Aortopathy in Patients with a Bicuspid Aortic Valve:

Determining Susceptibility for Aortic Complications

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The studies described in this thesis were performed at the Department of Cardiothoracic Surgery, the Department of Anatomy and Embryology and the Department of Molecular Cell Biology of the Leiden University Medical Center, Leiden, The Netherlands.

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Layout: Jasper Koning

Layout frontpage: Simran Grewal & Charlotte Blokhuis

Printed by: Ipskamp Drukkers

ISBN: 978-94-6259-551-4

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged. Publication of this thesis was financially further supported by: the department of Cardiothoracic Surgery, LUMC, Leiden, ABN AMRO, Stichting Hartpatiënten Nederland and St. Jude Medical

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Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 19 februari
klokke 13:45 uur
door

Nimrat Grewal

geboren te Amsterdam
in 1988

Promotiecommissie

Promotor:	Prof. Dr. R.J.M. Klautz
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“Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning.”

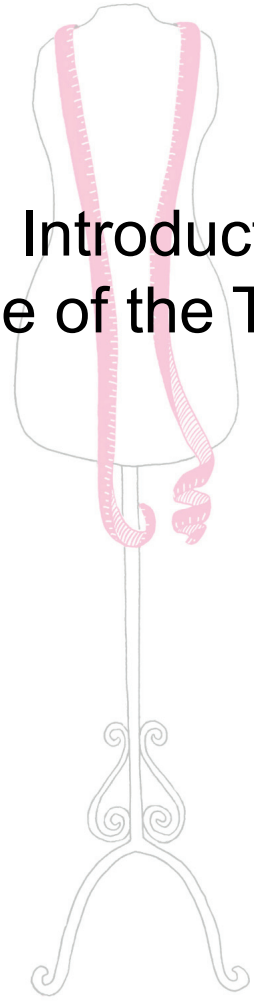
- Albert Einstein (14 March 1879 – 18 April 1955)

For my parents and sister

TABLE OF CONTENTS

CHAPTER

1



General Introduction and Outline of the Thesis

1. BACKGROUND

Bicuspid aortic valve (BAV) is the most common congenital heart defect, being present in almost 1% of the general population (1-3). The prevalence of BAV depends on ethnicity and gender, as BAV is more common in Caucasians and males, however these factors have not always been considered in analyses of BAV prevalence (4;5). The male to female ratio of BAV is estimated to be approximately 2:1 (3;6).

BAV is associated with an increased risk of aortic valve stenosis, aortic regurgitation, as well as thoracic aortic dilation, occurring in approximately 40-60%, and aortic dissection, as compared to patients with a tricuspid aortic valve (TAV) (7;8). Aortic dilation was long thought to be secondary to the valve disease itself, but increasing evidence points to a common origin for both sites of pathology (3;6;9;10). Although the relative risk of aortic dissection is lower than in patients with Marfan syndrome (MFS), there are likely as many, if not more, dissections in patients with BAV given the significantly greater prevalence of this disease (11). Hence, BAV-associated aortic wall pathology has important public health implications and has therefore long been a subject of intensive clinical and basic research. Optimal management of patients with BAV and associated ascending aortic dilation requires an approach, carefully assessing various risk factors of the aortic valve and wall and discerning individual indications for ongoing surveillance, medical management, and surgical intervention.

Operative management of aortic dilation presents a complicated clinical problem given the unpredictable lifetime risk of morbidity and mortality related to aortic wall pathology in BAV and major surgical intervention required to address these risks (11-13). This is not a new clinical issue; the complexities of aortic wall disease have long been appreciated (14;15) but our understanding and ability to diagnose and intervene have also evolved considerably. Recommendations on when to intervene surgically for thoracic aortic dilation for BAV patients have been progressively expanded over the past 15 years. BAV is now widely considered to be an independent risk factor for an acute aortic event, which has led surgeons to consider whether an additional concomitant aortic procedure should be performed at the time of aortic valve replacement.

The fact that some BAV patients can present with severe forms of aortic wall pathology (16), with an early onset in life (17), and clear familiar inheritance

for aortic complications (10) has led to greater general aggressiveness towards all BAV-related vascular wall pathology. With some arguing that aortic dimensions indicating surgical intervention in BAV should be similar to those used for other genetic diagnosis with an increased risk for aortic complications, such as MFS (18-20). Absolute aortic diameter remains the most used clinical parameter to guide intervention, although indexed and non-size predictors have also been proposed (21-23). Guideline recommendations for surgical intervention based on the ascending aortic diameter have decreased from ≥5 cm (24-27) to ≥4.5 cm for patients with BAV undergoing concomitant aortic valve replacement, with others proposing even lower thresholds for intervention (21;28-30). However, these recommendations remain controversial (13;31;32) as there is still no clear understanding of how the dimensions of the ascending aorta change over long term in patients with BAV. A great number of BAV patients show a less severe natural clinical course (12). The debate will only calm down when our ability to stratify BAV patients will improve, so that indications will be less subjective and more individualized. Therefore among the most important tasks at hand for the research in this field is to define and validate tools and criteria for risk stratification, in order to more rationally guide surgical management.
To reach this, a detailed knowledge of the pathophysiology of aortic wall pathology in BAV and the clinical and genetic factors increasing the risk of aortic complications is necessary.

As an introduction to this thesis, an overview of the current status of knowledge of aortic wall pathology in bicuspidy will be provided. First, the cardiac anatomy will be described, along with the embryologic development of the cardiac valves. Furthermore, the two most discussed possible pathogenetic mechanisms of aortic wall pathology in BAV: haemodynamics and intrinsic wall abnormalities will be discussed and compared. Finally, the aims of this thesis and the chapter outline are presented.

2. CARDIAC ANATOMY

The main function of the cardiovascular system is to transport nutrients and oxygen to the entire body. The heart can be thought of as two pumps in

series that send a fluid (blood) through tubes (vessels) that eventually return to the pump.

Each 'pump' in the heart is made up of two chambers; an atrium and a ventricle, giving the heart a total of four chambers. The atria receive blood returning from the circulation and pass it to the ventricles. The ventricles make up most of the heart's volume, with the left ventricle being the larger of the two. The ventricles receive blood from the atria and pump it through arteries to the body.

The heart uses a series of valves to ensure that blood flows in one direction into and out of the heart. Heart valves are made of tough, flexible fibrous tissue that are oriented in such a way that blood can only go through the valve in one direction. A valve only opens when the blood exerts enough pressure on it, forcing it to open for blood to flow through. When this pressure drops, the valve returns to its originally closed position, preventing blood from flowing in the wrong direction. A pressure gradient is developed as blood flows through the body, and blood only flows from a high pressure to a lower one.

Like the heart chambers there are four heart valves, two atrioventricular valves and two semilunar valves. An AV valve is located between each atrium and ventricle, with the tricuspid valve on the right and the mitral valve on the left. The valve opens when the atrial pressure is greater than ventricular pressure. When ventricular pressure exceeds atrial pressure, the valve closes again. The area of the valve cusps are about twice that of the passageway they cover, creating a large overlap of the cusps when they close. This overlap helps to prevent the backflow of blood (regurgitation) into the atrium.

A semilunar valve is located between the right ventricle and pulmonary trunk (pulmonary valve) and the left ventricle and the ascending aorta (aortic valve). Similar to the AV valves, when left or right ventricular pressure exceeds aortic or pulmonary artery pressure, the valve opens. When ventricular pressure decreases, the three cusps of the valves close, preventing blood from flowing back into the ventricle.

The heart has two atria and two ventricles because there are two different blood flow circulation paths. The circulation path controlled by the right side of the heart is a low-pressure system known as the pulmonary circulation. Oxygen depleted blood enters the right atrium through the caval veins and coronary sinus and is pumped to the lungs by the right ventricle through the pulmonary trunk. The blood receives oxygen in the lungs and is sent back to

the left side of the heart through the pulmonary veins and is returned to the left ventricle through the left atrium. The left ventricle pumps the oxygen-rich blood through the aorta to the rest of the body. As the blood flows farther from the body, oxygen concentration is diminished by exchange with CO2 in the body tissues and organs. The blood returns to the heart through the caval veins in the right atrium, depleted of oxygen, completing the cycle. This circulation path is known as the systemic circulation. Due to the large distance the blood travels, this is a high-pressure system. After entering the right atrium, the blood repeats the two circulatory paths.

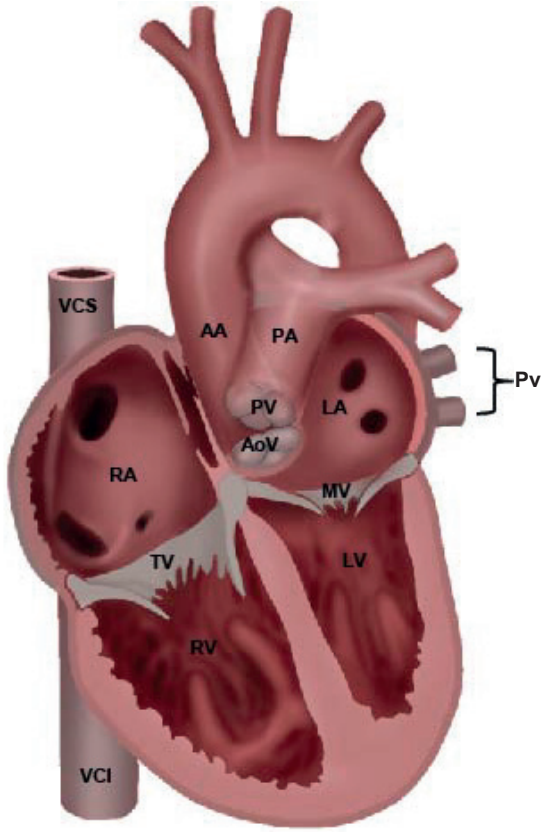


Figure 1. *Schematic overview of the heart.*
AA: ascending aorta; AoV: aortic valve, LA: left atrium; lPA: left pulmonary artery; LV: left ventricle; MV: mitral valve; PT: pulmonary trunk; PV: pulmonary valve; Pv: pulmonary vein; RA: right atrium; rPA: right pulmonary artery; RV: right ventricle; TV: tricuspid valve; VCI: vena cava inferior; VCS: vena cava superior.

Throughout the cardiac cycle, blood pressure increases and decreases. The aorta is an elastic artery (with a large number of collagen and elastin filaments in the tunica media), which gives it the ability to stretch in response to each pulse to maintain a relatively constant pressure in the aorta despite the pulsating nature of the blood flow. This is called the 'Windkessel effect' of the elastic artery.

3. CARDIAC EMBRYOLOGY

a. Semilunar valves

The heart is derived from the anterior splanchnic mesoderm. It forms from two crescent-like cardiogenic plates. After fusion of these plates in the midline, a primary heart tube is formed (33) that shows peristaltic contraction at 3 weeks of development in a human embryo. The primary cardiac tube is lined on the inside by endocardium and on the outside by myocardium consisting of about two cell layers. A thick basement membrane is sandwiched in between referred to as cardiac jelly, containing water-binding extracellular matrix molecules including hyaluronic acid. At a later stage the cardiac jelly is restricted to endocardial cushions lining the myocardial outflow tract and the atrioventricular canal (34). The myocardium-lined primary heart tube initially consists of a small atrial component (connected to the sinus venosus), an atrioventricular canal, a ventricular inflow tract and a small outflow tract (connecting to the aortic sac). On the borderline of the ventricular inflow tract and the outflow tract a bulboventricular or primary fold is present. These cardiac components are derived from the mesoderm of the first heart field. The addition of dorsal cardiac mesoderm positioned between the primary heart tube and the primitive gut, the so-called second heart field (SHF) mesoderm, is essential for the subsequent development of all cardiac components. This SHF mesoderm can reach the heart tube at both the arterial (anterior SHF) and venous poles (posterior SHF). At the arterial pole the anterior SHF-derived mesoderm supplies the myocardium of the right ventricle up to the right side of the ventricular septum. The SHF also contributes to the semilunar valves and the walls of the great arteries.

For subsequent remodeling, septation, valve formation and coronary vascular development two other cell populations are added to the heart, being the neural crest cells and the epicardium. Semilunar valve formation

includes remodeling of the distal end of the endocardial outflow tract cushions that derive from the cardiac jelly and receive mesenchymal cells from the overlying endocardium. During normal development, separation of the arterial orifice level results in three semilunar valve cusps in the aortic and pulmonary orifices. Abnormal development includes both the occurrence of deficient numbers (as well as excessive numbers) of valve cusps (35). Several gene mutations have now been reported in the human population to play a role in the development of BAV, which will be further discussed in the paragraph *'Genes related to BAV in animal models and human population'*. However, the exact cause of BAV has not been elucidated, there are even indications that the anterior SHF is involved and that not only the aortic valve is abnormal but the wall of the ascending aorta is also included (35).
The mature cusps of the aortic valve are usually less than 1 mm thick and consist of three layers: the collagen-rich fibrosa located at the aortic side of the valve, the ventricularis with an abundance of elastin and located at the ventricular side of the valve, and, in between these, the spongiosa, which rich in proteoglycans.

b. Ascending aorta

Recent lineage tracing studies in transgenic mice with Nkx2.5 (5) have shown that SHF progenitor cells give origin to three specific cell lines: (1) outflow tract and right ventricular myocardium, (2) endothelial-derived endocardial cushion cells, which are in part derived from the endothelium and (3) vascular smooth muscle cells (VSMCs) of the great arteries (36). Recently, Harmon et al presented data on the boundary where SHF-derived VSMCs meet neural crest cell-derived VSMCs at the base of the aorta (5). The SHF contribution to the aortic media then forms a vertical seam complementary with neural crest-derived VSMCs (36). Next to contributing to the vascular wall, a population of neural crest cells migrates to the outflow tract cushions where they are important for semilunar valve formation and outflow tract septation (37;38). Preliminary data show a contribution of the arterial epicardium to the VSMCs of the ascending aorta (39).
The wall of the ascending aorta consists of three basic layers: the internal layer, tunica intima, which contains a single layer of endothelial cells and a subendothelial thin layer of connective tissue and VSMCs; the middle layer, tunica media, which mainly contains VSMCs, elastic fibers and collagen; and the outer layer, tunica adventitia, which predominantly contains loosely

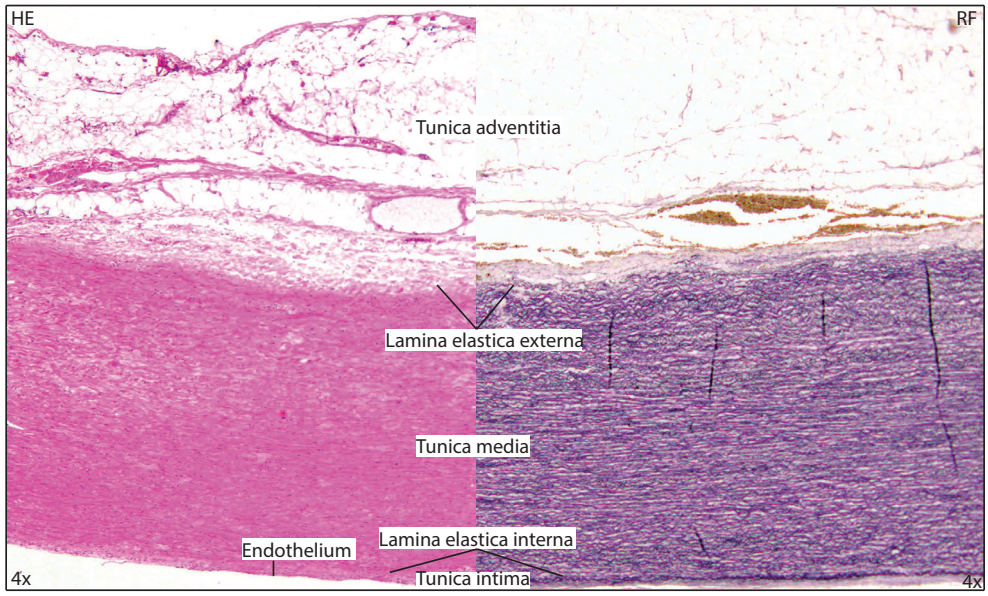


Figure 2. Transverse sections of the aortic wall stained with hematoxylin-eosin (HE) and resorcin fuchsin (RF).

organized collagen fibers, small blood vessels and fatty tissue. The intima and the media are separated by the lamina elastica interna and the media is separated from the adventitia by the lamina elastic externa.

The aortic media is arranged in lamellar units where two elastic lamellae enclose VSMCs, which are surrounded by extracellular matrix that contains microfibrils, small elastic fibers, collagen, and proteoglycans. The extracellular matrix interconnects the two elastic lamellae as well as connecting the elastic lamellae with the VSMCs (40-42). Elastin fibers from the lamellae protrude between thick collagen fibers (containing collagen I, III, and V) and together with microfibrils of fibrillin-1 and collagen VI (also containing some fibronectin) facilitate the VSMCs-elastin interaction (43).

4. GENETIC BASIS OF BAV

In 1866 it was already suggested by Peacock that BAV is congenital in origin (44). It has since then been confirmed that BAV is indeed related to other cardiac malformations. BAV is reportedly found in 25-85% of patients with

aortic coarctation (45). BAV further is found co-existing with hypoplastic left heart syndrome and these two conditions are to some extent genetically linked (46). Shone's syndrome is characterized by a supravalvular mitral ring, parachute mitral valve, subaortic stenosis, and aortic coarctation. Bolling et al. reported that 63% (19/30) of their patients with Shone's syndrome had a BAV (47). William's syndrome is a neurodevelopmental disorder that is also associated with cardiovascular conditions such as supravalvular stenosis, peripheral pulmonary artery stenosis, aortic coarctation, and BAV (48). BAV is found in about 25% of individuals with Turner's syndrome and is present in 95% of patient with Turner's syndrome who suffer from aortic dissection (49;50). BAV has been noted in conjunction with ventricular septal defects (51), patent ductus arteriosus, (52;53) and atrial septal defects. BAV is also associated with left coronary artery dominance (54;55).

Heritability of BAV formation is suggested to be as high as 89% (56). BAV has an inheritance consistent with an autosomal dominant pattern with reduced penetrance (57;58) and about 9% of first-degree relatives of BAV individuals will also have the malformation (56;59). BAV formation has been linked to several different chromosomes and gene mutations, which suggests complex inheritance (57;60-62).

5. GENES RELATED TO BAV IN ANIMAL MODELS AND HUMAN POPULATION

Since BAV is a congenital malformation, it is reasonable to expect that mutations in genes encoding transcription factors, extracellular matrix components, and proteins of signaling pathways that are implicated in valvulogenesis are important in BAV formation (Table 1) (63-67).

Linkage analyses revealed an association between BAV and chromosome 9q34-35 and subsequently NOTCH1 in humans (60). NOTCH1 encodes a transmembrane receptor and its signaling pathway is important in developmental processes and organogenesis. NOTCH signaling is highly conserved in evolution and, regardless of what animal model is used, perturbations in the pathway inevitably lead to developmental abnormalities (review (68). NOTCH1 is expressed in endocardial cells found in the common outflow tract in mouse embryos (69) and NOTCH signaling is suggested to have a role in BAV formation and valve calcification (60). Smad6 (SMAD

Table 1 *Genes related to valvulogenesis in BAV formation*

Gene	Description	Function
Acvr1	Activin A receptor, Type 1 (Alk2)	Member of the TGFβ superfamily
EGF superfamily	Epidermal growth factor	Signaling pathway
eNOS	Endothelial nitric oxide, NOS3	Signaling pathway
ErbB	V-erb-b2 erythroblastic leukemia viral oncogene homolog	Epidermal growth factor receptor signaling pathway/family of receptor tyrosine kinases
GATA5	GATA binding protein 5	Transcription factor
KCNJ2	Inward- rectifying potassium channel Kir2.1	Voltage-gated potassium channel activity involved in cardiac muscle action potential repolarization
NF-1	Neurofibromin 1	Ras signaling pathway
NFATc1	Nuclear factor of T cells cytoplasmic 1	Transcription factor
Nkx2-5	NK2 homeobox 5	Transcription factor
NOTCH1	Family of transmembrane receptors	Signaling pathway
Smad6	SMAD family member 6	Signal transducer and transcriptional modulator in BMP signaling
Sox9	SRY (sex determining region Y)-box 9	Transcription factor
TGF-β superfamily	Transforming growth factor beta	Signaling pathway
Tbx20	T-box 20	Transcription factor
Twist-1	Twist homolog 1	Transcription factor
VEGF	Vascular endothelial growth factor	Signaling pathway
Wnt/β-catenin Canonical Wnt signalling pathway.	Wingless type MMTV integration site family/cadherin-associated protein beta	Wnt, growth factor; β-catenin, co- activator of transcription factors

family member 6) encodes a protein that functions as a signal transducer and transcriptional modulator in BMP signaling (bone morphogenetic protein, a member of the TGF-β superfamily). Smad6 is expressed in the embryonic outflow tract in mice and genetic variants of Smad6 predispose for BAV in humans (70). BAV has also been linked to chromosomes 18q22, 5q21 and

13q34; however which specific genes within these loci are associated with BAV is unknown (61). The empirical association of BAV and hypoplastic left heart syndrome has also been confirmed genetically (46).The mutation of the inward-rectifying potassium channel Kir2.1 (KCNJ2) found in patients with Andersen's syndrome (a combination of characteristic physical features and arrhythmias) has also been suggested in BAV disease (62).
A number of other genes are implicated in BAV formation in mice but their relevance in humans remains to be shown. These include Nkx2-5 (NK2 homeobox 5, transcription factor) (71), eNOS (endothelial nitric oxide, Nos3) (72), GATA5 (GATA binding protein 5, transcription factor) (73), and Acvr1 (activin A receptor, Type 1, alias Alk2, member of the TGF-β superfamily) (74). There is a marked phenotypic variability in individuals with BAV; it is not always familial and/or associated with other cardiac malformations. Furthermore, not all individuals with BAV develop valve and/or aortic disease and therefore might remain unidentified (75-77).

6. BAV PHENOTYPE

It is now recognized that BAV should not be considered as one single entity, but that distinct morphological phenotypes are distinguishable based on the presence and number of raphe, as classified by Sievers et al. (6) Most BAVs consist of one free and two cusps that are conjoined (or have failed to separate during embryonic development). The term 'raphe' defines the conjoined area of the two underdeveloped cusp turning into a malformed commissure between both cusps (6). Variable orientation of the raphe/ fused commissure in relation to the sinus are seen: fusion of the right (RCC) and left coronary cusp (LCC) with a raphe (RCC/LCC), fusion of the right and non-coronary (NCC) cusp with a raphe (RCC/NCC), fusion of the left and non-coronary cusp with a raphe (LCC/NCC). This orientation of the raphe defines the subcategory in the classification of Sievers et al. referring to three types: type 0, valve with no raphe; type 1, valves with one raphe; and type 2, valves with two raphe. In our pathology papers we used a modified classification: type 1: RCC/LCC, type 2: RCC/NCC, type 3: LCC/NCC (Fig. 3).
BAV phenotype is suggested to have an incomplete heritability (78) i.e. families with several BAV individuals will not all have the same BAV phenotype. The etiology of the different BAV phenotypes seems to differ. Mice

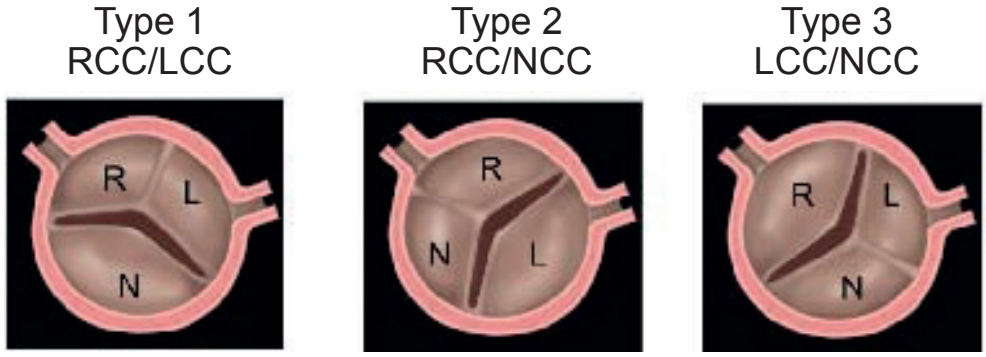


Figure 3. *Schematic overview of BAV morphologies.*
Drawings are oriented in echocardiographic, i.e. inferior, view of the aortic valve. The three major valve morphologies observed in BAV patients are described as three different types based on the valve cusp orientation: fusion of the right (RCC) and left coronary cusp (LCC) with a raphe (RCC/LCC) (type 1), fusion of the right and non-coronary (NCC) cusp with a raphe (RCC/NCC) (type 2), fusion of the left and non-coronary cusp with a raphe (LCC/NCC) (type 3).

deficient in eNOS or Gata5 develop RCC/NCC BAVs (72;73;79).The RCC/NCC phenotype is thought to be the result of perturbations in the formation of endocardial cushions of the common outflow tract that occurs before septation of the outflow tract (79). By contrast, the RCC/LCC phenotype is suggested to be the result of an abnormal septation of the common outflow tract and to be linked to neural crest cell function on the basis of studies in inbred Syrian hamsters (79).

7. BAV AND AORTIC WALL PATHOLOGY

a. General introduction

BAV is usually asymptomatic in children and young adults and is commonly an incidental finding. Symptoms and physical findings associated with BAV are mainly related to the associated valvular dysfunction, being aortic stenosis, aortic regurgitation or a combination of both conditions. Ascending aortic dilation is rarely symptomatic whereas aortic dissection is usually associated with an acute onset of severe chest and/or back pain and is accompanied by signs of organ dysfunction and shock.

Some BAVs have an accelerated progression of valve thickening and calcification which is generally evident in the fourth decade of life (77;80)

(5;81;82). The valvular changes frequently progress into clinically significant valve stenosis (83). Excessive folding and creasing as well as asymmetrical and turbulent flow patterns due to a morphological 'stenosis' are thought to cause increased stress and subsequently early regurgitation, (not secondary to infective endocarditis) (77;80) Furthermore, BAV individuals are predisposed to infective endocarditis (77;80). The risk of bacterial colonization is greater owing to increased shear stress and subsequent endothelial damage which in turn leads to platelet aggregation and fibrin deposition. Microorganisms tend to adhere to and multiply in these platelet-fibrin vegetations (84-86) (review (87)). BAV phenotype has been proposed to affect the susceptibility for valve disease; BAV individuals with a RCC/NCC configuration are prone to develop valve disease in childhood, whereas BAV individuals with a RCC/LCC configuration tend to develop valve disease in adulthood (78). All together approximately 50% of individuals with BAV will have to undergo aortic valve surgery (12).

BAV individuals have an age-adjusted relative risk of aneurysm formation of approximately 86% in comparison with the general population, and about 25% of BAV individuals will develop indication for ascending aortic replacement (12).

Aortic dissection is caused by disruption of the intimal layer of the aortic wall, which results in bleeding between the aortic wall layers and creation of a dissection plane (88;89). The condition is associated with malperfusion of vital organs and predisposes for aortic rupture. The risk of aortic dissection is eight-fold higher in BAV individuals than in the general population (12).

b. Aortic valve stenosis

Aortic valve stenosis is the most frequent valvular dysfunction associated with BAV (80). The underlying mechanisms of aortic valve stenosis formation are believed to be similar to those of atherosclerosis (for review see (90)). A number of risk factors that are associated with aortic valve calcification have been identified and these are similar to those of atherosclerotic disease; however, a distinction between BAV and TAV has not been made (91;92). Even though BAV patients present with significant valve stenosis earlier in life than patients with TAV, the pathogenesis of the valve lesion is thought to be similar, however not identical (93-95). eNOS deficiency and signaling pathways such as NOTCH and Wnt/β- Catenin, which are implicated in BAV formation, have also been suggested to be important in valve calcification,

possibly providing a link between the malformation and its most frequent valvular pathology (60;96;97).
An association between aortic valve disease and BAV phenotype has been proposed; however there are conflicting results. Beppu et al. found a more rapid progression of valve stenosis in BAVs with a RCC/LCC BAV (82). Fernandes et al. found an association between valve disease (both stenosis and regurgitation) and RCC/NCC BAVs (98). Conversely, Tzemos et al concluded that BAV phenotype is not an independent predictor of cardiac events (including aortic valve disease) (80).

c. Aortic valve regurgitation
Aortic valve regurgitation is the second most common valvular lesion associated with BAV and is often secondary to valve calcification. BAV patients with isolated valve regurgitation are in general younger than those with combined valve stenosis/regurgitation. Further mechanisms of incompetence include incomplete closure of cusps, redundancy of the fused cusps leading to prolapse, infective endocarditis, dilation of the aortic root, and aortic dissection (5;86;99-102).

d. Aortic dilation: structural wall abnormalities or shear stress?
In patients with a normal TAV histological changes of the aortic wall related to age as well as aortic pathology (dilation/dissection) are associated with medial degeneration (focal loss of smooth muscle cell nuclei), regardless of aortic location (i.e., ascending, descending, or abdominal) (103;104). Histologic changes in BAV patients are controversial in the current literature. More severe histopathological features have been described in BAV patients as compared to TAV patients (105;106). Furthermore, elastic fragmentation is more pronounced in BAV patients than in TAV patients with isolated valve pathology (107) and an association of BAV phenotype and the severity of medial degeneration has been reported (108). Whereas others have shown that BAV exhibits less histopathologic features (109).
The pathogenesis of aortic dilation and aneurysm formation of the ascending, descending, and abdominal aorta differs (110;111). Experimental studies have shown that the embryonic origin of the cells populating the aorta in mammals differs with the aortic location. Neural crest cells give rise to the ascending aorta, aortic arch, pulmonary trunk, and ductus arteriosus. By contrast, cells originating from the mesoderm populate the descending

aorta and subclavian artery (112;113). Whether developmental differences have an impact on differences in the underlying pathogenesis of aortic dilation related to aortic location (i.e., ascending, descending, abdominal) is not known. Furthermore, ascending aortic dilation in TAV, but not BAV, is associated with inflammation and immune response (114).

In addition to differences in the severity of medial degeneration and inflammatory profile between BAV and TAV patients with aortic dilation, several other factors related to BAV-associated aortic dilation have been reported as follows: 1) dilation of the ascending aorta is more progressive in BAV, as compared to TAV, even after aortic valve replacement (30;115); 2) children (and young adults) with BAV have larger dimensions of the aortic root/ascending aorta and impaired aortic elastic properties compared with children with TAV (116-118) 3) approximately one third of first degree relatives of BAV patients (with normally functioning TAVs) have dilated aortic roots and abnormal elastic properties of the aorta (10) 4) there are differences in signaling pathways involved in extracellular matrix homeostasis between BAV and TAV patients with dilated aortas (119-125) 5) eNOS expression is lower in BAV patients than in TAV patients and is inversely correlated with aortic diameter (126) and 6) mutations in genes encoding components of the extracellular matrix are linked to aortic dilation and BAV (FBN-1(124;127), TGFBR-2 (127), ACTA-2 (128)).

Many aspects of what causes BAV formation and subsequent BAV disease are not known, but there are two predominating theories, i.e., genetics and haemodynamics. The description above entails the genetic theory; haemodynamics will now further be discussed.

The morphology of a BAV is evidently different from that of a normal TAV with the consequence of altered flow across the valve (129). A bileaflet valve causes a turbulent flow compared to a normal tricuspid valve, due to asymmetric movement of valve cusps. A turbulent flow, along with other haemodynamic factors, as an increased stroke volume (i.e. aortic regurgitation) and aortic curvature, could play a facilitative role in developing aortic complications, such as dilation in BAV individuals (6;130-132). In regions of turbulent flow matrix degrading enzymes are expressed and smooth muscle cells (SMCs) go into apoptosis (133). In response to degradation of the extracellular matrix local fibroblasts and SMC are considered to synthesize new connective tissue components. This progressive remodeling presumably results in a new extracellular matrix of a different structure. The combination

of remodeling and loss of SMCs, due to apoptosis, weakens the integrity of the vessel wall, which could lead to subsequent aortic dilation (134).

Though, on the other hand, several studies have confirmed that ascending aortic aneurysms can develop in the absence of valvular abnormality (7;106;135-142). Moreover, Yasuda et al. have reported development of aortic dilation after (an isolated) surgical repair of the diseased bicuspid aortic valve (115). These studies suggest that structural abnormalities occur at the cellular level. Haemodynamic factors alone seem therefore not sufficient to explain the pathogenesis of cardiovascular malformations associated with bicuspid aortic valves. Suggesting that genetically determined abnormalities of the aortic wall lead to a defect in the cellular microenvironment, causing, or at least contributing to the aortic pathology associated with BAV.

Although, besides a strictly genetic or haemodynamic theory, a combination of both is plausible as an alternative hypothesis. We postulate that the structurally altered vascular wall, might be prone for secondary haemodynamic changes observed in BAV.

AIM OF THIS THESIS

The **aim of this thesis** is to determine clinical, histological, molecular biologic and morphological factors that may predict the clinical course and explain the increased susceptibility for aortic wall pathology seen in the majority of patients with BAV. We also intended to compare the pathobiology of aortic wall pathology in BAV with MFS.

In **Chapter 2** we approach our hypothesis that aortic wall pathology associated with BAV could be the result of a shared development defect during embryogenesis. To address this hypothesis we provided an overview of embryonic cell lines involved in the normal development of the semilunar valves and the ascending aortic dilation, being neural crest cells, second heart field (SHF) progenitors and endocardial cushion derived cells. Genes involved in abnormal development of the aortic valve (BAV) are further discussed. To understand whether thoracic aortic dilation, not necessarily accompanied by BAV, is related to defects in neural crest signaling or SHF-derived cells. The genetic origin of syndromes associated with aortic dilation (including MFS, Ehlers-Danlos, Smad3 mutations and Loeys-Dietz) was reviewed in the view of a central role for TGFβ and linked to embryonic development.

In **Chapter 3** we approach our hypothesis that the aortic wall is structurally different in BAV as compared to the TAV. By studying the dilated and also particularly the non-dilated aortic wall specimen, we investigated the expression of smooth muscle cells markers of diverse differentiation states. Further Lamin A/C, with a pivotal role in myoblasts differentiation and progerin, a marker for cardiovascular aging, were studied.

In **Chapter** 4 valve phenotype and aortic dimensions of 255 BAV patients were evaluated retrospectively. Patient characteristics, the clinical course and echocardiographic parameters including morphology of the valve were obtained. The aim of this study was to more definitively characterize whether an association exists between the morphology of the BAV and the degree of aortic dilation. And to provide a risk profile for clinical complications based patient characteristics and echocardiographic measurements.

In **Chapter 5** we further investigated a panel of vascular wall markers that might distinguish within the non-dilated BAV group a susceptible and non-susceptible group for future aortic wall complications.

In **Chapter 6** we investigated the aortic wall composition in patients with BAV, MFS and TAV. Recent studies suggest that in BAV patients the risk of aortic catastrophes, although higher than in the general population, remains low (12;80) as compared to the MFS. Controversies regarding aortic similarities and differences between various types of aortic wall pathology (BAV, MFS, TAV) in regard to aortic dilation and dissection of the aorta are as yet unresolved. Therefore the aim of this study was to shed light on the pathogenetic mechanism of aortic wall complications seen in both syndromes and compare those to the aortic wall pathology in dilated TAV patients. Findings were correlated to the effectiveness of clinical treatment modalities.

In **Chapter 7** the activity of the arterial epicardium covering the ascending aorta is investigated in both non- and dilated aortic wall specimen of BAV, TAV and MFS. In chapter 5 a signaling cascade is presented consisting of markers which can aid in distinguishing BAV patients with an increased susceptibility for future aortic complications. However, the link between 2 markers in the cascade: eNOS and MMP9, could not be substantiated. In this paper we aimed to identify how these markers are associated with each other.

Chapter 8 provides a summary of this thesis and discusses future persepectives

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CHAPTER

2

Normal and abnormal development of the aortic wall and valve: correlation with clinical entities

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Modified after:
Neth Heart J. 2014;22(9):363-9.

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ABSTRACT

Dilation of the wall of the thoracic aorta can be found in patients with a tricuspid (TAV) as well as a bicuspid aortic valve (BAV) with and without a syndromic component. BAV is the most common congenital cardiovascular malformation, with a population prevalence of 0.5–2%. The clinical course is often characterised by aneurysm formation and in some cases dissection. The non-dilated aortic wall is less well differentiated in all BAV as compared with TAV, thereby conferring inherent developmental susceptibility. Furthermore, a turbulent flow, caused by the inappropriate opening of the bicuspid valve, could accelerate the degenerative process in the aortic wall. However, not all patients with bicuspidy develop clinical complications during their life. We postulate that the increased vulnerability for aortic complications in a subset of patients with BAV is caused by a defect in the early development of the aorta and aortic valve. This review discusses histological and molecular genetic aspects of the normal and abnormal development of the aortic wall and semilunar valves. Aortopathy associated with BAV could be the result of a shared developmental defect during embryogenesis.

2

INTRODUCTION

Aortic dilation is a pathological widening of the aorta, which can be found in a thoracic and abdominal form according to its location. In contrast to abdominal aortic dilation, thoracic aortic dilation is usually not related to atheroma and often occurs at a younger age (1). Different aetiologies have been described which predispose individuals for thoracic aortic dilation, involving monogenic syndromes, such as Marfan (MFS), Ehlers-Danlos, Smad3 mutations and Loeys-Dietz syndromes, sometimes accompanied by bicuspid aortic valve (BAV) as well as idiopathic causes (2), while BAV is also found as an isolated anomaly.

Although patients with isolated BAV may remain asymptomatic, in a significant proportion of the patients the clinical course is accompanied by aortic stenosis, aortic regurgitation, infective endocarditis, and thoracic aortic dilation which has a prevalence as high as 50–60 % (3). Particularly, thoracic aortic dilation forms a critical complication, as it carries a risk of dissection and rupture, making it a potentially lethal disease.

Considering these clinical complications, understanding of the development of the ascending aorta and both normal and abnormal aortic valves is mandatory. By sharing a number of embryonic cell types, the development of the ascending aorta is narrowly related to the development of the aortic valve. Hence, aortopathy associated with BAV could be the result of a combined developmental defect in early embryogenesis. It has to be kept in mind, however, that not all individuals with BAV develop thoracic aortic dilation. In search of the pathogenesis of aortic complications in BAV, the focus has recently shifted towards defining patients susceptible for aortopathy needing aortic intervention.

This review discusses several aspects of normal and abnormal development of the aortic wall and aortic semilunar valves. We hypothesise that the increased vulnerability for aortic complications in BAV is caused by a defect in the early development of the aorta and aortic valve.

GENERAL OVERVIEW OF NORMAL AND ABNORMAL AORTIC VALVE AND AORTA DEVELOPMENT

During organogenesis the first functional organ to form is the heart. The first sign of valvulogenesis is the formation of endocardial cushions in the atrioventricular canal and outflow tract. The atrioventricular cushions contribute to the atrioventricular (mitral and tricuspid) valve leaflets, whereas the outflow tract cushions contribute to the semilunar (aortic and pulmonary) valve leaflets (4). Development of semilunar valves is a complex process in which neural crest cells, second heart field (SHF) progenitors and endocardial cushion derived cells play a role (Fig. 1). The developmental origin of the endocardial cushion cells themselves has been a matter of debate in the past years. Recent lineage tracing studies with Nkx2.5 (5) have shown that SHF progenitor cells give origin to three specific cell lines: 1. VSMCs of the great arteries, 2. outflow tract and right ventricular myocardium, and to 3. the much discussed endothelial-derived endocardial cushion cells, which are in part derived from the endothelium (5). Recently, Harmon et al. presented data on the boundary where SHF-derived VSMCs meet neural crest cell-derived VSMCs at the base of the aorta (5). The SHF contribution to the aortic media then forms a vertical seam complementary with neural crest derived VSMCs (5). Next to contributing to the vascular wall, a population of neural crest cells migrates to the outflow tract cushions where they are important for semilunar valve formation and outflow tract septation (6, 7). Preliminary data show a contribution of the arterial epicardium to the VSMCs of the ascending aorta (8).

During valvulogenesis several signalling pathways such as Wnt/β-catenin, NOTCH, transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), vascular endothelial growth factor, NFATc1 and MAPK, as well as transcription factors, including Twist1, Tbx20, Msx1/2, and Sox9, are necessary for the regulation of cell migration, proliferation, and extracellular matrix deposition in the developing valves (9–12). As a consequence, more than one cell population that contributes to both aortic wall and semilunar valve formation may be involved in the development of bicuspidy. Clinically, several BAV subtypes are distinguished on the basis of the fused commissure or raphe position. Type 1: raphe between right coronary cusp (RCC) and left coronary cusp (LCC), type 2: between RCC and non-coronary cusp (NCC) and type 3: raphe between LCC and NCC (Fig. 2). Clinical outcome differs

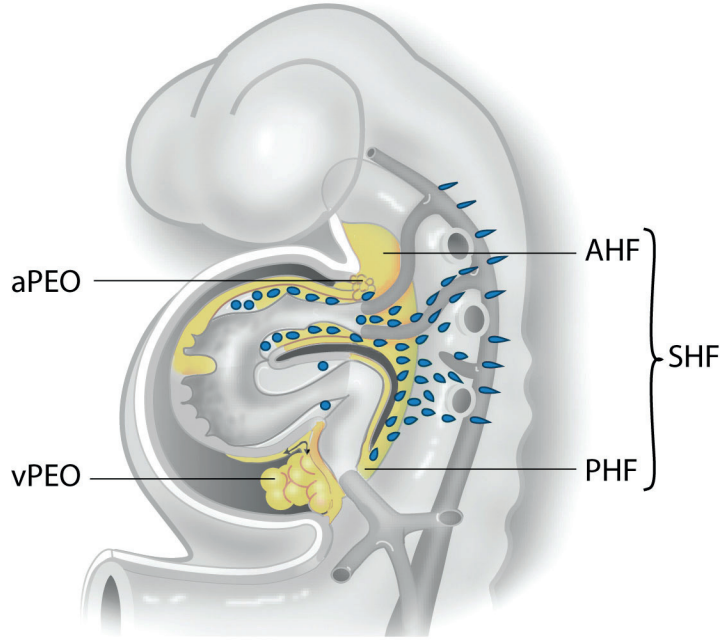

Figure 1. *Schematic overview of the developing heart tube.*
The second heart field (SHF) is indicated contributing to the arterial pole of the heart including the great vessels and the right ventricle by the anterior population (AHF). At the venous pole SHF cells are entering from the posterior population (PHF). Both at the venous (vPEO) and arterial (aPEO) pole a proepicardial organ provides the epicardial cells that cover the myocardium and the intrapericardiac part of the great vessels. Neural crest cells migrate from the neural tube primarily to the arterial pole of the heart.

between the valve types, supporting a different developmental background as underlying cause. Recent support for the role of deficient neural crest cell contribution in development of type 1 BAV was seen in the Rock 1,2 deficient mouse (6). Fernandez et al. argued that type 1 and type 2 BAVs have a different pathogenesis (13). An altered neural crest cell behaviour was suggested to be responsible for the development of type 1 BAVs and the endothelial nitric oxide (eNOS) mutation for type 2 BAV. eNOS is expressed by endocardial cells (14), cardiomyocytes (14) and VSMCs (Grewal et al. unpublished data), all SHF-derived cell types/populations, indicating a role for SHF progenitors in the development of type 2 BAV. Another role for SHF in the development of type 2 BAV was demonstrated by endocardial specific deletion of the gene encoding for the activin type I receptor (ALK2) (15). Other

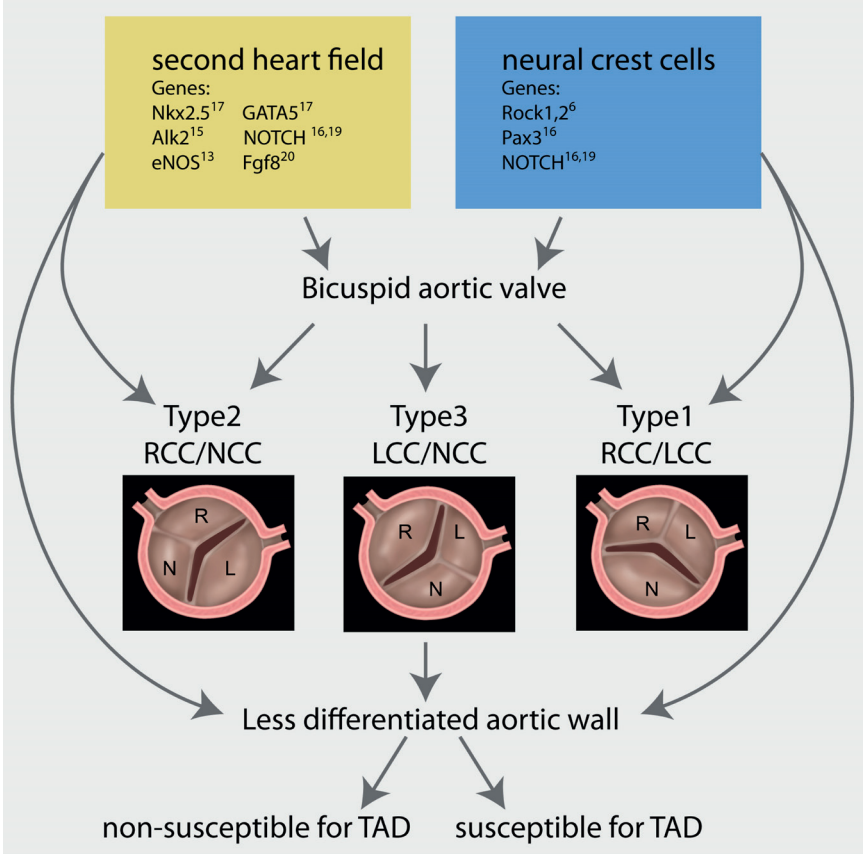

Figure 2. *Overview of our hypothesis on the developmental origin of the bicuspid aortic valve and aortic wall abnormalities.*

Figure 2 provides a schematic presentation of the aortic bicuspidy valve types based on the valve cusp orientation and the position of the raphe. Type 1: with fusion between the right coronary cusp (RCC) and left coronary cusp (LCC). Type 2: with fusion between the RCC and non-coronary cusp (NCC). Type 3 with fusion between the LCC and NCC. Furthermore an overview of our hypothesis on the developmental origin of the bicuspid aortic valve and aortic wall abnormalities is provided. Previously identified genetic mutations in mice (Nkx2.5, Alk2, eNOS, GATA5, NOTCH, Fgf8, Rock1,2 and Pax3) and in human (NOTCH) resulting in bicuspid aortic valves (BAV) are indicated in this figure. These genetic defects can be subdivided in either second heart field (SHF) or neural crest cell related. SHF and neural crest cells both contribute to the vascular smooth muscle cells in the ascending aorta as well as to the cells involved in semilunar valve formation. The SHF most probably also contributes to the endocardial cells of the cardiac outflow tract. Therefore these early developmental defects can cause bicuspidy, but also explain the less well differentiated aortic wall seen in all patients with a BAV. However, not every patientwith BAV has increased susceptibility for aortopathy. Therefore an additional factor needs to be identified to recognise patients with increased vulnerability for aortic complications.

genetic defects leading to BAV have been identified with selective knockout of genes in murine models but have not focused on the differentiation in type 1 or type 2 BAVs. From the recognised genes, Pax3 is a marker of neural crest cells (16). Furthermore, the identified SHF markers Nkx2.5 and GATA5 (endocardial cell-specific) are associated with the development of BAV (17). Abnormalities of NOTCH signalling in the neural crest (18) or SHF can also contribute to the development of abnormal semilunar valves (16, 19). Interestingly, inhibition of NOTCH in SHF impairs fibroblast growth factor 8 (Fgf8) signalling, which results in the development of BAV, but also in VSMCs abnormalities of the great arteries (20). Therefore, we postulate that a developmental defect of various progenitor cell lines may provide a common mechanism underlying aortic valvulopathy (BAV), as well as aortopathy. The next section focuses on genetic defects described in BAV and thoracic aortic dilation in human and their origin in embryogenesis.

GENETIC BASIS OF BAV AND THORACIC AORTIC DILATION

Consistent data have suggested a genetic cause of BAV disease (21, 22). Despite high heritability it remains challenging to determine the underlying mechanism of BAV in the human population, supported by murine data, as it is probably due to interacting mutations in diverse genes encoding transcription factors, extracellular matrix proteins and signalling pathways that regulate cell proliferation, differentiation, adhesion or apoptosis.

Although a remarkable reduction in eNOS levels was seen in BAV patients, this could not be correlated to a mutation in the eNOS gene (23). Moreover, mutations in the NOTCH1 gene, which is expressed by both neural crest and SHF, and mapped to chromosome 9q34, have been associated with the development and progression of BAV (18, 24). Further genetic haplotypes within the AXIN1-PDIA2 locus have been recognised that strongly associate with BAV. AXIN1 (Axis Inhibitor 1) is a critical member of the Wnt pathway, which regulates both heart valve formation (25) and cardiac neural crest development (26). Another haplotype within the Endoglin gene (known as a co-receptor in the TGFβ pathway) is required for differentiation of neural crest cells into VSMCs that populate the aorta (27).

From these genetic defects we conclude that there is a clear link to defects in neural crest as well as SHF-derived cell populations with elements for BAV.

Alterations in neural crest signalling are associated with the most common type 1 BAV (28), identified as the valve type with most severe aortic wall abnormalities as compared with the other valve types (13, 29, 30) while SHF-related genes seem to correlate more with BAV type 2.

This brings us to the next important question whether thoracic aortic dilation, not necessarily accompanied by BAV, is related to defects in neural crest signalling or alternatively, is there a more specific role for the SHF-derived cells? The following section concentrates on genetics of thoracic aortic dilation in syndromes including Marfan, Ehlers-Danlos, Smad3 mutations and Loeys-Dietz and their link to embryonic development.

Marfan syndrome (MFS) is a connective tissue disorder characterised by cardiovascular, skeletal and ocular manifestations. The progressive dilation of the aortic root culminating in dissection is a major cause of morbidity and mortality in MFS patients. This syndrome is the result of a defect in the fibrillin-1(FBN1) gene that localises on chromosome 15q21.1 and is inherited in an autosomal dominant manner (31). A second locus for Marfan syndrome (MFS2) has been mapped to chromosome 3p25-24.2, and a heterozygous mutation in TGFBR2 was subsequently identified as the genetic defect (32). The TGFBR2 mutations in MFS patients involve the serine-threonine kinase domain and reduce TGFβ-induced receptor signalling.

Loeys-Dietz is caused by another defect in the TGFβ signalling pathway. In this syndrome TGFBR1 and TGFBR2 mutations are mapped to chromosome 9q33-34 and 3p24-25 respectively. Cardiovascular lesions in Loeys-Dietz syndrome include aortic valve regurgitation and aortic root dilation, aneurysm formation and dissection. Other phenotypic characteristics include craniosynostosis, cleft palate, bifid uvula, congenital heart disease and mental retardation.

Another syndrome presenting with aneurysms, dissections and tortuosity throughout the arterial tree in association with mild craniofacial features and skeletal and cutaneous anomalies has recently been described by Van de Laar et al. (33). The genetic locus has been mapped to chromosome 15q22.2-24.2 and shows that the disease is caused by mutations in SMAD3, essential for propagation of the TGF-β signal to the nucleus and activation of downstream gene transcription.

An additional syndrome worth mentioning is Ehlers Danlos. Patients with vascular Ehlers-Danlos often present with dissection or rupture of the

thoracic aorta. Aortic dissections have been reported in at least 10 % of patients with Ehlers-Danlos (34). This syndrome is attributed to a mutation in the gene encoding type III procollagen (COL3A1), mapped to 2q24.3-q31 (35). Type I and type III collagen are the most abundant collagen fibers found in the media and adventitia of the aortic wall. In addition to providing mechanical strength, collagen has other functional properties, including activation of intracellular signalling cascades, storage of soluble factors, such as IL-2, and regulation of their local activity (36). Recently, a collagen crosslinking disorder has been reported specifically for isolated BAV (37).
Furthermore, thoracic aortic dilation occurs in association with an autosomal dominant disorder in the absence of syndromic features, termed familial thoracic aortic aneurysm and dissection (FTAAD). A variety of genetic loci have been identified in this regard, such as TAAD1 locus, on chromosome 5q 13-14 (38) and TAAD2 locus on chromosome 3p24-25 (39), the mutant gene associated with this locus is TGFBR2. Only 5 % of investigated families have this mutation, suggesting a relatively rare cause (39). TAAD3 locus on chromosome 15q24-26 (38, 39) and TAAD4 locus on chromosome 10q23-24 are also related to the development of aortic dissection. In the latter ACTA2 encoding for VSMCs a-actin is identified (40). The mutations impair the function of the VSMCs and this affects the integrity of the vessel wall, making it prone to dilation. This mutation has also been associated with an increased activity of the TGFβ pathway in the aorta (41). In TAAD5 the identified gene is TGFBR1, mapped to chromosome 9q33-34 (42).
In most of the above thoracic aortic dilation cases an increased TGFβ activity has been identified. However, TGFβ is not specific for neural crest, as it is also clearly involved in the endothelium and the SHF-derived cell populations (43). In syndromes as MFS, Ehlers-Danlos and Loeys-Dietz syndrome, bicuspidy is not an obligatory clinical manifestation, indicating that a defective TGFβ signalling is at least not the main factor causing BAV formation. Neural crest defects seem to cause the most frequently occurring type 1 BAV which is associated with most marked complications of the aortic wall, and often with an increased TGFβ activity (44). However, the clinical course is not complicated with thoracic aortic dilation in all BAV patients. Thus, neural crest involvement and TGFβ activity together are not sufficient to explain the variability within the pathogenesis of BAV and associated aortopathy. Additional pathogenetic factors need to be taken into account such as haemodynamics or a contribution of SHF.

PATHOGENESIS OF THORACIC AORTIC DILATION IN BAV: ROLE OF HAEMODYNAMICS AND SHF

The morphology of the bi-leaflet valve produces a nonaxial transvalvular turbulent flow jet within the aortic root (45). This turbulent flow, along with other haemodynamic factors, as an increased stroke volume (for instance aortic regurgitation), have been suggested to facilitate developing aortic complications, as the created abnormal biomechanics and helical flow alterations lead to an uneven wall stress distribution. However, several studies have confirmed that ascending aortic aneurysms can develop in the absence of valve abnormality (46). Moreover, Yasuda et al. have reported development of aortic dilation after surgical repair of the diseased bicuspid aortic valve (47). These studies suggest that structural wall abnormalities at the cellular level may be important for the onset of dilation. Therefore, haemodynamic factors alone are not sufficient to explain the pathogenesis of aortopathy associated with bicuspid aortic valves.

The alternative hypothesis is that genetically determined abnormalities of the aortic wall lead to a defect in the cellular microenvironment, causing or at least contributing to the aortic pathology and render the wall vulnerable to haemodynamic stress.

Several studies have focused on differences between the dilated aortic wall in BAV and TAV. Histopathological features of the aortic wall in BAV show decreased medial inflammation, elastin fragmentation and cystic medial necrosis, when compared with TAV (48). In addition, the aortic wall has a different composition in BAV, with a significantly thicker tunica media but significantly thinner tunica intima (49). In recent years research on extracellular matrix composition mainly established differences in the aortic media of dilated aortic wall in BAV and TAV (24, 37). To take a step further in unravelling a possible different pathogenetic mechanism of thoracic aortic dilation in BAV and TAV, we investigated non-dilated aortic walls of both valve types. The aortic media was specifically studied for maturation of VSMCs and ageing characteristics. We concluded that thoracic aortic dilation in TAV has aspects of ageing, whereas in bicuspidy there is a defective smooth muscle cell differentiation (Fig. 3) unrelated to ageing. These results suggest that the fundamental difference in the aortic wall make-up of BAV is found in less differentiated VSMCs as compared with TAV while still both neural crest cells and SHF contribute to the VSMCs in the aortic wall. Haemodynamic

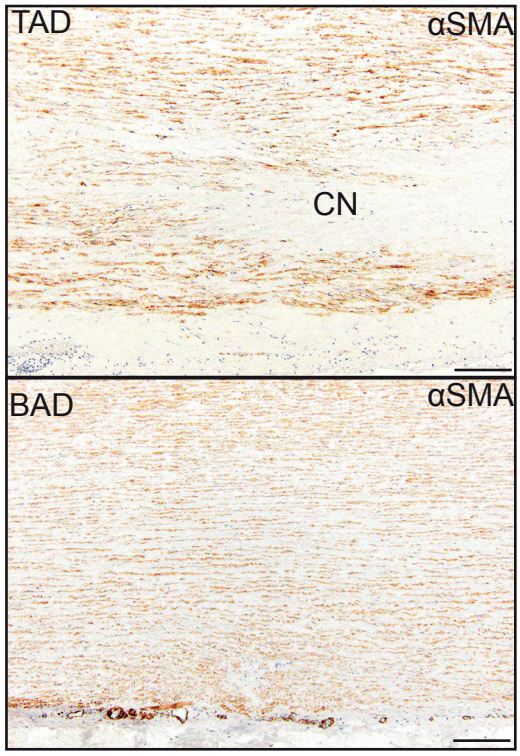

Figure 3. *Transverse histological sections of the media of the aortic wall stained for the smooth muscle cell marker alpha smooth muscle actin (αSMA).*
In patients with a tricuspid aortic valve and a dilated aortic wall (TAD) the expression of this marker is higher as compared with the expression in patients with a bicuspid aortic wall and dilated aortic wall (BAD). Furthermore the aortic media in TAD shows significantly more pathology as compared with BAD, with profoundly more cystic medial necrosis (CN) defined as loss of smooth muscle cell nuclei. Magnification bar: 500 μm.

factors might play a role in the aortic complications, but superimposed on the already present structurally immature aortic wall seen in BAV.

CONCLUSION AND FUTURE PERSPECTIVES

From a clinical point of view, aortic complications vary between the different BAV types. Aortic root diameters for instance have been analysed between BAV type 1 and 2 in several studies, all of which found larger aortic root diameters in the type 1 (13, 29, 50), being more vulnerable for degradation (30). Type 2 BAVs are responsible for valve dysfunction at a younger age (29, 50). Aortopathy seems most outspoken in BAV type 1, probably being caused by a defect in neural crest. Despite these recent findings however, clinical parameters have not been conclusive in distinguishing patients with BAV susceptible for aortopathy, suggesting that an alternative, molecular biological approach might be necessary.

In this review we describe that altered neural crest cell and second heart field contribution, separately or in combination, can account for a structurally different aortic wall in combination with bicuspidy (Fig. 2). Figure 2 summarises that defects in neural crest cells are mostly associated with type 1 BAV and defects in SHF with type 2. These contributions alone, however, are not sufficient to explain the clinical heterogeneity seen in BAV patients, as not all individuals with BAV develop aortic complications during their life. Therefore, additional factors make the aorta susceptible for ensuing complications. It is important to determine which developmental defect accounts for the additional pathology making the aortic wall susceptible for thoracic aortic dilation. Future research, therefore, needs to focus on identifying molecular pathways related to neural crest and SHF. These factors are required to distinguish a susceptible and a non-susceptible group for suspected aortic complications.

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CHAPTER

3

Ascending aorta dilation in association with bicuspid aortic valve: a maturation defect of the aortic wall

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Modified after: J Thorac Cardiovasc Surg. 2014;148(4):1583-90.

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ABSTRACT

Objective: Patients with a bicuspid aortic valve have increased susceptibility to the development of ascending aortic dilation and dissection compared with persons with a tricuspid valve. To unravel a possible different mechanism underlying dilation in bicuspidy and tricuspidy, a comparison of the structure of the aortic wall was made.

Methods: Ascending aortic wall biopsies were divided into 4 groups: bicuspid (n = 36) and tricuspid (n = 23) without and with dilation. The expression of vascular smooth muscle cell maturation markers including lamin A/C, which plays a pivotal role in smooth muscle cell differentiation, and its splicing variant progerin indicative of aging, were studied immunohistochemically. Attention was also paid to the inflammatory status.

Results: There is a significant difference in the structure and maturation of the aortic wall in bicuspidy, persisting in the dilated aortic wall, presenting with a thinner intima, lower expression of a smooth muscle actin, smooth muscle 22a, calponin, and almost absent expression of smoothelin. We show for the first time significantly lowered lamin A/C expression in bicuspidy. Progerin was found to be significantly increased in the media of the dilated wall in tricuspidy, also showing increased periaortic inflammation.

Conclusions: The structure of the non-dilated and dilated aortic wall in bicuspidy and tricuspidy are intrinsically different, with the latter having more aspects of aging. In bicuspidy there is a defective smooth muscle cell differentiation possibly linked to lowered lamin A/C expression. Based on this vessel wall immaturity and increased susceptibility to dilation, different diagnostic and therapeutic approaches are warranted.

INTRODUCTION

A bicuspid aortic valve (BAV) is characterized by an aortic valve with 2 semilunar leaflets. BAV is the most common congenital cardiovascular malformation with a prevalence of 0.5% to 2% (1). The incidence of thoracic aortic dilation or aneurysm formation and dissection in patients with BAV is considered to be 50% to 70% (2). Compared with patients with a tricuspid aortic valve (TAV), patients with BAV have larger aortic root dimensions and a higher progression rate of dilation (3,4) suggesting that the process of dilation of the thoracic aorta is different in BAV compared with TAV. The terms aneurysm and dilation for the aortopathy are used interchangeably. We have chosen to use the term dilation for clarity. Turbulent flow, as a result of asymmetric movement of valve leaflets in BAV, has been postulated as an essential determinant for the development of aortic dilation (5). An alternative hypothesis, that aortic dilation in BAV is mainly based on the intrinsic structure of the aortic wall, (6) is supported by a high incidence of dilation in asymptomatic patients with BAV as well as dilation observed after aortic valve replacement (7). The latter hypothesis is further supported by reported altered molecular and/or metabolic characteristics in the aortic wall and valve leaflets in BAV, differences in elastic lamellae, loose attachment of VSMCs to their surrounding elastic lamellae, and precocious VSMCs apoptosis (8-17). Most studies have focused on the differences between the dilated TAV and BAV wall. The exact mechanisms underlying the development and progression of an aorta with normal dimensions into dilation in patients with TAV versus BAV, however, have not been delineated.

In this study, we used a unique opportunity to compare non-dilated ascending aortic wall specimens from patients with BAV, representative of a specific architecture and possible early lesions rather than of end-stage disease, with the normal aortic wall in TAV.

Furthermore, these data could be compared with the histopathology of the dilated vessel wall in BAV and TAV. Histologic procedures using hematoxylin-eosin and resorcin fuchsin were applied to assess the general vessel wall architecture, that is, inflammation, vessel wall thickness, elastic lamellae, and cystic medial necrosis (focal loss of VSMC nuclei in the media). Expression of markers of differentiated VSMCs was investigated to determine differences in vessel wall maturation or differentiation between patients with BAV and TAV. Smooth muscle 22α (SM22α), smoothelin, and calponin were used

as markers for fully differentiated contractile VSMCs (18) and a smooth muscle actin (αSMA) was used as a marker for differentiation of VSMCs and myofibroblasts (19). Lamin A/C, was investigated to explain possible differences between VSMC differentiation in BAV and TAV, because it has a pivotal role in the differentiation of myoblasts (20). Progerin, a splice variant of lamin A/C, was studied to further elucidate differences in the pathogenesis of aortopathy between the 2 valve types, because it has been suggested that progerin not only plays a role in Hutchinson-Gilford progeria syndrome but also in cardiovascular aging (21-23). We hypothesize that the BAV vessel wall has a maturation defect that underlies a more aggressive form of dilation.

MATERIALS AND METHODS

Ethical Approval

Sample collection and handling was carried out according to the official guidelines of the Medical Ethical Committee of Leiden University Medical Center (LUMC), Leiden, and the code of conduct of the Dutch Federation of Biomedical Scientific Societies www.FMWV.nl). Six cryopreserved bicuspid human aortic valves were obtained from the Heart Valve Bank in Rotterdam (Erasmus University Medical Center, Rotterdam) originating from postmortem donors. These valves were declared unfit for implantation because of the bicuspid nature of the valves. The Advisory Board of the Heart Valve Bank allowed these valves to be included in the present project because the research was in line with the permission of the donation.

Tissue Samples

Samples of the ascending aorta were collected from individuals with BAV and TAV, with and without dilation. Material from patients with BAV without dilation was available in cases of stentless root replacement, the preferred technique for stentless valve implantation in LUMC. Dilation was clinically defined by reaching an ascending aortic wall diameter of 45 mm or more (24). The patients were divided into 4 groups: (1) TAV without dilation (TA, n = 11, mean age 64.5 ± 9.0 years) obtained post mortem; (2) TAV with dilation (TAD, n = 12, mean age 72.3 ± 11.2 years) collected during elective repair; (3) BAV without dilation (BA, n = 17, mean age 55.8 ± 9.8 years); (4) BAV with dilation (BAD, n = 19, mean age 60.7 ± 7.8 years) obtained during

elective repair. Patients with a proven genetic disorder (eg, Marfan disease) were excluded.

After excision, specimens were fixed in 4% formalin (24 hours), decalcified in a formic acid-formate buffer (120 hours), and embedded in paraffin. Transverse sections (5 μm) were mounted on precoated Starfrost slides (Klinipath BV, Duiven, The Netherlands). Histologic Parameters Sections stained with hematoxylin-eosin and resorcin fuchsin were analyzed quantitatively for (1) periaortic inflammation (presence of a cellular infiltrate in the adventitia), indexed from 0 (no inflammatory cells), 2 (a few cells), 4 (groups of cells) to 6 (large clusters of cells); (2) maximum intimal thickness in micrometers (the distance between the endothelial layer and the first major internal elastic lamella, excluding atherosclerotic areas; (3) maximum medial thickness in micrometers (the distance between the first and last elastic lamella on the borderline with the adventitia. Furthermore, the organization of the elastic content was studied qualitatively. All specimens were reevaluated by an independent, experienced histopathologist who was blinded to the clinical data.

Immunohistochemistry

After deparaffinization, antigen retrieval was performed in a microwave oven in citrate buffer (pH 6.0, 12 minutes), followed by treatment with 0.3% H2O2 in phosphate buffered saline (PBS, pH 7.3, 20 minutes) to extinguish endogenous peroxidase activity. Subsequently, sections were rinsed briefly twice in PBS and once in PBS with 0.05% Tween-20 (PBS-T). Sections were incubated overnight at room temperature with the primary antibodies diluted in PBS-T and 1% bovine serum albumin (BSA, Sigma, St. Louis, Mo) (Table 1). Between the incubation steps, the slides were rinsed in PBS (23) and PBS-T (13). Bound antibodies were detected using 1-hour incubation with a secondary antibody diluted in PBS-T (Table 1). Subsequently, all slides, except for αSMA, were incubated with ABC reagent (Vector Laboratories, Burlingame, Calif; PK 6100) for 45 minutes. Control stainings were performed using PBS-T and BSA as the first incubation step. Slides were incubated with 400 mg/mL 3,30-diaminobenzidine tetrachloride (Sigma-Aldrich Chemie, Buchs, Switzerland; D5637) dissolved in Tris-maleate buffer to which 20 mL of H2O2 were added (pH 7.6, 10 minutes). After rinsing, counterstaining was performed with 0.1% hematoxylin (Merck, Darmstadt, Germany) (5 seconds), followed by rinsing in tap water (10 minutes). After dehydration,

sections were mounted in Entellan (Merck, Darmstadt, Germany). Sections used for (semi)quantitative and morphometric analysis were stained in the same batch.

Double Immunofluorescence Staining

Deparaffinization, rehydration, and antigen retrieval were performed in the manner described earlier. Sections were incubated with the primary antibodies, progerin and lamin A/C (overnight, 4°C) (Table 1). The secondary antibodies used were 1:500 Cy3 donkey antimouse IgG (Jackson Immunoresearch,

Table 1 *Immunohistochemistry reagents*

Primary antibody	Vendor, order number	Concentration	Secondary antibody
Anti-lamin A/C	Chemicon (MAB3211)	1:50	HAM (1:200) & NHS (1:66) (Vector Laboratories, USA, BA-2000) (Brunschwig Chemie, Switserland, S-2000)
Anti-progerin	Kindly provided by K. Djabali (dpt. Of Dermatology, Colombia University, NY, USA)	1:50	GAR (1:200) & NGS (1:66) (Vector Laboratories, USA, BA-1000 and S1000)
Anti-αSMA	Sigma-Aldrich Chemie (A2547)	1: 5000	RAM-PO (1:250) (DAKO p0260)
Anti-SM22α	Abcam (-)	1:100	GAR & NGS
Anti-human Calponin	Sigma-Aldrich Chemie (C2687)	1:6000	HAM & NHS
Anti-Smoothelin	Progen Biotechnik (d4816101)	1:200	HAM & NHS

GAR (goat-anti-rabbit-biotin), NGS (normal goat serum), HAM (horse-anti-mouse-biotin), NHS (normal horse serum) and RAM-PO (peroxidase-conjugated rabbit anti-mouse).

Newmarket, England; 715-165-150) and 1:500 Alexa Fluor 647 donkey antirabbit IgG (Invitrogen, Grand Island,NY; A-31573) (1 hour, 20°C). The nuclei were counterstained for visualization with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). All slides were mounted with ProlonGold (Invitrogen, P36930).

Immunohistochemical and Morphometric Analyses

Sections were studied with a Leica BM5000 microscope equipped with plan achromatic objectives (Leica Microsystems, Wetzlar, Germany). The cytoplasmic level of expression of αSMA, SM22α, calponin, and smoothelin was indexed at 3 predetermined locations (left, middle, and right) of every tissue section, referred to as the microscopic field (MF), from 0 (no expression in VSMCs), 2 (expression in less than one-third of all VSMCs), 4 (expression in two-thirds of all VSMCs) to 6 (expression in all VSMCs). This was maintained for all stainings.

To determine the lamin-progerin balance, in each stained section the number of positively stained nuclei was counted using ImageJ in the 3 MFs. A threshold was applied to filter background noise. The total number of nuclei (positively stained and negative) was equal in all specimens. In each MF, the number of lamin- and progerin-positive nuclei was therefore normalized to the total number of nuclei per 100,000 μm^2. The number of normalized lamin- and progerin-positive nuclei was averaged between the 3 MFs.

Western Blot

Aortic tissue (60 mg) was homogenized in 300 mL of lysis buffer (Cell Signalling Technology,Beverly, Mass). Whole protein concentration in the cell lysate was measured using the BCA protein assay (ThermoFisher Scientific, Rockford, Ill). Lamin A/C expression was quantified following procedures described previously (25), by using lamin A/C rabbit polyclonal antibody (sc-20681) together with bovine antirabbit IgG AP (sc-2372) secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) to detect lamin A/C protein. Band intensity was analyzed using ImageJ software. To compare band intensity between different blots, an internal control sample (Rest) was run on every blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (sc-47724; Santa Cruz Biotechnology) was used as loading control and with the calibration sample.

Statistical Analysis

All numerical data are presented as the mean ± standard deviation of 3 MFs on each stained slide. Statistical differences were evaluated with the Mann-Whitney U test for comparison between the groups. SPSS 20.0 (SPSS Inc. Chicago, Ill) was used for the statistical analyses.

An additional 1-, 2- and 3-way analysis of variance test was performed to correct for age and gender and it was found that both factors were not confounding in this study. GraphPad software (GraphPad Software, Inc, San Diego, Calif) was used to create graphics of the statistical analyses.

RESULTS

Histopathology

The ascending aorta consists of a tunica intima, media, and adventitia, with endothelium lining the lumen (Fig. 1, A, D, H, and K). The border between the fine elastic fibers in the tunica intima and more regular thick elastic lamellae in the tunica media is delineated by an internal elastic lamella (Fig. 1, B and I). The media to adventitia border is marked by the last elastic lamella of the media. The absolute intimal thickness was significantly smaller in BA compared with TA and BAD compared with TAD ($P<0.001$, $P<0.0001$, respectively) (Fig. 1, B, I, and O). The media, however, was significantly thicker in all specimens in the BAV groups (BA and BAD) compared with the TAV groups (TA and TAD) ($P < .01$ and $P<0.001$, respectively) (Fig. 1, A, H, and P). Differences in the organization of the elastic lamellae were also observed. In the TA group, the elastic lamellae had a dense regular distribution (Fig. 1, C), whereas in the TAD group the lamellae appeared fragmented and the distance between the lamellae was enlarged (Fig. 1, E). The BA group contained well-organized lamellae, even more neatly regulated compared with the TA group (Fig. 1, J). In the BAD group, the architecture of the elastic lamellae remained regular (Fig. 1, L). Specimens from the BAD group also showed less cystic medial necrosis (focal loss of VSMC nuclei in the media) compared with specimen from the TAD group (Fig. 1, G and N). The adventitia in all specimens harbours fat cells, fibroblasts, nerve fibers, a few quiescent resident inflammatory cells, and vasa vasorum. The amount of adventitial fat cells (Fig. 1, M) was not different between the 4 groups (data not shown). Periaortic inflammation

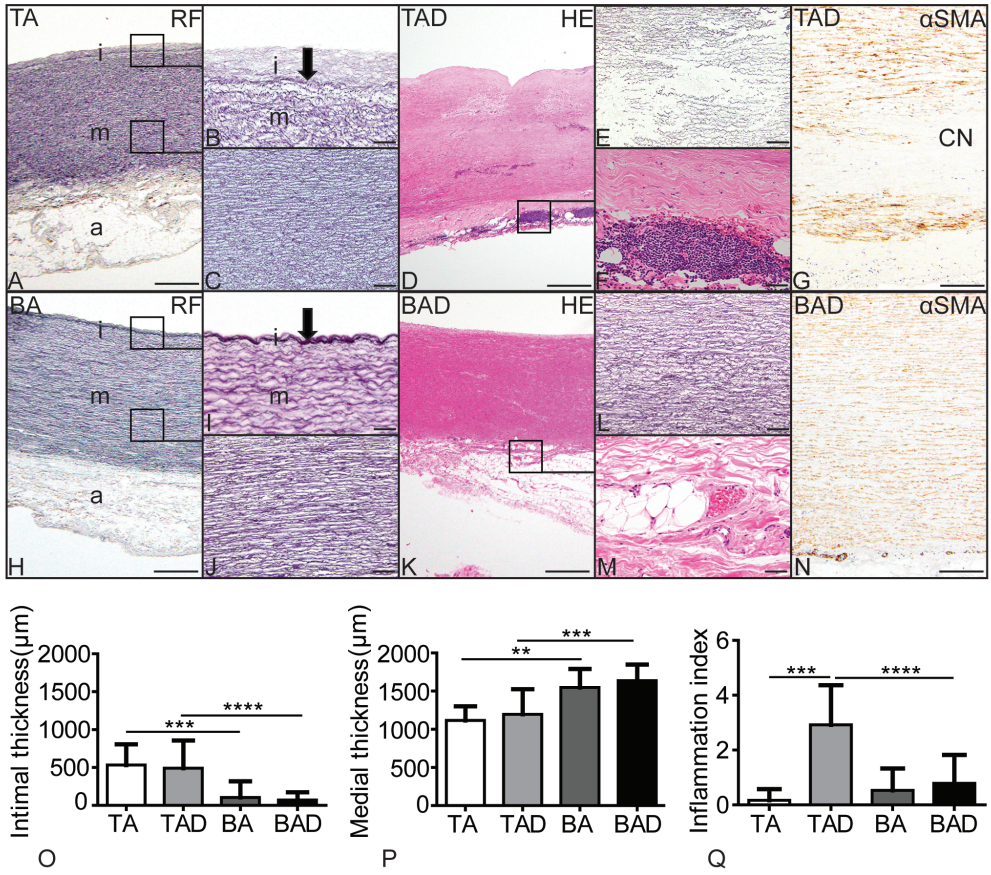

Figure 1. *Transverse sections of the aortic wall (A-N).*
The aorta consists of an intima (i), media (m), and adventitia (a) indicated in A and H. The media and intima are separated by the internal elastic lamella (arrows in B and I).A very compact layering of medial elastic lamellae is seen in TA (C) compared with the somewhat more loosely organized yet regular lamellae in BA (J). The latter is maintained in the BAD group (L), in contrast to the TAD where the lamellae become fragmented and disorganized after dilation (E). Medial thickness is significantly increased in BA and BAD compared with TA and TAD (P). The reverse is seen for the intimal thickness, which is significantly thinner in BA and BAD (O). αSMA staining (G, N) exhibits more severe cystic medial necrosis (CN) in TAD compared with BAD (G). Periaortic inflammation (box in D, indexed as 6) is significantly more severe in TAD compared with BAD (D, F, K, M,and Q). TA, Tricuspid valve, without dilation; TAD, tricuspid valve, with dilation; BA, bicuspid valve, without dilation; BAD, bicuspid valve, with dilation; RF, resorcin fuchsin; HE, hematoxylin-eosin; αSMA, a smooth muscle actin. Scale bars: A, D, G, H, K, and N, 500 μm; B, C, E, F, I, J, L, and M, 50 μm.

was significantly more profound in the TAD group compared with the TA and BAD groups (P<0.001 and P<0.0001, respectively) (Fig. 1, D, F, K, M, and Q).

Altered Pattern of Smooth Muscle Cell Expression in the Aorta of Patients With BAV Compared With TAV

Differences in the expression of actin-positive VSMCs were noted between the groups. The ascending aorta from BAV showed less αSMA expressing VSMCs compared with TAV, in both dilated and non-dilated specimens (P<0.05) (Fig. 2, A, B, and C). Less expression in BAV was not due to focal vessel wall degeneration, rather individual cells expressed less αSMA. The results obtained for αSMA were mirrored by the expression pattern of SM22α (P <0.01) (Fig. 2, D, E, and F) and calponin (data not shown). The intima of the aortic wall stained positive for SM22α, but not for αSMA (Fig. 2, A, B, D, and E). Smoothelin expression in the TA group was seen mainly in the outer media compared with the middle and inner media, with a significant degenerative loss of expression in the middle media of the TAD group (P<0.001) (Fig. 2, G, J, and I). Smoothelin expression in the BA and BAD groups was nearly absent (P<0.0001 and P<0.001, respectively) (Fig. 2, H, K, and I).

Lamin A/C and Progerin Expression

The expression of the nuclear protein lamin A/C was seen in the intima and media. Significantly less lamin A/C positive nuclei were observed in the BA group compared with the TA group (P <0.01) (Fig. 3, A, E, and G). We also investigated the expression of progerin. Confocal microscopy revealed progerin expression at the nuclear lamina (Fig. 3, C and F), whereas lamin A/C was also distributed in the nucleoplasm (Figure 3, B and D), confirming that the 2 antibodies recognize different proteins. Morphologic analyses of progerin expression patterns revealed a significant increase in the TAD group compared with the TA group (P<0.05) (Fig. 3, H), whereas no such increase was seen between the BAD and BA groups. Moreover, the expression in the BAD group was significantly lower than in the TAD group (P<0.05) (Fig. 3, H). Lamin A/C expression in the BA and TA groups was quantified by performing a Western blot. The level of GAPDH was used as calibration of the samples. The results of lamin A/C quantification showed the same trend as the immunohistochemistry data (Fig. 4).

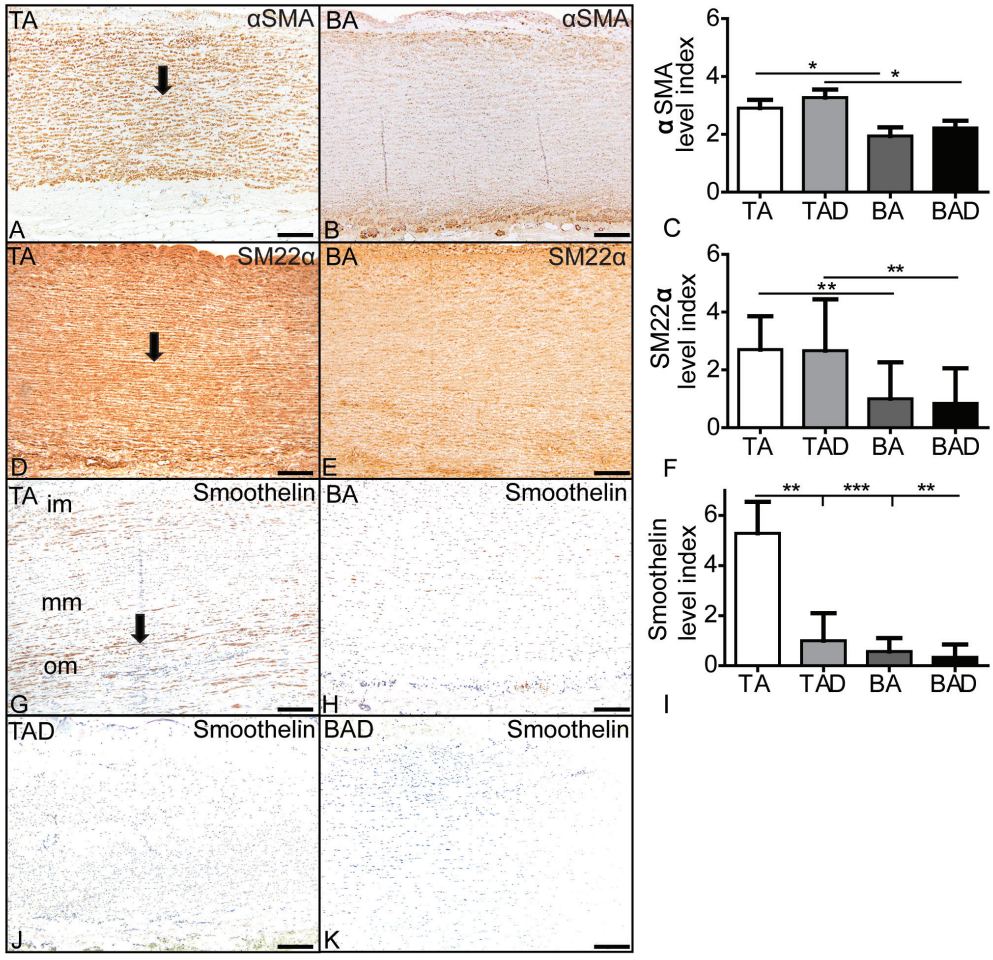

Figure 2. *Transverse sections of the aortic wall (A, B, D, E, G, H, J, K).*
Cytoplasmic 3,30-diaminobenzidine tetrachloride vascular smooth muscle cell (VSMC) staining, with positivity depicted by an arrow in A, D, and G. The number of αSMA expressing VSMCs is significantly lower in BA (B, indexed as 0) than TA (A, indexed as 6), which is maintained after dilation (BAD and TAD, respectively) (C). The number of SM22α expressing VSMCs is also significantly lower in BA (E, indexed as 2) than TA (D, indexed as 6) and in BAD compared with TAD (F). VSMCs expressing smoothelin were only seen in the TA, mainly in the outer (om) compared with the middle (mm) and inner media (im) (G, indexed as 4) and nearly absent in the other groups (H, J, and K, indexed as 0) (I). TA, Tricuspid valve, without dilation; TAD, tricuspid valve, with dilation; BA, bicuspid valve, without dilation; BAD, bicuspid valve, with dilation; αSMA, a smooth muscle cell actin; SM22α, smooth muscle cell 22a. Scale bars: 200 μm.

DISCUSSION

Previous studies demonstrated that patients with BAV have more severe aortic wall abnormalities than patients with TAV (14). The results of our study provide evidence that the aortic wall in BAV is intrinsically different and presents with a maturation defect compared with the aortic wall in TAV. This maturation defect is maintained in the dilated aortic wall, which also has a different basic architecture in BAV compared with TAV. The significant difference observed remained after correcting for age and gender.

Most literature data refer to comparison of the dilated wall in patients with TAV and BAV (11,12,14). We had the opportunity to compare the wall in 4 groups, including a unique non-dilated BA population, which allowed us to define its basic structural differences. Our observation that the inflammatory component is minimal in BAD compared with TAD, despite the presence of a similar degree of aortic dilation, confirms the findings of Matthias Bechtel and colleagues (26). The significantly thinner intima in all our BAV specimens compared with the TAV specimens is a novel finding and might explain the less frequent observation of atherosclerosis in the BAV aortic wall (26). The aortic media appeared significantly thicker in all patients with BAV compared with TAV, which was not found earlier (12). Specimens in the BA and BAD groups showed relatively well-organized, albeit thinner, elastic lamellae, with a larger interlamellar distance compared with those from patients with TAV, a finding previously described for the BAD group, but not for the non-dilated BA group (11,12,14).

We propose that the finer elastic lamellae observed combined with an increase in extracellular matrix in the non-dilated BA group might add to the increased susceptibility for dilation of the media as has been suggested in Literature (27).

VSMCs have the ability to undergo a phenotypic switch from a quiescent contractile state to a less mature, proliferative synthetic state. Disability of this switch has been shown to play a critical role in a variety of cardiovascular diseases (18,28). By using differentiation markers of contractile VSMCs, we showed less maturation in patients with BAV compared with TAV in both dilated and non-dilated specimens. This was particularly prominent for smoothelin, a specific marker of highly differentiated VSMCs, which was significantly less in the TAD group compared with the TA group, and, as we show for the first time, nearly absent in all BAVs. These findings correlate

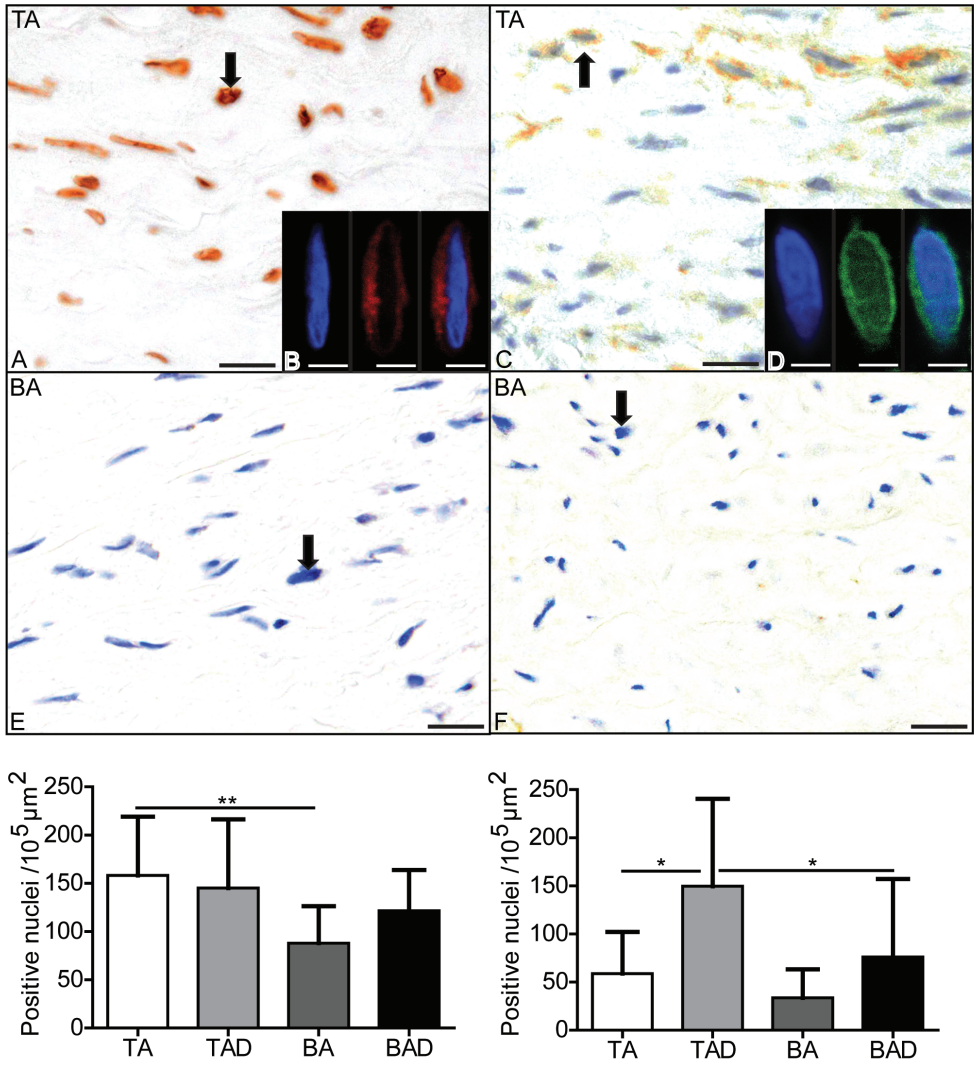

Figure 3. *Transverse histologic sections of the aortic wall (A-F).*
Nuclear 3,30-diaminobenzidine tetrachloride (DAB) staining of lamin A/C (A, brown, see arrow; E, absent staining, see arrow). The number of lamin A/C-expressing nuclei is significantly lower in the BA group compared with the TA group (G). Nuclear DAB staining of progerin is shown in C (brown, see arrow) and F (absent staining, see arrow). The number of progerin-expressing nuclei significantly increases with wall dilation in TAD (H), which is not seen in BAD. The number of progerin-expressing nuclei is significantly lower in BAD compared with TAD (H). Confocal images of consecutive sections (B and D). Nuclei are counterstained with diamidino phenylindole (B and D, blue). Immunofluorescence shows nuclear distribution of lamin A/C (red, B) and nuclear envelope localization of progerin (green, D). TA, Tricuspid valve, without dilation; TAD, tricuspid valve, with dilation; BA, bicuspid valve, without dilation; BAD, bicuspid valve, with dilation. Scale bars: A, C, E, and F, 20 µm; B and D, 1 µm.

with the structurally less well-differentiated BA wall compared with the normal TA wall, which could explain aortic wall vulnerability in bicuspidy.

This hypothesis is further supported by the novel finding that lamin A/C is significantly less expressed in all patients with BAV. The nuclear intermediate filament lamin A and C proteins are expressed from the same LMNA gene by alternative splicing. Lamin A/C are of utmost importance in regulating transcription, organizing the chromatin, and protecting the nucleus from mechanical stress during cell development (29). The role of lamin A/C in normal VSMC differentiation has not been reported so far, however mice with reduced expression show nuclear deformation and impaired transcription response to mechanical stress (30). Low expression and an altered lamin A/C ratio in the BA group, which is maintained in the BAD group, suggest that the lamins might also play a role in VSMC differentiation and maturation, and thereby affect the aortic dilation process. In line with these data is the remarkably increased progerin expression in the degenerated TAD group, which can be linked to normal cardiovascular aging (21-23). The relative lack of progerin in the BA group and the non-significant increase subsequently

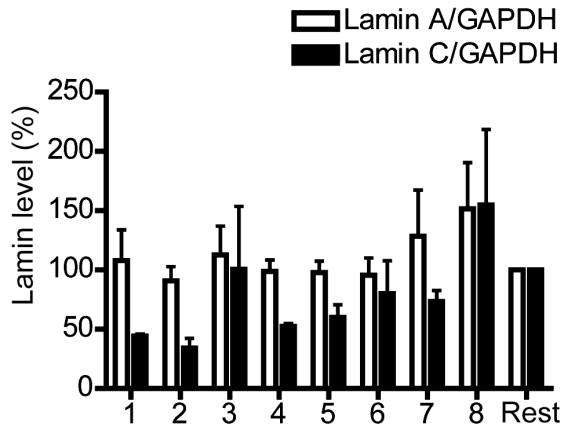

Figure 4. *Western blot of cell lysate from 8 samples of non-dilated aortic wall with a bicuspid aortic valve (BA, 1-4) and tricuspid valve (TA, 5-8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control sample (Rest).*

The lamin A/C protein level in each sample was determined by densitometry, performed using ImageJ software, and normalized. The band intensity of the Rest was used as calibrator. The Coomassie Blue stained gel showed equal loading of proteins with a molecular mass of 69 kDa (lamin A), and 62 kDa (lamin C). Bar diagram in white, lamin A level in %; and in black, lamin C level in %.

in the BAD group indicate that in the immature wall of patients with BAV, the degenerative changes are of a different nature compared with TAV and do not involve so much accelerated aging, emphasizing that BAV and TAV are clearly different entities. Although, in general, reduced levels of lamin A/C and associated proteins affect the transcription of genes (31) by, for example, histone modifications, (32) it is of utmost importance to understand its role in cell type-specific differentiation and dedifferentiation processes. Future studies should focus on the regulation of lamin A and C in VSMCs and the role of these lamins in vessel wall pathology. In conclusion, our data show that the aortic wall in patients with BAV is intrinsically different from patients with TAV, in whom inflammation and accelerated aging lead to aortic pathology. A maturation defect of the aortic wall in BAV, showing less well-differentiated VSMCs and low lamin A/C expression, might be the cause of the increased risk of aortic wall dilation. We have thereby shown with the amended markers that there is clearly a different substrate for aortopathy, seen more frequently and at a younger age, in BAV compared with TAV. As the non-dilated aortic wall has already been found to be different, there is a need for re-evaluation of the role of haemodynamics as a possible primary cause for the dilation in BAV. Future experimental research should focus on whether pharmacologic interventions can influence maturation in the aortic wall, which would be valuable in limiting clinical disease progression in the immature BAV wall.

STUDY LIMITATIONS

A limitation of our study is that we did not have aortic wall specimens from control patients (children and adolescents) to indicate whether the observed immaturity in BAV is the result of a maturation defect or a matter of non-progression of normal maturation. This can only be studied in the various existing BAV mouse models.

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CHAPTER

3A

Editorial: The aortic wall with bicuspid aortic valve: immature or prematurely ageing?

Commenting on the paper by Grewal N et al. "Ascending aorta dilation in association with bicuspid aortic valve: a maturation defect of the aortic wall"

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Modified after:
J Thorac Cardiovasc Surg. 2014;148(5):2439–2440

Dear Editor,

We read with interest the study (1) by Grewal and co-workers, suggesting a maturation defect of the aortic wall in patients with bicuspid aortic valve (BAV). A number of recent publications have addressed the pathobiology of BAV-associated aortopathy, however most of them just vaguely concluded that BAV and tricuspid aortic valve (TAV) must have different mechanisms driving aortic dilatation. Grewal *et al.* are commendable for their effort to define the nature of this difference.

However, their data are partially in contrast with previous studies, with which they failed to perform a critical comparison. We have reported (2) increased α-smooth muscle actin (α-SMA) mRNA expression and only mildly and non-homogenously increased overall protein levels, with sub-intimal areas of α-SMA-positive cell loss. This relative stability of the amount of α-SMA (in contrast with Grewal's data) coupled with smoothelin decrease (not absence, unlike in Grewal's results) was consistent with other studies both on samples from patients and on experimental models of aneurysm (discussed in our paper (2)), and suggests a loss of contractile-phenotype SMCs and the emergence of a differentiated myofibroblast line.

In our abovementioned study (2), to minimize confounding factors, we selected BAV and TAV aortic stenosis patients with maximal diameter <4 cm, and analysed both the concavity and the convexity of the vessel, to distinguish constitutive from stress-induced changes. Some of Grewal's co-Authors have previously borrowed in their studies this protocol of sample retrieval from different sites of each aorta, first introduced by us (3), and confirmed that aortic wall changes are asymmetrically expressed with BAV, suggesting a role for longstanding flow and stress pattern alterations in their development. The change of SMC orientation from circumferential to longitudinal direction we observed in the convexity (2) is known to occur in vessels submitted to altered tensile strain. The undefined site of sampling in the present study (1), conversely, could explain the above discrepancies. Similarly, the authors stated that the aortic wall specimens were obtained during full-root stentless implantation in non-dilated BAV patients (1), suggesting that the BAV specimens were taken from the root (sinuses of Valsalva) instead of the ascending tubular tract proper.

Where were the aortic specimens taken in the other patients, also considering that BAV aortopathy usually affects the ascending tract?

How was valve function in the four groups? Also, the mean diameters per subgroup were not acknowledged (1). Intimal thickness data (1) were at odds with previous investigations (2,4): could different diameters between BAV and TAV subgroups explain this?

The paradigms of flow-induced remodeling share many mechanisms with the vascular ageing process, including increased TGF-β receptor-II signalling (5), which has been evidenced in both BAV-associated and non-BAV-associated aortopathies (2). Functionally, one of the early signs of arterial ageing is impaired wall distensibility, which is known to occur in the BAV aorta, even before overt dilation. Thus, with the currently available data, an hypothesis of defective wall maturation is hardly sustainable, without ruling out the contribution of flow induced remodeling: a conceptually opposite theory of premature ageing of the aortic wall, prompted by altered biomechanical environment, could be drawn as well.

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CHAPTER

3B

Reply to Editorial: The aortic wall with bicuspid aortic valve: immature or prematurely ageing?

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Modified after
J Thorac Cardiovasc Surg. 2014;148(5):2440–2442

Dear Editor:

We thank dr. Della Corte for his insightful comments and the opportunity to clarify a number of points from our work and you as an Editor to give us this opportunity. We agree with authors of the letter that the mechanisms driving aortic dilation in bicuspid aortic valve (BAV) and tricuspid aortic valve (TAV) are different but not explained as yet. Following that line, also based on the expertise of most of our authors, we hypothesized that a developmental background linking BAV and the accompanying aortic wall might provide new insights into the matter. Haemodynamic differences could still play a role in the developing pathobiology but might not be the primary insult. This approach led to different selection criteria for our patient population. We did not primarily take into account the presence of aortic stenosis/regurgitation but focused on non-dilation (< 45 mm) and dilation of the aortic wall within the BAV and TAV groups. Additional characteristics of our study population are presented in Table 1, which also provides answers to a number of questions with regard to the material studied. The aortic valve pathology in non- and dilated BAV and dilated TAV varied from either stenosis or no stenosis, with or without regurgitation. In dilated BAV and TAV in some cases only an ascending aorta replacement was performed as there was no aortic root pathology that needed surgical correction. All biopsy specimen were taken from the ascending aortic wall adjoining the surgical transverse aortotomy site. In case of post-mortem or transplantation material the tissue was taken at identical sites. The results of the Forte paper (1) and our publication (2) can in part be appreciated as complementary. Some differences need an additional explanation from our side as provided below. The described results in expression of alpha-smooth muscle actin in the Forte paper (1) and our study do indeed not correlate. This is most probably due to a difference of technique in which mRNA expression based on RT-PCR analysis of the vascular wall (1) was compared to our immunohistochemistry of the three vessel wall layers (2). In case of increased mRNA detection a lowered translation into protein cannot be excluded. Dr Della Corte is correct that almost absent smoothelin expression cannot be detected by immunohistochemistry, so we agree with their results that smoothelin expression is lowered, which correlates with an immature smooth muscle cell (SMC) phenotype (3, 4). With regard to the cellular composition of the media and intima of the aortic wall we do not postulate a few resident fibroblasts to

Characteristic	TA	TAD	BA	BAD
	N=11	N=12	N=17	N=19
Specimen obtained from	Post mortem, LUMC	During elective repair of the ascending aorta, LUMC	During stentless root replacement, collected when waste material became available from the proximal anastomosis from the LUMC and six biopsies from the EMC.	During elective repair of the ascending aorta, LUMC
Age (years)	64.5 ± 9.0	72.3 ± 11.2	55.8 ± 9.8	60.7 ± 7.8
Males (%)	54.5%	33.3%	70.1%	84.2%
Females (%)	45.5%	66.7%	29.4%	15.8%
Ascending aorta diameter (mean)	*	55.0 ± 10.7	36.5 ± 7.4**	52.7 ± 6.2
Commissure position			Unicuspid N=1 RCC/LCC*** N=8 RCC/NCC*** N=4 LCC/NCC*** N=1 Unknown N=3	RCC/LCC*** N=15 Unknown N=4

TA: non-dilated tricuspid aortic valve, TAD: dilated tricuspid aortic valve, BA: non-dilated bicuspid aortic valve, BAD: dilated bicuspid aortic valve, LUMC: Leiden University Medical Center, EMC: Erasmus Medical Center
* data unavailable, clinically defined as non-dilated by pathologist. ** data unavailable for 5 patients, clinically defined as non-dilated by pathologist. *** RCC/LCC: fusion of the right and left coronary cusp; RCC/NCC: fusion of the right and non coronary cusp, LCC/NCC: fusion of the left and non coronary cusp

be present that, under experimental development of thoracic aortic dilation, replace the disappearing SMC population by myofibroblasts as indicated by Jones et al (5) and adopted by the Della Corte group (1). In our studies it suffices to have a mature contractile SMC phenotype that can switch to a synthetic (immature) phenotype that expresses most of the markers also attributed to the myofibroblast phenotype, including the fibronectin splice variants (6). Our assumption is supported by less apoptosis (caspase-3) in the wall of the non-dilated as well as the dilated BAV (data unpublished). Apoptosis was however detected (not shown) in the media of the dilated TAV

and was in our study (2) linked to the increased expression of progerin. On this aspect we will have further comments when discussing the aspect of ageing. As described above, our study population was primarily not selected on the valve pathology or on the difference in architecture of the convex versus concave site. The fact that our group consisted of patients with a non- and dilated aortic wall, variable aortic valve pathology and commissure position, underlines that the observed differences between all BAVs and TAVS are intrinsic to the BAV wall morphology. This is further supported in a recently accepted paper (7) in which we did address the differences between the convex and concave aortic sites of patients with a dilated aortic wall, provided by our German co-authors that have adopted the selection technique as described before (8). We did not disclaim the earlier published differences (8, 9) in the structure of the wall and the expression of markers between the convex and concave site of the aorta. We performed, however, an additional analysis on these specimen with Transforming-Growth-Factorβ and endothelial nitric oxide and found no difference in expression between the convex and concave sites (7). Haemodynamic influences are therefore not excluded from having an important influence but according to our studies cannot be considered as the primary source for the observed differences in dilation formation between BAV and TAV.

On basis of the above observations and additional arguments provided below we cannot endorse the concluding remarks of Dr Della Corte: 'Functionally, one of the early signs of arterial ageing is impaired wall distensibility, which is known to occur in the BAV even before overt dilation. Thus, with the currently available data, an hypothesis of defective wall maturation is hardly sustainable, without ruling out the contribution of flow induced remodeling: a conceptually opposite theory of premature ageing of the aortic wall, prompted by altered biomechanical environment could be drawn as well'. In this respect the definition and detection of ageing of the vascular wall is an issue. Loss of aortic wall distensibility, based on the increase of collagen and loss of elastin is adapted by some as a measure of vascular wall ageing (5) and used not only for the TAV models but also for BAV (1). We have not specifically studied the collagen content but confirm the diminished elastin structure in BAV (2). Additionally we have introduced a new set of ageing markers being the balance between lamin A/C and progerin (2, 10). Even physiologic ageing with increasing age has been correlated with the increased expression of progerin (10-12). We are excited about the fact that in this way we could differentiate

between TAV and BAV, showing increased physiologic ageing in TAV based on an increase of progerin and accompanying apoptosis resulting in cytolytic necrosis, while this was lacking in BAV. The decrease of distensibility as described (1, 5) might still hold for the aortopathy in both BAV and TAV but should in our opinion not be translated as an indication of premature ageing in BAV. In our study we had the relatively unique opportunity to show that the structural immaturity was present in both non- and dilated BAVs independent of the degree and type of underlying aortic valve pathology.

In conclusion we demonstrated that a different mechanism underlies aortic dilation in BAV and TAV patients. We postulate that secondarily haemodynamic influences, additionally triggered by the specific BAV aortic valve pathology, might have a different impact on the immature BAV wall as compared to the ageing TAV aortic wall.

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CHAPTER

4

Is morphology and extent of the raphe associated with clinical outcome in patients with bicuspid aortic valves?

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Submitted

4

ABSTRACT

Background: The clinical course of bicuspid aortic valve (BAV) disease is variable. Data on predictors of aortopathy and valve dysfunction are limited. This study sought to define a risk profile for BAV patients based on clinical patient characteristics.

Methods: Valve morphology and aortic dimensions of 255 BAV patients (179 men
(70.2%), 48±15 years) were evaluated retrospectively from the center's echocardiography database. Patient characteristics, clinical course and echocardiographic parameters including morphology were obtained.

Results: Type 1A BAV patients (n = 151, fusion right and left coronary cusps) showed significantly larger sinuses than type 2A BAV patients (n = 37, fusion right coronary and non-coronary cusps) (p= 0.001). Regurgitation was most apparent in type 1A BAVs with a complete raphe (p=0.017). All regurgitant valves showed larger sinuses (p=0.023) and patients with valve regurgitation had a larger LV mass (p<0.001). Male gender was associated with aortic valve regurgitation (p<0.001), larger aortic diameters and a larger LV mass (p=0.01). Patients with a history of aortic coarctation showed less valve regurgitation (p=0.048) and smaller diameters of the ascending aorta and arch (p<0.001). Patients with hypertension had faster dilation of the ascending aorta (p=0.049), statin use was associated with a smaller diameter of the ascending aorta (p=0.017).

Conclusions: This study provides a working model showing that males with a type 1A BAV with a complete raphe, hypertension and no statin use exhibit the highest risk of complications such as progressive aortic and valvular dysfunction necessitating intervention.

INTRODUCTION

Bicuspid aortic valve (BAV) is the most common congenital cardiac malformation, with a clear male predominance and an estimated prevalence of 0.5-1% (1-3). BAV can occur in an isolated form or in association with other congenital malformations, such as coarctation of the aorta (CoA). The prevalence of BAV in CoA patients is reported to be as high as 40-50% (4).
Although some patients with isolated BAV remain asymptomatic throughout their lifetime, others develop severe cardiac complications from an early age onwards, such as aortic valve stenosis, aortic insufficiency and/or endocarditis. However, the first presentation can also be a clinically relevant aortic wall abnormality, including ascending aortic dilation (reported to occur in 45% of patients (5)), aneurysm formation or rupture of the ascending aorta. Identifying patients who are prone to develop complications is a major, clinically relevant, challenge.
It is now recognized that BAV should not be considered as one single entity, but that distinct morphological phenotypes are distinguishable based on the presence and number of raphe, as classified by Sievers et al. (6) Most BAVs consist of one free cusp and two cusps that are conjoined (or have failed to separate during embryonic development). The term 'raphe' defines the conjoined area of the two underdeveloped cusps turning into a malformed commissure between both cusps (6).
There is discussion in the literature about the relationship between BAV morphology and long term clinical outcome. Several studies reported that BAVs with fusion of the right coronary cusp and left coronary cusp (Type 1A in this study) are associated with more aortic dilation in adults, whereas BAVs with fusion of the right and non-coronary cusp (Type 2A in this study) are responsible for valve dysfunction at a younger age (7-12). The third phenotype resulting from fusion between the left and non-coronary cusp (Type 3A in this study) is rare compared to the other two types (8). Some studies reported that type 1A BAVs are more stenotic than type 2A and 3A, whereas other studies described that the latter types are more stenotic and type 1 BAVs are more regurgitant (13-15). When no raphe is present, the valve is called strictly bicuspid (subtype B in our study). The raphe position has been linked to the area of dilation, type 2A BAVs are for instance responsible for dilation of the aortic sinus, whereas type 1A BAVs are related to dilation of the ascending aorta (16,17). In contrast, other studies reported

valve regurgitation as the main cause of sinus dilation, whereas stenosis causes a much milder ascending aortic dilation and valve morphology is proposed to be of little influence (18-21). The extent of the raphe (i.e. the extent to which the cusps are conjoined) however, has not been taken into account in the mentioned studies.
The purpose of this study was to determine whether valve morphology is associated with the degree of aortopathy and valvular dysfunction. By also taking the extent of the raphe and individual patient characteristics into account, a tailored risk stratification was developed.

METHODS

Study population

Consecutive patients who had undergone transthoracic echo-cardiography between 2005 and 2010 and had been diagnosed with BAV disease were identified retrospectively from the Leiden University Medical Center (LUMC) echocardiography database. Clinical and echocardiographic data were collected and analyzed from the departmental Cardiology Information System (EPD-Vision®, LUMC) and the echocardiography database respectively. For this analysis of clinically acquired data, the Institutional Review Board waived the need of patient written informed consent. A total of 255 patients was selected, all had situs solitus of the atria and concordant AV and VA connections. Other patient characteristics recorded included date of birth, height, weight, body surface area (calculated using the Du Bois formula (22)), other congenital malformations, genetic syndromes, history of arrhythmias, ischemic procedures, cardiovascular risk factors, use of medication, known valve dysfunction, history of valve surgeries and age at time of surgery (Table 1).

Classification of valve morphology

As from a developmental point of view a morphological spectrum may exist in which the extent of the raphe can be regarded as a continuum, in the current study we chose to describe the major valve morphologies observed in BAV patients as 3 different types (Type 1A, 2A and 3A, based on valve cusp orientation), in which the extent of the raphe can vary (Fig. 1). In this study, fusion of the right and left coronary cusp is defined as type 1A BAV, fusion

Table 1 *Patient Characteristics*

Variable	***n (%)***
Gender	
- Male	179 (70.2)
- Female	76 (29.8)
Bicuspid aortic valve	
- Type 1A	151 (59.2)
- Type 2A	37 (14.5)
- Type 3A	1 (0.4)
- Strictly bicuspid (type B)	66 (25.9)
o Type 1B	42 (63.6)
o Type 2B	24 (36.4)
Extent of the raphe	
- Complete	111 (43.5)
- Incomplete	78 (30.6)
Valve surgery	66 (25.9)
- Mitral valve replacement	1 (0.39)
- Pulmonary valve stenosis	1 (0.39)
- Pulmonary valve regurgitation	1 (0.39)
Arrhythmias	64 (25.1)
- Atrial fibrillation	39 (15.3)
- Supraventricular tachycardia	19 (7.5)
- Ventricular tachycardia	14 (5.5)
- Pacemaker	6 (2.4)
- Implantable cardioverter defibrillator	15 (5.9)
o Secondary prevention	10 (66.7)
o Primary prevention	5 (33.3)
Medication	140 (54.9)
- Angiotensin converting enzyme inhibitors	91 (35.7)
- Statins	61 (23.9)
- Carbasalate calcium	45 (17.6)
- Oral anticoagulants	43 (16.9)
- Antiarrhythmic drugs	33 (12.9)
Risk Factors	117 (45.9)
- Hypertension	65 (25.5)
- Mitral valve repair	2 (0.78)
- Aortic valve replacement	50 (19.6)
- Aortic valve repair	10 (3.9)
- Bentall	22 (8.6)
- Tricuspid valve repair	1 (0.39)
- Pulmonary valve replacement	1 (0.39)
Congenital defects	51 (20.0)
- Aortic coarctation	39 (15.3)
- Atrial septal defect	1 (0.39)
- Ventricular septal defect	11 (4.3)
- Marfan	2 (0.78)
- Common truncus	1 (0.39)
- Patent ductus arteriosus	5 (2.0)
Valvular dysfunction	189 (74.1)
- Aortic valve stenosis	120 (47.1)
- Aortic valve regurgitation	120 (47.1)
- Mitral valve regurgitation	16 (6.3)
- Tricuspid valve regurgitation	8 (3.1)
Smoking	54 (21.2)
Diabetes	11 (4.3)
Cerebrovascular accident	14 (5.5)
Hypercholesterolemia	33 (12.9)
Peripheral arterial disease	7 (2.7)

of the right and non-coronary cusp as type 2A BAV and fusion of the left and non-coronary cusp as type 3A BAV. As at this time there is no consensus on whether or not a strictly bicuspid valve (i.e. a valve without a raphe) should truly be regarded as a separate group, strictly bicuspid valves were defined as a subgroup B to facilitate analysis of data (Fig. 1).

Echocardiography

Transthoracic echocardiography was performed using a GE Vivid7 or E9 (GE-Vingmed, Horten, Norway) ultrasound machine with standard views from the parasternal, subcostal, suprasternal and apical windows. The aortic valve was examined in the two dimensional parasternal short-axis view and classified as bicuspid when two cusps could be clearly identified and the

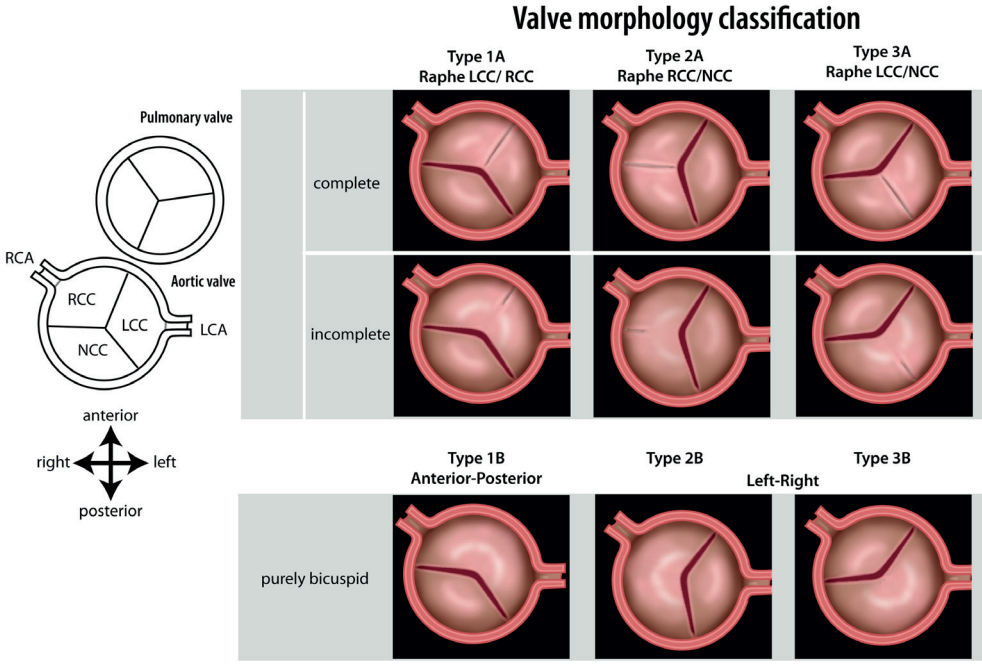

Figure 1. *Schematic overview of BAV morphologies.*
Drawings are oriented in echocardiographic, i.e. inferior, view of the aortic valve. Upper panel: The three major valve morphologies observed in BAV patients are described as three different types based on the valve cusp orientation: type 1,2 and 3. The extent of the raphe is indicated as analyzed in this study (<0.5, >0.5, complete). Lower panel: Strictly bicuspid valves (without a raphe) are defined as a subgroup B. BAV: bicuspid aortic valve

typical 'fish-mouth opening' of the valve was observed (Fig. 2). Diameters of the aortic root and ascending aorta were determined in the parasternal long axis view.

Echocardiographic analysis

All echocardiographs were evaluated by one experienced observer, using GE Medical Systems's EchoPac, Version 7 (110.0.0, GE-Vingmed, Horten, Norway). The aortic valve was evaluated in a cross-sectional view for the presence and extent of a raphe. For valves where a raphe could be distinguished (subgroup A), distinction was made between a complete raphe and an incomplete raphe. Cases where no raphe was detected (subgroup B) were defined as strictly bicuspid valves. Diameters of aortic sinus, ascending aorta and aortic arch were measured from leading edge to leading edge in end-diastole according to the European Association of Echocardiography recommendations (23). Aortic annular diameter was measured from inner edge to inner edge during systole. All measurements were made in mm, rounded to 2 significant figures. The ascending aorta was considered dilated at a diameter of >38mm. Subgroup analysis was performed in: 1) patients with pronounced dilation of the aortic wall, defined as an ascending aortic diameter >44 mm; 2) patients with a history of CoA; 3) patients who had undergone ascending aortic or aortic valve surgery. If a patient had undergone surgery of either the aortic valve or the ascending aorta, the last echocardiographic study before surgery was used to measure aortic diameters and to evaluate valve morphology.

Of 199/255 patients serial echocardiographic studies at least 6 months apart were available. Both the first and most recent studies were selected for quantitative evaluation of the dynamics of aortic dilation over time. To evaluate left ventricular hypertrophy, end-diastolic left ventricular mass was calculated by measuring the end diastolic outer and inner left ventricular wall thickness in the M-mode view using the Devereux formula (24).

Statistical Analysis

All collected data were registered in a Microsoft Office Access 2003 database. The database was exported into IBM SPSS Statistics Version 20 for computing variables and statistical analysis. Independent samples T-tests were used to compare means of numerical data in 2 categories. One-way ANOVA tests were used for comparing numerical data in more than 2 categories. Cross-

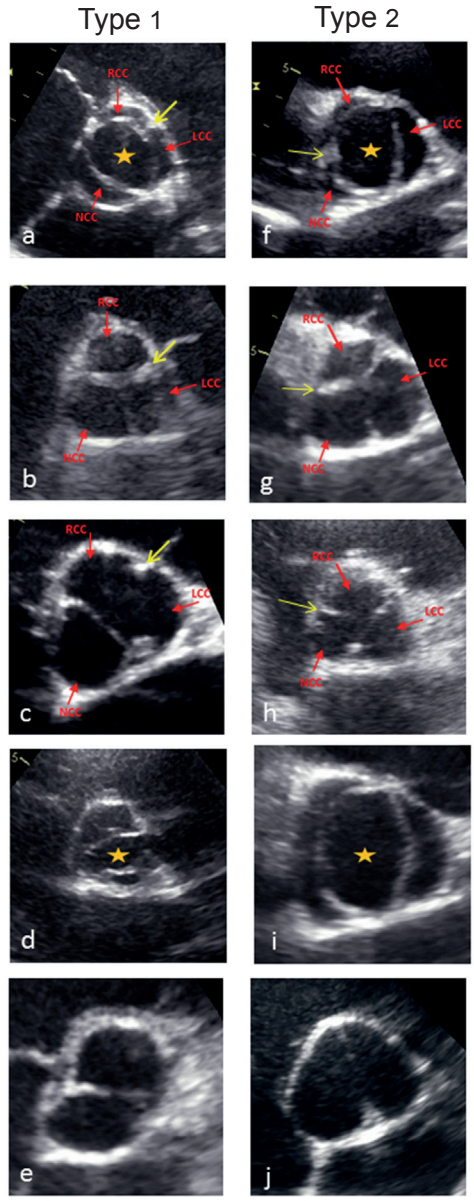

Figure 2. *Representative examples of different BAV types as seen from the short-axis parasternal view on echocardiography.*
The yellow arrow indicates the raphe. The orange star indicates the typical fish mouth opening of the valve. a: Type 1A in open position. b: Type 1A with complete raphe in closed position. c: Type 1A with incomplete raphe in closed position. d: Type 1B in open position. e: Type 1B in closed position. f: Type 2A in open position. g: Type 2A with complete raphe in closed position. h: Type 2A with incomplete raphe in closed position. i: Type 2B in open position. j: Type 2B in closed position.BAV: bicuspid aortic valve

tabulations were made for binary categorical data, on which chi-square goodness-of-fit-tests were performed to test for independence. For sets of independent numerical data linear regression analysis was used to evaluate trends. Similarly, trends for binary categories were evaluated with binary logistic regression to correct for possible confounding factors such as age and gender. To evaluate surgery-free survival for different patient groups, Kaplan-Meier survival curves were made whereby the age of a patient at the time of a surgical intervention of the aortic valve or ascending aorta was considered an uncensored 'event' and the age of an un-operated patient at the end of the study was considered censored data. All statistical analyses were two-tailed and considered significant if $p<0.05$.

RESULTS

Patient characteristics

A total of 255 patients with BAV (age 18-85 years, mean 48±15 years) were identified and analyzed. Of these, 179 were male (70.2%) and 76 female (29.8%). Patient characteristics and echocardiographic data are summarized in Table 1. The distribution of valve morphology is shown in figure 3.

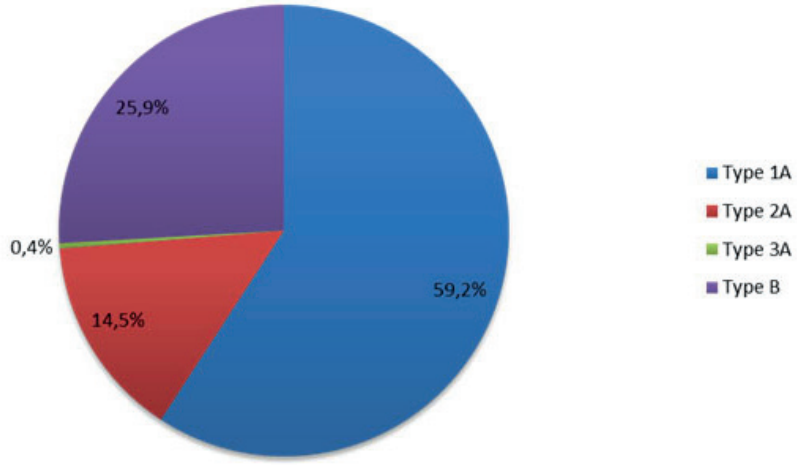

Figure 3. *Schematic overview of the distribution of valve morphologies in the study population.*

BAV morphology as related to valve function

120 patients were diagnosed with aortic stenosis, the same number of patients showed aortic regurgitation (Table 1). There were no significant differences in valve function between the different morphological subtypes. However, BAVs with a complete raphe had a significantly higher prevalence of valve dysfunction than BAVs with an incomplete raphe (82.9% vs. 66.7%, p=0.01, Table 2). When specifying this for type of valve dysfunction, this difference remained significant in patients with aortic regurgitation (56.8% vs. 42.3%, p= 0.05), whereas in patients with aortic stenosis there was no difference between patients with a complete or incomplete raphe (49.5% vs 43.6%, p=0.419, table 2). In type 1A BAVs with a complete raphe the difference in prevalence of aortic regurgitation was even more outspoken compared to the rest of the study population (57.6% vs. 41.8% p=0.017). Aortic valve regurgitation was a greater predisposing factor of valve surgery than aortic stenosis, as shown by the Kaplan-Meijer survival curves (Fig; 4a, b).

BAV function and left ventricular mass

In patients with valve regurgitation, left ventricular mass was larger compared to those without regurgitation (128g/m2 vs. 103g/m2, p<0.001). Both aortic valve regurgitation and a larger left ventricular mass were associated with male gender (53.6% in males vs. 31.6% in females, p= 0.001; 122.2 in males vs. 94.2 g/m2 in females, p<0.001 respectively). This remained significant even after correcting for body surface area (p=0.01). Surprisingly, aortic stenosis was not associated with either left ventricular mass, nor with gender.

BAV morphology as related to aortic diameters

All type 1A BAVs, compared to type 2A BAVs, showed a significantly larger

Table 2: *Valve morphology as related to dysfunction*

Valve dysfunction	**Complete raphe**	**Incomplete raphe**	***P* value**
Valve dysfunction (*n* = 144)	92 (82.9%)	52 (66.7%)	0.01
Aortic regurgitation (*n* = 96)	63 (56.8%)	33 (42.3%)	0.05
Aortic stenosis (*n* = 89)	55 (49.5%)	34 (43.6%)	Not significant

sinus (37.29 vs. 33.89mm, p=0.001, table 3a). Valves with a complete raphe had significantly larger aortic diameters at the level of the ascending aorta, as compared to valves with an incomplete raphe (p=0.041). At the level of the aortic sinus and aortic arch this difference was not significant (Table 3b). Type 1A BAVs and a complete raphe also showed a significant difference in sinus diameter (37.74 vs. 36.01 p=0.031) compared to the rest of the study population. Males had significantly larger absolute aortic diameters compared to females at all levels, but these gender differences became non-significant when corrected for body surface area.
Regurgitant valves showed a significantly larger sinus (37.5mm vs. 35.8mm, p= 0.023) compared to non-regurgitant BAVs. Stenotic valves on the other hand had a significantly smaller sinus (38.1mm vs. 39.9mm p<0.001) and annulus (20.93mm vs. 22.96mm, p<0.01) compared to the rest of the study population, but a larger aortic arch (30.2mm vs. 28.7mm, p=0.036).
When corrected for body surface area, age and gender, statin intake was associated with a significantly smaller diameter of the ascending aorta (p= 0.017) and with a significantly longer surgery free survival compared to no statin use, apparent on the Kaplan-Meijer survival curve for intervention-free lifespan and statin use (Fig. 4c). The latter finding corresponded with the mean age at the time of operation, BAV patients taking statins were almost 14 years older at the time of an intervention as compared to those who do not (56.0 vs. 42.2 years old, p<0.001) (Fig. 4c).

Table 3 *Valve morphology as related to aortic diameter*
a: Type 1A versus type 2A

Aortic diameter	Type 1A	Type 2A	P value
Sinus (mm)	37.29 ± 5.88	33.89 ± 4.84	0.001
Ascendens (mm)	37.34 ± 6.75	36.33 ± 7.03	Not significant
Arch (mm)	29.30 ± 5.45	30.94 ± 4.70	Not significant

b: In all types BAV with complete raphe versus all types BAV with incomplete raphe

Aortic diameter	Complete raphe	Incomplete raphe	P value
Ascendens (mm)	38.02 ± 6.53	35.91 ± 7.00	0.041
Sinus (mm)	36.91 ± 5.82	36.13 ± 5.81	Not significant
Arch (mm)	30.07 ± 5.16	29.21 ± 5.66	Not significant

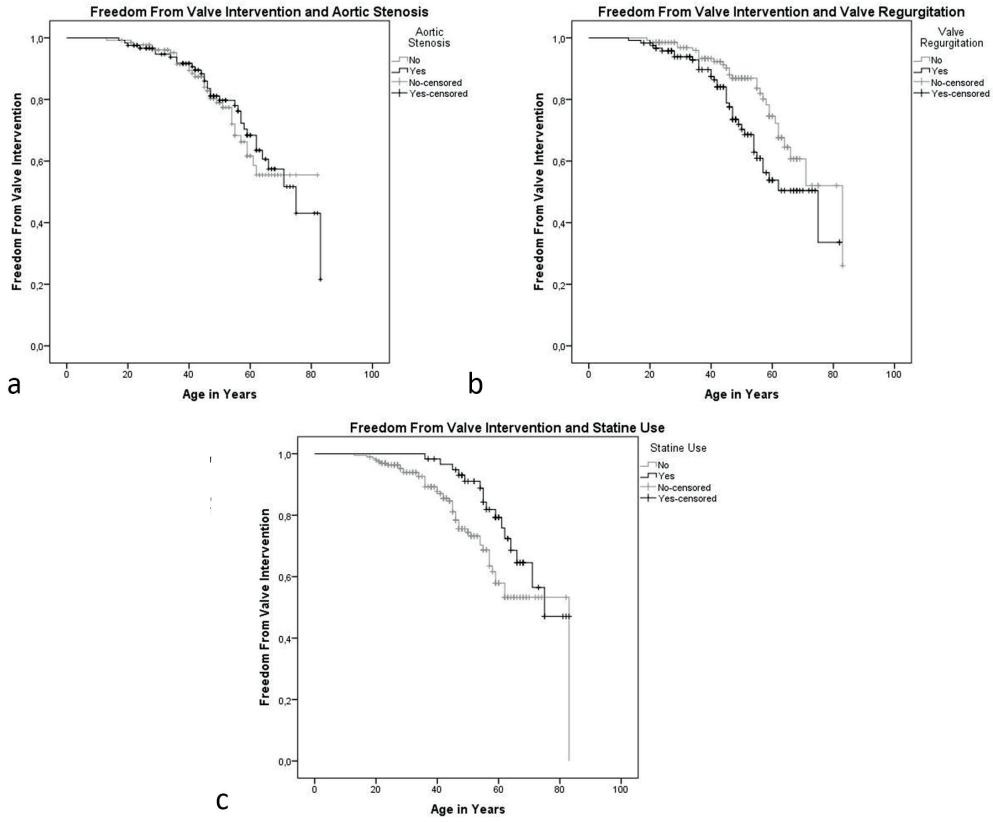

Figure 4. *Kaplan-Meijer curves of freedom from intervention with age in years* amongst a) patients with aortic stenosis versus patients without; b) patients with valve regurgitation versus patients without; c) patients with statin use versus patients without.

Aortic dimensions over time

The interval between the evaluated echoes ranged from 1 to 17 years with an average of 5.6 (SD 4.1) years follow-up. Dilation was most pronounced at the ascending aorta followed by the sinus, arch and annulus.

Increase in diameter of the ascending aorta, occurred more often in males than in females (46.9% vs. 28.9%, p= 0.008). Evaluation of the subgroup with dilation of the ascending aorta >44 mm, showed that these patients were on average 10 years older than patients with smaller degrees of dilation, indicating a progression of dilation with age. These patients also had more rapid aortic dilation per year at all locations, except for the aortic arch.

Patients with isolated aortic dilation received valve/aortic surgery 10 years later in life than patients with isolated valve disease who underwent surgery

(p<0.05), probably reflecting the fact that the latter group became symptomatic earlier in life. When studying the progression of dilation of the ascending aorta over time, hypertension was found as a predisposing factor; 14.6% of patients with hypertension showed dilation of more than 1.5 mm/year, compared to only 5.7% of patients without hypertension (p= 0.049 when corrected for age and gender).

BAV and Aortic coarctation

Patients with aortic coarctation constituted the largest group of patients with associated congenital cardiac malformations (n=39). Other congenital malformations included atrial or ventricular septal defect, and patent ductus arteriosus (numbers too small for sufficient power for analysis). Patients with a history of aortic coarctation were 9 years younger than those without (40.7 vs. 49.3 years old, p<0.001) and there was no statistical difference in gender (p=0.47).

Distribution of BAV morphology in patients with aortic coarctation was identical to the rest of the study population, the majority having a type 1A BAV (56.4% in patients with versus 59.7% in patients without aortic coarctation, p=0.321). However, in the majority (72.7%) of aortic coarctation patients and type 1A BAV an incomplete raphe was seen as compared to only 38.8% in the population without aortic coarctation. A complete raphe was found in only 27.3% of aortic coarctation patients, whereas 61.2% of the patients with type 1A BAV without aortic coarctation, had a complete raphe (Table 4).

Patients with aortic coarctation and type 1A BAV had significantly less valve regurgitation (13.6% vs. 55.8%, p<0.001) and significantly smaller diameters of the ascending aorta (33.7mm vs. 37.8mm, p<0.001) and aortic arch (25.8mm vs. 30.2mm, p<0.001) than patients with isolated BAV. Figure 5 shows a working model for the risk stratification of BAV patients, based on findings of this study.

DISCUSSION

Key findings of this study are 1) Patients with type 1A BAV and a complete raphe show more aortic regurgitation and root dilatation as compared to the rest of the study population 2) Patients with type 1A BAV have larger aortic sinuses than patients with type 2A BAV 3) aortic coarctation patients have

Table 4: *Characteristics of patients with type 1A BAVs with aortic coarctation versus without aortic coarctation*

Variable	Aortic coarctation	No aortic coarctation	P value
Type 1A BAV, n (%)	22 (56.4%)	129 (59.7%)	Not significant
Raphe			0.003
- Incomplete	16 (72.7%)	50 (38.8%)	
- Complete	6 (27.3%)	79 (61.2%)	
Ascendens (mm)	33.3 ± 6.06	37.8 ± 6.78	<0.001
Arch (mm)	25.5 ± 4.77	30.2 ± 4.98	<0.001
Valve regurgitation	3 (13.6%)	72 (55.8%)	< 0.001
Valve stenosis	10 (45.5%)	56 (43.4%	Not significant

smaller aortic root diameters and less valve regurgitation 4) Males have a higher prevalence of aortic regurgitation, larger aortic diameters 5) Use of statins is correlated with smaller aortic diameters and a delay in valve intervention.

Valve morphology and extent of the raphe as predictors of outcome.

The most important findings were related to the extent of the raphe. A complete raphe predisposed for larger aortic diameters and more valve regurgitation. However, the extent of the raphe could not explain the gender differences described above as the prevalence of complete raphe was the same in males and females. To our knowledge, the extent of a raphe in BAV disease has not been studied previously as a prognostic factor. The worse outcome observed in patients with a complete raphe is possibly due to the fact that BAVs with incomplete raphe have a more physiological, tricuspid-like opening and therefore function better. BAVs with complete raphe seem to have more uneven sized cusps and smaller openings which may predispose to valve dysfunction. With regard to the orientation of the raphe, type 2A BAVs showed a trend to more aortic valve stenosis and dilation of the ascending aorta over time than type 1A BAVs. The same is observed in patients with type B BAVs (no raphe), when comparing type 2B to type 1B, with about the same level of significance. This is supported by developmental findings of Fernandez et al, who state that the etiology underlying various BAV orientations is different (25). In this study eNOS knock-out mice developed only type 2 BAVs with abnormal development of cardiac valve cushions, suggesting that

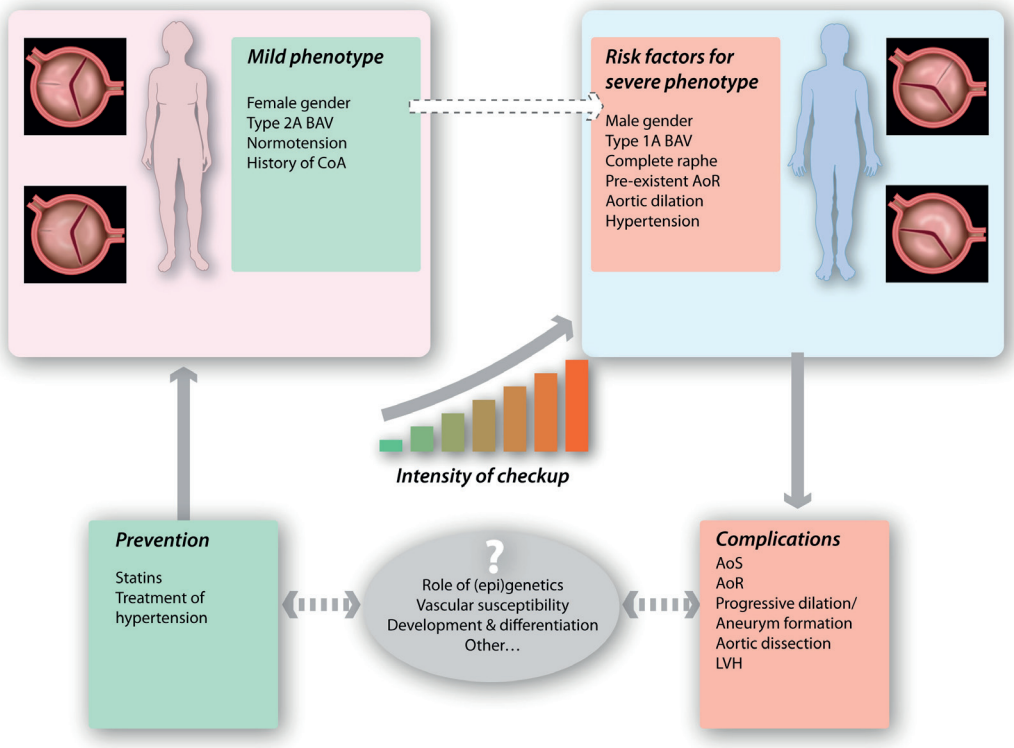

Figure 5. *Working model for risk stratification of BAV patients*. BAV: bicuspid aortic valve

the type 2 BAV may be a product of a developmental defect related to an exacerbated NO dependent epithelial-to-mesenchymal transformation. The Syrian hamster on the other hand develops only type 1 BAV, which seemed to be the product of an abnormal embryonic outflow tract septation and may therefore be related to alterations in neural crest cell behaviour (25).

Type 1A BAVs had a significantly larger sinus and these patients underwent 10% more operations as compared to type 2A BAVs. These data are in line with previous studies (6,7,10). Patients with type 1A BAVs and a complete raphe showed significantly more regurgitation and root dilation as compared to the rest of the study population. As freedom from valve and aortic intervention is an important criterion for a better prognosis in BAV disease, type 1A BAVs can be regarded as the valve orientation with the highest risk, which is in line with the above mentioned studies. This indicates that type 1A BAVs with a complete raphe are more susceptible for aortic dilation and should be monitored more closely for valve regurgitation and aortopathy.

Aortic valve regurgitation versus stenosis as predictors of outcome

Aortic valve regurgitation was a stronger predictor for adverse outcome than stenosis. Patients with regurgitant BAVs had larger sinus diameters, showed more left ventricular hypertrophy and underwent surgery earlier in life than patients without aortic regurgitation. In contrast, stenosis has little effect on the age at time of operation (Fig. 4). This corresponds to the data reported by Cotrufo et al. and by Della Corte et al., who found BAV regurgitation responsible for root dilation, whereas stenotic BAVs had an unaffected root (18,19). Hence, the type of valve dysfunction is an important tool for the clinician when monitoring BAV patients.

Exponential dilation and aneurysm formation of the ascending aorta

Dilation was most apparent in the ascending aorta, with a progressive growth per year seen with increasing diameters. This stresses the indication for careful follow up of patients with larger diameters, as they may show more rapid progression in the course of their disease. The age at the time of operation of patients with dilation of >44mm however, was 10 years older than other BAV patients. This may be explained by the fact that patients with isolated dilation can remain asymptomatic for many years, whereas patients with valve disease develop symptoms and are referred for a surgical intervention. Concomitant aortic surgery can be performed then, also in cases with a non-critical aortic diameter.

BAV patients with hypertension showed a higher incidence of rapid dilation of the ascending aorta than those without. This again stresses the detrimental effects of strain on the aortic wall caused by high blood pressure, predisposing for dilation and the necessity for careful regulation of blood pressure (26).

The use of statins was correlated with smaller aortic diameters and a delay in valve interventions. Based on these results the preventive use of statins could be considered in high risk patients with BAV. Statins are already known for limiting the progression of stenosis in BAV disease (27) as well as slowing aortic dilation in patients with BAV and stenosis (28). Further research is warranted to determine whether statins may also be used as preventive measure in patients with BAV without valve stenosis.

BAV and aortic coarctation

Subgroup analysis of the aortic coarctation group revealed that these patients are on average 9 years younger than the rest of the study population, which

may be explained by the fact that these patients usually show symptoms earlier and are often referred from the pediatric cardiologist as soon as they reach adulthood. The prevalence of BAV in aortic coarctation patients is an estimated 40-50%(4). The majority of patients in the current study had type 1A BAV, which corresponds to reports in literature (29). Aortic coarctation patients had smaller aortic root diameters and less valve regurgitation, which may be explained by the fact that less aortic coarctation patients had a BAV with complete raphe. The prevalence of stenosis was similar in the aortic coarctation group compared to the rest of the study population. This in contrast to earlier research which found an association between aortic coarctation and valve dysfunction (29).

Male gender and prognosis

Male patients with BAV had a higher prevalence of valve dysfunction, especially regurgitation. They also had larger absolute aortic diameters and more often increase in aortic diameter. More left ventricular hypertrophy was observed in male BAV patients as compared to females. These data indicate that male gender is a risk factor for a worse prognosis in patients with BAV, which is in line with previous studies (17,24). The larger degree of regurgitation may be a direct result of the larger aortic dimensions observed in male patients, although an opposite effect (regurgitation leading to dilation of the sinus and ascending aorta) may also be the case. The hypertrophy could be a result of chronic volume overload caused by aortic regurgitation, as reported previously (30). Why this is more pronounced in male patients, is not clear. Nistri and colleagues (31) have attributed aortic dilation, which was predominantly observed in young males, to a congenital weakness of the aorta, independent of valve dysfunction, age or body size. On the other hand, Della Corte and colleagues state that aortic dilation in young males should be attributed to varying degrees of regurgitation of the aortic valve (18). The current study indicates that this gender difference is not only seen in ‘young’ males, but remains significant at any age, indicating that males with BAV should be monitored more frequently than females throughout their lifetime.

STUDY LIMITATIONS

This was a retrospective analysis of clinically obtained patient data derived from a single center. A retrospective analysis is subjected to selection bias, as the investigator self-selects the cases. This bias was minimalized by including consecutive BAV patients who underwent an echocardiogram between 2005 and 2010.

CONCLUSIONS AND CLINICAL IMPLICATIONS

With regard to valve morphology in BAV patients, this study shows that the extent of the raphe is of clinical importance, as a complete raphe predisposes for more valve dysfunction and aortopathy.
This study provides a working model showing that males with a type 1A BAV with a complete raphe, hypertension and no statin use exhibit the highest risk of complications such as progressive aortic and valvular dysfunction necessitating intervention (Fig. 5).

ACKNOWLEDGEMENTS

We thank Ron Slagter for drawing Fig. 1 and Fig. 5 of this manuscript, Bert Wisse for designing the database and Ron Wolterbeek for reviewing the statistics.

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CHAPTER

5

Bicuspid aortic valve: phosphorylation of c-Kit and downstream targets are prognostic for future aortopathy

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Modified after: Eur J Cardiothorac Surg. 2014 Nov;46(5):831-9.

ABSTRACT

Objectives: The clinical course of many patients with a bicuspid aortic valve (BAV) is complicated by ascending aortic dilatation. Currently, the indication for aortic surgery is solely based on the aortic diameter and subsequently only a small proportion of BAV patients undergoing valve surgery require concomitant ascending aortic replacement based on these recommendations. Unfortunately, a substantial number of BAV patients still develop aortic dilatation in the future and would potentially benefit from a more aggressive approach towards ascending aortic replacement. We, therefore, designed this study to identify molecular biological markers in the aortic wall predictive of aortopathy in BAV.

Methods: Ascending aortic wall specimen of BAV (*n* = 36) and tricuspid aortic valve (TAV) (*n* = 23), both without and with (>44 mm) dilatation were investigated histologically and immunohistochemically for the expression of markers for vascular remodeling (transforming growth factor (TGF)-β, phosphorylated Smad2, matrix metalloproteinase 9 (MMP9)), cellular differentiation (c-Kit, phosphorylated-c-Kit, hypoxia-inducable factor-1 alpha (HIF1α)) and haemodynamic influences on the aortic wall (endothelial nitric oxide (eNOS)).

Results: All BAV patients showed significantly less inflammation ($P < 0.001$) and an altered intima/media ratio when compared with TAV patients. The expression of markers of a signalling pathway characteristic for cellular dedifferentiation, as exemplified by the marked expression of c-Kit, phosphorylated c-Kit and HIF1α; in the dilated BAV group was however completely comparable with only a subgroup of the non-dilated BAV (*BAb*), whereas the remainder of the non-dilated BAV group (*BAa*) was significantly distinct. This difference between the dilated BAV and *BAa* was further confirmed in the expression of TGF-β, phosphorylated Smad2, MMP9 and eNOS. Besides the expression pattern, similarity in the dilated BAV and *BAb* was also noted clinically in the most common variant of commissure position and conjoined raphe of the BAV. Based on these observations, we consider the *BAb* group a likely candidate for future dilatation as opposed to the *BAa* group.

Conclusions: Using a panel of molecular tissue markers, the non-dilated BAV patients can be divided into groups susceptible and non-susceptible to aortopathy.

INTRODUCTION

Bicuspid aortic valve (BAV) is the most common congenital cardiac defect, with a prevalence in the general population in the range 0.5–2% (1). The clinical course of BAV is often complicated by aortic regurgitation and/or stenosis, infective endocarditis and aortic dilatation. In particular, the latter one forms a critical complication in individuals with BAV, as aortic dilatation carries an increased risk of dissection and rupture. Fifty-nine percent of patients with BAV, below the age of 30 have ascending aortic dilatation, which rises to 88% in people over 80 years old (2). Currently, guidelines recommend ascending aortic replacement in patients with BAV and an ascending aortic diameter ≥50 mm, and concomitant aortic surgery if the diameter exceeds 45 mm (3). Unfortunately, aortic diameter alone as a selection criterion is not sufficient for identifying patients with inherent aortic weakness before a life-threatening complication occurs (4, 5). On the one hand, patients with a non-dilated aortic wall at the time of aortic valve surgery may still develop aortic dilatation in the future, while, on the other hand, some patients will never experience aortic wall dilatation. A preventive ascending aortic replacement would expose this last group of patients, not prone to develop dilatation of the aorta, unnecessarily to the risk of this procedure and ensuing postoperative complications. Therefore, there is an unmet need to identify genetic and/or molecular markers to improve a patient-tailored risk stratification for BAV individuals, applicable prior to or during surgery. Although histological and biochemical differences have been shown between dilated aortic walls of BAV and tricuspid aortic valve (TAV) patients, little is known about the difference in non-dilated aortas. We have previously shown marked histological differences and maturation defects in non-dilated aortas of BAV patients (6). We were, however, not yet able to demonstrate differences within this group, as would be expected based on the fact that some patients with BAV never develop aortic dilatation. The aim of this study was therefore to identify patients with BAV and a non-dilated aorta who are susceptible for future aortopathy. To identify these patients, we compared dilated and non-dilated aortas of both BAV and TAV patients as controls with respect to biochemical markers of vascular remodeling, cellular differentiation and haemodynamic modifiers. Since developmental data (7) and recent clinical diagnostic data based on commissure position (8) suggest that the orientation of the conjoined raphe is of relevance, we also studied this aspect.

MATERIALS AND METHODS

Aortic tissue samples

Ascending aortic wall biopsies were collected from individuals with BAV and TAV, with or without dilatation. Dilatation was clinically defined by surpassing an ascending aortic wall diameter of 44 mm, based on the American College of Cardiology/American Heart Association guidelines (3). The institutional ethics committee at the Leiden University Medical Centre (LUMC) approved this study. The Heart Valve Bank, Thoraxcenter, Erasmus Medical Center, Rotterdam, provided six BAV samples without aortic dilatation as these were not suitable for transplantation, as approved by their Scientific Advisory Board. Furthermore, we received five BAV aortic specimens from both the convex and concave side of the aortic wall of patients with dilatation, from the Universitätsklinikum Schleswig-Holstein, Lübeck, Germany. Patients were divided into four groups: (a) TAV without dilatation, termed TA (n = 11, mean age: 64.5 ± 9.0 years), obtained post-mortem, (b) TAV with dilatation, termed TAD (n = 12, mean age: 72.3 ± 11.2 years), collected during elective repair, (c) BAV without dilatation, termed BA (n = 17, mean age: 55.8 ± 9.8 years), representing the six BAV samples provided by the Heart Valve Bank Rotterdam and a group of patients who underwent elective stentless root replacement - our preferred technique - while they had no ascending aortic pathology and (d) BAV with dilatation, termed BAD (n = 19, mean age: 60.7 ± 7.8 years). A small subgroup of BAD (n = 5, mean age: 52.6 ± 7.9 years, BAD2 group) were selectively studied for differences in the protein expression patterns of the aortic wall between the convex and concave site, obtained during elective repair. We excluded patients with a proven genetic disorder identified by genetic tests (e.g. Marfan's disease, familial thoracic aortic aneurysm and dissection). Characteristics of enrolled patients are presented in Table 1. All patients in BAV undergoing surgery had a stenotic valve with either mild or no regurgitation. The patients thus had comparable valve pathology. We also investigated the orientation of the conjoined cusps in BAVs: the raphe was noted either between the right to non-coronary (RCC/NCC), right to left coronary (RCC/LCC) and left to non-coronary (LCC/NCC) cusp or was defined as unicuspid, with fusion at two commissure sites. Following excision, all specimens were fixed in formalin for 24 h, decalcified in Kristensen's solution (a formic acid–formate buffer) for 120h and subsequently embedded in paraffin. Transverse sections (5 µm) were mounted on precoated Starfrost

slides (Klinipath B.V., 3057-1, Duiven, The Netherlands) comparing different stainings on consecutive sections.

Immunohistochemistry

In this article, we focused on the expression of proteins known to be involved in vascular remodeling (transforming growth factor (TGF)-β, phosphorylated Smad2 (pSmad2) and matrix metalloproteinase 9 (MMP9) and cellular differentiation (alpha smooth muscle actin (αSMA) and c-Kit (3, 9)) and possible haemodynamic influences on the convex and concave area of the dilated BAV aortic wall (endothelial nitric oxide (eNOS)). For immunohistochemical staining, sections were deparaffinated and rehydrated before antigen retrieval in citrate buffer (microwave, 92-98°C, 12 min). Inhibition of endogenous peroxidase was performed with 0.03% H2O2 in phosphate buffered saline (PBS) (20 min). Non-specific staining was reduced by blocking with PBS-Tween-20 (PBS-T) with 1% bovine serum albumin (1% BSA, Sigma-Aldrich, USA). Subsequently, the slides were incubated overnight at room temperature (20°C) with diluted primary antibodies against: eNOS 1/100 (Thermo Scientific, PA1037), MMP9 1/100 (MCA2736), panTGF-β 1/1000 (MO-C40009E), pSmad2 1/250 (Cell Signaling, 3108), fibulin-1 1/100 (Santa Cruz, sc-20818), c-Kit 1/100 (DAKO, A4502) and phosphorylated c-Kit (pc-Kit) 1/100 (Abcam ab62154). All primary antibodies were dissolved in PBS-T with 1% BSA. Control staining was performed using PBS-T and 1% BSA as the primary step. Between subsequent incubation steps, all slides were rinsed with PBS (2×) and PBS-T (1×). The slides were incubated with secondary antibodies (45 min): for eNOS, c-Kit and pc-Kit with goat anti-rabbit biotin 1/200 (Vector Laboratories, USA, BA-1000) and goat serum 1/66 (Vector Laboratories, USA, S1000) in PBS-T; for MMP9, panTGF-β and pSmad2 with horse anti-mouse biotin 1/200 (Santa Cruz Biotechnology, Inc., CA, USA, SC-9996-FITC) in horse serum 1/66 (Brunschwig Chemie, Switzerland, S-2000) in PBS-T. Subsequently, slides were incubated with ABC reagent (Vector Laboratories, USA, PK 6100) (45 min). The slides were incubated with 400 µg/ml 3–3'di-aminobenzidin tetrachloride (DAB, Sigma-Aldrich Chemie, USA, D5637) dissolved in Tris-maleate buffer (pH7.6) to which 20 µl of 30% H2O2 was added (10 min). Counterstaining was performed with 0.1% hematoxylin (Merck, Darmstadt, Germany) (5 s), followed by rinsing with tap water (10 min). Finally, slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany). To differentiate

Table 1 *Clinical characteristics of all patients*

Characteristic	*TA*	TAD	BA	BAD	BAD2
	N=11	N=12	N=17	N=19	N=5
Age (years)	64.5 ± 9.0	72.3 ± 11.2	55.8 ± 9.8	60.7 ± 7.8	52.6 ± 7.9
Males (%)	6	4	12	16	2
Females (%)	5	8	5	3	3
Ascending aorta diameter (mean)	*	55.0 ± 10.7	36.5 ± 7.4**	52.7 ± 6.2	56.2 ± 10.5

Patient characteristics are shown in table 1. TA: tricuspid valve, without dilation; TAD: tricuspid valve, with dilation; BA: bicuspid valve, without dilation; BAD: bicuspid valve, with dilation. * data unavailable, clinically defined as non-dilated by pathologist. ** data unavailable for 5 patients, clinically defined as non-dilated by pathologist.

c-Kit positive cells from mast cells expressing c-Kit, deparaffinated sections were stained with 0.1% toluidine blue (2 min), rinsed in distilled water, quickly dehydrated and cover-slipped. Toluidine blue staining excluded the possibility that the observed cells were mast cells, which appeared as metachromatic reaction granules containing heparin or histamine. The mast cells had a unique morphological appearance, and were rare among the c-Kit positive cells lining the adventitial–medial border.

Double immunofluorescence

To detect co-expression, we performed double immunofluorescent stainings. Sections were deparaffinated, rehydrated and subjected to antigen retrieval as described. Tissue sections were incubated with primary antibodies pc-Kit 1/100 and HIF1α 1/50 overnight (4°C), followed by incubation with secondary antibody Cy3 donkey anti-mouse immunoglobulin G (IgG) (Jackson Immunoresearch, 715-165-150) for HIF1α and Alexa Fluor 647 donkey anti-rabbit IgG (Invitrogen, A-31573) for pc-Kit (1 h, 20°C). Cy3 and Alexa Fluor 647 were preferred secondary antibodies, because of green autofluorescence of the elastic lamellae. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Finally, slides were mounted with ProlonGold (Invitrogen, P36930).

Histological parameters, immunohistochemical analyses and morphometry

To provide a basis for histopathological characteristics, we followed the techniques as previously described in our study on the differentiation differences between the aortic wall of BAV and TAV patients (6). In addition, cytoplasmic expression levels of MMP9 and c-Kit, nuclear expression of pSmad2, pc-Kit and HIF1α, cytoplasmic and extracellular expression of TGF-β, and cytoplasmic and nuclear expression of eNOS were analyzed using a BM500 microscope with plan achromatic objectives (Leica Microsystems, Wetzlar, Germany) on three predetermined locations (left, middle and right) of every section, which we refer to as 'microscopic fields' (MFs), and preserved in the evaluation of all stainings. In each MF, the level of expression was indexed on the three anatomical layers of the aortic wall (tunica intima, media and adventitia) as 0 (no expression in the respective layer), 2 (expression in less than one-third of the layer), 4 (expression in two-thirds of the layer) and 6 (expression in the whole layer). To determine the level of eNOS expression, the number of positively stained nuclei and cytoplasm was counted and analyzed using ImageJ in the three fields for each stained section. A threshold was applied to filter background noise. The total number of cells (positively and negatively stained nuclei and cytoplasm) was not different between specimens. Therefore, in each MF the number of eNOS-positive cells was normalized to the total number of cells per 10^5 μm2. Finally, the number of normalized positive cells for each staining was averaged between the three MFs. All specimens were re-evaluated by an independent, experienced histopathologist who was blinded to the clinical data.

Statistical analyses

All numerical data are presented as mean ± standard deviation of 3 fixed MFs on each stained slide. Statistical differences were evaluated with the Mann-Whitney U-test for comparison between the groups. Significance was assumed when $P < 0.05$ using the SPSS 20.0 software program (SPSS, Inc., Chicago, IL, USA). We have performed a one-, two- and three-way analysis of covariance test to correct for age and gender. The Graphpad software was used to create graphics of statistical analysis.

RESULTS

Histological observations

The normal aortic wall is made up of three layers, the tunica intima, media and adventitia (Fig. 1A, B, F and G). The internal elastic membrane forms a border separating the fine elastic fibers in the tunica intima and lamellae in the tunica media, which are more regular and thick. The sometimes ill-defined border between the media and adventitia is delineated by the last elastic lamella of the media.

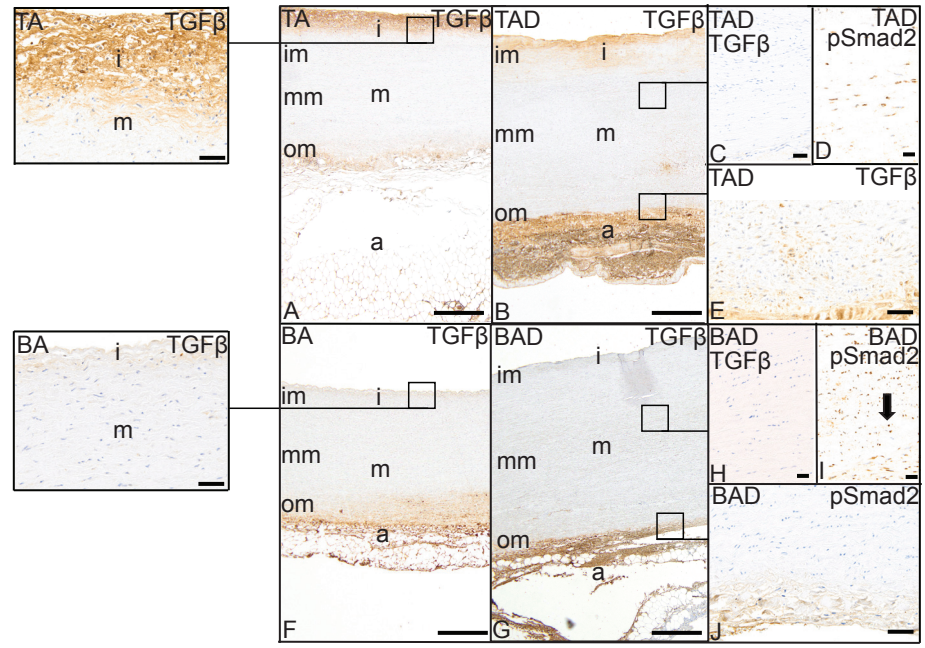
The adventitia consists of loose fibrous tissue containing nerve fibers, fibroblasts, adipocytes, a few quiescent resident inflammatory cells and vasa vasorum, lined by endothelial cells and vascular smooth muscle cells (VSMCs). Characteristics of the differences in histopathology between the TAV and BAV groups which we recently reported (6) are summarized in Fig. 1A, B, F, G, K–M.

Transforming growth factor-β, pSmad2 and endothelial nitric oxide

We examined two components of the TGF-β pathway, TGF-β and pSmad2, involved in vascular remodeling. TGF-β expression spread out from the intima to the inner media (Fig. 1A and B). Intimal expression was significantly lower in all specimens of the BAV (BA and BAD) when compared with all TAV groups (TA and TAD) ($P = 0.0058$ and $P = 0.0076$, respectively) (Fig. 1A, B, F, G and N). The middle media was completely devoid of TGF-β expression in both groups, but in the outer media, on the adjacent adventitial side, expression was visible as a gradient (Fig. 1A, B, E–G). In the outer media

Figure 1 *Transverse histological sections of the aortic wall (A-J), consisting of an intima (i), media (m), further subdivided in an inner (im), middle (mm) and outer media (om), and adventitia (a).*

The significantly thinner intima in BA and BAD (L) is devoid of TGF-β (F (with a detailed view of the intima),G) and pSmad2 expression compared to TA and TAD (A,B) (N,O). In the outer media TGF-β and pSmad2 level of expression is significantly higher in TAD compared to BAD (P,Q), TGF-β expression shown in B and G with a detailed view of TGF-β in E and of pSmad2 in J. No TGF-β expression is seen in the middle media of either group (A-C, F-H), whereas pSmad2 expression is present (D,I see arrow). In the significantly thicker media in TAD (M) significantly more severe inflammatory reaction is seen than BAD (K). TGF-β: Transforming Growth Factor-β, pSmad2: phosphorylated Smad2, TA: tricuspid valve without dilation, TAD: tricuspid valve with dilation, BA: bicuspid valve without dilatation, BAD: bicuspid valve with dilation. Scale bars: A,B,F,G 500 μm; E,J 100 μm; C,D,H,I 50 μm


TA
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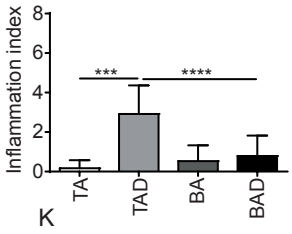
Inflammation index
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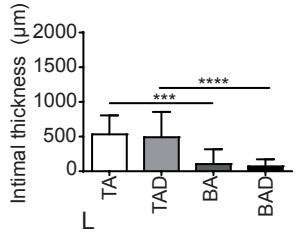
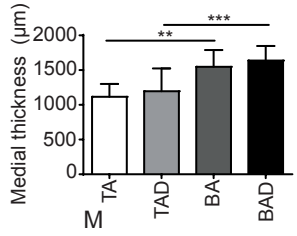
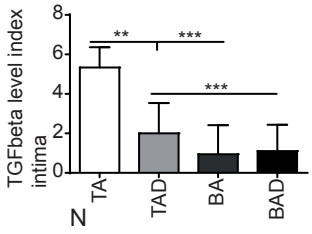
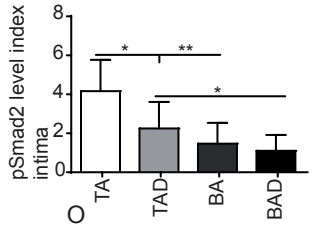
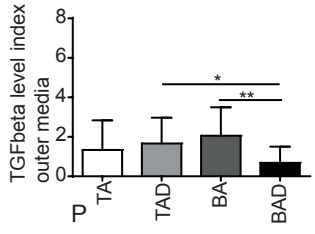
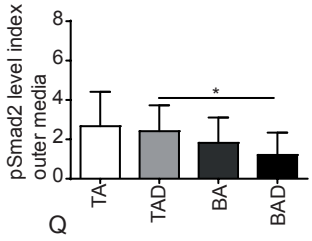

Intimal thickness (µm)
L


Medial thickness (µm)
M


TGFbeta level index intima
N


pSmad2 level index intima
O


TGFbeta level index outer media
P


pSmad2 level index outer media
Q

TGF-β expression was significantly lower in the BAD when compared with TAD and BA groups (P = 0.026 and P = 0.0035, respectively) (Fig. 1B, E–G, J and P).

The expression of the downstream mediator of the TGF-β signaling pathway, pSmad2, was in general comparable with its ligand TGF-β. PSmad2 expression was seen in the intima, and significantly lower expressed in all BAV when compared with all TAV groups (P = 0.0020 and P = 0.0179) (Fig. 1O). Expression of pSmad2 in the outer media was lower in the BAD compared with the TAD group (P = 0.012) (Fig. 1J and Q). Unlike TGF-β, which was only seen in a gradient in the inner and outer media, pSmad2 was observed in the complete media, including the middle media (Fig. 1C, D, H and I).

Pathological changes in the aortic wall during dilatation might be linked to haemodynamic changes, probably mainly acting on the endothelial cell layer, although no difference in endothelial eNOS localization was noted between the groups. A subset of VSMCs in the media, however, stained positive for eNOS (Fig. 2A and B). This media expression of eNOS was significantly less in all BAV (BA, BAD) when compared with TAV (TA, TAD) specimens (P = 0.023 and P = 0.035, respectively) (Fig. 2C). As shear stress plays an important role in the regulation of TGF-β and eNOS expression (10), we investigated whether TGF-β and eNOS levels were different between concave, with an increased turbulence of blood flow, and convex bicuspid aortic wall biopsies of the BAD group. No difference in expression was found between these two locations.

Vascular smooth muscle cells expression of c-Kit

To further elucidate the pathological changes in the aortic wall, we stained the sections for c-Kit (Fig. 2D and E), while c-Kit is well known as a stem cell marker. We observed staining solely in the cytoplasm of a subset of VSMCs in the media and no expression in the intima. The level of expression was significantly higher in the BA group when compared with the TA (P = 0.0024, (Fig. 2F), which was inverted in the dilated aortic walls where there was a significantly lower expression in the BAD compared with the BA group (P = 0.028); whereas in the TAD compared with the TA group (P = 0.021) (Fig. 2F), a significant higher level was noted. Toluidine blue staining of consecutive sections excluded the possibility that the observed cells were mast cells. As we recently described that the aortic wall in all patients with BAV is less well

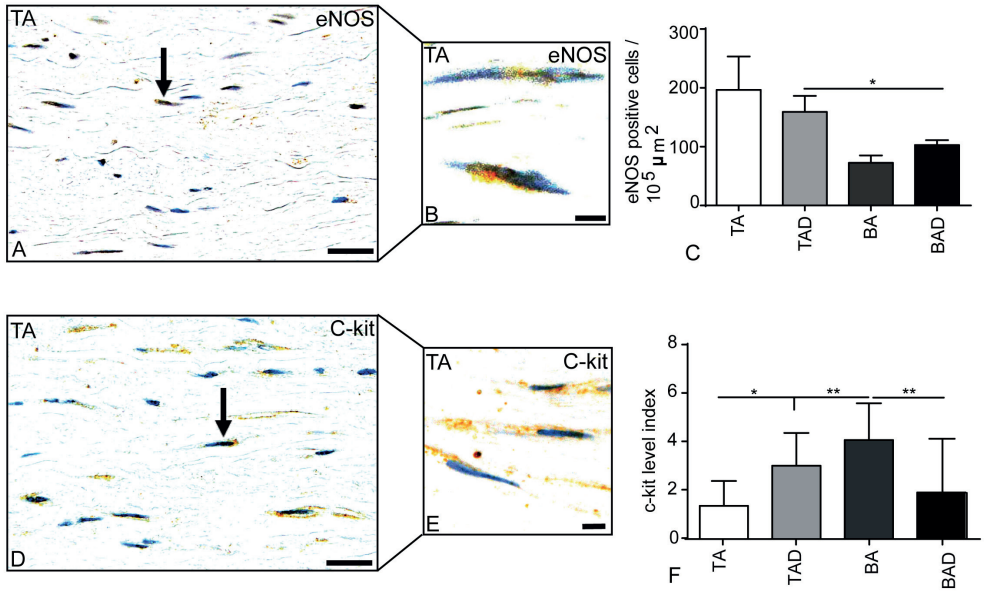


Figure 2 *Transverse histological section of the aortic wall (A,D) with details (B,E)*. eNOS positivity, defined as brown colored DAB staining, in a subset of vascular smooth muscle cells in the media is shown in A (arrow) and B. Level of expression of eNOS was significantly lower in the dilated aortic wall of patients with a bicuspid valve (BAD) compared to the tricuspid valve (TAD) (C). C-Kit level expression (2D see arrow, E) is significantly lower in the non-dilated aortic wall of patients with a tricuspid aortic valve, TA, compared to bicuspid aortic valve, BA (F). Lowering of c-Kit level of expression is seen after dilation in bicuspid aortic valve (BAD) compared to BA, whereas an increase in the dilated aortic wall of patients with a tricuspid aortic valve (TAD) is seen as compared to TA (F). Scale bar A,D: 50 µm B,E 10 µm

differentiated compared with the TAV (6) and c-Kit was highly present in the less differentiated vascular wall when compared with the mature walls, we conclude that the c-Kit expressing cells are not stem cells but dedifferentiated VSMCs (9).

Bicuspid valve without dilatation variability and vascular wall remodeling

While analysing the expression of TGF-β and pSmad2 in the outer media of the BAD group (Fig. 3C and G), we found a striking segregation of the expression level pattern within the BA group. Eight (coded as the BAa group) of the total 17 specimens consistently showed significantly different expression patterns when compared with the BAD group (Fig. 3A and E), whereas the remaining 9 (BAb) completely resembled the BAD group (Fig.

3B and F) with a lower expression of both markers (Fig. 3D and H). This was not only obvious for TGF-β and pSmad2 expression but was noted for other markers expressed in the media. c-Kit expression in the media was also significantly higher in the BAa when compared with the BAD and BAb groups (Fig. 3I–L), whereas eNOS expression was almost absent in the media of the BAa when compared with the BAD and BAb groups (Fig. 3M–P). This variance in the BA group was not seen in the intima.

To further analyse whether the differences found between the BAa and BAb groups could be associated with differences in vascular wall remodeling, we analysed the expression of αSMA and MMP9. While αSMA expression was significantly lower in both dilated and non-dilated BAVs when compared with all TAVs (P = 0.015 and P = 0.044, respectively) (6), its expression pattern was not variable within the BA group. This is in contrast to the expression of MMP9, for which the two BA subgroups could be distinguished. The BAa group showed almost absent expression of MMP9, whereas significant expression was seen in the BAD, comparable with the BAb group (Fig. 3Q–T). Hollenbeck et al. (9) have shown that MMP9 regulates the phosphorylation of c-Kit, by releasing its ligand stem cell factor from the cell membrane, and therefore can also be considered as a marker for differentiation. Pc-Kit in turn controls expression of HIF1α. As the MMP9 level was significantly lower and c-Kit expression significantly higher in BAa (Fig. 3L and T), we eliminated a potential difference in pathobiology between the BAa and BAb groups by studying the expression of pc-Kit and HIF1α in specimens from the BA and BAD groups. Expression of pc-Kit was seen in the nucleus (Fig. 4A, D and G) of a subset of VSMCs, while HIF1α was expressed at the cell membrane (Fig. 4B, E and H). Pc-Kit and HIF1α expression was abundantly present in the aortic media of patients with BAb and BAD (Fig. 4D–I). The BAa group, with the almost absent MMP9 expression, was nearly devoid of pc-Kit and HIF1α expression (Fig. 4A–C). Interestingly, HIF1α was only found coexpressed with pc-Kit in the BAb and BAD groups (Fig. 4A–I). In this cascade, however, the link of TGF-β with MMP9 could not be confirmed. The expression patterns of the discussed markers in the BAa, Bab and BAD groups are summarized in Fig. 4J.

Commissure position in bicuspid aortic valve

It has been reported that the commissure position is a predictor for future aortopathy (7). Provided that the presented similarities between the BAb

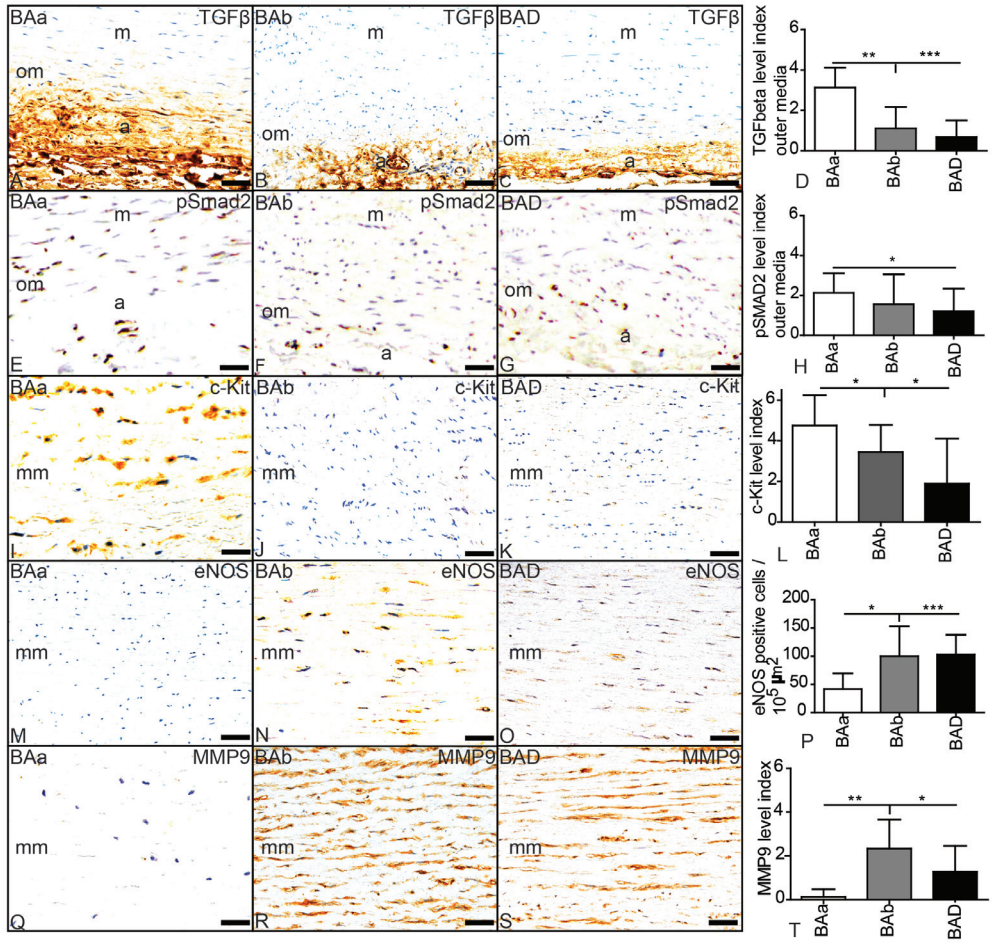


Figure 3 *Subdivision of the group patients with a bicuspid aortic valve, without dilation (BA) in a BAa and BAb group.*

Between the BAa, BAb and patients with a bicuspid aortic valve and dilated aorta (BAD) groups the stainings and level of expression of Transforming Growth Factor-β (TGF-β) (A-D) and phosphorylated Smad2 (pSmad2) (E-H) in the outer media (om), further c-Kit (I-L), endothelial nitric oxide (eNOS) (M-P) and matrix metalloproteinase-9 (MMP9) (Q-T) in the middle media (mm) were compared. TGF-β, pSmad2, c-Kit expression was significantly higher in the BAa group as compared the BAD group. eNOS and MMP9 expression was significantly lower in the BAa than the BAD group. The Bab group showed marked similarity with the BAD for all markers. Whereas he BAb group was significantly different from BAa for all, except for pSMAD2 (E). a: adventitia m: media Scale bar: 50 µm

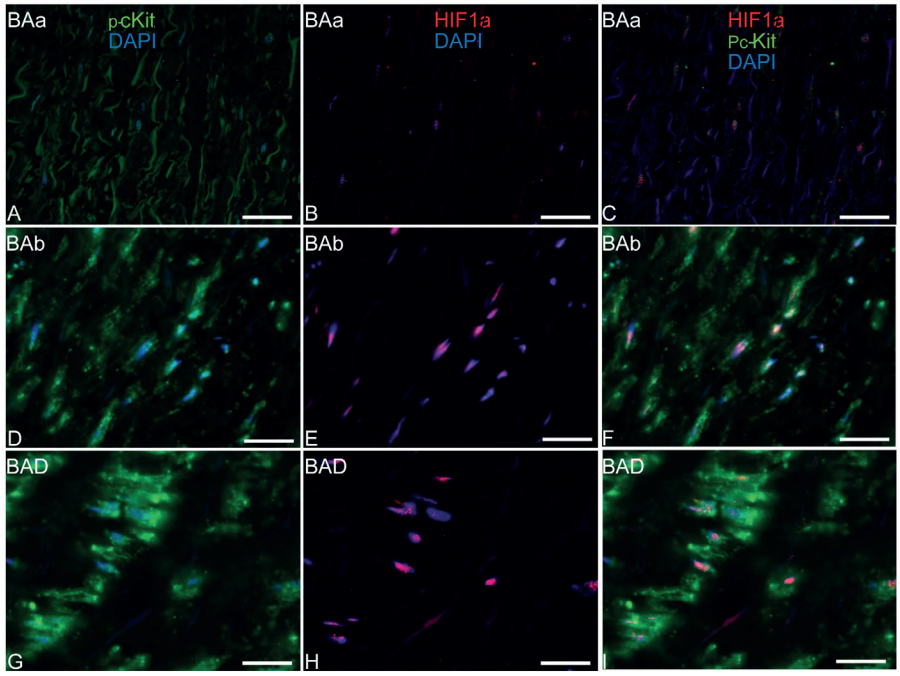
and the BAD group are valid, we expect that the commissure position would match between the two groups. Therefore, we assessed the commissure position in the BAa, BAb and the BAD groups. In the BAD group, of 4 patients the orientation was unknown and the remainder (n = 15) had an RCC/LCC-type BAV. Although the groups are too small to obtain significant data, the RCC/NCC was most apparent in the BAa, while the RCC/LCC was most present in the BAb group, similar to the BAD group (Table 2 and Fig. 5).

DISCUSSION

In this study, we found two distinct expression patterns in patients with BAV without apparent dilatation. One pattern is completely comparable with the expression pattern seen in BAV patients with aortic dilatation, suggestive of a different state of vulnerability. To date, decision-making for preventive aortic root replacement in patients with BAV is solely based at the population level on the aortic diameter. However, although thoracic aortic dilatation forms the most critical complication, not all BAV patients are at risk for dissection and rupture. Additional criteria are needed to identify patients at risk as early as possible. Earlier, clinical parameters, such as the morphological appearance of the commissure position of BAV, have been considered (8, 11); however, these tools are not conclusive. Therefore we searched for additional morphological features of the aortic wall, suggestive of vulnerability for future complications, which could either alone or in combination with the commissure position be applied as a patient-tailored risk stratification.

When analysing the aortic wall histologically, we could not substantiate the observation made by many thoracic surgeons of a thinner aortic wall in BAV. Although this is a striking discrepancy with the clinical findings, other studies also reported that the total wall thickness, excluding the adventitia, is not different between BAV and TAV groups (12). The difference in the ratio between the intimal and medial thickness might therefore have been interpreted as difference in the wall thickness.

We previously confirmed that inflammation was much more pronounced in the dilated TAV groups than in any other group (6). We also showed that the aortic wall in BAV patients is intrinsically different from those in TAV patients, as in TAV inflammation and accelerated ageing led to aortic pathology (6). In all cases with BAV, in both the dilated and non-dilated groups, there is a



	BAa	BAb	BAD
TGβ	+++	+/-	+/-
pSmad2	++	+	+
c-Kit	+++	+	+
eNOS	-	++	++
MMP9	-	+	+
pc-Kit	-	++	++
Hif1α	-	++	++

J

Figure 4 *Transverse histologic sections of the aortic wall (A-I).*
Immunofluorescent staining of pc-Kit (green), HIF1α (red) and DAPI (blue) is shown in the BAa (A-C), BAb (D-F) and BAD (G-I) groups. Expression of pc-Kit was seen in the nucleus (A,D,G) of a subset of VSMCs and HIF1α was expressed in the cellular membrane (B,E,H). Pc-Kit and HIF1α is richly expressed in the BAb and BAD group as compared to the BAa group which was nearly devoid of pc-Kit and HIF1α expression (C,F,I). VSMCs of only stained positive for HIF1α co-expressed with pc-Kit (A-I). The expression patterns of the discussed markers in the BAa, BAb and BAD group are shown in J. Expression patterns of TGFβ, pSmad2, c-Kit, eNOS, MMP9, pc-Kit and HIF1α in BAb and BAD resembled each other whereas BAa was significantly different. Scalebar: 25 μm

maturation defect of the aortic wall, showing less well-differentiated VSMCs and a low lamin A/C and progerin expression (6). As the inflammatory status and the intima/medial thickness ratio are similar in all BAVs, these factors could not serve as a marker to identify the subset of BAV patients susceptible to future aortopathy. We, therefore, then focused on differences in the expression of markers/proteins involved in vascular remodeling (TGF-β, phosphorylated Smad2, MMP9), cellular differentiation (c-Kit (9), phosphorylated-c-Kit, MMP9, HIF1α) and eNOS as a marker of possible haemodynamic influences. We could distinguish a marked variability in the expression pattern of the above-indicated set of differentiation markers within the non-dilated BAV group. We see a comparable expression pattern in the BAD and BAb subgroups, different from that in the BAa group. We, therefore, postulate that the BAb is susceptible to dilatation, while the BAa group is non-susceptible to aortopathy. We hypothesize that the enhanced expression of the dedifferentiation markers and eNOS induces a cascade that will lead to a less stable aortic wall which is accompanied by a decrease in the expression of TGF β and pSmad2 correlating with (future) aortic wall dilatation. The precise mechanism causing the decrease in expression of the vascular remodeling markers in the BAb and BAD groups is not yet

Table 2 *Commissure position in BA*

Commissure position	*BA susceptible*	BA non-susceptible
	N=9	N=8
Unicuspid (n) (%)		1 (13%)
RCC/LCC (n) (%)	6 (67%)	2 (25%)
RCC/NCC (n) (%)		4 (50%)
LCC/NCC (n) (%)	1 (11%)	
Unknown (n) (%)	2 (22%)	1 (13%)

Commissure position in subdivided groups with bicuspid aortic valve, without dilation: BA-susceptible and BA-non-susceptible. RCC/LCC right to left coronary cusp fusion. RCC/NCC right to non-coronary cusp fusion. LCC/NCC left to non-coronary cusp fusion.

identified, and is a focus for future research. Further study also is necessary to determine the developmental and environmental factors that initiate the cascade and thus the distinction in a susceptible and non-susceptible group. The findings and hypothesis are summarized in Fig. 5.

Finally, we considered the morphological appearance of the commissure position as a determinant of susceptibility, since commissure position, which can clinically be analysed echocardiographically, is reported to be important in predicting future aortopathy (8, 11). Previously, several studies reported that BAVs with an RCC/LCC BAV, which is the most common variant, are

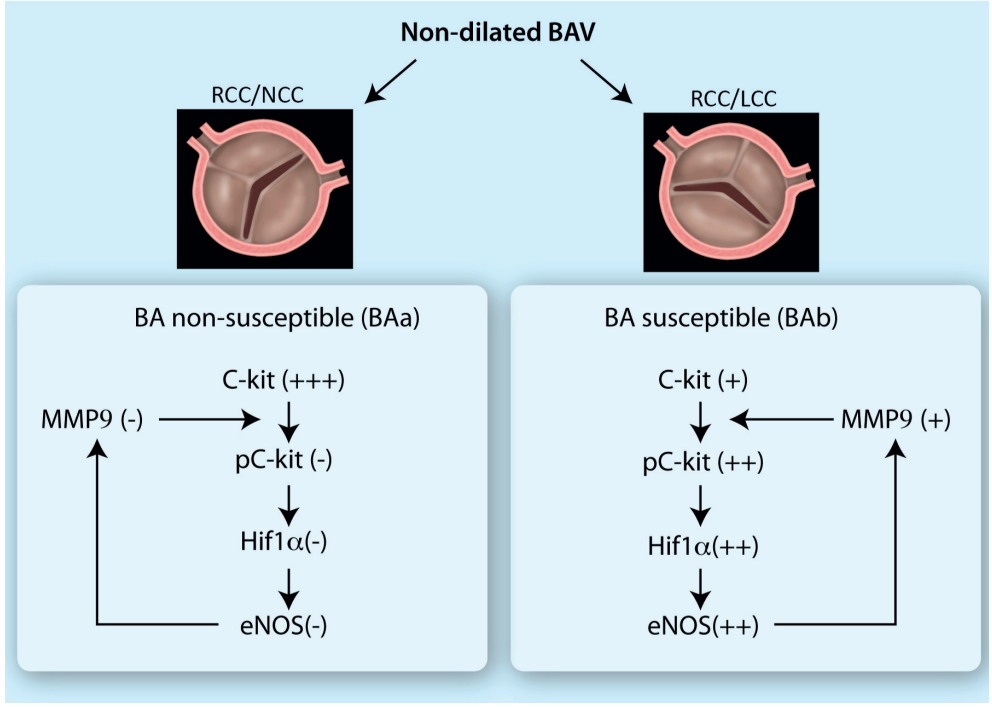


Figure 5 *The observed differences in expression of molecular biologic markers are presented in a cascade.*

The non-susceptible group (BAa) showed significant higher expression of c-Kit as compared to the susceptible group (BAb). MMP9, scarcely expressed in the non-susceptible group and abundantly in the susceptible, is involved in the phosphorylation of c-Kit. Lower expression of c-Kit in the susceptible group as shown in the cascade, could therefore be the result of its conversion to pc-Kit. Besides pc-Kit, HIF1α was richly expressed in the susceptible group, in contrast to the non-susceptible. HIF1α was only found co-expressed with pc-Kit. eNOS, regulated by HIF1α, showed a similar expression pattern of low expression in the non-susceptible and significantly higher expression in the susceptible group.

associated with more aortic dilatation in adults, whereas BAVs with fusion of the RCC/NCC are responsible for valve dysfunction at a younger age (13–18). Although the groups were small in our study, the orientation of the commissure and position of the raphe was in line with previous findings: RCC/NCC was most apparent in the BAa (the considered non-susceptible group), while the RCC/LCC was seen more often in the BAb group (the considered susceptible group) and in BAD patients (Table 2). Recent preclinical studies showed that RCC/NCC and RCC/LCC likely have a different pathogenesis (19, 20). RCC/NCC BAVs are observed in eNOS −/− mouse embryos (19, 21, 22), which is comparable with our BAa-non-susceptible group, as this group also shows almost absent expression of eNOS and has the RCC/NCC BAV type as the most common variant. Although more research is needed, we suggest that identifying patients with an RCC/LCC commissure type could be the first step in selecting patients for a preventive aortic root replacement; however, as some variation in commissure position is apparent, patient selection could not solely be based on valve morphology. The proposed activation cascade should also be taken into account and the combination is a possible protocol for decisionmaking. To choose the best markers for clinical applications, we first need to question whether decision-making for aortic replacement surgery will be possible before or only during surgery, as this influences the choice of markers. The clearest difference in expression level between the susceptible and non-susceptible BA group was seen for MMP9, phosphorylated c-Kit and Hif1α, and these would be most appropriate to serve as clinical biomarkers. Analysis of these markers would be relatively easy to perform using a quick histological frozen section during surgery. A disadvantage is that a biopsy from the aortic wall is only possible after the patient is on extracorporeal circulation and the aorta is clamped. Immunohistochemical analyses are time consuming and increases the time the patient is on bypass. Preoperative decision making would be preferable, and we will therefore be concentrating in future studies on factors detectable in blood. Not only will the decision be formed preoperatively, a blood test is also less invasive. Therefore, we will explore if any of the markers identified in this study can be measured, is sensitive enough to distinguish the susceptible patients and specific enough to recognize patients without expression and thus being non-susceptible.

STUDY LIMITATIONS

We designed our study by comparing the expression of a panel of markers distinguishing cases of non-dilated aorta of BAV with BAV that will progress to dilatation over time. A limitation of our study is that we did not have frozen tissue samples of all the aortic wall specimens we received fixed in formalin from the various groups.Therefore, we could not perform a western blot to correlate this to our findings of immunohistochemistry. To secure greater confidence in the obtained results, an animal model that recapitulates the c-Kit phosphorylation and downstream HIF1α biology, using knock-down or c-Kit inhibition strategies is needed.

ACKNOWLEDGEMENTS

We thank the Heart Valve Bank Rotterdam, The Netherlands, for providing the described cryopreserved valves.

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CHAPTER

6

Histopathology of aortic complications in bicuspid aortic valve versus Marfan syndrome: relevance for therapy?

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Submitted

ABSTRACT

Background: Patients with bicuspid aortic valve (BAV) and patients with Marfan syndrome (MFS) are more prone to develop aortic dilation and dissection compared to patients with a tricuspid aortic valve (TAV). To elucidate potential common as well as distinct pathways of clinical relevance, we compared the histopathological substrates of aortic pathology.

Methods and results: Ascending aortic wall biopsies were divided in five groups: BAV (n=36) and TAV (n=23) without and with dilation and non-dilated MFS (n=8). General histologic features, apoptosis, and the expression of markers for VSMCs maturation, markers predictive for ascending aortic dilation in BAV and expression of fibrillin-1 were investigated. Both MFS and BAV showed an altered distribution and decreased fibrillin-1 expression in the aorta and a significantly lower level of differentiated VSMCs markers. Interestingly, markers predictive for aortic dilation in BAV were not expressed in the MFS aorta. The aorta in MFS was similar to the aorta in dilated TAV with regard to the presence of medial degeneration and apoptosis, while other markers for degeneration and ageing like inflammation and progerin expression were low in MFS, comparable to BAV.

Conclusions: Both MFS and BAV aortas have immature VSMCs, while MFS and TAV patients have a similar increased rate of medial degeneration. However, the mechanism leading to apoptosis is different, being *fibrillin-1* mutation induced increased angiotensin-receptor-pathway signaling in MFS and cardiovascular ageing and increased progerin in TAV. Our findings could explain why angiotensin inhibition is successful in MFS and less effective in TAV and BAV patients.

INTRODUCTION

Bicuspid aortic valve (BAV) is the most common congenital cardiac malformation, with a prevalence of 1% in the general population (1). This anomaly is associated with complications as aortic stenosis and/or regurgitation as well as critical aortic dilation, with an increased risk of dissection and rupture. Aortic dilation is also a key feature of the clinical presentation in patients with Marfan syndrome (MFS). In MFS, mutations in the *fibrillin-1* gene, encoding for the fibrillin-1 protein, account for approximately 70-93% of patients who meet the diagnostic criteria (2). Due to the *fibrillin-1* mutation, the aorta in MFS exhibits markedly abnormal elastic properties which are assumed to lead to a decrease in compliance and progressive increase in dilation (3).

Both patients with MFS and BAV show aortic dilation but the anatomic site of vulnerability is distinct in both conditions. While maximal aortic dilation is observed above the sinotubular junction in BAV, in the MFS population it is mainly found at the level of the sinuses of Valsalva, also referred to as aortic root (4).

To unravel the pathogenetic mechanism leading to aortic wall pathology in BAV, differences between the diseased aortic wall of patients with BAV and patients with a tricuspid aortic valve (TAV) were studied previously (5). We found that the ascending aorta in BAV is intrinsically different from TAV patients. The vascular smooth muscle (VSMC) cell layer is less well differentiated in BAV while inflammation and accelerated ageing attribute to the aortic pathology in TAV (5).

Despite the VSMC immaturity, not all patients with BAV carry an increased risk for aortic dilation. In a previous study we defined a panel of markers which could differentiate the non-dilated BAV patients, in a susceptible and non-susceptible subgroup for future dilation, being c-Kit, a marker for dedifferentiated VSMCs, and its phosphorylated state (pc-Kit) triggered by the presence of matrix metalloproteinase-9 (MMP9) influencing Hypoxia-Inducible-Factor-1alpha (HIF1α) and endothelial nitric oxide synthase (eNOS) (6).

The aortic wall in MFS and BAV has been described to have similarities, like an increased MMP activity and decreased fibrillin-1 expression (7,8). However, cytolytic necrosis, (also termed medial degeneration), defined as VSMC dropout, apoptosis and elastic fiber degeneration, highly characteristic for

MFS and the dilated aorta in TAV, are far less obvious in BAV (5,7). Although clinically, symptoms of aortic wall pathology in MFS and BAV overlap, still some striking differences remain less well understood. For instance, BAV patients rarely possess Marfanoid characteristics, conversely in patients with MFS the risk of concomitant BAV syndrome is only slightly increased (4.7%) (9). Furthermore, the risk for aortic dilation in BAV, although higher than in the general population, remains low when compared to MFS where the majority of patients is prone to severe aortic wall disease. In MFS moreover, not only aortic dilation is a critical aortic complication, but also dissections in a non-dilated aortic wall and at a younger age, as compared to the BAV, have been reported (10,11). In MFS furthermore, angiotensin-receptor-blockers (ARBs) as Losartan have been identified as a potentially therapeutic agent to prevent progressive dilation of the ascending aorta in (12-18). A similar positive effect in preventing aortic complications, by reducing the angiotensin pathway signaling is however not seen in the BAV population (19). It is therefore interesting to study which similarities are actually present immunohistochemically between the ascending aortic wall in both diseases and the dilated aortic wall in the TAV, despite possible different clinical sequelae in future. What factors cause increased weakness of the aortic wall in MFS and are these similar in BAV? Why is aortic wall pathology seen in BAV and MFS not comparable to the aortic dilation complications in patients with TAV?

To study this we compared the aortic wall between MFS, BAV and TAV, starting with the investigation of general histopathological features, including cytolytic necrosis, inflammation, elastin lamellae degradation and VSMC apoptosis. The level of expression of fibrillin-1 was also studied in these groups. As VSMCs play a role in the synthesis and assembly of fibrillin-1, we compared our findings of fibrillin-1 expression to that of differentiation and maturation of the vascular wall, being the VSMC differentiation markers lamin A/C and progerin (5). We further studied the expression of the pc-Kit pathway (c-Kit, pc-Kit, MMP9, HIF1α and eNOS) to investigate whether these markers, indicative of BAV aortic dilation (6) are also applicable in the MFS group.

MATERIAL AND METHODS

Ethical statement

The institutional ethics committee at the Leiden University Medical Centre, Leiden approved this study. The Academic Medical Center (AMC), Amsterdam provided us with eight MFS biopsy aorta specimen, with approval of the Medical Ethical Committee. The Heart Valve Bank, Thoraxcenter, Erasmus Medical Center (EMC), Rotterdam, provided six non-dilated BAV aortic wall samples which were not suitable for transplantation, approved by their Scientific Advisory Board.

Patients and tissue samples

Ascending aortic wall samples were collected from non-MFS individuals with TAV and BAV, with and without dilation. Based on the ACC/AHA guidelines, dilation was clinically defined by surpassing an ascending aortic wall diameter of 44 mm (20). The study population was divided in five groups: 1) TAV without dilation, termed TA (n = 11, mean age 64.5 ± 9.0 years) obtained post mortem, 2) TAV with dilation, termed TAD (n = 12, mean age 72.3 ± 11.2 years) collected during elective replacement, 3) BAV without dilation, termed BA (n = 17, mean age 55.8 ± 9.8 years) representing a unique group from patients with stentless root replacement (the biopsy material was collected as residue waste material from the proximal anastomosis), and the 6 biopsies received from the EMC, 4) BAV with dilation, termed BAD (n = 19, mean age 60.7 ± 7.8 years), collected during elective replacement and 5) MFS aortic wall without ascending aorta dilation, termed MFS (n = 8, mean age 34.2 ± 11.0 years), derived after elective replacement of the dilated root (the biopsy material was collected as residue waste material from the proximal anastomosis). Group one to four have been reported previously (5,6) and in the current study additional staining with fibrillin-1 and apoptosis markers were performed for comparison with the newly described MFS group. In this study we paid additional attention to the underlying aortic valve pathology of the study population. The TA group showed no valve pathology and the TAD group showed variably either no valve pathology or aortic valve stenosis or regurgitation. In the non-dilated BAVs six patients had a non-pathologic aortic valve, these were the specimen we received from the EMC, the remainder of the BA group showed aortic valve stenosis, aortic valve regurgitation or a combination of both. In the dilated BAV group 3 patients had a non-

pathologic aortic valve. All MFS patients had a non-pathologic valve and a dilated aortic root, specimen were hence obtained during a valve sparing root replacement, which was performed in this patient group.

Sample processing and routine histology

Specimens were sectioned and stained as described previously (5,6). Briefly, following excision, all specimens were fixed, decalcified and paraffin embedded. Transverse sections (5 μm) of the paraffin-embedded tissue were deparaffinated and rehydrated after which they were stained with hematoxylin-eosin (HE) and resorcin fuchsin (RF) to study the morphology of the vessel wall. Aortic inflammation was quantified using the HE stained sections, indexed from zero (no inflammatory cells) to 6 (large clusters of cells). In RF stained sections the maximum intimal thickness was quantified in μm and the organization of the elastic lamellae in the media was evaluated.

Immunohistochemistry

Sections were stained following the protocol described previously (5,6). An overview of the primary and secondary antibodies used is given in Table 1.

Histologic parameters, immunohistochemical analyses and morphometry

Sections were studied with a Leica BM500 microscope equipped with plan achromatic objectives (Leica Microsystems, Wetzlar, Germany). Cytolytic necrosis and elastic fiber degeneration were defined qualitatively, in alpha-smooth muscle actin (αSMA) and Resorcin Fuchsin (RF) stained sections respectively. The cytoplasmatic level of expression of αSMA, smooth-muscle22-alpha (SM22alpha), smoothelin and MMP-9, intra- and extracellular expression of fibrillin-1, cytoplasmatic and extracellular matrix expression of Transforming Growth Factor- beta (TGFβ), nuclear expression of lamin A/C, progerin, cleaved-caspase-3, eNOS, pc-Kit and Hif1α were analyzed on three predetermined locations (left, middle and right) of every section, that we refer to as ‘microscopic fields’ maintained in evaluation of all staining on sister sections. In each microscopic field the level of expression was indexed on the three anatomical layers of the aortic wall (tunica intima, media, adventitia) as 0 (no expression in the respective layer), 2 (expression in less than one third of the layer), 4 (expression in two thirds of the layer) and 6 (expression in the whole layer). To determine the level of lamin

Table 1 *Immunohistochemistry reagents*

Primary antibody	Vendor, order number	Concentration	Secondary antibody	Mechanism
Anti-αSMA	A2547, Sigma-Aldrich Chemie, Darmstadt, Germany	1:5000	RAM-PO (1:250) (DAKO p0260)	Smooth muscle cell differentiation
Anti-cleaved Caspase-3	9661, Cell Signaling, Beverly, United States	1:250	GAR (1:200) & NGS (1:66) (Vector Laboratories, USA, BA-1000 and S1000)	Apoptosis
Anti-SM22α	AB10135, Abcam, Cambridge, United Kingdom	1:100	GAR & NGS	Smooth muscle cell differentiation
Anti-Smoothelin	16101, ProgenBiotechnik, Heidelberg, Germany	1:200	HAM (1:200) & NHS (1:66) (Vector Laboratories, USA, BA-2000) (Brunschwig Chemie, Switserland, S-2000)	Smooth muscle cell differentiation
Anti-lamin A/C	MAB3211, Millipore, Billerica, USA	1:50	HAM & NHS	Myoblast differentiation
Anti-progerin	SC-81611, Bio-Connect, Huissen, The Netherlands	1:50	GAR & NGS	Cardiovascular ageing
Anti-eNOS	PA1037, Thermo scientific, Rockford, USA	1:100	GAR & NGS	Susceptibility for aortopahty in BAV
Anti-TGFβ	MO-C40009E, Anogen, Ontario, Canada	1:1000	HAM & NHS	Susceptibility for aortopahty in BAV
Anti-MMP9	MCA2736, ThermoFisher, Waltham, USA	1:100	HAM & NHS	Susceptibility for aortopahty in BAV
Anti-c-Kit	A4502, Dako, Heverlee, Belgium	1:00	GAR & NGS	Susceptibility for aortopahty in BAV
Anti-pc-Kit	ab62154, Abcam, Cambridge, United Kingdom	1:100	GAR & NGS	Susceptibility for aortopahty in BAV
Anti-HIF1α	SC-53546, Santa Cruz Biotechnology, Texas, USA	1:500	HAM	Susceptibility for aortopahty in BAV
Anti-*FBN1*	MAB1919, Millipore, Billerica, Germany	1:100	HAM & NHS	Fibrillin-1 expression

αSMA: alpha smooth muscle actin, SM22α: smooth-muscle-22-alpha,eNOS: endothelial nitric oxide, TGFβ: transforming growth factor-beta, MMP9: matrix metalloproteinase-9, pc-Kit: phosphorylated c-Kit, HIF1α: Hypoxia-Inducible-Factor-1-alpha, FBN1: fibrillin-1, GAR: goat-anti-rabbit-biotin, NGS: normal goat serum, HAM: horse-anti-mouse-biotin, NHS: normal horse serum and RAM-PO: peroxidase-conjugated rabbit anti-mouse

A/C, progerin, cleaved-caspase-3, eNOS, pc-Kit and Hif1α expression, the number of positively stained nuclei was counted following previously described methods (6). All specimens were re-evaluated by an independent, experienced histopathologist who was blinded to the clinical data.

Statistical analyses

All numerical data are presented as mean ±SD of three microscopic fields on each stained slide. Statistical differences were evaluated with the Mann-Whitney U-test for comparison between the groups. We also performed a one, two and three way ANCOVA test to correct for age and gender. Significance was assumed when $p<0.05$ using SPSS 20.0 software program (SPSS Inc. Chicago, USA). Graphpad software was used to create graphics of statistical analysis.

Table 2 *Clinical characteristics of all patients*

Characteristics	TA	TAD	BA	BAD	MFS
	N=11	N=12	N=17	N=19	N=8
Age (years)	64.5 ± 9.0	72.3 ± 11.2	55.8 ± 9.8	60.7 ± 7.8	34.1 ± 11.8
Males (%)	54.5%	33.3%	70.1%	84.2%	62.5%
Females (%)	45.5%	66.7%	29.4%	15.8%	37.5%
Ascending aorta diameter (mean)	*	55.0 ± 10.7	36.5 ± 7.4**	52.7 ± 6.2	28.4 ± 12.8
Aortic root diameter (mean)	***	***	***	***	48.1 ± 3.0
Aortic valve pathology					
-No valve pathology	N=11	N=6	N=6	N=3	N=7
-Aortic stenosis	N=0	N=1	N=4	N=8	N=0
-Aortic regurgitation	N=0	N=5	N=1	N=5	N=1
-Aortic stenosis and regurgitation	N=0	N=0	N=5	N=3	N=0

* data unavailable, clinically defined as non-dilated by pathologist. ** data unavailable for 5 patients, clinically defined as non-dilated by pathologist. *** aortic root diameters unavailable

RESULTS

Patient characteristics

Patient characteristics of all five groups are shown in Table 2. The MFS patients were evidently the youngest, followed by the BAV patients. In MFS, male and female were almost equally affected, the BAVs, however, showed a marked male predominance. There was thus a noticeable variance in age and gender distribution in our study. Statistically, both age and gender were not found confounding in our study. All MFS patients showed typical aortic root dilation (diameter 48.1 ± 3.0 mm), with a non-dilated ascending aorta (diameter 28.4 ± 12.8 mm). Marked root dilation was not present in the other four groups (TA, BA, TAD, BAD).

General histopathologic features in aortic walls of MFS, BAV and TAV

The ascending aortic wall, consisting of a tunica intima, media and adventitia, was compared between the MFS, BAV (BA, BAD) and TAV (TA, TAD) groups. The total vessel wall thickness, excluding the highly variable adventitia, was not different between the 5 groups.

Intima: Similar to BAV (5), in MFS the intima was significantly thinner as compared to the TAV groups (MFS vs TAD $p<0.001$) (Fig1A,B, graph 1F). Previously we described that in all specimens from BAV patients, the intima also showed a significantly lower intimal expression of TGFβ as compared to TAV (6). When analyzed in the MFS group, we found that in this group the intima also had a very low TGFβ expression (Fig1C).

Media: Of all groups only the aortic wall of MFS and TAD showed significant pathology in the media, with more profound cytoclytic necrosis (Fig 1D) and a more fragmented pattern of the elastic lamellae in which the inter-lamellar distance was enlarged (Fig 1E). These signs of pathology were not observed in the TA, BA, and BAD group (not shown). Expression of the apoptosis marker cleaved-caspase-3 was markedly elevated in the media of MFS and TAD as compared to the BAD ($p=0.033$ and $p=0.0286$ respectively) (Graph 1H).

Adventitia: The adventitia consisted of loose fibrous tissue containing nerve fibers, fibroblasts, adipocytes and vasa vasorum, lined by endothelium and VSMCs in all groups. Adventitial inflammatory cells were most outspoken in the TAD group as compared to the MFS and BAD group ($p<0.0001$, $p<0.001$ respectively) (Fig1A, graph 1G).

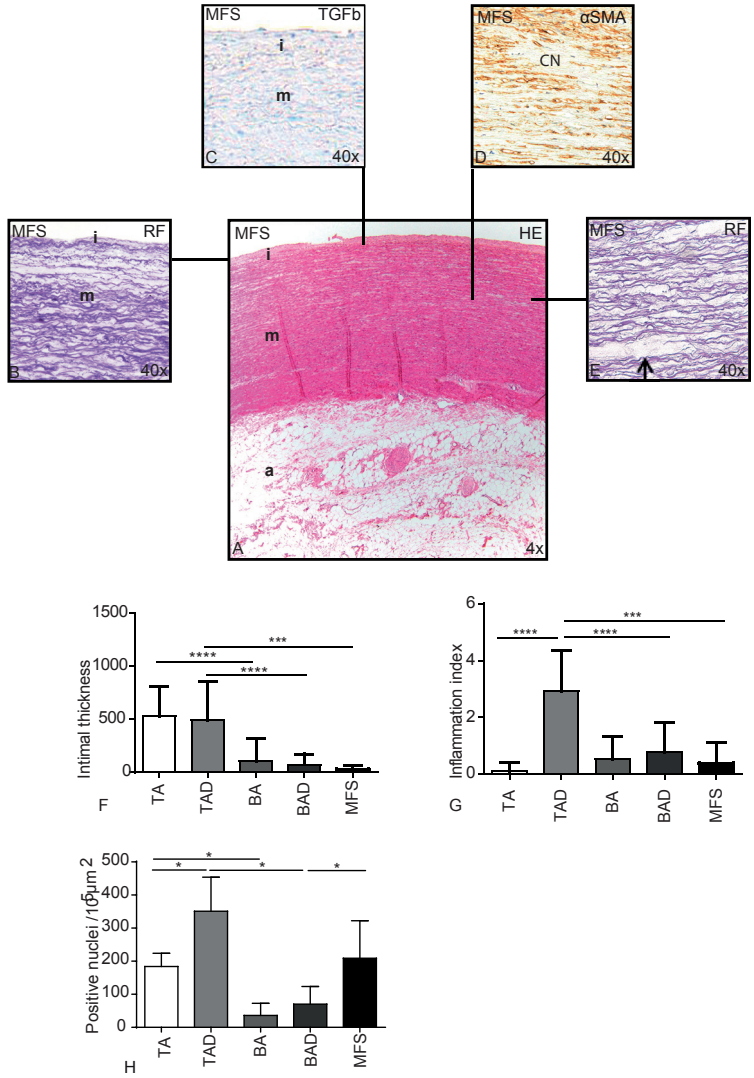


Figure 1 *Transverse histologic sections (5µm) stained with Resorcin Fuchsin (RF), Hematoxylin-Eosin (HE) and alpha Smooth Muscle Actin (αSMA) in Marfan syndrome (MFS).* HE stained overview section (A) shows the tunica intima (i), which was significantly thinner (B), (graph F) and lacked TGF-β expression in all MFS (C) and BAV patients as compared to the TAV groups, tunica media (m) and tunica adventitia (a). The aortic media of MFS and TAD showed significant pathology in the media, with more profound cytolytic necrosis (CN) (D) and a more fragmented pattern of the elastic lamellae in which the inter-lamellar distance (arrow) was enlarged (E). Adventitial inflammatory cells were absent in the MFS (A) and most outspoken in the TAD group (graph G). Expression of the apoptosis marker cleaved-caspase-3 was significantly elevated in the media of MFS and TAD as compared to the BAD (graph H). Magnification: A 4x; B-E 40x; *** = p<0.001; **** = p<0.0001

In conclusion intimal thickness and lack of adventitial inflammation were similar between BAV and MFS. Aortic media pathology was comparable between the non-dilated MFS and dilated TAV.

Fibrillin-1, differentiating and mature VSMCs, lamin A/C, progerin and the pc-Kit pathway

To further understand the pathobiology we focused on the differentiation state of the aortic wall and the expression of the pc-Kit pathway and fibrillin-1 in all patient groups.

Fibrillin-1 expression was identified in the aortic media of all groups. The level of expression was significantly lower in all patients with MFS and BAV (BA, BAD) as compared to TAV (TA, TAD) ($p<0.05$) (Fig. 2A-E, graph 2F). The localization of the staining was different between the groups. In the TAV (TA and TAD) the staining was mainly seen extracellular, whereas in the MFS and BAV besides being decreased, the expression was mainly observed intracellular (cytoplasmic) in the VSMCs (Fig. 2A-E).

We further observed that in MFS the expression of αSMA (Graph 3E), SM22α (Fig. 3A, graph 3F) and smoothelin (Fig. 3B) was similar to the BAVs and significantly lower as compared to the TAVs group. Expression of lamin A/C (Fig. 3C, graph 3G) and progerin (Fig. 3D, graph 3H) in MFS was as seen in BAV, being lower as compared to the TAVs. Thus the aortic wall in MFS shows features of less differentiation comparable with the BAV group. The results are summarized in Table 3. We further investigated markers predictive for ascending aortic dilation in BAV, including pc-Kit (6). We found that these markers were not expressed in the MFS group (data not shown).

DISCUSSION

Our study describes an in-depth effort to compare the ascending aortic wall of patients with MFS (non-dilated), BAV (non- and dilated) and TAV (non- and dilated) in one study. A specific aortic wall architecture associated with aortic wall pathology in both MFS and BAV is an extensively discussed, yet controversial, subject. Previous studies compared the aortic wall of MFS and BAV histologically and found that the media was characterized by cytolytic necrosis and elastic fiber degeneration in both diseases (21). An increased MMP activity and decreased fibrillin-1 in the aortic wall, without comparable

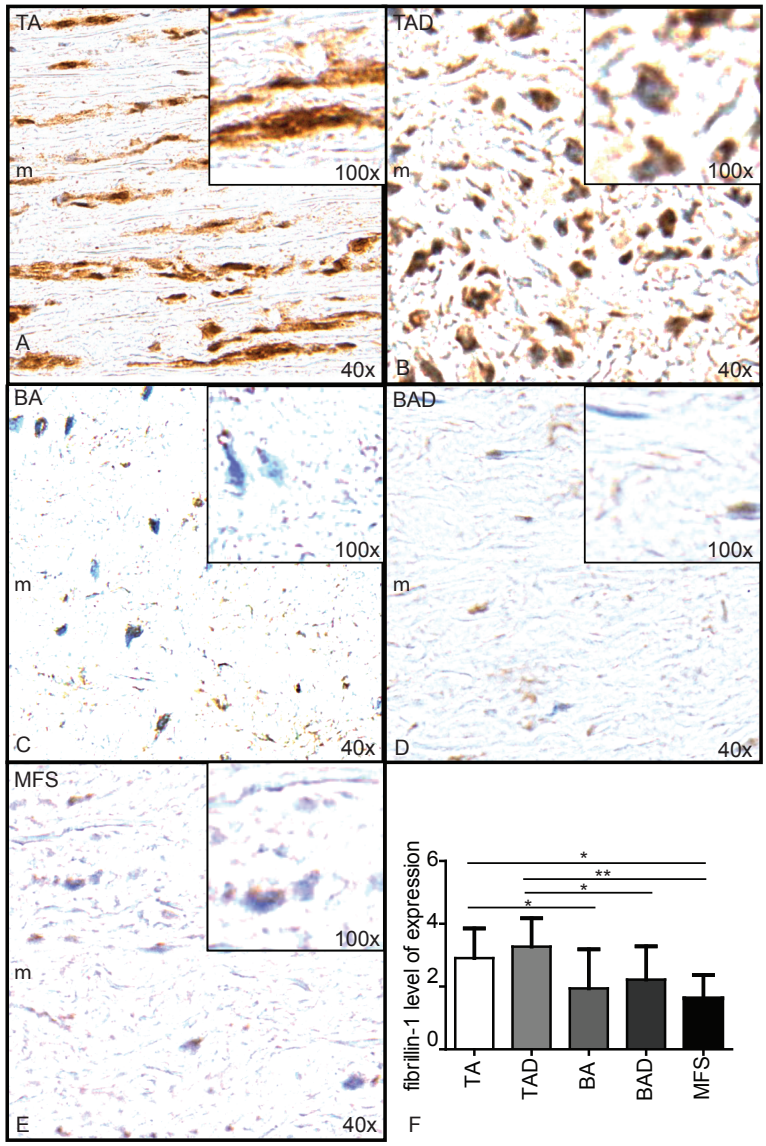
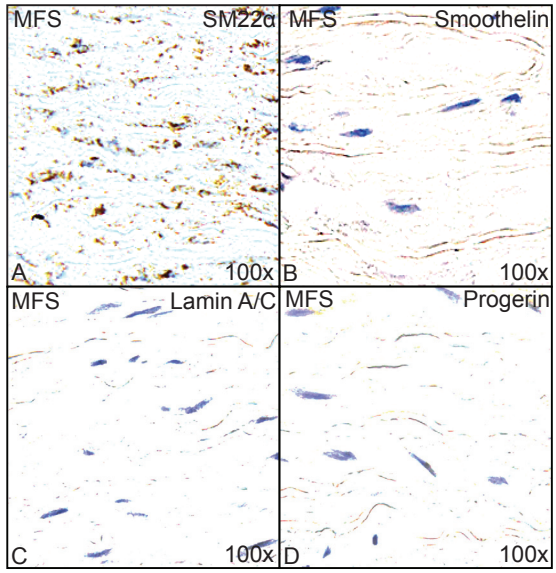


Figure 2 *Transverse histologic sections (5µm) stained with fibrillin-1. TA (tricuspidy without dilation), TAD (tricuspidy with dilation), BA (bicuspidy without dilation), BAD (bicuspidy with dilation), MFS (Marfan syndrome without dilation).*
FBN-1 expression was observed in the aortic media (M) of all groups, with each picture (A-E) showing an insert with magnification 100x to illustrate the expression on cellular level. The level of expression was significantly lower in all patients with MFS (E), BA (C) and BAD (D) as compared to TA (A) and TAD (B) (Graph F). Staining was mainly extracellular in the TA and TAD, whereas in the MFS and BAV, the decreased expression, was mainly observed intracellular (cytoplasmic) in the VSMC. Magnification: A-E 40x; * = $p<0.05$; ** = $p<0.01$

reduction in matrix components elastin and collagen was further noticed (4,7,8). Similarities have thus been noted, though without getting grip on a possible common defect. In this study we attempted to shed light on intrinsic defects of the aortic wall leading to the observed similarities by comparing the ascending aortic wall of both diseases with each other and with patients with TAV without MFS. As recently several publications focused on a difference in dilation progress after aortic valve repair dependent on whether the diseased aortic valve was stenotic or regurgitant with concomitant root dilation (22) in the latter, we also paid additional attention to the underlying aortic valve pathology besides structural histopathologic features in the patient groups. In the literature it is argued that the aortic dilation in the stenotic type is a functional haemodynamic induced problem, while the aortic wall problem in the root phenotype is genetically determined. Therefore, an isolated aortic valve replacement in stenotic BAV patients is believed to halt the aortic wall dilation. Girdauskas et al further state that BAV patients with root dilation require a more aggressive surgical approach, being a genetic, connective tissue disorder–like form of aortic disease which is independent of transvalvular flow perturbations (22). We however do not agree on this differentiation into two main groups for a several reasons. Firstly, all BAVs are intrinsically a congenital malformation and most probably also involve the wall of the ascending aorta independent of a stenotic or a root phenotype (23). Secondly, as shown in table 2, in our study population aortic valve pathology in non- and dilated BAV and dilated TAV was highly variable showing either stenosis or no stenosis, with or without regurgitation. The dilated BAV and MFS group even consisted of patients without valve pathology, which indicates that even in the absence of specific aortic valve pathology aortic complications can occur. We can however not completely refute the influence of haemodynamics on the development of aortic wall complications, as we do not have follow up data of the operated patients. This is a limitation of our study and an important aspect which should be taken into account in future research.

Histopathologically, in our study patients in the BAV group did not show marked degenerative features in the aortic media (5), which has also been reported by Bechtel et al (24). The media of the ascending aorta in MFS however showed resemblance with the TAD with significant cytolytic necrosis, VSMC apoptosis and degradation of the elastic lamellae, which could predispose the aortic wall for dissection. The MFS group thus showed



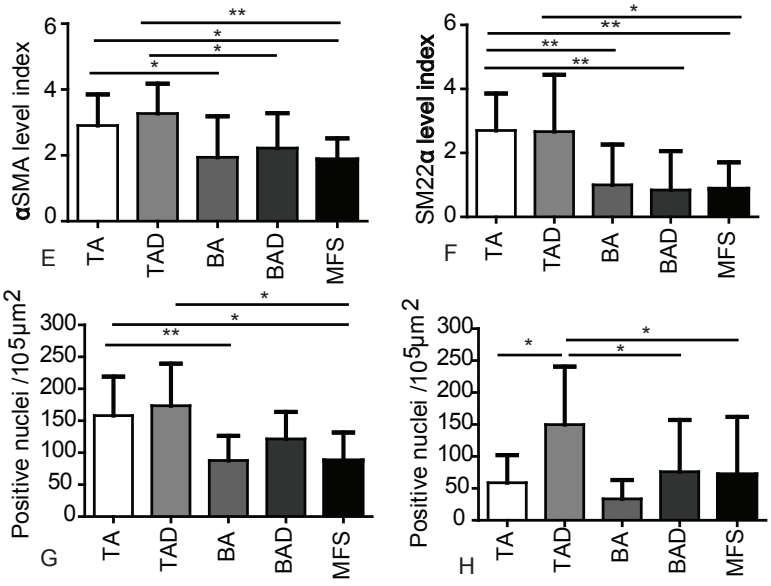


Figure 3 *Transverse histologic sections (5µm) stained with smooth muscle 22 alpha (SM22α), smoothelin, lamin A/C and progerin, markers for differentiation state of VSMCs.*
SM22α (A, graph 3F), αSMA (Graph E), and smoothelin (B) expression in MFS was similar to BAD and significantly lower as compared to TAD. Expression of lamin A/C (C, graph 3G) and progerin (D, graph 3H) in MFS was also significantly lower than in TAD. Magnification: A-D 100x; * = p<0.05; ** = p<0.01

Table 3 *Summary of the results*

	TA	TAD	MFS	BA	BAD
Intimal thickness	+++	+++	+/-	+/-	+/-
TGFβ intima expression	++	++	-	-	-
Inflammation	+/-	+++	+/-	+/-	+/-
Cytolytic necrosis	-	+	+	-	-
Apoptosis	++	+++	++	+	+
Elastic lamellae degeneration	-	+	+	-	-
Fibrillin-1	+++	+++	+	+	+
Smooth muscle cell expression (αSMA, SM22α, smoothelin)	+++	+++	+/-	+/-	+/-
Lamin A/C	+++	+++	+	+	+
Progerin	+	+++	+	+	+

characteristics of a weak aortic wall, although strikingly the pathologic aorta was not even markedly dilated in the MFS study population as compared to the TAD (Table 2).

In order to further elucidate the high incidence of aortic wall pathology in MFS and BAV, we studied the level of fibrillin-1 expression. The immature aortic wall of BAV and MFS shared a marked resemblance in the level of expression of fibrillin-1. Decreased *fibrillin-1* mRNA and fibrillin-1 protein have been demonstrated before in BAV individuals (8) and can lead to dissociation of VSMCs from medial matrix components (25). The diagnosis of MFS is however clinical and relies on a set of defined clinical criteria (the Ghent nosology) (2). The new diagnostic criteria emphasize cardiovascular manifestations of the disorder, in which aortic root aneurysm is one of the prime features. Recently Pape et al. (26) identified two *fibrillin-1* mutations in a population of eight BAV patients. These mutations had never been detected in MFS patients before. Moreover, these BAV patients did not meet the clinical MFS criteria; therefore they were not diagnosed with MFS (26). We can conclude that in the present study and previous studies a decrease in fibrillin-1 has been reported in four conditions: patients with BAV without a *fibrillin-1* mutation (8) (current study), patients with BAV and a *fibrillin-1* mutation with (9) or without (26) clinical MFS features and finally patients with clinically MFS and a *fibrillin-1* mutation but no BAV (27).

As fibrillin-1 is produced by VSMCs (28), a significant decrease (of structurally normal) fibrillin-1 is plausible in an aortic wall which constitutes less well differentiated VSMCs, also without apparent *fibrillin-1* mutations. As described in this study, in both MFS and BAV the aortic wall is primarily less well differentiated which can explain the secondary decrease in the (structurally normal) expression of fibrillin-1. Besides a decreased amount

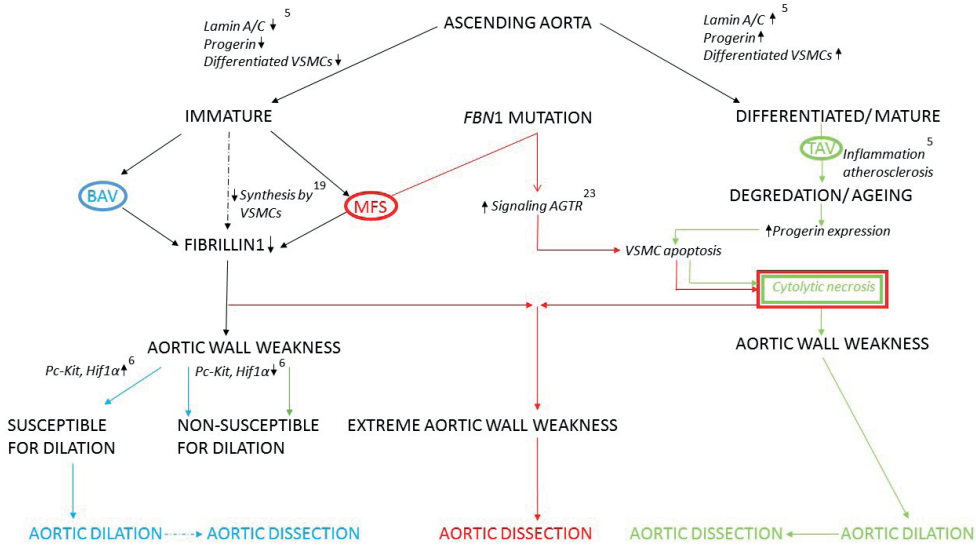


Figure 4 *Schematic overview of our hypothesis regarding similarities and differences in aortic wall pathology between bicuspid aortic valve (BAV), tricuspid aortic valve (TAV) and Marfan syndrome (MFS).*

According to this schematic overview the ascending aortic wall can be classified as being either mature or less well differentiated. Patients with a TAV have a differentiated/ mature vascular wall (differentiated vascular smooth muscle cells (VSMCs) and lamin A/C) in which cardiovascular ageing (increased expression of progerin with increased apoptosis, atherosclerose and inflammation) accompanies degeneration with features of cytolytic necrosis (CN) This progressive aortic wall pathology in TAV thus leads to a weakened aortic media causing complications as aortic dilation and dissection.

In BAV and MFS weakness of the aorta is however caused by immaturity of the aortic wall (deficient differentiated VSMCs and lamin A/C expression) instead of ageing. Fibrillin-1, pivotal for structural stability of the vessel wall is produced by VSMCs. Immaturity of the vessel thus leads to a quantitative decrease of fibrillin-1 in both BAV and MFS. In MFS additionally the FBN1 (fibrillin-1) mutation leads to VSMC apoptosis through an increased signaling of Angiotensin II receptors (AT2 receptor). CN, caused by VSMC apoptosis, in combination with the immature state of the aortic media renders the vascular wall extremely weak. Most probably presenting a different pathogenesis of aortic dissection in MFS as compared to the TAV.

of fibrillin-1 in the aortic wall, in line with earlier research we found that the distribution and localization of fibrillin-1 was different in the MFS and BAV, with more accumulation within the VSMCs (7,29,30), whereas it was mostly seen extracellular in the TAVs. As earlier described by Nataatmadja et al. the few extracellular fibers observed in BAV and MFS were thick and short (7). Hollister et al. also found a deficiency in the amount of microfibrillar fibers, analyzed immunohistochemically (30), comparable with our results.

As described above, cytolytic necrosis and VSMC apoptosis are observed in both MFS and TAV. However, VSMC apoptosis, which leads to cytolytic necrosis, seems to occur due to a different pathogenetic mechanism in MFS as compared to TAV. In TAV ageing, accompanied by an increased progerin expression, and atherosclerosis causes VSMC apoptosis (5,31), whereas as we have seen in this study in MFS these features are not apparent. In MFS the media of the aortic wall contains less well differentiated VSMCs similar to BAV. Therefore there must be a different pathway leading to cytolytic necrosis in MFS, which is on the one hand not related to cardiovascular ageing, but on the other hand can also not be associated directly to the immature state of the VSMCs as in BAV where cytolytic necrosisis scarce (5.24).

VSMC apoptosis and subsequent cytolytic necrosis in MFS has earlier been described to occur at a much younger age as compared to the TAV and as a direct consequence of the *fibrillin-1* mutation which leads to an increased Angiotensin II (AT2) receptors signaling and subsequently induction of TGFβ signalling (32-34). Two different pathologic pathways can thus be distinguished leading to aortic dissections. For clarification we have schematically hypothesized (Figure 4) how the various differentiation pathways and the involved gene pathways can lead to the observed histopathological similarities and differences we have seen between the investigated groups. In MFS there is thus a combination of immaturity of the aortic media and cytolytic necrosis, due to increased VSMC apoptosis, related to increased AT2 receptor signaling (31,32), rendering the aortic wall very weak. These observations could explain why angiotensin-receptor-blockers (ARBs), as Losartan, have been identified as a potentially therapeutic agent to prevent progressive dilation of the ascending aorta in MFS. ARBs reduce the signalling that occurs through both AT receptors, (AT1 and AT2 receptor). Many trials have emerged in recent years investigating the efficacy of ARB's in human patients with MFS (12-18). The first results from these studies show reduced dilation of the aortic root in MFS with Losartan (17).

A similar positive effect has not been reported in a population of BAV patients that were treated with an angiotensin inhibitor (ACE inhibitor) (19) which can be understood on the basis of our current results. In BAV medical treatment is not effective because the aortic wall in BAVs is not characterized by apoptosis and cytolytic necrosis. In conclusion, comparison of the aortic wall samples in BAV, MFS and TAV demonstrates that aortic dissections in MFS have a different pathogenesis as compared to the TAV, explaining the higher incidence and the younger age of occurrence. These findings could be relevant for understanding why medical treatment to inhibit angiotensin are seemingly successful in MFS and less effective in TAV and BAV patients also being prone for aortic wall pathology.

ACKNOWLEDGEMENTS

We thank the Heart Valve Bank Rotterdam, The Netherlands, for providing the described cryopreserved valves.

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CHAPTER 7

Wt1 expression in epicardium and vascular smooth muscle cells as a marker for aortic wall pathology in bicuspid aortic valve and Marfan syndrome

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Submitted

ABSTRACT

Objective Patients with a bicuspid aortic valve (BAV) and patients with Marfan syndrome (MFS) have increased susceptibility for aortopathy. In the heart, epicardial cells expressing Wilms tumor suppressor protein (Wt1) are known to become activated after myocardial infarction. We hypothesize that the arterial epicardium might show a similar response in pathologic conditions. Furthermore, variability of Wt1 activity in BAV patients without dilation might aid in distinguishing patients susceptible for future aortopathy.

Methods: Ascending aorta specimen of BAV (n=36) and tricuspid aortic valve (TAV) (n=23), non- and dilated (>45 mm) and non-dilated MFS specimen were investigated. The aorta was studied by immunohistochemistry, using Wt1, Retinaldehyde dehydrogenase-II (RALDH2), transcriptional activation of this enzyme is regulated by Wt1, and endothelial nitric oxide (eNOS), which regulates Wt1 expression.

Results: Endothelial cells, epicardial cells and VSMCs stained positive for Wt1, RALDH2 and eNOS. The endothelium did not show a difference in the ratio of positive compared to negative cells between the groups. In epicardial cells all groups exhibited Wt1 positive cells in a quiescent stage. Significantly increased Wt1 activity was observed in the epicardium and VSMCs of the dilated TAV group as compared to the dilated BAV group. Furthermore, a subset of non-dilated BAV patients showed a significantly increased Wt1 expression in the VSMCs.

Conclusion: In all non-dilated patients, a baseline activity of Wt1 is present in epicardial and VSMCs. In the TAD features of cardiovascular ageing as inflammation and increased progerin expression, which are not present in the BAD, lead to an increased Wt1 activity. In BAV, susceptibility for future aortopathy is probably associated to an increased MMP9 expression regulated by Wt1 through an eNOS mediated pathway.

INTRODUCTION

In the early 20th century thoracic aortic dilation in patients with a tricuspid aortic valve (TAV) had already been labelled as a degenerative disease, characterized by cytolytic necrosis (CN) (also termed medial degeneration) of unknown aetiology (1). Excessive medial degeneration thereby has been associated with destruction of the extracellular matrix (2) and loss of VSMCs (3), which can progressively weaken the aortic wall and ultimately lead to dilation.

Patients with a bicuspid aortic valve (BAV) and patients with Marfan syndrome (MFS) carry an increased risk for aortic dilation as compared to TAV. Earlier research has shown that both syndromes have similarities in the aortic media architecture, characterized by immature VSMCs ((4), Marfan submitted). This is in contrast to the aortic wall in TAV, which harbours differentiated VSMCs (4). However, prevalence of aortic complication is higher in the MFS as compared to the BAV, as nearly 30% of all patients with BAV will never experience progressive dilation of the ascending aorta. A different pathobiological mechanism thus seems to underlie aortic wall pathology in BAV and MFS, probably superimposed on the immature state of the aortic media. In BAV we could identify markers possibly associated with an increased susceptibility future complications The markers included c-Kit, a marker for dedifferentiated VSMCs, and its phosphorylated state (pc-Kit) triggered by the presence of matrix metalloproteinase-9 (MMP9), Hypoxia-Inducible-Factor-1alpha (HIF1α) and endothelial nitric oxide (eNOS).

The aortic wall in MFS did not show a similar expression of the susceptibility markers (Marfan submitted), on the contrary showed features of CN, which together with the immaturity could render the aortic media extremely weak and susceptible for aortic complications (Marfan submitted). We postulated earlier that the mechanism leading to CN is an increased angiotensin-receptor signaling due to the *FBN1* mutation in MFS rather than an increased progerin expression as seen in TAV (Marfan submitted). These findings could have implications for the applied preventive treatment modalities in both syndromes, such as the administration of angiotensin-receptor blockers (Marfan submitted). However, some issues still remain unexplained, which we aim to address in the current paper.

Firstly, most research on aortic dilation in BAV, TAV and MFS mainly approached the media or adventitia (5-17) of the vessel wall. The outermost covering of the ascending aorta, the arterial epicardium, has received hardly any attention

so far (18). In the heart, epicardial cells fulfill many important functions during embryogenesis and adult life (18;19). During the development of the heart, the epicardium undergoes a process called epithelial-to-mesenchymal transition. During epithelial-to-mesenchymal transition the morphologic appearance of the epicardial cells alters from squamous to cuboidal and the basement membrane delaminates. The epicardial cell delaminates becoming an epicardium derived cell (EPDC) which invades the subepicardial layer and subsequently migrates into the myocardium (20;21). EPDCs are mandatory for the development of the VSMCs of the coronary vasculature, contribute to the atrioventricular valves (reviewed: (18)). A large proportion of EPDCs differentiate into the cardiac interstitial fibroblasts of the heart, contributing to the fibrous skeleton. In adult life the epicardium and EPDCs are in a quiescent stage, but it has been shown that these inactive cells can become active in response to pathological processes such as myocardial ischemia (22). We postulate that a similar effect of the arterial epicardium is plausible, wherein aortic wall pathology might drive the activation of these cells. It has also been shown that endothelial nitric oxide (eNOS) regulates the expression of Wilms tumor suppressor protein (Wt1, which is an early epicardial marker for epithelial-to-mesenchymal transition (23)) and a deficiency of eNOS inhibits EPDC migration (24). Hence, we investigated the arterial epicardium on the expression of 1. Wt1; 2. Retinaldehyde dehydrogenase type II (RALDH2), Wt1 controls retinoic acid signalling through transcriptional activation of the enzyme Raldh2 (25) and is a specific marker for the epicardium (26); and 3. eNOS.

Another question which remained unexplained in our previous study was which trigger leads to an increased expression of MMP9 and thereby induces signaling of the pc-Kit cascade in the susceptible BAV group (27). It is known that Wt1 shuttles continuously between the nucleus, where it is active, to the cytoplasm, where it is in a quiescent stage (28). Marcet-Pollocios concluded that MMP9 expression is regulated by shuttling of Wt1 through a nitric oxide (NO) mediated pathway (29). Since all the identified susceptibility markers are found expressed in the aortic media, it is plausible that the marker which induces the expression of MMP9, might also be expressed by the VMSCs. However, the endothelial cells also express eNOS and Wt1 (13), and might be able to induce MMP9 expression paracrine. To address the missing link in the pc-Kit cascade, expression of Wt1, RALDH2 and eNOS was therefore also studied in the endothelial cells in the intimal layer and the VSMCs in the aortic media.

MATERIAL AND METHODS

Ethical approval and tissue samples

Ascending aortic wall samples were obtained from individuals with BAV and TAV, both with and without dilation and individuals with MFS, without dilation. Dilation was clinically defined by reaching an ascending aortic wall diameter of 45 mm and above (4;30). Sample collection and handling was carried out according to the official guidelines of the Medical Ethical Committee of the Leiden University Medical Centre (LUMC), Leiden, the Netherlands and the code of conduct of the Dutch federation of Biomedical Scientific Societies. The Heart Valve Bank, Thoraxcenter, Erasmus Medical Center (EMC), Rotterdam, provided 6 BAV samples without aortic dilation as these were not suitable for transplantation, as approved by their Scientific Advisory Board. Furthermore the Academic Medical Center (AMC) provided us with 8 MFS biopsy specimen, with approval of the Medical Ethical Committee. The study population was divided in five groups: 1) TAV without ascending aorta dilation, termed TA 2) TAV with ascending aorta dilation, termed TAD 3) BAV without ascending aorta dilation, termed BA 4) BAV with ascending aorta dilation, termed BAD 5) MFS without ascending aorta dilation, termed MFS shown in table 1.

Following excision, all specimen were fixed in 4% formalin for 24 hours, decalcified in Kristensen's solution (a formic acid buffer) for 120 hrs. and subsequently embedded in paraffin. Transverse sections (5 µm) were mounted on pre-coated Starfrost slides (Klinipath B.V., 3057-1, Duiven, The Netherlands) to allow comparing different expression profiles on sequential sections.

Immunohistochemistry

Immunohistochemical staining was performed following the protocol for deparaffination, antigen retrieval and staining as described in our previous study (4). Primary antibodies applied to the slides were Wt1 1/300 (Product number CA1026, Calbiochem, Billerica, USA), RALDH2 1/100 (Product number ab75674, Abcam, Cambridge, UK) and eNOS 1/100 (Product number, PA1037, Thermo scientific, Rockford, USA). Secondary antibodies applied were: 1/200 goat-anti-rabbit-biotin (GAR), (Product number BA-100, Vector Laboratories, Burlingame, USA) and 1/66 normal goat serum (NGS), (Product number S1000, Vector Laboratories, Burlingame, USA).

Table 1 *Patient characteristics are shown in table 1*

Characteristic	TA	TAD	BA	BAD	MFS
	N=11	N=12	N=17	N=19	N=8
Specimen obtained from	Post mortem, LUMC	Elective repair of the ascending aorta, LUMC	Stentless root replacement in the LUMC and six biopsies from the EMC.	Elective repair of the ascending aorta, LUMC	Elective repair of a dilated aortic root (>45mm), AMC.
Exclusion criterion	MFS	MFS	MFS	MFS	BAV
Age (years)	64.5 ± 9.0	72.3 ± 11.2	55.8 ± 9.8	60.7 ± 7.8	34.1 ± 11.8
Males (%)	54.5%	33.3%	70.1%	84.2%	62.5%
Females (%)	45.5%	66.7%	29.4%	15.8%	37.5%
Ascending aorta diameter (mean)	*	55.0 ± 10.7	36.5 ± 7.4**	52.7 ± 6.2	28.4 ± 12.8
Aortic root diameter (mean)	***	***	***	***	48.1 ± 3.0

TA: tricuspid valve, without dilation; TAD: tricuspid valve, with dilation; BA: bicuspid valve, without dilation; BAD: bicuspid valve, with dilation; MFS: Marfan syndrome, without dilation; LUMC: Leiden University Medical Center, EMC: Erasmus Medical Center, AMC: Academic Medical Center.

* data unavailable, clinically defined as non-dilated by pathologist. ** data unavailable for 5 patients, clinically defined as non-dilated by pathologist. *** aortic root diameters unavailable

Immunofluorescence staining

To detect co-expression, we performed double immunofluorescent stainings. Sections were deparaffinated, rehydrated and subjected to antigen retrieval as described. Tissue sections were incubated with primary antibodies Wt1 1/100, RALDH2 1/100 and eNOS 1/100 overnight (4°C), followed by incubation with secondary antibody Cy3 donkey anti mouse IgG (Product number 715-165-150 Jackson Immunoresearch, 715-165-150) for Wt1 and Alexa Fluor 647 donkey anti-rabbit IgG (Invitrogen, A-31573) for eNOS (1

hr, 20°C). Cy3 and Alexa Fluor 647 were preferred secondary antibodies, because of green autofluorescence of the elastic lamellae. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Finally, slides were mounted with ProlonGold (Invitrogen, P36930).

Morphometric analyses

Sections were studied with a Leica BM5000 microscope equipped with plan achromatic objectives (Leica Microsystems, Wetzlar, Germany).

As handling during excision of aortic tissue can easily damage epicardial cells, we first identified Wt1 stained specimen in which the adventitia showed an intact monolayer of epicardial cells: TA n=4, TAD n= 4, BA n=4, BAD n=8 and MFS n=2. The number of MFS patients was to less to allow an adequate statistical analysis. Analysis of epicardial Wt1, RALDH2 and eNOS expression was therefore performed on the BA, BAD, TA and TAD group. In the MFS group, only the morphologic appearance of the epicardial cells could be described. To determine the level of expression of the Wt1, eNOS and RALDH2 markers in the aortic media, the number of positively stained nuclei (for Wt1 and eNOS) and cytoplasmic positive cells (for RALHD2) was counted using ImageJ on three predetermined locations (left, middle and right) of every section. These we refer to as 'microscopic fields' and maintained in evaluation of all sections. A threshold was applied to filter background noise. The total number of cells (positively stained and negative nuclei and cytoplasm) was not different in all specimens. In each microscopic field the number of cells positive for Wt1, RALDH2 and eNOS was therefore normalized to the total number of cells per 10^5 μm^2. Finally, the number of normalized positive cells for each staining was averaged between the three microscopic fields. All specimens were re-evaluated by an independent, experienced histopathologist who was blinded to the clinical data.

Statistical Analysis

All numerical data are presented as mean ±SD of 3 fixed microscopic fields on each stained slide. Statistical differences were evaluated with the Mann-Whitney U-test for comparison between the groups. Significance was assumed when $p<0.05$ using SPSS 20.0 software program (SPSS Inc. Chicago, USA). We have performed a one, two and three way ANCOVA test to correct for age and gender. Graphpad software was used to create graphics of statistical analysis.

RESULTS

Wt1, RALDH2 and eNOS expression pattern in the ascending aorta

The aortic wall consists of three layers: the tunica intima, media and adventitia. Expression of Wt1, RALDH2 and eNOS was seen in all three layers, as will be presented in the next segments. Table 2 gives an overview of the stained markers in the endothelial cells, vascular smooth muscle cells and the epicardial cells (cytoplasmic and/or nuclear staining)

Endothelium

Endothelial cells lining the luminal surface of the tunica intima stained predominantly positive for Wt1 in the nucleus. Morphologic appearance of all Wt1 positive cells was similar, being squamous. We analysed the total Wt1 positive population in each sample of the five groups and calculated the ratio of Wt1 positive compared to Wt1 negative cells. No significant difference was seen between the five groups (Table 2 and 3).

All Wt1 positive endothelial cells also showed eNOS and RALDH2 expression, with no difference in the ratio of positive compared to negative cells between the five groups (Table 2 and 3).

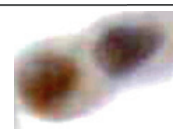
Media

In the aorta a subset of VSMCs variably stained positive for Wt1 in the nucleus and/or cytoplasm. For analysis nuclear Wt1 level of expression was considered.

The number of positively stained VSMCs in the aortic media was significantly highest in the TAD group. In the BAD however the VSMCs stained significantly less as compared to the TAD ($p<0.0001$), the expression was higher than in the BA group ($p=0.015$). Between the BA, TA and MFS non-dilated aortic wall groups no significant difference was found in medial expression of Wt1 between the BA and TA group, whereas the MFS showed significantly less expression as compared to both BA and TA ($p=0.0022$ and $p=0.0018$ respectively) (Fig. 1A, Table 2 and 3). All Wt1 positive VSMCs also stained cytoplasmic for RALDH2 (Fig. 1B, Table 2 and 3).

Nuclear eNOS staining of the VSMCs was markedly seen in Wt1 and RALDH2 positive cells (Fig. 1A-G), however some cells stained positive for only eNOS. The number of eNOS positive cells was significantly higher in all TAVs (TA and TAD), as compared to all BAVs (BA and BAD) ($p=0.0234$ and

Table 2 *overview of the stained markers*

Antigen	Location	Staining	Positive staining	Negative staining
Wt1	Endothelial cells	Nuclear and cytoplasmic		
	Media (VSMCs)	Nuclear and cytoplasmic		
	Epicardial cells	Nuclear and cytoplasmic		
RALDH2	Endothelial cells	Nuclear and cytoplasmic		
	Media (VSMCs)	Cytoplasmic		
	Epicardial cells	Cytoplasmic		
eNOS	Endothelial cells	Nuclear		
	Media (VSMCs)	Nuclear		
	Epicardial cells	Nuclear		

Wilms tumor suppressor protein (Wt1), Retinaldehyde dehydrogenase-II (RALDH2) and endothelial nitric oxide (eNOS), in the endothelial cells, vascular smooth muscle cells (VSMCs) and the epicardial cells. Positive and negative staining is indicated, either cytoplasmic or nuclear.

p=0.0353 respectively). In the MFS group, the number of eNOS positive cells was comparable to the BA group (p=0.1529) (Fig, 1A-C, Table 2,3).

Adventitia and epicardial cells

The adventitial side of the vessel wall, bordering the pericardial cavity, is lined by arterial epicardial cells. Wt1 expression was variably present in the

nucleus and/or cytoplasm. An epicardial cell was identified as positive for Wt1 if either the nucleus or cytoplasm stained positive. The epicardial cells could morphologically be distinguished in three types: Wt1 negative squamous, Wt1 positive squamous and Wt1 positive cuboidal phenotype. The Wt1 positive cells were often found in epicardial invaginations/ inpocketings (Fig. 2A, B). We first analysed the total Wt1 positive population (both squamous and cuboidal) in each sample and calculated the ratio of Wt1 positive compared to Wt1 negative cells. No significant difference was seen between the four groups (mean ratio 0.44). The Wt1 positive squamous epicardial cells represent an initial phase of epicardial activity, whereas cuboidal cells are prone for epithelial-to-mesenchymal transition. Therefore we assessed the epicardial activity, defined as the transition from an initial quiescent phase to active epicardium, by the ratio of Wt1 positive squamous and cuboidal epicardial cells. A significant increase in epicardial activity based on number of cuboid cells was seen in the TAD as compared to the TA (p=0.0286), and the epicardial activity was significantly greater in TAD compared to BAD (p<485) (G, Table 2 and 3). The epicardial cells of the ascending aorta of the MFS group were mostly squamous in appearance and showed minimal Wt1 expression and when present only in the cytoplasm. In dilated specimen of

Table 3 *An overview of the results and the statistical significance.*

Antigen	Location	TA	TAD	BA	BAD	MFS	P-value
Wt1, RALDH2 and eNOS	Endothelial cells	0.54*	0.54*	0.54*	0.54*	0.54*	ns
Wt1, RALDH2	Media (VSMCs)	346***	1207***	344***	581***	100***	TA-TAD and TAD-BAD: p<0.0001 BA-BAD: p=0.015; TA-MFS: p=0.0018; BA-MFS: 0.0022
eNOS	Media (VSMCs)	197***	159***	73***	103***	48***	TA-BA: p=0.023, TAD-BAD: p=0.0353, TA-MFS: p=0.02
Wt1, RALDH2 and eNOS	Epicardial cells	0.73**	6.54**	1.05**	2.99**	-	TA-TAD: P=0.0286 TAD-BAD: P=0.0485

Wilms tumor suppressor protein: Wt1, Retinaldehyde dehydrogenase-II: RALDH2, endothelial nitric oxide: eNOS. TA: tricuspid valve, without dilation; TAD: tricuspid valve, with dilation; BA: bicuspid valve, without dilation; BAD: bicuspid valve, with dilation; MFS: Marfan syndrome, without dilation. *: mean ratio positive/ negative cells; ** ratio positive squamous/ positive cuboidal cells; *** mean number of positive cells/ 10^5 µm2

TAV invasion of epicardial cells into the subepicardial layer was also seen (not quantified), demonstrating epithelial-to-mesenchymal transition resulting in EPDCs entering the adventitia, which was not prominent in BAV and MFS patients. Wt1 positive epicardial cells also stained positive cytoplasmic for its downstream mediator RALDH2 in all groups (Table 2). Moreover expression of eNOS was also observed in the same epicardial cells (Fig. 2C-F, Table 2).

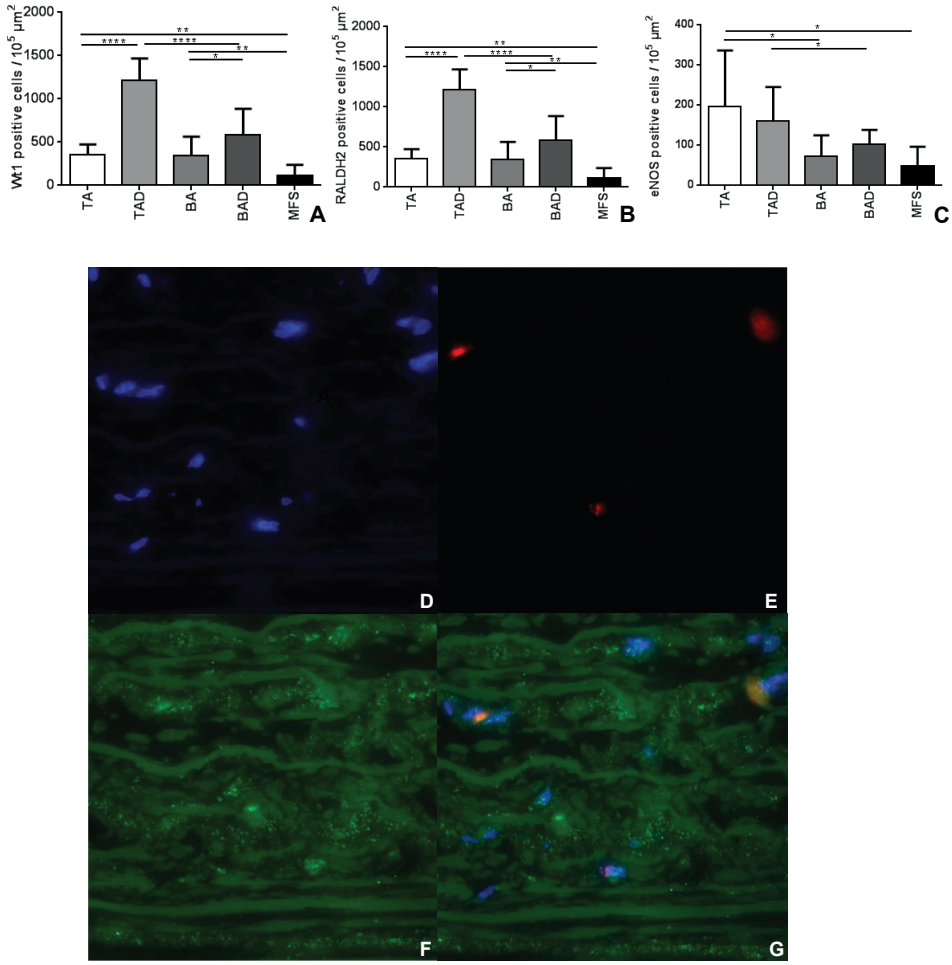


Figure 1 *Expression of Wilms tumor suppressor protein (Wt1) (Graph A), Retinaldehyde dehydrogenase-II (RALDH2) (Graph B) and endothelial nitric oxide (eNOS) (Graph C) in the VSMCs of the aortic media.*
Transverse histologic sections of the aortic wall (D-G). Immunofluorescent staining of Wt1 (red), eNOS (green) and DAPI (blue) in the VSMCs. Scale bar: 25 µm

Medial expression of Wt1 in the BAsusceptible and BAnon-susceptible group

As shown in Figure 3, the group of patients prone for future aortopathy (BAsusceptible) showed an increased phosphorylation potential of c-Kit under influence of MMP9. Pc-Kit in turn led to an increase in HIF1α and eNOS expression as compared to the non-susceptible BA group. In this study we found that the expression of Wt1 was higher in the BA-susceptible as compared to the non-susceptible group ($p<0.001$) (Fig. 3).

DISCUSSION

Thoracic aortic dilation is a relatively common medical problem. The fact that the first presentation can be life threatening in the form of dissection or rupture of the aortic wall makes it even more crucial to understand the underlying pathobiology. The results of this study provide evidence that an increased expression of Wt1 in the epicardial cells and VSMCs might be a marker of progressive pathology of the aortic wall.

eNOS and Wt1 in the arterial endothelium

We describe for the first time that Wt1 is expressed in the arterial endothelium, indicating that this marker is not specific for the epicardium (23). Functionality of Wt1 was confirmed by the co-expression of RALDH2 in the same cells.

Kispert et al. have earlier shown that Wt1 is expressed in endogenous endothelium of the developing heart (31). It has however not been described in adult vasculature before. In our study we found that Wt1 is expressed in a similar fashion in the non-dilated aortic wall of BAV, TAV and MFS.

Besides during normal development, an enhanced cardiac endothelial Wt1 expression has also been described in pathologic conditions in a rat model (32). We however did not observe differences in Wt1 or RALDH2 expression in the endothelium of the dilated aortic walls of BAV, TAV and MFS as compared to the non-dilated aorta.

We also studied the eNOS expression in the endothelial cells. The level of expression was not different between the non- and dilated groups of BAV and TAV, as described before (26). In this study we found that the endothelial eNOS expression in the MFS group was also similar. Expression of Wt1 and eNOS has been linked in the epicardial cells (33), however the observed co-expression in the endothelium has not been described before.

Wt1 in the arterial epicardium

In the epicardium of the heart an increased Wt1 activity had been noted as a response to pathology. We hypothesized that the epicardial cells covering the aorta might show a similar mechanism. In the healthy adult heart, the epicardium always harbours some Wt1 activity. In this study we found that in all five groups the epicardial layer exhibited patches of quiescent epicardial cells alternating with cuboid epicardial cells that expressed Wt1 and RALDH2. The dilated aortic specimens showed an increase of epicardial activity, possibly as a response to pathologic processes. This increase was significantly greater in the TAD as compared to the BAD.

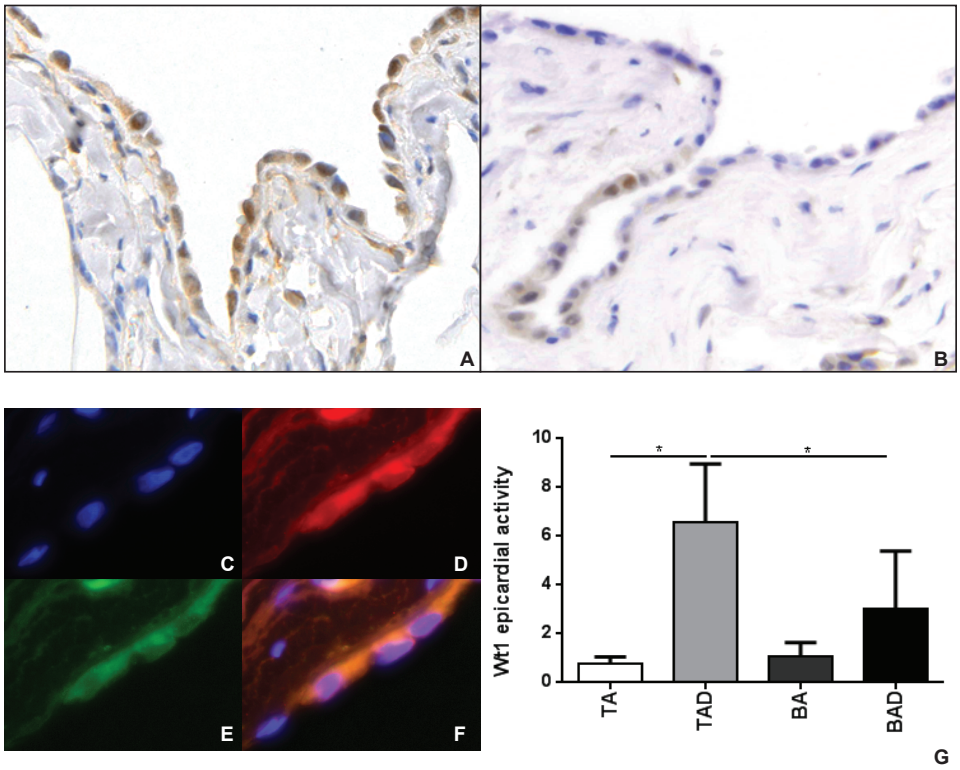


Figure 2 *Transverse histologic sections of the aortic wall.*
DAB staining (brown) of Wilms tumor suppressor protein (Wt1) is shown in the TAV group, with aortic dilation (TAD) and BAV, with aortic dilation (BAD). Immunofluorescent staining (C-F) of Wt1 (red), endothelial nitric oxide (green) and DAPI (blue). Epicardial activity, defined as the ratio of Wt1 positive squamous and cuboidal epicardial cells, is shown in Graph G for TA: tricuspid valve, without dilation; TAD: tricuspid valve, with dilation; BA: bicuspid valve, without dilation; BAD: bicuspid valve, with dilation.

The less apparent increase of Wt1 activity in BAD might be associated with the immaturity of the aortic wall and the lack of cardiovascular ageing. We base our assumption on the observation that the pathologic dilated aortic wall in TAD shows features of cardiovascular ageing as inflammation, atherosclerosis and an increased progerin expression. In the coronary vasculature comparable characteristics of cardiovascular ageing lead to myocardial ischemia (34) and subsequent activation of the epicardial Wt1 (22).

As the absolute diameters of the ascending aorta are somewhat similar in BAD and TAD, Wt1 expression in the epicardium is not likely to be associated with the degree of dilation, rather with the pathologic conditions leading to it.

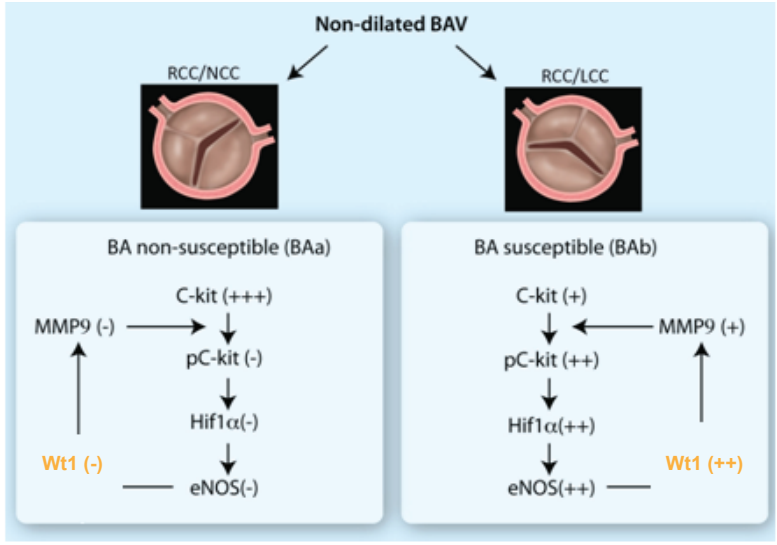
As we performed our stainings on the complete vessel wall, we also found for the first time expression of eNOS in the epicardial cells. The epicardial cells which expressed Wt1 and RALDH2 were also positive for eNOS. Although in eNOS deficient mice an inhibition of EPDC migration has been noted (24), such prominent role of eNOS in the arterial epicardium of BAV cannot be concluded from this study. Future research should focus on arterial epicardial cells in BAV mouse models, to elucidate a possible role of eNOS related to the decreased Wt1 activity.

Wt1, RALDH2 and eNOS in the aortic media

We describe in this study the co-expression of Wt1, RALDH2 and eNOS in the VSMCs of the aorta. As described for the endothelial and epicardial cells, co-localization of Wt1 with RALDH2 confirmed the functionality of Wt1 in the VSMCs. Wt1 and RALDH2 positive cells were variably co-expressed with eNOS. VSMCs could on the other hand also only be positively stained for eNOS.

In a previous study, we suggested that a subset of BAV patients without apparent ascending aorta dilation, but with an increased susceptibility for future aortic wall pathology can be identified on basis of a panel of markers (27). The identified markers could be linked to each other in a cascade, which was found active only in the BA susceptible group. MMP9 is an important regulator of the cascade as its presence triggers the phosphorylation of c-Kit. Pc-Kit in turn induces the expression of HIF1α, which then stimulates the expression of eNOS in the BA- susceptible group (Fig. 3) (27).

In this study we found that the VSMCs expression of Wt1 is significantly higher in the BA-susceptible as compared to the non-susceptible group.



	BA non-susceptible	BA susceptible	BAD	MFS
C-Kit	+++	+	+	+++
Pc-Kit	-	++	++	-
HIF1α	-	++	++	-
eNOS	-	++	++	-
Wt1	-	++	++	-
MMP9	-	+	+	-

Figure 3 *The observed differences in expression of molecular biologic markers are presented in a cascade.*
In the bicuspid aortic valve group, without aortic dilation (BA) two subgroups are distinguishable: BAa and BAb. The non-susceptible group (BAa), which has a similar expression pattern as the MFS group (Marfan syndrome, without aortic dilation), showed significant higher expression of c-Kit as compared to the susceptible group (BAb), which has a similar expression pattern as the BAD (Bicuspid aortic valve, with aortic dilation). MMP9, scarcely expressed in the non-susceptible group and abundantly in the susceptible, is involved in the phosphorylation of c-Kit. Lower expression of c-Kit in the susceptible group as shown in the cascade, could therefore be the result of its conversion to pc-Kit. Besides pc-Kit, HIF1α was richly expressed in the susceptible group, in contrast to the non-susceptible. HIF1α was only found co-expressed with pc-Kit. eNOS, regulated by HIF1α, is less expressed in the non-susceptible and significantly higher expressed. Wt1 showed a similar expression pattern of low expression in the non-susceptible and significantly higher expression in the susceptible group.

This suggests a positive link between eNOS, Wt1 and MMP9 which has not been described before. It has earlier been shown however that Wt1 regulates MMP9 through a nitric oxide (NO) mediated pathway (29). Wt1 is a transcriptional factor that acts as a gene repressor (35-37), which can participate in transcriptional regulation of MMP9 too (29). NO is an important regulator of MMP9 (38-41). Mice with mutant inducible NOS (iNOS), produce significantly less MMP9 than wild-type mice (42;43). Also in human NO is shown to be an important up-regulator of MMP9 (29). Considering this, Marcet-Pollocios concluded that through a NO mediated pathway shuttling of Wt1 occurs: from the nucleus, where it is active, to the cytosol, where it loses its repression potential, leading to a de-repression (and thus an increase) of the MMP9 expression (29). These findings can be applied to the described panel of susceptibility markers as shown in Fig. 3, and previously by Grewal et al. (27). Expression of eNOS is significantly higher in the BA-susceptible as compared to the BA-non-susceptible and MFS, which can explain the shuttle of Wt1 from the nucleus to the cytoplasm in the susceptible group. As a consequence MMP9 expression was higher in the BA-susceptible and can lead to an increase in phosphorylation of c-Kit.

Comparable to the epicardium we further see a difference in the VSMCs Wt1 activity between the BAD and TAD. We postulate that that this difference, like in the epicardial cells, is based on the immature state of the vessel wall and the lack of degenerative features, like in the epicardial cells.

In this study we could thus complete the cascade by adding Wt1 to the panel of susceptibility markers. Further research is however needed to understand what triggers the activation of this cascade.

ACKNOWLEDGEMENTS:
We thank the Heart Valve Bank Rotterdam, The Netherlands, for providing the described cryopreserved valves.

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CHAPTER

8

Summary and Future Perspectives
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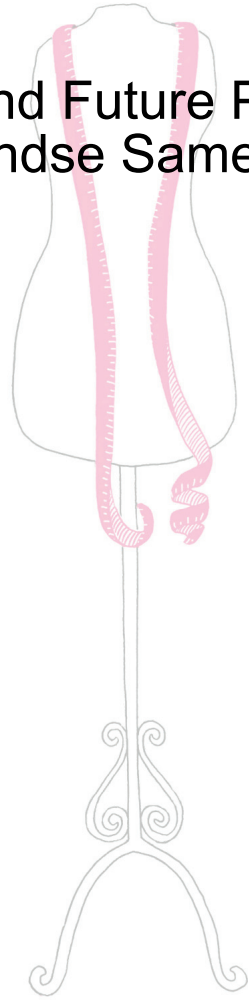

SUMMARY AND FUTURE PERSPECTIVES

The prevalence of aortic dilation and related complications as rupture and dissection is higher in patients with a bicuspid aortic valve (BAV) as compared to patients with a tricuspid aortic valve (TAV), although not every individual carries an increased risk. It is therefore essential to identify those patients who are less susceptible for aortic wall pathology, as preventive ascending aortic surgery would not be necessary in this group. Since aortic diameter as a criterion for surgery is only decisive at population level, it would be very valuable to have tailored risk stratification at patient level.
The purpose of this thesis was therefore to investigate the possibility to identify patients with BAV, without apparent dilation, with an increased susceptibility for future complications as aortic dilation and dissection. Furthermore the biological mechanism underlying aortic wall pathology in BAV was compared to a known genetically determined syndrome with an increased risk of aortopathy being Marfan syndrome.

In **Chapter 1** the development of BAV is described. An overview is provided of commonly observed complications in patients with a BAV. Also, the aim of this thesis is presented and an outline of the chapters is given.

In **Chapter 2** histological and molecular genetic aspects of the normal and abnormal development of the aortic wall and semilunar valves are discussed. In this review we describe how a defect during early embryogenesis can distort the contribution of neural crest cells and second heart field derived cells in the valvulogenesis and development of the ascending aortic wall. The genetic origin of syndromes associated with aortic dilation (including Marfan syndrome, Ehlers-Danlos syndrome, Smad3 mutations and Loeys-Dietz syndrome) are discussed. The central role of transforming growth factor β (TGFβ) is presented and linked to the embryonic development.
We concluded that aortic wall pathology associated with BAV could be the result of a developmental defect during embryogenesis. These contributions alone, however, are not sufficient to explain the clinical heterogeneity seen in BAV patients, as not all individuals with BAV develop aortic complications during their life. To identify the patients susceptible for dilation, we first aimed for a more in-depth understanding of the pathobiology leading to aortopathy in BAV as compared to TAV, which is discussed in the next chapter.

In **Chapter 3** differences in the histopathology of the aortic wall of patients with a BAV and TAV are described. Uniquely, not only dilated aortic wall specimen were investigated but also non-dilated specimen of both groups, representative for early lesions rather than of end-stage disease. The expression of vascular smooth muscle cell maturation markers, lamin A/C, which plays a pivotal role in vascular smooth muscle cell differentiation, and its splicing variant progerin indicative of aging, were studied. The results of this study show that the structure of the non-dilated and dilated aortic wall in bicuspidy and tricuspidy are intrinsically different. In bicuspidy lower lamin A/C expression is possibly linked with a defective smooth muscle cell differentiation, seen in these patients. This vessel wall immaturity, which can account for the increased weakness of the aortic wall, is encountered in all patients with BAV, including those who are less susceptible for future aortic complications. To identify the subset of patients which has an increased susceptibility for aortic complications we subsequently searched for markers, both clinical and immunohistochemical, predictive for aortic wall pathology in the non-dilated BAV patients.

Chapter 4 concerns the recognition of BAV patients with an increased vulnerability for aortic wall pathology, based on clinical characteristics. A study population of 255 patients was evaluated. Analysis of patient characteristics, clinical course and echocardiographic parameters including valve morphology led to the identification of predictors for future complications. In this study a clinical risk stratification model is presented to detect patients with an increased susceptibility. This working model shows that males with a BAV with fusion of the right and left coronary cusp, a complete raphe, hypertension and no statin use exhibit the highest risk of complications and should be monitored more closely.

Chapter 5 details the search for molecular biologic markers in identifying patients with an increased vulnerability for aortic complications, which can aid in patient selection for surgery. The aortic wall in patients with BAV and TAV, both dilated and non-dilated were studied. We studied a signaling pathway characteristic for cellular dedifferentiation, including the markers c-Kit, a marker for dedifferentiated vascular smooth muscle cells, and its phosphorylated state phosphorylated c-Kit (pc-Kit) triggered by the presence of matrix metalloproteinase-9 (MMP9), as well as the Hypoxia-Inducible-Factor-1alpha (HIF1α) and endothelial nitric oxide (eNOS). We

found that this pathway was markedly expressed in the dilated BAV group and expression was completely comparable to only a subgroup of the non-dilated BAV group. Whereas the remainder of the non-dilated BAV group was significantly distinct. This difference between the dilated BAV group and the susceptible non-dilated BAV group was further confirmed in the expression of TGFβ and phosphorylated Smad2. Next to the expression pattern, similarity in the dilated and the susceptible non- dilated BAV group was also noted clinically in the most common variant of commissure position and conjoined raphe of the BAV, being fusion of the right and left coronary cusp. This was in line with our findings described in **Chapter 4**. Based on these observations we considered the susceptible non-dilated BAV group a likely candidate for future dilation as opposed to the non-susceptible non-dilated BAV group. In this paper we also discussed the role of haemodynamic influences on development of aortic wall pathology in BAV by comparing expression of haemodynamic related markers in specimen obtained from the convex and concave side of the ascending aortic wall. A significant effect of shear stress on aortic complications in BAV was not observed.

In **Chapter 6** we compared the aortic wall in BAV and TAV to a syndrome which is highly prone for aortic wall pathology, being Marfan syndrome. Mutations in the *fibrillin-1* gene are found in 90-95% of all Marfan syndrome cases. In this study we investigated whether histopathological similarities are present between the aortic wall of BAV, TAV and Marfan syndrome, despite important clinical differences. We sought to identify an immunohistochemical explanation for the increased susceptibility for aortic dissections in Marfan syndrome, even in a non-dilated aortic wall, and at a younger age as compared to the BAV and TAV. The aortic media in Marfan syndrome showed a similar immature state of the vascular smooth muscle cells as in BAV. In both patient groups the level of expression of fibrillin-1 was also found decreased together with an altered distribution and localization of the protein as compared to the TAV. As fibrillin-1 is produced by vascular smooth muscle cells, a significant decrease of fibrillin-1 is plausible, also without apparent *fibrillin-1* gene mutations, due to the less well differentiated vascular smooth muscle cells.
The media in Marfan syndrome however also showed some histopathological resemblance with the dilated TAV group being significant cytolytic necrosis also referred to as medial necrosis, vascular smooth muscle cell apoptosis (programmed cell death) and degradation of the elastic lamellae. Vascular

smooth muscle cell apoptosis, which leads to cytolytic necrosis, however seems to occur due to a different pathogenetic mechanism in Marfan syndrome as compared to the dilated TAV group. In the dilated TAV ageing, accompanied by an increased progerin expression, and atherosclerosis causes vascular smooth muscle cell apoptosis, whereas these features were not apparent in the Marfan syndrome. In Marfan syndrome, vascular smooth cell apoptosis and subsequent cytolytic necrosis can occur as a direct consequence of the *fibrillin-1* gene mutation which leads to an increased Angiotensin II receptor signaling in Marfan syndrome. This combination of immaturity of the aortic media and cytolytic necrosis, renders the aortic wall very weak. These observations could explain why angiotensin-receptor-blockers as losartan, which reduce signaling through Angiotensin II receptor, have been identified as a potentially therapeutic agent to prevent progressive dilation of the aorta in Marfan syndrome. In BAV such medical treatment is not as effective because the pathology in the aortic wall in BAVs is not characterized by cytolytic necrosis.

In **Chapter 7** we investigated the expression of Wilms tumor suppressor protein (Wt1), which has been found to become active in response to pathologic conditions in epicardial cells covering the myocardium of the heart. We hypothesized that the arterial epicardium might show a similar response in pathologic conditions. Wt1, retinaldehyde dehydrogenase-II (RALDH2), a downstream target of Wt1, and eNOS, which regulates Wt1 expression, were studied in the arterial epicardium in the BAV, TAV and Marfan syndrome. In search of the missing link between eNOS and matrix metalloproteinase (MMP9) in the phosphorylated c-Kit cascade described in Chapter 5, Wt1, RALDH2 and eNOS were also studied in the endothelial cells and medial vascular smooth muscle cells. From this study we could conclude that in all non-dilated patients, a baseline activity of Wt1 is present in epicardial and vascular smooth muscle cells. In the dilated TAV group features of cardiovascular ageing as inflammation and increased progerin expression, which are not present in the dilated BAV group, lead to an increased Wt1 activity.
We have shown earlier (Chapter 5**)** that within the non-dilated BAV group a subset of patients can be distinguished with an increased susceptibility for future aortic wall pathology on basis of the expression pattern of a panel of markers. The ‘susceptible group’ showed an increased expression of phosphorylated c-Kit, HIF1α, eNOS and MMP9. In this study we found that the Wt1 expression in the susceptible group is also significantly higher as

compared to the remainder of the non-dilated BAV group. Wt1 is a transcription factor that acts as a gene repressor, which can participate in transcriptional regulation of MMP9 too. It has been reported earlier that eNOS stimulates the shuttle of Wt1 from the nucleus (where it is active) to the cytoplasm (where it loses its repressor activity). It is therefore plausible that the increased eNOS expression in the susceptible group leads to an enhanced MMP9 expression in this group, which subsequently stimulates the phosphorylation of c-Kit.

In this thesis we have added some new perspectives to the problem of decision making regarding patients with BAV and associated aortic wall pathology. The recommending guidelines for surgery are up till now mainly based on aortic diameter, although already some exceptions for patients with BAV are made in practice (1). In this guideline a distinction between BAV patients with and without risk factors is made, eluding to the fact that not all BAV patients are equal. Our studies underline that exclusive use of aortic wall dimensions is not sufficient in selecting patients vulnerable for future aortic wall complications. It is however not possible as yet to make a direct extrapolation from our histopathology observations in a patient to the clinical practice. We should therefore, in future research, focus on the translation of the histopathology findings to 1) blood borne markers, 2) on site biopsy evaluation during surgery, as performed in research of malignancies, 3) refinement of imaging techniques, which can be applied in the clinical practice, to distinguish the susceptible from the non-susceptible cases with BAV.

An important aspect which has not received sufficient attention in our current work is the follow up of patients after the aortic valve surgery. Several studies have indicated that in cases of an isolated aortic valve replacement the risk of postoperative ascending aortic dilation is minimized (2-5). Whereas Yasuda et al. indicated in their study that the aorta grows faster in BAV patients even after an aortic valve replacement has been performed (6).

Recent publications take the discussion on the role of haemodynamics in BAV patients in the development of aortic wall dilation to another level. This report focusses on a difference in dilation progress dependent on whether the diseased aortic valve was stenotic or regurgitant with concomitant root dilation (7) in the latter. It is argued that the aortic dilation in the “stenotic phenotype” is a functional haemodynamic induced problem, while the aortic wall problem in the “root phenotype” is genetically determined. We, however, do not completely agree on this differentiation into two main groups, as this cannot

be supported from a developmental point of view. All BAVs are intrinsically a congenital malformation and we have shown that this developmental disorder most probably also involves the wall of the ascending aorta independent of a stenotic or a root phenotype. From our studies we could also not divide our cases in pure stenotic and regurgitant phenotypes as in many cases both phenomena are encountered. In future, besides a prospective clinical follow up of the study population after aortic valve replacement, it would thus also be valuable to expand the histopathology research subdivided on clinically well-defined stenotic and root phenotype patients. Thereby investigating in more detail the influence of the jet stream site using the opposite site of the aortic wall as a control. Most importantly this should include patients without an overt dilation as this category is not mentioned in the literature as yet. Haemodynamic markers should include shear stress responsive factors such as Krüppel like factor 4 (KLF4) (8).

These detailed studies might also shed more light on our consistent observation of diminished expression of maturation markers including alpha smooth actin both in the dilated and the non-dilated BAV aortic walls. This can confirm or exclude the possibility that shear stress, which is the frictional force acting in the direction of blood flow on the inner surface of blood vessels, is the cause of an inhibition of the expression of maturation vascular smooth muscle cell markers as has been observed in *in vitro* studies (9).

The localization of aortic wall dilation is typically different between patients with Marfan syndrome and BAV patients (10). Molecular biological research has shown that in Marfan syndrome *fibrillin-1* mutations, typical for Marfan patients, lead to an increased Angiotensin II receptor signaling and subsequent induction of TGFβ signaling (11). In BAV patients a reduction of fibrillin-1 protein expression and rarely a mutation in the *fibrillin-1* gene has also been described (Chapter 6, (12;13)). We, however, showed in our study that the expression level of TGFβ is significantly lower in the dilated BAVs as compared to controls, thus differentiating them from the Marfan patient with a *fibrillin-1* mutation. It is therefore interesting to investigate whether the reported Angiotensin receptor increase in Marfan patients is also found in the wall of BAV patients or that this phenomenon is purely linked to the mutation and not to the observed diminished expression of fibrillin-1 protein as seen in both Marfan and BAV. The results might be relevant for the understanding of the difference in treatment results between Marfan and BAV patients after use of Angiotensin receptor blockers.

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NEDERLANDSE SAMENVATTING

Bij patiënten met een bicuspide aortaklep (BAV), bestaat de klep uit twee klepblaadjes in plaats van drie (tricuspide aortaklep, TAV). Patiënten met BAV hebben vergeleken met TAV patiënten een verhoogd risico op complicaties van de aortaklep en van de grote lichaamsslagader, de aorta. Een veel voorkomende complicatie is een verwijding van het eerste deel van de aorta, de aorta ascendens, met bijbehorende vaak lethale problemen als een ruptuur of een dissectie (een scheur van de binnenste laag van de vaatwand). Bij een deel van de BAV patiënten wordt echter nooit pathologie van de aorta gezien. Het is daarom van essentieel belang om de patiënten die minder gevoelig zijn voor aorta pathologie te identificeren, aangezien een preventieve ingreep om de aorta te vervangen in deze groep patiënten niet noodzakelijk is. Tot op heden is de besluitvorming voor aortachirurgie gebaseerd op de maximale doorsnede van de aorta ascendens. Uit eerder onderzoek is echter gebleken dat alleen de diameter onvoldoende is om patiënten met een verhoogd risico te selecteren voor chirurgie. Het zou dus waardevol zijn als er patiënt specifieke criteria opgesteld kunnen worden voor de selectie van patiënten voor een (preventieve) operatieve ingreep van de aorta.
Het doel van deze studie is het onderzoeken van klinische, morfologische en moleculair biologische factoren die BAV patiënten kunnen identificeren, met op dit moment een niet-gedilateerde aorta maar wel een verhoogd risico op aorta dilatatie in de toekomst.
Daarnaast wordt het pathobiologische mechanisme dat leidt tot complicaties van de aorta vergeleken tussen BAV patiënten en patiënten met het Marfan syndroom (MFS), die ook een verhoogde kans hebben op aorta pathologie.

In **Hoofdstuk 1** wordt de ontwikkeling van BAV beschreven en een overzicht gegeven van veel voorkomende complicaties. Het doel van deze studie wordt verder toegelicht en de indeling van de hoofdstukken wordt weergeven.

In **Hoofdstuk 2** worden histologische en moleculair genetische aspecten van de normale en abnormale ontwikkeling van de aortawand en de aortaklep bediscussieerd. In dit review beschrijven we hoe een defect in de vroege ontwikkeling kan leiden tot een verstoring van de bijdrage van twee belangrijke embryonale cel populaties, namelijk de neurale lijst cellen

en cellen van het “second heart field”, aan de zich ontwikkelende klep en aortawand.
De genetische oorsprong van syndromen die geassocieerd zijn met aorta dilatatie (waaronder MFS, Ehlers-Danlos, Smad3 mutaties en Loeys-Dietz) wordt verder besproken. De centrale rol van TGFβ hierin wordt bediscussieerd en gekoppeld aan de embryonale ontwikkeling. In dit review concluderen we dat BAV en de aorta pathologie die met deze klepafwijking geassocieerd is het gevolg kan zijn van een defect tijdens de embryonale ontwikkeling. Deze ontwikkelingsbiologische verklaring is echter niet voldoende om de klinische heterogeniteit in BAV te verklaren, aangezien niet alle BAV patiënten aorta complicaties ontwikkelen gedurende hun leven. Om de patiënten te kunnen identificeren die een verhoogd risico lopen op aortadilatatie, moeten we eerst de pathobiologie van de vaatwand die alle BAV patiënten gemeen hebben onderzoeken en vergelijken met de TAV patiënten. Dit wordt beschreven in het volgende hoofdstuk.

In **Hoofdstuk 3** worden verschillen in de aortawand beschreven tussen BAV en TAV patiënten. Biopten van de aortawand zijn onderzocht van zowel gedilateerde (BAD en TAD respectievelijk) als niet-gedilateerde (BA en TA respectievelijk) vaten. De vaatwanden werden onderzocht op de expressie van gedifferentieerde gladde spiercelmarkers, lamin A/C, dat een belangrijke rol speelt in de gladde spiercel differentiatie, en progerin, een marker indicatief voor cardiovasculaire veroudering. Uit dit onderzoek bleek dat de structuur van zowel de gedilateerde als de niet-gedilateerde aortawand intrinsiek anders is in de BAV vergeleken met de TAV. In the BAV is de gevonden lagere lamin A/C expressie mogelijk geassocieerd met een defecte gladde spierceldifferentiatie. Deze vaatwand immaturiteit, die de aortawand kan verzwakken, wordt dus in alle BAV patiënten gezien, inclusief de patiënten die minder gevoelig zijn voor aorta complicaties. Om de patiënten te kunnen identificeren die verhoogd kwetsbaar zijn voor pathologie van de aortawand, zijn we verder gaan zoeken naar voorspellende klinische en immunohistochemische markers.

Hoofdstuk 4 richt zich op klinische kenmerken die kunnen bijdragen aan het identificeren van BAV patiënten met een verhoogde gevoeligheid voor pathologie van de aorta. Een studiepopulatie van 255 patiënten werd onderzocht op demografische kenmerken, het klinisch beloop en

echografische parameters, waaronder de morfologie van de aortaklep. In dit hoofdstuk wordt een klinisch risicomodel gepresenteerd om patiënten met een verhoogde gevoeligheid op complicaties te identificeren. Dit werkmodel laat zien dat mannen met een complete fusie van het rechter en linker coronaire klepblad, met een hoge bloeddruk en zonder gebruik van cholesterolremmers het hoogste risico lopen en frequenter gemonitord moeten worden.

Hoofdstuk 5 beschrijft de zoektocht naar moleculair biologische markers die gebruikt kunnen worden om patiënten met een verhoogde kans op aortacomplicaties te identificeren. Hiervoor werden niet- en gedilateerde ascenderende aortawand biopten van BAV en TAV patiënten onderzocht. De markers die geïdentificeerd werden konden gekoppeld worden in een signaleringscascade die kenmerkend is voor cellulaire de-differentiatie. De markers zijn: c-Kit, een marker voor ongedifferentieerde gladde spiercellen en de gefosforyleerde vorm gefosforyleerde-c-Kit (pc-Kit). De fosforylatie van c-Kit wordt aangedreven door de aanwezigheid van matrix metalloproteinase-9 (MMP9). Pc-Kit stuurt vervolgens de expressie van Hypoxia-Inducible-Factor-1alpha (HIF1α) en endothelial nitric oxide (eNOS) aan. Deze cascade was geactiveerd in de gedilateerde BAV groep en de expressie was volledig vergelijkbaar met slechts een subgroep van de BA groep. Terwijl de rest van de BA groep een significant afwijkende expressiepatroon toonde. Dit verschil was ook te zien in de expressie van TGF-β en gefosforyleerde Smad2. Naast het expressiepatroon, waren overeenkomsten tussen de gedilateerde BAV groep en de subgroep van de niet-gedilateerde BAV groep ook duidelijk te zien in commissuur positie en de plaats van de raphe, namelijk een fusie tussen het rechter en linker coronair klepblad. Dit kwam overeen met onze bevindingen in **Hoofdstuk 4.** Op grond van deze observaties beschouwen we de BAb groep in tegenstelling tot de BAa groep als mogelijke kandidaat voor aortadilatatie in de toekomst.

In dit hoofdstuk beschrijven we ook de rol van hemodynamiek in de ontwikkeling van aorta pathologie. Hiervoor zijn biopten van de vaatwand onderzocht en vergeleken die verkregen waren van de convexe en concave zijde van de aorta. We hebben in de BAV patiënten geen significant effect van een veranderde hemodynamiek gevonden op het ontwikkelen van aortacomplicaties.

In **Hoofdtuk 6** hebben we de aortawand van BAV en TAV patiënten vergeleken met een syndroom dat een zeer verhoogd risico op aortawand pathologie heeft, namelijk het MFS. Van alle MFS gevallen wordt 90-95% veroorzaakt door mutaties in het *FBN1* (fibrilline-1) gen. In dit hoofdstuk werden de histopathologische overeenkomsten onderzocht tussen de aorta van de BAV, TAV en MFS patiënten. We hebben verder gezocht naar een immunohistochemische verklaring voor de verhoogde gevoeligheid van MFS patiënten voor het ontwikkelen van een aortadissectie, die zelfs gezien wordt in een niet gedilateerde aorta en op een jongere leeftijd vergeleken met de BAV en TAV patiënten. In MFS waren de gladde spiercellen in de media van de aorta, net als in de BAV, immatuur. In beide patiënten groepen was de expressie van fibrillin-1 ook verlaagd samen met een veranderde distributie en lokalisatie van het eiwit in vergelijking tot de TAV. Aangezien fibrillin-1 wordt geproduceerd door gladde spiercellen is het aannemelijk dat een verminderde expressie van fibrillin-1 ook kan voorkomen in minder gedifferentieerde vaten, terwijl er geen *FBN1* mutatie is zoals in de BAV het geval is. De MFS liet echter ook histopathologische overeenkomsten zien met de gedilateerde TAV zoals significante cytolytische necrose (CN) (verlies van gladde spiercelkernen in de media), gladde spiercel apoptose en degradatie van de elastische lamellen. De oorzaak van gladde spiercel apoptose, dat leidt tot CN, is echter anders in de MFS dan in de gedilateerde TAV groep. In de gedilateerde TAV veroorzaakt cardiovasculaire veroudering, met een verhoogde expressie van progerin, atherosclerose en inflammatie, terwijl deze verschijnselen in de MFS niet voorkomen, In MFS veroorzaakt de *FBN1* mutatie direct gladde spiercel apoptose, via een verhoogde signalering van de Angiotensin II receptoren (AGTR). In MFS is er dus een combinatie van immaturiteit van de media en CN die de vaatwand extreem zwak maakt. Deze bevindingen verklaren ook waarom angiotensin-receptor-blokkers (ARBs) zoals losartan, die de signalering via de AGTRs verminderen, in MFS als preventief medicijn werken om dilatatie van de aorta tegen te gaan. In BAV werken deze medicijnen niet of nauwelijks omdat de aortwand in deze groep patiënten geen tekenen van CN laat zien.

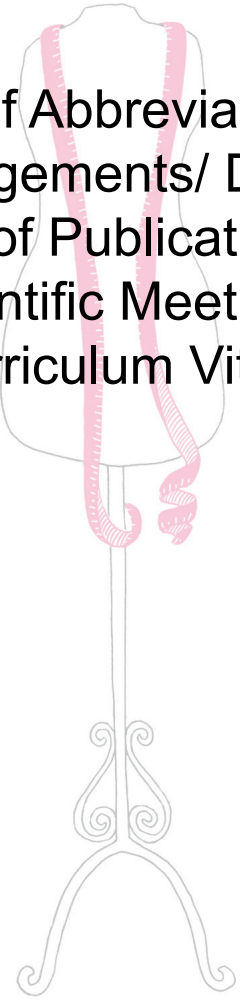
In **Hoofdstuk** 7 hebben we het expressiepatroon van Wilms tumor suppressor protein (Wt1) onderzocht, waarvan uit eerder onderzoek is gebleken dat het als reactie op pathologische omstandigheden geactiveerd wordt in de epicardcellen die het hart omgeven. Wij hypothetiseren dat de buitenste

cellaag van de ascenderende aorta, het arteriële epicard, een zelfde mechanisme vertoont onder pathologische omstandigheden. We hebben het arteriële epicard in BAV, TAV en MFS onderzocht op de expressie van Wt1, Retinaldehyde dehydrogenase-II (RALDH2), dat onder transcriptionele regulatie staat van Wt1 en eNOS, dat de Wt1 expressie reguleert. De expressie van deze drie markers werd ook in de endotheelcellen en de gladde spiercellen onderzocht, met als doel het kunnen linken van eNOS en MMP9 beschreven in de cascade in Hoofdstuk 5**.** In deze studie konden we concluderen dat in de epicardcellen en gladde spiercellen van alle niet-gedilateerde vaten een basis Wt1 activiteit aanwezig is. In de gedilateerde TAV leiden kenmerken van cardiovasculaire veroudering zoals inflammatie en een verhoogde progerin expressie, die niet aanwezig zijn in de gedilateerde BAV groep, tot een significant hogere Wt1 activiteit.

We hebben eerder beschreven (Hoofdstuk 5**)** dat er binnen de niet-gedilateerde BAV groep, op basis van het expressiepatroon van een moleculair biologisch pad, een subgroep geindentificeerd kan worden met een verhoogde kans op complicaties van de aorta. Deze groep vertoont een verhoogde expressie van pc-Kit, HIF1α, eNOS en MMP9. In deze studie hebben we aangetoond dat Wt1 expressie in de ‘ gevoelige patiënten’ ook significant hoger is vergeleken met de rest van de niet gedilateerde BAV groep. Wt1 is een trancriptiefactor dat een belangrijke rol speelt als onderdrukker van genexpressie, waaronder dat van MMP9. Daarnaast is eerder ook beschreven dat eNOS de verplaatsing van Wt1 uit de kern (waar Wt1 actief is) naar het cytoplasma (waar Wt1 de onderdrukkende functie verliest) stimuleert. Het is daarom aannemelijk dat de toegenomen eNOS expressie in de ‘gevoelige patiënten’ kan leiden tot een verhoogde MMP9 expressie, dat vervolgens de fosforylering stimuleert van c-Kit.

8

CHAPTER 9



List of Abbreviations
Acknowledgements/ Dankwoord
List of Publications
Scientific Meetings
Curriculum Vitae

LIST OF ABBREVIATIONS

ACEinhibitor:	Angiotensin-converting-enzyme inhibitor
ALK2:	Activin type I receptor
AMC:	Academic Medical Center
aPEO:	Arterial proepicardial organ
ARBs:	Angiotensin-receptor-blockers
αSMA:	α Smooth muscle actin
AT2 receptor:	Angiotensin II receptor
AXIN1:	Axis Inhibitor 1
BA:	Bicuspid aortic valve without dilation
BAa:	Bicuspid aortic valve without dilation, non-susceptible group
BAb:	Bicuspid aortic valve without dilation, susceptible group
BAD:	Bicuspid aortic valve with dilation
BAV:	Bicuspid aortic valve
BMP:	Bone morphogenetic protein
BSA:	Bovine serum albumin
CN:	Cytolytic necrosis
DAB:	Diaminobenzidine tetrachloride
DAPI:	4’,6-diamidino-2-phenylindole
EMC:	Erasmus Medical Center
eNOS:	Endothelial nitric oxide
FBN1:	Fibrillin-1
Fgf8:	Fibroblast growth factor 8
NO:	Nitric oxide
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GAR:	Goat-anti-rabbit-biotin
HAM:	Horse-anti-mouse-biotin
HE:	Hematoxylin-Eosin
HIF1α:	Hypoxia-Inducible-Factor-1alpha
IgG:	Immunoglobulin G
LCC:	Left coronary cusp
LUMC:	Leiden University Medical Center
MF:	Microscopic field
MFS:	Marfan syndrome
MMP9:	Matrix metalloproteinase-9

NCC:	Non-coronary cusp
NGS:	Normal goat serum
NHS:	Normal horse serum
PBS:	Phosphate buffered saline
PBS-T:	Phosphate buffered saline with 0.05% Tween-20
pc-Kit:	Phosphorylated c-Kit
pSmad2:	Phosphorylated Smad2
RALDH2:	Retinaldehyde dehydrogenase-II
RAM-PO:	Peroxidase-conjugated rabbit anti-mouse
RCC:	right coronary cusp
RF:	Resorcin-Fuchsin
SD:	Standard deviation
SHF:	Second heart field
SM22α:	Smooth muscle 22α
TA:	Tricuspid aortic valve without dilation
TAD:	Tricuspid aortic valve with dilation
TAV:	Tricuspid aortic valve
TGF-β:	Transforming growth factor β
Type 1 BAV:	Fusion between the RCC and LCC
Type 2 BAV:	Fusion between the RCC and NCC
Type 3 BAV:	Fusion between the LCC and NCC
VSMC:	Vascular smooth muscle cell
Wt1:	Wilms tumor suppressor protein

ACKNOWLEDGEMENTS/ DANKWOORD

Promoveren doe je niet alleen en daarom besluit ik mijn proefschrift met het bedanken van iedereen zonder wiens hulp, steun en bijdrage dit proefschrift niet tot stand was gekomen. Een aantal mensen wil ik hierbij in het bijzonder noemen.

Allereerst wil ik mijn proefschrift opdragen aan mijn allerliefste ouders en zus. Mom en Papa, jullie hebben mij groot gebracht met veel liefde, mij discipline bijgebracht en hebben mij laten zien dat je alleen met hard werken iets kan bereiken. Ik ben er heel trots op dat ik jullie dochter ben! Lieve Simran, di, je bent de beste zus die iemand kan hebben! My granny, you inspired me to choose this career. Love you loads!

Beste promotoren, Prof. Dr. R.J.M. Klautz en Prof. Dr. M.C. de Ruiter, hartelijk dank voor alle begeleiding en de geboden kansen. Prof. Dr. A.C. Gittenberger- de Groot, beste Adri, voor u ligt het proefschrift waar u de grondlegger van bent. U ontving mij met open armen en enthousiasme, het is fantastisch dat we dit samen hebben bereikt!

Prof. Dr. J.A. Rauwerda, uw woorden: ‘U bent een uitzonderlijk talent, u wilt zeker chirurg worden’ zal ik nooit vergeten. Veel dank voor uw bijdrage aan mijn eerste stappen in de heelkunde.

Ik heb de afgelopen jaren van een fijne samenwerking genoten tussen alle afdelingen die onderdeel uitmaken van ons onderzoeksgroep, zowel nationaal als internationaal: Beste Dr J.H.N. Lindeman, hartelijk dank voor uw onmisbare bijdrage aan dit onderzoek. Dear Prof. Dr. H.H. Sievers and Dr. S. Mohamed, thank you for your indispensable contribution to different manuscripts in this thesis. Beste prof. Dr. A.J.J.C. Bogers, uw expertise en het aortaweefsel dat wij van de Kleppenbank Rotterdam hebben mogen gebruiken, was onmisbaar voor dit proefschrift en hebben geleid tot mooie publicaties. Prof. Dr. B.J.M. Mulder en Romy Franken, veel dank voor de fijne samenwerking, we hebben mooie resultaten verkregen. Prof. Dr. R.E. Poelmann, beste Rob, uw plezier in het onderzoek is aanstekelijk. Dr. M.R.M. Jongbloed, beste Monique, het is heel fijn om met iemand te kunnen samenwerken die ook zo snel en fanatiek is. Mijn collega’s van de afdeling Anatomie en Embryologie, ondanks de korte periode waarin ik hier werkzaam was, kan ik terug kijken op een gezellige tijd. Bert, bedankt voor het aanleren van de labtechnieken. Sjoerd, als ik even vastliep kwam je met goede ideeën en fantastische tips.

Mijn collega's van de thoraxchirurgie, ontzettend bedankt voor jullie enthousiasme en vertrouwen in mij. 'Dit aortaweefsel moet bewaard worden voor Nimrat' is een belangrijk onderdeel geworden van de aortachirurgie in het LUMC, waarvoor veel dank.
Beste Charlotte, veel dank voor het vormgeven van de omslag, het is heel mooi geworden. Lieve vrienden (onnodig om jullie bij name te noemen), bedankt voor de afleiding wanneer ik die nodig had. Beste Danielle, mijn paranimf, dank voor het mij ter zijde staan bij de verdediging van mijn proefschrift.

LIST OF PUBLICATIONS

Normal and abnormal development of the aortic wall and valve: correlation with clinical entities
N. Grewal, M.C. DeRuiter, M.R.M. Jongbloed, M.J.T.H. Goumans, R.J.M. Klautz, R.E. Poelmann, A.C. Gittenberger-de Groot
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N. Grewal, W..M.C. Koenraadt, O.Y. Gaidoukevitch, M.C. DeRuiter, A.C. Gittenberger- de Groot, M.M. Bartelings, E.R. Holman, R.J.M. Klautz, M.J. Schalij, M.R.M. Jongbloed
* Authors contributed equally to this paper
Submitted

Bicuspid aortic valve: phosphorylation of c-Kit and downstream targets are prognostic for future aortopathy
N. Grewal, A.C. Gittenberger-de Groot, M.C.DeRuiter, R.J.M. Klautz, R.E. Poelmann, S.N. Duim, J.H.N. Lindeman, W.M.C. Koenraadt, M.R.M. Jongbloed, S.A. Mohamed, H.H. Sievers, A.J.J.C. Bogers, M.J.T.H. Goumans
Eur J Cardiothorac Surg;46(5):831-9

Aortic complications in bicuspid aortic valve and Marfan syndrome: histopathologic comparison and therapeutic relevance
N. Grewal, R. Franken, B.J.M. Mulder, M.J.T.H. Goumans, J.H.N. Lindeman, M.R.M. Jongbloed, M. C. DeRuiter, R.J.M. Klautz, A.J.J.C. Bogers, R.E. Poelmann, A.C. Gittenberger-de Groot
Submitted

Wt1 expression in epicardium and vascular smooth muscle cells as a marker for aortic wall pathology in bicuspid aortic valve and Marfan syndrome
N. Grewal, M.J.T.H. Goumans, M.C. DeRuiter, R.J.M. Klautz, R.L.P. Roscam Abbing, R.E. Poelmann, J.H.N. Lindeman, M.R.M. Jongbloed, B.J.M. Mulder, A.J.J.C. Bogers, A.C. Gittenberger-de Groot
Submitted

Colectomy enhances tumor cell adhesion in the liver and subsequent tumor outgrowth in rats.
S. Grewal, N. Gul, M. Bogels, R. Braster, S. Pouw, **N. Grewal**, M.van Egmond.
Submitted

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S. Grewal, N. Gul, M. Bogels, R. Braster, S. Pouw, **N. Grewal**, S.J. Oosterling, R.H.J. Beelen, M.van Egmond.
Submitted

Carotid stenosis: new insights in pathophysiology and diagnostic imaging.
N. Grewal N, C.G. Vos, J.A. Rauwerda
In process

SCIENTIFIC MEETINGS

2014 September

4th International meeting on Aortic Diseases, Liège, Belgium

Aortic complications in bicuspid aortic valve and Marfan syndrome: histologic comparison

Nimrat Grewal, Romy Franken, Barbara J.M. Mulder, Marie-Jose Goumans, Johannes H.N. Lindeman, Monique R.M. Jongbloed, Marco C. DeRuiter , Robert J.M. Klautz, Robert E. Poelmann, Ad J.J.C. Bogers, Adriana C. Gittenberger-de Groot

Oral presentation

2014 September

4th International meeting on Aortic Diseases, Liège, Belgium

Bicuspid aortic valve: molecular tissue factors identified prognostic for future aortopathy

Nimrat Grewal, Adriana C. Gittenberger-de Groot, Marco C.DeRuiter, Robert J.M. Klautz, Robert E. Poelmann, Sjoerd Duim, Johannes H.N. Lindeman, Wilke M.C. Koenraadt, Monique R.M. Jongbloed, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marie-José Goumans

Oral presentation

2014 September

aLondon Heart Valve 2014, London, Great Britain

Bicuspid Aortic Valve: Determining Susceptibility for Dilation of the Ascending Aorta by Histopathology Nimrat Grewal, Adriana C. Gittenberger-de Groot, Robert J.M. Klautz, Robert E. Poelmann, Marie-José Goumans, Sjoerd N. Duim, Johannes H.N. Lindeman, Monique R.M. Jongbloed, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marco C. DeRuiter

Oral presentation

2014 April
American Association for Thoracic Surgery, Aortic Symposium, New York, USA
Bicuspid Aortic Valve: Determining Susceptibility for Dilation of the Ascending Aorta by Histopathology
Nimrat Grewal, Adriana C. Gittenberger-de Groot, Robert J.M. Klautz, Robert E. Poelmann, Marie-José Goumans, H.G. Smeenk, S.N. Duim, Johannes H.N. Lindeman, M.R.M. Jongbloed, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marco C.DeRuiter
Invited presentation
Selected as 'Director's Choice' presentation

2013 November
Symposium Experimenteel Onderzoek Heelkundige Specialismen 2013, Maastricht, The Netherlands
Determining susceptiblity for aortopathy in patients with abicuspid aortic valve by histopathology
Nimrat Grewal, Adriana C. Gittenberger-de Groot, Robert E. Poelmann, Robert J.M. Klautz, Johannes H.N. Lindeman, Marie-José Goumans, M.R.M. Jongbloed, Meindert Palmen, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marco C.DeRuiter
Poster presentation

2013 November
Rembrandt Symposium, Noordwijkerhout, The Netherlands
Determining susceptibility for aortopathy in patients with a bicuspid aortic valve by histopathology
Nimrat Grewal, Adriana C. Gittenberger-de Groot, Robert E. Poelmann, Robert J.M. Klautz, Johannes H.N. Lindeman, Marie-José Goumans, M.R.M. Jongbloed, Meindert Palmen, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marco C.DeRuiter
Poster presentation

2013 November **Nederlandse Verenging Voor Cardiologie, Papendal, The Netherlands**

Aortopathy in bicuspid aortic valve, genes involved in immaturity of the aortic wall

N. Grewal, M.R.M. Jongbloed, M.C. DeRuiter, R.J.M. Klautz, R.E. Poelmann, M.J. Goumans, S.N.Duim, J.H.N. Lindeman, S.A. Mohamed, H.H. Sievers, A.J.J.C. Bogers, A.C. Gittenberger-de Groot

Oral presentation

First prize in recognition of best oral presentation

2013 October **European Association Cardio-Thoracic Surgery, Vienna, Austria**

Determining susceptibility for aortopathy in patients with bicuspid aortic valve

Nimrat Grewal, Adriana C. Gittenberger-de Groot, Robert E. Poelmann, Robert J.M. Klautz, Johannes H.N. Lindeman, Marie-José Goumans, M.R.M. Jongbloed, Meindert Palmen, Salah A. Mohamed, Hans-Hinrich Sievers, Ad J.J.C. Bogers, Marco C.DeRuiter

Oral presentation

Presented by prof. dr. A.C. Gittenberger- de Groot

2013 September **European Society of Cardiology 2013, Amsterdam, The Netherlands**

Bicuspid aortic valve and aneurysm formation: immaturity of the aortic wall

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Poster presentation

Selected as excellent poster presentation

CURRICULUM VITAE

Nimrat Grewal was born on March 8th, 1988 in Amsterdam, The Netherlands. From 2000 to 2006 she attended high school, at the Hervormd Lyceum Zuid in Amsterdam where she graduated summa cum laude.

Nimrat started studying medicine in 2006 at the Vrije University Medical Center (VUMC). She received her bachelor degree cum laude in 2010, after which she started her clinical rotations. In the same year she joined the department of Molecular Cell Biology and Immunology at the VUMC as a student researcher.

In 2012 she did her scientific internship at the department of cardiothoracic surgery and at the department of Anatomy and Embryology at the Leiden University Medical Center (LUMC) under supervision of prof. dr. R.J.M. Klautz, prof. dr. A.C. Gittenberger-de Groot and prof. dr. M.C. DeRuiter. In 2013 she started her PhD project. Nimrat did her final clinical rotation at the department of cardiothoracic surgery at the LUMC. In July 2013 she graduated from the medical caollege cum laude. Currently, she is working as a resident at the department of cardiothoracic surgery at the LUMC. She continues to do basic and clinical research.

In her free time she loves dancing (ballet and Bharatnatyam, classical Indian dance), traveling and shopping. Since 2000 she is a volunteer at the Missionaries of Charity in Amsterdam. After graduating from secondary school, she also took over the management of the charitable organization ‘Educational support’ in India, which was set up by her parents.