Defective NOTCH signalling drives increased vascular smooth muscle cell apoptosis and contractile differentiation in bicuspid aortic valve aortopathy: a review of the evidence and future direction.

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Abstract

Bicuspid aortic valve (BAV) disease remains the most common congenital cardiac disease and is associated with increased risk of potentially fatal aortic aneurysms and dissection. Despite a growing appreciation of the genetic and haemodynamic origins of this association, many questions remain unanswered. Mutations in the *NOTCH1* gene are one of a few genetic anomalies identified in BAV disease. NOTCH signalling orchestrates neural crest cell migration during cardiogenesis. These cells constitute the first vascular smooth muscle cells (VSMC) in the ascending aorta. It is hypothesised that a common genetic defect affects both valve and ascending aorta in BAV disease, predisposing to aortopathy. However, evidence for defective NOTCH signalling, and its involvement in the characteristic histological changes of VSMC apoptosis and differentiation in BAV aortopathy is lacking. Given its the central role in cell fate decisions, we hypothesise that changes in NOTCH signalling may underlie increased VSMC apoptosis seen in BAV aortopathy. Furthermore, a concurrent influence on cell differentiation may underlie the failure of VSMCs to respond to, and repair the degenerated extracellular matrix, which is characteristic of BAV aortopathy. This review scrutinises the evidence for the interactions of NOTCH signalling, cellular differentiation and apoptosis in the context of aortic VSMCs and provides focus for future research efforts in the diagnosis and treatment of BAV aortopathy. We propose that manipulation of the NOTCH signalling represents a therapeutic opportunity for influencing VSMC apoptosis and differentiation in the context of BAV aortopathy, and thus prevent the catastrophic complications of aortic dissection and rupture.

Keywords

Bicuspid aortic valve, aortopathy, vascular smooth muscle cells, NOTCH signalling, apoptosis, cell differentiation

Introduction

Bicuspid aortic valve (BAV) disease results when the aortic valve forms with just two leaflets (cusps), in place of the normal three. The incidence worldwide is between 0.4 – 2.25% making it the most common congenital cardiac anomaly in humans, with males more frequently affected at a ratio of 3:1[7, 34, 79]. Since its first documentation over 500 years ago by Leonardo da Vinci, an appreciation has grown of its tendency to predispose individuals to associated cardiovascular disease. Although comprising just a few percent of the population, BAV patients make up over 40% of the patients who die from or require an operation for aortic valve disease[70]. Furthermore, at least one third of BAV patients will develop complications including valve narrowing (stenosis) or leaking (regurgitation). Consequently, BAV disease accounts for more morbidity and mortality than all other congenital cardiac defects combined[81]. The disease presents a significant financial burden for healthcare systems across the world, and despite increasing research interest, little progress has been made towards defining the pathophysiological mechanisms.

BAV disease is also a major risk factor for ascending aortic aneurysm and aortic dissection (collectively termed BAV aortopathy). The curious link between valve morphology and ascending aortic pathology was first described by Abbott in 1928[1]. Microscopic examination of the aortic wall reveals the histological hallmark of BAV aortopathy, first termed Erdheim’s cystic medial necrosis on account of the cyst-like appearance of accumulated ground substance[56]. Medial necrosis is accompanied by loss of fibrillin, elastic laminar fragmentation and vascular smooth muscle cell (VSMC) apoptosis[24, 25, 64]. Loss of extracellular matrix (ECM) integrity is compounded by overexpression and activity of matrix metalloproteinases (MMPs), which contributes to cell detachment and apoptosis[13, 25, 37].

Conflicting evidence exists regarding the contribution of haemodynamic stress (‘post-stenotic’ dilation) and genetics to the pathognomonic changes of BAV aortopathy[10, 32, 66]. BAV disease is a largely heritable condition with between 10 – 35% of first degree relatives being affected in an autosomal dominant fashion[36, 42]. BAV commonly occurs in association with other genetic syndromes including Marfan, Ehlers-Danlos and Turner, and with other congenital cardiac abnormalities including aortic coarctation and hypoplastic left heart[57]. Furthermore, mutations in the *NOTCH1* gene have been identified in BAV populations[29, 55]. The NOTCH signalling pathway is an evolutionarily-conserved cell signalling mechanism that dictates cell fate decisions[4]. In addition to its implication in the development of BAV disease, NOTCH signalling is identified as a key effector of neural crest cell migration during cardiogenesis coordinating differentiation of the first VSMC population in primitive ascending aorta[38, 51, 65]. Thus, it is hypothesised that a common genetic defect may affect both valve and ascending aorta in BAV disease predisposing to aortopathy[15, 85].

However, there is little evidence for defective NOTCH signalling in BAV aortopathy, and its contribution to VSMC apoptosis and differentiation has yet to be elucidated[73]. Given the central role of NOTCH signalling in cell fate decisions, and its implication in BAV disease, we hypothesise that changes in NOTCH signalling may underlie increased VSMC apoptosis in BAV aortopathy. Furthermore, the influence of NOTCH signalling on cellular differentiation may underlie the failure of VSMCs to respond to and repair the degenerated ECM, which is also characteristic of BAV aortopathy. In this review, we scrutinise the evidence for the interactions of NOTCH signalling, cellular differentiation and apoptosis in the context of the aortic VSMC and provide focus for future research efforts in the diagnosis and treatment of BAV aortopathy. We propose that manipulation of the NOTCH signalling pathway may represent a therapeutic opportunity to reduce VSMC apoptosis and control cell differentiation and so prevent the catastrophic complications of aortic dissection and rupture.

VSMC apoptosis in BAV aortopathy

VSMCs are the only cell type found in the healthy aortic media. They are an essential prerequisite for normal development of the ascending aorta and maintenance of ECM homeostasis in the mature vessel. VSMCs provide support to the structure of the vessel wall and in smaller arteries contract and relax to regulate blood flow in response to physiological stimuli. VSMCs are capable of contraction, secretion and maintenance of the ECM components and can undergo apoptosis (programmed cell death), which is a physiologically event critical for maintaining vascular wall homeostasis. The consequence of reduced apoptosis is evident in cancer where mutations in tumour suppressor genes (e.g. p53) instigate uncontrolled growth. Conversely, excessive apoptosis is also associated with disease processes, including aneurysm formation.

There are two major pathways of apoptosis in VSMCs, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways (Figure 1)[54]. The extrinsic pathway is initiated by activation of membrane-bound ‘death receptors’ including tissue necrosis factor receptor (TNF-R); TNF-related apoptosis-inducing ligand receptor (TRAIL-R), FAS (first apoptosis signal) ligand receptor and death receptors (DR3, 4 and 5). Subsequent step-wise activation of proteolytic caspases (the caspase cascade) ensues, which cleaves intracellular substrates required for cell survival[18, 76]. Caspase-3, a major effector of the caspase cascade, is responsible for the hallmarks of apoptosis including DNA fragmentation, nuclear condensation and apoptotic body formation[40].



Figure 1 Schematic summary of the key pathways of apoptosis. Light blue oval = extrinsic (death receptor) pathway; pale red oval = intrinsic (mitochondrial) pathway. BAX = BCL-2-associated X; BCL-2 = B-cell lymphoma 2 protein; BID = BH3 interacting domain death agonist; DR = death receptor; IAPs = inhibitor of apoptosis proteins; TNF-R = tissue necrosis factor receptor; TRAIL-R = TNF-related apoptosis-inducing ligand receptor. Author’s illustration.

Conversely, the intrinsic pathway utilises mitochondria, and may be activated by either the extrinsic pathway (described above), or by a p53 dependent response to DNA damage[45, 46, 71]. Activation of pro-apoptotic BCL-2 (B-cell lymphoma 2) protein family members (BCL-2-associated X, BAX; BCL-2-interacting killer, BIK; and BCL-2 homologous antagonist/killer, BAK; and BID) initiates their translocation to the mitochondrial membrane where they activate mitochondrial membrane channels. This facilitates the movement of cytochrome c into the cytoplasm, activating caspases and triggering apoptosis[74]. BCL-2 family anti-apoptotic proteins are able to bind to these channels and prevent activation. Finally, cytosolic inhibitors of apoptosis proteins (IAPs) bind and inhibit caspases, inhibiting apoptosis independently of the mitochondrial pathways[22]. An example is X-chromosome linked IAP (XIAP), which inhibits caspase-3 and -7 reducing apoptosis through BAX mediated, cytochrome c release pathway[22]. Thus, XIAP does not reduce expression of BAX and cytochrome c but inhibits their action of activating important caspases in the cytoplasm.

VSMC apoptosis was first quantified in BAV aortopathy by Bonderman *et al.*, who identified that apoptotic neural crest-derived VSMCs appeared to be concentrated around areas of medial degeneration (MD)[10]. Moderate grade MD was seen in all patient groups (including TAV patients with non-aneurysmal aortas), however BAV patients with both aneurysmal and non-aneurysmal aortas had significantly higher apoptotic indices than non-aneurysmal TAV patients. Of note the aneurysmal TAV group had a higher apoptotic index than both BAV groups, reinforcing the observation that MD and VSMC apoptosis is not exclusive to BAV disease. Furthermore, it suggests that the mechanism of BAV aortopathy is active before aneurysm occurs, and that BAV aortas are inherently different from TAV.

Subsequently, Schmid *et al.* examined aortic samples from BAV and TAV patients with aortic aneurysms and compared them with donor control tissue[72]. Similarly to Bonderman *et al.*[10] they demonstrated MD in both TAV and BAV aneurysmal tissue, however MD was more severe in the BAV group. Apoptotic indices were no different between TAV and BAV, but were both significantly higher than control, which is concurrent with previous findings. Similarly, assessment of cellularity revealed a significant decrease in cell nuclei number in the TAV group and BAV group (25% and 32% respectively) compared to healthy control. In addition, expression of pro-apoptotic proteins FAS and Perforin (PRF) were found to be elevated in aneurysmal TAV and BAV tissue versus control. These proteins are associated with activation of the extrinsic pathway of apoptosis typically triggered by extracellular ligands. Interestingly, infiltration of inflammatory cells was seen in both BAV and TAV groups, which suggests a possible role of activated inflammatory cells releasing FAS and PRF to induce VSMC apoptosis.

Della Corte *et al.* support these earlier findings demonstrating a consistent increase in apoptotic VSMCs in BAV patients when minimal aortic dilation was present[21]. Again, this differed from TAV patients who displayed high variability in apoptotic indices. The authors also quantified VSMC density demonstrating significantly decreased VSMC numbers in normal or mildly dilated BAV aortas, but similar numbers in aneurysmal aortas compared to control. They also measured expression of pro-apoptotic BCL-2-modifying factor (BMF)-binding protein, which triggers apoptosis in response to ECM disruption, and anti-apoptotic BCL-2 mRNA expression as a marker of the molecular tendency to apoptosis. In the BAV non-aneurysmal group, elevated BMF-BCL-2 binding was observed suggesting that cytoskeletal disruption is occurring at an early stage. However, this did not increase further in BAV aneurysmal patients despite marginal increases in apoptotic index, which suggests differing mechanisms may underlie apoptosis in early and late BAV aortopathy. Differences in apoptotic index, cell density and expression of synthetic VSMC proteins between early and late aortopathy is interesting. A possible explanation is that normal contractile VSMCs are susceptible to flow-induced apoptosis, whereas phenotypically-changed synthetic VSMCs are not[8]. However, evidence for this switch in BAV aortopathy is lacking.

VSMC differentiation in BAV aortopathy

VSMCs retain a degree of plasticity allowing them to carry out specialised functions including contraction, proliferation and ECM synthesis[2]. The cells are capable of modifying their phenotype in a continuous and dynamic fashion between a more differentiated ‘contractile’ phenotype, and a less differentiated ‘synthetic’ phenotype, which allows them to achieve their function (Figure 2). The phenotypic state of the VSMC may be defined by the expression of characteristic proteins. α-smooth muscle actin (αSMA), smooth muscle myosin heavy chain (MYH11), calponin-1 (CNN1) and smooth muscle protein 22-alpha (SM22α) are key proteins expressed in the contractile phenotype. Downregulation of these, and upregulation of synthetic proteins (e.g. non-muscle myosin heavy chain; MYH10) is an indication that the VSMC is more towards the synthetic phenotype[17]. In reality, VSMC phenotype represents a spectrum between the contractile and synthetic type. Phenotype modification of VSMCs is a key aspect of vascular remodelling implicated in injury, atherosclerosis and aortic aneurysms[53, 68].

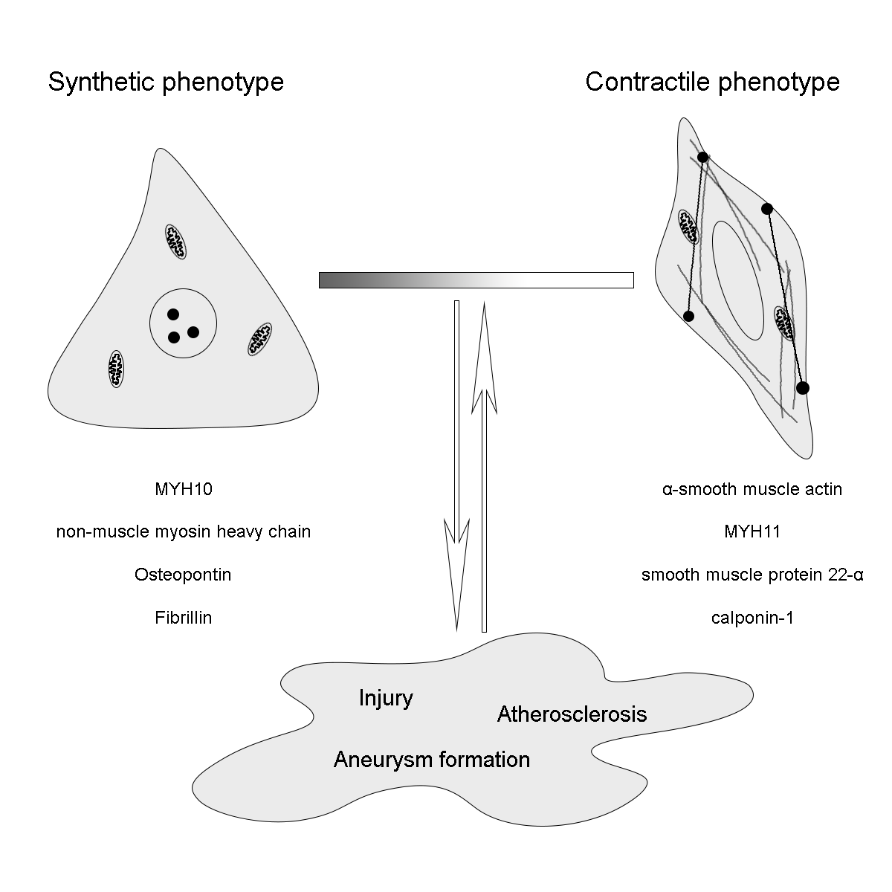


Figure 2 Phenotypic states of the vascular smooth muscle cell and key proteins specific for each state. Author’s illustration.

The extent to which VSMC differentiation is a feature of BAV aortopathy, and how this links to apoptosis remains to be elucidated. A few groups have highlighted differences in protein expression between BAV and TAV aortic samples which may indicate VSMC phenotype modification. Significantly elevated levels of osteopontin (OPN) and tenacin C (TNC), produced by synthetic VSMCs, have been demonstrated in TAV versus BAV aneurysms in a number of studies[26, 52, 83]. Folkersen *et al.* showed significant higher expression of TNC and SPP1 (osteopontin) genes in dilated aortas from TAV samples compared to BAV samples suggesting VSMCs in BAV aortopathy remain in the contractile state[26]. Non-dilated aortic samples from both TAV and BAV patients showed little difference in gene expressions suggesting changes in VSMC phenotype occur over time. Contrary to these findings, Cotrufo *et al.* found elevated TNC, and significantly decreased laminin (LAM) expression (a protein known to promote the contractile phenotype) in BAV patients versus healthy TAV controls[19].

In summary, there is some evidence to support a role of altered VSMC differentiation in BAV aortopathy all be it conflicting. Furthermore, no studies to date have compared differences in phenotypic markers between non-aneurysmal and aneurysmal aortic specimens from BAV and TAV patients. It is likely that changes in the state of VSMC differentiation occur as aortopathy progresses, which could provide further insight into the underlying mechanisms. In addition, previous studies have not looked specifically the expression of the contractile genes MYH11 and CNN1 as markers of differentiation in BAV and TAV patients. Since it is these proteins that provide the machinery necessary to function as well-differentiated contractile VSMCs, it seems appropriate to focus specifically on these genes when making inferences about the phenotypic state of VSMCs in the aortic wall.

The NOTCH signalling pathway

NOTCH signalling was first described in the laboratory of Thomas Hunt Morgan in 1913[60, 61]. During the late 1980s and early 1990s, evidence emerged to suggest that NOTCH works as an intercellular signalling mechanism via a transmembrane protein, with large extracellular and intracellular domains[3, 27, 30]. Activation of this protein triggers transcriptional intracellular changes leading to a variety of effects, including cell proliferation, differentiation and apoptosis[16, 58, 62]. Therefore, mutations in the NOTCH protein have been demonstrated to underlie a number of developmental disorders, whilst a dysregulation of the NOTCH signalling mechanism appears to result in tumour development in a number of tissue types types[50]. In comparison to other signalling mechanisms, NOTCH signalling does not occur in a paracrine way, mediated by ligands secreted distantly, but in a juxtacrine manner, the process only taking place between two adjacent cells and requiring the cells to be in direct contact (Figure 3)[43, 84].



Figure 3 NOTCH signalling pathway. NICD (NOTCH intracellular domain), CSL (CBF1, Suppressor of Hairless, Lag1), MAM (Mastermind), HES (Hairy enhancer of splice), MMP ADAM (a disintegrin and metalloproteinase). Author’s illustration.

Four NOTCH receptors (NOTCH 1-4) have been described in humans and represent large multidomain type I transmembrane proteins[43, 44]. Along with these receptors, three Delta-family ligands (Dll1, Dll3 and Dll4) and two Serrate-family ligands (Jagged1 and Jagged2) have been found in mammals. These are also type I transmembrane proteins but have a large extracellular domain with a short intracellular domain[20, 43]. The NOTCH receptor binds to the ligands expressed on the adjacent cell. This activates a proteinase, γ-secretase, which cleaves the NOTCH intracellular domain (NICD), releasing it. Following the NICD cleavage, the extracellular domain of the NOTCH receptor is endocytosed by the sending cell. The NICD then translocates to the nucleus where it interacts with a DNA binding transcriptor factor CSL (CBF1, Suppressor of Hairless, Lag1), and a coactivator Mastermind (MAML1-MAML3)[11, 78]. This cascade leads to the disassembling of the corepressor complex and derepression of the gene targets, with activation of transcription complexes[6, 14, 59]. The NICD is then phosphorylated by kinases (CDK8)[28] followed by polyubiquitination via E3 ubiquitin ligases like SEL10 or FBXW7[35], leading to degradation of NICD and termination of the signal, thus preventing continuous signal activation[31, 67, 82].

NOTCH signalling is thought to play a central role in the orchestration of aortic valve development. A key stage in formation of the primitive endocardial cushions is infiltration of migrating neural crest cells (NCC). During migration, a proportion of these cells differentiate into VSMC, which populate the wall of the developing ascending aorta, aortic arch, and head and neck vessels. Together with cells of the secondary heart field and mesenchyme, the NCC orchestrate many important aspects of cardiac outflow tract and ascending aortic development drawing many to hypothesise that a common defect may be responsible for BAV disease and the associated aortopathy[38]. In support of this, it is NCC-derived VSMCs that undergo increased apoptosis in BAV aortopathy[10].

Characterising NOTCH signalling in BAV aortopathy is of particular interest because *NOTCH1* mutations are implicated in the pathogenesis of BAV disease[29, 55]. To date however, relatively few studies have examined NOTCH signalling changes in human aortic aneurysms. Decreased expression of both NOTCH1 and NOTCH3 are reported in aortic samples from abdominal aortic aneurysms versus control, in parallel to decreased expression of contractile VSMC phenotype markers[9, 23]. Conversely, upregulation of NOTCH1, NICD and HES1 was reported in the wall of descending thoracic aortic aneurysms, but decreased expression of these proteins was shown when VSMC populations are examined in isolation[86]. To the best of our knowledge, only one study has quantified NOTCH signalling in ascending aortic tissue. Sciacca *et al.* demonstrated significantly decreased mRNA and protein expression of several regulators of NOTCH signalling in BAV versus TAV aortic tissue (including *NOTCH1* & *HES1*), although no reference to aortic dimension is given[73]. In summary, changes in NOTCH signalling may be a significant factor in the development of BAV aortopathy. Such changes may impact on VSMC apoptosis and/or differentiation, however more evidence is needed to confirm this.

The role of NOTCH signalling in altered VSMC apoptosis and differentiation in BAV aortopathy

Given the pivotal role of NOTCH signalling in aortic valve and ascending aortic development, there is remarkably little evidence to link NOTCH signalling with increased VSMC apoptosis and differentiation seen in BAV aortopathy. However, a few studies have investigated the effect of NOTCH signalling on apoptosis and differentiation in cultured VSMCs. Overexpression of *NOTCH1* and *NOTCH3* in rat VSMCs resulted in a significant decrease in cell apoptosis in association with a decrease in *BAX:BCL-xL* mRNA expression ratio[63, 75]. This observation concurs with the work of Sciacca *et al.* who demonstrated decreased NOTCH1 signalling in BAV aortas, which is consistent with the observation of increased VSMC apoptosis[73]. Similar findings from T-cell hybridoma work demonstrated that NOTCH1 receptor activation upregulated anti-apoptotic *BCL-2* expression[39]. Liu *et al.* demonstrated NICD upregulated X-linked inhibitor of apoptosis protein (XIAP) in Jurkat T leukaemia cells by direct interaction with the protein[49]. NICD appears to bind and prevent ubiquitin-dependent degradation of XIAP thereby potentiating its effect of inhibiting apoptosis. Incidentally, significantly reduced *XIAP* mRNA expression has also been demonstrated in patients with BAV and Turner syndrome versus those with TAV[41]. It is not clear whether this occurs in a NOTCH-dependent manner but may contribute to increased VSMC apoptosis in the ascending aorta. Increased expression of NOTCH3 but not NOTCH2 in human aortic VSMCs promoted cell survival genes *BCL-2*, *BIRC5* and *CFLAR* (cFLIP)[5]. Supporting these findings, Boucher *et al.* demonstrated reduced proliferation of human aortic VSMC when *NOTCH2* was activated, via upregulation of the cell cycle regulatory gene p27[12]. Together these observations suggest NOTCH1 & NOTCH3 activation are pro-survival, and NOTCH2 activation is pro-apoptotic. Given the observation of decreased NOTCH1 signalling in BAV aortas, we hypothesise that defective NOTCH1 signalling in BAV patients may contribute to increased apoptosis and ascending aortic aneurysm formation.

NOTCH signalling may also play a key role in cell differentiation. Endothelial cell-induced activation of NOTCH signalling in VSMCs is central to normal cardiovascular development, promoting VSMC development and maturation[33]. *In-vitro*, simulated activation of NOTCH signalling with Jagged1 ligand promotes the contractile phenotype in cultured human aortic VSMCs, as indicated by upregulation of αSM actin, SM22α and CNN1[77]. Lin *et al.* co-cultured vascular endothelial cells with human aortic VSMCs and demonstrated similar upregulation of contractile phenotype transcripts and cell quiescence related to upregulation of the *NOTCH3* mRNA expression[47]. However, they also showed upregulation of synthetic markers Caldesmon-1 (CALD1), Retinol binding protein-1 (RBP1), and Vimentin (VIM). Furthermore, inhibition of NOTCH signalling with the γ-secretase inhibitor DAPT blocked endothelial-induced contractile differentiation of VSMCs and decreased the expression of *NOTCH3* mRNA, suggesting NOTCH activation is key to this process. Interestingly however, NOTCH inhibition did not affect synthetic phenotype transcript expression suggesting that other factors may be responsible for promoting this phenotype. Consistent with these findings, Liu *et al.* demonstrated that repression of NOTCH3 in culture human aortic VSMCs stimulates proliferation, apoptosis and cell migration[48]. Conversely, Proweller *et al.* demonstrated inhibition of myocardin-induced VSMC differentiation in rat aortic VSMC transfected with constitutionally activate NOTCH1, as represented by decreased expression of αSM actin, SM22α and SM MyHC[69]. Myocardin has been identified as an essential co-factor for maintenance of the differentiated (contractile) VSMC phenotype[80]. Therefore, as for apoptosis, opposing effects of different NOTCH receptors on VSMC differentiation are seen, with NOTCH3 promoting the well-differentiated (contractile) phenotype and NOTCH1 promoting the de-differentiated (synthetic) phenotype. Whether or not these observations hold true for VSMCs in BAV aortas remains to be elucidated. There is a clear lack of evidence for the role of defective NOTCH signalling in apoptosis and differentiation in human aortic smooth muscle cells and BAV aortopathy.

Summary and future directions

NOTCH signalling is key to cell survival and differentiation, and mutations in the *NOTCH1* gene are implicated in BAV disease, a condition associated with abnormal apoptosis and differentiation of VSMCs. Yet a pathophysiological association between NOTCH signalling, apoptosis and differentiation in VSMCs from BAV aortas has not been established. Given the limited evidence available we hypothesise that inherent defective NOTCH1 activation in neural crest cell-derived VSMCs of the BAV ascending aorta promotes pro-apoptotic and inhibits anti-apoptotic protein expression. This imbalance drives VSMC apoptosis, which in turn disrupts the extracellular matrix homeostasis, fuelling catabolic degeneration of the ascending aortic wall, which over time thins and weakens predisposing to aneurysm and dissection. This process is perpetuated by defective NOTCH1 activation simultaneously promoting the contractile, well-differentiated VSMC phenotype which when driven to quiescence, fail to appropriately upregulate extracellular matrix synthesis and repair the thinning aortic wall. A summary of these hypotheses can be seen in Figure 4.

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| **A** |
| **B** |

Figure 4 Summary of the hypothesised mechanism for defective NOTCH signalling causing BAV aortopathy through increased apoptosis (A) and promotion of the contractile VSMC phenotype (B). Authors illustration.

There is clearly a need to design and implement meaningful basic research to further quantify NOTCH signalling in the ascending aorta of BAV patients and include consideration of differing aortic dimensions. Concurrent quantification of key apoptotic gene and protein expression (e.g. BAX and BCL-2) should also be made, together with markers of VSMC differentiation (e.g. MYH11, CNN1, MYH10). Furthermore, VSMCs should be isolated from the ascending aortas of BAV patients and subject to inhibition and activation of NOTCH signalling, and the effect on apoptotic and differentiation gene expression quantified. Our hope would be that therapeutic modulation of the NOTCH signalling pathway, may provide a means to reverse the pathological mechanism of increased VSMC apoptosis and differentiation, preserving the ascending aortic wall integrity, and preventing the potentially fatal complications of aneurysm and dissection.

References

1. Abbott ME (1928) Coarctation of the aorta of the adult type II. A statistical study and historical retrospect of 200 recorded cases, with autopsy, of stenosis or obliteration of the descending arch in subjects above the age of two years. American Heart Journal 3:381-421 doi:10.1016/S0002-8703(28)90386-5

2. Alexander MR, Owens GK (2012) Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annual review of physiology 74:13-40 doi:10.1146/annurev-physiol-012110-142315

3. Artavanis-Tsakonas S, Matsuno K, Fortini ME (1995) Notch signaling. Science 268:225-232

4. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770-776

5. Baeten JT, Lilly B (2015) Differential Regulation of NOTCH2 and NOTCH3 Contribute to Their Unique Functions in Vascular Smooth Muscle Cells. J Biol Chem 290:16226-16237 doi:10.1074/jbc.M115.655548

6. Barolo S, Stone T, Bang AG, Posakony JW (2002) Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. Genes Dev 16:1964-1976 doi:10.1101/gad.987402

7. Basso C, Boschello M, Perrone C, Mecenero A, Cera A, Bicego D, Thiene G, De Dominicis E (2004) An echocardiographic survey of primary school children for bicuspid aortic valve. The American journal of cardiology 93:661-663 doi:10.1016/j.amjcard.2003.11.031

8. Birney YA, Sweeney CH, Cappadona CR, Sitzmann JV, Cummins PM, Redmond EM, Cahill PA (2004) Pulse pressure-induced transmural fluid flux increases bovine aortic smooth muscle cell apoptosis in a mitogen activated protein kinase dependent manner. Journal of vascular research 41:364-374 doi:10.1159/000080700

9. Biros E, Walker PJ, Nataatmadja M, West M, Golledge J (2012) Downregulation of transforming growth factor, beta receptor 2 and Notch signaling pathway in human abdominal aortic aneurysm. Atherosclerosis 221:383-386 doi:10.1016/j.atherosclerosis.2012.01.004

10. Bonderman D, Gharehbaghi-Schnell E, Wollenek G, Maurer G, Baumgartner H, Lang IM (1999) Mechanisms Underlying Aortic Dilatation in Congenital Aortic Valve Malformation. Circulation 99:2138-2143 doi:10.1161/01.cir.99.16.2138

11. Borggrefe T, Liefke R (2012) Fine-tuning of the intracellular canonical Notch signaling pathway. Cell Cycle 11:264-276 doi:10.4161/cc.11.2.18995

12. Boucher JM, Harrington A, Rostama B, Lindner V, Liaw L (2013) A receptor-specific function for Notch2 in mediating vascular smooth muscle cell growth arrest through cyclin-dependent kinase inhibitor 1B. Circulation research 113:975-985 doi:10.1161/CIRCRESAHA.113.301272

13. Boyum J, Fellinger EK, Schmoker JD, Trombley L, McPartland K, Ittleman FP, Howard AB (2004) Matrix metalloproteinase activity in thoracic aortic aneurysms associated with bicuspid and tricuspid aortic valves. J Thorac Cardiovasc Surg 127:686-691 doi:10.1016/j.jtcvs.2003.11.049

14. Bray S, Furriols M (2001) Notch pathway: making sense of suppressor of hairless. Curr Biol 11:R217-221

15. Cecconi M, Manfrin M, Moraca A, Zanoli R, Colonna PL, Bettuzzi MG, Moretti S, Gabrielli D, Perna GP (2005) Aortic dimensions in patients with bicuspid aortic valve without significant valve dysfunction. The American journal of cardiology 95:292-294 doi:10.1016/j.amjcard.2004.08.098

16. Cereseto A, Tsai S (2000) Jagged2 induces cell cycling in confluent fibroblasts susceptible to density-dependent inhibition of cell division. J Cell Physiol 185:425-431 doi:10.1002/1097-4652(200012)185:3<425::AID-JCP13>3.0.CO;2-U

17. Chamley JH, Campbell GR, Burnstock G (1974) Dedifferentiation, redifferentiation and bundle formation of smooth muscle cells in tissue culture: the influence of cell number and nerve fibres. J Embryol Exp Morphol 32:297-323

18. Chinnaiyan AM, Dixit VM (1996) The cell-death machine. Curr Biol 6:555-562

19. Cotrufo M, Della Corte A, De Santo LS, Quarto C, De Feo M, Romano G, Amarelli C, Scardone M, Di Meglio F, Guerra G, Scarano M, Vitale S, Castaldo C, Montagnani S (2005) Different patterns of extracellular matrix protein expression in the convexity and the concavity of the dilated aorta with bicuspid aortic valve: preliminary results. J Thorac Cardiovasc Surg 130:504-511 doi:10.1016/j.jtcvs.2005.01.016

20. D'Souza B, Meloty-Kapella L, Weinmaster G (2010) Canonical and non-canonical Notch ligands. Curr Top Dev Biol 92:73-129 doi:10.1016/S0070-2153(10)92003-6

21. Della Corte A, Quarto C, Bancone C, Castaldo C, Di Meglio F, Nurzynska D, De Santo LS, De Feo M, Scardone M, Montagnani S, Cotrufo M (2008) Spatiotemporal patterns of smooth muscle cell changes in ascending aortic dilatation with bicuspid and tricuspid aortic valve stenosis: Focus on cell–matrix signaling. The Journal of Thoracic and Cardiovascular Surgery 135:8-18.e12 doi:10.1016/j.jtcvs.2007.09.009

22. Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300-304 doi:10.1038/40901

23. Doyle AJ, Redmond EM, Gillespie DL, Knight PA, Cullen JP, Cahill PA, Morrow DJ (2015) Differential expression of Hedgehog/Notch and transforming growth factor-beta in human abdominal aortic aneurysms. Journal of Vascular Surgery 62:464-470 doi:10.1016/j.jvs.2014.02.053

24. Fedak PWM, de Sa MPL, Verma S, Nili N, Kazemian P, Butany J, Strauss BH, Weisel RD, David TE (2003) Vascular matrix remodeling in patients with bicuspid aortic valve malformations: implications for aortic dilatation. The Journal of Thoracic and Cardiovascular Surgery 126:797-805 doi:10.1016/s0022-5223(03)00398-2

25. Fedak PWM, Verma S, David TE, Leask RL, Weisel RD, Butany J (2002) Clinical and Pathophysiological Implications of a Bicuspid Aortic Valve. Circulation 106:900-904 doi:10.1161/01.cir.0000027905.26586.e8

26. Folkersen L, Wagsater D, Paloschi V, Jackson V, Petrini J, Kurtovic S, Maleki S, Eriksson MJ, Caidahl K, Hamsten A, Michel JB, Liska J, Gabrielsen A, Franco-Cereceda A, Eriksson P (2011) Unraveling divergent gene expression profiles in bicuspid and tricuspid aortic valve patients with thoracic aortic dilatation: the ASAP study. Mol Med 17:1365-1373 doi:10.2119/molmed.2011.00286

27. Fortini ME, Artavanis-Tsakonas S (1993) Notch: neurogenesis is only part of the picture. Cell 75:1245-1247

28. Fryer CJ, White JB, Jones KA (2004) Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell 16:509-520 doi:10.1016/j.molcel.2004.10.014

29. Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, Grossfeld PD, Srivastava D (2005) Mutations in NOTCH1 cause aortic valve disease. Nature 437:270-274 doi:10.1038/nature03940

30. Greenwald I, Rubin GM (1992) Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. Cell 68:271-281

31. Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, Ciechanover A, Israel A (2001) Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. J Biol Chem 276:34371-34378 doi:10.1074/jbc.M101343200

32. Hahn RT, Roman MJ, Mogtader AH, Devereux RB (1992) Association of aortic dilation with regurgitant, stenotic and functionally normal bicuspid aortic valves. J Am Coll Cardiol 19:283-288

33. High FA, Lu MM, Pear WS, Loomes KM, Kaestner KH, Epstein JA (2008) Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proceedings of the National Academy of Sciences 105:1955-1959 doi:10.1073/pnas.0709663105

34. Hoffman JIE, Kaplan S (2002) The incidence of congenital heart disease. Journal of the American College of Cardiology 39:1890-1900 doi:10.1016/S0735-1097(02)01886-7

35. Hubbard EJ, Wu G, Kitajewski J, Greenwald I (1997) sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. Genes Dev 11:3182-3193

36. Huntington K, Hunter AG, Chan KL (1997) A prospective study to assess the frequency of familial clustering of congenital bicuspid aortic valve. J Am Coll Cardiol 30:1809-1812

37. Ikonomidis JS, Jones JA, Barbour JR, Stroud RE, Clark LL, Kaplan BS, Zeeshan A, Bavaria JE, Gorman JH, 3rd, Spinale FG, Gorman RC (2007) Expression of matrix metalloproteinases and endogenous inhibitors within ascending aortic aneurysms of patients with bicuspid or tricuspid aortic valves. J Thorac Cardiovasc Surg 133:1028-1036 doi:10.1016/j.jtcvs.2006.10.083

38. Jain R, Engleka KA, Rentschler SL, Manderfield LJ, Li L, Yuan L, Epstein JA (2011) Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves. J Clin Invest 121:422-430 doi:10.1172/jci44244

39. Jang MS, Miao H, Carlesso N, Shelly L, Zlobin A, Darack N, Qin JZ, Nickoloff BJ, Miele L (2004) Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. J Cell Physiol 199:418-433 doi:10.1002/jcp.10467

40. Janicke RU, Sprengart ML, Wati MR, Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273:9357-9360

41. Jevalikar GS, Zacharin M, White M, Yau SW, Li W, Ijspeert C, Russo VC, Werther GA, Sabin MA (2015) Turner syndrome patients with bicuspid aortic valves and renal malformations exhibit abnormal expression of X-linked inhibitor of apoptosis protein (XIAP). Journal of pediatric endocrinology & metabolism : JPEM 28:1203-1208 doi:10.1515/jpem-2014-0208

42. Joziasse IC, Vink A, Cramer MJ, van Oosterhout MF, van Herwerden LA, Heijmen R, Sieswerda GT, Mulder BJ, Doevendans PA (2011) Bicuspid stenotic aortic valves: clinical characteristics and morphological assessment using MRI and echocardiography. Neth Heart J 19:119-125 doi:10.1007/s12471-010-0060-6

43. Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216-233 doi:10.1016/j.cell.2009.03.045

44. Kovall RA, Blacklow SC (2010) Mechanistic insights into Notch receptor signaling from structural and biochemical studies. Curr Top Dev Biol 92:31-71 doi:10.1016/S0070-2153(10)92002-4

45. Lakin ND, Jackson SP (1999) Regulation of p53 in response to DNA damage. Oncogene 18:7644-7655 doi:10.1038/sj.onc.1203015

46. Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491-501

47. Lin CH, Lilly B (2014) Notch signaling governs phenotypic modulation of smooth muscle cells. Vascul Pharmacol 63:88-96 doi:10.1016/j.vph.2014.09.004

48. Liu N, Li Y, Chen H, Wei W, An Y, Zhu G (2015) RNA interference-mediated NOTCH3 knockdown induces phenotype switching of vascular smooth muscle cells in vitro. Int J Clin Exp Med 8:12674-12684

49. Liu W HH, Tsou W, Lai M (2007) Notch inhibits apoptosis by direct interference with XIAP ubiquitination and degradation. The EMBO journal 26:1660-1669 doi:10.1038/

50. Louvi A, Artavanis-Tsakonas S (2012) Notch and disease: a growing field. Semin Cell Dev Biol 23:473-480 doi:10.1016/j.semcdb.2012.02.005

51. Majesky MW (2007) Developmental basis of vascular smooth muscle diversity. Arterioscler Thromb Vasc Biol 27:1248-1258 doi:10.1161/ATVBAHA.107.141069

52. Majumdar R, Miller DV, Ballman KV, Unnikrishnan G, McKellar SH, Sarkar G, Sreekumar R, Bolander ME, Sundt TM, 3rd (2007) Elevated expressions of osteopontin and tenascin C in ascending aortic aneurysms are associated with trileaflet aortic valves as compared with bicuspid aortic valves. Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology 16:144-150 doi:10.1016/j.carpath.2006.12.001

53. Mao N, Gu T, Shi E, Zhang G, Yu L, Wang C (2015) Phenotypic switching of vascular smooth muscle cells in animal model of rat thoracic aortic aneurysm. Interact Cardiovasc Thorac Surg 21:62-70 doi:10.1093/icvts/ivv074

54. McCarthy NJ BM (2000) The regulation of vascular smooth muscle cell apoptosis. Cardiovascular Research 45:747–755

55. McKellar SH, Tester DJ, Yagubyan M, Majumdar R, Ackerman MJ, Sundt TM, 3rd (2007) Novel NOTCH1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. J Thorac Cardiovasc Surg 134:290-296 doi:10.1016/j.jtcvs.2007.02.041

56. McKusick VA (1972) Association of congenital bicuspid aortic valve and erdheim's cystic medial necrosis. Lancet 1:1026-1027

57. Michelena HI, Prakash SK, Della Corte A, Bissell MM, Anavekar N, Mathieu P, Bosse Y, Limongelli G, Bossone E, Benson DW, Lancellotti P, Isselbacher EM, Enriquez-Sarano M, Sundt TM, 3rd, Pibarot P, Evangelista A, Milewicz DM, Body SC, Investigators BA (2014) Bicuspid aortic valve: identifying knowledge gaps and rising to the challenge from the International Bicuspid Aortic Valve Consortium (BAVCon). Circulation 129:2691-2704 doi:10.1161/CIRCULATIONAHA.113.007851

58. Miele L, Osborne B (1999) Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol 181:393-409 doi:10.1002/(SICI)1097-4652(199912)181:3<393::AID-JCP3>3.0.CO;2-6

59. Morel V, Schweisguth F (2000) Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. Genes Dev 14:377-388

60. Morgan TH (1916) Sex-linked inheritance in Drosophila. Carnegie Institution of Washington

61. Morgan TH (1917) The Theory of the Gene. The American Naturalist 51:513-544 doi:10.1086/279629

62. Morimura T, Goitsuka R, Zhang Y, Saito I, Reth M, Kitamura D (2000) Cell cycle arrest and apoptosis induced by Notch1 in B cells. J Biol Chem 275:36523-36531 doi:10.1074/jbc.M006415200

63. Morrow D, Sweeney C, Birney YA, Cummins PM, Walls D, Redmond EM, Cahill PA (2005) Cyclic strain inhibits Notch receptor signaling in vascular smooth muscle cells in vitro. Circ Res 96:567-575 doi:10.1161/01.RES.0000159182.98874.43

64. Nataatmadja M, West M, West J, Summers K, Walker P, Nagata M, Watanabe T (2003) Abnormal extracellular matrix protein transport associated with increased apoptosis of vascular smooth muscle cells in marfan syndrome and bicuspid aortic valve thoracic aortic aneurysm. Circulation 108 Suppl 1:II329-334 doi:10.1161/01.cir.0000087660.82721.15

65. Niessen K, Karsan A (2008) Notch Signaling in Cardiac Development. Circulation research 102:1169-1181 doi:10.1161/circresaha.108.174318

66. Niwa K, Perloff JK, Bhuta SM, Laks H, Drinkwater DC, Child JS, Miner PD (2001) Structural abnormalities of great arterial walls in congenital heart disease: light and electron microscopic analyses. Circulation 103:393-400

67. Oberg C, Li J, Pauley A, Wolf E, Gurney M, Lendahl U (2001) The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. J Biol Chem 276:35847-35853 doi:10.1074/jbc.M103992200

68. Owens GK KM, Wamhoff BR (2004) Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. Physiological reviews 84:767-801

69. Proweller A, Pear WS, Parmacek MS (2005) Notch signaling represses myocardin-induced smooth muscle cell differentiation. J Biol Chem 280:8994-9004 doi:10.1074/jbc.M413316200

70. Roberts WC, Ko JM (2005) Frequency by decades of unicuspid, bicuspid, and tricuspid aortic valves in adults having isolated aortic valve replacement for aortic stenosis, with or without associated aortic regurgitation. Circulation 111:920-925 doi:10.1161/01.cir.0000155623.48408.c5

71. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. The EMBO journal 17:1675-1687 doi:10.1093/emboj/17.6.1675

72. Schmid F-X, Bielenberg K, Schneider A, Haussler A, Keyser A, Birnbaum D (2003) Ascending aortic aneurysm associated with bicuspid and tricuspid aortic valve: involvement and clinical relevance of smooth muscle cell apoptosis and expression of cell death-initiating proteins. European Journal of Cardio-Thoracic Surgery 23:537-543 doi:10.1016/s1010-7940(02)00833-3

73. Sciacca S, Pilato M, Mazzoccoli G, Pazienza V, Vinciguerra M (2013) Anti-correlation between longevity gene SirT1 and Notch signaling in ascending aorta biopsies from patients with bicuspid aortic valve disease. Heart & Vessels 28:268-275 doi:10.1007/s00380-012-0238-5

74. Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483-487 doi:10.1038/20959

75. Sweeney C, Morrow D, Birney YA, Coyle S, Hennessy C, Scheller A, Cummins PM, Walls D, Redmond EM, Cahill PA (2004) Notch 1 and 3 receptor signaling modulates vascular smooth muscle cell growth, apoptosis, and migration via a CBF-1/RBP-Jk dependent pathway. FASEB J 18:1421-1423 doi:10.1096/fj.04-1700fje

76. Takahashi A, Hirata H, Yonehara S, Imai Y, Lee KK, Moyer RW, Turner PC, Mesner PW, Okazaki T, Sawai H, Kishi S, Yamamoto K, Okuma M, Sasada M (1997) Affinity labeling displays the stepwise activation of ICE-related proteases by Fas, staurosporine, and CrmA-sensitive caspase-8. Oncogene 14:2741-2752 doi:10.1038/sj.onc.1201131

77. Tang Y, Urs S, Boucher J, Bernaiche T, Venkatesh D, Spicer DB, Vary CP, Liaw L (2010) Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. J Biol Chem 285:17556-17563 doi:10.1074/jbc.M109.076414

78. Tanigaki K, Honjo T (2010) Two opposing roles of RBP-J in Notch signaling. Curr Top Dev Biol 92:231-252 doi:10.1016/S0070-2153(10)92007-3

79. Tutar E, Ekici F, Atalay S, Nacar N (2005) The prevalence of bicuspid aortic valve in newborns by echocardiographic screening. American Heart Journal 150:513-515 doi:10.1016/j.ahj.2004.10.036

80. Wang YW, Ren HL, Wang HF, Li FD, Li HH, Zheng YH (2015) Combining detection of Notch1 and tumor necrosis factor-alpha converting enzyme is a reliable biomarker for the diagnosis of abdominal aortic aneurysms. Life Sciences 127:39-45 doi:10.1016/j.lfs.2015.02.009

81. Ward C (2000) Clinical significance of the bicuspid aortic valve. Heart (British Cardiac Society) 83:81-85

82. Wu G, Lyapina S, Das I, Li J, Gurney M, Pauley A, Chui I, Deshaies RJ, Kitajewski J (2001) SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol Cell Biol 21:7403-7415 doi:10.1128/MCB.21.21.7403-7415.2001

83. Yamamoto M, Aoyagi M, Azuma H, Yamamoto K (1997) Changes in osteopontin mRNA expression during phenotypic transition of rabbit arterial smooth muscle cells. Histochemistry and cell biology 107:279-287 doi:10.1007/s004180050113

84. Yamamoto S, Schulze KL, Bellen HJ (2014) Introduction to Notch signaling. Methods Mol Biol 1187:1-14 doi:10.1007/978-1-4939-1139-4\_1

85. Yasuda H, Nakatani S, Stugaard M, Tsujita-Kuroda Y, Bando K, Kobayashi J, Yamagishi M, Kitakaze M, Kitamura S, Miyatake K (2003) Failure to prevent progressive dilation of ascending aorta by aortic valve replacement in patients with bicuspid aortic valve: comparison with tricuspid aortic valve. Circulation 108 Suppl 1:II291-294 doi:10.1161/01.cir.0000087449.03964.fb

86. Zou S, Ren P, Nguyen M, Coselli JS, Shen YH, LeMaire SA (2012) Notch signaling in descending thoracic aortic aneurysm and dissection. PLoS One 7:e52833 doi:10.1371/journal.pone.0052833