Endothelial Dysfunction and Inflammatory Activation in Patients with Bicuspid Aortic Valves

Master of Clinical Science

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Abstract

Bicuspid aortic valve (BAV) is found to affect 1-2% of the Western population and represents the most common congenital cardiac disorder. BAV is associated with valvular dysfunction and aortopathy and its main clinical significance lies in its association with increased variable rates of progressive valve calcification and/or dilatation of the ascending aorta. Often significant aortic stenosis and/or regurgitation ensue. Sometimes BAV is associated with other forms of congenital heart disease particularly that of coarctation of the aorta. Furthermore the natural history of BAV often results in the need for extensive, corrective valvular and/or aortic surgery before the age of 60. Both inflammatory activation and endothelial dysfunction have been considered as potential modulators of these changes; however the predominant pathophysiological bases' are unclear. Data from endothelial nitric oxide synthase (eNOS) -/- mice and aortic biopsies in patients undergoing surgery suggest an association between eNOS deficiency and BAV though detailed evaluation of NO signalling in BAV is lacking. Furthermore, valvular and aortic degeneration varies widely among individuals with BAV. Both aortic stenosis and aortic dilatation in the context of BAV have shown to be associated with an inflammatory process. Therefore the relative impacts of inflammatory infiltration and endothelial dysfunction on valvular function and aortic dilatation in a cohort of patients with BAV were examined.

Methods:

A case-control study of patients with BAV was performed together with a multivariate analysis within the BAV group in order to identify factors associated with:

(a) Development of significant valvular disease.

(b) Dilatation of the ascending aorta.

(c) Differential valve: aortic disease.

BAV patients and controls underwent evaluation of endothelial function with flow mediated dilatation (FMD) and plasma concentrations of asymmetric dimethylarginine (ADMA). Correlations with inflammatory markers, myeloperoxidase (MPO) and high sensitivity C-reactive protein (HsCRP), endothelial progenitor cell counts (EPC) were also examined. Morphological and physiological assessment of the valve and ascending aorta was performed with transthoracic echocardiography (TTE) and magnetic resonance imaging (MRI).

Results:

Patients with BAV (n=43) and controls (n=25) were age and gender-matched. FMD was significantly lower in the BAV patient group (7.85% ± 3.48% vs 11.58%± 3.98%, p = 0.001) and these differences were age-independent on ANOVA. Within the BAV cohort, upon

multivariate analysis, correlates of peak aortic valve velocity (peak AV_{max}) were ADMA and MPO plasma concentrations (both $p< 0.01$), while increasing age was noted as an independent correlate of ascending aortic diameter ($p<0.05$). Furthermore, both low FMD and inflammatory activation were multivariate correlates of selectivity for valvular over aortic disease.

Conclusions:

While BAV is associated with endothelial dysfunction evident from low FMD and inflammatory activation (specifically MPO release), its structural impact primarily acts on the integrity of the valve, rather than the aortic structure. Confirmatory therapeutic interventions should be directed at reversal of these pathophysiological changes as well as slowing of disease progression.

Declaration

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Publication / presentation list

2014 Chapman MJ, Henthorn R, Surikow S, Zoontjens J, Stocker B, Mclean T, Zeitz CJ. Rheumatic mitral valve disease diagnostic tissue quantification (backscatter) *Euro Echo-Imaging* 2014 abstract 90680. Vienna Austria.

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Ali OA, Chapman MJ, Chirkov YY, Horowitz JD Physiological correlates of progression of aortic and valve disease in patients with type I bicuspid aortic valve. *European Journal Echocardiography* 2011. Abstracts Supplement December 2011. P253.

Presentations Conferences

2014 European echocardiography and other imaging modalities. Vienna Austria 3-7 December 2014. Poster presentation. **Rheumatic mitral valve disease diagnostic tissue quantification (backscatter).**

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Abbreviations

Adenosine 5`–diphosphate	ADP
Angiotensin II	ANGII
Angiotensin converting enzyme	ACE
Angiotensin receptor blockers	AT1
Aortic valve	AV
Aortic valve replacement	AVR
Aortic sclerosis	Asc
Ascending aorta	AscAO
Asymmetric dimethyl arginine	ADMA
Bicuspid aortic valve	BAV
Cardiac magnetic resonance imaging	CMRI
Colour flow doppler	CFM
Continuous wave	CW
Cyclic guanosine monophosphate	cGMP
Electrocardiogram	ECG
Endothelial dysfunction	ED
Flow mediated dilatation	FMD
High sensitivity C-reactive protein	HsCRP
Inflammatory activation	IA
Left ventricular	LV
Low density lipoproteins	LDL's
Left ventricular outflow tract	LVOT
Mast cell	MC
Matrix metalloproteinases	MMP's

Myeloperoxidase	MPO
Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH
Nitric oxide	NO
Pulsed wave	PW
Parasternal long axis view	PLAX
Parasternal short axis	PSAX
Sodium nitroprusside	SNP
Thioredoxin Interacting Protein	TXNIP
Trans-aortic valve implantation procedures	TAVI
Transforming growth factor β_1	$TGF_{\beta 1}$
Tricuspid aortic valve	TAV
Two dimensional	2D
Valvular endothelial cells	VECs
Velocity time interval	VTI

1.1 Clinical perspectives in contemporary western society

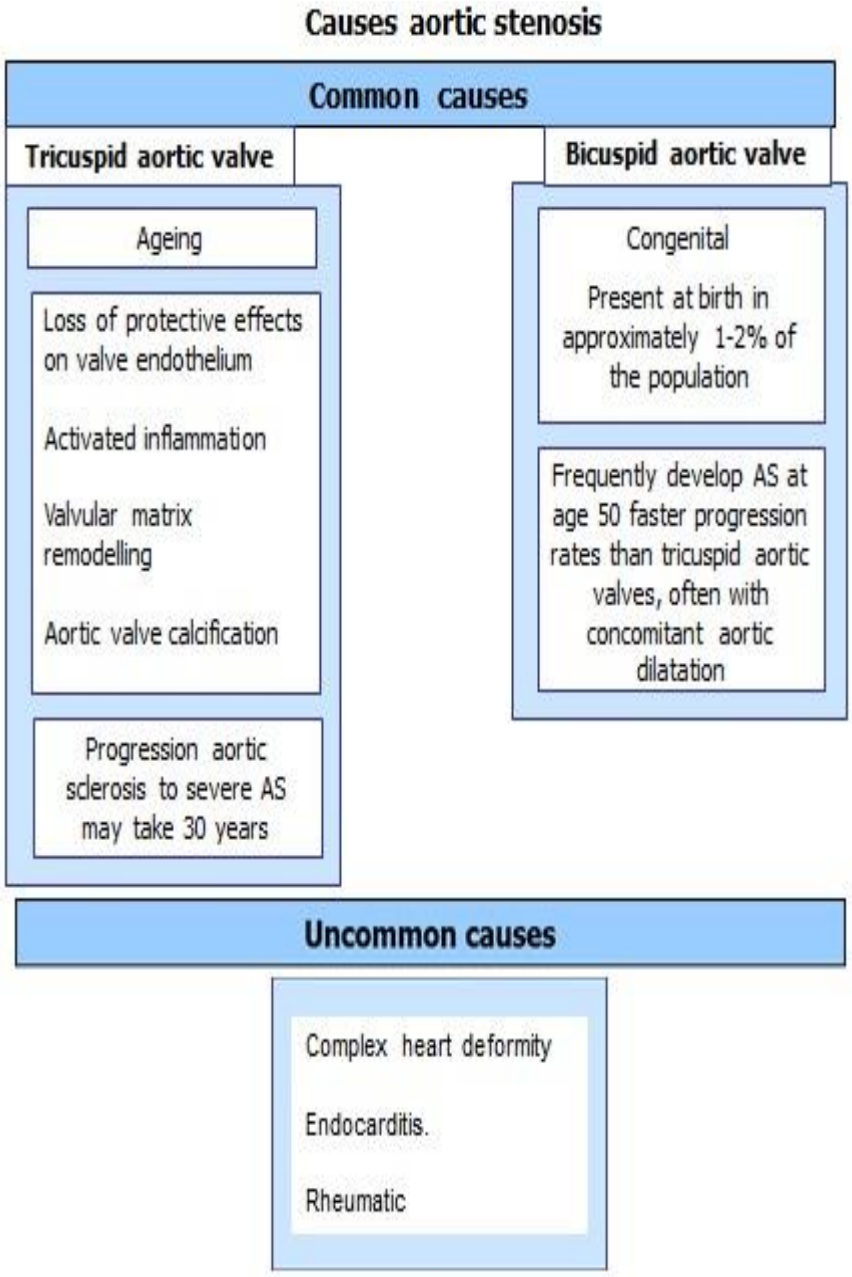
Aortic stenosis (AS) is the most frequent type of valvular heart disease in the western world - it is defined as a narrowing of the aortic valve (AV) orifice area. This resultant progressive narrowing contributes to an increase in left ventricular (LV) after load (Sverdlov AL et al., 2011). Its precursor, aortic valve sclerosis (ASc), is defined as an irregular thickening often with focal calcification on the valve leaflets. Conversely sclerotic AV leaflet excursion is generally not restricted, nor are the commissures fused resulting in the absence of significant LV outflow obstruction (Sverdlov AL et al., 2011).

While AS may occur as a complex congenital disorder or as part of rheumatic heart disease, there are two main causes of AS in the western world (see table 1.1) The most common being that of degenerative calcific AS arising in individuals with advancing age and tricuspid aortic valves (TAV) (Lindroos M et al., 1993). In this condition changes are found within an otherwise normal valvular matrix and typically the onset of the process is around 50 years of age (Braunwald E & Goldman L., 2003). The time-course for commencement of ASc (early valvular inflammation) progressing to that of severe AS is slow and may take 30 years or longer (Sverdlov AL et al., 2012). Moreover some degree of valve calcification is found in 75% of the population aged over 85 years and the prevalence of critical AS increases with age to approximately 3% for ages > 85 years (Lindroos M et al., 1993).

The second major cause of AS is congenital bicuspid aortic valve (BAV) which presents at birth and is found to affect 1-2% of the western population (Braverman AC et al., 2005 &

Samuel C et al., 2010). Interestingly patients with BAV frequently develop significant progressive AS by age 50 and are also prone to have concomitant problems such as dilatation of the aorta, aortic regurgitation and coarctation of the descending aorta (Braverman AC et al., 2005).

Table 1.1.



*Rheumatic heart disease remains a common finding in the presence of social deprivation.

1.2 Inflammatory activation and the progression of AS

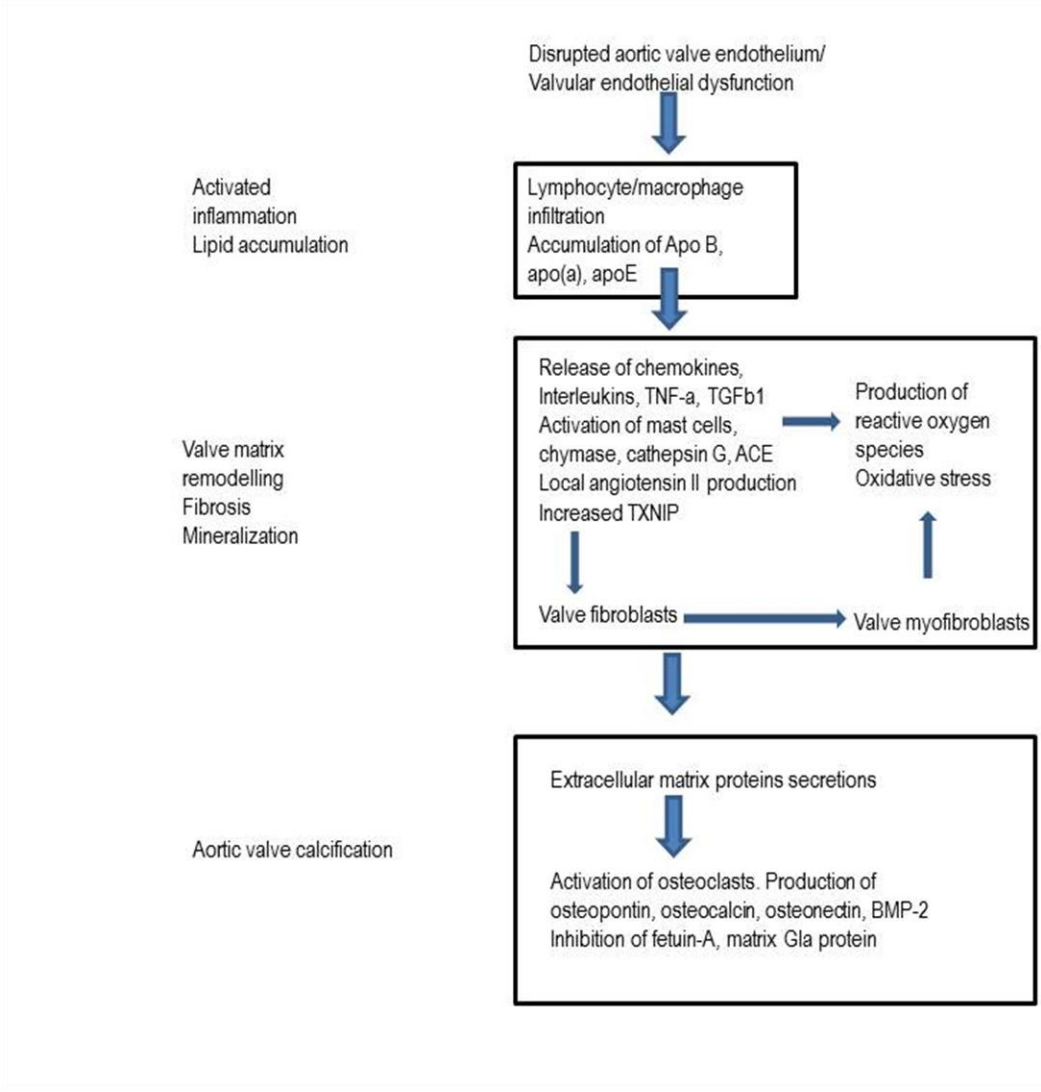
AS is associated with an inflammatory infiltration within the valvular matrix (schematized in figure 1.1) (Chen K et al., 2003 & Otto CM et al., 1994). Furthermore there is evidence of systemic inflammatory activation. A critical issue is whether this low-grade inflammation is distinct in origin from that associated with atherosclerosis. A number of investigations suggest that all stages of aortic valve disease are also associated with impaired vascular endothelial function (El-Hamamsy I et al., 2009).

The normal aortic valve endothelium is a potent generator of nitric oxide (NO) and prostacyclin (El-Hamamsy I et al., 2009 & Pompilio G et al., 1998). However with development of ASc/AS valvular tissue exhibits potential attenuation of NO release, due in part to the denudation of the valve endothelium (Pompilio G et al., 1998) but also potentially due to impairment of end-organ resistance to NO effect (Chirkov YY et al., 2002). Moreover, in patients with AS, platelets manifest both an increase in aggregation in response to adenosine 5`–diphosphate (ADP) and also inhibition of aggregation with the NO donor sodium nitroprusside (SNP) is reduced (Chirkov YY et al., 2002).

A contributing mechanism to the earliest stages of AS development is increasing tissue concentrations of angiotensin II (ANGII), an important mediator of inflammation and fibrosis (Sverdlov AL et al., 2011). ANGII accumulates as a result of the effects of angiotensin converting enzyme (ACE) and possibly mast cell (MC) - derived neutral protease chymase. ANG II supports a pro-fibrotic and pro-inflammatory environment, further contributing to injury of the valvular matrix. (Sverdlov AL et al., 2011). Thioredoxin Interacting Protein (TXNIP) is a

pro-inflammatory intracellular protein which is expressed in response to non-laminar flow, redox stress, hyperglycaemia and lack of NO (Chong C et al., 2014). Accumulation of TXNIP within calcifying aorta valve cells has been demonstrated in a rabbit model of AS (Ngo DMT et al., 2011). In this model, ACE inhibition with Ramipril limited the development of AS and also prevented AS progression. However it remains unclear whether TXNIP is critically important regarding progression of human AS. See summary figure 1.1

Figure 1.1 Postulation of inflammation and calcification in the progression of AS



(Sverdlov et al., 2011)

1.3 Available therapy and economic impact.

In patients with severe AS, therapeutic options are limited apart from aortic valve replacement (AVR). However in patients with mild to moderate AS strategies to retard progression of disease would potentially be of considerable advantage in fact, because severe AS usually develops in old age and co morbidities may preclude valve replacement in many patients. Trans-aortic valve implantation procedures (TAVI) may partially obviate this problem.

During the 2010 – 2011 financial year the median cost for AVR (per patient) in Australia was $45 000 - $50 000, including hospital and medical service related charges (Neyt M et al., 2011). Moreover TAVI procedures (an increasing option for very frail patients), have been reported to cost ~ $50 000 - $60 000, with the actual cost of the TAVI valve itself reaching approximately ~ $25 000 - $30 000 per patient (Neyt M et al., 2011). Thus if it were possible to reduce the likelihood of AS progression to that of severe obstructive stage, the benefits in health care cost would be considerable.

2.1 BAV: a special case.

Congenital bicuspid aortic valve (BAV) may be defined on the basis of the presence of two leaflets, often of unequal size. There is resultant fusion at the commissures with the larger leaflet contributing to a raphe (Braverman AC 2005 & Samuel C et al., 2010). Rarely are the aortic cusps of equal size with no raphe, known as pure bicuspid valve.

BAV, a congenital anomaly, affects the heart with a prevalence of 1- 2% of the population (Braverman AC et al., 2005 & Samuel C et al., 2010) and is more common in males. The major pathophysiological consequence of BAV is the risk of progressive valvular dysfunction with both valvular stenosis and regurgitation occurring frequently (Otto CM., 2002). In patients with BAV, valvular gradients increase more rapidly compared to tricuspid aortic valves (TAV) (Bepu S et al., 1993). On the basis of this propensity for rapid development of stenosis, BAV underlies 70-80% of stenotic aortic valves in children and at least 40% of aortic stenosis in adults (Schoen FJ., 2008 & Cripe L et al., 2004). The most common complication in BAV patients, the early development of AS, often occurs in the 4th decade of age (Yener N et al., 2002). In contrast TAV AS development rarely occurs before the 6th to 7th decade (Sverdlov AL et al., 2011).

The pathology of BAV is not restricted to the valvular tissue. Subjects with BAV tend to have a larger and stiffer aortic sinus and, are also at increased risk of developing aortic dilatation, aneurysm and dissection. Furthermore these aortic abnormalities represent the most common basis for aortic valve replacement under the age of 70 years (Braverman AC et al., 2005). There is also an association with coarctation of the aorta, which is described more extensively on page 24.

As regards progression of aortopathy a BAV study by Thanassoulis G et al (2008), demonstrated a mean increase in the diameter of the ascending aorta of 0.37mm/year and 0.17mm/year for the aortic sinus of Valsalva. As regards progression of aortic valve stenosis Tzemos N et al (2008), suggested that a mean annum increase in aortic valve gradient of 0.7 mmHg arrived based on a prospective cohort of 642 subjects.

BAV is not structurally a homogeneous disorder (Sabet HY et al., 1999). First, the pattern of cusp fusion varies: type 1 right/left coronary cusp fusion gives rise to anterior-posterior leaflet orientation making up to 70% of BAV cases (Table2) and (figure 2). The implications of the presence of BAV on cardiovascular anatomy also vary with the pattern of cusp fusion; specifically the known association between BAV, aortic sinus enlargement and the presence of coarctation is stronger for the right and left cusp fusion pattern than for other forms of BAV (Fernandes B et al., 2004 & Schaefer BM et al., 2007). This pattern of cusp fusion interestingly is associated with a greater risk of aortic dissection and rupture.

Type II BAV comprises fusion of the right and non-coronary cusps and is observed in approximately 25% of BAV cases. This morphology is associated with a higher prevalence of significant aortic stenosis, regurgitation and arch dilatation, including a higher incidence to eventual AVR (Otto CM., 2002 & Schaefer BM et al., 2007).

Approximately 1% of cases give rise to left and non-coronary cusp fusion – type III BAV. (Schaefer BM et al., 2007). Table 2 summarises the major anatomical features and potential physiological associations (on the basis primarily of findings in animal models) on BAV subtypes.

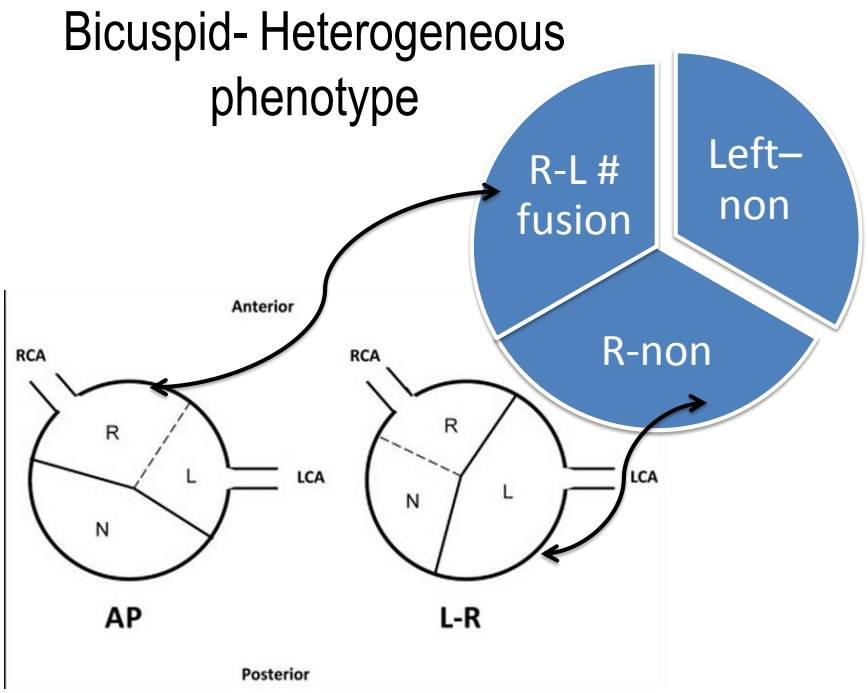
Table 2: Subtypes of bicuspid aortic valves - their anatomical associations and laboratory model features to the different BAV subtypes (Sverdlov AL et al., 2011 & Abdulkareem N et al., 2013).

Subtypes of BAV
Anatomical, associated and genetic features.

Subtype / prevalence	Anatomical features	association	Laboratory model Genetics
Type I 70-75%	Right and left coronary cusp fusion. Resultant anterior – posterior orientation	Male predominance. Aortic root dilatation and co-arctation prevalence	Inbred Syrian hamster. Likely an nomalous septation of the proximal portion of the outflow tract, which is caused by distorted behaviour of the neural crest cells
Type II 25-30%	Right and non coronary cusp fusion. Resultant right and left commissural orientation.	More common in females. Higher progressive aortic stenoisis and aortic regurgitation.	GATA 5 -/- Mice eNOS -/- Mice Morphological defect occurring before cardiac outflow-tract septation. ? Reliance on NO dependent epithelial to mesenchymal transformation.
Type III ~1%	Fusion Non and left coronary cusp.	No known association	Not known

Finally, there are limited data suggesting associations between BAV and some systemic syndromes, specifically Turner's and Williams' syndromes (Siu SC. & silversides C K., 2010).

Figure 2.1 Schematic: Types of BAV



Type 1 BAV **Type II BAV**

2.2 Clinical diagnosis

The majority of patients with BAV are asymptomatic for many years and indeed the diagnosis in childhood is most likely to be made on the basis of hypertension due to associated coarctation, alternatively, BAV may be an incidental finding on routine examination. Specifically cardiac examination of a systolic ejection murmur or that of aortic insufficiency may be heard. Suspicion of BAV clinically, generally requires further modality analysis, such as that of echocardiography.

2.3 Imaging studies

(a) Echocardiography

Two dimensional (2D) echocardiography; generally provides an accurate assessment for confirmation of BAV. The parasternal short axis (PSAX) view is used to determine valvular morphology as seen in (Figure 3.1). In optimal images the echocardiographic sonographer can determine, cusp fusion and the presence of a raphe or incidence of a pure BAV. In addition assessment of the possibility of coarctation through the descending AO can be imaged from a suprasternal view located near the suprasternal notch.

Continuous wave (CW) and pulsed wave (PW) Doppler in addition to 2D echocardiography is applied during the examination for the determination of the degree of aortic stenosis. The 2-D echocardiography view of apical-5 and apical 3 chambers are utilised. Furthermore a smaller footprint probe is used in numerous cardiac windows to obtain maximal CW Doppler through the AV. Valvular gradients can be determined utilising the simplified Bernoulli equation ($4V^2$). The aortic valve area AVA is determined by utilising the continuity equation - dividing the left ventricular stroke volume by the aortic valve velocity time integral (VTI). The AVA and pressure gradients assist in grading the degree of stenosis to that of mild, moderate or severe.

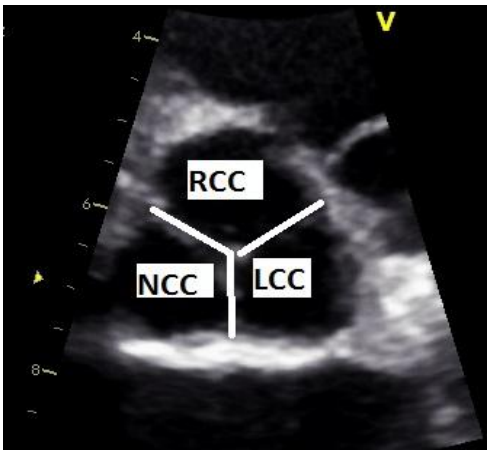
In the determination of the degree of aortic insufficiency, colour flow mapping (CFM) is utilised. CFM flow reversal in the suprasternal and abdominal aorta provide qualitative assessment for severity of the regurtitant lesion to that of moderate or severe.

The parasternal long axis view (PLAX) determines if there is systolic doming of the aortic valve, an indicator of BAV. The PLAX view also allows accurate measurements of the sinus of Valsalva, the sinotubular junction and the ascending aorta. In this view the determination and classification of aortic dilatation can be determined such as normal aorta (type N), dilatation of the ascending aorta (Type A) and effacement of the aorta (Type E). Examples of echographic images of normal valves with type 1 and type 2 are shown in figure 3.1.

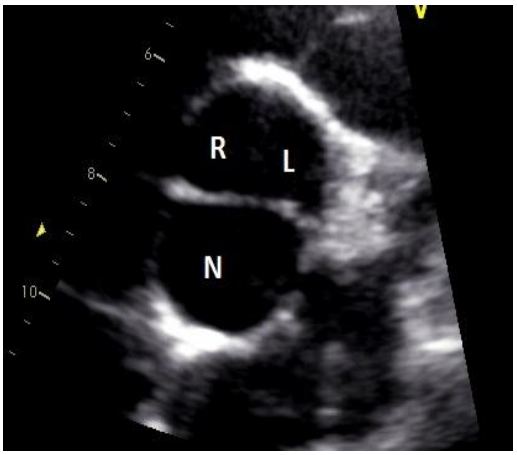
(b) Magnetic resonance imaging MRI and computerised tomography CT

Cardiac magnetic resonance imaging (cMRI) can evaluate functional assessment of the LV with sequences in a standard short and long axis cardiac view. Gating to electrocardiogram (ECG) is provided and timed to mid-diastole. Dimensions of the aortic root and ascending aorta are obtained from a dedicated short and long axis view. Ascending aortic dimension, measured at the level of pulmonary artery bifurcation is obtainable and measurements of the aorta and pulmonary artery trunk are of gold standard.

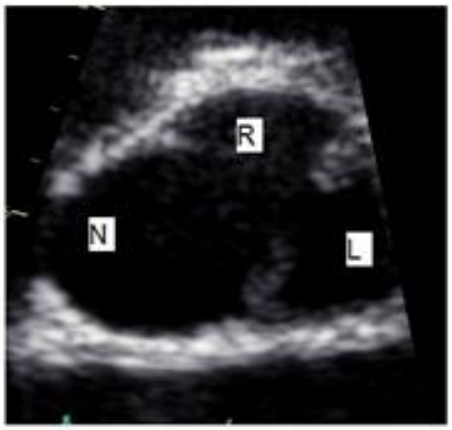
Figure 3.1



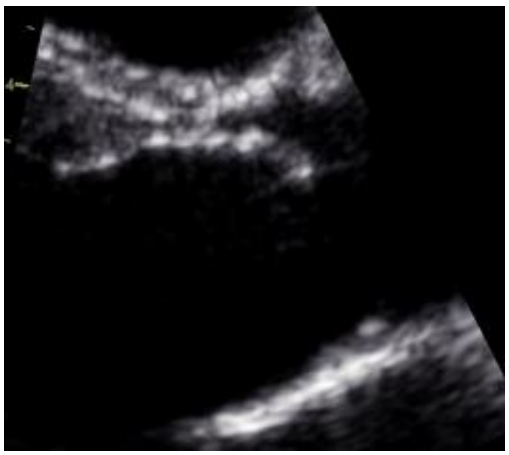
Echocardiographic features of TAV



Echocardiography features BAV type I



Echocardiographic features BAV type II



Systolic doming Echocardiography

2.4 Genetics

It is clear that BAV may be familial (Cripe L et al., 2004) but current understanding of genotype-phenotype relationship is incomplete**.** Overall genetic investigations suggest an autosomal dominant inheritance with a 3:1 male prevalence. The NOTCH 1 gene is known to be expressed in Cardiac embryogenesis in development of the cardiac outflow tract and the aortic valve; it may be responsible for abnormal valvular development potentially giving rise to BAV (Samuel C et al., 2010). Interestingly the NOTCH 1 gene has been linked to calcified AS progression (Acharya A et al., 2011).

2.4.1 Laboratory models of BAV: Clinical correlates

A number of unique findings have emerged regarding BAV. Lee TC et al (2000), found that homozygous deletion of the endothelial nitric oxide synthase (eNOS) gene in mice was often associated with bicuspid aortic valve. Fernandez B et al (2009), supported this concept of nitric oxide (NO) deficiency, utilizing eNOS -/- mice showing a predominance to type II BAV (the right-non coronary cusp fusion). Furthermore Laforest B et al (2011), showed that a targeted deletion of gata 5 gene leads to mice displaying fusion of the right and non-coronary cusps type II BAV.

It appears type II BAV may result from a morphological defect that arises before the cardiac outflow tract septation. The separation of the valve leaflets in the prenatal heart has therefore been linked to an exacerbated nitric oxide-dependent endothelial-to-mesenchymal transformation (Fernandez B et al., 2009).

Type 1 BAV (right – left coronary cusp fusion) has been shown to occur in inbred Syrian hamsters with no apparent association to eNOS deficiency and as yet no evaluation of NO – mediated physiology (Fernandez B et al., 2009). Type I BAV may result from the anomalous septation of the proximal portion of the outflow tract, due likely to the distorted behavior of neural crest cells (Ratnasari P et al., 2012). This may also explain the association of Type I BAV to an increased incidence of associated coarctation of the aorta and pronounced aortic wall degeneration when compared to BAV with fusion of the right and non-coronary cusp type II BAV (Ratnasari P et al., 2012).

A number of studies have established actual clinical correlates of the above animal model physiology. Aicher D et al (2007), found that in BAV, endothelial cells of the aorta exhibited reduced eNOS protein expression when compared to TAV. In this study among BAV patients, there was a significant inverse correlation between aortic diameter and eNOS expression. Moreover Tzemos N et al (2010), revealed that young men with BAV and dilated proximal aortas exhibit systemic endothelial dysfunction as expressed utilising flow mediated dilatation (FMD). Therefore based on results of aortic biopsies (Aicher D et al., 2007) and by analogy with the occurrence of BAV in endothelial nitric oxide synthase (eNOS) knockout mice (Lee TC et al., 2000) an apparent nexus between BAV and endothelial dysfunction appears evident.

2.4.2 Interaction with pro-inflammatory stimuli.

Macrophage infiltration and neovascularization is increased in BAV when compared to that observed in TAV (Wallby L et al., 2002). Furthermore excised aortic tissue in BAV suggests both the valve and aorta exhibit an inflammatory process which is more aggressive in BAV

(Wallby L et al., 2002). Some, but not all, investigators (LeMaire SA et al., 2005 & Tzemos N et al., 2010) have also found evidence of systemic inflammatory activation in BAV.

There is currently a growing body of evidence implicating an increased expression and activity of the matrix metalloproteinases (MMP), a large family of zinc-dependant endopeptidases responsible for degradation of the extracellular matrix (in particular MMP 2 and MMP 9), however this increase has been reflected in some (Tzemos N et al., 2010), but not all studies. This degradation affects type IV collagen and elastin (Katie L. Losenno et al., 2009).

2.5 Endothelium and valve homeostasis

(a) Protective role

The normal aortic valve matrix consists of valvular endothelial cells (VECs), which line the surface of the valve and are phenotypically different from other endothelial cell populations (Pompilio L et al., 1998). The homeostatic role of the aortic valve endothelium is known to exhibit a potent source of anti-inflammatory and anti-thrombotic autocoids such as NO and prostacyclin, both generating vasodilator effects. In addition, the VECs in a normal valvular matrix generate vasoconstriction such as endothelin-1 factors. Together these factors affect the homeostatic milieu status of the valvular matrix (Pompilio L et al., 1998). However data are fragmentary as to the physiological effectiveness of NO / prostacyclin release in:-

(a) Limiting potential for thrombus formation on valve leaflets and

(b) Stabilising subjacent fibroblasts against transformation to myofibroblasts. Chirkov YY et al (2006), demonstrated attenuation of anti-aggregatory effect in valves excised from patients with AS.

2.6 Determinants of anti-inflammatory role of nitric oxide

Nitric oxide, apart from its vasomotor and anti-aggregatory effects, is an important anti-inflammatory agent. For example, Liberts EA et al (2006), demonstrated that NO donors especially in combination with perhexaline, limited superoxide (0_{2^-}) release for circulating white blood cells. Furthermore, Kennedy JA et al (2009), demonstrated that NO donors' suppress pro-inflammatory / pro-calcific changes in the aortic valve matrix (see below). Finally, recently it was shown that there is a reciprocal relationship between expression of the pro-inflammatory agent thioredoxin interacting protein (TXNIP) and tissue responsiveness to NO (Sverdlov AL et al., 2013).

The integrity of NO signalling in valve tissue, as elsewhere, is subject to a number of biomedical modulators. For example, asymmetric dimethyl arginine (ADMA) functions in part to inhibit NO synthases: plasma concentrations of ADMA are elevated in AS (Ngo DMT et al., 2007). Furthermore NO effects are attenuated by oxidative stress due to "scavenging" of NO $^{\cdot}$ free radical and also to oxidation / haem depletion of the NO "receptor" soluble guanylate cyclase (see Cirkov YY & horowitz JD 2007 for review).

2.7 Replacement of valve endothelium – role of endothelial progenitor cells

The maintenance of endothelial integrity and function is vital to the preservation of a healthy vasculature. Following a mechanical or chemical injury, the endothelium undergoes a process of repair (Asahara T et al., 1999). This process depends on new endothelial cells recruited from bone-marrow derived circulating endothelial progenitor cells (EPC's) (Asahara T et al., 1999). In 2003 Aicher D et al, showed that EPC's isolated from eNOS -/- mice exhibit impaired function. Furthermore Vaturi M et al (2011) compared EPC counts and function in patients with BAV. In this study patients with at least moderate aortic regurgitation and/or stenosis had impaired EPC functional properties as compared to that of BAV patients without significant aortic stenosis or regurgitation. Interestingly these patients appeared to have reduced levels of circulating EPCs defined on the basis of co expressed CD133 or CD34.

2.8 Transformation of valve matrix and development of inflammation / calcification

Calcification is present early on in the pathogenesis of AS. Co-localisation of calcification occurs in areas of the valve where increased lipids, particularly apolipoprotein (a), B and E accumulate (Freeman RV and Otto CM., 2005). Additionally within the valvular matrix oxidised low density lipoproteins (LDL's) accumulate and are taken up by macrophages to form foam cells. Within the early stages there is additional inflammatory cell predominance and activation of T lymphocytes, which trigger cytokine release with an association of pro-inflammatory and growth factor-stimuli. In particular activation of transforming growth factor β_1 ($TGF_{\beta1}$) is notably involved. Kennedy JA et al (2009), demonstrated that in a cell culture model of porcine aortic valve fibroblasts that $TGF_{\beta1}$ induced calcific nodule formation.

Inflammatory activation and expression of mediators such as TXNIP, additional release of reactive oxygen species (ROS), along with chemical mediators supporting a pro-inflammatory and pro-fibrotic milieu all support valvular fibroblasts to promote cell proliferation and matrix synthesis. The stimulation of a pro-inflammatory and pro-fibrotic environment drives valvular fibroblasts to differentiation towards myofibroblasts, which in turn tend to produce superoxide readily in response to stimulation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase activity.

Within the valvular matrix myofibroblasts exhibit an osteoblast phenotype and secrete increased collagen and promote disorganised fibrous tissue accumulation (Dwek MR et al., 2012). Additionally these myofibroblasts in association with inflammatory cells secrete matrix metalloproteinases (MMP's), which have an important role in the restructure of the valvular matrix (Dwek MR et al., 2012). The differentiation of the myofibroblasts to phenotypic osteoblasts encourages further accumulation of calcification within the aortic valve. The osteoblasts under a highly regulated process promote a likeness to skeletal bone formation (Sverdlov AL et al., 2011). Furthermore expression of an inhibitor of calcification, fetuin A, has been found to be diminished in the progressive stages of AS (Freeman R V and Otto C M., 2005).

This process: - valvular fibroblasts -> myofibroblasts -> Osteoblasts are reviewed in figure 1.1. The process of bone formation is largely at the "end stage" of AS disease where a reduction in aortic valve area (AVA) ensues due to bone-like calcific nodules (Sverdlov AL et al., 2011).

2.9 Can we improve treatment?

2.9.1 Improve prediction of rapid progression:

Prediction of valvular disease progression would be beneficial to the BAV patient as the outcome in severe symptomatic AS is often AVR. Patients with bicuspid aortic valve exhibit variable rates of development of aortic valve dysfunction and also of dilatation of the ascending aorta (AscAO) although valve dysfunction is usually the dominant process. Both inflammatory activation (IA) and endothelial dysfunction (ED) have been considered as potential modulators of these changes (Tzemos et al., 2008). The progression towards severe aortic stenosis is substantially accelerated in BAV compared to TAV. There are a number of interesting potential pathological determinants of disease progression. However to date no study has correlated these parameters with either valvular degeneration or valvular aortopathy in the BAV patient.

2.9.2 Slowing progression.

Theoretically, promising pharmacological targets in reducing the progression of AS have included lipid lowering drugs such as statins. However studies have proved disappointing reviewed by (Ngo DTM et al., 2012). There is increasing evidence that both angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor (AT1) blockers may prove effective. For example in a rabbit model Ngo DTM et al (2011), showed that ACE inhibition with Ramipril limited the development of AS and also prevented AS progression with concomitant reduction in calcification and macrophage infiltration. Experimental data showed a diminution in TXNIP accumulation and preservation of NO signalling. Other evidence in

favour of the potential utility of ACE inhibitors / AT_1 antagonists in Asc / AS is that angiotensin II accumulates in the valvular matrix and suggests a potential therapeutic target. Clinical evidence in favour of ACE inhibitor / AT1 antagonist therapy in limiting AS progression remains fragmentary, with no large prospective clinical trials. However utility of these drugs appear to include reduced morbidity in AS (Ngo DTM et al., 2012). Sverdlov A et al (2011), demonstrated that in an ageing normal population therapy with ACE inhibitors / AT1 antagonists predicted retention of normal aortic valve anatomy, rather than development of aortic sclerosis. Thus it is possible that such therapy is selectively useful in the early stages of the valve fibrosis / calcification process.

3.0 Scope of present study

BAV phenotypically is a heterogeneous disorder with valvular dysfunction and aortopathy. As previously mentioned the majority of BAV cases (approximately 75% of bicuspid aortic valves), involve fusion of the right and left coronary cusps termed type I BAV and this type is associated with, prominent aortic dilatation and descending aortic coarctatation. Type II BAV (predominantly the remainder of subtypes) involves right and non coronary cusp fusion and association with early development of valve stenosis and or regurgitation being a common finding (Siu SC and Silversides CK., 2010).

As regards pathogenesis, uncertainty still remains due probably to significant heterogeneity. Data from endothelial nitric oxide synthase (eNOS) -/- mice and aortic biopsies in patients undergoing surgery suggest an association between eNOS deficiency and BAV. However,

detailed evaluation of NO signalling in BAV is lacking, both inflammatory activation (IA) and endothelial dysfunction (ED) have been considered as potential modulators of valvular disease changes, however to date no association with disease progression has been investigated. Furthermore, the rate of progression of valve disease and aortopathy is not well predicted by subtype, nor by any previously identified clinical or biochemical parameters (Rossi A et al., 2013 and Schaefer BM et al., 2008).

The impact of clinical heterogeneity of disease progression is such that while some patients require surgery in early adult life, others will never require either valve or aortic surgery (Siu SC and Silversides CK., 2010). The rate of progression of valve disease appears to be largely dissociated from that of aortic dilatation, suggesting the existence of different pathophysiological processes (Kang JW et al., 2013).

The main purpose of the present study is to report the results of a case-control study of patients with BAV, which sought to test the hypothesis that; BAV is associated with both endothelial dysfunction and inflammatory activation. Furthermore, the present study will sought to identify biochemical correlates within the BAV cohort of (i) valvular dysfunction (ii) ascending aortic dilatation and (iii) selective occurrence of valvular dysfunction.

Endothelial function: Will be quantified utilising flow mediated dilatation (FMD), asymmetric dimethylarginine (ADMA) and tissue responsiveness to nitric oxide (NO) measured as response to sodium nitroprusside (SNP) (expressed as a percentage of ADP aggregation response).

Inflammatory activation: Evaluated utilising myeloperoxidase (MPO), high sensitivity C-reactive protein (HsCRP) and matrix metallopeptidase 2 (MMP2).

Structural assessment: Morphological and physiological assessment of the valve and ascending aorta will be performed with transthoracic echocardiography (2D, colour flow mapping and Doppler) and MRI.

3.1 Hypothesis to be tested

a) Primary

Bicuspid Aortic Valve disease is associated with endothelial dysfunction, as evidence by reduced FMD and increased ADMA levels as compared to age-matched controls.

Bicuspid Aortic Valve disease is associated with inflammatory activation, as evidence by increased MPO, MMP2 and HsCRP levels as compared to age-matched controls.

b) Secondary

1. Tissue responsiveness to NO : anti-aggregatory effects

i. Inhibition of ADP-induced aggregation by the NO donor sodium nitroprusside (SNP) (expressed as a percentage of ADP aggregation response).

2. Endothelial progenitor cell production

i. BAV is associated with increased counts of progenitor cells.

3. Anatomic correlates

BAV is associated with increased ascending AO diameter compared to control patients.

3.2 STUDY DESIGN

Case control study of adolescents and adults with proven bicuspid aortic valve versus age-matched controls with tricuspid aortic valve.

3.2.1 Subject / patient selection

(a) BAV: Adult patients with BAV recruited from clinical data bases across South Australia. The only criterion for selection: definitive echocardiographic documentation of BAV.

(b) Criteria for exclusion: concomitant complex congenital heart disease and terminal malignancy. Individuals with known ischemic or structural heart disease.

(c) Controls: age and gender-matched patients.

3.2.2 Clinical data

Assessment of patients / subjects will include history, pharmacotherapy and prior management of BAV with a physical examination for BMI and blood pressure.

3.2.3 Statistical methods

Comparisons between patients and controls will be performed utilizing non-paired t-tests or Wilcoxon tests as appropriate for quantitative data and $\chi 2$ tests for categorical data. Differential interactions between age and patients physiological / biochemical parameters will be evaluated by ANCOVA. Determinants of valve dysfunction, ascending aortic dilatation and the ratio

AVmax : AscAo will be sought via univariate comparison followed by backwards stepwise multiple logistic regression.

Comparisons utilising a multivariate analysis within the BAV group will be sought in order to identify factors associated with:

(a) Development of significant valvular disease.

(b) Dilatation of the ascending aorta.

(c) Differential valve: aortic disease AVmax : AscAo.

Correlations between MRI and echo-based measures of aortic dimensions will be evaluated by linear regression and by Bland-Altman plots. Data will be expressed throughout as mean ± SD unless otherwise stated.

3.2.4 Methods

(a) Echocardiography

Transthoracic echocardiography (TTE) studies, will be performed on all subjects utilising, M-mode, 2-dimensional (2D) echocardiography (with the intention of strain and strain rate for LV functional analysis) and Doppler analysis. The peak and mean pressure gradients across the aortic valve will be calculated utilising the simplified Bernoulli equation provided by analysis software from GE. A continuous-wave Doppler (CWD) recording requires attempts from all

cardiac windows to obtain the highest peak flow across the AV. As per American Society of Echocardiography (ASE) guidelines, the aortic valve area (AVA) will be determined using the continuity equation utilising the peak AV gradient. Dimensionless performance index (DPI) is calculated by dividing the left ventricular outflow tract (LVOT) velocity time interval (VTI), pulsed wave Doppler (PWD) by the CWD peak flow across the aortic valve VTI and is utilised as a supplementary to AVA. This dimensionless index is known as an accurate method of determining the degree of aortic valve stenosis and is particularly useful in concomitant valvular regurgitation such as that seen in BAV patients. Measurements of the aorta at the different levels, the sinus of valsalva, sinotubular junction and ascending aorta will be utilised from the parasternal long axis view. For these measurements the leading edge to leading edge technique as per ASE guidelines will be performed. The degree of valvular regurgitation utilising colour Doppler flow mapping (CFM) and any significant regurgitation will be further analysed with PW and CW in the descending aorta looking for flow reversal, to further quantify regurgitant lesions. Coarctation will be sought by CW down the descending aorta looking for a gradient across the potential narrowing.

(b) Cardiac magnetic resonance imaging

Cardiac magnetic resonance imaging (CMRI) was sought by a 1.5 T Philips Integra and Achieva scanner (Philips Medical Systems, Best, The Netherlands), with a five-element cardiac phased-array surface coil with electrocardiographic gating. Imaging parameters as follows: field of view 360 mm, TR 2 x RR interval, TE 80 milliseconds, turbo factor 35, matrix 240/560. Functional assessment of the left ventricle will be assessed using a cine-CMR Balanced Turbo Field Echo - sequences in standard short and long axis cardiac views. Gating will be prospective and timed to mid-diastole. MRI quantitative analysis will be performed on

a Philips View-forum workstation by an experienced CMR cardiologist. Dimensions of the aortic root and ascending aorta will be obtained in triplicate from dedicated short and long axis views. The ascending aortic dimensions will be measured at the level of the pulmonary artery bifurcation.

3.2.5 Investigations

Endothelial function / NO signalling assessment.

(a) Flow mediated dilatation (FMD)

Maintenance of vascular tone relies heavily on the vascular endothelium and a balance between endothelium derived relaxation and contraction factors. Many blood vessels respond to an increase in blood flow, generating sheer stress. This is designated flow-mediated dilatation (FMD) (Celermajer DS et al., 1992). This change in blood flow affects the endothelial cells to respond with variable release of NO such that vasodilatation ensues (Celermajer DS et al., 1992).

FMD is frequently used as a physiological index of the integrity of the NO pathway. Synthesised from L-arginine by endothelial nitric oxide synthase (eNOS), NO is a potent endogenous vasodilator and plays a critical role in endothelial derived vasodilatation (Böger RH and Ron ES., 2005). NO vasodilator effects are exerted largely via the release of cyclic

guanosine monophosphate (cGMP) through activation of soluble guanylyl cyclase (Böger RH and Ron ES., 2005).

In response to shear stress, endothelial cell membranes activate a specialised calcium-activated potassium channel. The calcium released also activates eNOS, and the NO generation sequentially accounts for FMD (Coretti MC et al., 2002). Interestingly there is some redundancy in the system. Treatment with nitric oxide synthase (NOS) inhibitors do not completely abolish FMD, suggesting the presence of more than one mediator with the capability of acting as a signal between the endothelium and the smooth muscle influence FMD (Coretti MC et al., 2002). It is possible for example that vasodilator prostanoids play a significant part.

(b) FMD Technique-measurement

Numerous factors affect FMD: including temperature, food, drugs and sympathetic stimuli (Coretti MC et al., 2002). Subjects should be fasted, scanning should take place in a quiet and temperature controlled room. High resolution ultrasound is used to scan the brachial artery in a longitudinal section. Flow increase is induced by a forearm cuff inflated up to 260 mmHg for 5 minutes. Upon cuff deflation reactive hyperaemia is induced and ultrasound images ensue. The Increase in vessel diameter emerges approximately 45-60 seconds post deflation (Coretti MC et al., 2002). The increase in diameter at this time has been shown to be an endothelium-dependent process mediated by NO (Pleiner J & Woltz M., 2007). A sudden increase in blood flow into the vascular bed following release of the cuff triggers endothelial mediated dilatation. As a control the response of the brachial artery to sublingual glycerol trinitrate (50 mcg GTN) is recorded. Brachial ultrasound is performed continuously for 3

minutes post deflation. Measurements of arterial dimensions are performed by leading edge technique during peak diastole (R wave on ECG) in triplicate and averaged.

(c) Endothelial progenitor cell (EPC) counts

EPC counts; determined using flow cytometric analysis (FACScan, Becton Dickinson). Mononuclear cells positive for cell surface antigens, CD34 fluorescein isothiocyanate and CD133 phycoerythrin (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and quantitated.

(d) NO responsiveness / Platelet study

This was performed at Basil Hetzel institute as previously described see Chirkov et al., 1999 for review.

Blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate); acidified citrate was used in order to minimize deterioration of platelet function during experiments. Blood was centrifuged at 250*g* for 10 minutes at room temperature to obtain platelet-rich plasma. Platelet-poor plasma was prepared by further centrifugation of the remaining blood at 2500*g* for 20 minutes. Platelet counts were performed on the STKS Coulter Counter (Coulter Electronics Inc) and the platelet-rich plasma was adjusted with platelet-poor plasma to a constant count of 250 000/μL(Chirkov et al., 1999).

Aggregation in whole blood and platelet-rich plasma was examined using a dual-channel impedance aggregometer (Model 560, Chrono-Log). Tests were performed at 37°C and

stirring speed of 900 rpm. Samples of blood or platelet-rich plasma were diluted 2-fold with normal saline (final volume 1 mL) and prewarmed for 5 minutes at 37°C. Aggregation was induced with adenosine 5'-diphosphate (ADP) (final concentration of 1 µmol/L) in experiments with whole blood and 0.5 µmol/L ADP with platelet-rich plasma. Aggregation was monitored continually for 7 minutes, and responses were recorded (RO-3 Rikadenki chart recorder) for electrical impedance, in ohms. SNP and NTG (final concentration of 10 and 100 µmol/L, respectively) were added to samples 1 minute before ADP. SOD and catalase (final concentration of 300 U/mL for both enzymes) were added immediately before NTG or SNP. 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) (1 µmol/L) was added 5 minutes before NTG or SNP. The duration of incubations were estimated as those optimal in preliminary experiments. In control tests, physiological saline was added in appropriate volumes. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied (Chirkov et al., 1999).

Platelet aggregation induced by ADP (final concentrations of 2.5µmol/L) and platelet responsiveness to NO, inhibition of aggregation by the NO donor sodium nitroprusside (SNP) (expressed as a percentage of ADP aggregation response) were examined utilizing a dual-channel impedance aggregometer (Model 560, Chrono-Log, Havertown, PA, USA (Chirkov et al., 1999).

Fig 4.0 NO responsiveness / Platelet study

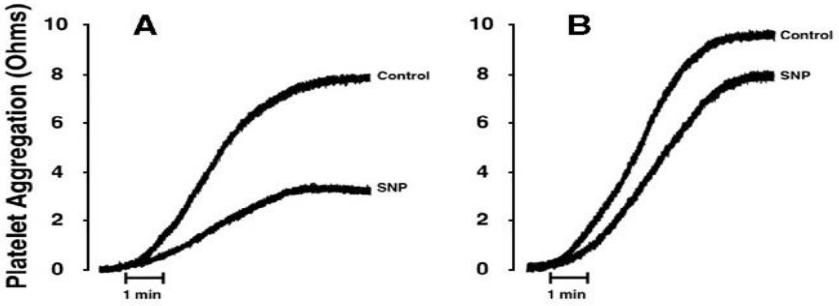


Figure shows. Inhibition of ADP (1 μM)-induced platelet aggregation in whole blood by SNP (10 μM): demonstration of NO resistance. (A) Normal subject. (B) Patient with unstable angina pectoris, showing both hyperaggregability and reduced responsiveness to antiaggregatory effect of SNP), image taken from, (Yuli Y and Horowitz JD 2007)

3.2.6 Biochemical evaluations

(a) Asymmetric dimethylarginine – ADMA

Concentrations of plasma ADMA were measured by high-performance liquid chromatography (HPLC) using the derivatisation reagent AccQ-Fluor after solid phase extraction as previously described see Heresztyn T et al.,2004. Examination was performed in the Basil Hetzel institute.

(b) High sensitivity C-reactive protein – hsCRP

High sensitivity C-reactive protein (hs CRP) concentrations were measured utilizing a latex-enhanced immunoturbidometric assay (Olympus au5400, Dallas, Texas, USA). This test was performed at the Queen Elizabeth Hospital South Australia. Examination was performed in the Basil Hetzel institute.

(c) Myeloperoxidase – MPO

Concentrations of MPO from EDTA-stored plasma were determined using a MPO ELISA assay (Mercodia, Uppsala, Sweden) as manufacturer's instructions. Examination was performed in the Basil Hetzel institute.

(d) Matrix metallopeptidase 2 MMP2

MMP2 levels were measured using a MMP2 ELISA kit (GE Health Care, Amersham, UK). Examination was performed in the Basil Hetzel institute.

3.3 Results

3.3.1 Patient / subject characteristics

The study compared 43 BAV patients and 25 control subjects as summarised in Table 1A. The two groups were well-matched, with a relatively low prevalence of conventional risk factors for endothelial dysfunction. However the age of both BAV and controls varied widely.

Table 1 A. Clinical characteristics

Clinical characteristics	BAV (n = 43)	Controls (n = 25)
Age: Mean ± SD	45 ± 16	45 ± 15
Females	33%	48%
HT	18%	16%
Diabetes	2%	0%
Hyperlipidemia	4%	0%
Smoking	2%	0%

Among the BAV patients, 86% had Type 1 BAV, where two patients had previous surgery or stenting for coarctation. One patient had previously undergone an aortic valve replacement - data from this patient was utilized only for physiological/biochemical comparisons with control subjects.

Patients were classified as BAV once definitive echocardiography evidence was obtained; In contrast control patients with no known history of BAV were also confirmed to have normal valvular structure by echocardiography.

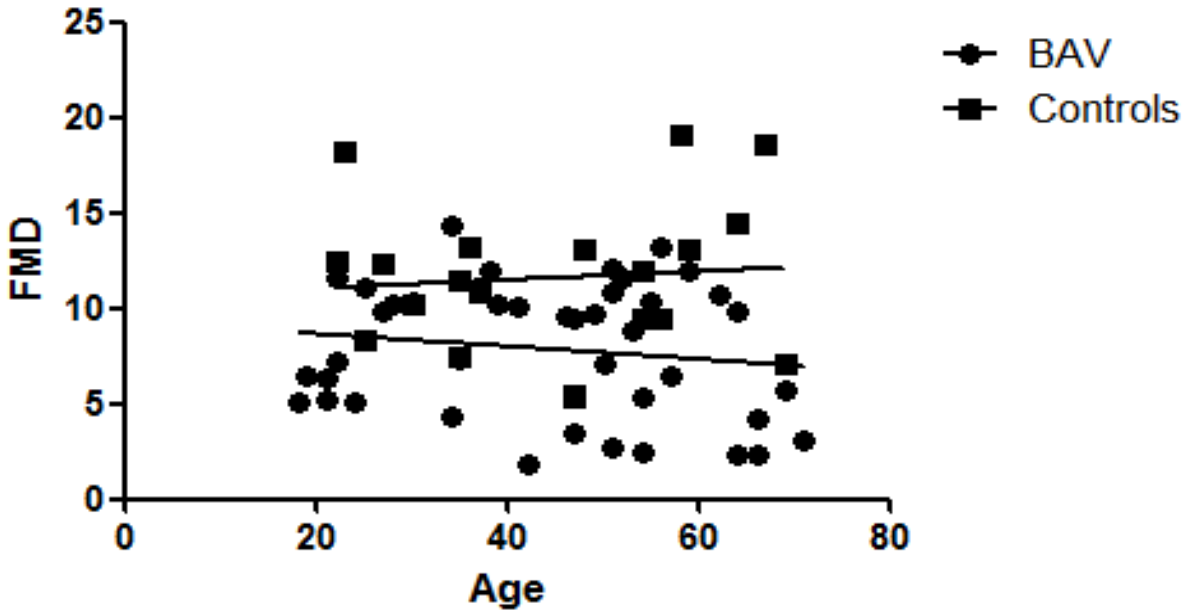
3.3.2 Comparisons: BAV patients Vs Controls

(a) Parameters of endothelial function / NO signaling

The major difference between the BAV and Control groups was that FMD (%) was substantially lower in the BAV patient cohort as compared to controls 7.85 ± 3.48 / 11.58 ± 3.98 respectively (p= 0.001); see table 1 B. These differences were age-independent as depicted in figure 1 analysis by ANCOVA shows the relationship between age and FMD for BAV and control subjects. FMD was significantly greater for controls than BAV patients (F = 10.5, p = 0.002) with no significant impact of age or age: FMD interaction.

Fig 1. Relationship between age and FMD for BAV and control subjects

(F = 10.5, p = 0.002)



Interestingly ADMA (μM) concentrations, although marginally higher than control patients, did not differ significantly between the 2 groups: BAV 0.54 ± 0.08 and control 0.51 ± 0.07 (table 1 B). These data therefore suggest that FMD difference between groups reflects clearance, rather than formation, of NO.

Other physiological/biochemical parameters related to NO signalling, for instance platelet response to SNP %, did not differ significantly between BAV patients (table 1 B).

Although EPC counts tended to be marginally higher in the BAV group, this did not reach statistical significance (table 1 B).

Table 1.B Comparison of physiological and biochemical parameters of nitric oxide function between groups.

Comparisons	BAV	Controls
1. Endothelial function		
FMD (%)	7.85 ± 3.48	11.58 ± 3.98**
ADMA (μM)	0.54 ± 0.08	0.51 ± 0.07
Platelet response to SNP (%)	53 ± 28	42 ± 34
EPC counts	72 (43-110)	57 (36-86)

** statistically significant p = 0.001.

(b) Parameters of inflammatory activation

As summarised in Table 1 C, there were no significant differences regarding markers of inflammatory activation between the BAV and control groups. Importantly, both MPO and MMP were assayed as enzyme concentrations rather than activity.

Table 1 C Markers of inflammatory activation

Inflammatory markers	BAV	Controls
hs-CRP (mg/L)	1.8 (0.8-4.1)	1.0 (0.7-4)
MPO (ng/ml)	67 ± 13	62 ± 20
MMP2 (ng/ml)	840 ± 388	909 247

3.3.3 Determinants of valvular dysfunction

(a) Parameters of endothelial function / NO signaling

Valvular dysfunction in BAV patients was expressed by aortic valve peak flow. This was determined by Doppler echocardiography and expressed as $AV_{max.}$ In addition dimensionless performance index (DPI) was expressed utilizing Doppler echocardiography as previously described.

It was found that DPI was directly correlated with FMD (r = 0.45, p = 0.003; Fig 2) and that AV_{max} was also directly correlated with ADMA concentrations (r = 0.43, p = 0.006 Fig 3). However, parameters of valvular dysfunction were not significantly correlated with either platelet response to NO or to EPC count (data not shown).

Fig 2. Univariate correlates of valvular dysfunction: FMD

(r = 0.45, p = 0.003)

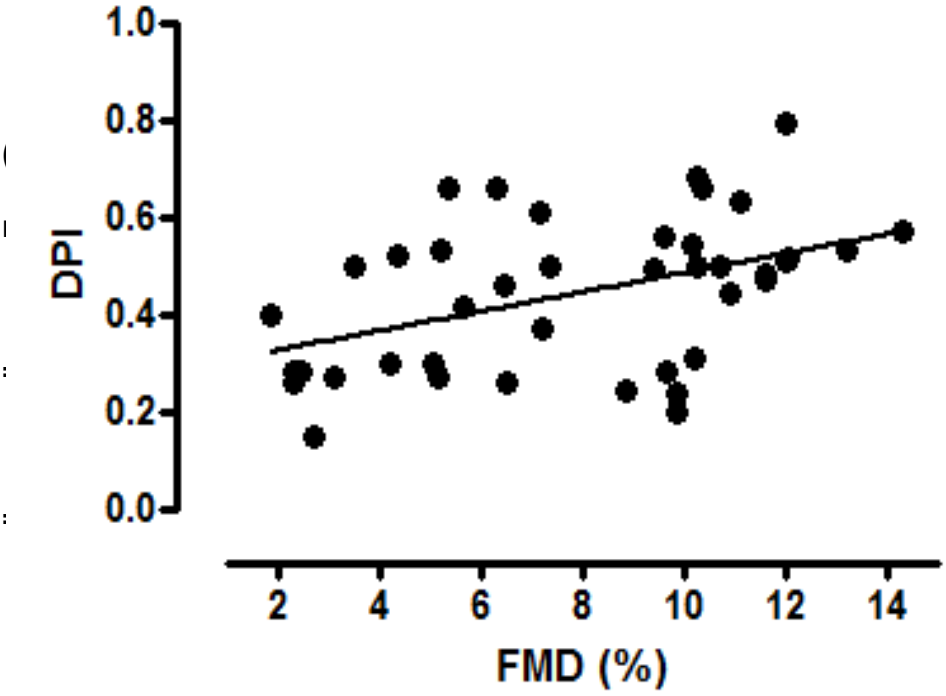
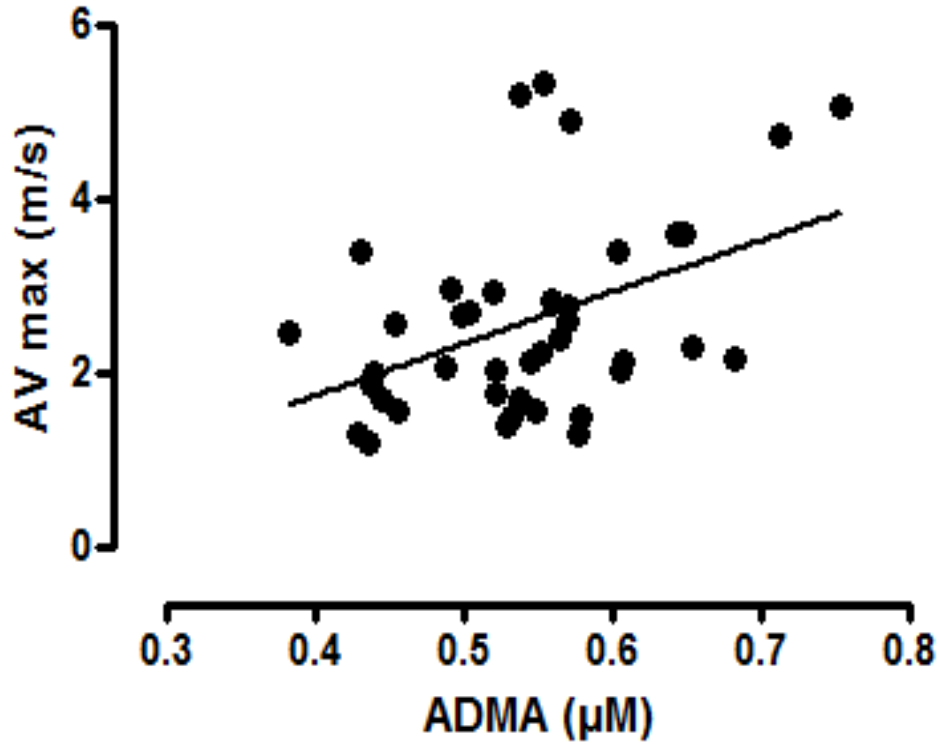


Fig 3. Univariate correlates of valvular dysfunction: ADMA

(r = 0.43, p = 0.006)



(b) Inflammatory activation

As regards inflammatory activation markers: a direct and strong correlation between peak AV_{max} and CRP was observed (r = 0.49, p = 0.001), including a direct correlation with MPO (r = 0.49, p = 0.001); see figures 4 and 5 respectively.

Fig 4 Univariate correlates of valvular dysfunction: CRP

(r = 0.49, p = 0.001),

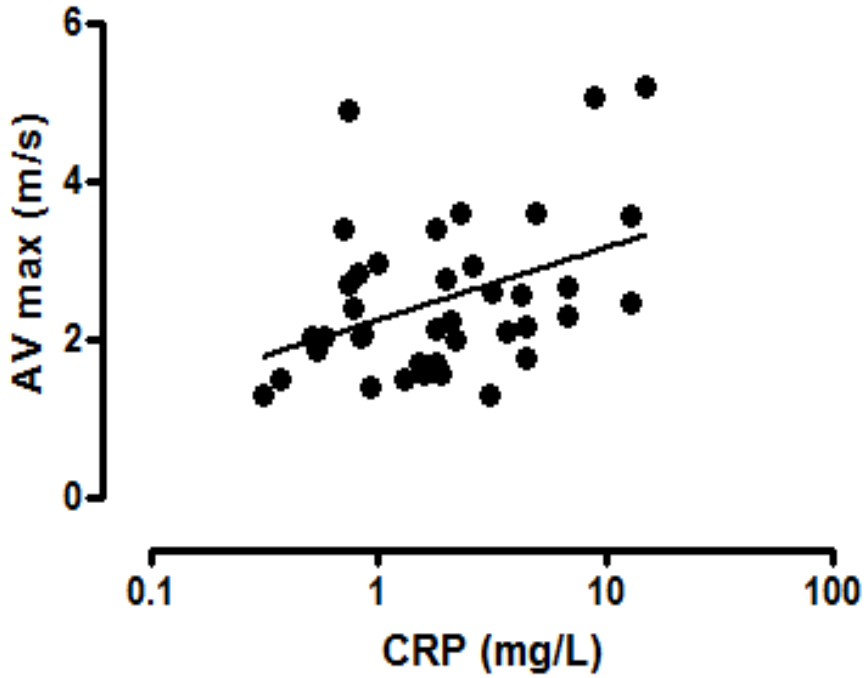
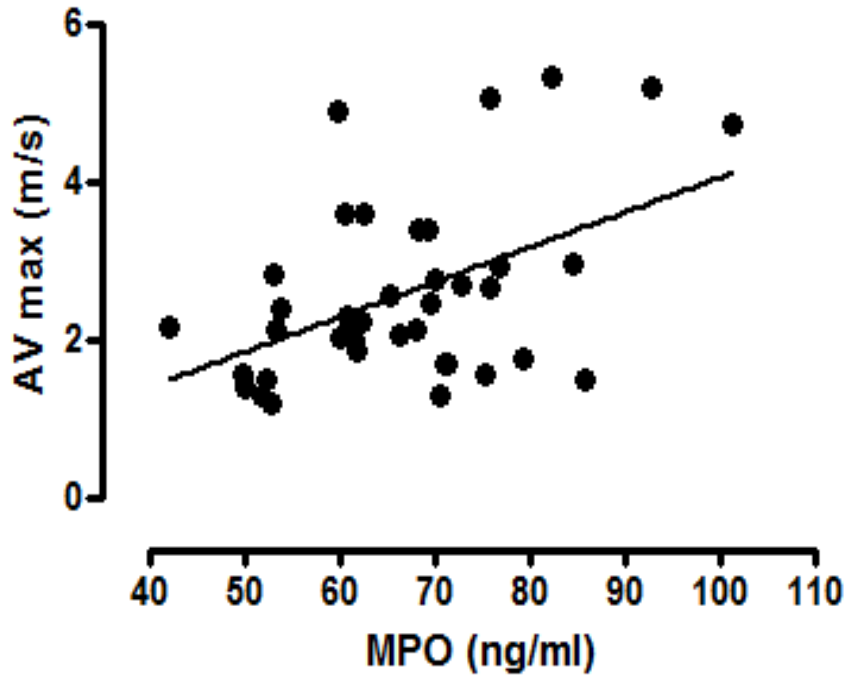


Fig 5 Univariate correlates of valvular dysfunction: MPO

(r = 0.49, p = 0.001)



In addition to direct univariate analysis, results of backwards stepwise multiple logistic regression for peak AV_{max} were as follows:

Endothelial function and NO signalling.

Backwards stepwise multiple logistic regression showed a significant correlation of peak AV_{max} and ADMA, while a negative association with FMD was of borderline statistical significance (table 2 A).

Inflammatory activation

A significant correlation was seen for MPO concentration, p = 0.002

(table 2A).

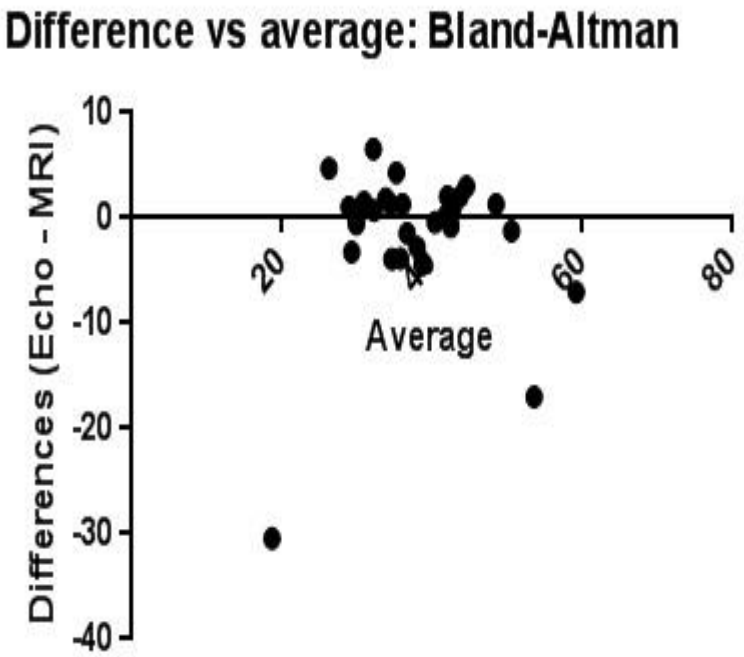
Table 2 A: Summary: Predictors of valve dysfunction on multivariate analysis: - Results of backwards stepwise multiple logistic regressions for peak AV_{max}

Dependent variable	Predictors	β	p
Valvular dysfunction AV_{max}	ADMA	3.2	0.003
	MPO	3.4	0.002
	FMD	-1.8	0.07

3.3.4 Determinants of ascending aortic dilatation

Ascending aortic dimensions were measured on MRI and echocardiography and were closely related (r= 0,72, p < 0.00001). Relationships between MRI and echo-based measurements of aortic dimensions were evaluated by linear regression and by Bland-Altman plots (see figure 6).

Fig 6 Bland – Altman plot MRI and Echocardiography



Univariate analysis revealed the major clinical correlate of increasing ascending aortic diameter was that of increasing age (figure 7). On multivariate analysis (table 2 B), increasing age correlated with ascending aortic diameter. Interestingly no biochemical parameters predicted aortic dilatation.

Fig 7. Univariate correlates of aortopathy: Age

(r = 0.47, p = 0.002)

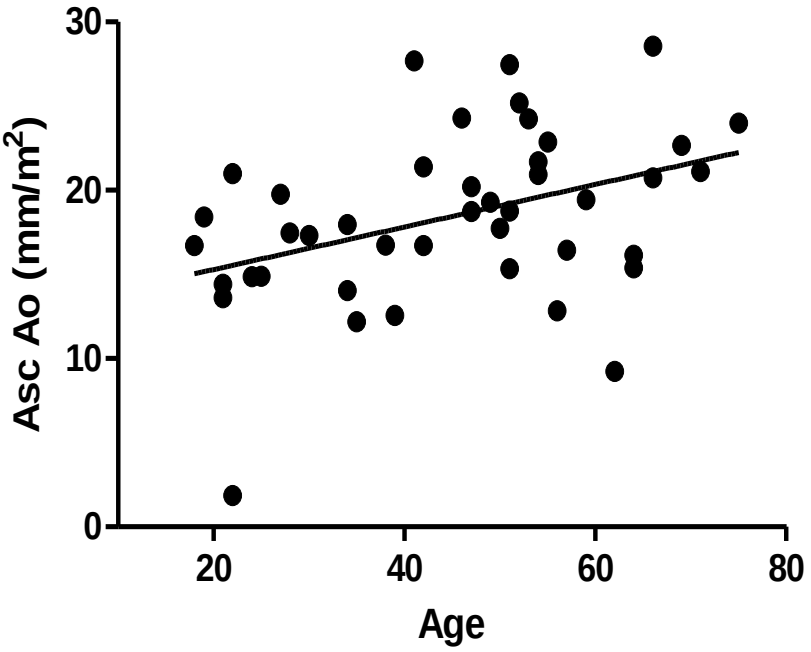


Table 2 B. Age correlation with ascending aortic diameter

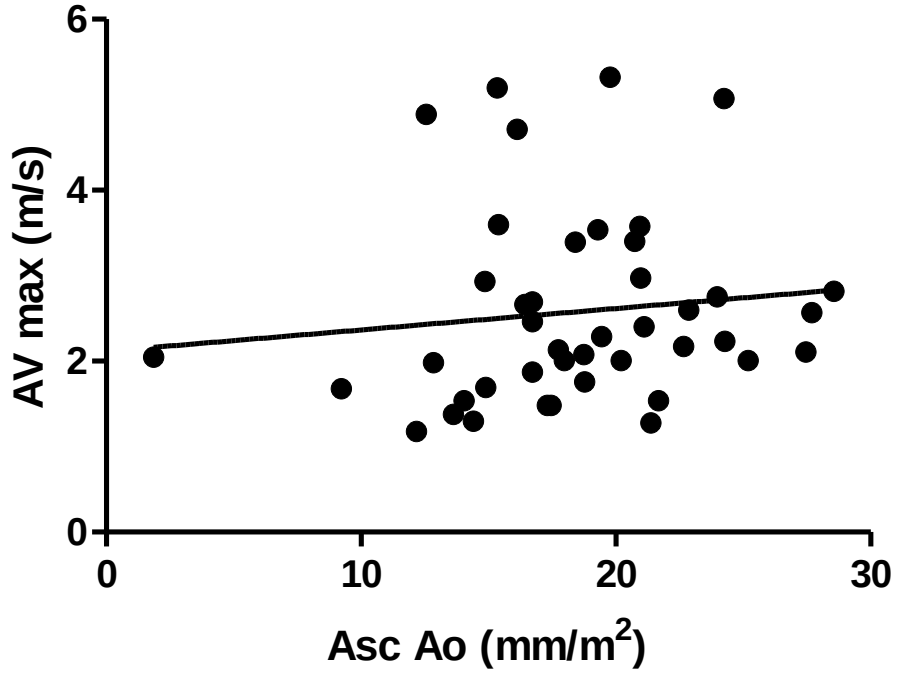
Ao dilatation	Predictors	β	p
AscAo	Age	0.35	0.04
Aortic Sinus	Age	0.45	0.006

3.3.5 Determinants of "selective" aortic valve degeneration

In the BAV patient group, there was no significant overall correlation between AV_{max} and ascending aortic diameter (figure 8).

Fig 8: Relationship between AscAo diameter and AV_{max}

(r = 0.1,p = 0.5)



The ratio AV_{max} : AscAo was utilised to show selective valvular degeneration of the BAV group. Increased MPO (Figure 9 A and table 2 C) and impaired FMD (Figure 9 B and table 2 C) respectively were notably selective for valvular disease, with a similar trend for increased ADMA (p = 0.057). Furthermore, type 2 BAV patients also exhibit selective valvular degeneration (Table 2 C).

Fig 9 A Relationships between plasma MPO concentrations

(r = 0.61, p = 0.0001)

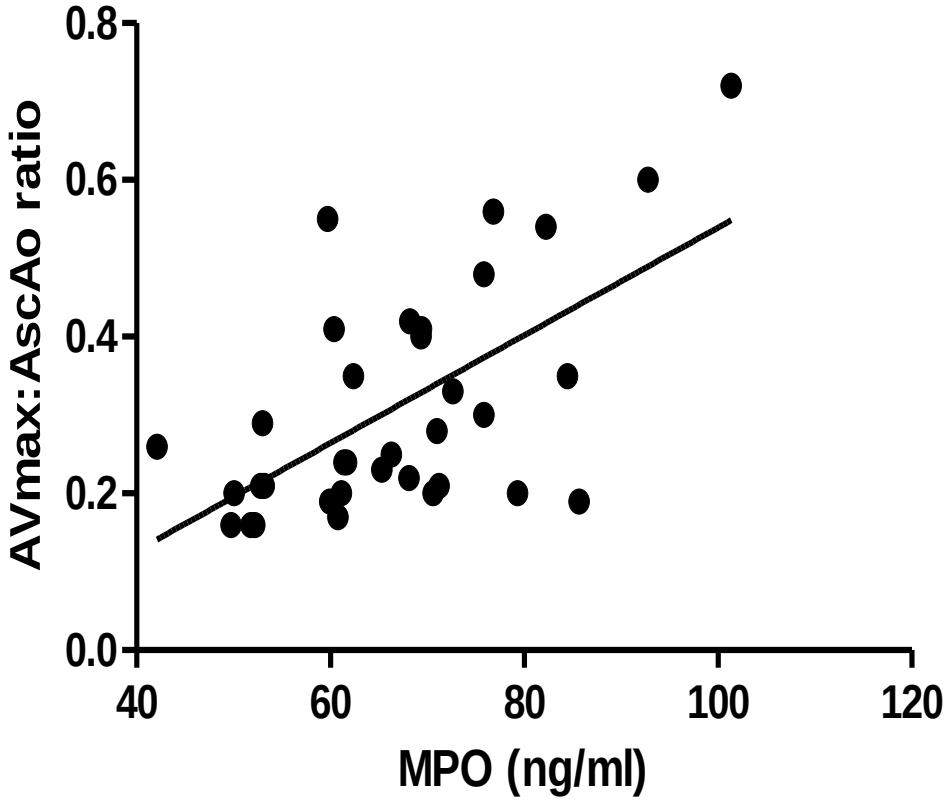


Fig 9 B Relationships between FMD (%)

(r = -0.39, p = 0.016)

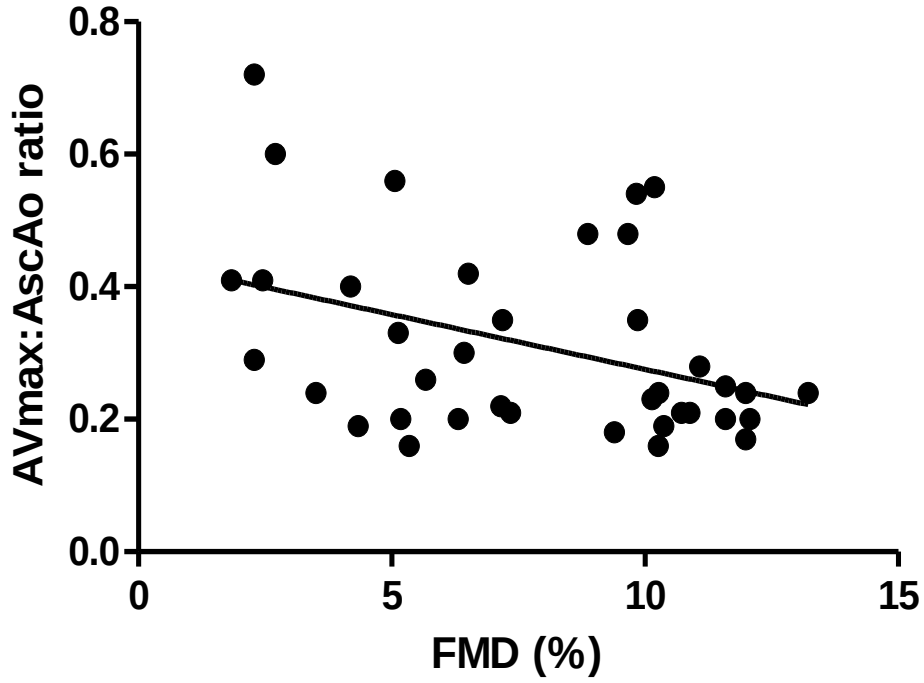


Table 2 C Multivariate analysis: significant correlates of selective involvement of aortic valve over aorta, as measured by AV_{max}:AscAO

Independent variable	Predictors	β	p
AV_{max}: AscAo	ADMA	2.0	0.057
	MPO	4.2	0.0001
	FMD	-3.5	0.002
	BAV type	0.27	0.03

3.4 Discussion

Results from the current study demonstrate that BAV is associated with vascular endothelial dysfunction as evidenced by increased plasma ADMA concentration and reduction in FMD. In addition BAV is associated with inflammatory activation as measured by increased plasma MPO concentrations and hsCRP; moreover these parameters correlate independently with the extent of valvular dysfunction.

On the other hand this study has failed to evaluate and identify biochemical and/or physiological correlates of BAV aortopathy. The current data signify the extent of systemic inflammatory activation and of endothelial dysfunction correlate with valvular, rather than aortic disease in bicuspid aortic valve.

Flow-mediated dilatation is currently used as a non-invasive physiological index for quantifying function of the nitric oxide (NO) pathway, encompassing both NO synthase (NOS) and distal activation of soluble guanylate cyclase (sGC) (Böger RH and Ron ES., 2005). The concept that BAV might be related to impairment of NO signalling arises from observations that eNOS -/- mice demonstrate an increased incidence of BAV (Leet TC., et al 2000). Furthermore, a comparison of eNOS expression in punch biopsies of ascending aorta in patients undergoing simultaneous aortic valve and coronary surgery also suggested that eNOS expression was reduced in patients with BAV (Aicher D et al., 2007). The current study extends these observations and has also confirmed that BAV is associated with FMD impairment to a similar extent to the findings reported in a study by Tzemos N et a (2010). In the current study, in addition to FMD being reduced relative to valves in controls, FMD was

found to correlate with valvular disease progression, utilising DPI measurements by echocardiography. The data from this study therefore suggest a strong relationship between vascular endothelial dysfunction (utilizing FMD as a physiological correlate) and progression of valvular disease. Moreover the current study utilized the biochemical marker of endothelial dysfunction ADMA, and showed that increased plasma levels directly correlated with valvular dysfunction. ADMA is known as a competitive inhibitor of nitric oxide synthase (NOS) (vallance et al 1992), thus this study further contributes to the current literature emphasising that BAV is associated with impaired NO signalling. Interestingly there was no significant correlation between ADMA concentrations and FMD, raising the possibility that both NO generation and signalling may have been affected. However the design of the study did not permit assessment of valvular endothelial function: this would be impossible except in animal models of aortic valve disease. Furthermore, the results did not delineate exactly how impaired FMD with BAV adds to the impact of normal ageing.

BAV remains a major cause of cardiovascular morbidity and mortality and is known to be associated with the early development in some patients of severe valvular dysfunction thus contributing to the costly requirements for valvular repair or replacement. Furthermore while there is a smaller risk of rupture of aneurysmal aorta in BAV (Siu SC and Silversides CK., 2010), in general the prognosis of BAV is determined by the rate of progression of valvular disease (Ferencik M and Pape L A., 2010). Therefore the identification of physiological correlates of deterioration of valvular function is potentially useful for clinical descision making in BAV patients.

Inflammatory activation

The concept that BAV is also associated with inflammatory activation comes from studies by Forte A et al (2013), who evaluated histology of explanted aortae from BAV patients. There was increased expression and / or activity of MMP – 2 and MMP - 9. This increase has been reflected in plasma MMP levels in some Tzemos N et al (2010), but not all studies.

In the current study, there was no significant difference in hsCRP, MMP-2 and MPO concentrations between patients and controls. However, within the BAV cohort elevated plasma MPO concentrations strongly correlated with valvular dysfunction. Interestingly It has previously been demonstrated that aortic stenosis is associated with inflammatory infiltration (Chen K, et al., 2003 and Sanchez PL et al., 2006), but the possibility that neutrophil infiltration and activation with associated release of MPO might play a pivotal role has previously received little attention.

It is possible that these two categories of biochemical anomaly may be interdependent. These data are consistent with recent findings that;

(a) nitric oxide (where formation is inhibited by ADMA) (noted to be increased in BAV and correlate with valvular dysfunction), stabilises neutrophils, inhibiting MPO release while

(b) MPO inhibits the enzyme dimethylarginine dimethylaminohydrolase (DDAH) which degrades ADMA (Von Leitner EC et al., 2011). Importantly, although MPO is released mainly from neutrophils, it has been shown that once released it binds to the endothelium in a neutrophil-independent manner, and there oxidises NO, as well as inhibiting NO generation by

"uncoupling" NO synthase (Xu J et al 2006). The possibility of using MPO inhibitors to decrease inflammatory activation within bicuspid valves is raised by the data, but has not yet been evaluated by any research group.

Of particular interest is the concept that inflammation and endothelial dysfunction together are responsible for deterioration of valvular function this has not previously been specifically considered, but is strongly suggested by the current data. Conversely these data should not be regarded as evidence of an "all or none" relationship: there is biopsy evidence of decreased eNOS expression (Aicher D et al., 2007) and of increased MMP activity (Tzemos N et al ., 2010 & LeMaire SA et al., 2005) in the aortae of BAV patients.

While there is some degree of aortic root / ascending aortic dilatation occurring in all patients with BAV, there are somewhat greater rates of dilatation in the ascending aorta than at the aortic sinus level (Kim et al., 2012). Rates of development of aortopathy are largely, but not entirely independent of the extent of valvular regurgitation, and are far greater for patients with type I BAV (Schaefer BM et al., 2008). Therefore it might have been argued that an approach specifically targeting measurement of intra-aortic inflammation, for example by PET scanning, might have facilitated a focus on this aspect of pathogenesis.

The main limitation of the current study is a failure to identify biochemical and physiological correlates of age-related dilatation of the ascending aorta. Despite their being a strong probability that inflammation and endothelial dysfunction play a role in this process, this study showed no convincing evidence to support this notion. Interestingly, two studies (LeMaire SA

et al (2005) & Balistreri CR et al (2013), have commented specifically on the limited nature of inflammatory activation in the aortae of BAV patients.

The current study showed no significant correlation between EPC numbers and aortic dilatation. Although there was a trend for higher counts in the BAV group compared to controls there was no statistically significant difference in evaluation. Interestingly, one study demonstrated an association between EPC dysfunction in BAV (Vaturi M et al., 2011). This study evaluated a group of BAV patients without significant valvular disease (BAV control) and BAV patients with at least moderate aortic regurgitation and/or stenosis (dysfunctional group). Patients in the valvular dysfunction group appeared to exhibit an impaired functional property of EPCs after 7 days of culture. Specifically, EPCs from patients within the dysfunctional valve group had decreased capacity to form colonies and to migrate compared to patients with normal functioning valves. Additionally, patients with dysfunctional BAVs tended to have a lower level of circulating EPCs represented by co expressed CD133 or CD34.

As the patients studied had predominantly type 1 BAV, it is possible that the conclusions of this study may be specific for that subtype. Furthermore we recently showed that an increase specific to aortic sinus diameter (AS_D) were associated with an increase in EPC count (Ali OA et al., 2011). However the current study did not support the concept that increased AS_D diameter statistically correlates with EPC count, despite a similar trend. The current study also did not examine the function of EPCs, so it is possible that EPC dysfunction might have been present.

Finally the current study's findings are purely correlative with the degree of dysfunction at the valvular level: the study was not potentially able to demonstrate any cause and effect relationship in this setting.

3.4.5 Conclusion and future perspectives

The current study has established a number of novel pathophysiological correlates with variable rates of progression of aortic stenosis severity in BAV, but has failed to identify anything beyond subject ageing as a basis for progression of aortopathy. Nevertheless it has been documented that in most cases it is valve disease rather than aortopathy which, precipitates surgery in BAV (Siu SC & Silversides CK., 2010).

The current results do not provide precise mechanisms mediating the association between BAV and MPO concentrations and AS progression rates. However MPO indicates leukocyte infiltration, which might accelerate degeneration and valvular dysfunction. In theory, MPO inhibitors and direct soluble guanylate cyclase activators could be utilised to identify the precise roles of each of these individual factors.

The final issue which remains to be considered is whether both ACE inhibitors and angiotensin receptor (AT_1) blockers may fulfil promise towards reducing disease progression (Ngo DTM et al., 2012 & Nadir MA et al., 2012). For example Ngo DTM et al., 2011: in experimental AS, showed that ramipril, an ACE inhibitor, retards the development of valvular calcification and prevents TxNIP accumulation within the valve with the preservation of NO signalling in a rabbit model. A large series of patients with moderate AS appeared to have

more favourable outcomes with treatment of ACE inhibitors or angiotensin receptor blockers (O'Brien et al., 2005), however this was not a randomised study but supports the need for prospective, randomized trials of ACEIs in calcific aortic valve disease.

The proper testing of therapeutic assumptions inherent in this thesis remains a high clinical priority.

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