From the Department of Medicine, Solna
Division of Cardiovascular Medicine
Karolinska Institutet, Stockholm, Sweden

PATHWAYS LINKING ATHEROSCLEROSIS TO AORTIC STENOSIS

Oscar Plunde

Karolinska Institutet

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Cover illustration: By Cajsa Åkerlund.
An obstructed flow is a signature feature of aortic valve stenosis and an advanced atherosclerotic lesion. Here it is illustrated in the heart, where a tightened rope symbolizes a stenotic aortic valve yielding turbulent flow.

Pathways Linking Atherosclerosis to Aortic Stenosis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Oscar Plunde

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Principal Supervisor:
Professor Magnus Bäck
Karolinska Institutet
Department of Medicine, Solna
Division of Cardiovascular Medicine

Co-supervisor(s):
Professor Anders Franco-Cereceda
Karolinska Institutet
Department of Molecular Medicine and Surgery
Cardiothoracic Surgery Unit

Opponent:
Professor Konstantinos Stellos
University of Heidelberg
Department of Cardiovascular Research

Examination Board:
Professor Thomas Kahan
Karolinska Institutet
Department of Clinical Sciences
Danderyd Hospital

Docent Pontus Andell
Karolinska Institutet
Department of Medicine, Solna

Professor Gustav Smith
Gothenburg University
Department of Molecular and Clinical Medicine

POPULAR SCIENCE SUMMARY OF THE THESIS

Every minute, the heart pumps approximately five liters of oxygenated blood via the circulatory system. The left part of the heart receives oxygenated blood from the lungs and pumps it further out to the body via the left ventricle. This vital system relies on valves acting as gatekeepers ensuring a unidirectional blood flow. The aortic valve guards the passage between the left ventricle and the largest artery of the body, the aorta. To properly guard the passage, the aortic valve needs to adapt to every heartbeat, being open when the heart is pumping out the blood and completely closed when the left ventricle is once again filled with oxygenated blood. The most common severe valvular heart disease is aortic valve stenosis (AVS) which means that the aortic valve is narrowed and therefore obstructs the blood flow from the heart to the aorta. It is a common disease among elderly and leads to symptoms like chest pain and syncope and if untreated, death. Unfortunately, the only treatment is invasive replacement of the valve and therefore we need to increase our knowledge about the disease to discover new therapeutic targets and improve patient care. Atherosclerosis shares many features with AVS. It is a lipid and inflammation driven disease that causes heart attack and stroke. The have common risk factors, pathophysiology, and partly overlap but can also exist exclusively.

In this thesis, the interplay between AVS and atherosclerosis was explored.

The results in **Article I** suggest that AVS might mask an increased arterial stiffness which may be important for risk stratification, prognosis and could potentially be used for AVS screening. In **Article II**, a genetic variation previously linked to both atherosclerosis and AVS was associated with aortic valve gene expression, important in the metabolism of polyunsaturated fatty acids (PUFA). In addition, patients carrying the genotype linked to decreased risk of AVS had higher aortic valve proportions of the omega-3 PUFA docosahexaenoic acid (DHA) which correlated inversely with aortic valve calcification. The results suggest that DHA or its metabolites may be a possible future AVS treatment. In **Article III**, antiphospholipid autoantibodies (aPL) previously linked to myocardial infarction were significantly more prevalent in AVS patients compared to matched healthy controls. aPL positive patients had lower aortic valve gene expression involving interferon signaling compared with aPL negative patients. The results provide a rationale for future studies investigating aPL as a risk factor for AVS and could be used in precision AVS therapy. In **Article IV**, coronary artery disease (CAD) was present in about 50% of AVS patients undergoing surgical aortic valve replacement. Patients with concomitant severe CAD demonstrated upregulated pathways related to atherosclerosis, determined by aortic valve gene expression. The results indicate that AVS might be classified depending on concomitant severe CAD and therefore may benefit from different medical treatments.

In conclusion, this thesis demonstrates links between atherosclerosis associated factors and AVS that may be used to improve patient care and provide future precision therapeutic targets.

ABSTRACT

Cardiovascular disease is the most common cause of death world-wide where atherosclerosis is the main culprit and aortic valve disease accounts about two percent of all CVD deaths. Atherosclerosis is a lipid and inflammation driven disease that share many features with aortic valve stenosis (AVS). Globally, the prevalence of AVS has been estimated to over 10 million patients and the incidence to over 12 500 new cases annually which is likely increasing due to increased longevity, yet no medical treatment is available. A link between atherosclerosis and AVS has previously been established by overlapping prevalence and common pathobiological hallmarks including lipid infiltration, inflammation, and calcification. Recent genetic studies have demonstrated several loci in which single nucleotide polymorphisms are associated with both diseases. However, there is also evidence pointing to separate etiologies including disease specific genetic risk factors, histopathological differences, and isolated clinical presentation.

The aim of this thesis was to establish the interplay between atherosclerosis and AVS. A physiologic part was covered in **Article I**, specific mechanisms in **Article II-IV** and molecular epidemiology in **Article IV**.

In **Article I,** arterial stiffness was determined in a cohort with ascending aortic dilatation and/or aortic valve disease before and after cardiac surgery. Arterial stiffness correlates with atherosclerotic cardiovascular disease and aggravates the increased left ventricular stress in AVS. Cardio-ankle vascular index (CAVI) measures arterial stiffness from the heart to the ankle and was lower in subjects with AVS compared with aortic regurgitation and ascending aortic dilation, before surgery, despite being older. In contrast, aortic stiffness assessed by carotid femoral pulse wave velocity (cfPWV) was not different between the groups. After surgery, CAVI but not cfPWV increased in patients with AVS but remained unchanged in patients undergoing aortic surgery. Age, diabetes, lower body mass index, decreased ejection time and lower preoperative CAVI was associated with an increased CAVI after surgery. The results suggest that AVS may mask an increased arterial stiffness if peripheral arteries are included in the measurement. Also, ejection time emerged as an important variable to account for when measuring arterial stiffness in aortic valve disease patients. Future work should aim to establish if arterial stiffness may be used to risk-stratify AVS patients.

In **Article II,** the impact of a single nucleotide polymorphism (SNP) within *FADS1* on aortic valve gene expression and fatty acid composition was identified. Fatty acid desaturase (*FADS)1* and *FADS2* encode rate limiting enzymes in the metabolism of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) and the SNP within this locus is associated with lower risk of both AVS and CAD. The SNP rs17547 was associated with FADS2 mRNA expression in calcified aortic valve tissue and the enzymatic activity of both FADS1 and FADS2. In addition, the aortic valve omega-3 PUFA docosahexaenoic acid proportion was higher in non-calcified compared with calcified tissue and positively correlated with the SNP. The results indicate that the protective effects of the SNP might be mediated via an increased DHA proportion in the aortic valve and/or possibly via downstream mediators from

DHA such as specialized pro-resolving mediators which have been shown to dampen inflammation.

Further pathophysiological evidence of shared pathways between CAD and AVS was obtained in **Article III.** The presence of antiphospholipid antibodies (aPL) in the general population is higher in patients with a recent myocardial infarction. Positivity for antibodies against β2-glycoprotein I and/or cardiolipin of IgG isotype was identified to be 8-fold higher in AVS patients compared with matched controls. In aortic valve tissue, aPL positivity was associated with downregulated interferon pathways and upregulated pathways related to mechanosensory signaling. Importantly, the differentially expressed genes could predict resilient (healthy), thickened (fibrotic) and calcified aortic valve tissue with high accuracy using supervised machine learning models suggesting a tight relationship between aPL related genes and local disease progression. The overall results imply that aPL IgG in the general population (without rheumatic disease) could be a risk factor for AVS and may potentially be used guide AVS precision medicine.

In **Article IV**, CAD associated gene expression in aortic valve tissue was identified. First, the prevalence of CAD in a contemporary surgical tricuspid AVS cohort was established at 49% and was associated with claudication, smoking, male sex, and diabetes. An exploratory analysis of aortic valve transcriptomic data from 74 patients revealed that severe CAD, affecting 2 or 3 vessel territories, was associated with the most prominent difference in gene expression. The differentially expressed genes were primarily found in non-calcified tissue and were enriched in pathways related to oxidative stress, inflammation, and lipids. Furthermore, a supervised machine learning model could predict if aortic valve tissue stemmed from patients with severe CAD, at high accuracy. The most important gene predictors of severe CAD could further be used to predict atherosclerotic or macroscopically normal carotid artery tissue. The results suggest that AVS patients with concomitant severe CAD exhibit more atherosclerosis related mechanisms in non-calcified tissue, ultimately leading to a common end-stage disease with severe AVS.

In summary, the results in this thesis demonstrate that AVS may be a cause of masked systemic arterial stiffness. Furthermore, pathways related to fatty acid metabolism and aPL are implicated in the pathophysiology of AVS and patients with severe CAD exhibit upregulated pathways related to atherosclerosis in the aortic valve. Collectively, pathways linking and differentiating aortic valve and vascular atherosclerotic disease were unraveled which open up for novel precision treatment regiments to halt AVS.

LIST OF SCIENTIFIC PAPERS

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Submitted manuscript

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Atherosclerotic Calcific Aortic Valve Stenosis Occurs in Multi Vessel Coronary Artery Disease
Submitted manuscript

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CONTENTS

LIST OF ABBREVIATIONS

AAD	Ascending aortic dilatation
aPL	Antiphospholipid antibodies
ASc	Aortic sclerosis
AVA	Aortic valve area
AVD	Aortic valve disease
AVC	Aortic valve calcium
AVcoup	Arterial-ventricular coupling
AVR	Aortic valve replacement
AVS	Aortic valve stenosis
baPWV	Brachial-ankle pulse wave velocity
BAV	Bicuspid aortic valve
BMI	Body mass index
BMP	Bone morphogenetic protein
CABG	Coronary artery bypass graft
CAC	Coronary artery calcium
CAD	Coronary artery disease
CAVI	Cardio ankle vascular index
CfPWV	Carotid femoral pulse wave velocity
CKD	Chronic kidney disease
CL	Cardiolipin
COX	Cyclooxygenase
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
D5D	Delta-5-desaturase
D6D	Delta-6-desaturase
DBP	Diastolic blood pressure
DEGs	Differentially expressed genes
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid

DM	Diabetes mellitus
DPP4	dipeptidyl peptidase 4
DT	Decision tree
ECM	Extracellular matrix
eGFR	Estimated glomerual filtration rate
EPA	Eicosapentaenoic acid
FADS	Fatty acid desaturase
GLA	Gamma-linolenic acid
GPCR	G-protein coupled receptor
GSEA	Gene set enrichment analysis
HR	Heart rate
hsCRP	High-sensitive C-reactive protein
ICA	Invasive coronary angiography
IFN	Interferon
IQR	Interquartile range
LDA	Linear discriminant analysis
LDL	Low-density lipoprotein
Lp-PLA2	lipoprotein-associated phospholipase A1
LTB	Leukotrien B
LV	Left ventricle
LVEF	Left ventricle ejection fraction
MAP	Mean arterial pressure
MGP	Matrix Gla protein
MVD	Multivessel disease
NES	Normalized enrichment score
NOS	Nitric oxide synthase
Nox	Nicotinamide adenine dinucleotide phosphate oxidases
OPG	Osteoprotegrin
OxPLs	Oxidized lipoproteins
PAD	Peripheral artery disease
PALMD	Palmdelphin

PCA	Principal component analysis
PCI	Percutaneous coronary intervention
PET	Positron emission tomography
PG	Prostaglandin
PP	Pulse pressure
Ppi	Pyrophosphate
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
Rv	Resolvin
SAVR	Surgical aortic valve replacement
SBP	Systolic blood pressure
SLE	Systemic lupus erythematosus
SML	Supervised machine learning
SNP	Single nucleotide polymorphism
SPMs	Specialized pro-resolving mediators
TAVI	Transcatheter aortic valve implantation
TG	Triglyceride
TTE	Transthoracic echocardiography
TXA	Thormboxane
VD	Vessel disease
VECs	Valvular endothelial cells
VHD	Valvular heart disease
VICs	Valvular interstitial cells
Vmax	Transaortic peak jet velocity
VSMCs	Vascular smooth muscle cells
vWF	Von-willebrand factor
β2GPI	Beta-2-glycoprotein I
5-LO	5-Lipoxygenase
^{18}F-FDG	^{18}F-fluorodeoxyglucose
^{18}F-NaF	^{18}F-sodium fluoride

1 INTRODUCTION

Cardiovascular disease (CVD) is currently the most common cause of death worldwide taking about 18 million lives annually and accounts for one third of all deaths[1] with the majority accounted for by atherosclerosis. CVD also comprises valvular heart disease (VHD) including aortic valve stenosis (AVS), the most common cause of mortality and morbidity in the spectrum of VHD[2]. AVS and atherosclerosis share many clinical risk factors and pathophysiological processes and research efforts have led to successful treatments against significant atherosclerosis albeit medical treatments against AVS have failed to prove beneficial effects. Instead, patients rely on interventional aortic valve replacement (AVR) as treatment. Further interrogation of the interplay between AVS and atherosclerosis may aid in understanding the lack of medical treatment against AVS and provide new therapeutic opportunities for AVS and improved patient care.

1.1 THE AORTIC VALVE AND ARTERIES

The aortic valve is composed of three cusps, the right and left, named so due to their proximity to the ostia of right and left coronary arteries whereas the third cusp is the non-coronary cusp[3, 4]. Each leaflet is attached to an area with condensed collagenous tissue, the annulus fibrous, in a semilunar fashion. The space between the aortic root and the corresponding areas covered by each cusp are named aortic sinuses and serve as a reservoir for blood during diastole, encompassing the ostia of the coronary arteries[4, 5]. Healthy aortic valve leaflets are thin (<1mm), sparingly vascularized and gracile. The aortic valve leaflet is stratified into three layers, fibrosa, spongiosa and ventricularis where the fibrosa is located on the aortic side, the ventricularis on the ventricular side with spongiosa in between[6] (Figure 1).

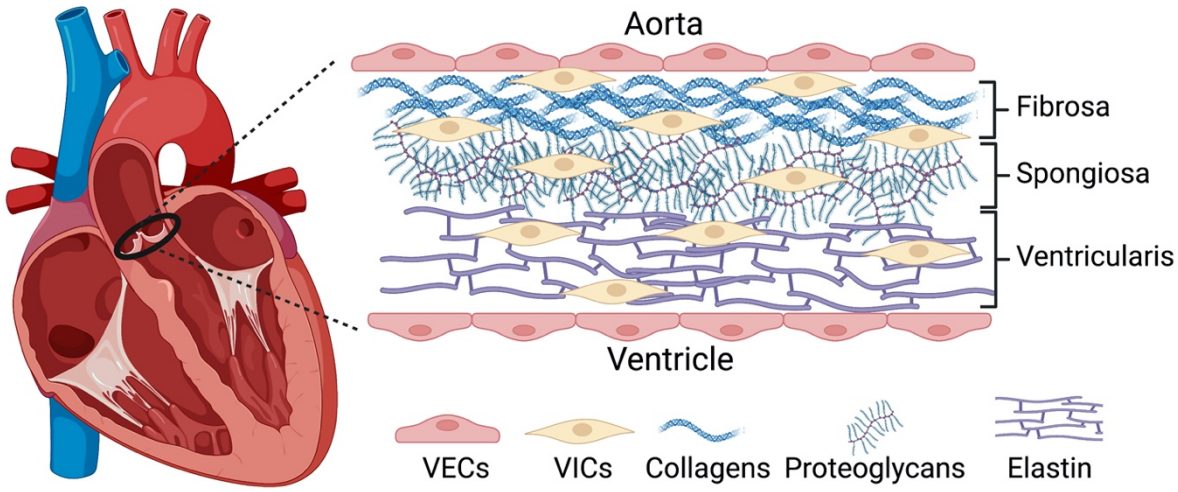

Figure 1. Architecture of the aortic valve. Created with BioRender.com.

As blood flow during diastole puts pressure on the aortic valve, the fibrosa is the load-bearing layer and is made up of type I and type III fibrillar collagens[7, 8]. A network of proteoglycans and glycosaminoglycans scattered with collagen fibers constitute the foundation of spongiosa,

which has to maintain the interlayer structure when being exposed to the tensile stress[9]. Ventricularis is primarily composed of radially oriented elastin fibers that facilitate the maintenance of the leaflet structure when the valve is fully open. Valvular endothelial cells (VECs) line the outer surface of the leaflets and provide protection to the underlying structure against mechanical and biochemical stimuli[10]. The dominating cell type in aortic valves, valvular interstitial cells (VICs) reside in all three layers of the valve. They exist in many different subsets, regulate extra cellular matrix (ECM) components, and can respond to mechanical stimuli[11, 12]. Therefore, VICs play a pivotal role in maintaining the aortic valve homeostasis but have also been shown to contribute to the development of AVS, which will be discussed below.

Arteries are adapted to cope with high pressure and transfer oxygenated blood from the heart to the organs. The coronary arteries supply the heart in diastole from the left and right coronary arteries. The left coronary artery further divides into two branches, the circumflex, and the left anterior descending. Like the aortic valve, the arterial wall is composed of three layers organized from the lumen: tunica intima, tunica media and the adventitia. Tunica intima is composed of a single layer endothelial cells in contact with the circulating blood and may therefore respond to circulating proteins and flow disturbance. Tunica media is the muscular layer of the artery containing vascular smooth muscle cells (VSMCs) and elastin fibers, important factors to maintain an adequate tonus of the artery. The outermost adventitia supports the arterial structure and contains connective tissue, fibroblasts, and vasa vasorum in large arteries like the aorta.

1.2 AORTIC VALVE STENOSIS AND ATHEROSCLEROSIS

AVS represents the significant obstruction caused by a calcified aortic valve leading to several hemodynamic and structural changes e.g., increased after load, higher transvalvular flow velocity, diastolic dysfunction, reduced left ventricular ejection fraction (LVEF), impaired coronary perfusion and left ventricle (LV) hypertrophy[13, 14]. Angina, syncope, and dyspnea are three classical symptoms of significant AVS, all of which reflect the hemodynamic consequences of AVS[15] and correlate with outcome[16]. Angina can result from the increased unmet need of oxygenated blood to the hypertrophied ventricle. Syncope often occurs during increased workload and is the result of insufficient blood supply to the brain which in turn is caused by an insufficient cardiac output response and vasodepressor response to decreased peripheral resistance[17]. Dyspnea may be a symptom of heart failure which also may cause orthopnea and edema and relate to LV hypertrophy, fibrotic remodeling and systolic and diastolic dysfunction[18]. Some of these symptoms may also arise from significant coronary artery disease (CAD) which highlights the importance of retrieving a rigorous clinical examination, medical history, and tests to determine the physiology of the aortic valve and coronary arteries before determining the optimal treatment.

Similar to AVS, atherosclerosis develops over a long period of time and leads to obstruction of arteries and may contribute to arterial stiffness[19]. In analogy, the disease is often clinically asymptomatic for many years until significant obstruction occurs. Atherosclerosis can affect

several different arterial beds which clinically may result in CAD and acute coronary syndrome, stroke, peripheral artery disease (PAD) which may demand lower limb amputation, renal artery stenosis leading to hypertension and intestinal ischemia[20].

1.3 ETIOLOGY OF AORTIC VALVE STENOSIS

AVS is a slow progressive process leading to severe calcification, a hallmark of the disease. The process includes gradual thickening and fibrocalcific remodeling of the aortic valve and as long as this process does not cause a significant stenotic hinder to the LV, it is referred to as aortic sclerosis (ASc)[21]. If the valve is further calcified rendering significant hemodynamic LV outflow stenosis, it is referred to as AVS (Figure 2).

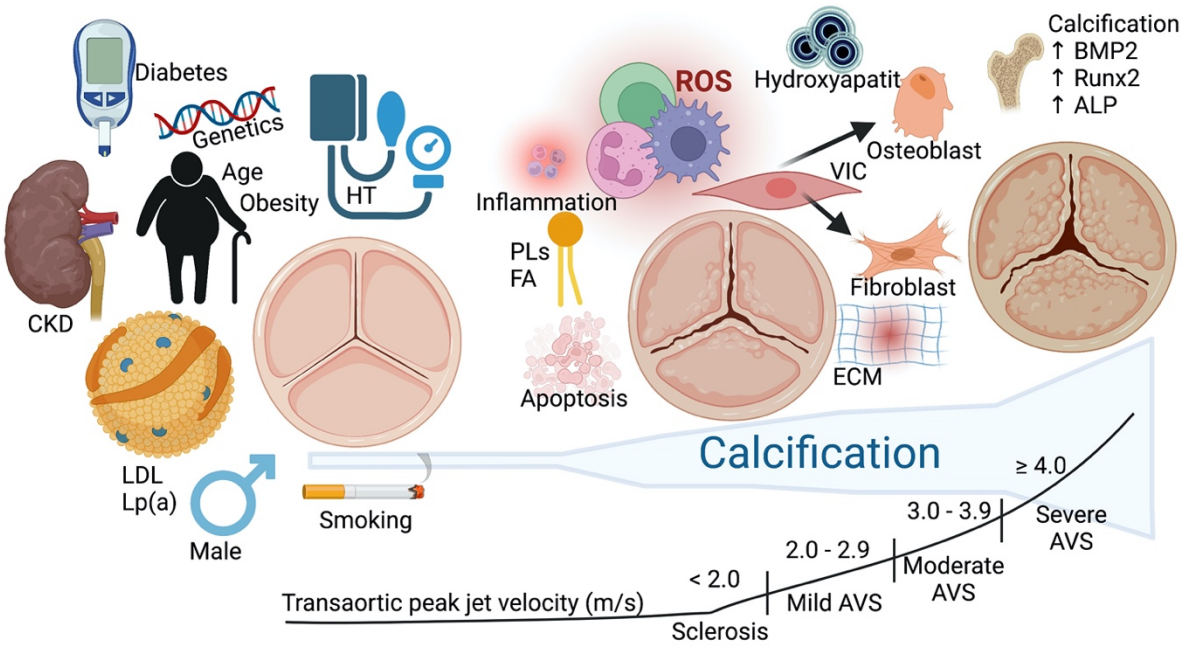

Figure 2. Representation of the AVS disease stages and selected accompanying factors. Created with BioRender.com.

The etiology is traditionally divided into rheumatic heart disease, bicuspid aortic valve (BAV) and degenerative calcific aortic valve stenosis. In low-income countries, rheumatic heart disease remains a significant contributor to AVS but in high-income countries, it is considered a neglectable source[22]. The mechanism behind rheumatic heart disease leading to AVS is not completely understood but is thought to involve an inadequate immune response to group A streptococci with lack of antibiotic treatment and the amount of bacterial load being important risk factors to develop severe disease[23].

BAV is a congenital defect present in about 1-2 % of the population where the normal tricuspid aortic valve is instead composed of two leaflets[24]. There are several classifications of BAV depending on morphology and if and what leaflets are fused. In general, patients with BAV develop severe symptoms earlier and stand for a majority of cases in patients up to

70 years old[25]. They more often have ascending aortic dilatation (AAD)[26] and even if the end stage disease is similar to tricuspid AVS, the mechanism behind the calcification is in part different[27-30]. Therefore, it is important to distinguish between AVS in BAV and TAV patients when discussing mechanisms, epidemiology, and the interplay with atherosclerosis.

1.4 METHODS TO ASSESS VALVULAR AND VASCULAR ANATOMY AND FUNCTION

1.4.1 Transthoracic Echocardiography

Transthoracic echocardiography (TTE) is the most common method to assess the hemodynamic severity of AVS and therefore timing of intervention. TTE further provides insight into LV function and hypertrophy, valve calcification, valve morphology and other associated pathologies like AAD and pulmonary hypertension[31]. The most important TTE variables in AVS (Level 1 recommendations) include peak transaortic jet velocity (Vmax), mean transvalvular pressure gradient (Pmean) and aortic valve area (AVA) by continuity equation[32]. Vmax is defined as the highest transvalvular velocity signal obtained and is assessed using continuous-wave Doppler (CWD). Vmax increases with AVS severity, is the strongest predictor of clinical outcome and a value ≥4 m/s is defined as severe AVS. Pmean represents the mean systolic pressure difference between the left ventricle and aorta and is calculated from velocity using the Bernoulli equation. Pmean ≥40 mmHg is considered severe. AVA is calculated with transvalvular velocity with CWD, left ventricular outflow tract (LVOT) diameter and LVOT velocity using the continuity-equation. AVA <1cm^2 marks a severe stenosis and is of particular interest when assessing a low-flow low-gradient AVS since this measure is not dependent on flow. A summary of the measurements is provided in Table 1.

1.4.2 Computed Tomography

Calcification can be visualized and quantified with computed tomography (CT) and can be performed prior to AVR since it provides important information that helps guide decision to surgical aortic valve replacement (SAVR) or transcatheter aortic valve implantation (TAVI)[31]. CT without contrast can assess calcium burden of the aortic valve and coronary arteries and with contrast, obstructive CAD can be identified[33]. CT-derived aortic valve calcium (AVC) correlates with TTE derived AVS hemodynamics and predicts adverse events[34]. The calcification is most often quantified by a method developed by Agatston[35] which yields a calcium score in arbitrary units or Agatston Units. An AVC score ≥2000 in men and ≥1200 in women is indicative for severe AVS[31]. A coronary artery calcium (CAC) score >100 is often considered indicative for more intense risk factor optimization and a score >400 has been suggested to be followed by a non-invasive test to assess CAD[36].

1.4.3 Positron Emission Tomography

Although not vastly used in clinical practice, positron emission tomography (PET) together with CT (PET/CT) is growingly demonstrating usability by assessing aortic valve disease

(AVD) activity. Using radioactive tracers, inflammation can be detected with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and calcification activity with ^{18}F-sodium fluoride (^{18}F-NaF)[37].

	Mild AVS	Moderate AVS	Severe AVS
Vmax (m/s)	2.0 - 2.9	3.0 - 3.9	≥4.0
Pmean (mmHg)	<20	20 - 39	≥40
AVA (cm2)	> 1.5	1.0 - 1.5	< 1.0
^{18}F-NaF	⇔ / ↑	↑ / ↑↑	↑↑↑
^{18}F-FDG	↑↑	↑/ ↑↑	↑
AVC (AU), male	<1600	≥2000	≥3000
AVC (AU), female	<800	≥1200	≥1600

Table 1. Simplified summary of measurements and their value indicating degree of AVS. AVC indicate AVS "unlikely", "likely", and "very likely".

1.4.4 Invasive Coronary Angiography

Despite an arsenal of available non-invasive tests to assess CAD, invasive coronary angiography (ICA) is gold standard[38]. ICA is often performed prior to SAVR and TAVI to assess potential need for revascularization. ICA offers a good ocular assessment of the coronary arteries however, not all visible obstructive atherosclerotic plaques are significant which has led to the recommendation to add fractional flow reserve (FFR) to assess CAD severity[39, 40]. FFR is an invasive pressure measurement taken proximal and distal to the obstructive area simultaneously, under pharmacologically induced hyperemia. The ratio of the mean pressure distal and proximal to the obstructive area yield the FFR where a ratio ≤0,8 indicates significant obstruction[41].

1.5 EPIDEMIOLOGY

1.5.1 Aortic Valve Stenosis

In the United States, AVS causes 15 of 100 000 deaths in the age span 75 – 84 and 153 of 100 000 deaths in patients ≥85 years old[42]. It is a common disease among elderly and has a 5-year mortality rate exceeding 60%[43]. The prevalence has been assessed in a few studies with randomly selected participants from the community. Using TTE, the Tromsø study reported an AVS prevalence ranging from 0.2% in the age group 50 – 59 which increased to 9.8% in octogenarians and older[44]. However, the whole spectrum of disease severity was included, from mild to severe AVS. In 1993, Lindroos et al. carried out a similar study in Finland with participants aged 75 – 86 years, where 5% had at least moderate and 3% severe AVS[45]. A meta-analysis including patients over 75 years of age, demonstrated a prevalence of 3.4% for severe AVS[46].

In Sweden, AVS is the most common diagnosis of VHD with a diagnosis incidence rate ranging from 100 and 130 per 100 000 person-years in women and men respectively with average age around 60 years[47] to 350 per 100 000 person-years for subjects over 85 years old[48].

The prevalence of ASc is higher and was assessed with TTE in the Finish study which reported some degree of valve thickening or mild calcification in over 50% of the participants over the age of 50[48]. A more recent meta-analysis stated that ASc was prevalent in between 9 to 42 per cent of the population, depending on age, and almost 2% of the participants with ASc progressed to AVS per year[49]. Even though ASc does not cause LV outflow obstruction, it independently associates with all-cause mortality and cardiovascular death[50], possibly due to a correlation with subclinical atherosclerosis.

1.5.2 Coronary Artery Disease

In the US, the prevalence of coronary heart disease is around 6 % in adults and about 805 000 heart attacks occur annually in the US[51]. The prevalence of CAC is largely dependent on the cohort studied. In a meta-analysis including over 6000 women (mean age ~54) with low risk of CAD, CAC was found in 36 % of the population and only showed a small prognostic impact on CVD mortality compared to traditional risk factors[52]. When over 16 000 British subjects (average age 53 and 75 % males) of which one in four had a risk factor, some degree of CAC was found in 56 % of males and 36 % of the females[53]. The CAC score increased significantly with aged and the CAC score in women lagged 10 years behind. In Sweden, over 25 000 participants without known CAD were assessed with coronary CT angiogram in which some degree of atherosclerosis was found in almost half of the subjects, ≥50% stenosis was detected in 5% and 3 vessel disease in 2 %[54].

1.5.3 Epidemiological Overlap Between Coronary Artery Disease and Aortic Valve Stenosis

The prevalence of AVC and CAC largely overlaps with a reported CAC prevalence of 82 % in subjects with AVC although less overlap is found in subjects with CAC of which 22 % have AVC[55]. In end-stage renal disease patients, the prevalence of concomitant AVC and CAC is 4 times higher and the overlap is more pronounced with AVC being prevalent in 53 % of patients with CAC and 95 % of patients with AVC also have CAC[56]. CAC and AVC also correlate with arterial stiffness which may indicate common pro-calcifying mechanisms[57, 58].

Many studies state that about 50 % of AVS patients have concomitant CAD however, the exact proportion vary a lot. Most studies include patients undergoing coronary artery bypass graft (CABG) surgery and in Table 2, studies reporting CAD prevalence in AVS is summarized. The varying prevalence of AVS with concomitant CAD is possibly due to non-stringent definition of CAD, varying cohort characteristics, comorbidities, and of importance, valve morphology.

Study	Mean age	Cohort and CAD definition	N (% female)	CAD %	AV morphology	Comments
Ramsdale et al[59] 1984	56	ICA on consecutive patients with AVS. ≥ 50 % obstruction = CAD	149 (28.9)	26.8	NA	
Vandeplas et al[60] 1988	59	Patients undergoing ICA 1976-1985 and patients with severe isolated AVS. ≥ 50% obstruction = CAD	192 (37.0)	24.5	NA	CAD associated with age and male sex
Akins et al[61] 1997	82	Consecutive patients ≥ 80 years old, undergoing cardiac surgery. CABG = CAD	216 (52.7)	51.4	NA	
Kvidal et al[62] 2000	65	Patients undergoing AVR in Uppsala, Sweden between 1980-1995. CABG = CAD	2359 (36.0)	38.9	NA	51 % CAD in patients ≥71 years old
Rapp et al[63] 2001	61	Patients undergoing ICA 1978-2000 and patients with severe isolated AVS were included. ≥ 70 % obstruction = CAD	272 (51.8)	37.5	NA	CAD associated with angina
Ozaki et al[64] 2020	73	Cardiac CT performed in low-risk patients enrolled in a TAVI trial in France. ≥ 50 % obstruction = CAD	169 (43)	27.8	NA	97 % had some degree of atherosclerosis

Table 2. Studies showing prevalence of CAD in AVS patients.

If the aim is to assess the interplay between AVS and atherosclerosis, TAV patients should be included since BAV patients have, at least in part, different pathophysiology[27]. Poggio et al. performed a meta-analysis of all available studies with data on CAD status and valve morphology. 4586 TAV patients were identified of which 30,3% had CAD[65]. However, no information regarding the morphology assessment was provided nor the definition of CAD.

This prevalence is in line with the PARTNER 3 trial, only including TAV patients[66]. However, in earlier PARTNER trials, CAD prevalence was higher as was the age and number of comorbidities (Table 3).

In conclusion, AVS and CAD show some degree of overlapping prevalence ranging from 10 to over 50 % depending on valve morphology, location, age of the cohort, and is in line with a suggested shared mechanistic pathophysiology. Noteworthy, a large proportion does not have concomitant significant CAD which might favor the notion of AVS specific calcification mechanisms. This is also supported by the large group av CAD patients without AVS.

	PARTNER[67]		**PARTNER 1[68]**		**PARTNER 2[69]**		**PARTNER 3[66]**	
	TAVI	Medical management	TAVI	SAVR	TAVI	SAVR	TAVI	SAVR
N	179	179	348	351	1011	1021	496	454
Age (mean)	83.1	83.2	83.6	84.5	81.5	81.7	73.3	73.6
CAD (%)	67.6	74.3	74.9	76.9	69.2	66.5	27.7	30.3
Important exclusion criteria	Uni-/bicuspid valve. Untreated clinically significant coronary artery disease requiring revascularization. AMI ≤ 1 month before the intended treatment.							

Table 3. Comparisons between overlapping CAD prevalence in the PARTNER trails.

1.6 THE PATHOPHYSIOLOGY OF AVS AND THE LINK TO ATHEROSCLEROSIS

Lipids and inflammation are involved in both AVS and atherosclerosis pathophysiology. The initiation of the atherosclerotic plaque includes upregulation of adhesion molecules in response to altered shear stress in tandem with deposition of low density lipoprotein (LDL) particles in tunica intima[70]. Here, LDL undergoes oxidation, further promoting an inflammatory response. Monocytes recruited to the site transdifferentiate into macrophages and transform into foam cells, a hallmark of atherosclerosis. Following recruitment of inflammatory cells and foam cell formation, VSMCs migrate from the media to the intima and synthesize ECM components[71]. The VSMCs migration results in a fibrous cap, isolating

the atherosclerotic core from the bloodstream. Calcification mechanisms similar to those described in AVS also contribute to atherosclerotic calcification, which is an independent predictor of coronary heart disease[72]. Atherosclerotic calcification can be divided into micro- and macrocalcification where microcalcification occurs early in the inflammatory cascade and promote destabilization of the plaque whereas macrocalcification can be part of a macrophage orchestrated healing process which promote plaque stabilization[73]. Depending on a number of different factors including calcification, the plaque can rupture and cause myocardial infarction[74].

The calcification of the aortic valve leading to AVS is now being considered an active process in contrast to past assumptions of a passive degenerative ossification of the tissue, an obsolete view as undisputable evidence suggest otherwise. Like atherosclerosis, the initial phase is influenced by blood flow. In AVS, the tensile stress of the diastolic flow on the fibrosa increase susceptibility to lipid retention and endothelial cell activation which promote extravasation of inflammatory cells like macrophages and lymphocytes[6, 75]. Studies from the 90's also described lipids that co-localized with LDL-derived apolipoprotein B in early stages of the fibrocalcific transformation[6, 76]. Noteworthy, lipid infiltration is increased in stenotic compared to normal valves and preferentially occurs in areas with low shear stress on the aortic side of the valve. These early findings enforced the notion of an atherosclerotic like process underlying the disease and might explain the large continued scientific investigation of hyperlipidemia as a risk factor and causal role of AVS. Moreover, oxidative stress has been found to be a vital part of atherosclerosis and has now been suggested to be part of the initiation of AVC[77, 78]. Nevertheless, examination of aortic valves in the initiating phase showed some distinguishing features from atheroma by containing more pronounced mineralization in addition to less smooth muscle cells[6]. The mineralization observed in the early studies might be represented by microcalcification that co-localizes with lipids and contribute to deposition of hydroxyapatite which creates a vicious cycle of calcification and inflammation[79-81]. Several pathways have been linked to calcification in both AVS and atherosclerosis of which some are described briefly in this introduction.

1.6.1 Inflammation

The role of inflammation in AVS has been intensely studied and seems to be involved throughout disease progression albeit with decreasing significance in advanced stages. C-reactive protein (CRP), a member of the pentraxin family and common marker of inflammation, associates with both atherosclerosis[82] and AVS[83]. However, CRP provided no prognostic value for development of AVS[84]. An *in vivo* imaging study in ApoE deficient mice, known for its hyperlipidemic inflammatory phenotype, displayed a strong association of an active calcification process and markers of inflammation[85]. The authors showed that endothelial cells preferentially within sites of increased mechanical stress became active with an upregulation of vascular cell adhesion molecule 1 which could propagate further inflammatory cells to the site. Others have revealed that calcified aortic valve tissue express

proinflammatory cytokines such as interleukin 1, receptor of nuclear factor κ-B and tumor necrosis factor-α (TNF-α)[8].

1.6.1.1 Interferons

Type I interferons (IFNs) are important in host defense mechanisms against viral infections, are produced by many cell types, and the most well-characterized members include IFN-α and IFN-β[86]. Type II interferon consists of IFN-γ which is produced by T cells[87]. In atherosclerosis, IFNs have been widely studied and shown to promote foam cell formation, endothelial cell activation, and inflammation[88]. In AVS, INF-γ is upregulated in calcified compared to non-calcified aortic valve tissue and associates with malfunctioning osteoclasts which promote calcification[89]. In VICs, IFN-α induced osteogenic differentiation, a modest upregulation of inflammatory adhesion molecules and activated calcification signaling cascades[90]. Moreover, several of the responses linked to calcification were evoked or aggravated with concomitant LPS treatment.

1.6.1.2 Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) are involved in preserving homeostasis by regulating intracellular signaling pathways, defense against pathogens, vascular tonus, adhesion properties etc.[91]. Endogenous ROS stem from the mitochondrial electron transport chain, during generation of ATP or are catalyzed via certain enzymes such as nitric oxide synthase (NOS) or nicotinamide adenine dinucleotide phosphate oxidases (Nox)[92]. The primary ROS formed is superoxide (O_2^-) which can further give rise to other secondary ROS, such as hydrogen peroxide (H_2O_2). In a mouse model of AVS, increased superoxide was present in the aortic valve prior to the development of significant stenosis[93]. In human aortic valves, increased levels of superoxide and hydrogen peroxide were detected in calcified compared with non-calcified areas which was accompanied by a decreased level of antioxidant enzymes[94]. Furthermore, inhibiting NOS reduced superoxide levels in contrast to a specific Nox inhibitor, suggesting a role of NOS uncoupling in superoxide formation in AVS. The authors argued that the role of ROS in AVS differ from atherosclerosis with regards to (i) decreased antioxidants (ii) greater degree of NOS uncoupling and (iii) limited importance of Nox. However, others have demonstrated increased Nox expression in calcified areas [77] and alleviated calcification and improved cardiac function after Nox2 inhibition in a rabbit model of AVS[95]. In human aortic valves, ROS do not co-localize with macrophages[77, 94] which contrasts findings in atherosclerosis[96]. Hence, the precise differences in ROS pathways between AVS and atherosclerosis merits elaboration although several common ROS factors have been identified.

1.6.2 Lipids and Fatty Acids

Lipid infiltration is a key common entity in both AVS[6] and atherosclerosis[97] and is highly linked with inflammation. Since the discovery of cholesterol in atherosclerosis, LDL have been a main focus of studies during the last 50 years[98]. Indeed, oxidized LDL, a key driver of atherosclerosis[97] is also found in early AVS development[76] and contribute to the

inflammation and calcification in AVS[99]. Unfortunately, statin treatment failed to achieve any halting effect on AVS[100], in contrast to the success in atherosclerotic disease. Fortunately, recent advances in lipid research have yield new promising targets for AVS prevention and treatments[101].

1.6.2.1 Lipoprotein (a)

Lp(a), a lipoprotein almost indistinguishable from an LDL particle where the difference lays in the presence of apo(a), covalently bound to apo B-100[102]. Variants within the gene *LPA* which controls Lp(a) levels is associated with AVC and incident AVS[103, 104]. Also, there is a strong correlation between Lp(a) levels and incident AVS[105]. Although Lp(a) associates with CAD, elevated levels seems to contribute more to AVS since treating elevated Lp(a) levels potentially could reduce the incidence of myocardial infarction by 7 % but almost 14 % of AVS[106]. AVS progression is faster in patients carrying high levels of Lp(a) and accompanying oxidized phospholipids (OxPL)[107]. Results from VICs treated with Lp(a) indicate that the pro-calcifying effect of Lp(a) result from bound OxPL as gene expression of inflammatory and osteogenic factors were decreased with inhibitors of OxPL-binding[108]. The aortic valve uptake of 18-NaF was not associated with Lp(a) levels in mild/moderate AVS[109]. However, favoring the notion of Lp(a) as a causal factor for AVS, the lipoprotein-associated phospholipase A2 (Lp-PLA2) pathway and downstream signaling generating bioactive lipids offers mechanistic explanation to the observed effects on calcification[110]. This pathway is known to promote atherosclerosis[111], although Lp-PLA2-inhibition did not prevent major adverse cardiovascular events in CAD cohorts[112, 113]. Taken together, the large bulk of evidence suggests that Lp(a) with its associated OxPL are causal factors that promote calcification in AVS. Furthermore, CAD could be important in future AVS trials as Lp(a) only predicted future AVR in patients with AVS and concomitant CAD[114].

1.6.2.2 Fatty Acids

In the 1970s, lower mortality from myocardial infarction was observed in Greenland compared to Denmark, despite similar total fat intake[115]. This finding spurred future studies to underpin the underlying cause. The omega-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA), present in fish oil, was appointed responsible for the favorable outcome in Greenland. EPA is a substrate for the cyclooxygenases (COX) which give rise to prostaglandin (PG)I3 and thromboxane A (TXA)3, and the 5-lipoxygenase (5-LO) catalyzed formation of leukotriene B (LTB)5[116]. In contrast, omega-6 PUFA arachidonic acid (AA) form more pro-thrombotic and pro-inflammatory mediators such as TXA2 via COX and LTB4 via 5-LO. Hence, EPA-derived mediators can act as competing substances to hinder the formation of more active mediators produced by AA which are associated with AVS and athersoclerosis[117, 118]. Fatty acids may also form bioactive lipids called specialized pro-resolution mediators (SPMs) that promote the resolution of inflammation[119]. EPA gives rise to resolvin (Rv)E1 which exercise its effects via specific G-protein coupled receptors (GPCR)[120]. In human aortic valves, higher omega-3 PUFA content is associated with slower progression of AVS, and calcified areas have less RvE1 compared to non-calcified areas,

suggesting SPMs suppress the pro-calcifying milieu in AVS yielding less pronounced disease[121]. This notion was further supported in mice models of AVS where higher systemic levels of omega-3 PUFAs led to alleviated markers of AVS. EPA may also counteract atherosclerosis by supporting macrophage efferocytosis of LDL[122]. A clinical benefit has been proposed since high-dose ethyl ester of EPA decreased the risk of major adverse cardiovascular events by 25% in a randomized control trial in a cohort of high-risk patients with moderately elevated triglyceride (TG) levels[123]. Although the beneficial effect of omega-3 PUFAs is supported by other studies[123-125], omega-3 PUFA supplementation for CVD prevention remains controversial since several studies have shown no significant benefit compared to placebo[126-129]. The dissimilarities may be explained by different dosage (favoring higher doses) and DHA/EPA ratio (favoring higher EPA).

1.6.2.3 Fatty Acid Genetics, Aortic Valve Stenosis and Atherosclerosis

DHA and EPA are apart from supplied by ingestion, also metabolized from longer fatty acids. Fatty acid desaturases (FADS) are a family of enzymes catalyzing insertion of double bonds on PUFAs at specific positions[130]. *FADS1* encodes the delta-5-desaturase (d5d) activity enzyme FADS1 that catalyzes insertion of a double bond at carbon 5, yielding AA from dihomo-γ-linolenic acid (DGLA) and EPA from eicosatetraenoic acid (ETA). *FADS2* encodes the delta-6-desaturase (d6d) activity enzyme that catalyzes insertion of a double bond at carbon 6, yielding the omega-6 PUFA γ-linolenic acid (GLA) from linolenic acid (LA) and the omega-3 stearidonic acid (SDA) from α-linolenic acid (ALA)[130]. These enzymes are rate-limiting in the metabolism of PUFAs and have been implicated in several diseases including AVS[131, 132], CAD[133-135], stroke[135], type 2 diabetes mellitus (DM)[136] and markers of inflammation in adipose tissue[137]. However, the mechanisms behind these findings have not been determined.

1.6.3 Osteogenic Factors

In human aortic valves, macrophages associate with VICs expressing an osteoblast-like activity in addition to several markers of osteogenesis like alkaline phosphatase activity, osteopontin, runt-related transcription factor 2 (RUNX2) and cleaved Notch 1[85]. In response to inflammatory stimuli, VICs expressed bone morphogenic protein 2 (BMP2), RUNX2 in addition to intracellular adhesion molecule 1 which suggest a tight link between inflammation and calcification on a mechanistic level[138]. This *in vitro* study further provided evidence that there is a synergistic effect of inflammation together with mechanical stress. The mRNA expression of osteogenic genes was increased in a dose dependent manner with cyclic stretch and osteogenic medium. Hence, the initiation of the fibrocalcific process leading to increased mechanical stress may promote inflammatory and osteogenic factors that drive the disease forward. Importantly, in later stages of the disease, the inflammatory component appears less pronounced in AVS compared with atherosclerosis, measured by 18-FDG PET tracer[139]. Instead, the marker of bone metabolism 18-NaF was more pronounced in calcified aortic valves compared to atherosclerotic lesions, and AVC score was not associated with calcium

score in the aorta, indicating a degree of differences between valvular and vascular calcification.

1.6.3.1 Extracellular Matrix

The extracellular matrix provides a supporting structure to maintain the organized valve architecture needed to comply with the pressure alterations that differ between the ventricularis and fibrosa (the main location for calcification). TNF-α has been demonstrated to induce VICs transdifferentiation into valvular myofibroblasts[140] which associates with fibrosis[141] and expression of matrix metalloproteinases[142]. In calcified aortic valves, ECM proteins are significantly altered at an early stage of calcification and accentuated with more advanced calcification[143]. The pro-inflammatory milieu also perturb the balance of ECM synthesis and degradation which together with matrix vesicles from macrophages and VICs may serve as foundation for dystrophic microcalcification[144]. *In vitro* data in VSMCs suggests that apoptotic bodies too can act as foundation site for microcalcification[145]. Apoptosis precede calcification and when inhibited, calcification decrease and when stimulated increase, indicating a role in the initial steps of calcification[145]. In contrast, autophagy may reduce calcification[146] and promote cell viability[147].

1.6.3.2 Inhibitors of Calcification

The highly regulated calcification process not only include pro-calcifying factors but also a dysregulation of inherent inhibitors of calcification that may be important in AVS. Osteoprotegrin (OPG) regulates osteoclast differentiation and works as a decoy receptor for receptor activator of nuclear factor κ B ligand which regulate the formation of bone and calcification[148]. However, the mechanisms of OPG in AVS merit further research since increased circulating levels of OPG was found in AVS patients compared to controls[149], implying that OPG might act as a compensatory factor in late disease stages.

Pyrophosphate (Ppi) inhibits calcification by preventing hydroxyapatite formation[150]. Ppi is released from ATP by a reaction catalyzed by ectonucleotide pyrophosphatase phosphodiesterase and degraded by TNAP[151]. Increased calcification may be evoked by both increased degradation by TNAP and decreased synthesis[152].

Fetuin A is a circulating protein that forms colloidal complex with calcium and phosphate which prevents deposition of calcium phosphate in the tissue[153]. Indeed, Fetuin A is lower in AVS patients compared with controls[154]. However, Fetuin A failed to prove any prognostic significance for progression of AVS[155].

Matrix Gla protein (MGP) inhibits calcification by binding directly to calcium and BMP2. MGP is initially produced locally in an uncarboxylated form and requires Vitamin-K dependent γ-carboxylation to exert its effect[156]. MGP correlates with CAC[157], outcome in AVS[158], increased aortic stiffness[159], and might serve as a surrogate marker for CKD[160], a known risk factors for both AVC and CAC.

1.7 RISK FACTORS AND ASSOCIATED PATHOLOGIES

As the case of pathophysiological mechanisms, several risk factors identified for AVS are shared with atherosclerosis albeit not true for all. Many of these risk factors are modifiable and therefore pose an opportunity to possibly delay or prevent AVS which have large implications on the healthcare systems in an ageing population. The Cardiovascular Health Study published in 1997 was a prospective longitudinal study including more than 5000 participants[161]. AVS was present in almost 3 % of the patients ≥75 years and age, male sex, Lp(a), LDL, hypertension and smoking were deemed significant risk factors. In another study assessing smoking as a risk factor for AVS, groups stratified by time since cessation, demonstrated a dose-response pattern where participants that had quit smoking >10 years prior to the inquiry had nearly the same HR as the group that never smoked which emphasizes the benefit of early smoking cessation[162]. Several risk factors presented in the Cardiovascular Health Study have been replicated in a more recent Canadian study emphasizing hypertension, dyslipidemia and DM[163], all common with atherosclerosis[164]. DM has been shown to increase the risk of myocardial infarction and heart failure in a prospective population-based cohort study[165]. DM is also associated with a rapid progression of AVS[166] in addition to increased valvular mRNA gene expression related to calcification[167]. Interestingly, anti-diabetic treatment with dipeptidyl peptidase 4 (DPP4)-inhibitors is associated with slower AVS progression and in vitro, DPP4 contributes to calcification[168]. Finally, risk factors for incident AVC were established in over 5000 participants in the Multi-Ethnic Study of Atherosclerosis[169]. Apart from the common risk factors for AVS presented above, body mass index (BMI) and high creatinine were independently associated with incident AVC.

1.7.1 Diet and Exercise

Exercise, obesity, hypertension, and type 2 DM are often counteracted with healthy diet and physical activity which are actively promoted in the clinic. However, neither physical activity nor dietary patterns have been associated with lower risk of AVS[170, 171] which might be explained by the use of questionnaires to assess physical activity but also that merely diet and exercise is not enough to counteract the above-mentioned risk factors. This stands in contrast to CAD where beneficial effects of diet and exercise have been reported[172-175].

1.7.2 Antiphospholipid Syndrome and Systemic Lupus Erythematosus

Antiphospholipid syndrome (APS) is defined by antiphospholipid antibodies (aPL) against β2-Glycoprotein I (anti-β2GPI) and/or Cardiolipin (anti-CL) and/or the lupus anticoagulant in combination with arterial and/or venous thromboembolism and/or defined obstetric morbidities[176]. In systemic lupus erythematosus (SLE), aPL is present in up to 50% of patients of which half exhibit clinical manifestation of APS[177]. VHD presented as Libman-Sacks endocarditis, is one of the first described cardiac manifestation in patients with APS and/or SLE, most often affecting the mitral and aortic valve with increased valve thickness leading to stenosis and/or regurgitation[178]. In the general population, aPL are present in about 5-15 % and associates with subclinical atherosclerosis and previous cardiovascular events[179].

A Swedish study identified higher prevalence of positivity for aPL IgG in myocardial infarction patients compared to matched controls[180, 181]. Hence, aPL could be a contributing factor in both atherosclerosis and AVS.

1.7.3 Arterial Stiffness

Atherosclerosis is associated with arterial stiffness[182] and can be defined as the amount of resistance offered by the artery in response to the pulsatile pulse wave[183]. The non-invasive gold standard arterial stiffness measurement carotid-femoral pulse wave velocity (cfPWV)[184] independently predicts future cardiovascular events and all-cause mortality[185]. CAVI is a less blood pressure dependent measurement compared to cfPWV that incorporates peripheral arteries. A meta-analysis found a hazard ratio of 1.2 (95% CI 1.05 – 1.36) per each increased standard deviation of CAVI for cardiovascular events[186]. In the cross-sectional material, CAVI was on average 1.28 units higher in subjects with established CVD compared to CVD free subjects. However, a majority of the studies were conducted in Asia, calling for large prospective studies in the general population.

Increased measures of arterial stiffness have been associated with AVC[187], AVSc[188], AVS[189], and poor prognosis after AVR[190], suggesting a role in the clinical evaluation of AVS patients. Furthermore, increased arterial stiffness affects the interaction between the forward pressure wave initiated by the LV and the reflective wave from branching points and arterioles yielding additive higher central systolic aortic pressure and decreased diastolic pressure[191] (higher pulse pressure) and impaired coronary perfusion[192]. The impact of AVS and AVR on arterial stiffness has not reached consensus based on available studies[193]. Several studies indicate increased arterial stiffness after AVR, assessed with cfPWV[194-196], aortic stiffness index[197], and augmentation index[198]. However, others have demonstrated decreased or unchanged measures of arterial stiffness in AVS subjects following AVR[195, 199-205] calling for further investigations.

1.8 TREATMENTS FOR AORTIC VALVE STENOSIS

Before the introduction of TAVI, SAVR was the only treatment option. The last decade, the ratio between SAVR and TAVI has changed dramatically as TAVI has broken new grounds, demonstrating feasibility in low-risk patients[206]. Therefore, TAVI now accounts for about half of aortic valve replacement interventions in Sweden (Figure 3).

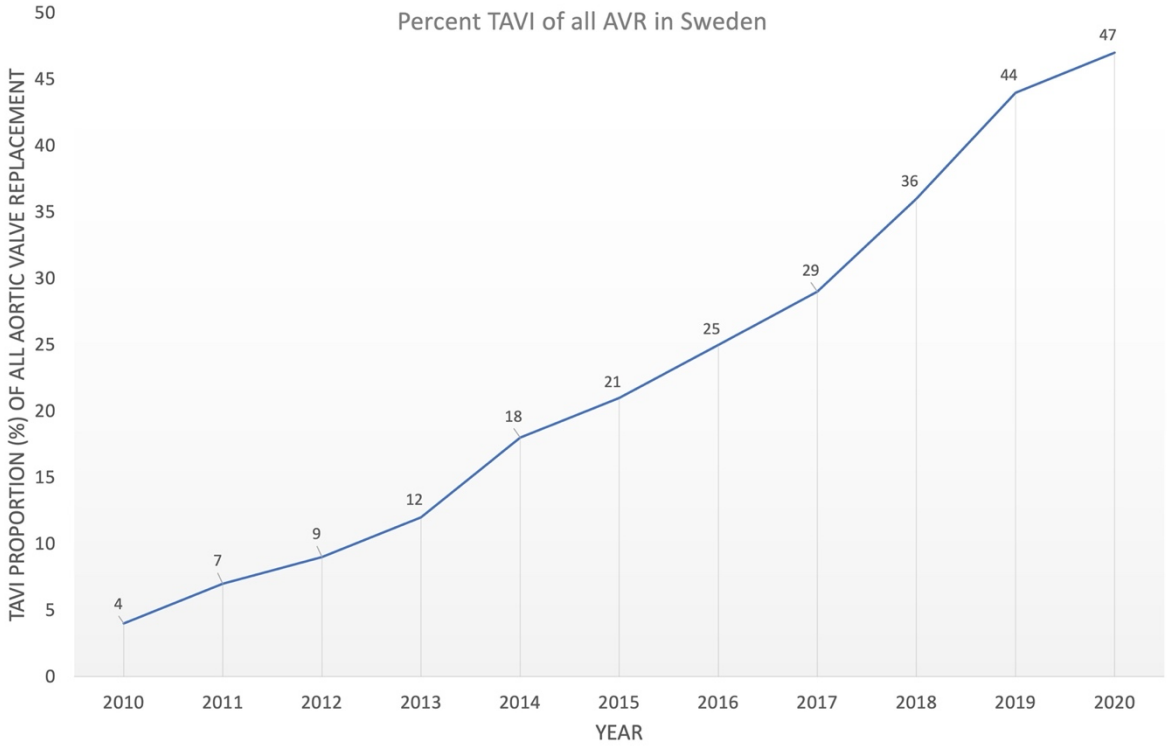

Figure 3. The TAVI proportion of all AVR, based on Swedish data from socialstyrelsen.se, calculated from procedure codes FMD12 (TAVI) / FMD (AVR).

The implanted valve prosthesis may be mechanical (requires SAVR) or biological. The main reason for choosing a biological valve, in the context of SAVR, is a riddance of life-long warfarin treatment with the downside of inferior durability with the risk of a new intervention needed. The choice can be particularly difficult in young patients. In Sweden, 60 % of the patients between 50-69 years of age received a mechanical valve which was associated with improved long-term survival[207]. Despite TAVI breaking new ground the last decades, it is associated with risks such as stroke, paravalvular leakage, atrio-ventricular block and comes with a substantial cost to the healthcare system[208].

Nonetheless, some promising strategies are in the pipeline. For example, in a proof-of concept study including 99 patients, Vitamin K supplementation showed a significant attenuation of AVC and a trend to slower progression of peak flow velocity[209]. Targeting the formation of hydroxyapatite in CKD, the inositol hexaphosphate compound SNF472 attenuated AVC progression compared to placebo treatment (14% vs. 98%)[210]. Importantly, these results would need to be translated into clinical benefit. Denosumab (RANKL antibody) and alendronic acid (bisphosphonate) were tested in the recent "Study Investigating the Effect of Drugs Used to Treat Osteoporosis on the Progression of Calcific Aortic Stenosis (SALTIRE2)" but failed to significantly retard disease progression measured by AVC, Vmax and 18-Na-F uptake[211]. Fortunately, several other studies are planned or ongoing, targeting Lp(a), Vitamin-K/MGP, DPP4, oxidative stress, hydroxyapatite formation and bone-remodeling drugs[99].

2 RESEARCH AIMS

The overall aim of this thesis was to elucidate the interplay between AVS and atherosclerosis by exploring physiology, biological pathways, and epidemiological factors. Specifically, the aims were:

- To determine systemic and aortic stiffness in AVS patients before and after AVR (**Article I**)

- To identify the impact of a *FADS1* genotype on aortic valve fatty acid composition and associated changes in aortic valve gene expression (**Article II**)

- To determine the prevalence of aPL in AVS and aPL associated gene expression signature in aortic valves (**Article III**)

- To establish epidemiological and aortic valve transcriptome characteristics of AVS with and without CAD (**Article IV**

3 MATERIALS AND METHODS

3.1 DAVAACA COHORT

All articles in this thesis involve the ongoing study Disease of the Aortic Valve Ascending Aorta and Coronary Arteries (DAVAACA). It is a single-center study that includes patients referred for elective open heart surgery for AAD and/or AVS with or without CABG[212]. The study started in 2013 at the Department of Cardiothoracic Surgery, Karolinska University Hospital, Stockholm, Sweden. Exclusion criteria include concomitant replacement of another valve, surgery related to endocarditis, and previous valve surgery. In **Article I**, all DAVAACA patients were screened for inclusion (Table 4). In **Article II**, only AVS patients with tricuspid aortic valves were included. In **Article III-IV**, a detailed examination of the whole tricuspid sub-cohort was carried out, which excluded 1 patient with rheumatic AVS and 1 patient with subaortic membrane as cause of the stenosis. Valve morphology was determined by the operating surgeon. Patients recruited between 2013-2019 were included in **Article III,** and patients recruited 2013-2021 were included in **Article IV**.

Article	Inclusion	Exclusion	N
I	All patients undergoing surgery. CAVI and cfPWV at baseline.	Atrial fibrillation, BMI>40, PAD	88
II	Tricuspid AVS. For eQTL, only valves with available genotyping and array data.		67
III	Tricuspid AVS. 2013-2019	Rheumatic valve, congenital cause	233
IV	Tricuspid AVS.2013-2021	Rheumatic valve, congenital cause	256

Table 4. DAVVACA sub-cohorts included in the articles.

Clinical information about each participant was obtained from electronic medical records (EMR) and questionnaires. TTE parameters were obtained from latest available preoperative examination (used in the decision to preform SAVR) or as stated in the treatment decision, documented in EMR. Estimated glomerular filtration rate (eGFR), measured in ml/min/1,73m2, was obtained from the Karolinska University Hospital laboratory using the revised Lund-Malmö formula[213]. CAD was defined by significant coronary artery stenosis subject to concomitant CABG and/or previous acute coronary syndrome and/or previous percutaneous coronary intervention (PCI). CAD was further characterized by vessel disease (VD) defined as number of significantly stenotic coronary artery territories on the preoperative coronary angiogram, stated in the treatment decision. All patients underwent coronary angiograms as part of the preoperative assessment and not due to symptoms related to myocardial infarction.

3.2 VALVE PREPARATION – FROM SURGERY TO RNA

The aortic valve biobank consists of 74 tricuspid valves derived from AVS patients in DAVAACA. During SAVR, the excised valves were collected in RNA-later (Qiagen. Hilden. Germany) which hinders the degradation of RNA. The valves were further weighted and

photographed followed by dissection. After scrutinizing the valve, pieces of resilient, thickened, and calcified tissue were cut and collected in a tube followed by freezing to -81°C or immediately continued to further RNA extraction. Approximately 100µg of non-calcified tissue (resilient and thickened) and 200µg of calcified tissue were used for further processing. Each piece of tissue was manually shattered followed by mechanical breakage using a TissueLyzer (Qiagen. Hilden. Germany). The valve homogenate was then used for RNA extraction with RNeasy Tissue Mini Kit (Qiagen. Hilden. Germany). The concentration and purity (260/280) of the RNA was determined by spectrophotometer NanoDrop (Thermo Scientific. Waltham. MA. USA). Also, the RNA integrity number (RIN) was determined with a 2100 Bioanalyzer (Agilent. Santa Clara. CA. USA). Extracted RNA was then transported to the core Bioinformatics and Expression Analysis for microarray analysis (Figure 4).

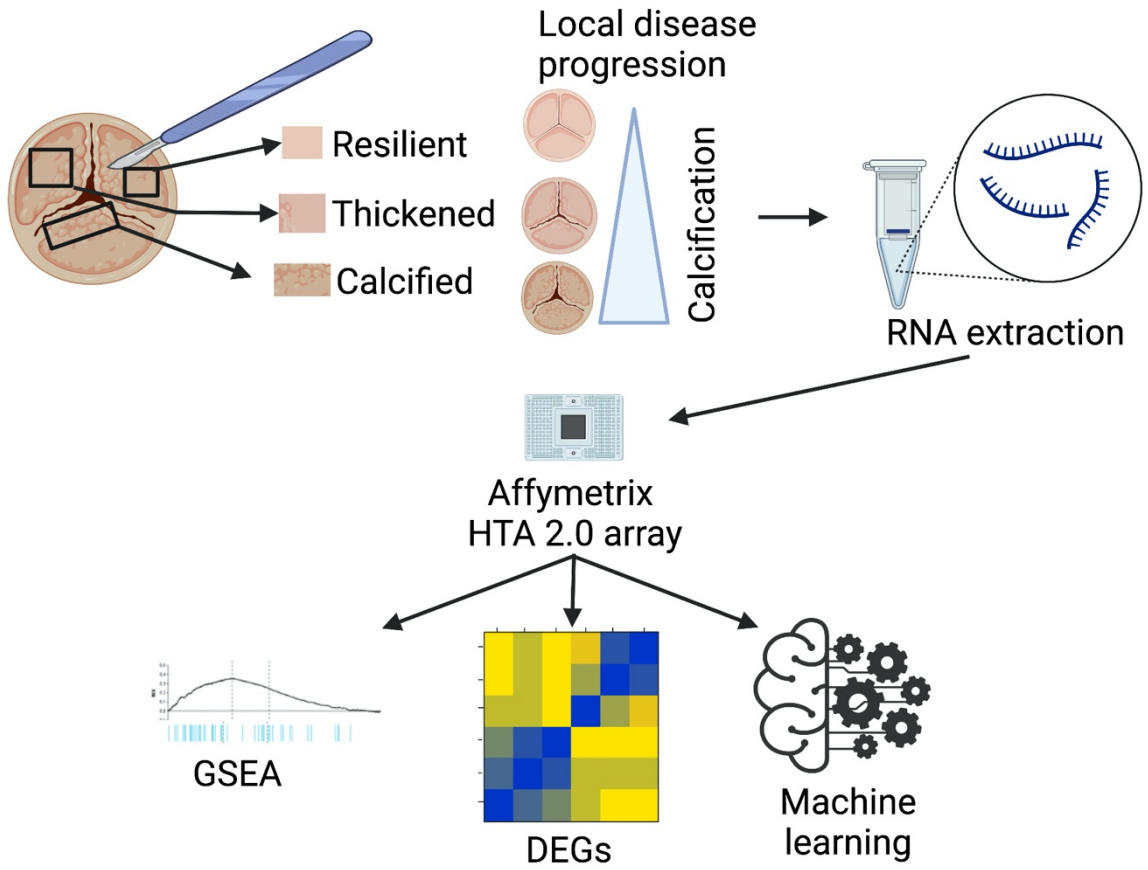

Figure 4. Schematic illustration of the biobank workflow from stenotic tricuspid aortic valves to transcriptomic data. Created with BioRender.com.

3.2.1 From mRNA to Gene Expression – Transcriptome Array

The transcriptome includes all transcripts and their quantity at a certain moment of time[214] and in this thesis, Affymetrix Human Transcriptome Array 2.0 microarrays were used to obtain the aortic valve transcriptome. The microarray is a plastic platform with hundreds of

thousands of spots in which several copies of a single-stranded DNA fragment is anchored. Each DNA strand (probe) contains the sequence of a specific part in the human transcriptome. This means that each gene transcript is covered by several probes. Before loading the sample to the array, RNA conversion to cDNA is performed by reverse transcription. In this process, the nucleotides are labeled using biotin. The cDNA is then transferred to the array, allowing hybridization to the probes. A type of laser scanner is then used to detect fluorescence from each spot, emitted from the biotin-labelled nucleotides. The detected fluorescence is then proportional to the expression level of the transcript complementary to that particular probe.

3.2.1.1 SST-RMA Normalization

The data from the microarrays are stored in .CEL files that needs to be normalized to get the actual expression levels of each gene. This was done with Signal Space Transformation-Robust Multi-Chip Analysis (SST-RMA). SST-RMA is a robust multi-chip analysis (RMA) normalization with additional pre-processing of the .CEL files in order to improve background noise correction, taking GC-content into consideration (GC-bonds are stronger)[215] which improves the capability to detect fold change. RMA is a widely used normalization method that includes background correction, quantile normalization, and summarization by median polishing[216]. The goal with background correction is to adjust the intensities for background noise, non-complementary binding and to get the expression estimates in a unifying scale[217]. Simplified, the expression value of each probe is subtracted by the average expression of the lowest expressed probes. The quantile normalization step gives each array the same expression distribution in log2-scale. Step 3 summarizes all probes covering a gene into an expression value by calculating probe median and sample median values followed by subtraction of those medians. The result is an expression value which comes from probe values in which the median is 0 for each sample.

3.2.1.2 Quality Control

After normalization, the expression value of each probe set (gene) is given in log2-scale. Next, a quality control of the data was performed with transcriptome analysis console (TAC, ThermoFisher Scientific). The array holds probe sets complementary to genes, not present in eukaryotic samples. To control the labeling of the nucleotides, Poly-A RNA controls complementary to these non-eukaryotic samples are added to the RNA at given concentrations. After normalization, these control Poly-A RNAs were detected at specific concentrations ensuring adequate labeling. The same principle is used to control for the hybridization in which biotinylated non-eukaryotic cRNA is added in known concentrations which allow detection of the transcripts at anticipated levels. This was also carried out. Other quality controls included comparing the expression of introns and exons (which should be different), looking at the box plots of global expression values in each sample (should be equal) and assessing a principal component analysis (PCA) to look for outliers.

3.3 FINDING BIOLOGICAL RELEVANCE IN TRANSCRIPTOME DATA

Several bioinformatic methods have been developed to aid the biological interpretation of array data. In **Article III-IV**, gene set enrichment analysis (GSEA) and STRING functional enrichment analysis were used.

3.3.1 Gene Set Enrichment Analysis

GSEA was developed in 2005 to counter the difficulties of interpreting differentially expressed genes (DEGs)[218]. The advantage with GSEA is that relevant pathways may be detected even if individual genes were not marked differentially expressed between two conditions. If several genes belonging to the same pathway are changed but not individually to the extent reaching statistical significance, it may still be more biologically relevant compared to one single DEG.

First, all genes in the analysis are ranked based on a chosen method, in this thesis, based on *t-tests* comparing two groups. The ranked list is then used to calculate an enrichment score (ES) which gives a value based on representation of the genes in the top or the bottom of the list. The score is based on a running sum statistic and is further normalized based on the number of genes in each gene list, yielding normalized enrichment score (NES). A p-value of the ES is generated from a null-distribution which is calculated based on 1000 permutations where the samples (phenotype) or genes are randomly assigned. The false discovery rate (FDR) is calculated by comparing the NES from the null distribution and the observed. The gene lists used consisted of gene ontology biological processes which include about 10 000 gene lists that describe biological processes carried out by the included genes[219]. A selection of lists including 15-500 genes were used.

3.3.2 STRING Functional Enrichment Analysis

The starting point in STRING functional enrichment is a set of genes that have been deemed interesting, here fetched after supervised machine learning (SML), based on importance. The genes were imported into Cytoscape[220] which has the STRING database[221] as a built in app for enrichment analysis. The imported genes are mapped onto a network with previously known protein-protein interaction so that genes belonging to common pathways are grouped with edges. The enrichment analysis is based on overrepresentation of the imported genes in certain pathways and a p-value is calculated based on the genome as background. The enriched pathways were clustered together based on similarities (included genes in each pathway) with Enrichment Map[222].

3.4 ARTIFICIAL INTELLIGENCE AND MACHINE LEARNING

SML was used to gain relevant biological information and to test basic hypotheses about differential gene expression pattern depending on phenotype. In **Article III**, several SML models were used to classify tissue type, using aPL associated genes. SML is a subcategory of artificial intelligence and differ from deep learning by having transparent algorithms which allow one to gain information about which variables (here genes) are used and how. The

importance of the variables used in each model can therefore be exported and used to gain biological information by for example enrichment analysis. In the current work, the gene expression from the biobank was used as features in all models to predict a certain instance. All SML algorithms were built in R 4.1.0 for Windows with the Caret package[223] that provides a framework to utilize a number of different algorithms from different packages. All models were built using default tuning parameter settings and variable importance were fetched using "VarImp" in Caret.

3.4.1 Random forest

Random forest (RF) was used in **Articles III-IV**, using the package "randomForest". RF is based on multiple decision trees (DT) which encapsulate an inherent feature selection[224]. A DT first establish the feature providing the best separation between the groups[225]. The best performing feature is then assigned to a decision node. If not perfect separation of the samples is accomplished, the same process is iterated yielding another node. This is repeated until no further separation is gained. The RF algorithm consists of several DTs which are built with a random subset of features and samples, so that the DTs don't contain the same starting information. The final classification label set by the algorithm stems from a majority vote of the DTs.

3.4.2 Gradient boosted trees

In **Article III**, a gradient boosted trees (GBT) model was used with the method "gbm_h20" in the package "h20" within Caret in R. In contrast to random forest, GBT tries to optimize the performance of each DT in a froward, stagewise procedure (boosting)[226]. Instead of building DTs independent of each other, GBT learn from each DT yielding a collection of DTs that collectively should outperform a single independent DT.

3.4.3 Support Vector Machines

In **Article III**, a support vector machines (SVM) model was built with the function "svmRadial" in the package "kernlab". A non-linear support vector classifier is built to determine unknown samples. svmRadial uses the radial Kernel function to modify the data aiming to increase the capability to group the samples in a multi-dimensional space to find an optimal separation[227]. The classification of new samples is then based on proximity of the already known samples[228]. Samples located near the unknown sample are given higher priority to the decision compared to samples that are located further away.

3.4.4 Linear Discriminant Analysis

Linear discriminant analysis (LDA) share similarity with a PCA but instead of dimension reduction based on variance, LDA reduces the dimensions while separating different groups, by combining the features in a linear model[229]. The goal of the separation is to have a large difference between the mean value of the groups and a small variance within the group. The resulting plot is then used to find linear discriminant lines to separate the different groups.

The result is a plot that can classify unknown samples based on where the new samples locate on the plot and the relation to the linear discriminant.

3.4.5 Performance Evaluation of Machine Learning Models

The goal of the SML was to predict unseen samples by learning from already known samples. To evaluate the performance of the models, k-fold cross-validation (kfCV) was used and the results from this procedure were used to gain different performance metrics.

3.4.5.1 Cross-validation

In kfCV, the complete data set is randomly sliced in *k* number of folds creating subsets of the data. When training the model, *k*-1 subsets are used following validation on the subset that was not used for training. Hence, *k* models are built and tested on "new" data. Depending on the size of the data, different *k* was chosen to get an equal number of samples in each fold.

3.4.5.2 Performance metrics

From the kfCV results, a confusion matrix was obtained including the predictions of the unseen samples and the true value (observed) of those samples. Hence the number of true positives, false positives, true negatives, and false negatives was determined. This information was used to calculate accuracy and kappa which was done automatically in the caret package. The Kappa metric is a more robust variant of accuracy as it takes the probability of agreement by chance into account (Figure 5).

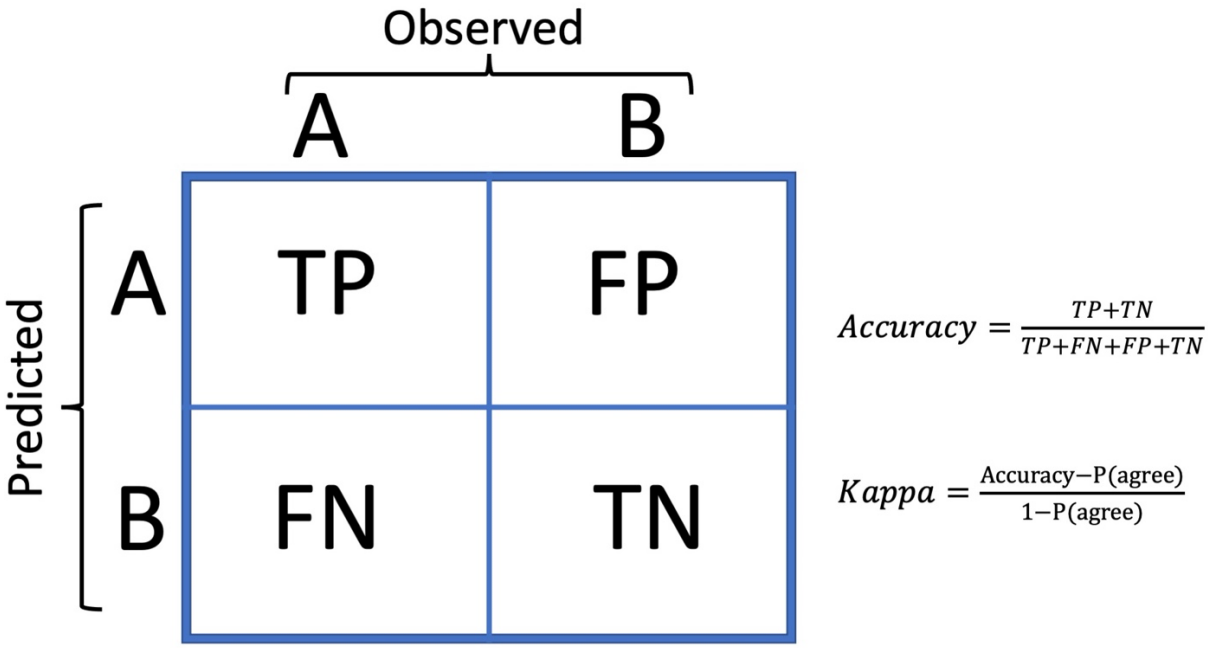

Figure 5. Confusion Matrix where A is positive and B negative. P(agree) denotes the probability of agreement on A or B.

3.5 ASSESSING GENES RELATED TO ATHEROSCLEROSIS

In **Article IV**, carotid artery gene expression collected from 32 patients were downloaded from Gene Expression Omnibus, number GSE43292. The set includes 2 samples per patient, one from macroscopically intact tissue with a Stary classification I-II and one sample containing tunica media and intima from the atherosclerotic plaque[230]. The downloaded .CEL files were normalized as described above and the gene expression of interest was used in an RF model predicting if tissue stemmed from atherosclerotic or macroscopically normal sites.

3.6 FATTY ACID COMPOSITION

In **Article II**, non-calcified (resilient or thickened) and calcified aortic valve tissue were pulverized in liquid nitrogen followed by characterization of fatty acids which was carried out by Omegametrix (Munich, Germany), using gas chromatography and yielded a semi-quantitative estimation of fatty acid content in the tissue[231]. The results are presented in weight percentage of total identified fatty acids. Calcified tissue from 25 patients and non-calcified tissue from 15 patients were used, of which 13 were paired (calcified and non-calcified tissue from the same patient).

3.7 GENOTYPING AND EXPRESSION QUANTITATIVE TRAIT LOCUS

In the TAV AVS DAVAACA cohort (n=256), 179 patients have also been genotyped of which 58 were included in the biobank and used for expression quantitative trait locus (eQTL) in **Article II.** Genotyping was performed with Illumina Human610-Quad BeadChip and Infinium Global Screening Arrays[232].

The single nucleotide polymorphism (SNP) within *FADS1* was interrogated for any *cis*-eQTL. Based on previous knowledge[232], genes within 400kb of the SNP were included in the analysis. GRCh37 (hg19) was used as reference genome. This was carried out in resilient, thickened, and calcified tissue separately. The gene expression was correlated with genotype in a linear regression model, adjusting for age and sex. All analyses were performed in Qlucore Omics Explorer (Lund, Sweden).

3.8 ARTERIAL STIFFNESS

Arterial stiffness was determined 1 day before (median 1, IQR 1-1) and 3 days after (median 3, IQR 3-3) open heart surgery. All measurements were obtained in the patient's private room after 10 minutes of rest, in supine position, at room temperature. Important exclusion criteria were atrial fibrillation or other arrythmias, BMI>40, ABI<0.9, limb amputation.

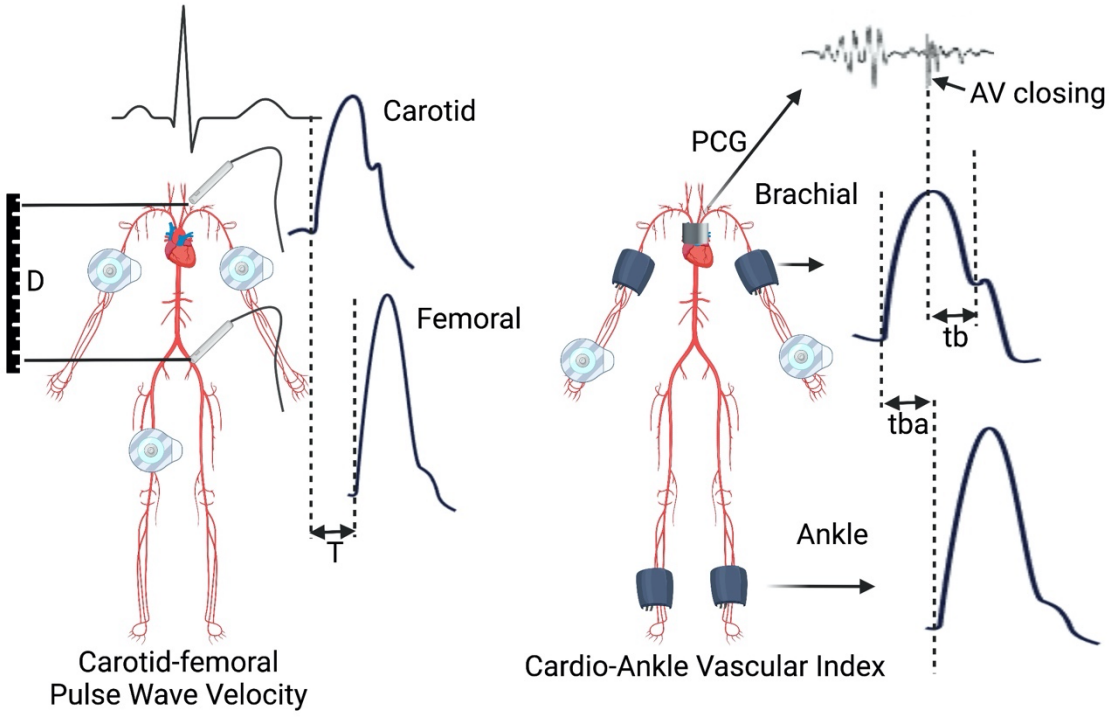

Figure 6. The principles of measuring cfPWV and CAVI with Sphygmocor and VaSera-1500 respectively. The figure was created with BioRender.com including illustrations from smart.servier.com.

3.8.1 Carotid-femoral Pulse Wave Velocity

CfPWV describes the velocity of the forward pulse wave in the aorta and was captured by applanation tonometry with the Sphygmocor device (AtCor Medical, Sydney, Australia), straight after CAVI. Importantly in the context of AVS, the foot of the pulse wave is used to detect the arrival of the pulse wave (Figure 6), which was determined with an intersecting tangent algorithm in the Sphygmocor device. The distance from the suprasternal notch (SN) to the carotid (d1) and femoral (d2) capture sites were used to determine distance traveled (D), by d2-d1, performed by the device. The time elapsed from LV ejection to pulse capture in carotid (t1) and femoral (t2) artery were determined with concomitant ECG recording. Finally, the distance divided by time (D / (t2-t1)) yielded the cfPWV (m/s). Since the majority of the measured artery in cfPWV consists of the aorta, it is also referred to as aortic stiffness and despite being gold standard, it has some drawback being highly dependent on blood pressure in addition to a relatively high inter-observer variability[233].

3.8.2 CAVI and baPWV

CAVI was developed to yield a more blood pressure independent measure of arterial stiffness and is calculated by the equation:

$$CAVI = a\left\{\frac{2\rho}{PP}\ x\ \left(ln\frac{SBP}{DBP}\right)\ x\ \left(\frac{L}{tba + tb}\right)^2\right\}\ x\ b$$

where ρ = blood density, PP =pulse pressure, SBP and DBP = systolic and diastolic blood pressure respectively, L = the length from the aortic valve to the ankle, tba = difference in time from start of the brachial pulse and time to ankle pulse and tb = time from aortic valve closure to the reflective wave seen in the brachial artery. L=0.77685 x height - 1.7536[234]. CAVI was determined with VaSera-1500 (Fukuda, Denshi) using brachial and ankle cuffs inflated to 50mmHg, and a phonocardiogram (PCG). The average CAVI from left and right were used as final CAVI value.

In addition to CAVI, blood pressure measurements were obtained from the VaSera-1500 and pulse pressure (PP) and mean arterial pressure (MAP) were calculated based on those measurements. Also, systolic time intervals upstroke time (from the pulse wave starting point to peak) and ejection time (time of blood flow across the AV) were captured with the device. Ejection time was normalized for heart rate with the formula ejection time / RR-interval[235] (calculated from heart rate).

baPWV was calculated with a formula to estimate the distance between the brachial and ankle (0.5934 x height (cm) + 14.4724[236], divided by *tba*).

3.9 ANTIPHOSPHOLIPID ANTIBODIES AND CONTROL COHORT

In **Article III**, aPL and extracted nuclear antigens (ENA) were measured from plasma with multiplexed beads (BioPlex 2200 Multiplex Testing, Bio-Rad). aPL were defined as autoantibodies targeting cardiolipin (CL) and/or β2-glycoprotein I (β2GPI) of IgG, IgM, and IgA isotypes. For aPL, values >99% of controls were defined as positive (>10U/ml IgG, >20U/ml IgA, >30U/ml IgM). For ENA, positive results were defined according to the manufacturer´s instructions.

The PAROKRANK (Periodontitis and Its Relation to Coronary Artery Disease) study cohort[180] was used as a disease-free control group to compare the results from DAVAACA. To obtain age- and sex matched cohorts, females ≥68 years (n=52) and males ≥69 years (n=124), (median age 71.0, IQR 3) from the PAROKRANK were included. From DAVAACA, females ≤76 years (n=48) and males ≤77 years (n=126) with median age 71.5, IQR 7 were included. Matching was performed blinded to results and to minimize the age- and sex differences between the cohorts while including all patients within each age category.

3.10 STATISTICS

Median and interquartile range (IQR) or mean with standards deviation (SD) were used to describe continuous measurements. Shapiro Wilk test was used to test gaussian distribution and Levene's test to assess equal variance. Log2-transformation was applied to some non-parametric data in **Article I-II** to yield normal distribution. Student t-test or Mann-Whitney U test was used to compare continuous variables between groups and Chi-square or Fisher's Exact test to compare categorical variables. When >2 groups were compared, ANOVA or the non-parametric Kruskal-Wallis test was used. When groups were compared whilst controlling for covariates, ANCOVA was performed in **Article I**. When matching was performed in

Article III-IV, the groups were compared with mean (SD), median (IQR) and standardized mean difference. DEGs were identified with unpaired or paired Student t-tests as appropriate. The FDR yielding q-value was used to control for multiple comparisons of genes. A p/q-value <0.05 was considered significant unless otherwise stated.

3.11 ETHICAL CONSIDERATION

All studies included in this thesis were approved by the ethics committee with numbers 2012/1633-31/4 for DAVAACA with amendment 2016/2346-32 for measuring arterial stiffness and nr 2008/152-31/2 for PAROKRANK. All studies were conducted in agreement with the declaration of Helsinki[237] and all participants gave informed written consent.

3.11.1 Ethical Considerations in Biobanks

The aortic valve biobank is involved in **Article II-IV** and has been built during the work of this thesis. In general, a biobank composes biological samples from an individual that are saved for more than two months together with general data regarding this individual. Samples and data are linked together to enable adequate analyses to answer research questions. This type of set-up is well established but also carries several ethical considerations to bear in mind. One needs to consider what type of personal data is gathered, what the informed consent should contain and who should ask for it. The personal consent from the study subject is fundamental to preserve autonomy. In line with a deontological principle, researchers are obliged to respect autonomous decision even if it might not act in favor to science or the society. The written consent should not be obtained from a person of whom the study subject is dependent on e.g., a surgeon performing the planned operation. In the DAVAACA cohort, the consent is obtained by a dedicated research nurse not a partaking in the clinical care of the patient, which is a great advantage. The consent itself needs to be (as said before) informed meaning that the study subject needs to be given full information about how the data will be handled and what it may be used for. Here, the research nurse has a big responsibility to make sure the study subject understands the study protocol. This is made by presenting the information both orally and written and by giving the study subject enough time to thoroughly read and reflect on the information, and by providing answers to potential questions. This is also the reason why insufficient knowledge in Swedish is an important ethical exclusion factor in DAVAACA.

The information gathered in this specific context is considered sensitive since it involves detailed information about medical aspect of an individual. It is of utter most importance that the data handling complies with General Data Protection Regulation (GDPR) and that measures are being taken to remove the risk of the information being spread. In DAVAACA, this is done by pseudonymization, assuring that a specific person cannot be coupled to the data. Also, adequate computer security must be in place and the data should be deleted whenever it is no longer of use.

When the results are finalized, another ethical question emerge. Should individual data be disclosed to the study participants? For example, should study subjects be contacted if a

genetic variant increasing the risk for a certain disease is detected? Today, it is not common practice to do so and would be difficult to carry out with pseudonymized data. This is also the case for the DAVAACA study. Although, with growing interest in personal health, commercially available genetic tests and increasing publicly available information about medical science, it might become more commonly asked for by patients.

4 RESULTS AND DISCUSSION

The work in this thesis has focused on the interplay between atherosclerosis and AVS and provide data regarding epidemiological overlap (**Article IV**), prevalence of aPL in AVS (**Article III**), arterial stiffness (**Article I**), and atherosclerotic mechanisms in AVS (**Article II-IV**).

4.1 CONTEMPORARY EPIDEMIOLOGICAL OVERLAP OF CORONARY ARTERY DISEASE AND CALCIFIC AORTIC VALVE STENOSIS

Data on contemporary prevalence of concomitant CAD in AVS patients with verified tricuspid aortic valves are scarce. The prevalence of CAD was therefore determined in the TAV AVS DAVAACA sub-cohort, at 49 % (n=125) and was associated with smoking, claudication, DM, and male sex when eGFR, age, hsCRP, and Vmax were held constant (Figure 7). The prevalence was in line with a meta-analysis which reported a prevalence of 45.7 % of concomitant CABG in tricuspid AVS[65]. Importantly, Vmax did not differ between the groups indicating no difference in AVS severity.

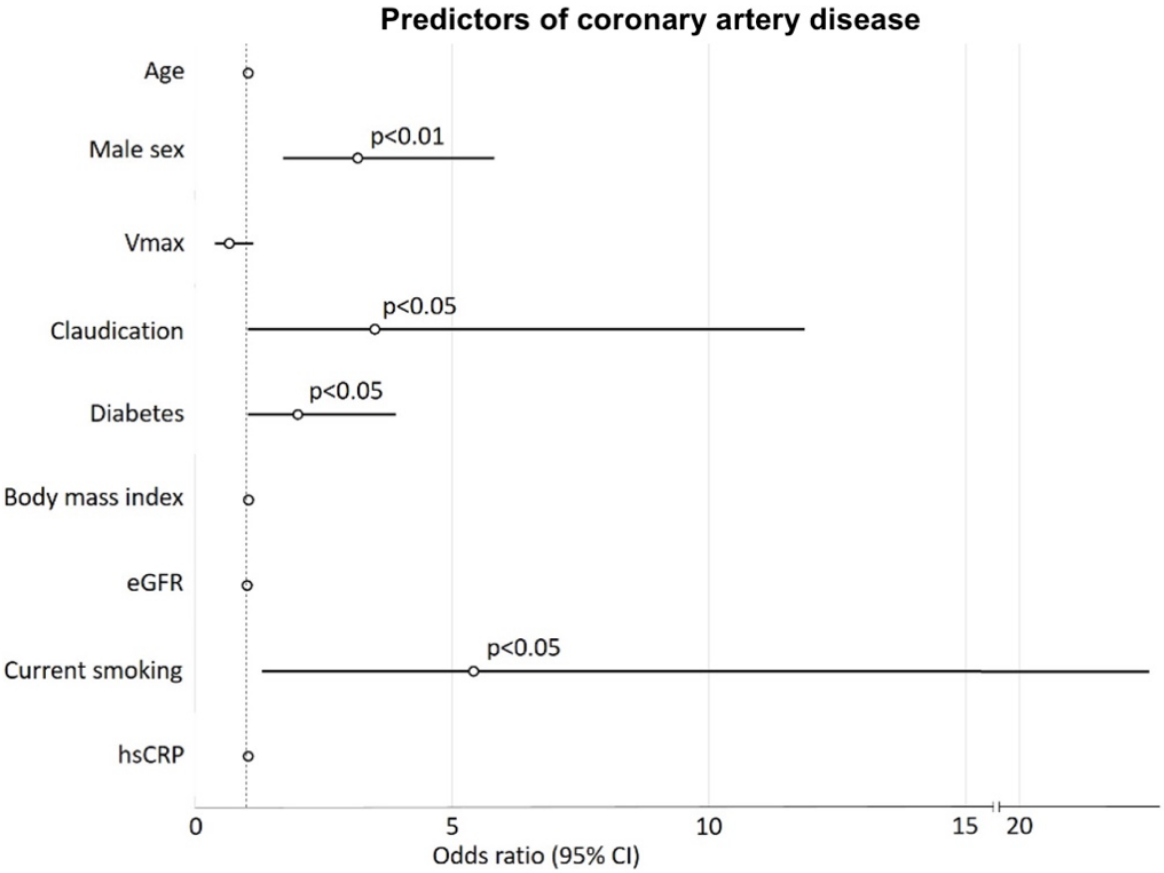

Figure 7. Predictors of CAD in AVS patients with TAV, undergoing SAVR. Points represent odds ratio from logistic regression with 95% confidence interval lines.

Smoking is a well-known risk factor for atherosclerotic disease and has recently been shown to also increase the risk for AVS (HR 1.3 [95% CI 1.12 – 1.51])[162]. In a mendelian randomization study, genetic predisposition to smoking initiation demonstrated higher odds ratio (OR) for CAD (OR 1.36 [95% CI 1.27-1.45]) compared to AVS (OR 1.22 [95% CI 1.01-1.48]).

Claudication was self-reported and no objective data regarding peripheral artery disease (PAD) was obtained. However, in a vast majority of the vases it is reasonable to assume PAD as underlying cause. PAD is more than twice as common in patients with previous myocardial infarction compared to patients without established CAD[238], while data in AVS are limited.

Male sex is associated with AVC (OR 1.87)[169], AVS and/or ASc (OR 2.03)[161] and AVS (Hazard ratio [HR] 1.53)[163]. The impact of sex on CAD is complex and myocardial infarction often occurs earlier in life compared to AVS. On average, myocardial infarction in women debut nine years later compared to men[239] and the HRs for incident myocardial infarction comparing men to women are between 3.7 and 2.2, with an age associated decrease[240].

The risk of AVS is higher in patients with type 2 DM (HR 1.34 [95% CI 1.05 – 1.71])[165]. However, the same study demonstrated a higher HR for myocardial infarction which was even greater in type 1 DM (HR 3.26 [95% CI 2.47 – 4.30]). Moreover, DM is an independent predictor of future surgery for AVS but only when not adjusting for CAD[241].

Collectively, all variables that were associated with concomitant CAD in AVS are more strongly associated with CAD.

The risk factors not associated with concomitant CAD in AVS may be due to a strong correlation with AVS. Obesity defined as BMI >25 often co-exists with dyslipidemia, diabetes, hypertension, and obstructive sleep apnea in the context of metabolic syndrome. Interestingly, obese patients even without history of diabetes, hypertension or dyslipidemia have increased risk of developing AVS[47]. Genetically predicted BMI displayed a convincing causal link to AVS where each genetically predicted 1 kg/m^2 increase conveyed 13% higher risk for AVS compared with 7 % for CAD[242].

CKD patients display markedly increased risk of both atherosclerotic disease as well as AVS. In fact, even a mild decrease in kidney function (eGFR 60-90) proved to be associated with increased risk of incident AVS in a dose-dependent manner[243]. The lack of association with hsCRP and age might be explained by a homogenous cohorts with low age variablitiy and relatively few cases with high CRP with a median value below 2 mg/L although it should be acknowledge that CRP is a strong risk marker for CAD[244] and is associated with AVS[83].

4.2 ANTIPHOSPHOLIPID ANTIBODIES IN CALCIFIC AORTIC VALVE STENOSIS

aPL are proposed risk factors for myocardial infarction in the general population, link to thrombosis and VHD in SLE and APS, albeit not previously identified in a AVS cohort from the general population. In **Article III**, positivity for any aPL was determined at 6.4 % in 233 tricuspid AVS patients (Figure 8). aPL positivity was associated with CAD, higher eGFR and lower cholesterol, although the use of lipid lowering drugs did not differ (64 % vs 73 %, p=0.58). To compare the aPL results to controls, age- and sex matched cohorts were used.

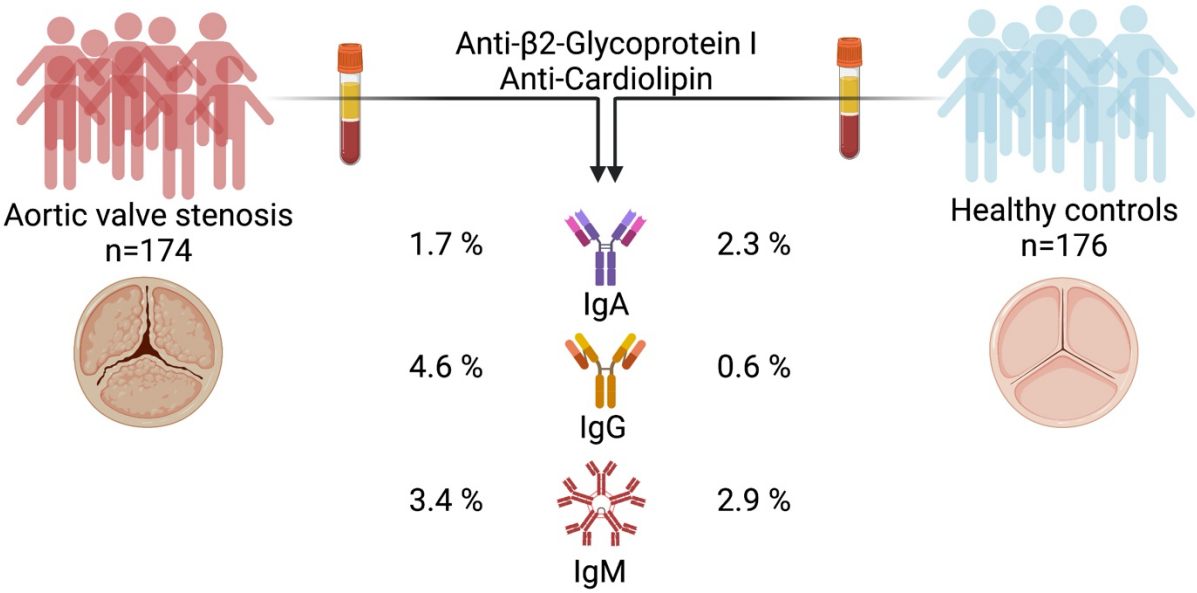

Figure 8. Schematic presentation of the molecular epidemiology of antiphospholipid antibodies in calcific aortic valve stenosis patients and healthy controls. Created with BioRender.com.

Positivity for ENA did not differ between the groups. AVS patients displayed significantly higher prevalence of aPL IgG-positivity compared with controls (4.6 % vs. 0.6 %, p=0.0037), corresponding to an OR of 8.4 (95% CI 1.04 – 68.2). Importantly, the IgG isotype has a stronger association with thrombotic manifestations compared to IgM, in APS[245] and was 10-fold higher in patients from the general population with a recent myocardial infarction compared with controls. The OR presented here is in par with results from SLE patients in which anti-CL IgG positive subjects have almost 6 times higher risk of VHD compared with aPL negative.

anti-β2GPI may promote foam cell formation[246], induce endothelial dysfunction by inhibiting endothelial Nox[247] which spur a pro-inflammatory thrombotic milieu[248]. aPL activate platelets[249] which can promote an osteogenic phenotype in VICs[250, 251]. Collectively, all these effects may contribute to fibrocalcific remodeling in the aortic valve. However, the interplay between aPL and atherosclerosis is not straight-forward. While the presence of aortic atherosclerosis was higher in APS and SLE/APS compared with controls, there was no difference between aPL positivity between APS and/or SLE patients with or without

atherosclerotic plaques[252]. The same inconsistent pattern was observed in studies investigating carotid atherosclerosis[253, 254]. Hence, the precise link between aPL, atherosclerosis and AVS remains to be determined.

The samples for aPL measurement were collected pre-surgery and hence the duration of positivity cannot be assessed. However, a significant overrepresentation of homozygous carriers of a SNP within *STAT4*, previously associated with both SLE and APS[255, 256] was demonstrated in AVS patients positive for aPL IgG/IgM. 33% (n=2) of the homozygous carriers of the risk allele were aPL IgG/IgM positive and thus genetically predisposed aPL positivity was replicated in our AVS cohort free from established SLE and/or APS and suggest long duration of aPL positivity prior to SAVR.

4.3 ARTERIAL STIFFNESS IN AORTIC STENOSIS

Arterial stiffness has been linked to both atherosclerosis[57, 182, 257] and AVD[258] and contributes to the increased load on the LV. The TTE derived valvulo-arterial impedance incorporates the arterial and aortic valve components of the LV afterload, which is increased in AVS and associated with poor outcome[259]. Nonetheless, the exact contribution of arterial stiffness cannot be assessed, calling for reliable methods that can be used to determine arterial stiffness in AVS patients.

Previous results in arterial stiffness and AVS have been inconclusive and mainly focused on aortic stiffness. The aims of **Article I** were therefore to determine systemic arterial stiffness before and after AVS surgery, to compare CAVI with cfPWV, and find predictors of arterial stiffness and its hypothesized alteration after surgery. In contrast to **Article II-IV, Article I** included all DAVAACA patients undergoing surgery which enabled a control group free of AVS, not previously been described in this context. The stratification was based on main indication for surgery according to EMR yielding an AVS group (n=45, median age 69), an AR group (n=30, median age 59) and one AAD group (n=13, median age 63).

	Aortic valve stenosis		**Aortic Regurgitation**		**Ascending aortic dilatation**		
	N	Median (IQRs)	N	Median (IQRs)	N	Median (IQRs)	p-value
cfPWV (m/s)	45	8.0 (7.2 - 9.7)	30	7.1 (6.0 - 9.2)	13	8.5 (6.2 - 10.8)	0.067
CAVI	45	7.85 (7.07 - 8.59)	30	7.35 (6.59 - 8.46)	13	8.65 (7.23 - 10.17)	0.053
baPWV (cm/s)	43	1292 (1190 - 1473)	29	1220 (1112 - 1380) *	12	1585 (1361 - 1896)	**0.008**

Table 5. Preoperative stiffness measures. *Indicate adjusted p-value <0.05 compared with ascending aortic dilation group.

At baseline, only baPWV differed significantly between the groups (Table 5). After adjusting for confounders (age, sex, MAP, DM, eGFR, CRP), preoperative CAVI was lower in the AVS (estimated marginal mean 7.60, p=0.005) and AR (estimated marginal mean 7.78, p=0.015) compared to AAD patients (estimated marginal mean 8.93), indicating that arterial stiffness might be affected by AVD. Lower CAVI in AR subjects compared to age matched controls has previously been described[260] but not in AVS. Preoperative cfPWV was not different between the groups, indicating less influence of AVD. Interestingly, both ejection time and ankle upstroke time differed significantly between the groups and were numerically the longest in AVS patients. These are variables related to a classic finding in AVS, a long and week pulse, *pulsus parvus et tardus*[261].

After surgery, the most prominent changes were increased CAVI and baPWV in AVS and AR subjects. Median increase in AVS subjects were 1.33 (IQR 0.74 – 2.26) for CAVI and 1,441 cm/s (1,275–1,586) for baPWV (Figure 9).

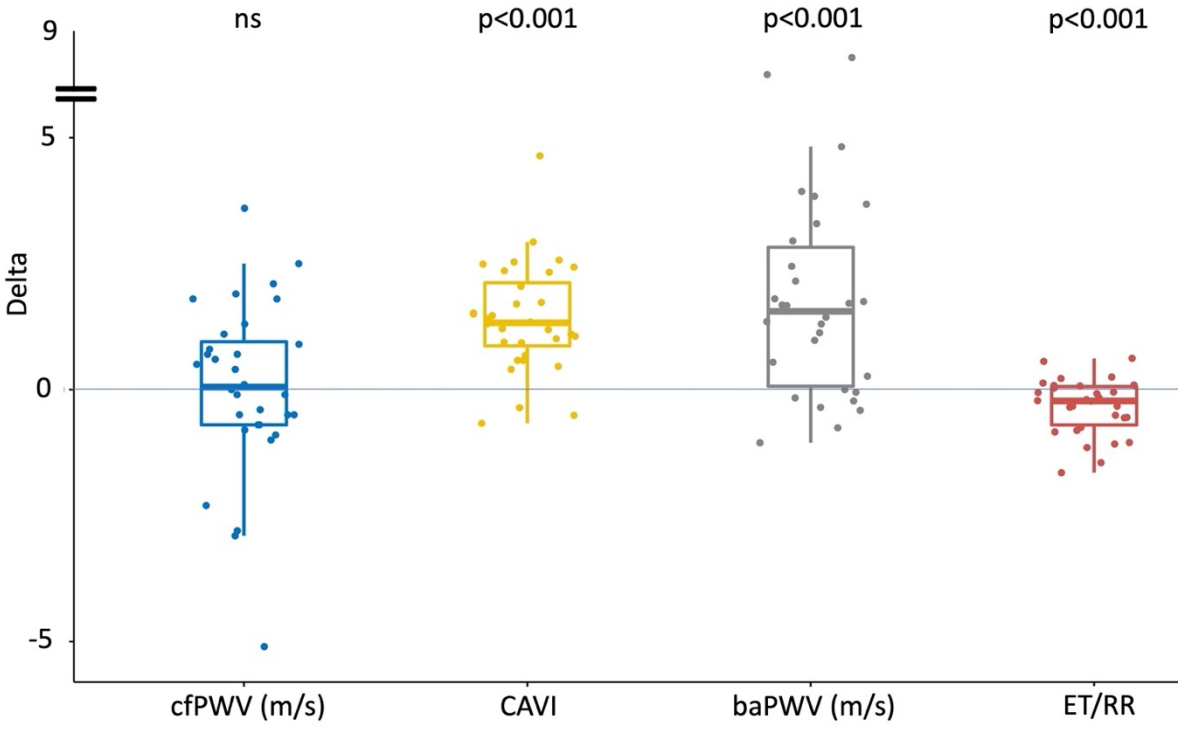

Figure 9. Delta values for AVS subjects (after – before surgery) for cfPWV (m/s), CAVI, baPWV (m/s), and ET/RR denotes ejection time divided by RR. Blue line = 0 (no change between before and after surgery).

In AVS subjects, this was accompanied by the numerically largest decrease in ejection time, upstroke time, and consequently, the preoperative between-groups difference of these variables was diminished. In contrast to CAVI and baPWV, cfPWV was not significantly

altered in any group. Hence, only systemic arterial stiffness including peripheral segments were increased after SAVR, solely in AVD patients. Last, low preoperative CAVI, decreased ejection time, lower BMI, diabetes, and age were independent predictors of increased CAVI after SAVR.

The decreased ejection time as a predictor of increased postoperative arterial stiffness has not been described before. Importantly, the relationship between the forward and reflective pressure waves is also influenced by the LV ejection time[191] which is prolonged in aortic stenosis[262]. However, the precise influence of this phenomenon on different measures of arterial stiffness is not fully elucidated.

Diabetes and age are both risk factors for atherosclerosis and AVS, and associated with increased arterial stiffness, which subsequently could be captured after AVD treatment. The negative association between BMI and increased CAVI after surgery is counterintuitive although previous studies have reported the same association[263, 264].

4.3.1 Differences between CAVI and cfPWV in AVS

Peripheral muscular arteries are stiffer in nature compared to large elastic conduit arteries like the aorta[265]. If a prolonged ejection time leads to longer elapsed time the pulse wave propagates, it seems plausible that CAVI rather than cfPWV is affected by the AVS resulting in lower stiffness measure with a subsequent larger postoperative increase. Importantly, this effect was not driven by surgery itself since AAD patients did not exhibit the same response. Augmentation index also include more peripheral arterial segments by using the reflective wave augmented pressure as an indicator of arterial stiffness and is associated with arterial-ventricular coupling (AVcoup), in contrast to cfPWV[266]. AVcoup has previously been demonstrated to decrease to normal levels after TAVI[267] and henceforth, CAVI might reflect this phenomenon. Certainly, the intrinsic property of the arteries including collagen composition is not likely altered by AVR and hence the explanation should lie in the interplay between the LV, aortic valve, and the arteries.

One might consider cfPWV to be superior to CAVI as no change was observed and thus might provide a more accurate estimation of large arterial stiffness, which has more robust evidence supporting a predictive value for future cardiovascular events[265]. Although, the cfPWV result in **Article I** is in line with a previous study in 30 patients undergoing AVR[205], three other studies with a total of 278 patients indicated increased cfPWV[194-196] (Table 6).

Study	Bruschi[205]	Chirinos[196]	Terentez[195]	Raimundo[194]	Article I
Year	2016	2019	2020	2021	2021
N	30	38	90	150	32
Mean age (SD)	79.3 (6.3)	72 (9.3)	80.2 (8.1)	72.5 (5.6)	67.3 (6.7)
Mean BMI (SD)	25.2 (3.6)	30.7 (6.4)	27.1 (4.3)	28.6 (4.3)	27.3(4.0)
Females (%)	50	31.6	50	48.7	33
CAD (%)	33.3	0	40	12.7	25
Hypertension (%)	73	87	70	83	41
Diabetes (%)		32	12	35	13
Mean Vmax (m/s) (SD)	4.6	4.4	4.4 (0.5)	NA	4.57 (0.5)
Mean cfPWV-1 (m/s) (SD)	11 (3.6)	8.8 (4.2)	7.5 (1.5)	9 (2.1)	8.2 (1.9)
Mean cfPWV-2 (m/s) (SD)	NA	11.2 (4.7)	8.4 (1.7)	9.9 (2.2)	8.2 (1.7)
Mean delta cfPWV (m/s) (SD)	0.4 (2.4)	2.41*	0.9*	0.9*	0.04 (1.7)

Table 6. Previous studies assessing cfPWV before and after AVR. *p-value <0.05

Consequently, AVS independent cfPWV cannot be assured given available data. Comparing patient characteristics between the available studies gives few clues to the conflicting observations. Patients in the other cohorts were older which could have an effect given that cfPWV increase with age. However, age was not an independent predictor of delta cfPWV in **Article I** (data not shown) nor in the study by Terentes-Printzios et al[195]. Nonetheless, age influence the etiology to AVS and in our cohort, younger BAV patients were mixed with older TAV patients. This may certainly affect the results as peripheral arterial stiffness does not seem to increase with age in the same manner as cfPWV[265]. The deltas for cfPWV are rather discrete and the coefficient of variation for intra-observation has been reported to be 9.5 %[233] which my hinder a detection of small differences although paired measures by the same investigator limits the plausibility for such interference. Last, insufficient power with only 32 patients available for paired measurements may have prevented a detection of a small increase.

4.3.2 Patients Not Available for Postoperative Measurements

29% of the AVS patients were lost in follow up, mostly due to postoperative atrial fibrillation. Patients excluded from the postoperative measurement had higher baseline MAP, cfPWV, and lower baseline ABI compared to patients that completed postoperative measurement (Table 7). Since low preoperative cfPWV is associated with postoperative increase[195], it did most likely not affect the results in **Article I**, albeit suggests that high cfPWV might predict postoperative atrial fibrillation.

	AVS		
	Not completed n=13	Completed n=32	p-value
MAP (mmHg)	108 (102 - 114)	102 (96 - 108)	**0.025**
cfPWV (m/s)	8.8 (7.9 - 13.4)	7.8 (7.1 - 9.0)	**0.033**
CAVI	8.2 (7.4 - 8.9)	7.6 (7.0 - 8.4)	0.215
ABI	1.10 (1.02 - 1.16)	1.18 (1.12 - 1.21)	**0.004**
HR (bpm)	66 (55 - 71)	66 (59 - 77)	0.437

Table 7. Comparison of baseline measures between patients that were excluded from post-operative measurement.

4.4 ATHEROSCLEROTIC RISK FACTORS AND ALTERATIONS IN LOCAL AORTIC VALVE BIOLOGY

In **Article II-III**, the impact of risk factors for AVS and/or atherosclerosis on local aortic valve changes were determined and in **Article IV,** the impact of established coronary artery disease on aortic valve gene expression was identified.

4.4.1 Polyunsaturated Fatty Acids and FADS genetics in Calcific Aortic Valve Stenosis

To seek mechanistic understanding of the rs174547 effect on AVS, its associations with aortic valve omega-3 and omega-6 PUFAs were determined. Aortic valve proportion of GLA, DGLA and DHA demonstrated the strongest association with rs174547 in aortic valve tissue and the activity of the FADS enzymes (FADS1 D5D and FADS2 D6D) were associated with decreased activity in C-allele carriers (Figure 10). Of note, data on fatty acid intake was not available although previous reports indicate scarce relation between proportion of fatty acids in erythrocytes and food intake[268]. Still, the enzyme activity also relies on the quantification of individual PUFAs, as demonstrated in the schematic metabolism in Figure 10. The specific activity of D5D and D6D may therefore be difficult to determine as both products and precursors depend on upstream and downstream enzymatic reactions.

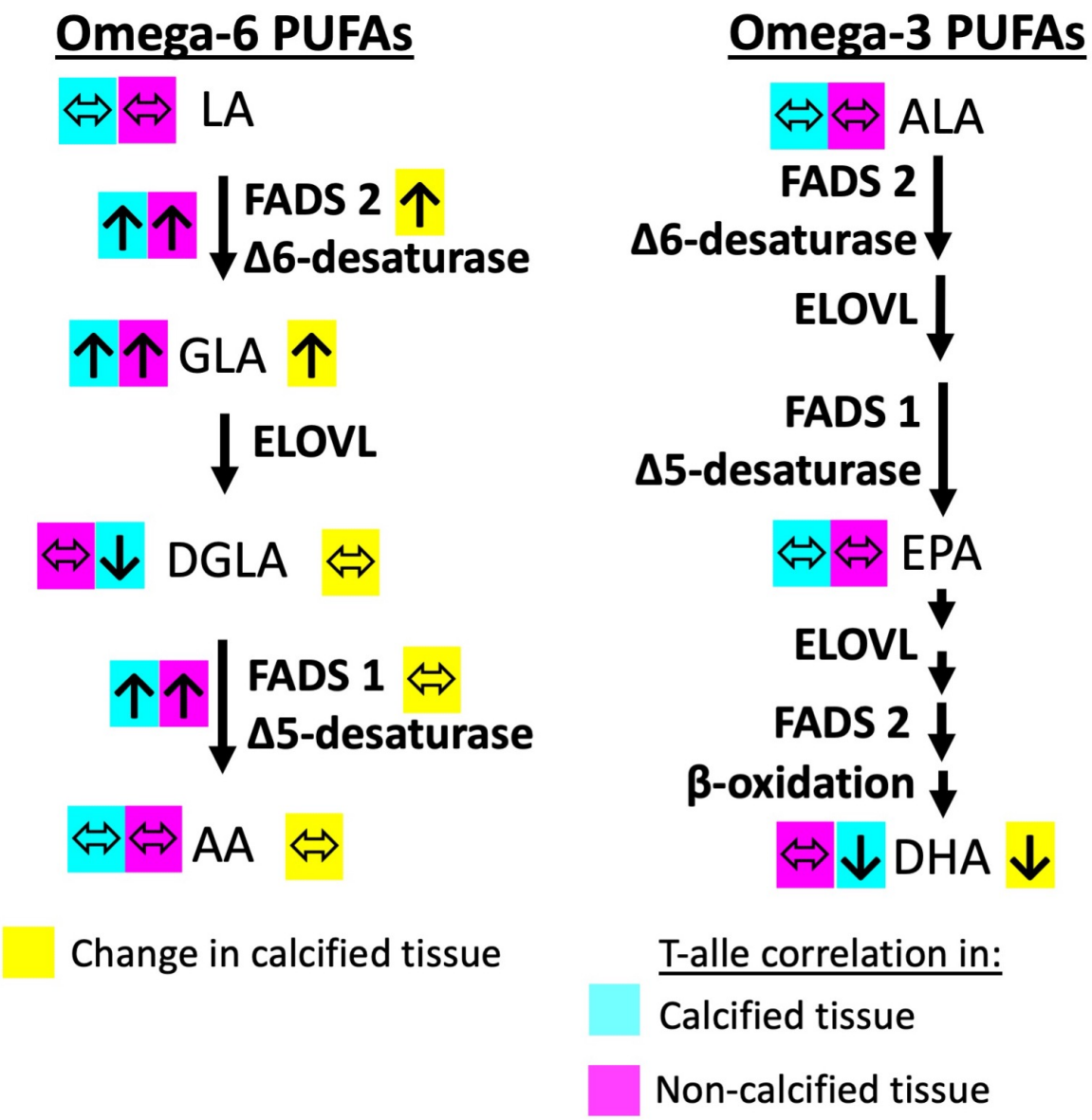

Figure 10. PUFA metabolism and associations between aortic valve PUFAs, calcification and rs174547 T-alle.

GLA has not been extensively studied but a recent investigation found a positive correlation between a high proportion of erythrocyte GLA and carotid intima-media thickness, in an Asian population[269]. However, a study from the United States did not demonstrate the same association using plasma proportions[270]. DGLA has been associated with a protective role in atherosclerosis by ameliorating foam cell formation, LDL uptake, and ROS substrates[271]. Albeit not investigated in an AVS context, the previously explored mechanisms have all been described in AVS pathophysiology. DHA is a known precursor of SPMs and have been associated with decreased risk of CAD[272] and decreased incidence of carotid artery atherosclerosis[269]. A link to aortic valve calcification was therefore hypothesized.

Calcified aortic valve tissue had higher GLA proportion (fold change 1.24, 95% CI 1.02 – 1.45) and lower DHA proportion (fold change 0.81, 95% CI 0.67 – 0.95) compared to non-calcified tissue. Although, both GLA and DHA were associated with both rs174547 and

aortic valve calcification, only DHA displayed a Bonferroni-adjusted significant rs174547 association in calcified tissue in which an eQTL was demonstrated. Furthermore, the overall conversion rate of DHA from ALA, involving both FADS enzymes and elongase activity, was significantly higher in aortic valve tissue from patients homozygous for the protective allele compared to CT/TT carriers.

Two studies have demonstrated the AVS protective association of the rs174547 C-allele within *FADS1* (OR per effect allele 0.92 [95% CI 0.86 - 0.98][135] and 0.91 [95% CI 0.88-0.94][131]). Dietary levels of omega-6 PUFA LA did not interact with rs174547 for the association with incident AVS or prevalent AVC[131], suggesting that genetically determined PUFA levels supersede the effect of dietary intake. The OR for rs174547 is slightly lower compared to other reported genetic risk factors for AVS (Table 8). It should however be noted that both *FADS* and *PALMD* have markedly higher effect allele frequency compared with *LPA*.

SNP within	Study	HR per effect allele	Effect allele frequency
LPA	Thanassoulis[103]	1.68 (1.32 - 2.15)	
LPA	Helgadottir[273]	1.46 (1.37–1.56)	6.2
FADS1	Chen[274]	0.91 (0.88 - 0.94)	34
PALMD	Helgadottir	1.20 (1.16–1.25)	51.2
PALMD	Thériault[275]	1.24 (1.18 - 1.30)	
TEX41	Helgadottir	1.15 (1.11–1.20)	37.5
IL-6	Thériault	1.16 (1.10–1.23)	
ALPL	Thériault	1.15 (1.10–1.21)	
NAV1	Thériault	1.13 (1.08–1.19)	

Table 8. Genetic risk factors for aortic valve stenosis

4.4.2 Impact of Atherosclerotic Risk Factors and Established Coronary Artery Disease on Aortic Valve Gene expression

Changes in aortic valve gene expression were analyzed with respect to the atherosclerotic/AVS risk factors rs174547, aPL IgG/IgM, and established CAD to determine if they were associated with any distinguishing pathways linking atherosclerosis to AVS.

4.4.2.1 rs174547 Associated Changes in Aortic Valve Gene expression

Only FADS2 mRNA expression in calcified aortic valve tissue demonstrated a rs174547 *cis*-eQTL with higher mRNA expression in carriers of the protective C-allele, suggesting that FADS2 rather than FADS1 exert the effect of rs174547. Also, mRNA expression of both FADS1 and FADS2 were decreased in calcified tissue. The PUFA ratio results suggested that increased enzyme activity of FADS2 link to calcification. However, the mRNA expression suggests a reverse association. Indeed, mRNA expression can be modified by post-transcriptional changes and might not reflect the protein end-product but also the enzymatic activity measure has drawbacks (*cf. supra*).

4.4.2.2 aPL Associated Changes in Aortic Valve Gene expression

aPL associated changes in aortic valve gene expression were identified by matching 5 aPL IgG/IgM positive patients to 5 aPL negative patients, based on age, sex and CAD. Only males were positive in the biobank cohort which limits the external generalizability of the study. To minimize multiple testing and to yield relevant results, probes annotated as non-coding were removed followed by exclusion of probes with expression values below median (if true in >50% of the samples), yielding 12 593 genes included in the analyses.

GSEA identified several enriched pathways where downregulated (negative NES) pathways related to IFN signaling and antigen processing in aPL positive samples were top hits (Table 9). Assessing individual genes, 46 downregulated and 54 upregulated DEGs with aPL positivity were identified (q-value <0.1 and fold change <0.8 or >1.2).

Name	**Size**	**Matches**	**NES**	**q**
GO_RESPONSE_TO_TYPE_I_ INTERFERON	96	66	-2,64	0
GO_INTERFERON_GAMMA_ MEDIATED_SIGNALING_PATHWAY	90	70	-2,62	0
GO_ANTIGEN_PROCESSING_ AND_PRESENTATION	223	179	-2,46	0
GO_DEFENSE_RESPONSE_ TO_VIRUS	239	157	-2,44	0
GO_MUSCLE_CONTRACTION	360	181	-2,3	0

Table 9. Selected top results from GSEA comparing aortic valve tissue from aPL positive and aPL negative patients.

4.4.2.2.1 aPL Associated Gene Expression and Aortic Valve Calcification

The link between calcification and aPL related genes were first established by demonstrating that 33 of the 54 upregulated DEGs in aPL were also higher expressed in calcified compared to non-calcified tissue (q-value <0.05). STRING functional enrichment analysis of these genes indicated enriched pathways involved in intraflagellar transport and dynein complex, both vital parts of primary cilia.

41 of the 46 downregulated aPL DEGs were also lower expressed in calcified compared with non-calcified aortic valve tissue. STRING functional enrichment confirmed GSEA results including several pathways related to IFN signaling.

To further strengthen the link between aortic valve calcification and aPL positivity, all aPL DEGs were used as predictors in SML models aiming to predict resilient, thickened, and calcified aortic valve tissue (Figure 11). 64 patients not selected for aPL matching were used. Four different algorithms were used and evaluated with kfCV, and linear discriminate analysis was able to predict all disease stages at 100% accuracy, indicating a firm association between aPL related genes and aortic valve calcification.

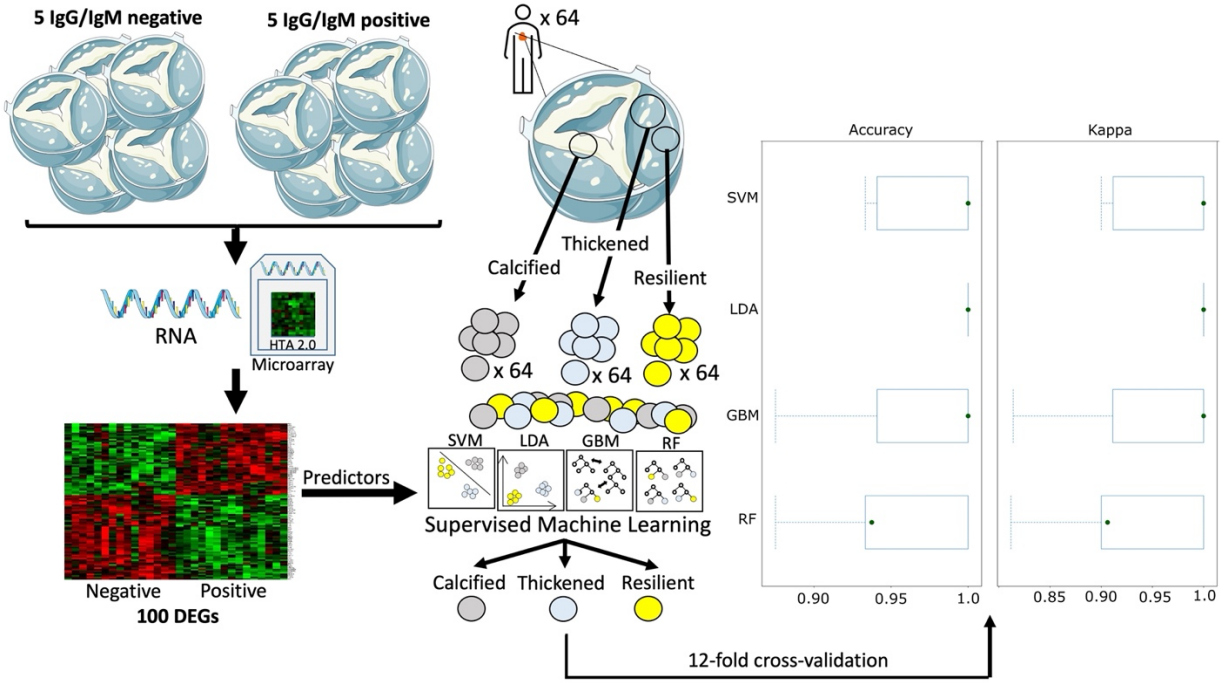

Figure 11. Schematic flow of the material used when predicting local aortic valve disease progression with supervised machine learning. The performance was evaluated during 12-fold cross-validation and the results are presented in box plots. Created with illustrations from smart.servier.com.

4.4.2.3 Impact of Concomitant Coronary Artery Disease on Aortic Valve Gene Expression

74 patients in the biobank were stratified based on CAD which was slightly overrepresented with a prevalence of 57 %. Before transcriptomic analyses were performed, the number of probe sets included were reduced by removing non-coding genes and redundant probes, and by applying an unbiased variance filter. The remaining 5152 genes were kept for further analysis. DEGs were identified by a q-value <0.05, after adjustments for sex and tissue type.

Since no previous study has determined aortic valve transcriptomics in relation to CAD, both CAD as a dichotomous variable and different degrees of CAD defined by VD were used to stratify the patients for comparisons. Based on heat map separation and number of DEGs, a comparison between patients with and without multi vessel disease (MVD) defined as ≥2 VD

yielded best separation (Figure 12). The results suggest that severe CAD is needed to evoke a prominent transcriptomic change in the aortic valve.

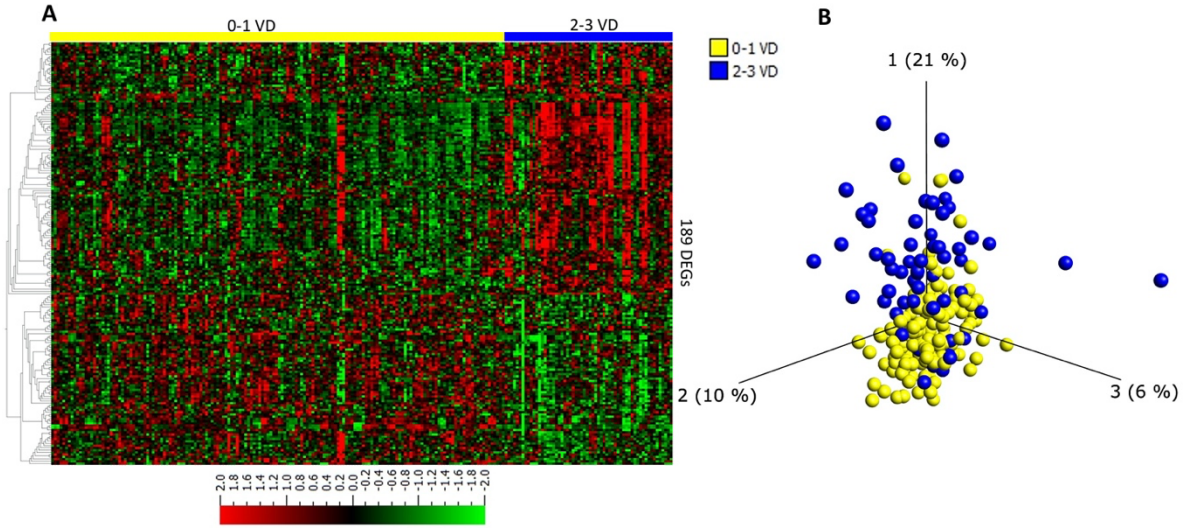

Figure 12. (A) Heatmap of the differentially expressed genes (DEGs) with q-value <0.05 comparing aortic valve tissue from patients with and without multi vessel disease. (B) Principal component analysis of the samples including the 189 DEGs as variables.

Next, GSEA identified 65 significantly enriched biological processes in aortic valves originating from MVD patients, including upregulated pathways related to mitogen-activated protein kinase cascade, organophosphorus, reactive oxygen species, and lipids, of which many have been described in an atherosclerotic context before[276-278]. When analyzing each tissue separately, almost all enriched pathways were found in non-calcified tissue, which indicates separate paths to a common end-stage disease.

To verify the feasibility of the MVD stratification and to reduce the influence of confounders, 20 patients with MVD were matched with 20 patients without MVD, based on age, sex, DM, HbA1c, BMI and Vmax. The matched cohorts were then used to predict aortic valve tissue from MVD patients, based on tissue adjusted aortic valve gene expression, using a random forest algorithm. kfCV was used to obtain model performance metrics which previously has been used for validation of sex-specific transcripts in AVS[279]. The current random forest model could predict aortic valve tissue from MVD patients at almost 90% accuracy as demonstrated in the confusion matrix in Table 10, which indicate feasibility of the stratification and a distinguished aortic valve transcriptome depending on MVD.

	Reference	
Predicted	No multi vessel disease	Multi vessel disease
No multi vessel disease	54	8
Multi vessel disease	6	52

Table 10. Confusion matrix including the predictions of the held-out samples during 10-fold cross validation.

Several previous studies have denoted joint mechanisms in AVS and atherosclerosis[280] but the results from the random forest model suggest that AVS patients with severe CAD defined by MVD may display in part different mechanisms. To further search for pathways involved in this potential atherosclerotic AVS phenotype, STRING functional enrichment analysis on the 30 most important genes used in the random forest model was performed (Figure 13).

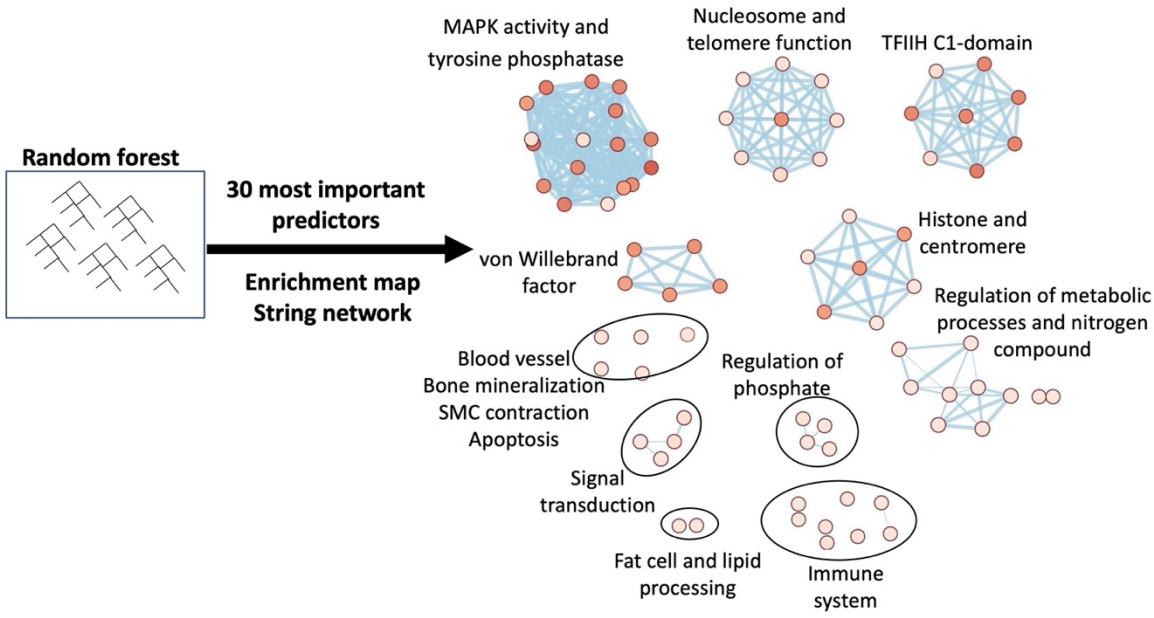

Figure 13. Results from STRING enrichment of the 30 most important genes used to predict aortic valve tissue from patients with or without concomitant multi vessel disease. Each node represent an enriched pathway connected by nodes based on similirities. The name of the clusters indicate a key term included in the pathways in each cluster.

Several enriched pathways identified in the GSEA were in common with the STRING enrichment analysis (n=22) including “Response to lipid”, “Response to organophosphorus”, “Response to oxidative stress” which indicated limited influence by known confounders in the first exploratory analysis. To further test if the genes were involved in atherosclerosis, the

carotid tissue expression of the 30 most important genes in the MVD random forest model were used to predict if carotid tissue were atherosclerotic or macroscopically normal (Figure 14). Using the MVD related genes, atherosclerotic carotid tissue could be predicted with 99 % accuracy, indicating a high relavence in the pathogenesis of atherosclerosis.

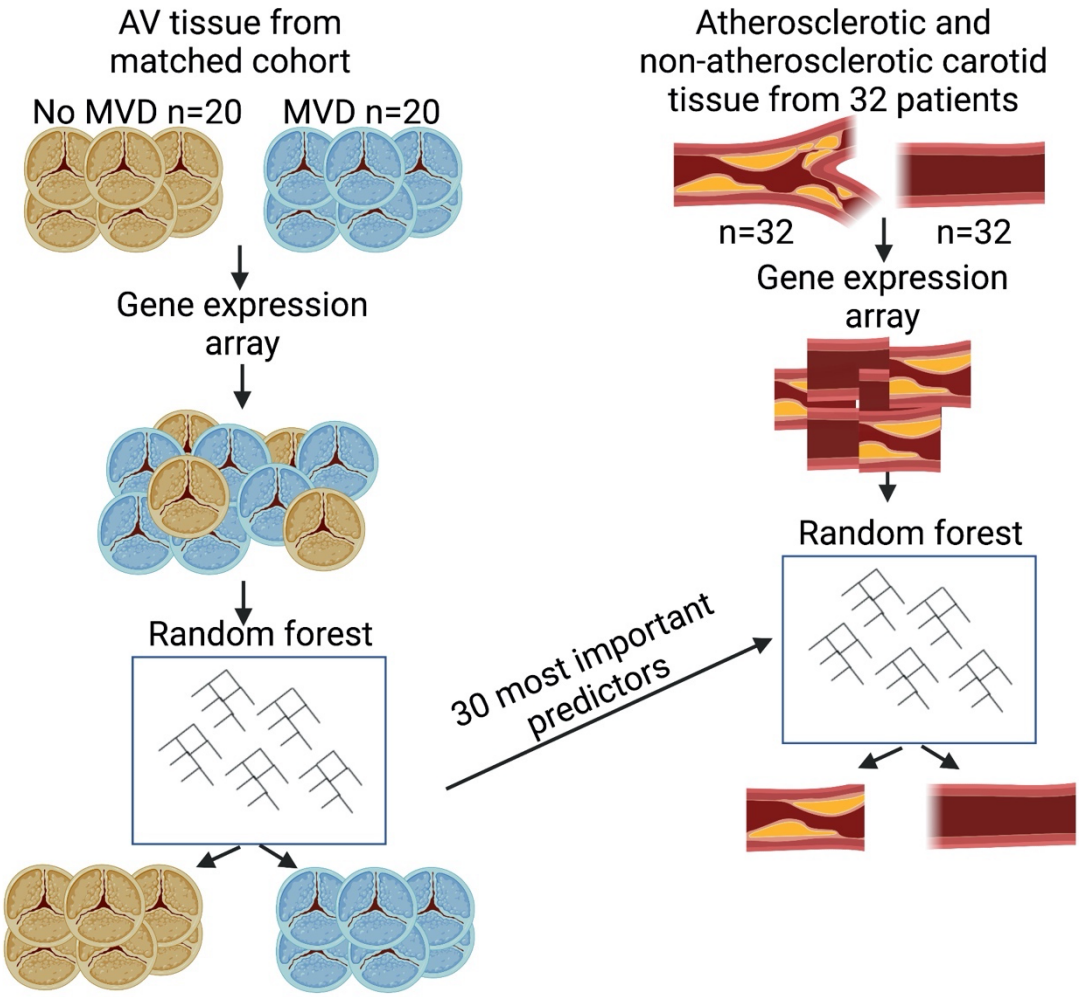

Figure 14. Schematic illustration of how the AVS with MVD related genes were used to predict carotid atherosclerosis. Created with BioRender.com.

In summary, the results propose that AVS can be stratified into an atherosclerotic and non-atherosclerotic phenotype which trudge on partly different paths reaching a common end-stage disease.

4.5 ATHEROSCLEROSIS PATHWAYS IN STENOTIC AORTIC VALVES

The results from **Articles II-IV** indicated altered pathways in aortic valve tissue related to inflammation, lipids, and phosphate among others, depending on concomitant severe CAD and/or atherosclerotic risk factors.

4.5.1 PUFAs and Inflammation

Supported by the results from **Article II**, the beneficial effect of rs174547 in AVS may be mediated by DHA (Figure 15). A previous suggested mechanism of the rs174547 effect on AVS is the pro-inflammatory mediators derived from arachidonic acid (AA). Higher levels of AA and higher desaturase activity (AA/LA) were both associated with AVC (OR 1.12 and 1.19, respectively)[131]. In contrast, the activity measured on the omega-3 PUFAs (EPA/ALA) did not associate with AVC[131]. The authors speculate that proinflammatory mediators from AA are responsible for the observed AVS rs174547 interaction. The results presented in this thesis challenge this notion by demonstrating an association between rs174547 and DHA locally in aortic valves. Indeed, AA is present in stenotic aortic valves,[117, 281] and its metabolites are involved in atherosclerotic mechanisms[282]. However, in **Article II**, aortic valve AA proportion was not associated with rs174547 nor associated with calcification (median paired delta in calcified compared to non-calcified tissue: -1.36, IQR 2.4, p=0.15, n=13). The lack of rs174547 and AA associations in aortic valves may depend on further conversion into pro-inflammatory mediators which might explain the trend to lower AA proportion in calcified tissue although the same reasoning cannot be applied to the findings in plasma. In addition, the short half-life of AA metabolites[283] highlights the importance of local aortic valve effects. In contrast to AA, DHA may dampen an inflammatory response *in vitro* via its GPR120 receptor[284] and an elevated DHA serum level associates with lower CAC score[285]. DHA act as substrate to generate the SPMs Rvs, protectins and maresins, catalyzed by acetylated COX and lipoxygenases[286]. They counterbalance a pro-inflammatory response via its GPCRs GPR32, GPR18, and FPR2 by promoting clearance of apoptotic bodies and mitigation of neutrophil and leukocyte recruitment[287]. While omega-3 PUFAs are known to reduce TG levels, subgroup analysis in REDUCE-IT demonstrated that the favorable effect was independent of baseline TG levels, which could indicate a potential explanatory role for SPMs[123]. Recently, the RvD1 receptor GPR32 was found to reduce atherosclerosis associated inflammation and mitigate atherosclerotic plaque burden in a mouse model[288]. Low GPR32 expression in carotid atherosclerotic plaque compared to non-atherosclerotic tissue further strengthened the possible relevance in humans. However, the supportive evidence for the protective role of EPA is somewhat more robust compared to DHA in both CAD prevention[123] and AVD[121]. On the other hand, more rapid AVS progression is associated with lower aortic valve omega-3 PUFA proportion[121] which together with the effects of DHA, fortify a plausible beneficial effect of DHA in aortic valve calcification. Nevertheless, this might not exclude an additive systemic AA effect.

Of note, high DHA intake, rs174547 C-allele and high FADS2 mRNA expression in whole blood have been associated with lower cfPWV, indicating a beneficial effect also on aortic stiffness[289]. Importantly, DHA intake was only associated with decreased cfPWV in CT and TT carriers and therefore highlights the importance of FADS genotype in omega-3 PUFA supplementation therapy. The association between high FADS2 mRNA expression and lower cfPWV also supports the DHA hypothesis presented above.

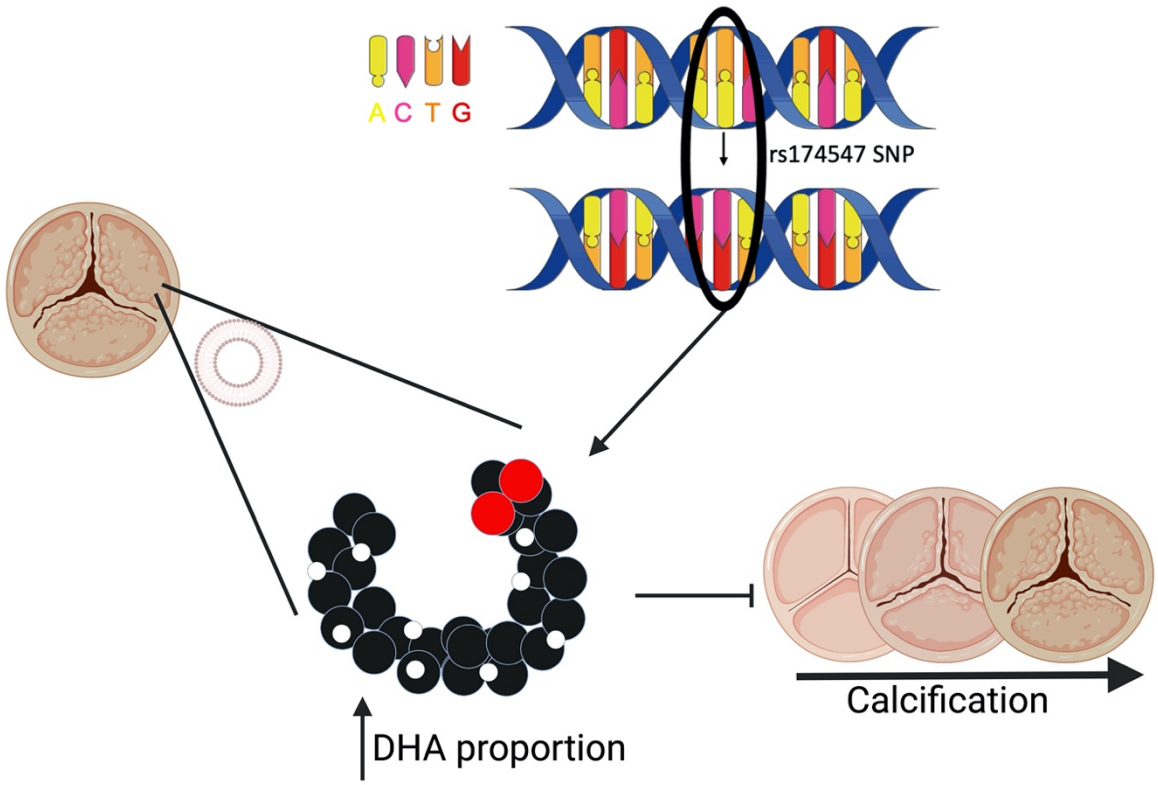

Figure 15. Schematic illustration of the proposed rs174547 effect on aortic valve calcification. Created with Biorender.com and with images from smart.servier.com

4.5.2 Interferons, Autoimmune Disorders, and Aortic valve Calcification

Supported by increased atherosclerotic lesion size and macrophages when boosting type I or type II IFNs in mice, IFNs have been suggested to contribute to atherosclerosis[88]. In line with these observations, a link to aortic valve calcification was provided when IFN-α treated VICs demonstrated upregulated mRNA expression of RUNX2 and BMP2, and decreased expression of MGP[90]. Moreover, high type I IFN score, based on gene expression in peripheral blood mononuclear cells, has been associated with primary APS and SLE[290]. The finding of downregulated IFN pathways in aortic valve tissue from aPL positive patients in **Article III** was therefore unexpected. However, in SLE patients, there was no association between aPL and high type I interferon score, measured by IFN-related gene expression after stimulating Wish-cells with patient serum[291]. The results indicate that the interferon response may differ depending on material and method used to detect the IFN response. Nevertheless, Singleton-Merten syndrome, a rare genetic disorder conferring constant interferon activation is associated with aortic valve calcification and SLE[292]. Also, the proteomic signature in AVS is shared with SLE[293]. Taken together, a link between aPL, calcification and interferon signaling is therefore reinforced and supports the results in **Article III,** albeit no causality or specific mechanisms could be attributed.

4.5.3 Mechanosensing and Nuclear Envelope

Genes upregulated with aPL IgG/IgM positivity were enriched in pathways related to mechanosensing function and dynein complex. Primary cilia can induce intracellular signaling in response to mechanical stimuli[294] and are prone to locate in arteries in areas with disturbed shear stress and atherosclerosis[295], suggesting mechanosensing properties with implications in atherosclerosis. Palmdelphin (PALMD) is part of the paralemmin family which have been proposed in controlling cell shape, cell motility and filopdoia[296-298]. A transcription wide association study proposed a SNP within *PALMD* as a causal AVS risk variant which was associated with decreased PALMD mRNA expression and hemodynamic markers of AVS severity[299]. In aortic valves, PALMD was found in VECs and silencing PALMD led to disruption of the nuclear integrity and nucleocytoplasmic transportation which was associated with altered gene expression[300]. The communication between the cytoskeleton and nucleus is also important for the ability of endothelial cells to respond to mechanic stimuli such as disturbed flow[301], implicated in aortic valve fibrocalcific remodelling[302]. Interestingly, although involved in processes important in atherosclerosis, PALMD is not associated with CAD in contrast to many other genetic risk variants. Collectively, the upregulated pathways in aPL positive patients are involved in cellular functions that may have an impact on AVS pathobiology although the exact role in AVS remains to be established.

4.5.4 Pathways Linking Atherosclerotic Risk Factors to Concomitant Severe Coronary Artery Disease in Aortic Valve Stenosis

Several of the upregulated pathways in aortic valves from MVD patients are associated with the results obtained in **Article I-III**. Numerous lipid pathways were upregulated in MVD which may also involve fatty acid metabolism. One of the genes contributing to the enriched lipid pathway was *PTGS2* encoding COX-2. COX-2 expression is higher in calcified compared to non-calcified aortic valves and catalyze the conversion of AA to PGE2[303]. However, COX-2 inhibition in VICs have demonstrated conflicting results[303-305]. Nevertheless, the discovery of *PTGS2* and lipid associated pathways in **Article IV** indicates that lipid metabolism is important in atherosclerotic AVS and suggest that the results from **Article II** might be further strengthened in an atherosclerotic AVS phenotype.

Pathways related to phosphate and von Willebrand factor (vWF) were also enriched in MVD derived aortic valve tissue and associated with the 30 most important MVD predictors in the random forest model. vWF has been associated with aortic and carotid atherosclerosis[306] and is an important factor in thrombosis. In APS and SLE patients, aPL IgG promote the release of vWF by endothelial cells and thereby stimulate thrombosis[307]. Moreover, antibodies against β2GPI can bind to β2GPI leading to hampered ability of β2GPI to inhibit vWF-dependent platelet adhesion[308]. Taken together, the findings in **Article III-IV** offer a rational for future studies targeting thrombosis to alleviate AVS progression in aPL positive subjects with concomitant severe CAD.

Phosphate is pivotal in calcification by forming calcium-phosphate complex that can yield hydroxyapatite, a building block for calcification[309]. The release of phosphate from the membrane phospholipids contribute to calcification[277] and might also be promoted by aPL, a hypothesis partly supported by a positive correlation between aPL and AVC[310]. Extracellular phosphate is also a cornerstone in arterial medial calcification[277] which increases arterial stiffness[311]. Consequently, increased activity of phosphate pathways in AVS subjects with concomitant MVD also have implications in aPL related AVS mechanisms and arterial stiffness.

4.6 GENERAL LIMITATIONS

The cross-sectional design in **Articles II-IV** prevented any conclusions regarding causality. Also, the lack of mechanistic data limits the conclusions. Microarray have lower sensitivity compared to RNA-sequencing although the risk of type I error is lower. No validation cohort was used to confirm SML results in **Articles III-IV** although kfCV is a good surrogate since held-out samples are used. The AVS biobank, although one of the largest existing, is relatively small which may obstruct detection of discrete changes in aortic valve transcriptomics depending on patient phenotype. All included DAVAACA patients were accepted for surgery, met the inclusion criteria which all together create a bias. Furthermore, all included samples in the biobank suffered from severe AVS which prevented having aortic valve tissue from patients without VHD as controls. The use of bulk aortic valve tissue prevented interrogations about the role of specific cell types. Furthermore, the dissection into three different stages may have included a bias since severely calcified valves have been excluded due to missing or insufficient amount of non-calcified tissue.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

"Surgery of the heart has probably reached the limits set by nature to all surgery: no new method and no new discovery can overcome the natural difficulties that attend a wound of the heart"

Stephen Paget – 1896

Fortunately, I don't concur with Dr. Paget regarding the future of AVS treatment and I believe the articles in this thesis may offer new pieces to a very large puzzle. The notion of an atherosclerotic process taken place in the stenotic aortic valve is not a novel concept although the interplay between the two diseases is not yet deciphered, despite substantial efforts.

In **Article I**, CAVI was introduced to measure systemic (including peripheral arteries) arterial stiffness in AVS patients. The results indicate that AVD interfere with the measurement yielding falsely low systemic arterial stiffness parameters. Furthermore, postoperative decreased ejection time and cardiovascular risk factors were independent predictors of an increased postoperative CAVI. Since arterial stiffness contribute to the LV load and have been linked to poor outcome after AVR, it could provide a useful complement in the arsenal of diagnostic tests when the decision to perform AVR is difficult. For example, in asymptomatic patients with normal LVEF, active expectance has been employed with prompt AVR if symptoms arise[312]. However, a recent randomized control trial demonstrated substantial benefit for these patients if SAVR was performed[313]. Perhaps the addition of arterial stiffness could aid in these difficult treatment decisions. Moreover, one could take advantage the masked arterial stiffness in AVS patients and the measurement of ejection time. Systolic time intervals were of great interest decades ago, and were associated with CAD, heart failure, and AVS[314]. They could be used to diagnose AVS[315], assessing its severity[316] and predict outcome[317]. However, the impact of these early studies has fallen, probably due to increased TTE accessibility. Interestingly, a recent study demonstrated that increased time from peak LV pressure to aortic pressure can aid in diagnosing low-flow low-gradient AVS and was associated with AVC[318]. Hence, measuring arterial stiffness in tandem with systolic time intervals with the VaSera device could be of value in screening patients for AVS and treatment decisions. In addition, easy-to-use and cheap devices, detecting systemic arterial stiffness and systolic time intervals may be of value in rural and low-income settings in which the burden of CVD is increasing. I believe these potential benefits merits future investigations in prospective trials.

In **Article II**, the SNP rs174547 located in a *FADS1* intron was associated with higher FADS2 mRNA expression, higher conversion of ALA to DHA and higher DHA proportion. Furthermore, DHA proportion was lower in calcified compared to non-calcified tissue which implies a tight link to aortic valve calcification. The results thus indicate a potential benefit of DHA in AVS. However, EPA supplementation seems to be favored over DHA for secondary CAD prevention[319] so future investigations should be designed to identify precisely which omega-3 PUFA offers the best halting effect on AVS. Also, since the downstream

metabolites of EPA and DHA might be the beneficial culprits, SPMs could also serve as future treatment targets. Determining the rs174547 genotype could also be of importance to understand the effect of omega-3 supplementation[289].

aPL, a proposed risk factor for CAD were associated with AVS in matched cohorts, accompanied by changes in aortic valve gene expression, independent of concomitant CAD. Although these results should be replicated in a larger cohort including isolated AVS and CABG patients, the results open up new avenues for aPL as risk factors and for potential therapeutic targets. The ability of aPL to activate platelets[249], promote inflammation[247] and perhaps facilitate inorganic phosphate availability[277] supports the idea of aPL as a possible risk factor for AVS. Future work should therefore determine if any halting effect on AVS progression can be accomplished with antithrombotic treatment in aPL positive patients although caution must be taken since VitK-antagonists is the recommended treatment in APS patients with previous venous or arterial thrombosis[320]. Pathways in interferon signaling and mechanosensing functions may also be interrogated for new potential therapeutic targets.

The exploratory analysis in **Article IV** revealed upregulated pathways related to phosphate, ROS, and lipids, among others, in aortic valves from patients with severe multi vessel CAD. These pathways were also linked to the results in **Article I-III**. Furthermore, SML could predict if AV tissue originated from MVD patients and the genes that could predict MVD were suggested to be involved in carotid atherosclerosis. The previous stratification of AVS phenotypes focused on valve morphology, but the results from **Article IV** offer a rationale to a new concept of atherosclerotic and non-atherosclerotic AVS, supported by previously demonstrated distinguished and common genetic risk factors. This could potentially facilitate therapeutic discoveries by applying more targeted treatments for separate phenotypes. However, to make the inclusion/exclusion more feasible in future randomized control trials, reliable biomarkers, or imaging techniques of future MVD should be identified.

Collectively, all articles in this thesis have possible implications in the future of precision medicine in AVS and demonstrates pathways linking atherosclerosis to aortic stenosis.

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