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Genetic Basis of Aortic Valvular Disease

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Abstract

Purpose of Review—Aortic valve disease is relatively common and encompasses both congenital and acquired forms. Bicuspid aortic valve (BAV) is the most common type of cardiac malformation and predisposes to the development of calcific aortic valve disease (CAVD). Since the description of the link between *NOTCH1* and BAV and CAVD approximately a decade ago, there have been significant advances in the genetic and molecular understanding of these diseases.

Recent findings—Recent work has defined the congenital cardiac phenotypes linked to mutations in *NOTCH1* and in addition, novel etiologic genes for BAV have been discovered using new genetic technologies in humans. Furthermore, several mouse models of BAV have been described defining the role of endothelial *Notch1* in aortic valve morphogenesis while others have implicated new genes. These murine models along with other cell-based studies have led to molecular insights in the pathogenesis of CAVD.

Summary—These findings provide important insights into the molecular and genetic basis of aortic valve malformations, including BAV, specifically highlighting the etiologic role of endothelial cells. In addition, numerous investigations in the mechanisms of CAVD demonstrate the importance of developmental origins and signaling pathways as well as communication between valve endothelial cells and the underlying interstitial cells in valve disease onset and progression.

Keywords

Bicuspid aortic valve; aortic valve calcification; genetics; congenital heart disease

Introduction

Heart valve disease is responsible for over 24,000 deaths each year in the United States [1]. Valvular heart disease encompasses both congenital and acquired forms and in the United

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States has an overall prevalence of 2.5% [1]. Among the four cardiac valves, the most commonly affected is the aortic valve and significantly contributes to both morbidity and mortality. Prevalence of aortic valve dysfunction (stenosis or regurgitation) is increased with age affecting an estimated 2–3% of the population over 75 years of age, and when severe requires surgical or catheter-based aortic valve replacement [1,2]. The most prevalent disease affecting the aortic valve is calcific aortic valve disease (CAVD), where calcification of the normally thin valve cusp perturbs the valve's ability to open and close properly to maintain adequate unidirectional blood flow. In addition to advanced age, other clinical risk factors such as hypertension, hypercholesterolemia, and diabetes mellitus are associated with CAVD, but an additional important contributor is the presence of a congenital malformation, bicuspid aortic valve (BAV) [3]. BAV has a prevalence of 1.3% in the population and a significant contributor to the development of severe aortic stenosis [4, 5]. Although the mechanisms of congenital BAV and acquired CAVD are not fully understood, there is increasing evidence to suggest that genes critical for normal aortic valve development may play roles in the development of CAVD.

The aortic valve is a tricuspid structure and each mature valve leaflet, or cusp, is comprised of three organized layers of extracellular matrix (ECM). The fibrosa comprised of collagen, the proteoglycan-rich spongiosa, and the elastin fiber-containing ventricularis (Figure 1). At the cellular level, the aortic valve is composed of valvular interstitial cells (VICs) that deposit the specialized ECM components and a layer of overlying valve endothelial cells (VECs) encapsulates the cusp. Aortic valve develops between 7–9 weeks of gestation in the human embryo and studies in animal models have demonstrated the multiple cellular sources from which aortic VECs and VICs are derived [6,7]. The semilunar (aortic and pulmonic) valves are formed from the endocardial cushions that appear as "swellings" within the embryonic cardiac outflow tract. Cushion formation begins with endocardial-to-mesenchymal transformation (EMT) of cells largely derived from the second heart field (SHF) in response to TGF-β, WNT, Notch and VEGF signaling pathways. An additional cellular contribution to the valve leaflets is from migrating cardiac neural crest cells (CNC). The outflow tract (OFT) endocardial cushions undergo remodeling to form the tri-leaflet aortic and pulmonic valve primordia, which will continue to develop by a process of thinning, reshaping and elongation well into postnatal maturation to form the mature valve cusps.

In contrast to healthy, trilaminar adult aortic valve cusps, the identification of calcific nodules in the collagen-based fibrosa on the arterial side of the aortic valve and disruption of this trilaminar valve structure are hallmarks of CAVD, which occurs in individuals with bicuspid or tricuspid aortic valves, although individuals with BAV are predisposed to more severe disease (Figure 2) [8]. The molecular mechanisms driving the ECM disorganization and calcification are thought to be the result of abnormal gene expression and mediated by VICs and interestingly, these signaling pathways in diseased valves are also critical for normal valve development [9]. An increased understanding of mechanisms underlying aortic valve development and disease along with recent advances in human genetics have resulted in significant insights to the molecular basis of aortic valve disease and here, we review the recent highlights in the molecular genetics of BAV and CAVD.

Genetics of Bicuspid Aortic Valve

BAV is the most prevalent birth defect, and population based studies have supported a strong genetic component along with other left-sided cardiac malformations [10]. Initial insight into the genetic etiology of BAV came from studies of familial BAV where mutations in *NOTCH1* were discovered to segregate in families with autosomal-dominant valve disease [11]. Since this, the majority of *NOTCH1* mutations that have been reported are inherited from unaffected family members [12]. Recently, genetic screening of *NOTCH1* was performed in 428 probands with left-sided congenital heart disease (CHD) and 14 mutations in *NOTCH1* were identified, 11 of which were in familial CHD. Interestingly, among these cases, a family member also had a CHD affecting the right-sided cardiac outflow tract (i.e. tetralogy of Fallot)[13]. Additional reports support this finding that mutations in *NOTCH1* cause malformations of the right and left sided cardiac outflow tract within families [11,14,15]. The other gene linked to BAV in humans is *GATA5*, where rare sequence variants in *GATA5* were identified in patients with BAV by multiple groups [16,17,18]. Similar to *NOTCH1*, mutations in *GATA5* have reported in a spectrum of CHD, including tetralogy of Fallot [19,20,21]. Furthermore, a deleterious mutation in *NKX2.5* that completely abolished its interaction with GATA5 was found to segregate with disease in a family with BAV, further supporting these findings [22].

While these efforts have primarily focused on non-syndromic BAV, Quintero-Riviera et al. utilized the Developmental Genome Anatomy Project and identified a proband with a balanced translocation involving chromosomes 1 and 5 who had BAV, coarctation of the aorta, and patent ductus arteriosus along with pervasive development delay [23]. The translocation breakpoint was found to disrupt the 3'UTR of *MATR3*, which encodes the nuclear matrix protein Matrin 3. They found that *Matr3* was expressed ubiquitously in the OFT, and heterozygous disruption of *Matr3* in mice recapitulated the cardiac phenotypes of the proband. The findings for MATR3 are limited to a single patient and while supported by murine studies, future work is needed to determine if mutations in MATR3 contribute to non-syndromic BAV.

Molecular Pathways for Aortic Valve Development

While human genetic approaches have led to important insights into the genetics of BAV, molecular studies using mouse models have provided insights into the underlying mechanisms of BAV and also identified novel candidate genes for BAV. The discovery that humans with heterozygous mutations in *NOTCH1* displayed aortic valve disease was not surprising since Notch signaling plays a critical role in several cardiovascular diseases [24]. Binding of the Notch signaling pathway ligands (Jagged and Delta-like) to the Notch receptors (1–4) initiates a sequence of cleavages, releasing the Notch intracellular domain (NICD) for nuclear translocation and initiation of target gene expression. Notch1 is the predominate receptor that functions in endothelial cells during cardiovascular development, where it is required for initiation of EMT, and persists after EMT throughout valve remodeling and into adulthood. Disruption of several Notch pathway members results in dysregulated EMT, leading to embryonic lethality and/or abnormal valve development [24].

Unlike humans, *Notch1* heterozygous mice are phenotypically normal. By introducing *Notch1* haploinsufficiency into a nitric oxide synthase (*Nos3*)-null background ($Notch1^{+/-}$;$Nos3^{-/-}$), a highly penetrant mouse model of aortic valve disease consisting of BAV with thickened cusps, stenosis and regurgitation was generated. The disease severity was significantly greater than the ~30% incidence of BAV observed in *Nos3*-knockout mice alone [25,26]. These compound mutant mice also suffer from significant (~65%) postnatal lethality. Although normal Mendelian ratios of $Notch1^{+/-}$;$Nos3^{-/-}$ embryos were found at embryonic day(E) 18.5, thickened aortic valve cusps at E15.5 were suggestive of a defect in remodeling of the semilunar valve cushions. In addition, we found that heterozygosity of *Notch1* specifically in endothelial and endothelial-derived cells in a *Nos3*-null background recapitulate the congenital cardiac phenotype of $Notch1^{+/-}$;$Nos3^{-/-}$ embryos [27]. These compound mutant mice also presented with overriding aorta and ventricular septal defect (VSD), which is reminiscent of tetralogy of Fallot, a congenital defect observed in patients with Alagille syndrome caused by mutations in *JAG1*. CHD associated with Alagille syndrome, which is also characterized by bile duct abnormalities, dysmorphic facies, ophthalmologic findings and butterfly vertebrae, have been attributed to endothelial-derived Jag1 in mice [28]. Our data in conjunction with this mouse model of Alagille syndrome implies critical signaling between Jag1 and Notch1 for proper formation of the semilunar valves, ventricular septum, and position of outflow tract.

Studies have implicated Dll4 and Jag1 as the ligands expressed in endothelial cells that signal to Notch1 during EMT [24], but until lately the spatio-temporal patterns of expression and signaling had not been delineated. Recent work by MacGrogan et al utilizes various *Cre* systems to describe the functions of Notch signaling pathway members during valve development. Their studies suggest that endothelial-Dll4 is required for EMT, but shortly after, endothelial-Jag1 takes over as the predominant Notch ligand in the endocardial cushions. Deletion of endothelial-derived Jag1 in a subset of endothelial cells that do not undergo EMT leads to thickened arterial valves, BAV, and VSD that was shown to be mediated by endothelial-Notch1. Transcriptome analysis revealed increased BMP signaling and mesenchymal cell proliferation, and also led to the identification of a new Notch effector, Heparin binding epidermal growth factor (EGF) like growth factor, required to suppress mesenchymal cell proliferation and potentially play a role in embryonic valve hypertrophy [29].

A recent complimentary study by Wang et al also explored the post-EMT requirements of Notch1 in the valve endothelium and mesenchyme. Homozygous deletion of *Notch1* in post-EMT endothelial cells resulted in BAV and valve stenosis, which was accompanied by an increase in apoptosis and a reduction in proliferation of valve mesenchyme cells. Additionally, Tumor necrosis factor alpha (TNFα) was identified as a novel target of endothelial Notch1 that mediates apoptosis in the valve mesenchyme post-EMT, and loss of Tnf signaling resulted in hypertrophic semilunar valves [30]. This study along with the studies by MacGrogan et al and Koenig et al. further solidified the role for endothelial Notch1 in the development of BAV. Furthermore, previous work had demonstrated that deletion of endothelial-*Gata5* led to BAV in mice [31]. In these in vivo studies, the investigators identified downregulation of the Notch signaling along with other endothelial genes including *Nos3*. Additional work has described the Slit-Robo signaling pathway

upstream of Notch signaling in aortic valve development. This pathway, which is important for axonal guidance during nervous system development, was shown to be involved in the remodeling of the cardiac cushions as *Robo1* and *Robo2* compound mutant mice display BAV phenotypes [32]. It remains to be seen if these and other endothelial genes will be important in human BAV.

Molecular Pathways for Aortic Valve Calcification

The aortic valve cusp is comprised of VICs distributed throughout the three specialized layers of ECM and surrounded by a monolayer of VECs. This specific composition allows the valve to comply with hemodynamic stresses during normal function and the anatomical localization allows for communication between the VICs and the external environment via VECs. In a healthy aortic valve, VICs are quiescent. In CAVD, VICs adopt an activated state in which they begin to express αSMA and transform into osteoblast-like cells, expressing RUNX2 and other calcification promoting proteins like bone morphogenetic protein (BMP). VECs are subjected to laminar shear stress on the ventricularis side that is protective against CAVD, while oscillatory shear stress adjacent to the fibrosa is associated with calcification [33]. Although alterations in several signaling pathways in VECs and VICs have been implicated, the molecular mechanisms of CAVD remain poorly understood [34].

The discovery linking mutations in *NOTCH1* with CAVD in humans provided an entry point to elucidating the mechanistic basis for valve calcification [11,35]. Initial work using cultured aortic VICs demonstrated that inhibition of Notch1 promoted aortic valve calcification through repression of Bmp2 and Sox9, which have independently been linked to play a significant role in CAVD suggesting that Notch1 signaling functions upstream of these mediators of calcification [36,37,38,39]. Both VECs and VICs have important functions in maintaining healthy aortic valves, but it is important to study these cells in combination to properly consider the effects of VEC:VIC communication on cell signaling. Accordingly, nitric oxide (NO) from VECs was shown to regulate Notch1 in aortic VICs during the process of calcification using an in vitro system [26]. Similar evidence supporting the importance of VEC was shown by Huk et al., who demonstrated that endothelial TGFβ1 was necessary to maintain nuclear Sox9 expression in VICs and prevent calcification using both in vitro and in vivo models [40].

Recently, additional work has defined the mechanisms by which haploinsufficiency of *Notch1* leads to CAVD. In order to define the role of Notch1 in AVICs, immortalized aortic VICs were isolated from *Notch1*$^{+/-}$ Immortomice, a compound mutant mouse which is both heterozygous for *Notch1* and expresses the immortalizing oncogene simian virus 40 (SV40) large tumor (T) antigen (Ag), and were found to not only have molecular differences at baseline but also had an exaggerated response to cyclic mechanical strain, which mimics in vivo diastolic loading, becoming fully activated and resulting in calcification [41]. Further mechanistic insight was found by studying calcified human aortic valves, where the long non-coding RNA, lncRNA *H19*, was identified as a *NOTCH1* repressor with a direct role in calcification of human aortic VICs. *H19* expression was shown to inversely correlate with CpG methylation at the *H19* promoter, indicating epigenetic regulation of expression. Silencing *H19* inhibits RUNX2 and BMP2, classic markers of osteogenic calcification, and

reduced mineralization, while overexpression had the opposite effects. Furthermore, it was found that *H19* overexpression reduced activity on the *NOTCH1* promoter, identifying *H19* as a novel suppressor of *NOTCH1* and therapeutic target in CAVD [42].

While Notch1 is expressed in VICs, it is also expressed in VECs. Theodoris et al utilized iPSC-derived VECs from patients with a *NOTCH1* CAVD-associated mutation, subjected the endothelial cells to shear stress, and profiled the transcriptome. Hemodynamic shear stress caused an increase in anti-osteogenic and anti-inflammatory networks in wildtype, but not $NOTCH1^{+/-}$ endothelial cells, indicating that endothelial NOTCH1 mediates the protective effects of shear stress [43]. The authors also found a discrepancy in H3K27ac at NOTCH1-bound enhancers, which correlated with an alteration in the downstream transcriptome, suggesting that this mutation alters the epigenetic profile in endothelial cells [42]. In another related study, the same group discovered that Matrix Gla Protein (MGP) is a direct target of NOTCH1 in human aortic VECs and responds to shear stress in a NOTCH1-dependent manner. Furthermore, in vivo experiments show that mutation of the CSL binding sites on the MGP enhancer dramatically attenuates expression of *MGP* in the valves and arterial system [44].

In addition to shear stress and other environmental factors, VECs are subject to the effects of aging, which is coupled with a significant risk of CAVD [1]. Aging in vascular disease is attributed to endothelial cell dysfunction [45], but VECs have been shown to respond differently than vascular endothelial cells in response to the environment [46]. Anstine et al found that VECs from aging mice display decreased NO bioavailability, EMT, cell membrane repair and proliferation, along with increased permeability of the VEC barrier. These multifactorial changes are supported by transcriptome analysis of VECs across 4 different timepoints – embryonic, postnatal, young adult, old adult [47]. It has been well established that BMP2, BMP4 and pSMAD1/5/8 are associated with aortic valve calcification [33], but a recent study by Gomez-Stallons et al demonstrated that BMP signaling is also required for aortic valve calcification in the $Klotho^{-/-}$ mouse model, which exhibits CAVD and premature aging associated with hyperphosphatemia. It was found that BMP signaling precedes and localizes with calcification in the aortic valve, and inhibition of BMP in VICs in vitro and in vivo prevents calcification [48]. Additional recent studies have further support the role of cadherin signaling in CAVD. *Cad-11* deletion results in hyperplastic semilunar valves through inactivation of GTP-RhoA and Sox9 in VICs, but these valves do not calcify [49]. Accordingly, Sung et al investigated the potential role of Cad-11 in Rho/ROCK mediated CAVD through overexpression of Cad-11 in VEC-derived cells. Cad-11 overexpression resulted in calcification, upregulation of RhoA and Sox9, and ECM remodeling. In vitro, ROCK inhibition attenuated calcific nodule formation. Finally, examination of human aortic valves revealed increased expression of Cad-11, GTP-RhoA, and Sox9 in diseased valves. Together, this data describe a molecular pathway involving upregulation of Cad-11, Rho/ROCK and Sox9 in CAVD [50].

Conclusions

Since the seminal discovery that mutations in *NOTCH1* were linked to BAV and CAVD, there has been an increase in our understanding of the genetic causes of BAV. With the

continued advances in human sequencing technologies, it is anticipated that more insights into the genetic contributors of aortic valve malformations will be uncovered potentially as part of the International Bicuspid Aortic Valve Consortium (BAVCon), which has this as one of its goals [51]. With the discoveries of the roles of endothelial Notch1 and Gata5 in the development of BAV, it has opened new avenues of research into the mechanisms by which these genes function in valve remodeling. Additional insights into the mechanisms of CAVD have also been identified with the analysis of new mouse models, specifically the importance of VEC-VIC communication in the calcification process. Further dissection of these intercellular communications may lead to new targets for pharmacologic therapy in CAVD.

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* of special interest

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KEY POINTS

1. Mutations of *NOTCH1* are associated with not only bicuspid aortic valve but also a spectrum of congenital cardiac malformations affecting both the left and right-sided cardiac outflow tracts in humans.
2. Several cellular and mouse models have shown the requirement of Notch1 in endothelial cells for proper development of the aortic and pulmonary valves.
3. Communication via multiple signaling pathways, including Notch1, between valve endothelial and valve interstitial cells is critical for the process of valve calcification.

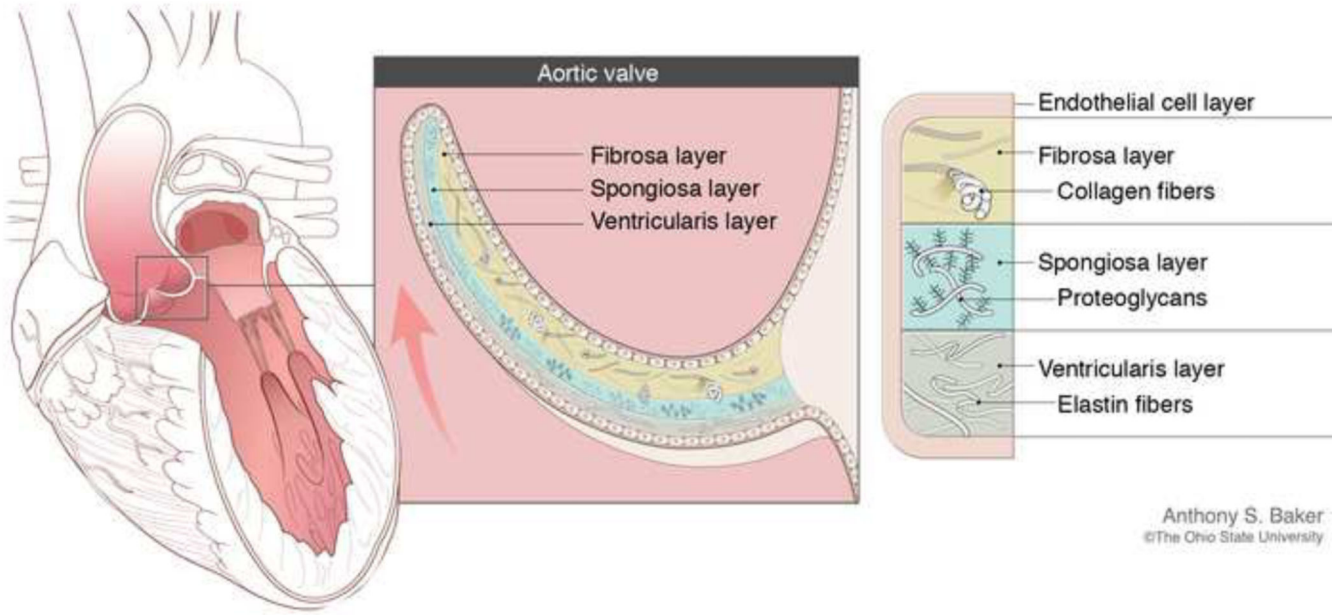

Figure 1. Overview of aortic valve structure

The aortic valve is a highly organized structure composed of cellular and extracellular components. Each valve cusp is surrounded by a continuous, single layer of valve endothelial cells and interspersed by valve interstitial cells. The extracellular matrix is highly organized and largely composed of elastin fibers in the atrialis/ventricularis (dark grey), proteoglycans in the spongiosa (blue) and collagens (yellow) in the fibrosa and these are arranged according to blood flow (red arrows).

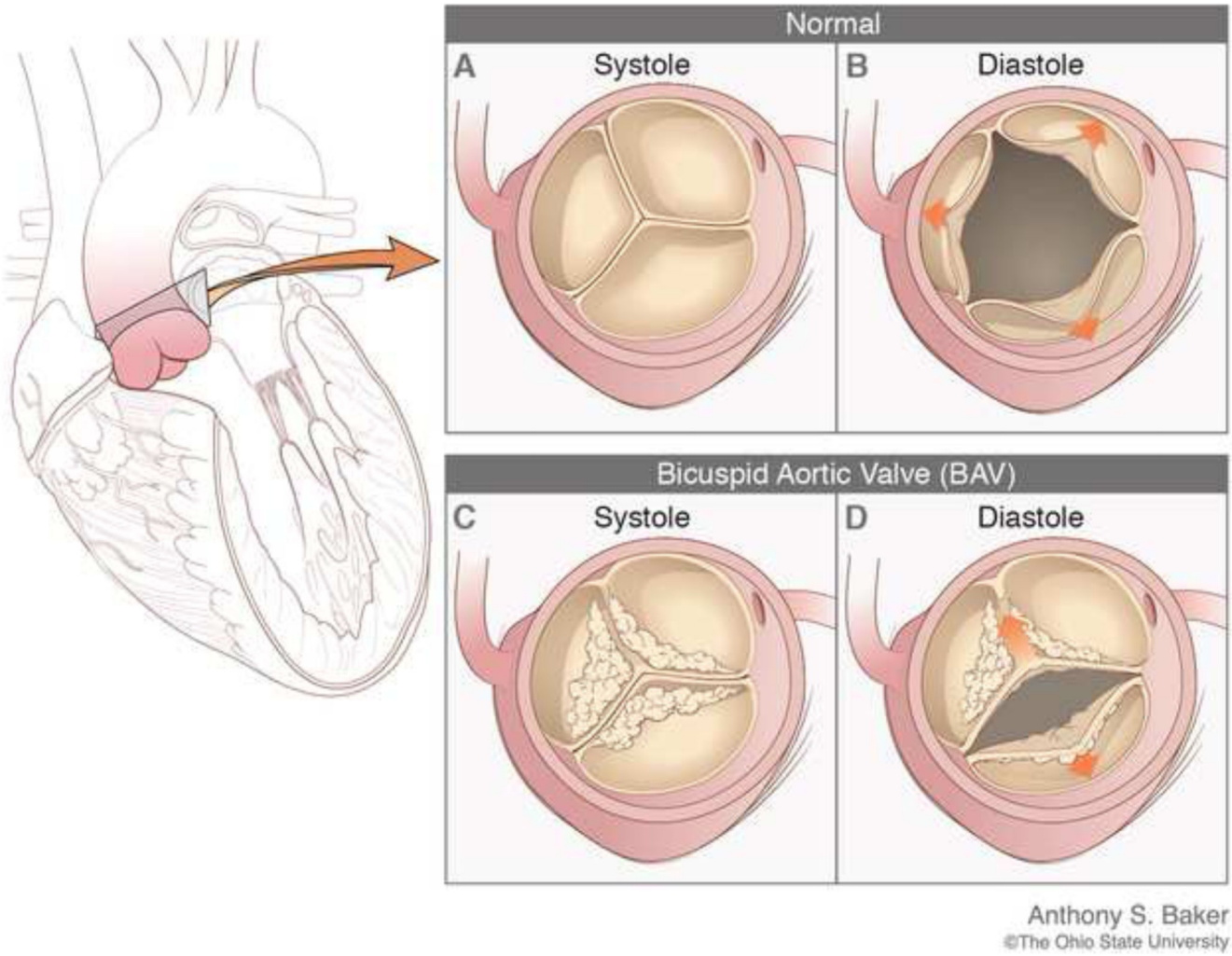

Figure 2. Congenital bicuspid aortic valve with the development of calcification

(A, B) The normal aortic valve consists of three cusps, and during diastole (A) and systole (B) the cusps sufficiently coapt and fully open respectively to regulate unidirectional blood flow. In contrast, bicuspid aortic valves consist of two cusps that results in narrowing (D) and leads to lifelong changes in valve biomechanics, stenosis and increases the risk of calcification (shown as nodules in C, D).