAVD undergoing surgical repair may be at risk for subsequently developing TAA, a not uncommon scenario that may be predicted by genotyping. McKellar et al recently described aorta complications in 1,286 aortic valve replacement patients with a median 12 year follow up, and reported that 10% demonstrate progressive aortic enlargement and only a minority of these lead to dissection or require further surgery [170]. However, in those patients, prophylactic replacement of the aorta would be warranted and would fundamentally change the overall approach to this group of patients. In addition, stratifying by genotype CAVD patients into those with and those without aorta abnormalities potentially informs type of surgical approach as well [171,172]. The ability to identify those patients at risk before the first surgery may substantially impact clinical decision-making, including for example a selective approach to combined valve and aorta replacement.

Genotype phenotype information will have important implications for clinical surveillance. For example, current recommendations for functional BAV patients include screening echocardiograms every 5 years for all first-degree relatives [13]. Recently it was shown that surveillance may be modified by morphology such that pediatric patients with RN morphology are screened every 2 years because they are at higher risk of developing new AVD, while individuals with RL BAV could be monitored less aggressively in early childhood as the risk of having AVD at this time is relatively low [26]. Family members of BAV patients may be at

risk for TAA or other cardiovascular disease (even if they don't have BAV), underscoring the importance of thoughtful monitoring. Since CAVD is a latent phenotype, continued surveillance is required. Since some individuals with BAV have progressive CAVD and others never develop disease, there is reason to think that genetic insights will clarify this phenomenon. Overall, refined screening strategies promise to provide opportunities for improved care.

Ultimately, genetic information will inform the identification of new pharmacologic based therapies for CAVD [173]. Genetics research in CAVD will lead to further basic research in animal models that can define the early pathogenesis and natural history of disease and therefore identify new therapeutic targets. This paradigm will have increasing significance as bioinformatics approaches overcome the challenges of extraordinary amounts of data. There has been considerable interest in applying CAD treatment paradigms to valve disease. However, while statin therapy showed early in vitro evidence of a potentially beneficial effect, a large clinical trial demonstrated that statin therapy does not positively impact either aortic valve disease progression or the need for surgery [174]. Recently, a strategy to use pediatric valve disease patients as a means to identify early genetic aspects of CAVD has been advanced because this population provides insight into the disease process that is not confounded by the common comorbidities of adulthood, such as CAD and HTN [127,175]. Increasingly, developmental paradigms will inform the search for etiology, new treatments and better bioprostheses. New therapies are likely to emerge from molecular biology fields, and innovative approaches to studying the genetic basis of CAVD will be needed to realize this goal.

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