

From The Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

THE BICUSPID AORTIC VALVE AND THORACIC AORTIC DISEASE

Carl Granath



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The Bicuspid Aortic Valve and Thoracic Aortic Disease

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*Till min familj - Anna och Sally, mamma, Evelina och pappa,
som är särskilt saknad en dag som denna.*

"I was taught that the way of progress is neither swift nor easy."

- Marie Curie

Populärvetenskaplig sammanfattning

Aortaklaffen är placerad mellan hjärtats vänstra kammare och stora kroppspulsådern, aortan. Den fungerar som backventil och säkerställer att blodet pumpas ut i kroppen och inte rinner tillbaka in i hjärtat. Aortaklaffen består normalt av tre tunna membran, så kallade klafffickor, som öppnas när blod pumpas ut i kroppspulsådern och stängs när hjärtats vänstra kammare fylls med syresatt blod från lungorna. En normal aortaklaff med tre klafffickor kallas trikuspid aortaklaff.

Ungefär 1% av befolkningen, oftast män, föds med en aortaklaff med enbart två klafffickor - en bikuspid aortaklaff. Bikuspid aortaklaff är kopplad till en kraftigt förhöjd risk att drabbas av sjukdom i aortaklaffen. Klaffsjukdom debuterar vanligen i 50-årsåldern men även barn kan drabbas. Hjärtklaffsjukdom innebär antingen att klaffen läcker och inte kan stängas ordentligt vilket gör att blod läcker tillbaka till hjärtat (insufficiens), eller att klaffen blir stel och trång så att hjärtat får svårare att pumpa ut blod till kroppen (stenos).

Den enda behandlingen för aortaklaffsjukdom är kirurgi, vanligen genom att klaffen byts ut mot en protes. Klaffproteser kan vara antingen biologiska från gris eller kalv, eller mekaniska tillverkade av kolfiber. Biologiska klaffproteser bryts till slut ned i kroppen och förkalkas, vilket gör att de kan behöva bytas ut igen, medan mekaniska klaffproteser kräver livslång behandling med blodförtunnande läkemedel. Det finns därmed ett stort behov av att utveckla nya alternativ för att ersätta en sjuk aortaklaff. Det gäller särskilt för individer som drabbas i ung ålder.

En metod som utforskas är att ersätta aortaklaffen med en stomme framställd i laboratorium. Förhoppningen har varit att en sådan stomme ska kunna växa in i kroppen och återskapa en ny, frisk klaff med hjälp av kroppens egna celler. Ett vanligt sätt att erhålla en klaffstomme är att tvätta bort cellerna i en hjärtklaff från en donator så att bara stödjevävnaden finns kvar. Metoden har testats för patienter men det har dock visat sig att stommen inte utvecklas till en ny klaff utan har begränsad hållbarhet precis som en konventionell biologisk klaffprotes. Orsaken till detta är till stor del okänd men mycket talar för att specifika molekyler på ytan av klaffens stödjevävnad har stor betydelse för att nya celler ska kunna växa in i stommen och bilda en ny levande klaff. Dessa är dock svåra att bevara under framställandet av en hjärtklaffstomme.

I delarbete I undersöktes den molekylära uppbyggnaden av ytan på hjärtklaffens stödjevävnad genom att studera hjärtklaffar från avlidna donatorer. Vi fann att stödjevävnaden på aortaklaffens yta liknar den på insidan av ett blodkärl. För att kunna studera stödjevävnadens ytas betydelse för nybildning av en frisk hjärtklaff utvecklade vi en metod för att tvätta bort cellerna från aortaklaffar från råttor, vilken i stor utsträckning bevarade molekyler på ytan. Metoden kan komma att användas i framtida forskning om stödjevävnadens yta och dess betydelse för nybildning av en frisk hjärtklaff.

Bikuspid aortaklaff ökar också risken för kroppspulsåderbräck (aortaaneurysm) nära hjärtat. Ett aortaaneurysm innebär en onormal vidgning av den stora kroppspulsådern. Det ger sällan symtom men resulterar i att kärlet blir skört och riskerar att leda till att aortan brister. Ingen medicinsk behandling har hittills visat sig vara effektiv utan den enda behandlingen för aortaaneurysm intill hjärtat är att ersätta den drabbade delen av aortan med en kärlprotes. Risken för att drabbas av aortaaneurysm eller aortaklaffsjukdom kan variera beroende på hur den bikuspid aortaklaffen ser ut. Den vanligaste formen av bikuspid aortaklaff är att två av de tre klafffickorna är sammanvuxna, men en bikuspid klaff kan också ha två jämnstora klafffickor utan spår av sammanväxning. Vad den bikuspid klaffens utseende har för betydelse för risken att utveckla aortaklaffsjukdom och aortaaneurysm är relativt välstuderat bland manliga patienter, men hur sambanden ser ut hos kvinnor har tidigare varit okänt.

I delarbete II undersöktes kopplingen mellan den bikuspid klaffens utseende, typ av klaffsjukdom samt förekomst av aneurysm i respektive kön bland patienter som genomgick hjärtkirurgi. Vi fann både likheter och avgörande skillnader mellan könen i hur klaffens utseende påverkar risken för klaffsjukdom och aneurysm vilket innebär att möjligheten att operera in en ny aortaklaff med en kärlkateter, ett allt vanligare alternativ till öppen hjärtkirurgi, kan skilja sig beroende på både den bikuspid klaffens utseende och kön.

Både individer med en normal aortaklaff och de med en bikuspid aortaklaff kan drabbas av aortaaneurysm nära hjärtat men sjukdomsprocessen i aortans kärlvägg skiljer sig avsevärt mellan de två patientgrupperna. Redan i nyföddhetsperioden kan individer med bikuspid aortaklaff ha vidgad aorta nära hjärtat, medan aneurysm hos individer med trikuspid aortaklaff uppstår i vuxen ålder. Uppkomstmekanismerna är till stora delar okända men skillnaderna mellan de två sjukdomsprocesserna är tydliga - kärlväggen hos patienter med bikuspid aortaklaff är ofta relativt välbevarad medan kärlväggen i aortaaneurysm hos patienter med trikuspid aortaklaff ofta är inflammerad och skörare. Skillnaderna i sjukdomsprocess kan ha stor betydelse för effekten av olika läkemedelsbehandlingar.

I delarbete III studerades sambandet mellan behandling med acetylsalicylsyra och förekomst av aortaaneurysm nära hjärtat. Acetylsalicylsyra (Trombyl®) är ett av de allra vanligaste läkemedlen för att behandla hjärt- och kärlsjukdomar och en del studier tyder på att det även kan vara verksamt som behandling av aortaaneurysm. Vi kunde visa att det fanns en tydlig koppling mellan acetylsalicylsyra och lägre förekomst av aortaaneurysm, men bara hos patienter med en normal trikuspid aortaklaff. I vävnadsprov som togs från aortaväggen i samband med operation sågs ett förändrat genuttryck i kärlväggen förenligt med en lokal inflammationshämmande effekt hos patienter som medicinerade med acetylsalicylsyra. En dämpad kärlinflammation hos patienter med acetylsalicylsyrabehandling skulle således kunna förklara den lägre förekomsten av aneurysm.

Om man har haft ett aortaaneurysm löper man ökad risk att utveckla ett nytt aneurysm i en annan del av aortan. Patienter som opererats genomgår därför röntgenkontroller av aortan även efter en operation för att upptäcka nya aneurysm i tid. Många studier tyder dock på att patienter med bikuspid aortaklaff bara utvecklar aneurysm närmast hjärtat. Det är också till stor del okänt

hur risken att utveckla nya aneurysm i olika delar av aortan skiljer sig åt beroende på om man har en normal eller bikuspid aortaklaff.

I delarbete IV studerades förekomsten av nya aneurysm och brusten aorta hos patienter som opererats för aneurysm nära hjärtat. Det kliniska förloppet och aortans storlek hos patienter med normal och bikuspid aortaklaff jämfördes genom granskning av sjukdomshistoria och röntgenbilder innan respektive tio år efter operationen. Vi fann att patienter med normal trikuspid aortaklaff har en avsevärd risk att drabbas av nya aneurysm eller brusten aorta medan risken var låg hos patienter med bikuspid aortaklaff. Vilken typ av aortaklaff en patient har bör därför tillmätas stor vikt vid operationsbeslut och uppföljning efter operation av kroppspulsåderbräck intill hjärtat.

Sammanfattningsvis har vi studerat möjligheter att förbättra behandlingen av patienter med bikuspid aortaklaff och aortaaneurysm nära hjärtat. Vi har upptäckt att ytan på aortaklaffens stödjevävnad liknar den som finns i blodkärl. Vi har också beskrivit en metod för framställning av en hjärtaortklaffstomme från råttor som möjliggör studier kring hur stödjevävnadens yta påverkar inväxten av levande celler. Dessa arbeten kan komma att bidra till utvecklingen av en hjärtaortklaffstomme som kan ombildas till en ny levande hjärtaortklaff hos patienter med hjärtaortklaffsjukdom. Vi har också studerat hur utseendet hos en bikuspid aortaklaff påverkar risken för aortaaneurysm nära hjärtat och funnit att betydelsen varierar beroende på kön. Detta kan ha stor betydelse för lämpligheten att behandla aortaklaffsjukdom med en kateterburen klaffprotes. Därutöver har vi konstaterat att acetylsalicylsyra är kopplat till både lägre förekomst av aortaaneurysm nära hjärtat och minskad inflammation i kärlväggen hos patienter med normal aortaklaff. Detta talar för att acetylsalicylsyra skulle kunna vara en verksamt behandling för aortaaneurysm men fler studier behövs. Slutligen upptäckte vi att patienter med normal aortaklaff som opererats för aortaaneurysm nära hjärtat har en hög risk att drabbas av ny allvarlig aortasjukdom, medan risken var förhållandevis låg för patienter med bikuspid aortaklaff.

Abstract

About 1% of the population is born with an aortic valve composed of only two cusps, called a bicuspid aortic valve. Bicuspid aortic valve is associated with a high risk of both aortic valve disease and ascending aortic dilatation, which develop at a significantly earlier age than in patients with a normal tricuspid aortic valve. No medical therapy exists for either of the conditions and current surgical treatments have substantial limitations. Tissue engineering is a potential future treatment option for valvular heart disease. However, clinical trials have shown that engineered valves exhibit limited durability similarly to conventional biological valves. A possible cause of their failure is an aberrant basement membrane in the scaffolds, but the normal basement membrane in the aortic valve has not been characterized. Moreover, the type and prevalence of valvular disease and aortic dilatation differs between anatomical variants, or phenotypes, of the bicuspid aortic valve, and potential sex differences have not been addressed in this context. Furthermore, the pathological process of ascending aortic dilatation differs between patients with bicuspid and tricuspid aortic valves, which has important implications for pharmacological targets, surveillance, and surgical management.

Study I investigated the expression of laminins, a key basement membrane component, in the aortic valve. Valves from deceased donors were studied with immunohistochemistry, which revealed expression of laminin chains α 4-5, β 1-2, and γ 1. A decellularized rat aortic valve scaffold with a retained basement membrane was developed to facilitate studies on the role of basement membrane components in valve regeneration.

Study II described sex differences in bicuspid phenotype in relation to valvular and aortic disease in a surgical cohort. Right-left cusp fusion was associated with aortic regurgitation in males, whereas right-non-coronary cusp fusion was associated with regurgitation in females. Furthermore, the 2-sinus phenotype was associated with root phenotype aortic dilatation in males, while females with the 2-sinus phenotype had small aortic roots and no root dilatation.

Study III demonstrated an inverse association between acetylsalicylic acid therapy and ascending aortic dilatation in patients with tricuspid aortic valve in a retrospective surgical series. Furthermore, acetylsalicylic acid was associated with decreased aortic intima-media gene expression of *Cyclooxygenase-2* in dilated aortas from patients with tricuspid aortic valve.

Study IV mapped the long-term outcome of the distal aorta (the arch to the abdominal aorta) after ascending aortic replacement in a prospective cohort of patients with bicuspid and tricuspid aortic valves. There was a high incidence of distal aortic complications in patients with tricuspid, but not bicuspid aortic, valves.

In conclusion, the present thesis provides novel insights about the aortic valve microstructure, clinical implications of bicuspid aortic valve disease and ascending aortic dilatation, and pharmacological therapy in ascending aortic dilatation. These findings may contribute to new treatment options for bicuspid aortic valve disease and ascending aortic dilatation, and an individualized clinical management.

List of scientific papers

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I. **Granath C**, Noren H, Björck H, Simon N, Olesen K, Rodin S, Grinnemo KH, Österholm C. Characterization of Laminins in Healthy Human Aortic Valves and a Modified Decellularized Rat Scaffold. *Biores Open Access*. 2020 Dec 7;9(1):269-278.
- II. **Granath C**, Mohamed SA, Olsson C, Grattan M, Mertens L, Franco-Cereceda A, Björck HM. Valve disease and aortopathy associations of bicuspid aortic valve phenotypes differ between men and women. *Open Heart*. 2021 Oct;8(2):e001857.
- III. **Granath C***, Freiholtz D*, Bredin F, Olsson C, Franco-Cereceda A, Björck HM. Acetylsalicylic Acid Is Associated With a Lower Prevalence of Ascending Aortic Aneurysm and a Decreased Aortic Expression of Cyclooxygenase 2. *J Am Heart Assoc*. 2022 May 3;11(9):e024346.
*Contributed equally.
- IV. **Granath C**, Dismorr M, Björck HM, Carlestål E, Olsson C, Bredin F. Fate of the Distal Aorta after Ascending Aortic Replacement in Patients with Bicuspid and Tricuspid Aortic Valves - A Ten-Year Follow-up.
Submitted manuscript

Scientific publications not included in the thesis:

1. Månsson-Broberg A, Rodin S, Bulatovic I, Ibarra C, Löfling M, Genead R, Wärdell E, Felldin U, **Granath C**, Alici E, Le Blanc K, Smith CIE, Salašová A, Westgren M, Sundström E, Uhlén P, Arenas E, Sylven C, Tryggvason K, Corbascio M, Simonson OE, Österholm C, Grinnemo KH. Wnt/ β -Catenin Stimulation and Laminins Support Cardiovascular Cell Progenitor Expansion from Human Fetal Cardiac Mesenchymal Stromal Cells. *Stem Cell Reports*. 2016 Apr 12;6(4):607-617.
2. Freiholtz D, Bergman O, Lång K, Poujade FA, Paloschi V, **Granath C**, Lindeman JHN, Olsson C, Franco-Cereceda A, Eriksson P, Björck HM. Bicuspid aortic valve aortopathy is characterized by embryonic epithelial to mesenchymal transition and endothelial instability. *J Mol Med (Berl)*. 2023 Jul;101(7):801-811.
3. Freiholtz D, Bergman O, Pradhananga S, Lång K, Poujade FA, **Granath C**, Olsson C, Franco-Cereceda A, Sahlén P, Eriksson P, Björck HM. SPP1/osteopontin: a driver of fibrosis and inflammation in degenerative ascending aortic aneurysm? *J Mol Med (Berl)*. 2023 Oct;101(10):1323-1333.

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List of abbreviations

AAA	abdominal aortic aneurysm
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AR	aortic regurgitation
ASA	acetylsalicylic acid
ASAP	Advanced Study of Aortic Pathology
ATAA	ascending thoracic aortic aneurysm
BAV	bicuspid aortic valve
BE	benzonase endoculease
BM	basement membrane
BSA	body surface area
CABG	coronary artery bypass graft
CI	confidence interval
COL IV	collagen IV
COX	cyclooxygenase
CT	computed tomography
DAPI	4',6-diamidino-2-phenylindole
DAVAACA	Diseases of the Aortic Vave, Ascending Aorta, and Coronary Arteries
DNA	deoxyribonucleic acid
EMT	epithelial-to-mesenchymal transition
FN	fibronectin
H&E	hematoxylin and eosin
HDL	high-density lipoprotein
HS	heparan sulfate glycosaminoglycans
hsCRP	high-sensitivity C-reactive protein
HSD	honestly significant difference
HSD	Honestly Significant Difference
IQR	interquartile range
L-N	left-non-coronary

LDL	low-density lipoprotein
LN	laminin
LVOT	left ventricular outflow tract
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NCC	neural crest cell
NETs	neutrophil extracellular traps
NSAID	non-steroid anti-inflammatory drug
OCT	optimal cutting temperature compound
PBS	phosphate-buffered saline
PLN	perlecan core protein
R-L	right-left
R-N	right-non-coronary
RMA	robust multichip average
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
TAV	tricuspid aortic valve
TAVR	transcatheter aortic valve replacement
TEE	transesophageal echocardiography
TTE	transthoracic echocardiography
UAV	unicuspid aortic valve
VE-cadherin	vascular endothelial cadherin
VIC	valvular interstitial cell

Introduction

The aortic valve is located between the left ventricle of the heart and the ascending aorta. A normal tricuspid aortic valve (TAV) consists of three thin tissue sheets, or cusps, but some individuals are born with a bicuspid aortic valve (BAV), which has only two cusps. BAV is the most common congenital cardiac malformation, with an estimated prevalence of ~1%^{1,2}. BAV is strongly associated with both aortic valve dysfunction and ascending thoracic aortic aneurysm (ATAA), and patients with BAV develop these conditions at a much earlier age than patients with a normal tricuspid aortic valve³⁻⁶.

The only available treatments for aortic valve dysfunction are surgery and endovascular procedures, usually entailing replacement with a biological or mechanical prosthetic valve, both with their respective limitations⁷. Future treatment options may include tissue-engineered valves, which could theoretically be composed of the recipient's own cells and develop into a new, healthy heart valve^{8,9}. However, the current generation of tissue-engineered valves suffers from the same limited durability as conventional biological valves, and the cause of their failure remains largely unknown¹⁰⁻¹², and there is a distinct need for improved knowledge of the heart valve microstructure and its role in valve regeneration and biology.

As many as 30-45% of individuals with BAV are at risk of ATAA, a silent and potentially fatal disease as it may lead to aortic dissection^{6,13-16}. It is currently unknown why many patients with BAV develop ATAA while others do not, though both genetics and the hemodynamic alterations associated with BAV likely have a role¹⁷. There are several anatomical variants, or phenotypes, of BAV; studies indicate that the risk of valvular and aortic disease differs between BAV phenotypes¹⁸⁻²². However, it is not known if sex differences affect the risk profile associated with a particular phenotype.

ATAA, which affects individuals with both BAV and TAV, can only be treated with surgical replacement of the affected segment²³. It has been increasingly recognized that BAV- and TAV-associated ATAAs are distinct clinical and pathological entities^{5,24-29}. BAV-associated ATAA appears to be a congenital lesion with a largely preserved aortic wall microstructure, whereas ATAA in TAV is characterized by extensive tissue remodeling and vascular inflammation^{1,5,24-27,30,31}. These differences could have significant implications for the efficacy of pharmacological therapy. Some studies suggest that acetylsalicylic acid (ASA), a platelet inhibitor and anti-inflammatory agent, may be beneficial in ATAA, but the findings have been conflicting and the pathological differences strongly imply that the efficacy may differ substantially depending on aortic valve phenotype³²⁻³⁴.

Patients who have had surgery for ATAA undergo long-term surveillance to identify residual aortic disease in other segments. No distinction is made between patients with BAV and TAV in current guidelines, despite the established differences in pathology²³. Moreover, emerging evidence demonstrates a more benign clinical course and indicate a low risk of distal aortic disease in BAV-associated ATAA^{29,35-37}. However, aortic growth rates and the long-term risk

of developing new aortic disease in distal segments after ATAA surgery in patients with BAV and TAV have not been reported.

1 Literature review

1.1 Anatomy and function of the aortic valve

The aortic valve is one of the two semilunar heart valves. It is located between the left ventricle of the heart and the ascending aorta (Figure 1). The ejected blood first passes from the left ventricle through the left ventricular outflow tract (LVOT) before crossing the aortic valve, which functions to prevent backflow of blood into the left ventricle during the diastole and thus maintain organ perfusion pressure. The valve consists of three thin tissue sheets, called cusps (or leaflets), which are attached to a crown-shaped fibrous structure known as the aortic annulus, immediately distal to the LVOT. The three cusps are separated by the three distal points of the annulus, called commissures. This normal phenotype, with three cusps and three commissures, is referred to as a TAV³. The aortic annulus is the first part of the structure known as the aortic root, which constitutes the most proximal part of the ascending aorta and is delineated by the annulus proximally and the sinotubular junction distally (Figure 2). The annulus is part of the fibrous framework of the heart; it provides structural support and maintains the circular orifice of the aortic root. Distal to the aortic annulus are the sinuses of Valsalva, which refers to the three bulging points of the proximal ascending aorta. Each sinus contains a valve cusp, and two of the sinuses contain the origins of the two coronary arteries. The sinotubular junction refers to the indentation between the sinuses of Valsalva and the tubular segment of the ascending aorta³⁸. The aortic valve cusps are denominated by their relationship to the coronary artery ostia: the right coronary cusp, the left coronary cusp, and the slightly larger non-coronary cusp³⁹.

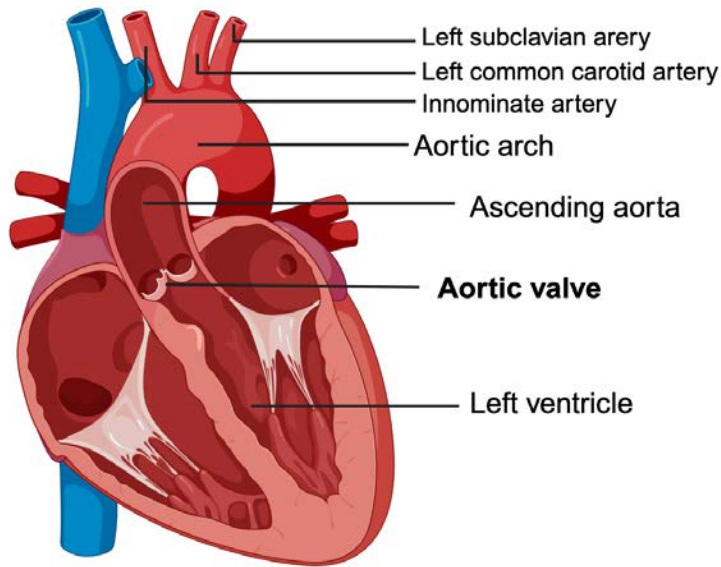


Figure 1. Anatomy of the heart

Cross-section of the human heart. Note the position of the aortic valve between the left ventricle and the aorta. Created with BioRender.com

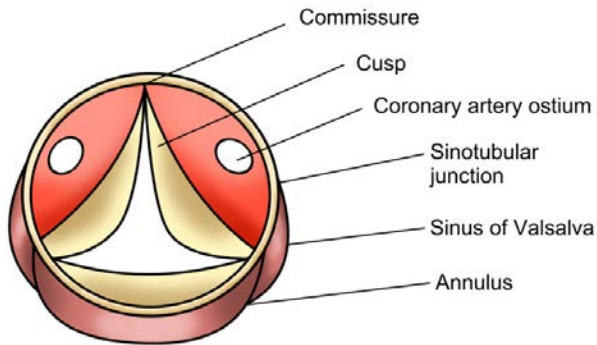


Figure 2. Anatomy of the aortic root

Schematic illustration of the aortic root, viewed from above. The aortic valve consists of three cusps, denoted by their relationship to the two coronary arteries: the right coronary cusp, the left coronary cusp, and the non-coronary cusp. The cusps are attached to the aortic root, which consists of the three aortic sinuses, and is delineated by the aortic annulus proximally and the sinotubular junction distally. The cusps are separated by commissures.

1.1.1 Microstructure of the aortic valve

The aortic valve cusps have a distinct layered structure (Figure 3). The cusps consist mainly of collagens, a class of structural extracellular matrix proteins, but also many other extracellular matrix proteins and specialized cells. Closest to the ascending aorta is the fibrosa (or arterialis), which consists mainly of collagen fibers that provide structural durability. The middle layer, termed the spongiosa, comprises a matrix with a high concentration of proteoglycans and glycosaminoglycans and has a gel-like structure due to the hydrophilic properties of its constituents. This serves to decrease and redistribute the strain the leaflet is subjected to when the blood flow reverses during diastole. Closest to the left ventricle is the ventricularis, with an extracellular matrix enriched with elastin fibers, which provides elasticity and thus facilitates proper coaptation of the cusps in diastole, reviewed in ⁴⁰. The entire valve cusp is covered by a monolayer of valvular endothelial cells, which have unique properties compared to aortic endothelial cells ^{41,42}. The endothelial cells are attached to the valvular basement membrane (BM) (described below), an interface between the endothelial layer and the underlying interstitium. The cells populating the interstitium are termed valvular interstitial cells (VICs), which constitute a diverse cell population that includes primarily fibroblasts and myofibroblasts, reviewed in ⁴³. Furthermore, heart valve cusps are also vascularized and contain both blood vessels, lymphatic vessels, and nerve fibers ⁴⁴⁻⁴⁶.

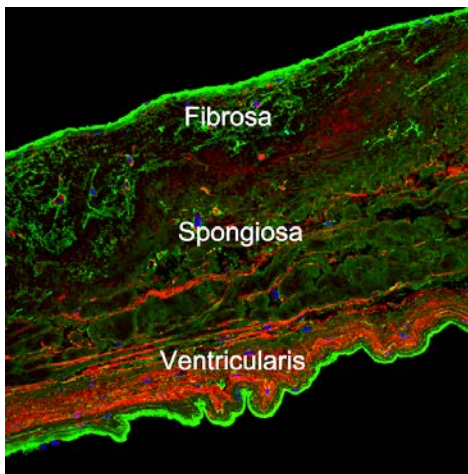


Figure 3. Histology of the aortic valve

Confocal microscopy image of a human aortic valve cusp. The cusp consists of three distinct layers: (1) the fibrosa on the aortic side, rich in collagen I (green), (2) the spongiosa in the middle, which contains a high concentration of proteoglycans, and (3) the ventricularis, rich in elastin (red). Blue color indicates cell nuclei. Adapted from Study I.

1.2 The bicuspid aortic valve

A BAV consists of two cusps rather than three as in the normal tricuspid aortic valve. BAV is the most common congenital cardiac malformation, and entails a considerably increased risk of both aortic valve dysfunction and ascending aortic disease (described below) ^{6,13,14}. The reported prevalence of BAV is 0.8-1.4% in the largest echocardiographic screening and necropsy series ^{1,2,47}. A male predominance of approximately 2.5-3:1 has been consistently reported ^{1,15,48}.

While BAV is often referred to as a single entity, it encompasses several distinct anatomic variants, or phenotypes (Figure 4), reviewed in ⁴⁹. The most common type of BAV (~90%) is a valve with two conjoined cusps, commonly separated by a raphe and an underdeveloped commissure ^{1,18,20,50}. These phenotypes are denominated by the cusp fusion pattern: right-left (R-L, prevalence ~70%), right-non-coronary (R-N, ~15-30%), or left non-coronary (L-N <5%) ^{1,18,20,39,49}. The cusp fusion may be complete or partial ("forme fruste") ⁴⁹. A BAV may also consist of two cusps without any raphe and only two aortic sinuses, termed a 2-sinus BAV (<10%). The 2-sinus phenotype was previously referred to as a "true BAV", and may be further classified as antero-posterior or lateral depending on the orientation of the cusps ^{1,18,20,39,49}. The different BAV phenotypes result in different flow perturbations in the ascending aorta ⁵¹⁻⁵³. Preclinical studies suggest that the different BAV phenotypes result from abnormalities at different stages of valve embryogenesis (see below).

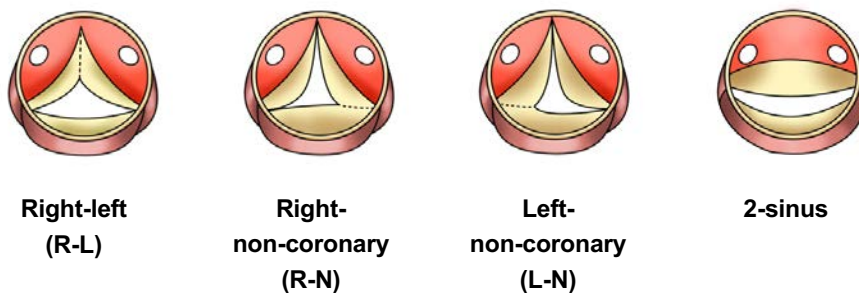


Figure 4. Bicuspid aortic valve phenotypes

Schematic overview of anatomical variants (phenotypes) of the bicuspid aortic valve. The right-left (R-L), right-non-coronary (R-N), and left-non-coronary (L-N) phenotypes are denominated according to which cusps are conjoined. The cusps are separated by a raphe and an underdeveloped commissure. The 2-sinus phenotype consists of two valve cusps without any raphe, with an antero-posterior (shown above) or a lateral cusp orientation. Note the presence of only two aortic sinuses and commissures in the 2-sinus phenotype.

1.2.1 Genetic origin of the bicuspid aortic valve

Several genetic syndromes are also associated with BAV and aortic disease, including Marfan syndrome (*FBN1*), Loeys-Dietz syndrome (*TGFBR1*), the vascular form of Ehlers-Danlos syndrome (*COL3A1*)⁴⁰, and Turner syndrome (karyotype 45, X)⁵⁴. Mutations in several genes have been linked to non-syndromic BAV, such as *NOTCH1*^{55,56}, *ROBO4*⁵⁷, *GATA6*⁵⁸, *SMAD6*⁵⁹, and *HOXA1*⁶⁰ to name a few. A common theme in several of the identified gene variants is that they appear to interfere with the signals between cardiac neural crest cells and other cells in the developing outflow tract^{55,59,60}. Interestingly, similar or identical genetic variants associated with BAV may sometimes lead to varying BAV phenotypes, which indicates an often complex interplay between different genetic and other developmental factors^{57,60}. Only a small minority of non-syndromic BAV cases (up to ~1%) can be explained by the individual genes identified so far^{57,61}, yet a high heritability of BAV and associated thoracic aortic disease has been demonstrated⁶².

1.2.2 Other anatomic variants

A related congenital aortic valve anomaly is the unicuspid (or unicomissural) aortic valve (UAV), in which the aortic valve consists of a single functional leaflet due to leaflet fusion at all but a single commissure or, rarely, all commissures^{3,63}. UAV is a very rare condition (<0.1%) that was previously considered a BAV phenotype due to its anatomical similarity^{1,2,39}. There are also significant similarities in clinical manifestations, with UAV being an even stronger risk factor for aortic valve and ascending aortic disease than BAV^{3,39,63}. An aortic valve may also have more than three cusps, such as in a quadricuspid aortic valve, which is an exceedingly rare congenital defect ($\leq 0.01\%$) with uncertain clinical implications².

1.3 Embryology

The primordial heart originates from the mesoderm, and is initially formed as a tube composed of a myocardial cell layer and an inner endothelial cell layer, reviewed in^{40,56}. The first stage of heart valve development is the formation of the endocardial cushions, which are budding protrusions in the cardiac jelly (named for its high proteoglycan and hyaluronan content) of the primitive heart tube^{40,56}. The cushions are formed by a process known as epithelial-to-mesenchymal transition (EMT), in which adjacent primordial endocardial cells traverse the underlying BM and migrate into the interstitium of the budding cushions⁵⁶. The aortic and pulmonary valves are derived from the cushions of the outflow tract. The semilunar valve development is intimately connected with the formation of the great arteries (the pulmonary trunk and ascending aorta) which develop through septation of the outflow tract⁵⁶.

The primitive heart is formed by two cardiac progenitor cell populations, denoted as the first and second heart fields^{40,56}. The great arteries and the heart valves are derived from the second heart field, originating from the pharyngeal mesoderm, but also from cardiac neural crest cells (NCCs), which originate from the ectoderm^{40,56}. These cardiac NCCs migrate into the endocardial cushions, where they form the aorticopulmonary septum in the outflow tract and

organize semilunar valve leaflet patterning, the latter by regulating proliferation of adjacent endocardial-derived mesenchymal cells within the endocardial cushions (Figure 5) ⁵⁶. The semilunar valves, pulmonary trunk, and proximal aorta thus have a distinct embryonic origin due to its formation by cells from two different germ layers. Blocking of specific pathways involved in interactions between the cardiac NCCs and surrounding mesenchymal cells results in BAV and/or congenital malformations in the ascending aorta and pulmonary trunk ^{55,56,64}. The R-L BAV phenotype is thought to be the result of defective NCC signaling, whereas R-N may be the result of defective endocardial cushion formation ⁶⁴.

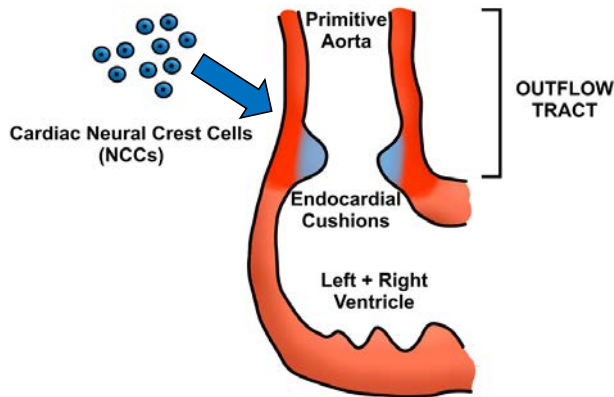


Figure 5. Embryonic development of the aortic valve and ascending aorta

The aortic valve and ascending aorta, together with the pulmonary valve and pulmonary trunk, originate from the outflow tract of the embryonic heart. Formation of the semilunar valves is initiated by budding of the endocardial cushions, with subsequent contributions of cardiac neural crest cells.

1.4 Valvular disease

Aortic valve disease is the most common complication of BAV. It can manifest as obstruction, known as aortic valve stenosis, regurgitation (also called insufficiency), or a combination of the two. Aortic valve disease has been estimated to affect up to ~50% of patients with BAV ^{14,15,65}, but a recent report estimates that the cumulative life-time risk of at least moderate aortic valve disease may be as high as 80% ⁶. Consequently, despite a prevalence of only ~1% ^{1,2}, about half of the patients in need of aortic valve replacement have BAV ^{3,66}. BAV-associated aortic valve disease is usually due to age-related degeneration or calcification of the valve cusps rather than a congenital lesion and is thus increasingly more common with higher age, but patients with BAV tend to present at least a decade earlier ^{3,6,13,14,67}. However, it may also present as a congenital valve disease in early childhood and infancy ^{1,20}. The risk of developing aortic valve disease varies somewhat depending on the BAV phenotype (see below). BAV may

also predispose to aortic valve infective endocarditis, a bacterial infection of the heart valve that leads to progressive aortic regurgitation and often septic embolism ^{13,14,68}.

1.4.1 Aortic valve stenosis

The most common cause of aortic valve stenosis in both BAV and TAV is calcific aortic valve disease, which overall is one of the most common cardiovascular diseases in the developed world ^{69,70}. It is a degenerative fibrocalcific disease with many similarities to atherosclerosis, reviewed in ⁷¹. Clinical risk factors for calcific aortic valve disease largely mirror those for other cardiovascular diseases, and include diabetes, hypertension, hypercholesterolemia, smoking, high age, and elevated levels of lipoprotein(a) ⁷¹. The progressive fibrosis and calcification of the aortic valve leaflets may eventually lead to valve failure due to obstruction, known as stenosis ⁷². Aortic valve stenosis leads to a compensatory hypertrophy of the left ventricle, gradually increasing both the oxygen demand and the perfusion pressure required to maintain adequate blood flow to the myocardium ⁷². Symptoms of aortic stenosis are secondary to decreased cardiac output and may include exertional dyspnea, angina pectoris, syncope and heart failure ⁷². Moderate to severe stenosis has a high mortality if left untreated ⁷³. Patients with a BAV have a very high risk of calcific aortic valve disease compared to patients with TAV, and they tend to present at least a decade earlier ^{3,67}.

1.4.2 Aortic valve regurgitation

Aortic regurgitation is less common than stenosis in both BAV and TAV and may be due to leaflet degeneration, aortic root dilatation or both ^{14,15,67,74}. Severe regurgitation leads to left ventricular overload and a significant drop in diastolic blood pressure. This leads to symptoms such as exertional dyspnea, angina pectoris, more severe symptoms of heart failure, and has a high mortality unless treated ^{75,76}.

1.5 Contemporary treatment for aortic valve disease

To this date there is no medical therapy for aortic valve disease, and the current treatment of choice is usually surgical or endovascular replacement with a biological or mechanical prosthetic valve ⁷⁵.

1.5.1 Surgical treatment

The most common surgical procedure is replacement with a biological or mechanical prosthetic valve under cardiopulmonary bypass. Biological prosthetic valves are glutaraldehyde-fixed xenografts, usually in the form of bovine pericardium mounted on a synthetic ring (stented), a porcine aortic valve mounted on a stent, or a whole porcine aortic root (termed stentless, or biological composite graft), reviewed in ⁷⁷. Less commonly, a cryopreserved allograft (a frozen aortic root from a deceased donor) may be used for biological valve replacement. Both types of biological valves have limited durability due to gradual structural deterioration, eventually

leading to graft failure and need for a new valve replacement procedure^{7,78}. The mechanism of failure has been largely attributed to immune-mediated rejection of the grafts^{78–81}. Mechanical valves, usually made of carbon, on the other hand offer long-term durability but necessitate life-long treatment with potent anticoagulants and are associated with complications related to thromboembolism and bleeding⁷.

An alternative approach in some cases (predominantly aortic regurgitation) is valve repair, which may entail narrowing of a dilated annulus (annuloplasty) and/or surgery on the cusps⁸². A further option, typically reserved for young patients, is the Ross procedure, in which the aortic valve is replaced by the patient's own pulmonary valve, which in turn is replaced by a cryopreserved pulmonary valve allograft. The procedure is technically challenging and only performed at expert centers, but the combination of good long-term durability and lack of need for anticoagulants can make it an excellent option in young adults⁸³. A final option is the Ozaki procedure, a more recently developed, technically challenging and rare operation, which involves the creation of new valve cusps using the patient's own pericardium⁸⁴.

1.5.2 Endovascular treatment

Transcatheter aortic valve replacement (TAVR) has become the intervention of choice for an increasing number of patients with aortic stenosis and TAV⁸⁵. Briefly, the femoral artery is cannulated and a large stent carrying a biological prosthetic valve is delivered with a catheter and deployed with an expanding balloon at the position of the aortic valve⁸⁵. Due to technical challenges related to anatomical differences and concerns regarding aortic outcome, TAVR has not been employed in patients with BAV except in selected cases⁸⁶.

1.6 Heart valve tissue engineering

The limitations of contemporary treatment options for aortic valve disease are especially problematic for patients with BAV, who may require surgical replacement at a young age, sometimes in early childhood^{20,87}. The limited durability of biological valves, particularly evident in young recipients, necessitates repeated cardiac procedures in children and young or middle-aged adults⁷⁵. Mechanical valves do not deteriorate over time, but the necessity of vitamin K antagonist treatment and the cumulative risk of bleeding and thromboembolic events is concerning for younger patients⁷. Furthermore, both types of prosthetic valves are associated with adverse maternal and fetal outcomes during pregnancy⁸⁸. Results from the Ross procedure demonstrate that a valve composed of the recipients own, living cells is superior to other forms of biological valves^{83,87,89}. The ideal replacement would be a storable valve scaffold with the potential to develop into a new, native aortic valve through reconstitution by the recipient's own cells. The process of creating such a heart valve, or other artificial tissues created through incorporation of living cells, is termed *tissue engineering*.

Tissue-engineered heart valves have been the subject of many studies, including both animal models^{90–96} and clinical trials^{97–99}. Tissue-engineered valves can be broadly divided into two categories: decellularized valves, which are essentially allografts from deceased donors that are

processed to remove the donor cells ^{8,97}, and *in vitro* synthesized valves, which are valve-shaped scaffolds of either synthetic or biological origin ^{9,95,100}. The resulting valve scaffolds may be repopulated with cells (recellularized) *in vitro* prior to implantation, or exclusively *in vivo*, most likely through migration and proliferation of adjacent cells ^{99,101,102}.

1.6.1 Decellularization

Decellularization refers to the process of removing the cellular component of an organ or tissue, thus creating a cell-free extracellular matrix scaffold. The rationale behind the method is two-fold: i) enable reconstitution of a tissue from a deceased donor with living cells, which may be guided by cues from the extracellular matrix scaffold, and ii) decrease tissue immunogenicity, reviewed in ¹⁰³. Decellularization has been employed in a variety of tissues and organs in regeneration experiments, including the heart, lung, and liver ^{104–106}. The theoretical advantage of decellularization compared to other methods is that it may preserve the gross anatomy, as well as part of the complexity of the native extracellular matrix, which is virtually impossible to recreate from the ground up. Many methods of decellularization have been described in the literature, but most protocols include processing of a tissue with detergents and/or enzymes. The various methods differ substantially in their mode of action, and the effectiveness of a particular reagent or protocol depends on tissue cellularity and vascularization, as well as extracellular matrix density, composition, and thickness ^{103–106}. Decellularization reagents may be combined in any fashion and applied to a tissue or organ through different techniques, but usually by immersion and agitation or perfusion ¹⁰³. An important limitation of the decellularization concept is that it is not possible to remove the cellular component without affecting the extracellular matrix ¹⁰⁷. For this reason, it is imperative to find an appropriate balance between cell removal and extracellular matrix preservation for the specific tissue and the intended application.

1.6.2 Clinical trials

Significant efforts have been made to translate decellularized heart valve scaffolds into the clinic. While some short- to mid-term results of aortic and pulmonary valve replacement with decellularized valve allografts have been promising ^{97–99,108,109}, a report on the long-term fate of decellularized aortic valves showed a strong trend toward higher reoperation rates when compared to conventional cryopreserved allografts ^{10,81}.

1.6.2.1 Modes of failure in tissue-engineered heart valves

The cause of failure of tissue-engineered heart valves remains largely unexplored ^{10,12}. However, it is well known that all organ and tissue transplants - including heart valve allografts - elicit an immune response that leads to graft degeneration and failure ^{81,92,110,111}. It is also increasingly recognized that decellularized valves remain immunogenic, despite minimal donor cell remnants ^{10,11,112}. Another major concern has been the ability of both decellularized and other engineered heart valve scaffolds to be repopulated with living cells *in vivo*, with

reports invariably describing aberrant cell phenotypes, incomplete, or virtually absent recellularization^{10,95,99,112}. This latter issue raises questions regarding how scaffold properties may impact recellularization potential, and especially how interactions between cells and the scaffold may affect cell phenotype, migration, and proliferation. Although extensive decellularization is desirable from an immunological perspective¹¹³, damage to the extracellular matrix may inhibit organized integration of cells into a decellularized scaffold^{107,114}. Of note, clinical studies of decellularized heart valves have typically focused on cell removal, likely at the expense of extracellular matrix preservation and integrity^{97–99,108,109}.

1.7 The basement membrane

All endothelial and epithelial cells are anchored to the underlying extracellular matrix through the BM (Figure 6). The BM is a thin, tissue-specific structure that may include a number of different molecules, but always consists of at least one of the following molecules, which are considered the core components: laminin, collagen IV, heparan sulfate proteoglycans, and nidogen, reviewed in¹¹⁵. Collagen IV is a network-forming collagen that stabilizes the BM, whereas nidogen (or entactin) serves as a bridging molecule between laminin and collagen IV by forming chemical bonds with both¹¹⁶.

1.7.1 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans, mainly perlecan in the vasculature, may sequester growth factors and cytokines through their heparan sulfate glycosaminoglycan side chains and hence regulate the bioavailability of these molecule, reviewed in¹¹⁷. These are important functions in vascular biology and tissue regeneration, and perlecan is critical for normal outflow tract development during cardiac embryogenesis^{114,118–121}.

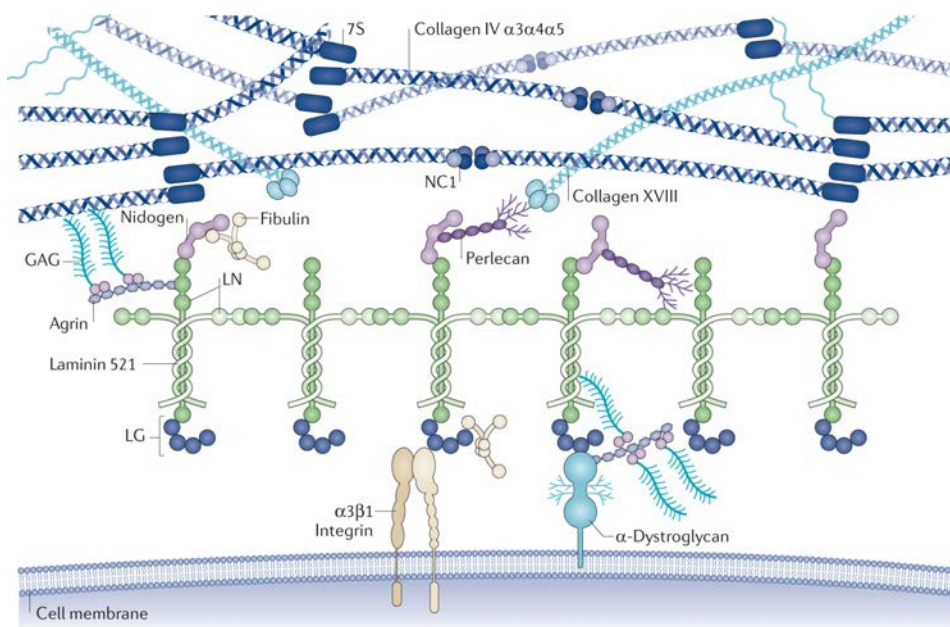


Figure 6. The basement membrane

Schematic illustration of a basement membrane, exemplified by the glomerular basement membrane of the kidney. Integrins and other cell surface receptors interact with laminins through the LG domain. Heparan sulfate proteoglycans (perlecan, agrin, collagen XVIII) and nidogen form chemical bonds with both laminin and the network of polymerized collagen IV, thus completing basement membrane assembly. Material from: 'Naylor et al, Complexities of the glomerular basement membrane, published 2021 Springer Nature, reproduced with permission of SNCSC' ¹²².

1.7.2 Laminins

Laminins are cruciform or anchor-shaped, large, trimeric glycoproteins, and the first extracellular matrix proteins to be expressed during embryogenesis ¹²³. They consist of an α chain (one of five isoforms), a β chain (one of three isoforms), and a γ chain (one of three isoforms), with the chain isoforms named according to Arabic numerals ¹²⁴. There are 16 known laminin isoforms in mammalian BMs, which are denominated by their constituent chains ^{125,126}. For example, laminin (LN) 521 denotes the laminin molecule composed of the $\alpha5$, $\beta2$ and $\gamma1$ chain ¹²⁶. Laminins interact with cell surface adhesion molecules, mainly integrins and dystroglycan, and the importance of laminins is such that several isoforms are essential for normal embryonic development, reviewed in ¹²⁴.

Endothelial BMs consist primarily of laminin chains $\alpha4$ and $\alpha5$, which combine with $\beta1$, $\beta2$, and $\gamma1$ chains to form LN 411, 511, 421 and 521 ^{127–133}. Blood vessels may also contain $\alpha2$ isoforms (LN 211 and/or 221) in the smooth muscle BMs of large arteries ¹³⁴. The expression of $\alpha4$ in relation to $\alpha5$ is known to regulate endothelial cell-cell junction stability, specifically vascular endothelial cadherin (VE-cadherin), and thus the permeability of the endothelium, with $\alpha4$ isoforms being permissive and $\alpha5$ isoforms being restrictive ^{135–139}. Furthermore, $\alpha4$

has an important role in angiogenesis and the promotion of endothelial cell survival^{138,140–142}. The $\alpha 5$ laminin chain (LN 511 and 521) on the other hand has been shown to be important in the shear stress response of resistance arteries¹⁴³. The β chain also affects the properties of a laminin molecule, and an increased endothelial $\beta 1$ expression in relation to $\beta 2$ has been associated with epithelial-to-mesenchymal transition¹⁴⁴. Additionally, LN 111 (or derivatives thereof) may affect the calcification potential and hemostatic properties of animal-derived valvular interstitial and endothelial cells *in vitro*^{145–147}. However, LN 111 is not expressed in blood vessels and the relevance of these findings, and the role of laminins in general, in human valve biology is uncertain as the expression of laminin chains in heart valves has not been reported¹⁴⁸.

1.7.3 Role in tissue regeneration

It is widely accepted that matrix preservation is desirable for the function of a tissue-engineered graft¹⁰³, but the role of an intact BM - and particularly its individual components - is mostly uncertain. The relative importance of a preserved BM may also vary between tissues^{114,149}, but an intact BM does appear to be important for endothelial cells¹⁰⁷. Further, heparan sulfate proteoglycans have been demonstrated to be key components in a decellularized lung scaffold and vital for proper differentiation of airway progenitor cells into mature airway epithelium¹¹⁴. Additionally, recombinant laminins can be used as a cell culture substrate, with a profound, isoform-specific impact on the phenotype of cardiac mesenchymal stromal cells and embryonic stem cells^{150,151}. There is also limited evidence suggesting that coating of a decellularized rat aortic scaffold with LN 111 may improve endothelialization *in vivo*⁹³. A small-animal aortic valve scaffold subjected to mild decellularization may facilitate both *ex vivo* and *in vivo* studies on the role of individual BM components and shed light on their role in valve regeneration^{114,152}.

1.8 The thoracic aorta

The thoracic aorta consists of the ascending aorta, which includes the aortic root and the ascending (or tubular) segment, the aortic arch, and the descending aorta, which runs along the vertebrae and is continuous with the abdominal aorta, located below the diaphragm. The ascending aorta and the aortic arch are delineated by the origin of the innominate artery (or brachiocephalic trunk). The arch also contains the origins of the left common carotid artery and the left subclavian artery, which marks the transition into the descending aorta¹⁵³. The innominate artery and left common carotid artery may have a common origin - an anatomical variant termed bovine aortic arch, which has been associated with thoracic aortic disease^{154,155}. Like all blood vessels, the aorta contains three distinct layers - the tunica intima, the tunica media, and the tunica adventitia (or externa) (Figure 7). The primary component of the intima is a single layer of endothelial cells. The media, by far the thickest layer of the aorta, consists predominantly of smooth muscle cells and elastic lamellae formed by elastin and collagen¹⁵⁶. Both the aortic endothelial and smooth muscle cells are attached to a BM¹³⁰. The adventitia, primarily composed of collagen, is a thin but highly durable sheath that covers the entire aorta

¹⁵⁶. The predominant cell types in the adventitia are fibroblasts and macrophages. The adventitia also contains nerve fibers and blood vessels that supply nutrients to the outer layer of the aortic wall (the vasa vasorum) ¹⁵⁷.

The aorta has a somewhat tapered morphology, with the average diameter decreasing from ~3.5 cm in the aortic root to ~2.5 cm in the distal descending aorta ^{158–160}. The normal dimensions of the ascending aortic segments vary according to age, sex, body size, and ethnicity ^{158–162}.

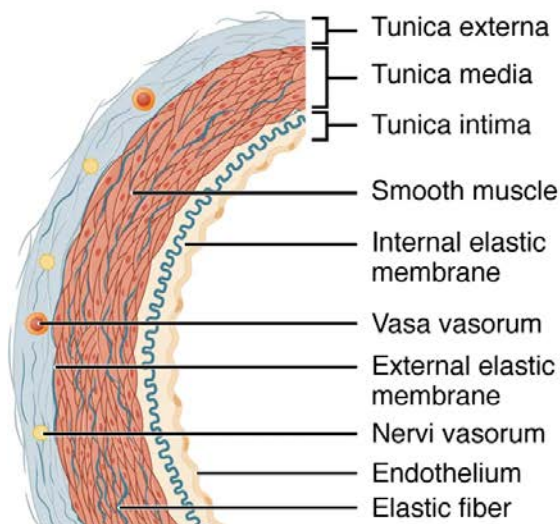


Figure 7. Structure of the aortic wall

Schematic illustration of the microstructure of an artery. The internal elastic membrane is usually indistinguishable from the media in the aorta. Adapted (cropped) from Figure 20.3, "Structure of blood vessels", in *Anatomy and Physiology 2e*, OpenStax 2022. © Jun 15, 2023 OpenStax. Access for free at <https://openstax.org/books/anatomy-and-physiology-2e/pages/1-introduction>. Reproduced under a Creative Commons license (CC BY 4.0 DEED).

1.9 Thoracic aortic disease

Thoracic aortic disease, or aortopathy, includes aneurysm or dissection in the ascending aorta, the arch, or in the descending aorta. An aneurysm is traditionally defined as an abnormal, localized dilatation of a vessel that exceeds $\geq 50\%$ compared to the expected diameter ¹⁶³. The terms "dilatation" and "aneurysm" are often used interchangeably, but dilatation is a more generic phrase that includes any degree of enlargement ⁴⁹. The pathology of ATAA is typically characterized by loss of medial smooth muscle cells, extracellular matrix fragmentation, inflammation, fibrosis, and often atherosclerosis ^{24–26,30,31}. The latter is also frequently found in imaging studies of descending aortic aneurysms, which are otherwise relatively poorly characterized ¹⁶⁴. There are substantial pathological and morphological similarities between ascending thoracic, and perhaps particularly descending, and abdominal aortic aneurysms (AAAs) ^{31,165,166} and it is not uncommon for an individual to develop both types of aneurysm

^{160,164,167}. Unlike AAAs, however, formation of an intraluminal thrombus inside the aneurysm is very rarely described in thoracic aortic aneurysms ¹⁶⁸.

Thoracic aortic aneurysms most frequently develop in the ascending aorta, followed by the descending aorta, and some are located in the arch ^{160,164}. They may occasionally extend into the abdomen and are then termed thoracoabdominal aortic aneurysms ²³. Risk factors include BAV (see below), male sex, hypertension, family history, and a coexisting aortic aneurysm ¹⁶⁰. The role of established risk factors for AAA, such as male sex, smoking, and coronary artery disease, is less clear in the thoracic aorta, whereas diabetes mellitus is inversely associated with all forms of aortic aneurysm ¹⁶⁰. Other risk factors include variant aortic arch anatomy (bovine aortic arch, described earlier) and renal cysts ^{154,169}. ATAAs are further classified according to the affected segments as i) root phenotype (confined to the root segments), ii) ascending phenotype (tubular ascending segment only), or iii) root extended phenotype (both root and tubular segment dilatation) (Figure 8) ⁴⁹. The clinical course of a thoracic aortic aneurysm is usually insidious as symptoms are rare until an aortic dissection or rupture occurs - catastrophic *aortic events* with a very high mortality ¹⁷⁰.

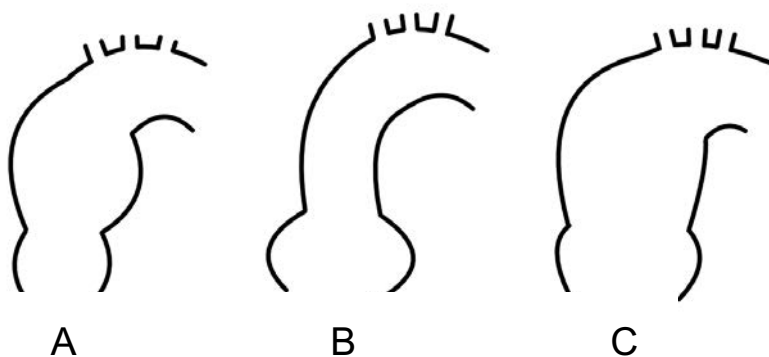


Figure 8. Patterns of ascending aortic dilatation

(A) Ascending phenotype. (B) Root phenotype. (C) Root extended phenotype.

1.9.1 Aortic dissection

Aortic dissection refers to an intimal tear that allows blood to spread inside the vessel wall, dividing (or dissecting) the intima from the media (Figure 9). The condition is incredibly painful and associated with a very high morbidity and mortality ¹⁷⁰. Risk factors (other than BAV and thoracic aortic aneurysm) include hypertension, connective tissue disorders, male sex, and other cardiovascular disease ¹⁷⁰. The Stanford classification is the most frequently used classification for aortic dissections, which are accordingly divided into two categories: type A (involves the ascending aorta), or ii) type B (involves any other segments). The size of the aorta

at the time of dissection varies considerably, and predicting which patients may be at risk consequently poses a remarkable clinical challenge. However, it has been established that the relative risk increases with larger aortic size and that dissections in normal-sized aortas are very rare ^{16,171}.



Figure 9. Aortic dissection

Schematic illustration of an aortic dissection (Stanford type A). The false lumen (red) extends from the ascending aorta to the descending aorta. Note the intimal tear in the ascending aorta. Image by www.MedicalGraphics.de. Reproduced under a Creative Commons license (CC BY-ND 4.0).

1.10 Bicuspid aortopathy

BAV has long been known for its association with ascending aortic disease, which was first described by M.E. Abbott in 1927 (referenced in ²) and is often referred to as bicuspid aortopathy ⁴⁹. Unlike most other forms of aortic aneurysm, bicuspid aortopathy may present in infancy and childhood. Sillesen et al. found that ascending aortic dilatation was present in 30% of neonates with BAV, and the prevalence is even higher in cohorts at pediatric referral centers ^{1,20}. Longitudinal studies in adult cohorts have found that the risk of aortic dilatation increases over time, eventually estimated to affect up to 45% of adult patients with BAV ^{14,15,65}. Remarkably, a recent study reports a cumulative lifetime risk of aortic dilatation as high as 75% ⁶. Thoracic aortic disease associated with BAV may affect the aortic root, ascending aorta, and occasionally the aortic arch, but does not extend into the descending or abdominal aorta ^{4,153,172}. Overall, individuals with BAV have a 8-9 times increased risk of aortic dissection compared to individuals with TAV ^{2,65}, but the absolute risk of aortic events in individuals with BAV is low. A systematic review reported an incidence rate of 0.4% over 2-16 years of follow-up and the lifetime risk of an aortic event has been estimated to 1.6% by others ^{6,173}. BAV is also associated with aortic coarctation, a congenital narrowing of the distal aortic arch ^{1,20,174}.

1.10.1 Pathology

The characteristic pathology of BAV ATAA was previously referred to as "cystic medial necrosis", a term that is now obsolete ¹⁷⁵. Compared to TAV-associated aortic aneurysms, BAV-associated ATAAs are typically characterized by less severe histological changes. There is a significant loss of smooth muscle cells, but typically absence of fibrosis, inflammation, and atherosclerosis ^{5,24–26}. Of note, the elastic lamellae appear largely intact in dilated BAV aortas ^{25,30}. Furthermore, recent biomechanical and longitudinal clinical studies have demonstrated that the aortic wall of an ATAA is more durable in patients with BAV compared to patients with TAV and may be less prone to dissection and rupture ^{28,29}. The underlying pathology of bicuspid aortopathy is unknown, but several studies have implicated a compromised intimal endothelium, characterized by loose cell-cell junctions, increased vascular permeability, and epithelial-to-mesenchymal transition ^{27,30,176}. These phenotypic changes, seen even in non-dilated BAV aortas, could be consistent with an altered laminin composition in the ascending aortic intimal BM ^{136,139,144}. Indeed, several other disease processes, including aging, myocardial ischemia, and atherosclerosis are associated with an altered endothelial laminin composition ^{144,177}.

1.10.2 Genetics or hemodynamics?

Two main theories have been proposed to explain the high prevalence of ATAA in patients with BAV; genetics and hemodynamics, reviewed in ¹⁷. The genes that have been linked to BAV, both syndromic and non-syndromic, are also frequently associated with ascending aortic dilatation ^{40,54,55,57,59}. Furthermore, the frequent presence of dilatation in neonates and the shared heritability between BAV and aortic dissection, which is aggravated if the relative with BAV also has ascending aortic dilatation, is highly consistent with a genetic basis for BAV aortopathy ^{1,62}.

However, there is also evidence for a role of altered hemodynamics in the pathogenesis of BAV aortopathy. Importantly, longitudinal studies have clearly identified valve disease as a risk factor for subsequent aortic complications ^{4,14,65}. Further, flow abnormalities in the ascending aorta differ between BAV phenotypes, and the aortic segments with the most perturbed flow appear to be more prone to aortic dilatation ^{51,52,178}. Although it has been demonstrated that ascending aortic dilatation can develop independently of valve dysfunction, even a normally functioning BAV may produce subtle alterations in flow in the ascending aorta ^{51,67}. Another compelling (albeit controversial) argument in favor of a role for hemodynamics is the low risk of aortic complications in patients with BAV who have undergone valve surgery ^{179–184}. It should be emphasized that the two theories are not mutually exclusive, and the current consensus is that both factors are likely to contribute to ascending aortic dilatation ¹⁷.

1.11 Sex differences in bicuspid aortic valve disease

The reason for the higher prevalence of BAV in males is largely unknown, but studies on patients with Turner's syndrome (karyotype 45, X) show that X-bound genes may interact with

autosomal genes and thus contribute to both BAV formation and aortopathy⁵⁴. Aortic valve stenosis is the most common type of valve disease in both sexes, but aortic valve regurgitation is more common in males than females^{19,21,185–187}. A concerning finding is that female patients with BAV tend to undergo aortic valve surgery with more advanced disease^{186,187}. As described earlier, normal thoracic aortic dimensions vary according to sex and body size. While studies have consequently shown that female patients with aortic dilatation (BAV or TAV) are at higher risk of aortic events for a given aortic diameter, most studies demonstrate that this may be corrected for by adjusting for body size (height or BSA)^{16,188,189}. In contrast to the guidelines for abdominal aortic aneurysm, a uniform size threshold for intervention for both sexes is advocated for thoracic aortic disease²³. Although size indexing is recommended for short (or tall) individuals of either sex²³, the continued use of a common size threshold for both sexes in thoracic aortic disease remains controversial¹⁹⁰. Aortic root dimensions are larger in male patients with BAV, even after correction for body size, and male patients are at higher risk of root and/or root extended phenotype aortic dilatation^{20,185,191}. Overall, aortopathy may be more common in male patients with BAV, but most studies have employed fixed diameter thresholds to define aortopathy^{185–187}. Of note, it has been established that BAV phenotypes differ in their associations with valve disease and aortic dilatation, but whether these associations are affected by sex remains unknown⁴⁸.

1.12 Clinical significance of the bicuspid aortic valve phenotype

Many studies have sought to determine the clinical implications of the different BAV phenotypes. The R-L phenotype has been associated with a higher prevalence of aortic root dilatation in children and adults, but also a higher overall prevalence of aortic dilatation in some studies^{18,20,21,191,192}. The R-N phenotype on the other hand has been associated with preferential dilatation at the ascending segment, a larger aortic arch, and valve dysfunction^{18–20,174,191}. Adult patients with 2-sinus BAV tend to present at an earlier age and may have a higher frequency of root phenotype aortic dilatation and a lower frequency of valve dysfunction^{18,22}. Due to their rarity, only a few studies include the 2-sinus and L-N phenotypes in analyses^{22,185}. Furthermore, R-L fusion and 2-sinus BAV are strongly associated with aortic coarctation in pediatric cohorts^{20,174}.

A significant limitation in most of the studies is the dependence on transthoracic echocardiography (TTE) rather than surgical inspection. While TTE is routinely used for diagnosis of valvular heart disease, its diagnostic accuracy when it comes to BAV has been contested and may vary between phenotypes^{193,194}. Most studies have also had a cross-sectional design, and the observed differences in aortic valve dysfunction, aortic dimensions, and aortic dilatation between phenotypes have generally been minor. On the other hand, the few studies that do include longitudinal data indicate that the BAV phenotype may have prognostic implications, both for the risk of valve and aortic surgery as well as ascending aortic growth, and could therefore be of considerable interest for the clinical management^{18,195}. It is thus important to discern if these clinical associations differ between the two sexes.

1.13 Contemporary management of ascending aortic disease

The only treatment for ATAA or type A aortic dissection is prophylactic or emergent surgical repair^{23,75}. The aim of elective aortic surgery is almost always to prevent an aortic event rather than alleviating symptoms, and the decision to operate is consequently an act of balance between the predicted survival benefit and the risk of the procedure. Although surgical outcomes for thoracic aortic disease have improved substantially over the years, the in-hospital mortality is still ~2% for elective ascending aortic surgery, stressing the need for accurate identification of patients at risk of aortic events^{196,197}. Surgical treatment of ATAA typically entails resection of the dilated segment and replacement with an interposed synthetic graft (usually made of Dacron) anastomosed end-to-end both proximally and distally^{36,82}. The aortic root may be replaced with a valved synthetic graft, a porcine aortic root (stentless porcine valve), a cryopreserved allograft, or through the Ross procedure, as described previously^{23,89}. Alternatively, the native valve may be retained in a valve-sparing root replacement procedure with a synthetic graft⁸². The anastomosis between the graft and distal ascending aorta may be performed either with continued cardiopulmonary bypass and the aortic cross-clamp in place, or in circulatory arrest (open distal anastomosis), which allows a more complete resection of the ascending aorta³⁶. Resection and graft replacement may be extended into the arch, or the arch and descending aorta, the latter through a one- or two-stage procedure called frozen elephant trunk. A more extensive aortic resection entails a progressive increase in surgical risk^{198,199}.

1.13.1 Pharmacological therapy

Aggressive blood pressure control is recommended (target blood pressure <130/80 mmHg), but no specific pharmacological therapy has been proven to affect disease progression in thoracic aortic aneurysms²³. Several agents have been investigated, including beta blockers, angiotensin II-receptor inhibitors, and statins. Current recommendations regarding specific classes of antihypertensives are based on studies of patients with Marfan syndrome and may not apply to other patient groups, and statins have only been associated with reduced risk of aortic dilatation in small cross-sectional studies^{23,200}. The rationale for medical therapy in current guidelines is often risk reduction related to comorbidities (other cardiovascular disease) rather than the aneurysmal disease itself, and it remains highly controversial²³. However, the significant pathological differences described previously strongly imply that the effects of a pharmacological agent may differ substantially depending on the aortic valve phenotype. One such medication that could have a differential effect in BAV- and TAV-associated ATAA is acetylsalicylic acid.

1.13.1.1 Acetylsalicylic acid

Acetylsalicylic acid (ASA), also known as aspirin, is one of the most common medications in the treatment and prevention of cardiovascular diseases^{201,202}. Its use as an anti-inflammatory agent dates back to ancient Greece, where the salicylate-containing willow bark was used as an analgesic²⁰². The primary effects of ASA are mediated by its inhibition of prostaglandin

and thromboxane A₂ synthesis by cyclooxygenase (COX)-1 and COX-2²⁰². Inhibition of COX-1, which is constitutively expressed by most cell types, effectively stops platelet production of thromboxane A₂ and thus limits platelet aggregation, which is the desired effect in cardiovascular prevention²⁰². In contrast, COX-2 expression is induced in conjunction with inflammation, and ASA blocks COX-2 messenger ribonucleic acid (mRNA) synthesis²⁰³. This leads to decreased synthesis of proinflammatory prostaglandins, which have been implicated in the pathology of aortic aneurysms^{32,204–208}.

Clinical studies on ASA and other COX inhibitors (non-steroid anti-inflammatory drugs, NSAIDs) have mostly focused on AAA, with results ranging from lower aneurysm growth and risk of rupture to higher mortality in ruptured AAA with ASA^{32,204,209,210}. Results have been equally varied in the three studies including patients with thoracic aortic aneurysms; one showed that ASA protects against aortic events, one showed an uncertain effect, and one showed an adverse effect on a composite outcome including both mortality, aortic events, and aortic surgery^{32–34}.

1.14 Distal aortic outcome after ascending aortic surgery

ATAA is associated with aneurysms elsewhere along the aorta^{160,164}. As a consequence, contemporary guidelines recommend surveillance of the remaining aorta after ascending aortic replacement to identify residual aortopathy^{23,211}. As described earlier, however, the pathology of BAV and TAV ATAA differ significantly, and there is no known relationship between BAV and aneurysms beyond the aortic arch, in which clinically significant dilatation is rare in patients with BAV^{4,153,172}. This has been corroborated in clinical studies, which demonstrate a very low risk of arch complications in patients with BAV^{35,36,212}. However, there have not been any systematic comparisons between BAV and TAV with regards to the development of the distal aorta after ascending aortic surgery, and not for segments distal to the aortic arch. Improved knowledge regarding differences in aortic outcome between BAV and TAV after ATAA surgery would facilitate a more individualized approach to the management of ATAA. This would benefit patients, as individualized management - based on risk stratification - could reassure individuals at low risk and ascertain adequate follow-up and risk factor management in patients with an increased risk. It would also ensure an accurate risk-benefit analysis for any extended aortic surgery.

2 Research aims

The overarching aim of this thesis was to investigate the clinical implications of BAV and ATAA, as well as potential treatments for BAV- and TAV-associated valve dysfunction and ascending aortic pathology. Specific aims were:

- I. To characterize the laminin isoforms in the BM of the healthy aortic valve, to ensure fidelity in heart valve tissue engineering experiments (Study I, first part).
- II. To develop a small animal decellularized scaffold with a preserved BM, for future mechanistic studies of the role of laminins and other BM components in valve regeneration (Study I, second part).
- III. To explore sex differences in valvular and aortic pathology in relation to BAV phenotype and the type of valve disease (Study II).
- IV. To investigate if ASA therapy may be associated with the prevalence of ascending aortic dilatation in patients with BAV and TAV (Study III).
- V. To map the long-term growth in the distal aorta and clinical outcomes after ascending aortic surgery in patients with BAV and TAV (Study IV).

3 Materials and methods

3.1 Human aortic valve specimens

Human aortic valves were obtained from deceased organ and tissue donors without a documented heart valve condition. Tissues were inspected to confirm a tricuspid phenotype and to assess for gross morphological abnormalities. The valve cusps were subsequently excised and embedded in optimal cutting temperature (OCT) compound for histological analysis.

3.2 Rat aortic valve scaffold

Female Sprague-Dawley rats aged 8-12 weeks were euthanized and all organs in the chest cavity were removed *en bloc*. The aortic valves were dissected as U-shaped valved aortic conduits as described by others^{111,152,213}, with the branches of the aortic arch ligated with sutures. Normal controls were embedded in OCT for histological analysis or processed for deoxyribonucleic acid (DNA) extraction and quantification.

3.2.1 Decellularization

A detergent-based decellularization method based on previously published protocols for other rat organs was used to produce a heart valve extracellular matrix scaffold^{104,105}. In brief, the aorta was cannulated with a conventional intravenous catheter and perfused with sodium dodecyl sulfate for 1-12 h, followed by 30 min perfusion with Triton X-100 through a tubing pump (Figure 10). Finally, the ascending aorta was divided at the origin of the innominate artery and the aortic valve, together with the remainder of the aorta, were treated with benzonase endonuclease on a see-saw rocker. Washing steps with phosphate-buffered saline (PBS) were included after processing with each reagent.

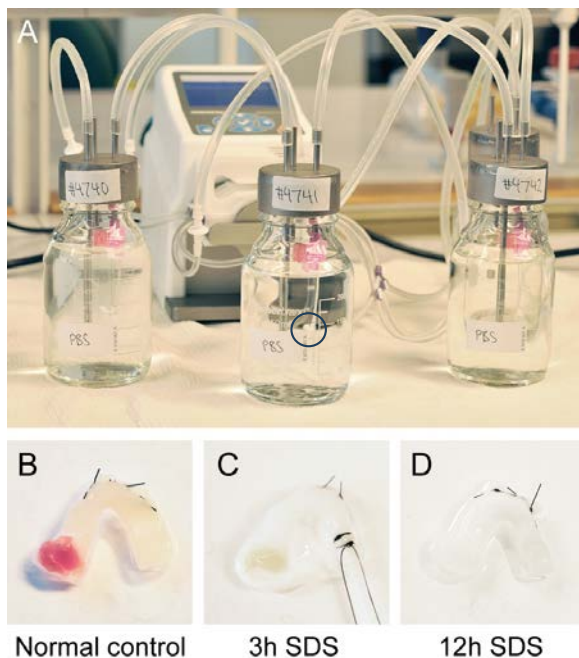


Figure 10. Overview of the perfusion decellularization process

(A) Three parallel decellularization experiments. Custom-built bottle caps with three pipes (inflow, drainage, air vent) were connected to a tubing pump to enable recirculation of liquids. The aortic valve conduit (encircled) was cannulated with an intravenous cannula and connected to the inflow pipe. (B) Aortic valve conduit before decellularization. (C) Aortic valve sample after 3 h processing with SDS, shown with the descending aorta cannulated. (D) Aortic valve after 12 h processing with SDS. I.V., intravenous; SDS, sodium dodecyl sulfate.

3.3 Assessment of human and rat aortic valves

3.3.1 Histology and immunohistochemistry

Human and rat aortic valves were sectioned in a cryostat and stained with hematoxylin and eosin (H&E) for general morphological studies. Immunohistochemistry was used to detect laminin chains in human and rat aortic valves, as well as other BM components (perlecan core protein, heparan sulfate glycosaminoglycans, collagen IV) and fibronectin in normal and decellularized rat aortic valves. Staining of nuclei with hematoxylin and 4',6-diamidino-2-phenylindole (DAPI) was used to confirm cell removal in decellularized rat aortic valves.

3.3.2 DNA quantification

Rat aortic valve cusps were excised at their base for comparison of the DNA content of normal and decellularized aortic valves. The valve cusps were processed for DNA extraction, after which the DNA content was quantified with a spectrophotometer. The mass of the valve cusps could not be reliably measured due to their small size and the DNA content was consequently presented per valve rather than per unit of mass.

3.4 Patient cohorts

Observational cohort studies were conducted on adult cardiac surgical patients from two institutions, Karolinska University Hospital Solna (Study II-IV) and the University Medical Center Schleswig-Holstein, Lübeck, Germany (Study II). All patients underwent elective, primary aortic valve surgery and/or ascending aortic surgery. Details regarding the surgical procedure were recorded and the aortic valve phenotype was determined by the surgeon. The medical history, including medications, was documented with the aid of a dedicated research nurse and/or retrieved from the medical records. An overview of the patient cohorts and the design of each study is shown in Table 1.

3.4.1 The Advanced Study of Aortic Pathology (ASAP) cohort

The ASAP cohort consists of 595 prospectively enrolled patients aged ≥ 18 years, of which 573 patients completed the inclusion process and underwent primary elective aortic valve and/or ascending aortic surgery at Karolinska University Hospital between February 2007 and November 2012. Exclusion criteria were significant coronary artery stenosis (according to the preoperative angiography) and monogenic syndromes. Surgical biopsies from the ascending aorta and other tissues were obtained for analysis, as was blood samples. An additional 298 patients were included without tissue biopsies (the "mini-ASAP" sub-cohort), constituting a total of 871 patients. All patients underwent preoperative transthoracic echocardiography and/or intraoperative transesophageal echocardiography to assess valve function and measure ascending aortic dimensions. A subset of patients underwent computed tomography (CT) to assess aortic dimensions along the length of the entire aorta. The original 573 patients were screened for inclusion in study III, whereas the subset of patients with BAV in the entire cohort ($n = 408$ of 871) were screened for eligibility in study II (the Redcap cohort, see below). Study IV was conducted on the subset of the original ASAP cohort who underwent aortic root and/or ascending aortic surgery and preoperative CT of the aorta.

3.4.2 The Diseases of the Aortic Valve, Ascending Aorta and Coronary Arteries (DAVAACA) cohort

The DAVAACA study is a continuation of the previously described ASAP study. Enrollment is ongoing, and the cohort consisted of 1,145 prospectively enrolled patients at the time of Study III in August 2021 (excluding phenotypes other than BAV and TAV). The inclusion and exclusion criteria were identical to those described above for the ASAP cohort, except that patients with coronary artery disease were not excluded. The subset of patients with BAV enrolled up to September 2015 ($n = 186$) were included in the Redcap cohort and reviewed for eligibility in study II.

3.4.3 The Redcap cohort

The Redcap cohort is the result of a multinational collaboration - the Mechanistic Interrogation of Bicuspid Aortic Valve-Associated Aortopathy (MIBAVA) Leducq consortium - in which Karolinska University Hospital and University Medical Center Schleswig-Holstein, Lübeck, Germany, contributed data for adult patients with BAV. A total of 1,291 patients with BAV, 594 from Karolinska University Hospital (derived from the ASAP and DAVAACA cohorts) and 697 from University Medical Center Schleswig-Holstein, who were enrolled between September 1995 and September 2015 at either of the two institutions were screened for eligibility. Inclusion criteria were age ≥ 18 years, BAV, and primary aortic valve and/or ascending aortic surgery. Exclusion criteria were genetic syndromes and diseases, history of endocarditis, subvalvular aortic stenosis, systemic inflammatory disease or vasculitis, aortic dissection, and indeterminate, missing, or unicuspid aortic valve phenotype.

Table 1. Overview of surgical cohort studies

	Study II	Study III	Study IV
Main outcomes	Ascending aortic dilatation, valve disease	Ascending aortic dilatation	Distal aortic outcome
Grouping variables	1) Sex 2) BAV phenotype 3) Valve disease	1) Aspirin treatment 2) Valve phenotype	1) Valve phenotype
Centers	Karolinska and Lübeck	Karolinska	Karolinska
Cohorts	Redcap	ASAP and DAVAACA	ASAP
Surgical procedure	Aortic valve and/or ascending aortic surgery	Aortic valve and/or ascending aortic surgery	Ascending aortic surgery
Aortic valve phenotype	BAV only	BAV and TAV	BAV and TAV
Screened patients	1,291	1,700	186
Included patients	1,045	1,468	127
Aortic imaging modality	TTE/TEE	TEE	CT
Gene expression analysis	No	Yes	No
Study design	Cross-sectional	Cross-sectional	Longitudinal

ASAP, Advanced Study of Aortic Pathology; BAV, bicuspid aortic valve; CT, computed tomography; DAVAACA, Diseases of the Aortic Valve, Ascending Aorta, and Coronary Arteries; TAV, tricuspid aortic valve; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography.

3.5 Echocardiography

All patients underwent preoperative TTE and/or intraoperative transesophageal echocardiography (TEE). The maximum diameter of the ascending aorta was measured at the level of the aortic annulus, sinus of Valsalva, sinotubular junction, and the tubular ascending aorta (Figure 11, segment 1-4). Transthoracic echocardiography was used to assess aortic valve function. Valve disease was classified as either aortic stenosis or aortic insufficiency based on the echocardiography findings or the primary surgical indication. Aortic stenosis was defined as a mean gradient ≥ 20 mmHg, $V_{\max} \geq 3.0$ m/s, or a surgical indication of aortic stenosis. Aortic insufficiency was defined as AI grade ≥ 2 of 4, or a surgical indication of aortic insufficiency. Aortic stenosis was considered the primary lesion in patients with mixed valve disease as it is the main determinant of treatment ⁷⁵.

3.6 Computed tomography

Most patients in the ASAP cohort who underwent ascending aortic replacement also underwent a preoperative CT of the aorta. The maximum aortic diameter, measured perpendicularly from outer wall to outer wall, was determined at each of ten points of measurement along the entire length of the aorta (Figure 11, segment 1-10). Patients were classified as having bovine aortic arch if the branching point between the origins of the innominate artery and left common carotid artery was cephalad to the transverse plane formed between the origins of the innominate and left subclavian arteries ¹⁵⁴. Patients were referred to repeat CT ten years after the primary surgical intervention and new measurements were obtained from the distal aorta (the proximal arch to the infrarenal aorta, Figure 11, segments 5-10). All CT images were reviewed by a single experienced thoracic radiologist.

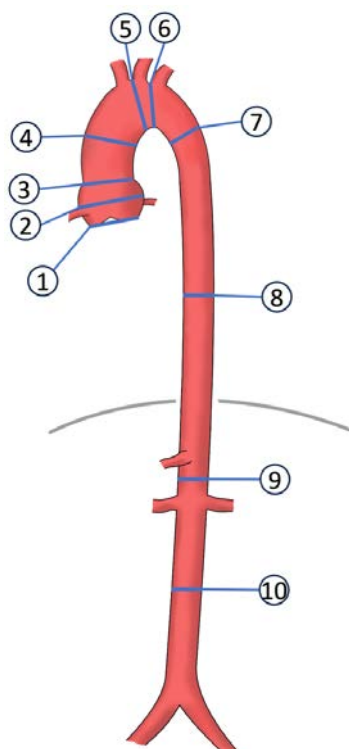


Figure 11. Measurement points along the aorta

1) The aortic annulus. 2) The sinus of Valsalva. 3) The sinotubular junction. 4) The ascending aorta. 5) The proximal arch. 6) The distal arch. 7) The aortic isthmus. 8) The descending aorta. 9) The suprarenal aorta. 10) The infrarenal aorta.

3.7 Aortic dilatation

Aortic dilatation was considered significant if any segment of the proximal aorta (sinus of Valsalva, sinotubular junction, or ascending aorta) were ≥ 4.5 cm in diameter, based on contemporary guidelines for the threshold of ascending aortic replacement^{23,75}. The type of aortic dilatation was classified as root phenotype if any of the root segments (sinus or sinotubular junction) were ≥ 4.5 cm and the ascending aorta < 4.5 cm, as ascending phenotype if the ascending aorta was ≥ 4.5 cm and both of the root segments were < 4.5 cm, and as the root extended phenotype if both a root segment and the ascending aorta were ≥ 4.5 cm⁴⁹. When the distal aortic segments (proximal arch to infrarenal aorta) were measured, these were categorized as aneurysmatic if the diameter was ≥ 5.5 cm, or ≥ 5.0 cm at the infrarenal segment for the female sex, based on current indications for aortic intervention²³.

3.8 Distal aortic outcome

The subgroup of patients who underwent ascending aortic surgery and a preoperative computed tomography at Karolinska University Hospital were followed for ten years, after which they underwent repeat CT of the aorta. Aside from growth measurements, patients were also

assessed with regards to aortic complications. Patients were considered to have experienced a negative distal aortic outcome if an aortic dissection or a new aortic surgical indication at a distal segment, according to the definitions described above, was found on the repeat CT. Furthermore, the medical records of deceased patients were reviewed to identify possible fatal aortic events, which were categorized as suspected if there were notes of chest and/or back pain followed by circulatory collapse immediately prior to death, or confirmed if corroborated by imaging.

3.9 Surgical biopsies of the ascending aorta

Full-thickness biopsies from the anterior aspect of the ascending aorta were procured intraoperatively from patients included in the ASAP and DAVAACA cohorts. The samples were obtained at the site of aortotomy in patients who underwent isolated aortic valve surgery, or from the resected ascending aorta or aortic root in patients who underwent isolated or concomitant aortic surgery. The samples were prepared for immunohistochemistry (as described above), and/or gene expression analysis as described elsewhere ²⁴. In brief, mRNA expression was measured in aortic intima-media using Affymetrix ST 1.0 exon microarrays at the Karolinska Institutet Affymetrix core facility. The raw data was pre-processed using robust multichip average (RMA) normalization and log2-transformation prior to analysis.

3.10 Statistics

Continuous variables are expressed as mean \pm standard deviation (SD), or as median [interquartile range (IQR)]. The only exception is the DNA content of rat aortic valves, which is expressed as mean \pm standard error of the mean as the value for each observation represents the mean of technical replicates. Categorical variables are expressed as frequencies and percentages. Independent samples t-test or one-way analysis of variance (ANOVA), with or without Welch correction as appropriate, were used to compare continuous variables between groups. Alternatively, the Mann-Whitney U-test was used if normal distribution could not be assumed. Analysis of covariance (ANCOVA) was used to adjust for confounders in comparisons of numeric variables between groups. χ^2 test or Fisher's exact test was used to compare categorical variables between groups. Post-hoc analyses were performed with Bonferroni corrections for multiple testing, or with Tukey Honestly Significant Difference (HSD) for ANOVA. Univariable and multivariable linear regression were used to model the relationship between two or more continuous variables. Multivariable logistic regression, or Poisson regression with robust standard errors, was used to model the relationship between multiple variables and a binary outcome. Variable selection was based on prior knowledge of risk factors for aortic dilatation, aortic growth, and/or aortic events ^{4,154,155,159,160,164}. Other pharmacological therapies (Study III) and variables related to the surgical procedure (Study IV) were also included as potential confounders. The primary exploratory variables were BAV phenotype (Study II), ASA therapy (Study III), and aortic valve phenotype (Study IV). Statistical analyses were performed in SPSS (IBM) or in the R programming language (R

Foundation for Statistical Computing) with RStudio (Posit Software PBC). A two-tailed pvalue <0.05 was considered statistically significant.

3.11 Ethical considerations

Institutional review board approvals were obtained for each study at the respective institution. The ethical concerns of the present thesis may be divided into three categories: i) animal research, ii) studies on deceased human subjects, iii) studies on live human subjects.

The ethics of a study using animal models depends on its adherence to the three R's of animal research: Replace, Reduce, and Refine²¹⁴. If replacement is not possible, the potential benefit to humans needs to outweigh the suffering that the animals are exposed to. While many studies on heart valve structure and valvular cells may be conducted *in vitro* using human-derived cell lines and tissues^{8,215,216}, an animal model is necessary to study heart valve regeneration and graft performance *in vivo*. The alternative would be to expose humans to an untested surgical intervention in a vital organ, which most people would find ethically unacceptable, especially if conventional treatment exists. In Study I, method development was performed with a single animal for each tested decellularization protocol, and only three in each group were used in the subsequent experiments as a bare minimum to demonstrate reproducibility. Further, animals had free access to food and were appropriately kept in groups with nesting material and other environmental enrichment in their cages. It is also generally accepted that the species with the lowest level of sentience, cognition, and social capacity should be used whenever possible, which in the case of heart valve tissue engineering is the rat^{93,111}.

Studies on human subjects on the other hand requires strict adherence to the Helsinki declaration²¹⁷. All patients, or their next-of-kin in the case of organ donors, provided written informed consent, could withdraw at any time without further explanation, and were offered the same standard of care regardless of whether they accepted or declined to participate in the research. If a study does not provide any immediate benefit to the subjects themselves, as is the case with those contained within the present thesis, it is vital that the research is not harmful to the subjects. An example of potential harm could be the ascending aortic biopsies from patients who did not undergo ascending aortic replacement. However, the biopsy does not affect closure of the aortotomy, and more than a decade of experience at Karolinska University Hospital and other institutions demonstrates the safety of the procedure. Another potential risk of harm is breach of patient confidentiality, which was managed through standard precautions and procedures. Furthermore, the subjects were not offered any compensation, which ensures that their financial situation did not affect their decision to participate in the research.

4 Results

4.1 Laminins in the aortic valve

4.1.1 Human aortic valves express laminin $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$

Tricuspid aortic valves from three deceased donors were used to study the expression of laminin chains in healthy aortic valves. All valves were morphologically normal upon inspection and routine histological studies. The three donors were men aged 50-55 years, one of them with obvious signs of cardiovascular disease (coronary atherosclerosis and ventricular hypertrophy; Study I Table 1). A single cusp of each type (right coronary, left coronary, non-coronary) was used in the analysis, each from a different donor. All cusps showed positive immunoreactivity for laminin $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$ in the endothelial BM lining the cusps, and to a lesser extent in the interstitium (Figure 12).

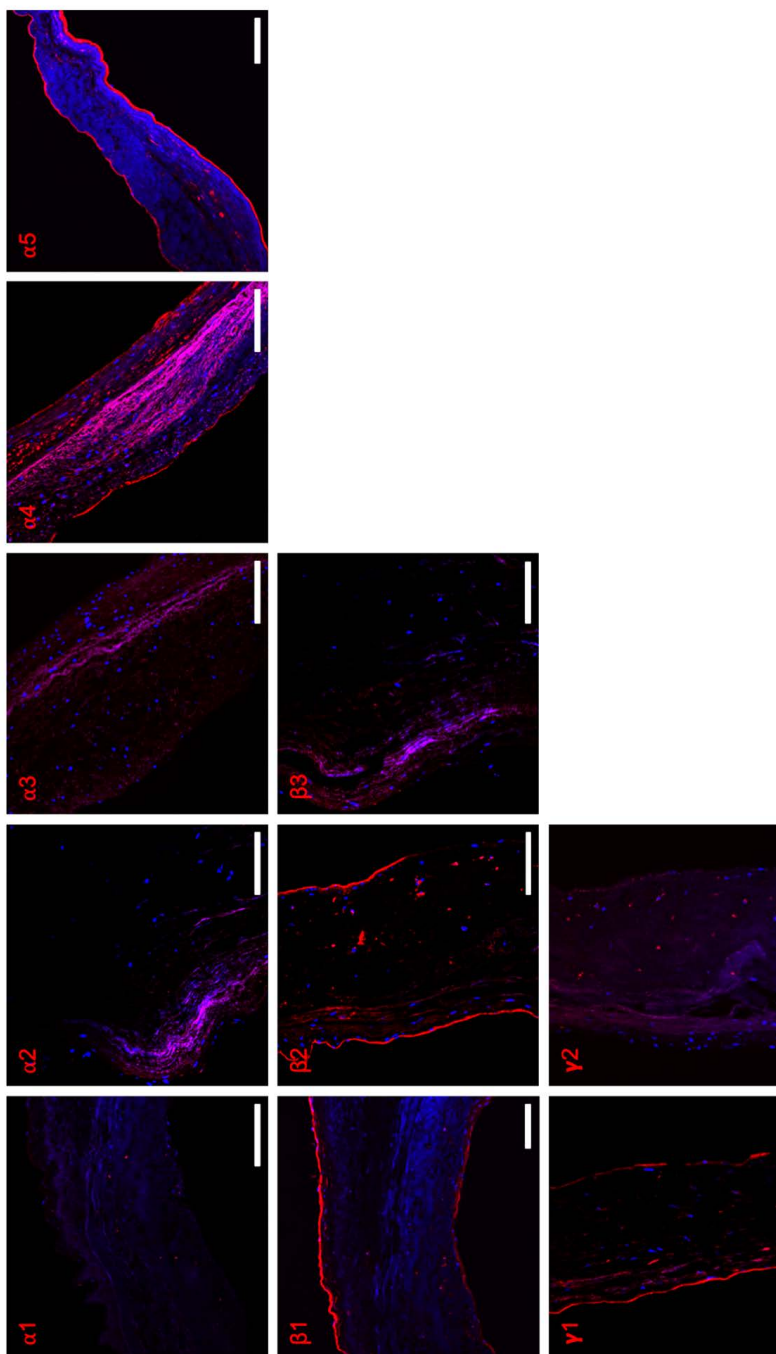


Figure 12. Laminin chain expression in normal human aortic valve cusps

The endothelial basement membrane showed positive immunoreactivity for laminin $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$, as did the endothelial lining of interstitial vessels and minor patches in the interstitium (red). There was no immunoreactivity for $\alpha 1$ - $\alpha 3$, $\beta 3$, or $\gamma 1$. Nuclei were counterstained with DAPI (blue). A representative image of $\alpha 3$ tissue sections from each of the three donor valve cusps is shown. Scale bar represents 100 μm . DAPI, 4',6'-diamidino-2-phenylindole.

4.1.2 Rat aortic valves express laminin $\alpha 4$, $\alpha 5$, and $\gamma 1$

To assess the suitability of a small-animal model for studying the role of laminins in heart valve regeneration, whole rat aortic valves were similarly sectioned and analyzed with immunohistochemistry. The rat valve cusps expressed the same α and γ laminin chains as the human valve cusps, which strengthens the validity and utility of the rat model (Figure 13).

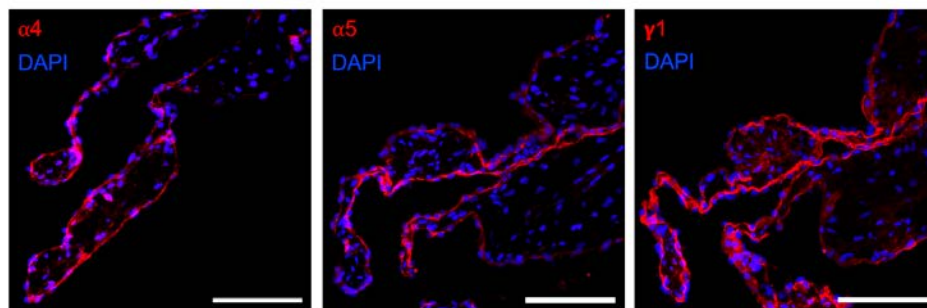


Figure 13. Laminin chain expression in rat aortic valve cusps

The endothelial basement membrane showed positive immunoreactivity for laminin $\alpha 4$, $\alpha 5$, and $\gamma 1$ (red). Nuclei were counterstained with DAPI (blue). A representative image of ≥ 3 tissue sections from each of the three donor valve cusps is shown. Scale bar represents 100 μm . DAPI, 4',6'-diamidino-2-phenylindole.

4.2 Small animal model for studies of heart valve regeneration

4.2.1 A modified decellularization protocol removes cells while preserving basement membrane components in rat aortic valves

A decellularization protocol for whole rat hearts¹⁰⁴ was adapted for rat aortic valves to facilitate studies of laminins and other BM components in the context of heart valve regeneration, *ex vivo* and potentially *in vivo*. A trial run with single samples using different detergent exposures (1 - 12 h of perfusion with 1% SDS) showed residual DNA in the valve cusps at all time points, with no clear difference between the samples (Study I, Supplementary Figure 3²¹⁸). Of note, even after 12 h, the fibrous aortic annulus still contained intact cell nuclei. Three hours was chosen as an intermediate exposure, and benzonase endonuclease was added as a final step, which resulted in minimal residual DNA upon histological inspection. Retention of BM components (laminin $\gamma 1$, the perlecan core protein, heparan sulfate glycosaminoglycans, and collagen IV), as well as fibronectin was confirmed with immunohistochemistry (Figure 14).

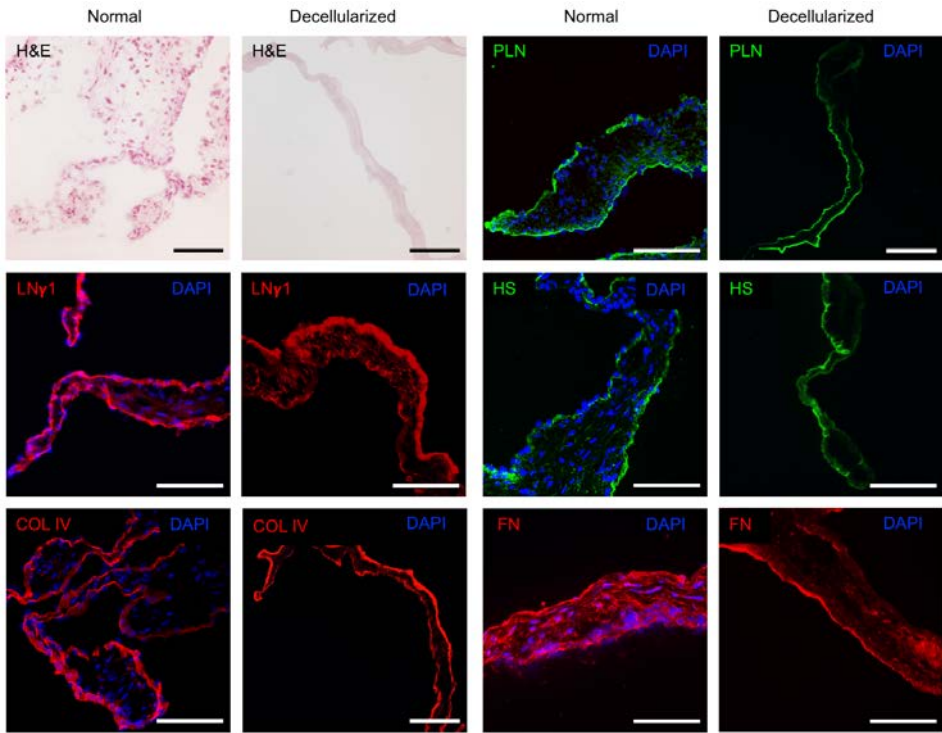


Figure 14. Morphology and extracellular matrix components in normal and decellularized rat aortic valves

H&E images and DAPI staining of cell nuclei show absence of intact nuclei and minimal residual DNA after decellularization. Laminin γ 1, collagen IV, the perlecan core protein, heparan sulfate glycosaminoglycans, and fibronectin were largely preserved, and their distribution patterns were intact after decellularization. Representative images of ≥ 3 tissue sections from each of three specimens per group are shown. Scale bar represents 100 μ m. DAPI, 4',6-diamidino-2-phenylindole; LN γ 1, laminin γ 1; COL IV, collagen IV; PLN, perlecan core protein; HS, heparan sulfate glycosaminoglycans; FN, fibronectin.

4.2.2 Decellularization with SDS and benzonase endonuclease results in minimal residual DNA

The amount of DNA in normal and decellularized aortic valves was quantified to estimate the amount of residual cellular contents after decellularization. The modified protocol with 3 h of exposure to SDS was compared to the protocol with 12 h of exposure previously described for whole hearts¹⁰⁴. Quantification of DNA in normal controls and decellularized valves revealed a significant reduction in the DNA content after decellularization, and the DNA content was further reduced to a minimum when benzonase endonuclease was added to the modified decellularization protocol (mean DNA content 63 ng compared to 1040 ng in normal controls, $p < 0.001$, Figure 15).

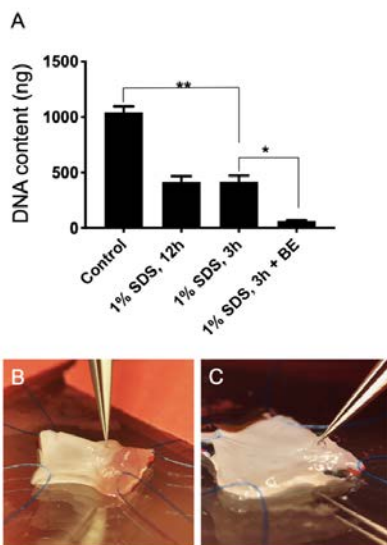


Figure 15. DNA quantification in normal and decellularized rat aortic valves

(A) DNA content is depicted in normal controls ($n = 6$) and decellularized valves after 3 ($n = 3$) or 12 ($n = 3$) h of exposure to SDS, or 3 h of SDS + BE ($n = 3$). Error bars represent the standard error of the mean. (B) Dissection technique for removal of intact cusps in a normal control. (C) Dissection technique in a decellularized specimen. BE, benzonase endonuclease; SDS, sodium dodecyl sulfate.

* $P < 0.05$; ** $P < 0.0001$.

4.3 Laminins and bicuspid aortopathy

A further project aimed to elucidate if a difference in laminin composition in the intimal endothelium of non-dilated BAV and TAV ascending aortas may explain the differences in endothelial cell phenotype, which have been implicated in the pathogenesis of BAV ATAA^{27,30}. These preliminary results were summarized in a master's thesis by a medical student²¹⁹. In brief, gene expression analysis of surgical biopsies from the human ascending aorta indicated that $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$ are expressed in the intima-media of the aortic wall of both non-dilated and dilated aortas from patients with BAV ($n = 77$) and TAV ($n = 46$)²¹⁹. Immunohistochemistry showed that the intima and media of non-dilated BAV and TAV ascending aortas contained laminin $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$, whereas $\alpha 4$ was only found in the intimal endothelial BM (Figure 16). Protein expression data from a previous study indicated that there may be a higher $\alpha 4$ expression in non-dilated BAV aortas ($n = 5$) compared to non-dilated TAV aortas ($n = 5$)²⁷, possibly indicating that the intimal endothelial $\alpha 4$ expression could differ between non-dilated BAV and TAV aortas. Attempts were made to study this hypothesis with further immunohistochemistry experiments and image analysis. However, the variability in immunoreactivity within both groups was too high to allow a robust

semiquantitative image analysis. The project was consequently put on hold pending further method development for quantitative analysis of specific regions of the aortic wall.

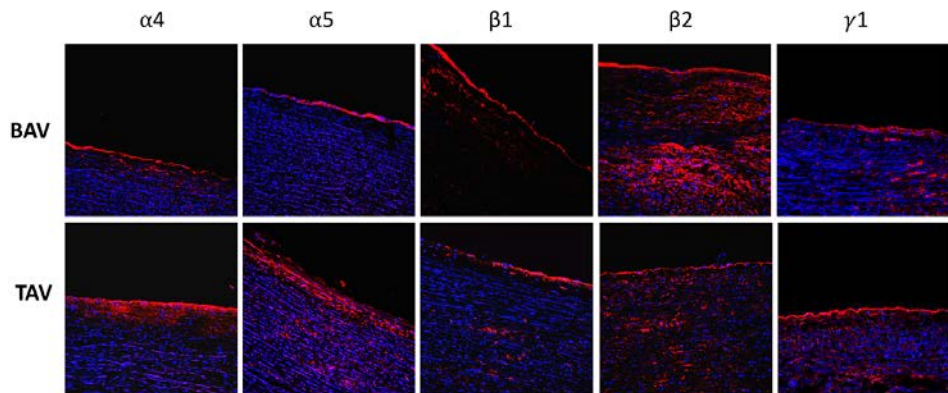


Figure 16. Laminin chain expression in non-dilated BAV and TAV aortas

Representative immunohistochemistry images of the laminin chain distribution (red) in non-dilated (<4.0 cm) ascending aortic wall in patients with BAV and TAV, modified from Freiholtz Åström²¹⁹. Nuclei were counterstained with DAPI (blue). Images were captured with 20× magnification. A representative image of one tissue section from each aortic specimen is shown (BAV n = 8, TAV n = 9). BAV, bicuspid aortic valve; DAPI, 4',6'-diamidino-2-phenylindole; TAV, tricuspid aortic valve.

4.4 Impact of the BAV phenotype and type of valve disease

4.4.1 The type of aortic valve disease and pattern of aortic dilatation differ between men and women with bicuspid aortic valve

The Redcap cohort, a large two-center surgical cohort of patients with BAV, was used to assess sex differences in the clinical associations of the different BAV morphologic phenotypes in patients who underwent aortic valve and/or aortic surgery. A total of 1,291 patients were screened for eligibility and 1,045 were included in the subsequent analyses (Study II, Figure 1²²⁰). Patient characteristics are shown in Table 2. In brief, female patients were older, more frequently had aortic stenosis, and less frequently had aortic regurgitation. Aortic root dimensions were smaller in females after adjustment for age and body size, and root dilatation was rare in female patients. The prevalence of the different BAV phenotypes; R-L, R-N, L-N, and 2-sinus did not differ between the two sexes.

Table 2. Patient characteristics of the multi-center BAV surgical cohort

	All (n = 1045)	Male (n = 794)	Female (n = 251)	P value
Age, years	56.4 ± 13.5	55.1 ± 13.9	60.3 ± 11.5	<0.001
BSA, m ²	1.99 ± 0.22	2.05 ± 0.19	1.81 ± 0.20	<0.001
Hypertension, n (%)	438 (41.9)	330 (41.6)	108 (43.0)	0.682
Diabetes mellitus, n (%; n = 583)	52 (8.9)	36 (8.5)	16 (10.1)	0.553
Dyslipidemia, n (%)	133 (12.7)	100 (12.6)	33 (13.1)	0.819
CABG, n (%)	51 (4.9)	47 (5.9)	4 (1.6)	0.006
Phenotype				
R-L, n (%)	769 (73.6)	590 (74.3)	179 (71.3)	0.634
R-N, n (%)	169 (16.2)	122 (15.4)	47 (18.7)	
L-N, n (%)	11 (1.1)	9 (1.1)	2 (0.8)	
2-sinus, n (%)	96 (9.2)	73 (9.2)	23 (9.2)	
Aortic valve disease				
Stenosis, n (%)	696 (66.6)	491 (61.8)	205 (81.7)	<0.001
Regurgitation, n (%)	276 (26.4)	252 (31.7)	24 (9.6)	<0.001
Dilatation only, n (%)	73 (7.0)	51 (6.4)	22 (8.8)	0.205
Aortic dimensions				
Annulus, cm	2.6 ± 0.4 (n = 768)	2.7 ± 0.4 (n = 574)	2.3 ± 0.3 (n = 194)	<0.001†
Sinus of Valsalva, cm	3.7 ± 0.6 (n = 887)	3.8 ± 0.6 (n = 666)	3.3 ± 0.6 (n = 221)	<0.001†
Sinotubular junction, cm	3.1 ± 0.6 (n = 660)	3.3 ± 0.6 (n = 492)	2.8 ± 0.6 (n = 168)	<0.001†
Ascending aorta, cm	4.2 ± 0.8 (n = 919)	4.2 ± 0.8 (n = 694)	4.0 ± 0.8 (n = 225)	0.581†
Aortopathy				
Any aneurysm, n (%; n = 1003)	351 (35.0)	284 (37.3)	67 (27.8)	0.007
Ascending phenotype, n (%)	271 (27.0)	208 (27.3)	63 (26.1)	0.725
Root phenotype, n (%)	19 (1.9)	18 (2.4)	1 (0.4)	0.058
Root extended, n (%)	61 (6.1)	58 (7.6)	3 (1.2)	<0.001

†P values adjusted for BSA and age. BAV, bicuspid aortic valve; BSA, body surface area; CABG, coronary artery bypass graft; L-N, left-non-coronary phenotype; R-L, right-left phenotype; R-N, right-non-coronary phenotype.

4.4.2 BAV phenotype associations with valve disease and aortopathy differ between male and female patients

There were significant differences between male and female patients with regards to BAV phenotype associations with valve disease, aortic dimensions, and aortic dilatation pattern (Table 3). Aortic valve regurgitation was most frequently observed in male patients with the R-L and L-N phenotypes. In contrast, R-N was associated with aortic valve regurgitation in female patients, whereas female R-L patients had a lower prevalence of aortic valve regurgitation. Further, the R-L phenotype was associated with isolated aortic dilatation in females, but a similar association could not be discerned in male patients. In male patients, the R-L phenotype was associated with larger aortic root dimensions than the R-N phenotype.

There was a trend in male patients toward lower prevalence of aortic dilatation with the R-N phenotype. Male patients with the 2-sinus BAV phenotype had a comparatively high prevalence of root phenotype aortic dilatation, whereas female patients with the 2-sinus BAV phenotype did not have root dilatation and even had smaller sinus of Valsalva dimensions than other female patients. Although there were few patients in the L-N group in either sex, only one of nine male patients with L-N had aortic dilatation (11.1%). There were two females with L-N, one with dilatation and one without. The main findings pertaining to BAV phenotype associations are summarized in Figure 17. Furthermore, differences in aortic dimensions between patients with aortic stenosis and patients with regurgitation were greater in females (Study II, Table 3 ²²⁰).

Table 3. Patient characteristics by bicuspid aortic valve phenotype in the two sexes

	Male				Female			
	R-L (n = 590)	R-N (n = 122)	L-N (n = 9)	2-sinus (n = 73)	P value	R-L (n = 179)	R-N (n = 47)	2-sinus (n = 23)
Age, years	55.5 ± 13.8	55.7 ± 13.5	51.8 ± 20.2	51.3 ± 13.7	0.080	61.0 ± 11.4	59.3 ± 12.6	56.0 ± 9.6
BSA, m ²	2.06 ± 0.19	2.05 ± 0.19	1.92 ± 0.20	2.07 ± 0.20	0.136	1.82 ± 0.20	1.78 ± 0.20	1.78 ± 0.15
Hypertension, n (%)	255 (43.2)	48 (39.3)	3 (33.3)	24 (32.9)	0.337	79 (44.1)	17 (36.2)	11 (47.8)
Diabetes mellitus, n (%)	29 (9.3)	4 (5.4)	1 (20.0)	2 (5.9)	0.400	10 (8.8)	5 (15.6)	1 (8.3)
Dyslipidemia, n (%)	72 (12.2)	17 (13.9)	3 (33.3)	8 (11.0)	0.264	22 (12.3)	6 (12.8)	5 (21.7)
Aortic valve disease								
Stenosis, n (%)	351 (59.5)	85 (69.7)	5 (55.6)	50 (68.5)	0.104	146 (81.6)	37 (78.7)	20 (87.0)
Regurgitation, n (%)	201 (34.1)	31 (25.4)	4 (44.4)	16 (21.9)	0.047	13 (7.3)*	10 (21.3)*	1 (4.3)
Dilatation only, n (%)	38 (6.4)	6 (4.9)	0 (0.0)	7 (9.6)	0.565	20 (11.2)*	0 (0.0)*	2 (8.7)
Aortic dimensions								
Annulus, cm	2.7 ± 0.4 (n = 421)	2.6 ± 0.3 (n = 90)	2.6 ± 0.3 (n = 8)	2.7 ± 0.3 (n = 55)	0.271†	2.3 ± 0.4 (n = 133)	2.3 ± 0.3 (n = 40)	2.1 ± 0.2 (n = 19)
Sinus of Valsalva, cm	3.8 ± 0.6 (n = 495)*	3.6 ± 0.5 (n = 101)*	3.5 ± 0.3 (n = 8)	3.7 ± 0.6 (n = 62)	0.002†	3.3 ± 0.6 (n = 156)*	3.2 ± 0.5 (n = 41)	2.9 ± 0.5 (n = 22)*
Sinotubular junction, cm	3.3 ± 0.6 (n = 412)*	3.1 ± 0.5 (n = 87)*	3.1 ± 0.5 (n = 7)	3.2 ± 0.5 (n = 55)	0.048†	2.9 ± 0.6 (n = 137)	2.7 ± 0.4 (n = 36)	2.7 ± 0.5 (n = 19)
Ascending aorta, cm	4.2 ± 0.9 (n = 515)	4.0 ± 0.7 (n = 107)	3.8 ± 0.7 (n = 8)	4.3 ± 0.7 (n = 64)	0.116†	4.1 ± 0.8 (n = 159)	3.9 ± 0.8 (n = 43)	3.7 ± 0.8 (n = 21)
Aortopathy								
Any dilatation, n (%)	222 (39.2)	33 (28.7)	1 (11.1)	28 (39.4)	0.065	52 (30.2)	9 (20.5)	5 (21.7)
Ascending phenotype, n (%)	160 (28.2)	27 (23.5)	1 (11.1)	20 (28.2)	0.569	48 (27.9)	9 (20.5)	5 (21.7)
Root phenotype, n (%)	13 (2.3)	0 (0.0)*	0 (0.0)	5 (7.0)*	0.031	1 (0.6)	0 (0.0)	0 (0.0)
Root extended, n (%)	49 (8.6)	6 (5.2)	0 (0.0)	3 (4.2)	0.433	3 (1.7)	0 (0.0)	0 (0.0)

*Groups are significantly different after Bonferroni corrections for multiple comparisons.

†P values adjusted for age and BSA.

BSA, body surface area; L-N, left-non-coronary phenotype; R-L, right-left phenotype; R-N, right-non-coronary phenotype.





	Male	Female
 <p>Right-left (R-L)</p>	<ul style="list-style-type: none"> • Large root • AR common* 	<ul style="list-style-type: none"> • Large root • Large ascending aorta • AR uncommon*
 <p>Right-non-coronary (R-N)</p>	<ul style="list-style-type: none"> • Small root • Dilatation less frequently • AR less common* 	<ul style="list-style-type: none"> • AR common*
 <p>Left-non-coronary (L-N)</p>	<ul style="list-style-type: none"> • Dilatation uncommon(?) 	<ul style="list-style-type: none"> • Uncertain
 <p>2-sinus</p>	<ul style="list-style-type: none"> • Root phenotype more frequently* 	<ul style="list-style-type: none"> • No root dilatation* • Small root*

Figure 17. Visual abstract of bicuspid aortic valve phenotype associations

*Significant sex difference. AR, aortic regurgitation.

4.5 Acetylsalicylic acid and ascending aortic dilatation

4.5.1 Patients with tricuspid aortic valves and ASA therapy have smaller aortic dimensions at the time of surgery

The ASAP and DAVAACA cohorts, two consecutive single-center cohorts including patients with both BAV and TAV, were used to study acetylsalicylic acid (ASA) therapy in relation to ascending aortic dilatation. A total of 1,700 patients were screened for eligibility and 1,468 were included in the analyses (Study III, Figure 1²²¹). Regardless of aortic valve phenotype, ASA therapy was associated with higher age, known vascular disease, hypertension, and lipid-lowering therapy (Table 4). There was also an inverse association between ASA and

anticoagulation. Furthermore, aortic valve regurgitation was more common in patients without ASA therapy and stenosis was more common in patients with ASA therapy. Both the aortic root and the tubular ascending aorta were smaller in patients with TAV and ASA therapy compared to those without, whereas a similar association could not be found in patients with BAV. Patients with diabetes mellitus were excluded due to the known inverse association between diabetes mellitus and aortic dilatation ^{160,222}.

Table 4. Characteristics of patients with and without ASA

	TAV		BAV	
	Without ASA (n = 458)	With ASA (n = 235)	Without ASA (n = 609)	With ASA (n = 166)
Male sex, n (%)	293 (63.6)	166 (71.6)	452 (74.2)	126 (75.9)
Age, years \pm SD	67.7 \pm 10.7	71.6 \pm 8.1	57.5 \pm 13.4	63.3 \pm 9.7
BSA, m ² \pm SD	1.98 \pm 0.24	1.98 \pm 0.21	2.0 \pm 0.22	1.99 \pm 0.23
hsCRP, mg/L \pm SD	3.4 \pm 7.8	3.6 \pm 7.5	2.22 \pm 5.2	2.14 \pm 3.7
HDL-cholesterol, mmol/L \pm SD	1.4 \pm 0.4	1.4 \pm 0.5	1.4 \pm 0.4	1.4 \pm 0.4
LDL-cholesterol, mmol/L \pm SD	2.7 \pm 1.0	2.6 \pm 2.5	2.9 \pm 0.9	2.6 \pm 1.0
Treated hypertension, n (%)	338 (73.3)	198 (85.3)	317 (52.1)	121 (72.9)
Known vascular disease, n (%)	99 (21.5)	134 (57.8)	58 (9.5)	71 (42.8)
Chronic inflammatory disease, n (%)	33 (7.3)	22 (9.5)	36 (6.2)	11 (6.7)
Current smoking, n (%)	22 (7.0)	11 (6.8)	45 (7.5)	23 (14.0)
Lipid-lowering agents, n (%)	149 (32.5)	153 (65.1)	134 (22.0)	94 (56.6)
Corticosteroids, n (%)	16 (3.5)	10 (4.3)	11 (1.8)	1 (0.6)
Anticoagulants, n (%)	93 (20.3)	7 (3.0)	55 (9.0)	2 (1.2)
Other thrombocyte inhibitors, n (%)	43 (9.4)	13 (5.5)	21 (3.4)	5 (3)
Aortopathy				
Sinus of Valsalva, cm \pm SD	3.7 \pm 0.8	3.5 \pm 0.6	3.6 \pm 0.6	3.6 \pm 0.5
Sinotubular junction, cm \pm SD	3.2 \pm 0.8	2.9 \pm 0.6	3.1 \pm 0.6	3.1 \pm 0.5
Ascending, cm \pm SD	4.0 \pm 1.1	3.6 \pm 0.8	3.9 \pm 0.8	3.9 \pm 0.9
Any aortic dilatation, n (%)	178 (38.9)	36 (15.8)	173 (28.9)	44 (27)
Root phenotype, n (%)	45 (9.8)	6 (2.6)	13 (2.2)	1 (0.6)
Dilatation without valve disease, n (%)	53 (11.6)	8 (3.4)	21 (3.4)	8 (4.8)
Aortic valve disease				
Stenosis, n (%)	173 (37.5)	159 (68.5)	411 (67.5)	133 (80.1)
Regurgitation, n (%)	224 (48.6)	66 (28.4)	191 (31.4)	31 (18.7)

ASA, acetylsalicylic acid; BAV, bicuspid aortic valve; BSA, body surface area; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; TAV, tricuspid aortic valve.

4.5.2 ASA therapy is independently associated with a lower risk of ascending aortic dilatation in patients with TAV

A Poisson regression was used to model the association between ASA therapy and aortic dilatation with adjustment for confounders (Table 5). The dependent variable was ascending aortic dilatation (any segment between the sinus and ascending aorta \geq 4.5 cm). There was an

independent, inverse association between ASA therapy and ascending aortic dilatation, which was not seen for anticoagulants or other thrombocyte inhibitors. Corticosteroid therapy was not included as an independent variable due to its scarcity, and NSAID treatment was even more uncommon ($n \leq 5$ in each respective group). Chronic inflammatory disease, used here as a surrogate for other anti-inflammatory and immunomodulatory medications, was likewise not associated with aortic dilatation. There was no effect of ASA in patients with BAV (relative risk 0.93, 95% CI 0.64 - 1.34, $p = 0.688$, Study III, Table 2²²¹).

Table 5. Poisson regression analysis of aortic dilatation in patients with TAV

	Relative risk	95% confidence interval		P value
		Lower	Upper	
Age, years	1.01	1.00	1.02	0.152
BSA, 0.1 m ²	1.63	0.94	2.82	0.084
Male sex	1.06	0.80	1.43	0.677
Known vascular disease	1.13	0.83	1.54	0.434
Treated hypertension	1.11	0.82	1.50	0.485
Chronic inflammatory disease	0.90	0.52	1.56	0.710
Anticoagulants	0.88	0.62	1.26	0.493
Other thrombocyte inhibitors	0.82	0.52	1.29	0.398
Lipid-lowering agents	0.94	0.71	1.24	0.648
ASA	0.68	0.48	0.95	0.026
Aortic regurgitation	23.60	10.91	51.06	<0.001

ASA, acetylsalicylic acid; BSA, body surface area; CI, confidence interval; TAV, tricuspid aortic valve.

4.5.3 ASA is associated with a lower COX-2 expression in the dilated ascending aorta

Gene expression analysis was performed in ascending aortic biopsies from a subset of patients ($n = 118$) to discern if decreased aortic *COX* expression could be a possible mechanistic explanation for the lower prevalence of ascending aortic dilatation observed in patients with TAV and preoperative ASA treatment. A roughly linear relationship between *COX* expression and ascending aortic diameter could be discerned in patients with TAV (*COX-1* $R^2 = 0.283$, $p < 0.001$, *COX-2* $R^2 = 0.088$, $p = 0.05$, Study III, Figure 2²²¹). The *COX-2* mRNA expression was found to be lower in dilated aortas from patients with ASA treatment, whereas no difference was found in non-dilated aortas (Figure 18). There was also a positive correlation between expression of *COX-2* and mRNA expression of pro-inflammatory cytokines and macrophage markers (Study III, Figure 4). In contrast, there was no difference in aortic *COX-1* expression between patients with TAV with and without ASA treatment, neither in

dilated nor non-dilated aortas (Study III, Figure S2²²¹). There was no association between ASA treatment and aortic *COX-2* expression in patients with BAV (Study III, Figure S3).

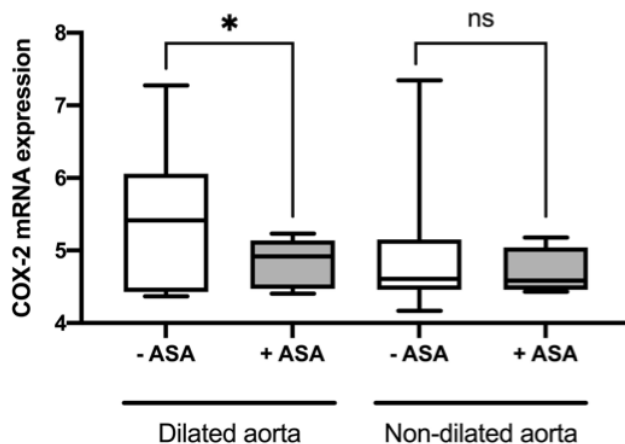


Figure 18. Aortic *COX-2* expression in patients with TAV

Cyclooxygenase-2 (*COX-2*) mRNA expression in dilated (≥ 4.5 cm) and non-dilated (< 4.0 cm) ascending aortic biopsies of patients with TAV with (+) and without (-) ASA treatment. N = 15 (dilated without ASA), n = 5 (dilated with ASA), n = 17 (non-dilated without ASA), n = 6 (non-dilated with ASA). *P = 0.034 (independent samples t test with Welch's correction). ASA, acetylsalicylic acid; ns, not significant; TAV, tricuspid aortic valve.

4.6 Distal aortic outcome after ascending aortic surgery

4.6.1 Patients with bicuspid aortic valves have smaller distal aortic dimensions

Patients in the ASAP cohort who underwent ascending aortic replacement, with or without concomitant aortic valve surgery, and a preoperative CT of the aorta were examined to assess differences in long-term distal aortic outcome and growth rates between patients with BAV and TAV. A total of 186 patients in the ASAP cohort underwent ascending aortic replacement, and 127 (BAV n = 85, TAV n = 42) fulfilled inclusion criteria (excluded n = 59, no CT n = 51, monogenic disease n = 4, preoperative dissection n = 2, aortic coarctation n = 1, multiple saccular aneurysms n = 1). Patients with BAV were younger and less frequently had hypertension (Table 6). Aortic stenosis and concomitant aortic valve surgery were more common in patients with BAV. The proximal aortic dimensions (sinus of Valsalva to ascending aorta) were mostly comparable between the two patient groups, but the distal aortic segments (proximal arch to infrarenal aorta) were all larger in patients with TAV at baseline. The pattern of ascending aortic dilatation was similar between the two groups.

Table 6. Characteristics of patients undergoing ascending aortic surgery

	Overall (n = 127)	BAV (n = 85)	TAV (n = 42)
Age, years (median [IQR])	61.1 [52.4, 67.1]	59.5 [50.9, 65.7]	63.2 [56.7, 72.8]
Male sex, n (%)	88 (69.3)	60 (70.6)	28 (66.7)
BSA, m ² (mean \pm SD)	2.00 \pm 0.21	2.00 \pm 0.21	2.00 \pm 0.22
Hypertension, n (%)	62 (48.8)	37 (43.5)	25 (59.5)
Smoking status, n (%)			
Current	9 (7.1)	7 (8.2)	2 (4.8)
Former	53 (41.7)	37 (43.5)	16 (38.1)
Aortic valve disease, n (%)	72 (56.7)	51 (60.0)	21 (50.0)
Regurgitation	33 (26.0)	14 (16.5)	19 (45.2)
Stenosis	39 (30.7)	37 (43.5)	2 (4.8)
Aortic dimensions and morphology			
Annulus, cm (median [IQR])	2.5 [2.4, 2.7] (n = 117)	2.6 [2.4, 2.7] (n = 77)	2.5 [2.3, 2.7] (n = 40)
Sinus of Valsalva, cm (median [IQR])	4.3 [3.9, 4.8]	4.3 [3.9, 4.7]	4.5 [3.7, 5.2]
Sinotubular junction, cm (median [IQR])	3.9 [3.6, 4.5]	3.8 [3.5, 4.3]	4.3 [3.7, 4.9]
Ascending, cm (median [IQR])	5.2 [4.8, 5.5]	5.2 [4.9, 5.4]	5.3 [4.7, 5.8]
Proximal arch, cm (median [IQR])	3.4 [3.2, 3.8] (n = 124)	3.3 [3.1, 3.7] (n = 84)	3.7 [3.4, 4.0] (n = 40)
Distal arch, cm (median [IQR])	3.1 [2.8, 3.4]	3.0 [2.8, 3.3]	3.3 [2.9, 3.6]
Isthmus, cm (median [IQR])	3.0 [2.8, 3.5]	2.9 [2.7, 3.3]	3.5 [3.0, 3.8]
Descending, cm (median [IQR])	2.8 [2.5, 3.2]	2.8 [2.5, 3.0]	3.2 [2.8, 3.5]
Suprarenal, cm (median [IQR])	2.4 [2.2, 2.6] (n = 120)	2.3 [2.1, 2.5] (n = 81)	2.6 [2.3, 2.8] (n = 39)
Infrarenal, cm (median [IQR])	1.9 [1.8, 2.2] (n = 95)	1.9 [1.7, 2.1] (n = 62)	2.2 [1.8, 2.3] (n = 33)
Bovine aortic arch, n (%)	27 (21.3)	17 (20.0)	10 (23.8)
Type of aneurysm, n (%)			
Ascending	69 (54.3)	50 (58.8)	19 (45.2)
Root	9 (7.1)	4 (4.7)	5 (11.9)
Root extended	49 (38.6)	31 (36.5)	18 (42.9)
Surgical details			
Aortic valve surgery, n (%)	44 (34.6)	32 (37.6)	12 (28.6)
Biological prosthesis	18 (14.2)	14 (16.5)	4 (9.5)
Mechanical prosthesis	12 (9.4)	11 (12.9)	1 (2.4)
Repair	14 (11.0)	7 (8.2)	7 (16.7)
Aortic root surgery, n (%)	67 (52.8)	45 (52.9)	22 (52.4)
Biological composite	26 (20.5)	18 (21.2)	8 (19.0)
Mechanical composite	22 (17.3)	19 (22.4)	3 (7.1)
Valve sparing	19 (15.0)	8 (9.4)	11 (26.2)
Circulatory arrest, n (%)	14 (11.0)	9 (10.6)	5 (11.9)
Open distal anastomosis	10 (7.9)	7 (8.2)	3 (7.1)
Arch/hemiarch	3 (2.4)	2 (2.4)	1 (2.4)
Frozen elephant trunk	1 (0.8)	0 (0.0)	1 (2.4)

BAV, bicuspid aortic valve; BSA, body surface area; CT, computed tomography; TAV, tricuspid aortic valve.

4.6.2 Growth rates are comparable between bicuspid and tricuspid aortic valve patients without distal aortic complications

96 patients (75.6%, BAV n = 67 and TAV n = 29) returned for repeat CT of the aorta after a median follow-up of 10.8 years (BAV median 10.9 years, TAV median 10.8 years, $p = 0.823$). A total of 31 patients were lost to follow-up, mostly due to the patient being deceased ($n = 20$, other reason $n = 11$). Three of the 96 returning patients had developed aortic dissection and were excluded from growth rate analyses. The highest observed growth at any distal segment (the proximal arch to the infrarenal aorta) was comparable between patients with BAV and

TAV (BAV median 0.2 [0.1, 0.3], TAV median 0.2 [0.1, 0.4], $p = 0.446$). There was no significant difference between the two groups at any distal aortic segment, although there was a trend toward higher growth in the proximal arch in patients with TAV (Figure 19).

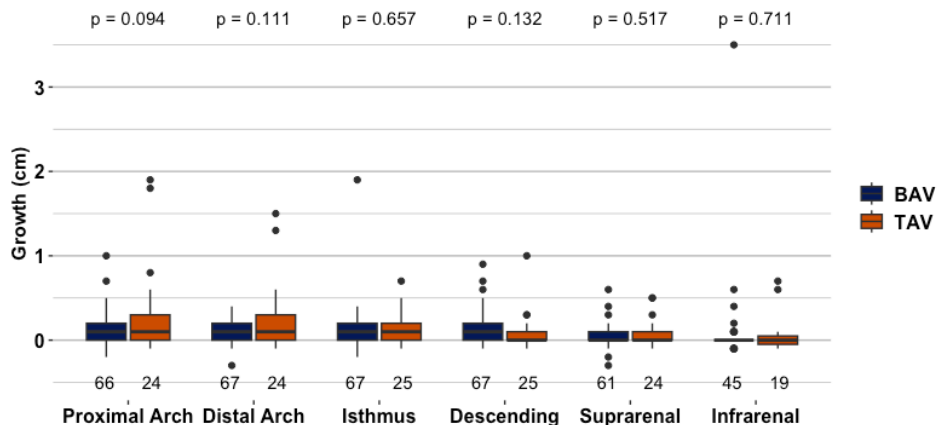


Figure 19. Aortic growth in each distal segment

Box-whisker plot of the unadjusted aortic growth in each distal segments after a median follow-up of 10.8 years. Blue boxes represent patients with BAV, orange boxes represent patients with TAV. Boxes represent interquartile range, horizontal lines denote median values, whiskers represent interquartile range $\times 1.5$, and dots represent outliers. The p values are for pairwise comparisons of growth between BAV and TAV at each segment (Mann-Whitney U test). The numbers below the boxes indicate the number of observations (n total = 93, BAV n = 67, TAV n = 26). BAV, bicuspid aortic valve; TAV, tricuspid aortic valve.

4.6.3 Patients with tricuspid aortic valve, but not bicuspid aortic valve, have a high risk of distal aortic complications

A composite endpoint, termed negative distal aortic outcome, was used to assess long-term risk of distal aortic complications. Negative distal aortic outcome was defined as any of the following findings: 1) aortic dissection, 2) intervention in a distal segment, 3) indication for aortic intervention (diameter ≥ 5.5 cm at any distal segment, ≥ 5.0 cm for the infrarenal aorta in females²³), or 4) medical records indicating a fatal acute aortic event. Only two patients with BAV (2.4%) compared to 10 patients with TAV (23.8%) experienced a negative distal aortic outcome ($p < 0.001$), both of which had other coexistent risk factors for aortic disease (Study IV, Supplemental Table 4). All acute aortic events and interventions occurred in the TAV group (n = 8). The observed negative outcomes were new distal aortic aneurysm (BAV, n = 2; TAV, n = 4), aortic dissection (TAV, n = 3), aortic intervention (TAV, n = 2), suspected fatal event (TAV, n = 2), and confirmed fatal event (TAV, n = 1). TAV-associated ascending aortic aneurysm remained a significant risk factor for negative distal aortic outcome after adjustment for confounders in a multivariable logistic regression model (Table 7). The diameter of the largest distal aortic segment and bovine aortic arch were also independently associated with a negative distal aortic outcome, whereas there was an inverse association between aortic valve surgery and negative distal aortic outcome. Stenosis was rare in the TAV group and aortic valve

surgery was predominantly valve repair for these patients, whereas stenosis was the dominant valve pathology in the BAV group and valve replacement was consequently the most frequent valve procedure in these patients.

Table 7. Logistic regression analysis of negative distal aortic outcome

	Odds ratio	95% confidence interval		P value
		Lower	Upper	
Age, years	1.03	0.89	1.20	0.668
Male sex	2.58	0.28	23.54	0.400
Largest distal segment, mm	1.58	1.12	2.23	0.009*
TAV	58.38	2.09	1632.76	0.017*
Bovine aortic arch	18.98	1.19	301.93	0.037*
Aortic root surgery	0.11	0.01	1.39	0.088
Aortic valve surgery	0.01	0.00	0.46	0.017*

n = 127; accuracy = 0.95; sensitivity = 0.58; specificity = 0.99.

*Statistically significant. TAV, tricuspid aortic valve.

5 Discussion

BAV is a multifaceted congenital cardiac malformation that affects a significant proportion of the population in all ages. The etiology, clinical implications, and management of both valvular and aortic complications remain matters of considerable debate^{29,62,86}. ATAAs are frequently associated with BAV, and the disease process of ATAA differs substantially between patients with BAV and a normal TAV^{5,6,15,24–27}. Both molecular and clinical studies are necessary to advance our understanding and management of patients with BAV and/or ATAA. The present thesis sought to address questions regarding the aortic valve microstructure and valve tissue engineering, the clinical implications of different BAV phenotypes, pharmacological treatment of ATAA with acetylsalicylic acid, and the long-term risk of residual aortic disease in patients who undergo surgery for ATAA.

5.1 Aortic valve laminins

Due to their significantly younger age at the onset of valve disease, patients with BAV would benefit from the development of tissue-engineered valves with long-term durability, lack of thrombogenicity, and growth potential^{3,20,83,87}. To develop a tissue-engineered heart valve that faithfully mimics a healthy valve, it is critical to discern the composition of the aortic valve BM. Laminin chains $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$ were expressed in the aortic valve cusps, indicating that the trimeric laminin molecules expressed in the valve are LN 411, 421, 511, and 521. These isoforms are also expressed in the endothelial BM of blood vessels and the endocardium^{128,131,150,177}. However, there may be quantitative differences in the laminin isoform composition between heart valves and other parts of the vasculature, which were not assessed. Regional differences in the laminin composition are known to occur throughout the vasculature^{130,136,139} and the unique structure and function of heart valves, as well as the specific properties of valvular endothelial cells, would be consistent with a distinct valvular endothelial BM^{41,42}. Laminin $\alpha 1$ was not found in the aortic valves, and previous *in vitro* studies of laminins and valvular interstitial and endothelial cells with animal-derived cells and LN 111 (or derivatives) are thus unlikely to reflect the role of valvular laminins in human heart valve biology^{143,146,147,223}.

Studies of laminins in blood vessels and endothelial cell cultures indicate that the identified valvular laminins likely have an important role in the integrity and function of the valvular endothelium^{136,139,141,142,177,224}. Laminins may also regulate valvular endothelial-to-mesenchymal transition¹⁴⁴, and could thus affect the replenishment of VICs in tissue-engineered valves, but also the propensity for calcification^{225,226}. The role of laminins in heart valve biology and regeneration remains to be discerned, and one method to study this could be a decellularized heart valve scaffold.

5.1.1 Decellularized scaffold

The decellularized rat aortic valve scaffold described herein resulted in retention of key BM components as well as fibronectin, indicating that the scaffold could be used to study the role

of laminins and other BM components in heart valve regeneration, similarly to how a decellularized lung scaffold has been used previously ¹¹⁴. However, the anticipated corresponding preservation of functional properties remains to be established through additional studies. While it is widely accepted that BM preservation is desirable in decellularized, tissue-engineered scaffolds ¹⁰³, the importance of laminins and other BM components for tissue regeneration is mostly unknown. Whereas some have reported that the lack of a BM may adversely affect endothelial cell confluence and phenotype ¹⁰⁷, others have shown that profound differences in the composition and integrity of the BM can have a negligible impact on recellularization potential in some contexts ¹⁴⁹. The importance of intact, tissue-specific BM components appears to depend on both the scaffold (or substrate) as well as the cell type ^{107,114,149–151,224}. However, the often poor or limited endothelialization observed in clinical trials of extensively processed decellularized heart valves ^{10,99,112}, as well as limited experimental evidence ^{93,107}, suggest that an intact BM is important in heart valve regeneration.

5.2 Bicuspid aortic valve phenotype associations

BAV is not a single entity, but rather a spectrum of congenital aortic valve malformations, reviewed in ⁴⁹. These different BAV phenotypes - R-L, R-N, L-N, and 2-sinus - are associated with different risks of valvular dysfunction and ascending aortic disease ^{18–20,50}. We could confirm the associations between R-L and large sinus and/or root dilatation ^{20,21,191,227,228}, and R-N and preferential dilatation of the tubular segment ^{19,191,228}. On the other hand, the previously reported association between R-N and aortic stenosis ^{19,174,191} was found exclusively in male patients. Likewise, the 2-sinus phenotype has previously been linked to root dilatation ⁵⁰, but females with the 2-sinus phenotype do not develop root dilatation and even have smaller root dimensions than females with other phenotypes. The lower prevalence of any aortic dilatation in R-N in both sexes is supported by some reports ^{18,19}.

The mechanisms behind the observed sex differences require further investigation, but the presence of aortopathy in neonates suggest that interactions between X chromosome-bound and autosomal genes during embryonic development may play a major role ^{1,48,54}. Another possible explanation could be the influence of sex hormones, which appear to have a role in AAA, whereas the literature is scarce for thoracic aortic disease ^{48,190,229}. An important limitation is that the results may not reflect all-comers with BAV due to surgical selection bias. Overall, the increasing body of evidence for differences between BAV phenotypes could help to inform patient management, particularly if the reported differences in aortic dimensions and dilatation pattern between the two sexes and phenotypes correlate with larger growth rates at specific segments, as some studies indicate ¹⁹⁵. The results presented here imply that patients with the R-N phenotype of either sex, females with 2-sinus, and possibly males with L-N, may be more suitable for TAVR due to the lower prevalence of aortic dilatation in general, and root dilatation in particular ⁸⁶.

5.3 Acetylsalicylic acid therapy and ascending aortic aneurysm

There is currently very limited evidence in support of pharmacological therapy for ATAA²³. A significant reduction in the risk of ATAA in patients with ASA therapy was observed exclusively in individuals with TAV. Concurrently, a reduced gene expression of *COX-2* was found in the aortic wall of patients with TAV and ATAA with ASA therapy, which is consistent with the mechanism of action of ASA²⁰³. There was no difference in *COX-1* expression, and the inhibitory effect of ASA on COX-1 and thrombocyte aggregation is unlikely to impact ATAA pathogenesis as it does not involve an intraluminal thrombus. The expression of *COX-2* had a strong positive correlation with gene expression of macrophage and inflammatory markers, illustrating a possible mechanistic explanation for the decreased prevalence of ATAA seen in patients with ASA therapy and TAV. Macrophages and other inflammatory cells have been implicated in the disease process of TAV-associated ATAA and may induce fibrosis and matrix metalloproteinase (MMP)-mediated degradation of the extracellular matrix in the aortic wall^{24,31,230}. This is supported by experimental studies, which demonstrate that prostaglandins increase macrophage recruitment and MMP activity in the aortic wall of animal models of aortic aneurysm^{32,205}. Prostaglandins can also adversely affect smooth muscle cells in the media directly by inhibiting proliferation and smooth muscle actin expression^{204,208}. Further, COX-2 inhibition mediated by ASA or other agents, or lack of the enzyme, attenuates aortic aneurysms in rodent models^{32,206,207,231}.

The findings are also consistent with the lower risk of rupture and dissection reported by Owens et al in patients with thoracic aortic aneurysms treated with ASA, but not in patients treated with clopidogrel³². In contrast, others have reported a negative impact of ASA on a composite endpoint including both mortality and aortic outcome, or an uncertain effect^{33,34}. Although the regression model included preexisting cardiovascular disease and valve disease, there may still be residual confounding caused by subclinical atherosclerosis and metabolic factors^{160,232–234}. In conclusion, evidence suggests that ASA may be a viable pharmacological therapy in patients with ATAA and TAV, whereas ASA is unlikely to benefit patients with BAV-associated aneurysms. The differential effect of ASA seen in patients with BAV and TAV further illustrates that the substantial differences in underlying aortic pathology between the two patient groups may have a profound impact on the effect of pharmacological therapies^{24–28}.

5.4 Distal aortic outcome after ascending aortic surgery

Although the pathological differences between BAV- and TAV-associated ATAAs are well established^{24,25,28,235}, consideration of valve phenotype is not advocated in the recent guidelines for clinical management for ATAA, published in 2022²³. We observed a high long-term incidence of distal aortic complications in patients with TAV, whereas the absolute risk was low in patients with BAV. The findings are consistent with previous results, which have demonstrated that bicuspid aortopathy is limited to the ascending aorta (including the root), with only occasional involvement of the arch^{4,35,153,164,236}. The apparent confinement of BAV-associated aneurysms to the ascending aorta, with occasional arch involvement, is highly

consistent with defective development pertaining to the unique, shared embryonic origin of the aortic valve and ascending aorta as the proposed underlying pathology of bicuspid aortopathy^{1,237}. The considerable overlap in prevalence between thoracic and abdominal aortic aneurysms reported by others^{160,164,167} is thus seemingly limited to TAV-associated ATAAs^{4,164}, as are the pathological similarities between ATAAs and AAAs^{24,25,31,165,230}. The high incidence of serious complications in the TAV group is concerning and has important implications for clinical practice, both for postoperative surveillance and, pending confirmation, the timing and extent of aortic surgery. The results are thus well in line with earlier studies and indicate that patients with BAV operated for ATAA rarely benefit from long-term CT surveillance of the distal aortic segments^{35,212}. One limitation, however, is that the remnant ascending aorta was not systematically assessed. The risk of residual ascending aortic dilatation has also been reported to be low^{36,37}, but on the other hand distal ascending aortic dilatation may be overlooked with conventional echocardiography²³⁸.

5.5 Methodological considerations

5.5.1 Choice of animal model

Various animal models have been used to study the long-term viability, *in vivo* recellularization potential, immune response, hemodynamics, and thrombogenicity of tissue-engineered heart valves as well as other valve replacement options. Sheep, pigs, and rats are among the most frequently described in the literature and may be used for both *in vivo* and *in vitro* studies of tissue-engineered heart valves^{94,95,111,152,239}. The sheep model is considered the gold standard for *in vivo* studies of heart valve grafts²⁴⁰. It provides human-sized proportions, a cardiac anatomy and physiology similar to humans, and tolerates anesthesia and orthotopic graft implantation well^{101,241,242}. On the other hand, sheep require large facilities and the perioperative management is complex²³⁹, making sheep costly in time and resources. Furthermore, biomedical research on large mammals requires particular attention to research ethics (see 3.11 Ethical considerations).

The rat is the smallest animal for which implantation of aortic valve grafts has been described¹¹¹. Several advantages of rat models have been proposed²⁴³, chief among them arguably being the considerably lower costs and fewer ethical concerns compared to large mammals. However, all animal models have inherent disadvantages due to differences between humans and other species. Firstly, the rat aortic valve is much smaller and the cusps are extremely thin compared to humans^{152,244}. Secondly, only heterotopic implantation is possible, which results in non-physiological hemodynamics across the valve, which does not appear to open and close properly during the cardiac cycle^{111,152}. Thirdly, there are significant differences in the immune system between species²⁴⁵. These differences may lead to a high frequency of valve cusp thrombosis in rat models, particularly in non-syngeneic recipients^{111,246,247}, further limiting their use. Nevertheless, rats may constitute cost-effective, relevant, and ethically defensible animal models for studies on specific aspects of aortic valve grafts. For example, key data on the mechanisms of bioprosthetic valve degeneration was obtained through studies on rats⁷⁹. In

the context of heart valve tissue engineering, rat models have been used to study the *in vivo* recellularization potential, immune response, tissue remodeling, effects of protein and heparin coating, and the growth potential of decellularized aortic valve conduits^{92,93,213,246,248,249}. Additionally, decellularized rat aortic valves may also be used to assess recellularization *ex vivo*¹⁵².

5.5.2 Decellularization reagents

Decellularization is usually accomplished with a detergent or an enzyme as the primary reagent^{104,105,152,246,250}. Detergents may be ionic (SDS, sodium deoxycholate), non-ionic (Triton X-100), or zwitter-ionic (CHAPS). They solubilize cell membranes and dissociate DNA from proteins but are also known to cause protein denaturation, reviewed in¹⁰³. In general, ionic detergents yield more effective cell removal at the cost of increased protein denaturation^{92,104,107}, and other detergents have mainly found use in less dense tissues^{105,106}. Although prolonged exposure to SDS inevitably leads to severe extracellular matrix damage¹⁰⁷, the controlled exposure reported in decellularization studies may result in higher preservation of non-collagenous proteins, such as laminin, collagen IV and fibronectin, as well as glycosaminoglycans, compared to other reagents¹⁴⁹. The addition of Triton X-100 minimizes the amount of residual detergent in the decellularized scaffold¹⁰⁴, which may have cytotoxic effects^{251,252}. Trypsin, a serine protease excreted by the pancreas to digest proteins in the gut²⁵³, is another common primary reagent^{152,249,250,254}, which is also widely used to detach cells from culture substrates^{255,256}. However, trypsin is known for its propensity to cause extensive extracellular matrix damage^{149,249,250}. Nucleases, a class of enzymes that cleave nucleic acids, are frequently applied to decellularized scaffolds to remove DNA and RNA^{105,249–251,254} and have limited side effects, reviewed in¹⁰³. While the impact of residual DNA in decellularized scaffolds is still controversial²⁵⁷, it has been increasingly recognized that DNA may be immunogenic in of itself, reviewed in²⁵⁸. Furthermore, cell-free DNA in the form of neutrophil extracellular traps (NETs) in the aortic valve has been linked to calcification and stenosis²⁵⁹.

5.5.3 Definition of ascending aortic dilatation

An enduring controversy in the field is how ascending aortic dilatation, or aneurysm, should be defined. The traditional definition of an aneurysm as a $\geq 50\%$ enlargement compared to the expected normal vessel diameter¹⁶³ immediately becomes problematic when applied to the ascending aorta²³. With a reported mean ascending aortic diameter of 3.7-3.8 cm in some male populations^{158,160}, the ascending aorta would have to grow to >5.5 cm to be classified as an aneurysm according to the traditional definition. In clinical practice, an aorta is usually considered aneurysmatic well before it reaches 5.5 cm in diameter regardless of the patient's body size²³, and a threshold of 5.5 cm is higher than the median diameter of 5.1 cm associated with type A aortic dissection¹⁶.

The rationale for a fixed diameter threshold is that a large absolute diameter has been consistently shown to be associated with the risk aortic events^{4,29,171}. Moreover, a steep

increase has been observed at diameters ≥ 4.5 cm¹⁷¹. However, the uniform threshold of ≥ 4.5 cm also has important limitations. First and foremost, the normal size of the adult ascending aorta differs significantly depending on the segment (root or tubular aorta), as well as the body size, age, sex, and ethnicity of the individual^{158–162}. Secondly, the normal variability in ascending aortic diameter is greater than that observed in other aortic segments in the same individuals (ascending aorta SD 0.4 cm compared to descending aorta diameter SD 0.3 cm)^{158,160}. Collectively, these limitations mean that a uniform diameter threshold will result in patients being diagnosed with ATAA at widely different stages of the disease depending on patient-related factors. The use of aortic size index, height index, or cross-sectional area/height ratio has been advocated to account for differences in expected aortic size imparted by body size, and may be useful for particularly tall or short individuals²³. Further, the use of indexed aortic size may effectively adjust for the increased risk associated with female sex^{16,189}, and sex-specific thresholds for surgical consideration are currently not recommended for thoracic aortic aneurysms²³. In conclusion, a diameter of ≥ 4.5 cm remains a simple and clinically relevant definition of ascending aortic dilatation for most patients, and limitations may be largely offset by indexing for BSA or height, or by including either as a covariate in risk estimation models^{23,188}.

6 Conclusions

- I. The aortic valve endothelial BM contains the same laminin isoforms as the intimal endothelial and endocardial BMs.
- II. A decellularization protocol based on SDS may effectively remove cells and retain key BM components in the rat aortic valve. The resulting scaffold may serve as a model for mechanistic studies of the role of the BM in heart valve tissue engineering.
- III. There are significant sex differences in the associations seen between BAV phenotype, type of valve disease, ascending aortic dilatation pattern, and aortic dimensions at the time of surgery.
- IV. ASA may decrease the risk of aortic dilatation in patients with TAV, but not BAV, possibly by attenuating COX-2-mediated inflammation in the aortic wall.
- V. ATAA in BAV is associated with a low risk of distal aortic complications after ascending aortic replacement, whereas patients with TAV have a high incidence of distal aortic complications.

7 Points of perspective

There is currently no valve replacement option that can replicate the key functions of a normal heart valve - life-long durability, lack of thrombogenicity, and growth capacity. Recent encouraging developments in the field of heart valve replacements include polymeric prosthetic valves ²⁶⁰ and calcification-resistant pericardial tissue valves ²⁶¹, although only a tissue-engineered valve has the theoretical possibility to mimic all aspects of a normal heart valve. However, concerns regarding their current long-term durability in the clinical setting warrant caution ¹⁰, and it is unlikely that the full potential of tissue-engineered heart valves will be realized before further mechanistic studies are conducted ¹². There is a distinct need to elucidate the impact of specific components and properties of a scaffold on the recellularization potential, and - ultimately - the long-term *in vivo* performance. Aside from the laminin isoforms reported in this thesis, the roles of other key BM and matrix components, such as heparan sulfate proteoglycans ^{114,119} and fibronectin ⁹¹, as well as the mechanical properties of the scaffold ²⁶², need to be further elucidated. Such mechanistic studies would also contribute to the overall knowledge of heart valve biology and pathology.

The sex-specific bicuspid aortic valve phenotype associations reported here may help to inform decision-making and patient selection for TAVR in patients who are not optimal surgical candidates, especially as some evidence suggests that they could have prognostic significance in relation to aortic growth rate ¹⁹⁵. Confirmation of this finding should be of considerable interest in any upcoming longitudinal studies, which could also provide additional data regarding the optimal timing of aortic surgery. One of the major concerns for adopting TAVR in patients with BAV is the risk of progressive aortic dilatation after valve intervention ²⁶³. Although more recent studies since indicate that it is uncommon ¹⁷⁹⁻¹⁸⁴, the identification of anatomical risk factors for progressive dilatation could further improve the evidence for TAVR in the BAV population. On the other hand, factors other than the predisposition for aortopathy may still favor surgical aortic valve replacement, such as the extent and location of cusp calcification, the size of the aortic annulus, and the geometry of the aorta and aortic root ⁸⁶.

The finding that ASA may lower the risk of ascending aortic dilatation specifically in patients with TAV is important in of itself, but together with contemporary knowledge of the differences in pathology between BAV- and TAV-associated ATAA, it also clearly demonstrates the need for distinction between the two entities in future studies of pharmacotherapy. The inverse association between ASA and ATAA in TAV needs to be confirmed in other settings. It also remains to be discerned if ASA can lower the growth rate of a dilated ascending aorta. While ASA is generally considered a safe medication, it is not completely innocuous and its role in the primary prevention of cardiovascular disease has been disputed ²⁰¹. Rather than a clinical trial, a logical next step would be to study longitudinal data of patients undergoing isolated aortic valve surgery and study how ASA may affect growth rate and risk of complications in relation to different baseline aortic dimensions.

The final study of this thesis demonstrates the fundamental role that aortic valve phenotype should have in informing an individualized surveillance strategy after ascending aortic surgery. Together with other recent studies^{16,28,29,212}, it also implies that a differentiated surgical decision-making process for patients with BAV and TAV and ascending aortic dilatation may be forthcoming. Further research should focus on the identification of other potential prognostic markers, such as additional clinical variables, detailed aneurysm geometry and aortic wall pathology, biochemical plasma markers, and genotype. The long-term goal should be a risk stratification model for individualized decision-making regarding the risk-benefit, and the optimal timing and extent of aortic surgery.

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9 References

1. Sillesen, A.-S. *et al.* Prevalence of Bicuspid Aortic Valve and Associated Aortopathy in Newborns in Copenhagen, Denmark. *JAMA* **325**, 561–567 (2021).
2. Larson, E. W. & Edwards, W. D. Risk factors for aortic dissection: A necropsy study of 161 cases. *Am. J. Cardiol.* **53**, 849–855 (1984).
3. Roberts, W. C. & Ko, J. M. Frequency by decades of unicuspid, bicuspid, and tricuspid aortic valves in adults having isolated aortic valve replacement for aortic stenosis, with or without associated aortic regurgitation. *Circulation* **111**, 920–925 (2005).
4. Davies, R. R. *et al.* Natural History of Ascending Aortic Aneurysms in the Setting of an Unreplaced Bicuspid Aortic Valve. *Ann. Thorac. Surg.* **83**, 1338–1344 (2007).
5. Eleid, M. F. *et al.* Type A aortic dissection in patients with bicuspid aortic valves: Clinical and pathological comparison with tricuspid aortic valves. *Heart* **99**, 1668–1674 (2013).
6. Yang, L.-T. *et al.* Bicuspid aortic valve: long-term morbidity and mortality. *Eur. Heart J.* **1**, 1–14 (2023).
7. Diaz, R. *et al.* Long-term outcomes of mechanical versus biological aortic valve prosthesis: Systematic review and meta-analysis. *J. Thorac. Cardiovasc. Surg.* **158**, 706–714.e18 (2019).
8. Cebotari, S. *et al.* Construction of autologous human heart valves based on an acellular allograft matrix. *Circulation* **106**, I63–I68 (2002).
9. Hoerstrup, S. P. *et al.* Tissue engineering of functional trileaflet heart valves from human marrow stromal cells. *Circulation* **106**, I143–I150 (2002).
10. Helder, M. R. K. K. *et al.* Late durability of decellularized allografts for aortic valve replacement: A word of caution. *J. Thorac. Cardiovasc. Surg.* **152**, 1197–1199 (2016).
11. Ebken, J. *et al.* Residual immune response towards decellularized homografts may be highly individual. *Eur. J. Cardio-Thoracic Surg.* **00**, 1–10 (2020).
12. Blum, K. M., Drews, J. D. & Breuer, C. K. Tissue-Engineered Heart Valves: A Call for Mechanistic Studies. *Tissue Eng. Part B Rev.* **24**, 240–253 (2018).
13. Cheng, C. *et al.* Clinical Outcomes of Adults With Bicuspid Aortic Valve: A European Perspective. *Mayo Clin. Proc.* **96**, 648–657 (2021).
14. Tzemos, N. *et al.* Outcomes in adults with bicuspid aortic valves. *JAMA* **300**, 1317–25 (2008).
15. Masri, A. *et al.* Characteristics and long-term outcomes of contemporary patients with bicuspid aortic valves. *J. Thorac. Cardiovasc. Surg.* **151**, 1650–1659.e1 (2016).
16. Perez, Z. G. *et al.* Aortic Size at the Time of Type A and Type B Dissections. *Ann. Thorac. Surg.* **116**, 262–268 (2023).
17. Rodríguez-Palomares, J. F., Dux-Santoy, L., Guala, A., Galian-Gay, L. & Evangelista, A. Mechanisms of Aortic Dilation in Patients With Bicuspid Aortic Valve: JACC State-of-the-Art Review. *J. Am. Coll. Cardiol.* **82**, 448–464 (2023).

18. Kong, W. K. F. *et al.* Prognostic implications of raphe in bicuspid aortic valve anatomy. *JAMA Cardiol.* **2**, 285–292 (2017).
19. Evangelista, A. *et al.* Anatomical and clinical predictors of valve dysfunction and aortic dilation in bicuspid aortic valve disease. *Heart* **104**, 566–573 (2018).
20. Grattan, M. *et al.* Predictors of Bicuspid Aortic Valve-Associated Aortopathy in Childhood: A Report From the MIBAVA Consortium. *Circ. Cardiovasc. Imaging* **13**, e009717 (2020).
21. Roman, M. J. *et al.* Aortic Dilatation Associated With Bicuspid Aortic Valve: Relation to Sex, Hemodynamics, and Valve Morphology (the National Heart Lung and Blood Institute-Sponsored National Registry of Genetically Triggered Thoracic Aortic Aneurysms and Cardiovascular Con. *Am. J. Cardiol.* **120**, 1171–1175 (2017).
22. Sievers, H. H., Stierle, U., Hachmann, R. M. S. & Charitos, E. I. New insights in the association between bicuspid aortic valve phenotype, aortic configuration and valve haemodynamics. *Eur. J. Cardio-thoracic Surg.* **49**, 439–446 (2016).
23. Isselbacher, E. M. *et al.* 2022 ACC/AHA Guideline for the Diagnosis and Management of Aortic Disease: A Report of the American Heart Association/American College of Cardiology Joint Committee on Clinical Practice Guidelines. *Circulation* **146**, E334–E482 (2022).
24. Folkersen, L. *et al.* Unraveling divergent gene expression profiles in bicuspid and tricuspid aortic valve patients with thoracic aortic dilatation: The ASAP study. *Mol. Med.* **17**, 1365–1373 (2011).
25. Blunder, S. *et al.* Characteristics of TAV- and BAV-associated thoracic aortic aneurysms-Smooth muscle cell biology, expression profiling, and histological analyses. *Atherosclerosis* **220**, 355–361 (2012).
26. Heng, E. *et al.* Comparative Histology of Aortic Dilatation Associated With Bileaflet Versus Trileaflet Aortic Valves. *Ann. Thorac. Surg.* **100**, 2095–101; discussion 2101 (2015).
27. Maleki, S. *et al.* Mesenchymal state of intimal cells may explain higher propensity to ascending aortic aneurysm in bicuspid aortic valves. *Sci. Rep.* **6**, 1–16 (2016).
28. Chung, J. C. Y. *et al.* Biomechanics of Aortic Dissection: A Comparison of Aortas Associated With Bicuspid and Tricuspid Aortic Valves. *J. Am. Heart Assoc.* **9**, e016715 (2020).
29. Zafar, M. A. *et al.* Bicuspid aortopathy does not require earlier surgical intervention. *J. Thorac. Cardiovasc. Surg.* (2023). doi:10.1016/j.jtcvs.2023.04.017
30. Freiholtz, D. *et al.* Bicuspid aortic valve aortopathy is characterized by embryonic epithelial to mesenchymal transition and endothelial instability. *J. Mol. Med.* **101**, 801–811 (2023).
31. Freiholtz, D. *et al.* SPP1/osteopontin: a driver of fibrosis and inflammation in degenerative ascending aortic aneurysm? *J. Mol. Med. (Berl)*. **101**, 1323–1333 (2023).
32. Owens, A. P. *et al.* Platelet inhibitors reduce rupture in a mouse model of established abdominal aortic aneurysm. *Arterioscler. Thromb. Vasc. Biol.* **35**, 2032–2041 (2015).

33. Jovin, I. S. *et al.* Comparison of the effect on long-term outcomes in patients with thoracic aortic aneurysms of taking versus not taking a statin drug. *Am. J. Cardiol.* **109**, 1050–1054 (2012).
34. Chen, C. Y., Huang, J. W., Lee, C. T. C., Lai, W. Ter & Huang, Y. Bin. Long-term outcome of patients with aortic aneurysms taking low-dose aspirin: A population-based cohort study. *J. Investig. Med.* **61**, 1004–1012 (2013).
35. Bilkhu, R. *et al.* Fate of the Aortic Arch Following Surgery on the Aortic Root and Ascending Aorta in Bicuspid Aortic Valve. *Ann. Thorac. Surg.* **106**, 771–776 (2018).
36. Pearsall, C. *et al.* The effect of adding an open distal anastomosis to proximal aneurysm repairs in bicuspid aortopathy. *Gen. Thorac. Cardiovasc. Surg.* **71**, 455–463 (2023).
37. Yoshioka, Y. *et al.* Does the residual aorta dilate after replacement of the bicuspid aortic valve and ascending aorta? *J. Thorac. Dis.* **15**, 994–1008 (2023).
38. Yacoub, M. H., Kilner, P. J., Birks, E. J. & Misfeld, M. The aortic outflow and root: a tale of dynamism and crosstalk. *Ann. Thorac. Surg.* **68**, S37–S43 (1999).
39. Sievers, H. H. & Schmidtke, C. A classification system for the bicuspid aortic valve from 304 surgical specimens. *J. Thorac. Cardiovasc. Surg.* **133**, 1226–1233 (2007).
40. Hinton, R. B. & Yutzey, K. E. Heart valve structure and function in development and disease. *Annu Rev Physiol* **73**, 29–46 (2011).
41. Butcher, J. T., Penrod, A. M., García, A. J. & Nerem, R. M. Unique morphology and focal adhesion development of valvular endothelial cells in static and fluid flow environments. *Arterioscler. Thromb. Vasc. Biol.* **24**, 1429–1434 (2004).
42. Butcher, J. T. *et al.* Transcriptional profiles of valvular and vascular endothelial cells reveal phenotypic differences: Influence of shear stress. *Arterioscler. Thromb. Vasc. Biol.* **26**, 69–77 (2006).
43. Wang, H., Leinwand, L. A. & Anseth, K. S. Cardiac valve cells and their microenvironment--insights from in vitro studies. *Nat. Rev. Cardiol.* **11**, 715–27 (2014).
44. Weind, K. L., Ellis, C. G. & Boughner, D. R. Aortic valve cusp vessel density: Relationship with tissue thickness. *J. Thorac. Cardiovasc. Surg.* **123**, 333–340 (2002).
45. Syväranta, S., Helske, S., Lappalainen, J., Kupari, M. & Kovanen, P. T. Lymphangiogenesis in aortic valve stenosis—Novel regulatory roles for valvular myofibroblasts and mast cells. *Atherosclerosis* **221**, 366–374 (2012).
46. Marron, K. *et al.* Innervation of human atrioventricular and arterial valves. *Circulation* **94**, 368–75 (1996).
47. Nistri, S., Basso, C., Marzari, C., Mormino, P. & Thiene, G. Frequency of bicuspid aortic valve in young male conscripts by echocardiogram. *Am. J. Cardiol.* **96**, 718–721 (2005).
48. Kong, W. K. F., Bax, J. J., Michelena, H. I. & Delgado, V. Sex differences in bicuspid aortic valve disease. *Prog. Cardiovasc. Dis.* **63**, 452–456 (2020).
49. Michelena, H. I. *et al.* International consensus statement on nomenclature and classification of the congenital bicuspid aortic valve and its aortopathy, for clinical, surgical, interventional and research purposes. *J. Thorac. Cardiovasc. Surg.* **162**, e383–

e414 (2021).

50. Sievers, H. H. *et al.* Toward individualized management of the ascending aorta in bicuspid aortic valve surgery: The role of valve phenotype in 1362 patients. *J. Thorac. Cardiovasc. Surg.* **148**, 2072–2080.e3 (2014).
51. Bissell, M. M. *et al.* Aortic dilation in bicuspid aortic valve disease: Flow pattern is a major contributor and differs with valve fusion type. *Circ. Cardiovasc. Imaging* **6**, 499–507 (2013).
52. Mahadevia, R. *et al.* Bicuspid aortic cusp fusion morphology alters aortic three-dimensional outflow patterns, wall shear stress, and expression of aortopathy. *Circulation* **129**, 673–682 (2014).
53. Dux-Santoy, L. *et al.* Low and Oscillatory Wall Shear Stress Is Not Related to Aortic Dilation in Patients With Bicuspid Aortic Valve: A Time-Resolved 3-Dimensional Phase-Contrast Magnetic Resonance Imaging Study. *Arterioscler. Thromb. Vasc. Biol.* **40**, e10–e20 (2020).
54. Corbitt, H. *et al.* TIMP3 and TIMP1 are risk genes for bicuspid aortic valve and aortopathy in Turner syndrome. *PLoS Genet.* **14**, e1007692 (2018).
55. Garg, V. *et al.* Mutations in NOTCH1 cause aortic valve disease. *Nature* **437**, 270–274 (2005).
56. MacGrogan, D., Münch, J. & de la Pompa, J. L. Notch and interacting signalling pathways in cardiac development, disease, and regeneration. *Nat. Rev. Cardiol.* **15**, 685–704 (2018).
57. Gould, R. A. *et al.* ROBO4 variants predispose individuals to bicuspid aortic valve and thoracic aortic aneurysm. *Nat. Genet.* **51**, 42–50 (2019).
58. Gharibeh, L. *et al.* GATA6 regulates aortic valve remodeling, and its haploinsufficiency leads to right-left type bicuspid aortic valve. *Circulation* **138**, 1025–1038 (2018).
59. Gillis, E. *et al.* Candidate gene resequencing in a large bicuspid aortic valve-associated thoracic aortic aneurysm cohort: SMAD6 as an important contributor. *Front. Physiol.* **8**, 1–10 (2017).
60. Odelin, G. *et al.* Variations in the poly-histidine repeat motif of HOXA1 contribute to bicuspid aortic valve in mouse and zebrafish. *Nat. Commun.* **14**, (2023).
61. Maleki, S. *et al.* Endothelial/Epithelial Mesenchymal Transition in Ascending Aortas of Patients With Bicuspid Aortic Valve. *Front. Cardiovasc. Med.* **6**, 1–12 (2019).
62. Glotzbach, J. P. *et al.* Familial Associations of Prevalence and Cause-Specific Mortality for Thoracic Aortic Disease and Bicuspid Aortic Valve in a Large-Population Database. *Circulation* **148**, 637–647 (2023).
63. Slostad, B. D. *et al.* Unicuspid Aortic Valve. *Circulation* **140**, 1853–1855 (2019).
64. Fernández, B. *et al.* Bicuspid Aortic Valves With Different Spatial Orientations of the Leaflets Are Distinct Etiological Entities. *J. Am. Coll. Cardiol.* **54**, 2312–2318 (2009).
65. Michelena, H. I. *et al.* Incidence of aortic complications in patients with bicuspid aortic valves. *JAMA* **306**, 1104–12 (2011).

66. Jackson, V. *et al.* Bicuspid aortic valve leaflet morphology in relation to aortic root morphology: A study of 300 patients undergoing open-heart surgery. *Eur. J. Cardio-thoracic Surg.* **40**, 118–124 (2011).
67. Michelena, H. I. *et al.* Natural history of asymptomatic patients with normally functioning or minimally dysfunctional bicuspid aortic valve in the community. *Circulation* **117**, 2776–2784 (2008).
68. Chambers, H. F. & Bayer, A. S. Native-Valve Infective Endocarditis. *N. Engl. J. Med.* **383**, 567–576 (2020).
69. Nkomo, V. T. *et al.* Burden of valvular heart diseases: a population-based study. *Lancet* **368**, 1005–1011 (2006).
70. Iung, B. *et al.* A prospective survey of patients with valvular heart disease in Europe: The Euro Heart Survey on valvular heart disease. *Eur. Heart J.* **24**, 1231–1243 (2003).
71. Lindman, B. R. *et al.* Calcific aortic stenosis. *Nat. Rev. Dis. Prim.* **2**, (2016).
72. Otto, C. M. & Prendergast, B. Aortic-valve stenosis--from patients at risk to severe valve obstruction. *N. Engl. J. Med.* **371**, 744–56 (2014).
73. Strange, G. *et al.* Poor Long-Term Survival in Patients With Moderate Aortic Stenosis. *J. Am. Coll. Cardiol.* **74**, 1851–1863 (2019).
74. Iung, B. & Vahanian, A. Epidemiology of valvular heart disease in the adult. *Nat. Rev. Cardiol.* **8**, 162–172 (2011).
75. Otto, C. M. *et al.* 2020 ACC/AHA Guideline for the Management of Patients With Valvular Heart Disease. *J. Am. Coll. Cardiol.* **77**, e25–e197 (2021).
76. Yang, L. T. *et al.* Outcomes in Chronic Hemodynamically Significant Aortic Regurgitation and Limitations of Current Guidelines. *J. Am. Coll. Cardiol.* **73**, 1741–1752 (2019).
77. Puri, R., Auffret, V. & Rodés-Cabau, J. Bioprosthetic Valve Thrombosis. *J. Am. Coll. Cardiol.* **69**, 2193–2211 (2017).
78. Fischlein, T. *et al.* Immunologic reaction and viability of cryopreserved homografts. *Ann. Thorac. Surg.* **60**, (1995).
79. Manji, R. A. *et al.* Glutaraldehyde-fixed bioprosthetic heart valve conduits calcify and fail from xenograft rejection. *Circulation* **114**, 318–327 (2006).
80. Senage, T. *et al.* The role of antibody responses against glycans in bioprosthetic heart valve calcification and deterioration. *Nat. Med.* **28**, 283–294 (2022).
81. Koolbergen, D. R. *et al.* The pathology of fresh and cryopreserved homograft heart valves: An analysis of forty explanted homograft valves. *J. Thorac. Cardiovasc. Surg.* **124**, 689–697 (2002).
82. Boodhwani, M. & El Khoury, G. Aortic Valve Repair. *Oper. Tech. Thorac. Cardiovasc. Surg.* **14**, 266–280 (2009).
83. Mazine, A. *et al.* Long-term outcomes of the ross procedure versus mechanical aortic valve replacement. *Circulation* **134**, 576–585 (2016).

84. Ozaki, S. *et al.* Midterm outcomes after aortic valve neocuspidization with glutaraldehyde-treated autologous pericardium. *J. Thorac. Cardiovasc. Surg.* **155**, 2379–2387 (2018).
85. Popma, J. J. *et al.* Transcatheter Aortic-Valve Replacement with a Self-Expanding Valve in Low-Risk Patients. *N. Engl. J. Med.* **380**, 1706–1715 (2019).
86. Vincent, F. *et al.* Transcatheter Aortic Valve Replacement in Bicuspid Aortic Valve Stenosis. *Circulation* **143**, 1043–1061 (2021).
87. Romeo, J. L. R. *et al.* Long-term Clinical and Echocardiographic Outcomes in Young and Middle-aged Adults Undergoing the Ross Procedure. *JAMA Cardiol.* **6**, 539–548 (2021).
88. Ng, A. P. *et al.* Maternal and Fetal Outcomes in Pregnant Patients With Mechanical and Bioprosthetic Heart Valves. *J. Am. Heart Assoc.* **12**, e028653 (2023).
89. El-Hamamsy, I. *et al.* Long-term outcomes after autograft versus homograft aortic root replacement in adults with aortic valve disease: a randomised controlled trial. *Lancet* **376**, 524–531 (2010).
90. Tudorache, I. *et al.* Decellularized aortic allografts versus pulmonary autografts for aortic valve replacement in the growing sheep model: Haemodynamic and morphological results at 20 months after implantation. *Eur. J. Cardio-thoracic Surg.* **49**, 1228–1238 (2016).
91. Flameng, W. *et al.* Coating with fibronectin and stromal cell-derived factor-1-alpha of decellularized homografts used for right ventricular outflow tract reconstruction eliminates immune response-related degeneration. *J. Thorac. Cardiovasc. Surg.* **147**, 1398-1404.e2 (2014).
92. Grauss, R. W., Hazekamp, M. G., Van Vliet, S., Gittenberger-De Groot, A. C. & DeRuiter, M. C. Decellularization of rat aortic valve allografts reduces leaflet destruction and extracellular matrix remodeling. *J. Thorac. Cardiovasc. Surg.* **126**, 2003–2010 (2003).
93. Toshmatova, M. *et al.* Influence of Laminin Coating on the Autologous In Vivo Recellularization of Decellularized Vascular Protheses. *Mater. (Basel, Switzerland)* **12**, (2019).
94. Iop, L. *et al.* Decellularized allogeneic heart valves demonstrate self-regeneration potential after a long-term preclinical evaluation. *PLoS One* **9**, (2014).
95. Syedain, Z. *et al.* 6-Month aortic valve implantation of an off-the-shelf tissue-engineered valve in sheep. *Biomaterials* **73**, 175–184 (2015).
96. Weber, B. *et al.* Off-the-shelf human decellularized tissue-engineered heart valves in a non-human primate model. *Biomaterials* **34**, 7269–7280 (2013).
97. Horke, A. *et al.* Early results from a prospective, single-arm European trial on decellularized allografts for aortic valve replacement: The ARISE study and ARISE registry data. *Eur. J. Cardio-thoracic Surg.* **58**, 1045–1053 (2020).
98. Da Costa, F. D. A. *et al.* The early and midterm function of decellularized aortic valve allografts. *Ann. Thorac. Surg.* **90**, 1854–1860 (2010).

99. Tudorache, I. *et al.* Decellularized aortic homografts for aortic valve and aorta ascendens replacement. *Eur. J. Cardio-thoracic Surg.* **50**, 89–97 (2016).
100. Schmidt, D. *et al.* Minimally-invasive implantation of living tissue engineered heart valves: A comprehensive approach from autologous vascular cells to stem cells. *J. Am. Coll. Cardiol.* **56**, 510–520 (2010).
101. Tudorache, I. *et al.* Orthotopic replacement of aortic heart valves with tissue-engineered grafts. *Tissue Eng. Part A* **19**, 1686–94 (2013).
102. Li, Z. *et al.* Single-cell transcriptome analyses reveal novel targets modulating cardiac neovascularization by resident endothelial cells following myocardial infarction. *Eur. Heart J.* **40**, 2507–2520 (2019).
103. Crapo, P. M., Gilbert, T. W. & Badylak, S. F. An overview of tissue and whole organ decellularization processes. *Biomaterials* **32**, 3233–3243 (2011).
104. Ott, H. C. *et al.* Perfusion-decellularized matrix: using nature’s platform to engineer a bioartificial heart. *Nat. Med.* **14**, 213–221 (2008).
105. Petersen, T. H. *et al.* Tissue-engineered lungs for in vivo implantation. *Science (80-.).* **329**, 538–541 (2010).
106. Takeishi, K. *et al.* Assembly and Function of a Bioengineered Human Liver for Transplantation Generated Solely from Induced Pluripotent Stem Cells. *Cell Rep.* **31**, 107711 (2020).
107. Faulk, D. M. *et al.* The effect of detergents on the basement membrane complex of a biologic scaffold material. *Acta Biomater.* **10**, 183–193 (2014).
108. Brown, J. W. *et al.* Performance of the CryoValve* SG human decellularized pulmonary valve in 342 patients relative to the conventional CryoValve at a mean follow-up of four years. *J. Thorac. Cardiovasc. Surg.* **139**, 339–348 (2010).
109. Cebotari, S. *et al.* Use of fresh decellularized allografts for pulmonary valve replacement may reduce the reoperation rate in children and young adults: Early report. *Circulation* **124**, 115–123 (2011).
110. Fukushima, S. *et al.* Long-term clinical outcomes after aortic valve replacement using cryopreserved aortic allograft. *J. Thorac. Cardiovasc. Surg.* **148**, 65-72.e2 (2014).
111. Oei, F. B. *et al.* A size-matching heterotopic aortic valve implantation model in the rat. *J. Surg. Res.* **87**, 239–244 (1999).
112. Sarikouch, S. *et al.* Early Insight Into In Vivo Recellularization of Cell-Free Allogenic Heart Valves. *Ann. Thorac. Surg.* **108**, 581–589 (2019).
113. Keane, T. J., Londono, R., Turner, N. J. & Badylak, S. F. Consequences of ineffective decellularization of biologic scaffolds on the host response. *Biomaterials* **33**, 1771–1781 (2012).
114. Shojaie, S. *et al.* Acellular lung scaffolds direct differentiation of endoderm to functional airway epithelial cells: Requirement of matrix-bound HS proteoglycans. *Stem Cell Reports* **4**, 419–430 (2015).
115. Pozzi, A., Yurchenco, P. D. & Iozzo, R. V. The nature and biology of basement membranes. *Matrix Biol.* **57–58**, 1–11 (2017).

116. Yurchenco, P. D. Basement membranes: Cell scaffoldings and signaling platforms. *Cold Spring Harb. Perspect. Biol.* **3**, 1–27 (2011).
117. Segev, A., Nili, N. & Strauss, B. H. The role of perlecan in arterial injury and angiogenesis. *Cardiovasc. Res.* **63**, 603–610 (2004).
118. Nugent, M. A., Nugent, H. M., Iozzo, R. V., Sanchack, K. & Edelman, E. R. Perlecan is required to inhibit thrombosis after deep vascular injury and contributes to endothelial cell-mediated inhibition of intimal hyperplasia. *Proc. Natl. Acad. Sci.* **97**, 6722–6727 (2000).
119. Tran, P. K. *et al.* Increased Intimal Hyperplasia and Smooth Muscle Cell Proliferation in Transgenic Mice with Heparan Sulfate-Deficient Perlecan. *Circ. Res.* **94**, 550–558 (2004).
120. Tran-Lundmark, K. *et al.* Heparan sulfate in perlecan promotes mouse atherosclerosis: Roles in lipid permeability, lipid retention, and smooth muscle cell proliferation. *Circ. Res.* **103**, 43–52 (2008).
121. Costell, M. *et al.* Hyperplastic conotruncal endocardial cushions and transposition of great arteries in perlecan-null mice. *Circ. Res.* **91**, 158–164 (2002).
122. Naylor, R. W., Morais, M. R. P. T. & Lennon, R. Complexities of the glomerular basement membrane. *Nat. Rev. Nephrol.* **17**, 112–127 (2021).
123. Leivo, I., Vaheri, A., Timpl, R. & Wartiovaara, J. Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* **76**, 100–114 (1980).
124. Domogatskaya, A., Rodin, S. & Tryggvason, K. Functional Diversity of Laminins. *Annu. Rev. Cell Dev. Biol.* **28**, 523–553 (2012).
125. Macdonald, P. R., Lustig, A., Steinmetz, M. O. & Kammerer, R. A. Laminin chain assembly is regulated by specific coiled-coil interactions. *J. Struct. Biol.* **170**, 398–405 (2010).
126. Aumailley, M. *et al.* A simplified laminin nomenclature. *Matrix Biol.* **24**, 326–32 (2005).
127. Sorokin, L., Girg, W., Göpfert, T., Hallmann, R. & Deutzmann, R. Expression of novel 400-kDa laminin chains by mouse and bovine endothelial cells. *Eur. J. Biochem.* **223**, 603–10 (1994).
128. Frieser, M. *et al.* Cloning of the mouse laminin $\alpha 4$ cDNA. Expression in a subset of endothelium. *Eur. J. Biochem.* **246**, 727–735 (1997).
129. Sorokin, L. M. *et al.* Developmental regulation of the laminin chain suggests a role in epithelial and endothelial cell maturation. *Dev. Biol.* **189**, 285–300 (1997).
130. Di Russo, J. *et al.* Vascular laminins in physiology and pathology. *Matrix Biol.* **57–58**, 140–148 (2017).
131. Roediger, M., Miosge, N. & Gersdorff, N. Tissue distribution of the laminin $\beta 1$ and $\beta 2$ chain during embryonic and fetal human development. *J. Mol. Histol.* **41**, 177–184 (2010).
132. Sasaki, T., Mann, K., Miner, J. H., Miosge, N. & Timpl, R. Domain IV of mouse laminin $\beta 1$ and $\beta 2$ chains: Structure, glycosaminoglycan modification and immunochemical

- analysis of tissue contents. *Eur. J. Biochem.* **269**, 431–442 (2002).
133. Petäjäniemi, N. *et al.* Localization of laminin $\alpha 4$ -chain in developing and adult human tissues. *J. Histochem. Cytochem.* **50**, 1113–1130 (2002).
 134. McLean, S. E., Mecham, B. H., Kelleher, C. M., Mariani, T. J. & Mecham, R. P. Extracellular matrix gene expression in the developing mouse aorta. in *Advances in Developmental Biology* **15**, 81–128 (2005).
 135. Wu, C. *et al.* Endothelial basement membrane laminin $\alpha 5$ selectively inhibits T lymphocyte extravasation into the brain. *Nat. Med.* **15**, 519–527 (2009).
 136. Song, J. *et al.* Endothelial Basement Membrane Laminin 511 Contributes to Endothelial Junctional Tightness and Thereby Inhibits Leukocyte Transmigration. *Cell Rep.* **18**, 1256–1269 (2017).
 137. Sixt, M., Hallmann, R., Wendler, O., Scharffetter-Kochanek, K. & Sorokin, L. M. Cell adhesion and migration properties of $\beta 2$ -integrin negative polymorphonuclear granulocytes on defined extracellular matrix molecules: Relevance for leukocyte extravasation. *J. Biol. Chem.* **276**, 18878–18887 (2001).
 138. Thyboll, J. *et al.* Deletion of the Laminin $\alpha 4$ Chain Leads to Impaired Microvessel Maturation. *Mol. Cell. Biol.* **22**, 1194–1202 (2002).
 139. Richards, M. *et al.* Intra-vessel heterogeneity establishes enhanced sites of macromolecular leakage downstream of laminin $\alpha 5$. *Cell Rep.* **35**, (2021).
 140. Gonzalez, A. M. *et al.* Complex interactions between the laminin $\alpha 4$ subunit and integrins regulate endothelial cell behavior in vitro and angiogenesis in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16075–16080 (2002).
 141. DeHahn, K. C. *et al.* The $\alpha 4$ laminin subunit regulates endothelial cell survival. *Exp. Cell Res.* **294**, 281–289 (2004).
 142. Knöll, R. *et al.* Laminin- $\alpha 4$ and integrin-linked kinase mutations cause human cardiomyopathy via simultaneous defects in cardiomyocytes and endothelial cells. *Circulation* **116**, 515–525 (2007).
 143. Russo, J. Di *et al.* Endothelial basement membrane laminin 511 is essential for shear stress response. *EMBO J.* **36**, 183–201 (2017).
 144. Wagner, J. U. G. *et al.* Switch in laminin $\beta 2$ to laminin $\beta 1$ isoforms during aging controls endothelial cell functions-brief report. *Arterioscler. Thromb. Vasc. Biol.* **38**, 1170–1177 (2018).
 145. Rodriguez, K. J. & Masters, K. S. Regulation of valvular interstitial cell calcification by components of the extracellular matrix. *J. Biomed. Mater. Res. - Part A* **90**, 1043–1053 (2009).
 146. Gu, X. & Masters, K. S. Regulation of valvular interstitial cell calcification by adhesive peptide sequences. *J. Biomed. Mater. Res. - Part A* **93**, 1620–1630 (2010).
 147. Balaoing, L. R. *et al.* Laminin peptide-immobilized hydrogels modulate valve endothelial cell hemostatic regulation. *PLoS One* **10**, 1–16 (2015).
 148. Virtanen, I. *et al.* Laminin $\alpha 1$ -chain shows a restricted distribution in epithelial basement membranes of fetal and adult human tissues. *Exp. Cell Res.* **257**, 298–309 (2000).

149. Akhyari, P. *et al.* The quest for an optimized protocol for whole-heart decellularization: a comparison of three popular and a novel decellularization technique and their diverse effects on crucial extracellular matrix qualities. *Tissue Eng. Part C. Methods* **17**, 915–926 (2011).
150. Månsson-Broberg, A. *et al.* Wnt/ β -Catenin Stimulation and Laminins Support Cardiovascular Cell Progenitor Expansion from Human Fetal Cardiac Mesenchymal Stromal Cells. *Stem Cell Reports* **6**, 607–617 (2016).
151. Rodin, S. *et al.* Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. *Nat. Commun.* **5**, 3195 (2014).
152. Kallenbach, K. *et al.* A novel small-animal model for accelerated investigation of tissue-engineered aortic valve conduits. *Tissue Eng. Part C. Methods* **16**, 41–50 (2010).
153. Fazel, S. S. *et al.* The aortopathy of bicuspid aortic valve disease has distinctive patterns and usually involves the transverse aortic arch. *J. Thorac. Cardiovasc. Surg.* **135**, (2008).
154. Hornick, M. *et al.* ‘Bovine’ aortic arch - A marker for thoracic aortic disease. *Cardiol.* **123**, 116–124 (2012).
155. Shalhoub, S. *et al.* Association of variant arch anatomy with type B aortic dissection and hemodynamic mechanisms. *J. Vasc. Surg.* **68**, 1640–1648 (2018).
156. Sherifova, S. & Holzapfel, G. A. Biomechanics of aortic wall failure with a focus on dissection and aneurysm: A review. *Acta Biomater.* **99**, 1–17 (2019).
157. Ross, M. H. & Pawlina, W. Chapter 13. Cardiovascular System. in *Histology - a text and atlas* 364–379 (Lippincott Williams & Wilkins, 2006).
158. Kälsch, H. *et al.* Body-surface adjusted aortic reference diameters for improved identification of patients with thoracic aortic aneurysms: Results from the population-based Heinz Nixdorf Recall study. *Int. J. Cardiol.* **163**, 72–78 (2013).
159. Chang, H. W. *et al.* Diameter and growth rate of the thoracic aorta-analysis based on serial computed tomography scans. *J. Thorac. Dis.* **12**, 4002–4013 (2020).
160. Obel, L. M. *et al.* Population-Based Risk Factors for Ascending, Arch, Descending, and Abdominal Aortic Dilations for 60-74-Year-Old Individuals. *J. Am. Coll. Cardiol.* **78**, 201–211 (2021).
161. Hu, X. *et al.* Comparisons of Two-dimensional Echocardiographic Aortic Dimensions Between Chinese, Japanese, and Europeans. *J. Thorac. Imaging* **38**, 399–406 (2023).
162. Patel, H. N. *et al.* Normal Values of Aortic Root Size According to Age, Sex, and Race: Results of the World Alliance of Societies of Echocardiography Study. *J. Am. Soc. Echocardiogr.* **35**, 267–274 (2022).
163. Johnston, K. W. *et al.* Suggested standards for reporting on arterial aneurysms. Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular. *J. Vasc. Surg.* **13**, 452–458 (1991).
164. Vapnik, J. S. *et al.* Characteristics and Outcomes of Ascending Versus Descending Thoracic Aortic Aneurysms. *Am. J. Cardiol.* **117**, 1683–1690 (2016).

165. Davis, F. M. *et al.* The Histone Methyltransferase SETDB2 Modulates Tissue Inhibitors of Metalloproteinase-Matrix Metalloproteinase Activity During Abdominal Aortic Aneurysm Development. *Ann. Surg.* **278**, 426–440 (2023).
166. Wang, S. K. *et al.* Osteopontin may be a driver of abdominal aortic aneurysm formation. *J. Vasc. Surg.* **68**, 22S–29S (2018).
167. Gouveia E Melo, R. *et al.* Synchronous and Metachronous Thoracic Aortic Aneurysms in Patients With Abdominal Aortic Aneurysms: A Systematic Review and Meta-Analysis. *J. Am. Heart Assoc.* **9**, e017468 (2020).
168. Ladich, E., Butany, J. & Virmani, R. Aneurysms of the Aorta. in *Cardiovascular Pathology* 169–211 (Elsevier, 2016). doi:10.1016/B978-0-12-420219-1.00005-7
169. Ziganshin, B. A. *et al.* Simple renal cysts as markers of thoracic aortic disease. *J. Am. Heart Assoc.* **5**, 1–11 (2016).
170. Pape, L. A. *et al.* Presentation, diagnosis, and outcomes of acute aortic dissection: 17-year trends from the international registry of acute aortic dissection. *J. Am. Coll. Cardiol.* **66**, 350–358 (2015).
171. Paruchuri, V. *et al.* Aortic Size Distribution in the General Population: Explaining the Size Paradox in Aortic Dissection. *Cardiol.* **131**, 265–272 (2015).
172. Cecconi, M. *et al.* Aortic dimensions in patients with bicuspid aortic valve without significant valve dysfunction. *Am. J. Cardiol.* **95**, 292–294 (2005).
173. Masri, A., Svensson, L. G., Griffin, B. P. & Desai, M. Y. Contemporary natural history of bicuspid aortic valve disease: A systematic review. *Heart* **103**, 1323–1330 (2017).
174. Blais, S., Meloche-Dumas, L., Fournier, A., Dallaire, F. & Dahdah, N. Long-Term Risk Factors for Dilatation of the Proximal Aorta in a Large Cohort of Children With Bicuspid Aortic Valve. *Circ. Cardiovasc. Imaging* **13**, e009675 (2020).
175. Verma, S. & Siu, S. C. Aortic dilatation in patients with bicuspid aortic valve. *N. Engl. J. Med.* **370**, 1920–1929 (2014).
176. Björck, H. M. *et al.* Altered DNA methylation indicates an oscillatory flow mediated epithelial-to-mesenchymal transition signature in ascending aorta of patients with bicuspid aortic valve. *Sci. Rep.* **8**, 2777 (2018).
177. Zegeye, M. M. *et al.* Interleukin-6 trans-signaling induced laminin switch contributes to reduced trans-endothelial migration of granulocytic cells. *Atherosclerosis* **371**, 41–53 (2023).
178. Minderhoud, S. C. S. *et al.* Wall shear stress angle is associated with aortic growth in bicuspid aortic valve patients. *Eur. Hear. J. - Cardiovasc. Imaging* **23**, 1680–1689 (2022).
179. Regeer, M. V. *et al.* Effect of Aortic Valve Replacement on Aortic Root Dilatation Rate in Patients With Bicuspid and Tricuspid Aortic Valves. *Ann. Thorac. Surg.* **102**, 1981–1987 (2016).
180. Kim, M.-S. *et al.* Long-term Fate of Dilated Ascending Aorta after Aortic Valve Replacement for Bicuspid Versus Tricuspid Aortic Valve Disease. *Am. J. Cardiol.* **129**, 53–59 (2020).

181. Hui, S. K. *et al.* The aortic root does not dilate over time after replacement of the aortic valve and ascending aorta in patients with bicuspid or tricuspid aortic valves. *J. Thorac. Cardiovasc. Surg.* **156**, 5-13.e1 (2018).
182. Abdulkareem, N. *et al.* Dilatation of the remaining aorta after aortic valve or aortic root replacement in patients with bicuspid aortic valve: A 5-year follow-up. *Ann. Thorac. Surg.* **96**, 43–49 (2013).
183. Charitos, E. I. *et al.* The fate of the bicuspid valve aortopathy after aortic valve replacement. *Eur. J. Cardio-thoracic Surg.* **45**, 128–135 (2014).
184. Hiraoka, T. *et al.* Non-aneurysmal ascending aorta diameter changes after aortic valve replacement in patients with stenotic bicuspid and tricuspid aortic valve. *Gen. Thorac. Cardiovasc. Surg.* **70**, 33–43 (2022).
185. Kong, W. K. F. *et al.* Sex Differences in Phenotypes of Bicuspid Aortic Valve and Aortopathy: Insights from a Large Multicenter, International Registry. *Circ. Cardiovasc. Imaging* **10**, 1–7 (2017).
186. Andrei, A. C. *et al.* Comparison of Outcomes and Presentation in Men-Versus-Women With Bicuspid Aortic Valves Undergoing Aortic Valve Replacement. *Am. J. Cardiol.* **116**, 250–255 (2015).
187. Michelena, H. I. *et al.* Sex differences and survival in adults with bicuspid aortic valves: Verification in 3 contemporary echocardiographic cohorts. *J. Am. Heart Assoc.* **5**, 1–13 (2016).
188. Davies, R. R. *et al.* Novel measurement of relative aortic size predicts rupture of thoracic aortic aneurysms. *Ann. Thorac. Surg.* **81**, 169–177 (2006).
189. Zafar, M. A. *et al.* Height alone, rather than body surface area, suffices for risk estimation in ascending aortic aneurysm. *J. Thorac. Cardiovasc. Surg.* **155**, 1938–1950 (2018).
190. Chung, J., Coutinho, T., Chu, M. W. A. & Ouzounian, M. Sex differences in thoracic aortic disease: A review of the literature and a call to action. *J. Thorac. Cardiovasc. Surg.* **160**, 656–660 (2020).
191. Della Corte, A. *et al.* Towards an individualized approach to bicuspid aortopathy: Different valve types have unique determinants of aortic dilatation. *Eur. J. Cardio-thoracic Surg.* **45**, 118–124 (2014).
192. Habchi, K. M. *et al.* Association between bicuspid aortic valve morphotype and regional dilatation of the aortic root and trunk. *Int. J. Cardiovasc. Imaging* **33**, 341–349 (2017).
193. Hillebrand, M. *et al.* Diagnostic accuracy study of routine echocardiography for bicuspid aortic valve: A retrospective study and meta-analysis. *Cardiovasc. Diagn. Ther.* **7**, 367–379 (2017).
194. Brandenburg, R. O. *et al.* Accuracy of 2-dimensional echocardiographic diagnosis of congenitally bicuspid aortic valve: Echocardiographic-anatomic correlation in 115 patients. *Am. J. Cardiol.* **51**, 1469–1473 (1983).
195. Lopez, A. *et al.* Predictors of ascending aorta enlargement and valvular dysfunction progression in patients with bicuspid aortic valve. *J. Clin. Med.* **10**, (2021).

196. Olsson, C., Thelin, S., Ståhle, E., Ekbom, A. & Granath, F. Thoracic aortic aneurysm and dissection: Increasing prevalence and improved outcomes reported in a nationwide population-based study of more than 14 000 cases from 1987 to 2002. *Circulation* **114**, 2611–2618 (2006).
197. Beyer, S. E., Secemsky, E. A., Khabbaz, K. & Carroll, B. J. Elective ascending aortic aneurysm repair outcomes in a nationwide US cohort. *Heart* **109**, 1080–1087 (2023).
198. Pearsall, C. *et al.* Long-term outcome of hemiarch replacement in a proximal aortic aneurysm repair: Analysis of over 1000 patients. *Eur. J. Cardio-thoracic Surg.* **62**, 1–10 (2022).
199. Preventza, O. *et al.* Neurologic complications after the frozen elephant trunk procedure: A meta-analysis of more than 3000 patients. *J. Thorac. Cardiovasc. Surg.* **160**, 20-33.e4 (2020).
200. Taylor, A. P. *et al.* Statin Use and Aneurysm Risk in Patients with Bicuspid Aortic Valve Disease. *Clin. Cardiol.* **39**, 41–47 (2016).
201. Antithrombotic Trialists' (ATT) Collaboration *et al.* Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet* **373**, 1849–1860 (2009).
202. Awtry, E. H. & Loscalzo, J. Aspirin. *Circulation* **101**, 1206–18 (2000).
203. Xu, X. M. *et al.* Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5292–5297 (1999).
204. Walton, L. J. *et al.* Inhibition of Prostaglandin E₂ Synthesis in Abdominal Aortic Aneurysms. *Circulation* **100**, 48–54 (1999).
205. Hiromi, T. *et al.* Excessive EP4 Signaling in Smooth Muscle Cells Induces Abdominal Aortic Aneurysm by Amplifying Inflammation. *Arterioscler. Thromb. Vasc. Biol.* **40**, 1559–1573 (2020).
206. King, V. L., Trivedi, D. B., Gitlin, J. M. & Loftin, C. D. Selective cyclooxygenase-2 inhibition with celecoxib decreases angiotensin II-induced abdominal aortic aneurysm formation in mice. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1137–1143 (2006).
207. Gitlin, J. M., Trivedi, D. B., Langenbach, R. & Loftin, C. D. Genetic deficiency of cyclooxygenase-2 attenuates abdominal aortic aneurysm formation in mice. *Cardiovasc. Res.* **73**, 227–236 (2007).
208. Ghoshal, S. & Loftin, C. D. Cyclooxygenase-2 Inhibition Attenuates Abdominal Aortic Aneurysm Progression in Hyperlipidemic Mice. *PLoS One* **7**, (2012).
209. Wemmelund, H. *et al.* Low-dose aspirin and rupture of abdominal aortic aneurysm. *J. Vasc. Surg.* **65**, 616-625.e4 (2017).
210. Lindholt, J. S., Sorensen, H. T., Michel, J. B., Thomsen, H. F. & Henneberg, E. W. Low-Dose Aspirin May Prevent Growth and Later Surgical Repair of Medium-Sized Abdominal Aortic Aneurysms. *Vasc. Endovascular Surg.* **42**, 329–334 (2008).
211. Borger, M. A. *et al.* The American Association for Thoracic Surgery consensus guidelines on bicuspid aortic valve-related aortopathy: Full online-only version. *J. Thorac. Cardiovasc. Surg.* **156**, e41–e74 (2018).

212. Iribarne, A. *et al.* Imaging Surveillance After Proximal Aortic Operations: Is it Necessary? *Ann. Thorac. Surg.* **103**, 734–741 (2017).
213. Assmann, A. *et al.* Acceleration of autologous invivo recellularization of decellularized aortic conduits by fibronectin surface coating. *Biomaterials* **34**, 6015–6026 (2013).
214. Russel, W. M. S. & Burch, R. L. *The Principles of Humane Experimental Technique*. (Universities Federation for Animal Welfare, 1959).
215. Zeng, Q. *et al.* Interleukin-37 suppresses the osteogenic responses of human aortic valve interstitial cells in vitro and alleviates valve lesions in mice. *Proc. Natl. Acad. Sci.* **114**, 1631–1636 (2017).
216. van Engeland, N. C. A. *et al.* Aortic calcified particles modulate valvular endothelial and interstitial cells. *Cardiovasc. Pathol.* **28**, 36–45 (2017).
217. World Medical Association. WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects [Internet]. (2022). Available at: <https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/>. (Accessed: 9th October 2023)
218. Granath, C. *et al.* Characterization of Laminins in Healthy Human Aortic Valves and a Modified Decellularized Rat Scaffold. *Biores. Open Access* **9**, 269–278 (2020).
219. Freiholtz Åström, D. Laminins in Bicuspid Aortic Valve associated Ascending Aortic Aneurysm. (Stockholm, Karolinska Institutet, 2020).
220. Granath, C. *et al.* Valve disease and aortopathy associations of bicuspid aortic valve phenotypes differ between men and women. *Open Hear.* **8**, e001857 (2021).
221. Granath, C. *et al.* Acetylsalicylic Acid Is Associated With a Lower Prevalence of Ascending Aortic Aneurysm and a Decreased Aortic Expression of Cyclooxygenase 2. *J. Am. Heart Assoc.* **11**, (2022).
222. Vignac, M., Ntika, S., Olsson, C., Franco-Cereceda, A. & Björck, H. M. Metformin therapy is not associated with the lower prevalence of ascending aortic aneurysm in diabetic patients. *Eur. J. Cardio-thoracic Surg.* **61**, 388–392 (2022).
223. Rodriguez, K. J., Piechura, L. M. & Masters, K. S. Regulation of valvular interstitial cell phenotype and function by hyaluronic acid in 2-D and 3-D culture environments. *Matrix Biol.* **30**, 70–82 (2011).
224. Ohta, R. *et al.* Laminin-guided highly efficient endothelial commitment from human pluripotent stem cells. *Sci. Rep.* **6**, 35680 (2016).
225. Hjortnaes, J. *et al.* Valvular interstitial cells suppress calcification of valvular endothelial cells. *Atherosclerosis* **242**, 251–260 (2015).
226. Xu, K. *et al.* Cell-Type Transcriptome Atlas of Human Aortic Valves Reveal Cell Heterogeneity and Endothelial to Mesenchymal Transition Involved in Calcific Aortic Valve Disease. *Arterioscler. Thromb. Vasc. Biol.* **40**, 2910–2921 (2020).
227. Merritt, B. A. *et al.* Association between leaflet fusion pattern and thoracic aorta morphology in patients with bicuspid aortic valve. *J. Magn. Reson. Imaging* **40**, 294–300 (2014).
228. Ruzmetov, M., Shah, J. J., Fortuna, R. S. & Welke, K. F. The Association Between

- Aortic Valve Leaflet Morphology and Patterns of Aortic Dilation in Patients With Bicuspid Aortic Valves. *Ann. Thorac. Surg.* **99**, 2101–7; discussion 2107–8 (2015).
229. Villard, C., Roy, J., Bogdanovic, M., Eriksson, P. & Hultgren, R. Sex hormones in men with abdominal aortic aneurysm. *J. Vasc. Surg.* **74**, 2023–2029 (2021).
 230. Jackson, V. *et al.* Matrix metalloproteinase 14 and 19 expression is associated with thoracic aortic aneurysms. *J. Thorac. Cardiovasc. Surg.* **144**, 459–466 (2012).
 231. Mukherjee, K., Gitlin, J. M. & Loftin, C. D. Effectiveness of cyclooxygenase-2 inhibition in limiting abdominal aortic aneurysm progression in mice correlates with a differentiated smooth muscle cell phenotype. *J. Cardiovasc. Pharmacol.* **60**, 520–9 (2012).
 232. Jackson, V., Eriksson, M. J., Caidahl, K., Eriksson, P. & Franco-Cereceda, A. Ascending aortic dilatation is rarely associated with coronary artery disease regardless of aortic valve morphology. *J. Thorac. Cardiovasc. Surg.* **148**, 2973–2980.e1 (2014).
 233. Ballegaard, C. R. *et al.* Aortic enlargement and coronary artery calcification in a general population cohort. *Eur. Hear. journal. Cardiovasc. Imaging* **23**, 855–862 (2022).
 234. Swahn, E., Lekedal, H., Engvall, J., Nyström, F. H. & Jonasson, L. Prevalence and determinants of dilated ascending aorta in a Swedish population: a case-control study. *Eur. Hear. J. open* **3**, oead085 (2023).
 235. Forsell, C., Björck, H. M., Eriksson, P., Franco-Cereceda, A. & Gasser, T. C. Biomechanical properties of the thoracic aneurysmal wall: Differences between bicuspid aortic valve and tricuspid aortic valve patients. *Ann. Thorac. Surg.* **98**, 65–71 (2014).
 236. Jackson, V., Olsson, C., Eriksson, P. & Franco-Cereceda, A. Aortic dimensions in patients with bicuspid and tricuspid aortic valves. *J. Thorac. Cardiovasc. Surg.* **146**, 605–10 (2013).
 237. Michelena, H. I. *et al.* Bicuspid aortic valve identifying knowledge gaps and rising to the challenge from the international bicuspid aortic valve consortium (BAVCON). *Circulation* **129**, 2691–2704 (2014).
 238. Evangelista, A. *et al.* Echocardiography in aortic diseases: EAE recommendations for clinical practice. *Eur. J. Echocardiogr.* **11**, 645–658 (2010).
 239. Baraki, H. *et al.* Orthotopic replacement of the aortic valve with decellularized allograft in a sheep model. *Biomaterials* **30**, 6240–6246 (2009).
 240. van der Velden, J. *et al.* Animal models and animal-free innovations for cardiovascular research: current status and routes to be explored. Consensus document of the ESC Working Group on Myocardial Function and the ESC Working Group on Cellular Biology of the Heart. *Cardiovasc. Res.* **118**, 3016–3051 (2022).
 241. Kheradvar, A., Zareian, R., Kawauchi, S., Goodwin, R. L. & Rugonyi, S. Animal Models for Heart Valve Research and Development. *Drug Discov. Today. Dis. Models* **24**, 55–62 (2017).
 242. Lintas, V. *et al.* Development of a Novel Human Cell-Derived Tissue-Engineered Heart Valve for Transcatheter Aortic Valve Replacement: an In Vitro and In Vivo Feasibility Study. *J. Cardiovasc. Transl. Res.* **11**, 470–482 (2018).

243. Sugimura, Y., Schmidt, A. K., Lichtenberg, A., Akhyari, P. & Assmann, A. A Rat Model for the in Vivo Assessment of Biological and Tissue-Engineered Valvular and Vascular Grafts. *Tissue Eng. - Part C Methods* **23**, 982–994 (2017).
244. Aikawa, E. *et al.* Human semilunar cardiac valve remodeling by activated cells from fetus to adult: Implications for postnatal adaptation, pathology, and tissue engineering. *Circulation* **113**, 1344–1352 (2006).
245. Bjornson-Hooper, Z. B. *et al.* A Comprehensive Atlas of Immunological Differences Between Humans, Mice, and Non-Human Primates. *Front. Immunol.* **13**, 1–17 (2022).
246. Assmann, A. *et al.* Development of a growing rat model for the in vivo assessment of engineered aortic conduits. *J. Surg. Res.* **176**, 367–375 (2012).
247. Zhou, J. *et al.* Development of Decellularized Aortic Valvular Conduit Coated by Heparin–SDF-1 α Multilayer. *Ann. Thorac. Surg.* **99**, 612–618 (2015).
248. Zhou, J. *et al.* Development of decellularized aortic valvular conduit coated by heparin-sdf-1 α multilayer. *Ann. Thorac. Surg.* **99**, 612–618 (2015).
249. Meyer, S. R. *et al.* Comparison of aortic valve allograft decellularization techniques in the rat. *J. Biomed. Mater. Res. - Part A* **79**, 254–262 (2006).
250. Yu, B. T., Li, W. T., Song, B. Q. & Wu, Y. L. Comparative study of the Triton X-100-sodium deoxycholate method and detergent-enzymatic digestion method for decellularization of porcine aortic valves. *Eur. Rev. Med. Pharmacol. Sci.* **17**, 2179–2184 (2013).
251. Rieder, E. *et al.* Decellularization protocols of porcine heart valves differ importantly in efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells. *J. Thorac. Cardiovasc. Surg.* **127**, 399–405 (2004).
252. Cebotari, S. *et al.* Detergent decellularization of heart valves for tissue engineering: Toxicological effects of residual detergents on human endothelial cells. *Artif. Organs* **34**, 206–210 (2010).
253. Whitcomb, D. C. & Lowe, M. E. Human pancreatic digestive enzymes. *Dig. Dis. Sci.* **52**, 1–17 (2007).
254. Grauss, R. W. *et al.* Histological evaluation of decellularised porcine aortic valves: Matrix changes due to different decellularisation methods. in *European Journal of Cardio-thoracic Surgery* **27**, 566–571 (2005).
255. Marin, V., Kaplanski, G., Grès, S., Farnarier, C. & Bongrand, P. Endothelial cell culture: Protocol to obtain and cultivate human umbilical endothelial cells. *J. Immunol. Methods* **254**, 183–190 (2001).
256. Gould, R. A. & Butcher, J. T. Isolation of Valvular Endothelial Cells. *J. Vis. Exp.* 6–10 (2010). doi:10.3791/2158
257. Gilbert, T. W., Freund, J. M. & Badylak, S. F. Quantification of DNA in Biologic Scaffold Materials. *J. Surg. Res.* **152**, 135–139 (2009).
258. Dholakia, S., De Vlaminck, I. & Khush, K. K. Adding Insult on Injury: Immunogenic Role for Donor-derived Cell-free DNA? *Transplantation* **104**, 2266–2271 (2020).
259. Kopytek, M., Kolasa-Trela, R., Ząbczyk, M., Undas, A. & Natarska, J. NETosis is

associated with the severity of aortic stenosis: Links with inflammation. *Int. J. Cardiol.* **286**, 121–126 (2019).

260. Stasiak, J. R. *et al.* Design, development, testing at ISO standards and in vivo feasibility study of a novel polymeric heart valve prosthesis. *Biomater. Sci.* **8**, 4467–4480 (2020).
261. Bavaria, J. E. *et al.* Five-year Outcomes of the COMMENCE Trial Investigating Aortic Valve Replacement With RESILIA Tissue. *Ann. Thorac. Surg.* **115**, 1429–1436 (2023).
262. Gould, S. T., Matherly, E. E., Smith, J. N., Heistad, D. D. & Anseth, K. S. The role of valvular endothelial cell paracrine signaling and matrix elasticity on valvular interstitial cell activation. *Biomaterials* **35**, 3596–3606 (2014).
263. Yasuda, H. *et al.* Failure to prevent progressive dilation of ascending aorta by aortic valve replacement in patients with bicuspid aortic valve: Comparison with tricuspid aortic valve. *Circulation* **108**, 291–294 (2003).