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AORTIC VALVE CALCIFICATION

IN VIVO AND EX VIVO EVALUATION

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Cover illustration

Heart amulets, Egyptian Dynasty 18–19 (ca. 1550–1186 b.c.) (10.130.1782_10.130.1804)

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Aortic valve calcification - in vivo and ex vivo evaluation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Jannah and Abdullah

“One thing I have learned in a long life: that all our science, measured against reality, is primitive and childlike -- and yet it is the most precious thing we have.”

Albert Einstein

ABSTRACT

Aortic valve calcification (AVC) or thickening is found in around one fifth of the general population between 65-75 years of age and increasingly thereafter. The process of aortic valve thickening and calcification is not only an aging (wear and tear) process of the valve leaflets. It is now considered to be closely related to atherosclerosis. The presence and the degree of AVC have been shown to have prognostic value in patients with cardiovascular diseases and in the general population. Despite its importance, there is no widely agreed upon scoring system that objectively quantifies AVC.

The aim of this project was to investigate different methods for evaluating AVC, using transthoracic and transoesophageal echocardiography (TTE and TOE), intra-operative assessment of the valve (IOS) and ex vivo evaluation based on computed tomography (CT) of the excised aortic valves and based on valve weight. A 5-grade scoring system was used for the visual assessment of AVC on real-time and still TTE and TOE images of the aortic valve, as well as intra-operatively. Computer-based greyscale measurement (GSM) software was used to obtain a quantitative-ultrasound-based AVC measure, which was compared with the visual AVC score from TTE and TOE, whereas IOS was used as the gold-standard method. We also aimed at identifying the most suitable ex vivo conditions and CT parameters for optimal AVC scanning by CT in a calcium hydroxyapatite (CaHA) phantom study. The TTE and TOE AVC scores and IOS were compared to the weight and the CT CaHA mineral mass (MM) index of the explanted aortic valves. The study cohorts were recruited among patients undergoing aortic valve replacement because of aortic valve disease and/or ascending aorta aneurysm.

In Study I, which included 185 patients, we showed that the visual evaluation of AVC using real-time TTE images yielded better correlations with IOS than did quantitative still frame measures based on GSM (r = 0.83 vs 0.64, respectively). In Study II, AVC scores based on TTE and TOE real-time images from 169 patients showed strong correlations with IOS (r = 0.83 and 0.82, respectively). GSM-based measures correlated less well with IOS, even for TOE (r = 0.52). In Study II, we also showed that TOE was more accurate than TTE in diagnosing the aortic valve phenotype. In the CT-phantom-based, methodological Study III, we identified optimal CT scanning and reconstruction parameters, as well as the most suitable medium (normal saline) for ex vivo tissue CT-based calcium scoring. In Study IV, 155 operatively explanted aortic valves were weighed and scanned ex vivo by CT, and CaHA MM was measured. The CaHA MM exhibited a strong correlation with valve weight (r = 0.91), whereas AVC scores based on TTE, TOE and IOS showed weaker correlations. Conversely, echocardiographic and intra-operative AVC evaluation showed a better correlation with haemodynamic parameters compared with ex vivo CT AVC scoring.

In conclusion, real-time echocardiographic images are crucial for accurate AVC scoring, regardless of whether TTE or TOE is used. Echocardiographic AVC scoring was as accurate as intra-operative assessment, according to valve weight and ex vivo CT. For ex vivo calcium scoring by CT, in addition to scanning and reconstruction parameters, using saline as the surrounding medium seems to be important. TOE is more accurate than TTE in detecting the bicuspid aortic valve.

SAMMANFATTNING

Aortaklaff-förkalkning (AVC) eller förtjockning förekommer hos ungefär en femtedel av befolkningen mellan 65-75 års ålder och ökar därefter. Förtjockning och förkalkning av aortaklaffen är inte bara degenerativa åldersförändringar. Senare rön har visat att det sannolikt finns en koppling mellan AVC och åderförkalkningssjukdom, med inflammation som en viktig gemensam faktor. Förekomst och grad av AVC har visat sig ha prognostisk betydelse hos patienter med hjärtkärlsjukdomar och i befolkningen generellt. Trots dess prognostiska värde finns det inga allmänt accepterade graderingsmetoder för AVC.

Syftet med denna avhandling var att på ett metodologiskt sätt utvärdera olika verktyg för gradering av AVC vid transthorakal (TTE) och transoesophageal (TOE) ekokardiografisk avbildning av aortaklaffen, i jämförelse med direkt intraoperativ kirurgisk bedömning (IOS) samt datortomografisk undersökning (CT) och vikt av bortopererade aortaklaffar. En 5-gradig skattningsskala användes för visuell bedömning av AVC på realtids- och stillbilder av aortaklaffen från TTE och TOE, samt intraoperativ kirurgisk bedömning. Programvara för datorbaserad gråskalemätning (GSM) användes för att erhålla en kvantitativ-ultraljudsbaserad AVC skattning som jämfördes med den visuella bedömningen av TTE och TOE, samt IOS som referensmetod. Vi ville identifiera lämpligaste ex vivo förhållanden och CT parametrar för optimal kalkbedömning med hjälp av CT i en experimentell studie av kalcium-hydroxyapatit (CaHA). AVC grad bedömd med TTE och TOE jämfördes med IOS, vikt och CT mätning av kalk i uttagna aortaklaffar. Studien omfattade patienter som genomgick klaffbyte på grund av aortaklaffsjukdom. I studie I, som omfattade 185 patienter, visade vi att visuell utvärdering av AVC med realtids TTE stämde bättre (r = 0,83) med IOS än mätning av gråskala i stillbilder (r =0,64). I studie II visades att AVC bedöming av rörliga TOE bilder från 169 patienter korrelerade väl med IOS (r = 0,83). GSM i stillbild korrelerade mindre bra med IOS, även för TOE (r = 0,52). I studie II visade vi också att TOE var mer exakt än TTE i att avgöra om aortaklaffen har två eller tre klaffblad (är bi- eller tricuspid; BAV eller TAV). I den CT-fantom-baserade studien III försökte vi identifiera optimala CT parametrar samt det mest lämpliga omgivande mediet (fysiologisk koksaltlösning) för CT-baserad kalkgradering av små preparat. I studie IV, vägdes och gjordes CT av 155 operativt uttagna aortaklaffar. CT mätning av klaffkalk korrelerade starkt till klaffvikt (r = 0,91), medan AVC skattning baserad på TTE, TEE och IOS visade svagare samband. Omvänt visade ekokardiografisk och intraoperativ AVC utvärdering en bättre korrelation med hemodynamiska parametrar relaterade till aortaklaffsjukdom, jämfört med CT.

Sammanfattningsvis, rörliga ekokardiografiska bilder är nödvändiga för noggrann bedömning av klaffkalk, oavsett om TTE eller TOE används. Ekokardiografisk kalkbedömning var lika exakt som intraoperativ bedömning, enligt klaffvikt och ex vivo CT. För CT mätning av kalk i uttagna klaffar synes, förutom CT parameterar, fysiologisk koksaltlösning viktig som omgivande medium. TOE är mer exakt än TTE för att detektera bicuspid aortaklaff.

LIST OF SCIENTIFIC PAPERS

I. **Real time imaging required for optimal echocardiographic assessment of aortic valve calcification**

Mohamed Yousry, Anette Rickenlund, Johan Petrini, Tomas Gustavsson, Ulrica Prahl, Jan Liska, Per Eriksson, Anders Franco-Cereceda, Maria J Eriksson, Kenneth Caidahl
Clin Physiol Funct Imaging. 2012 Nov;32(6):470-5

II. **Aortic valve type and calcification as assessed by transthoracic and transesophageal echocardiography**

Mohamed Yousry, Anette Rickenlund, Johan Petrini, Jonas Jenner, Per Eriksson, Anders Franco-Cereceda, Jan Liska, Maria J Eriksson, Kenneth Caidahl
Clin Physiol Funct Imaging. 2014 May 29. [Epub ahead of print]

III. **Quantification of calcium content in small objects by computed tomography: methodological aspects**

Mohamed Yousry, Maria J Eriksson, Kenneth Caidahl, Sven Nyrén
Submitted

IV. **Estimation of aortic valve calcification by echocardiography, surgical scoring, ex vivo computed tomography and valve weight**

Mohamed Yousry, Maria J Eriksson, Dianna Bone, Sven Nyrén, Per Eriksson, Anders Franco-Cereceda, Kenneth Caidahl
Manuscript

CONTENT

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AMS	Artery measurement system
AR	Aortic regurgitation
ARBs	Angiotensin receptor blockers
AS	Aortic stenosis
ASAP	Advanced study of aortic pathology
AVA	Aortic valve area
AVC	Aortic valve calcification
AVR	Aortic valve replacement
BAV	Bicuspid aortic valve
BMP	Bone morphogenic protein
CAD	Coronary artery disease
CaHA	Calcium hydroxyapatite
CT	Computed tomography
CV	Coefficient of variation
DES	Drug-eluting stents
EBCT	Electron beam computed tomography
EC	Endothelial cells
GSM	Greyscale measurement
GSMn	Greyscale mean
hsCRP	High sensitivity C-reactive protein
HU	Hounsfield unit
ICC	Intra-class correlation coefficient
IMT	Intima–media thickness
IOS	Intra-operative score (surgical score)
LDL	Low density lipoprotein
LV	Left ventricular
LVOT	Left ventricular outflow tract
MAC	Mitral annulus calcification
MM	Mineral mass
MRI	Magnetic resonance imaging
MSCT	Multi-slice computed tomography
PET	Positron emission tomography
PDA	Patent ductus arteriosus
OCT	optimum cutting temperature imbedding medium

QRM	Quantification of coronary calcium
RNA-later	Ribonucleic acid stabilization and storage reagent solution
SPECT	Single-photon emission computed tomography
STJ	Sinotubular junction
SVS	Still image AVC score
TAV	Tricuspid aortic valve
TAVR	Transcatheter aortic valve replacement
TGF-β	Transforming growth factor beta
TOE	Transoesophageal echocardiography
TTE	Transthoracic echocardiography
VEGF	Vascular endothelial growth factor
$\mathbf{V_{max}}$	Peak velocity measured across the valve measure in metres per second (m/s)

1 INTRODUCTION

1.1 HISTORICAL NOTES

The Edwin Smith Papyrus, which has been dated to 1700 B.C., is considered as the oldest known medical document that describes accurately several different types of body injuries, diagnostic methods and treatments (Figure 1). This ancient document was written on a 15-ft-long papyrus scroll by a single unknown Egyptian writer who was trying to copy an older document dated from 2500 to 3000 B.C. The document, which was never finished and was never signed by the author, was intended to be a surgical text-book and included 48 different types of surgical cases. It discussed in detail the sterilization of wounds and instruments, the use of bread moulds to improve healing, suturing, bone fixation and even surgical operations. Despite being aimed at surgery, the papyrus included the first ever known description of the function of some body organs, including the heart. The heart function was clearly described as pumping the blood in the blood vessels throughout the body to various organs, and not as producing blood, as was long thought in the ancient world. Moreover, it linked the heart-pumping function with peripheral pulsation. These facts were not firmly established until the 17th century, rendering this ancient surgical document the oldest discovered to date to note the actual function of the heart.[1, 2]

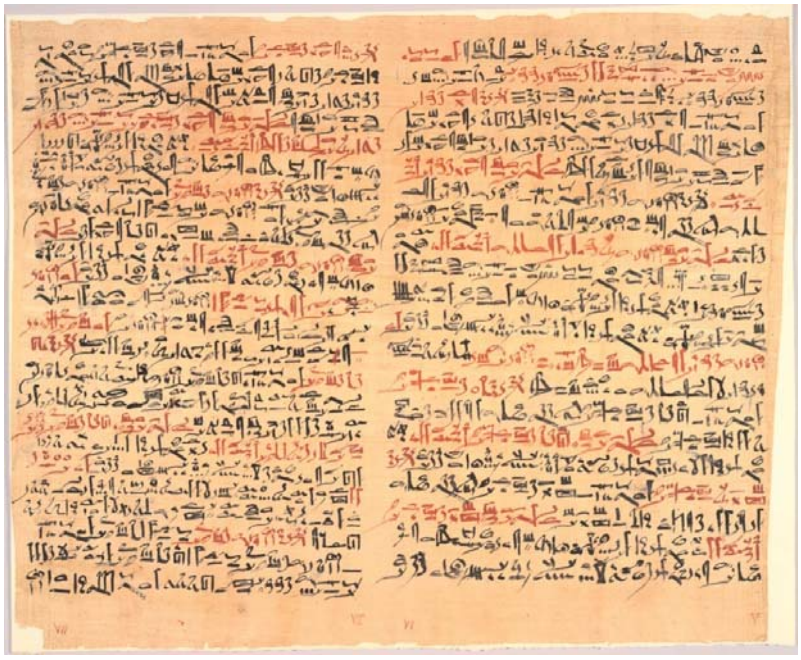

Figure 1.

A piece of the 4000-year-old Edwin Smith Papyrus scroll, currently preserved in the rare books vault at the New York Academy of Medicine.

Reused under "Fair use" conditions from the U.S. National Library of Medicine (http://archive.nlm.nih.gov/proj/ttp/smith_home.html)

Even Leonardo da Vinci (1452–1519), who studied and illustrated anatomy in an explicit way and worked in detail on the heart during his last years of life, never disputed the medieval idea of constant blood flow to the periphery via arteries and veins.[3] However, he applied the knowledge of flow in widening canals, and to study blood flow through heart valves, he built a glass model of the aorta with cusps. Furthermore, he produced the first depiction of a bicuspid aortic valve.[4]

Regarding cardiac valve calcification, the first case of a calcified aortic valve was described by Rayger in 1697 (published by T. Bonet in 1700). Cowper noted in 1706 a man with dyspnoea and, at autopsy, petrified aortic leaflets "…in as much that they could not approach each other"; in 1829, Lanneac presented a clinical description of valve calcification.[5] From the 1930s, further articles were published on this topic, and in 1949, Davies and Steiner provided an excellent historical overview, partially cited in the previous sentence, together with a study of 14 patients with "pure" calcified aortic valve.[5] Those authors described their aetiology, clinical symptoms, physical signs and radiological findings. Even as early as the 1930s, researchers aimed at using Röntgen imaging to detect aortic and mitral valve calcifications.[6] Several studies attempted to assess the association between the presence of cardiac valve calcification (mitral or aortic) and diet, lifespan and other cardiac pathologies.[7]

In the 1950s, the clinical need to determine the function of the mitral valve inspired Inge Edler – born in Malmö and head of the Department of Internal Medicine and director of the Cardiovascular Laboratory at the University of Lund for 10 years – to invent echocardiography.[8, 9] He achieved this with the help of another young physicist, Carl Hellmuth Hertz.[10] His primary aim was to rule out the presence of mitral regurgitation, which complicated commissurotomy of mitral stenosis. Based on the Siemens Ultrasound Reflectoscope (Ultraschall-Impulsgerät), the efforts of Edler and Hertz led to the first ultrasonic images of the moving heart, thus marking the dawn of echocardiography on October 29, 1953.[8, 11, 12] The echocardiographic M-mode appearance of mitral annulus calcification was described in 1975.[13]

1.2 AORTIC VALVE EMBRYOLOGY, ANATOMY AND FUNCTION

1.2.1 Embryology

Aortic valve formation starts in the 4th week of intra-uterine life, together with the appearance of the pulmonary valve (semilunar valve formation) (Figure 2). The process begins with the formation of two opposing cushions (the right dorsal and left ventral endocardial bulbar cushions) in the truncus arteriosus. The cushions fuse first at the truncoconal position, and then the fusion "zips" distally towards the outflow tract and proximally towards the ventricles. In addition, another two intercalated endocardial cushions develop at a right angle from the aforementioned cushions. This occurs as they spiral in a right-handed direction, leading to the pulmonary trunk and ending up anterior to the aorta.

The fusion of these cushions leads to the formation of the truncal septum, which undergoes differentiation to form two of the three aortic valve leaflets (right and left leaflets) and the two leaflets of the pulmonary valve. Subsequently, the previously formed right cushion forms the posterior (non-coronary) aortic valve leaflet, whereas the left cushion forms the anterior pulmonary valve leaflet. During this process, the truncus twists anti-clockwise and shifts caudally as the endocardial cushions differentiate, leading to mature valve leaflets. A normal aortic valve consists of three leaflets, i.e., a tricuspid aortic valve (TAV). Abnormal fusions or flaws in the aforementioned processes lead to anatomical congenital aortic and pulmonary valve anomalies.[14, 15]

Such anomalies include a congenital bicuspid aortic valve (BAV), which consists of only two cusps and is the most common congenital heart malformation.[16] BAV can occur in isolation, it can be associated with other cardiovascular malformations (such as coarctation of the aorta, patent ductus arteriosus and cardiac septal defects) or it can be a part of a clinical syndrome (such as Turner and Williams–Beuren syndromes).[17-22] The pathogenesis of BAV is multifactorial. Environmental factors have been suggested to cause the incorrect fusion of the leaflets.[23] Studies showing increased risk of BAV in first-degree relatives and in families further support the genetic heritable nature of isolated BAV.[20, 22] Studies performed in humans showed the association between the presence of isolated (non-syndromatic) BAV and the transcription regulatory gene *NOTCH1*, which is located on chromosome 9q34.[24, 25] Several other genes have been suggested to contribute to BAV occurrence, based on animal studies, [26, 27] however, none of them showed this type of association in humans.[28, 29] The dominant inheritance of cardiovascular defects associated

with BAV has been linked to chromosomes 18q, 13q and 5q; moreover, chromosome 15q25–26 in BAV patients is associated with ascending aortic aneurysm.[21, 30]

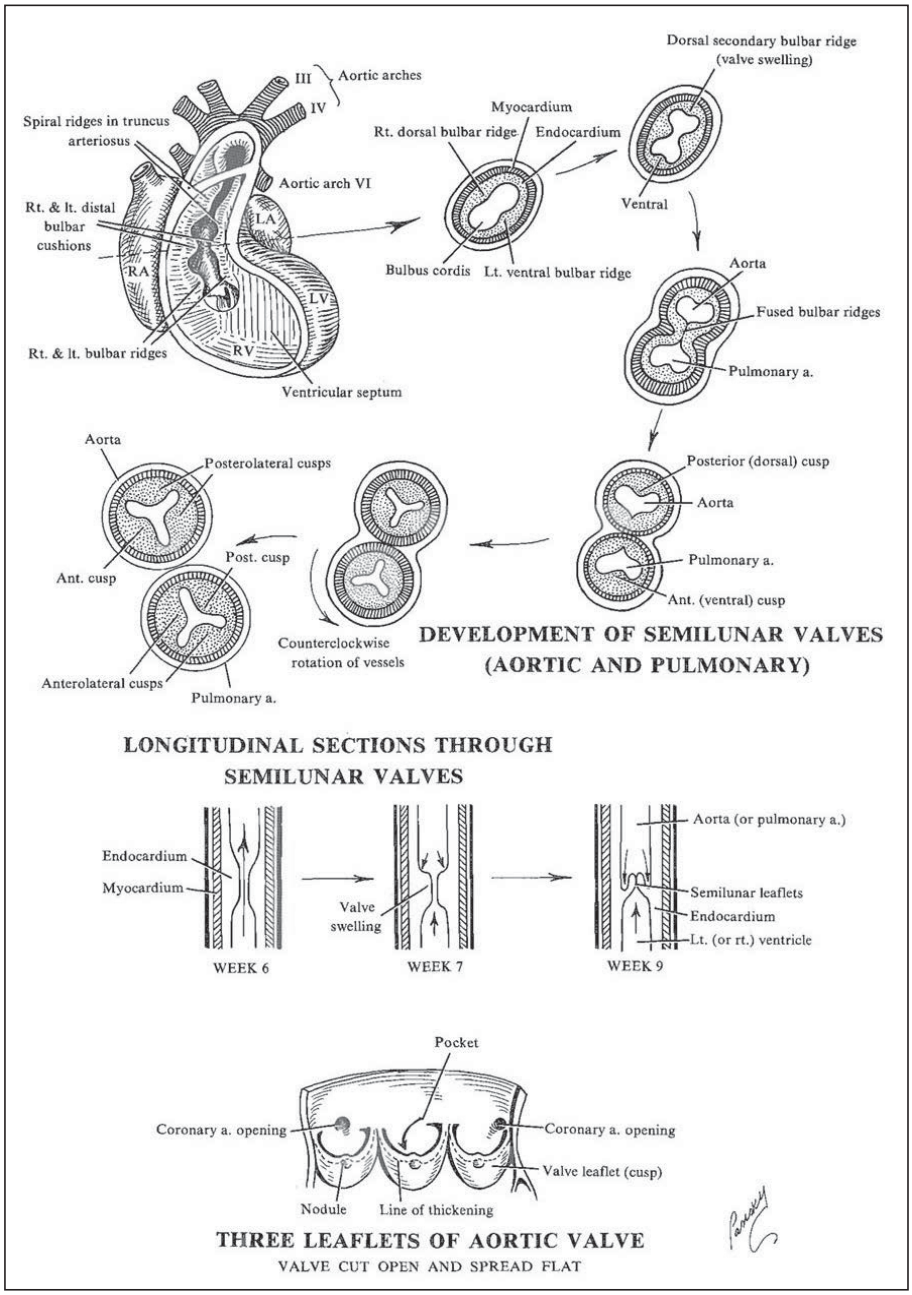

Figure 2. Embryology of the aortic valve. *Reprinted from the book "Review of Medical Embryology" by Ben Pansky MD PhD, 1982, with permission from The LifeMap Discovery Team http://discovery.lifemapsc.com* [31]

1.2.2 **Normal aortic valve anatomy and function**

Heart valves normally function to allow a unidirectional forward flow of the blood. Heart valves are in continuous motion throughout most of the cardiac cycle, thus requiring elasticity and strength to accomplish their function successfully and durably, as they withstand continuous stress and strain over the lifespan of an individual.[32]

The aortic valve is found at the junction between the left ventricular outflow tract (LVOT) and the aortic root. A collagenous aortic valve ring provides structural support to the aortic valve complex. The annulus is shaped like a crown and extends to the level of the aortic sinuses. The ring itself is further supported by its attachment to the vascular media distally and to the ventricular septum proximally and anteriorly.[33]

Thus, the aortic valve is normally tricuspid, with three semilunar leaflets. The aortic sinuses of Valsalva are small dilatations located just distal to the valve line, with a sinus corresponding to each leaflet (Figure 3). Each of the sinuses (and leaflets) is named after the corresponding coronary artery as the right, left and non-coronary sinuses (and leaflets). Normally, the left and right coronary arteries originate from the left and right aortic sinuses (in 91% and 93% of cases).[34]

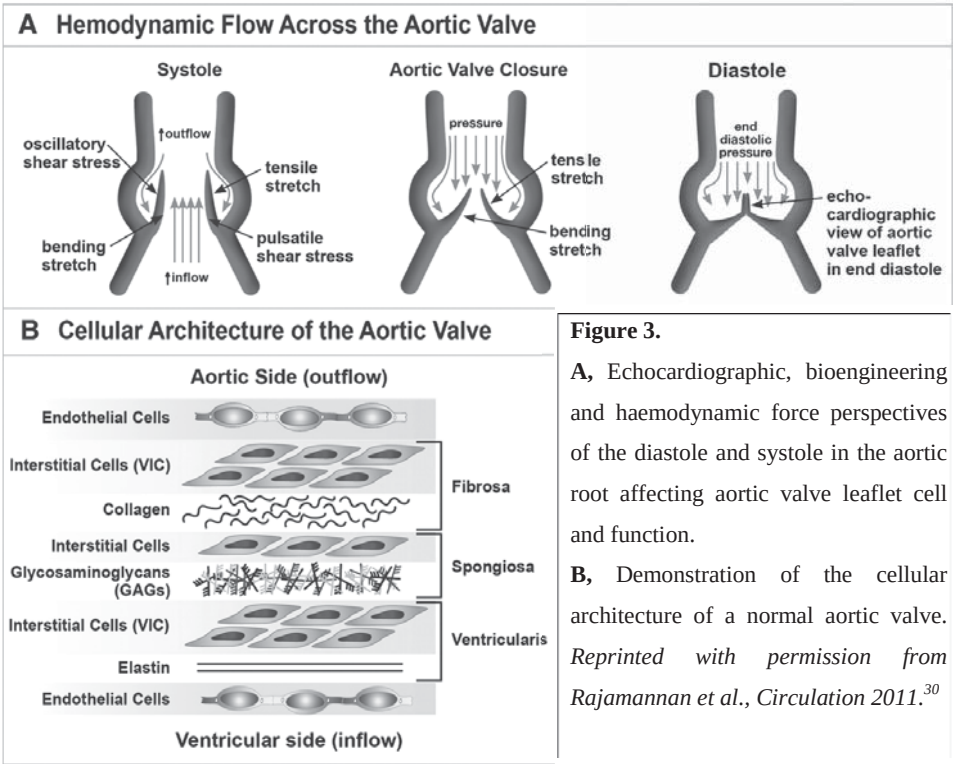

Figure 3.

A, Echocardiographic, bioengineering and haemodynamic force perspectives of the diastole and systole in the aortic root affecting aortic valve leaflet cell and function.

B, Demonstration of the cellular architecture of a normal aortic valve. *Reprinted with permission from Rajamannan et al., Circulation 2011.*[30]

At the centre of the free edge of each leaflet, there is a small fibrous bulge, named the nodule of Arantius. The rim (known as the 'lunula') of the free edge of each valve leaflet is slightly thicker than the body of the leaflet itself.. These lunulae of the different valve leaflets overlap at the time of valve closure in diastole to help seal the valve closure, in addition they provide leaflet support. The leaflet's rim can have fenestrations/holes, mostly located next to the commissures; however, these fenestrations have no known clinical significance.[34]

The leaflets of the valve have two distinctive surfaces, a ventricular surface and a vascular surface, each of which is exposed to different stress factors. During systole, blood flows with high velocity and opens the valve, whereas during diastole, the backward pressure of the blood fills the sinuses and closes the valve.[35]

1.3 CALCIFIC AORTIC VALVE DISEASE

Aortic valve sclerosis is the initial stage of progressive calcific aortic valve disease, especially calcific aortic valve stenosis (AS), which is the most common valvular heart disease in the Western world and accounts for most of the aortic valve replacements (see Table 1). Its number has doubled in the United States in the last 10 years, and figures are still on the rise.[36] Echocardiographic evidence of aortic valve sclerosis, with or without stenosis, can be found in around one-fifth of the population between 65-75 years of age [37, 38] and in around half of the population older than 80 years. [38, 39] In a post-mortem series of 72 patients published in 1951, 22 patients had aortic valve calcification (AVC) and 38 had coronary calcification.[40] The authors concluded that the higher age observed in AVC cases indicated that degeneration by age is an important factor in this condition. However, they pointed out that rheumatic infection was considered during the 1940s as a predominant reason for AVC, and that Mönckeberg suggested as early as 1904 that AVC had an atherosclerotic nature. Although aortic valve sclerosis and AVC were previously considered as degenerative phenomena and as natural consequences of aging, up to 50% of individuals older than 80 years have no echocardiographic evidence of AVC.[39]

Despite that not all studies find a relationship between AVC and lipids, smoking or diabetes,[39] several have found risk factors for AVC that were similar to those observed for atherosclerosis, such as old age, male sex, hypertension, elevated lipoprotein levels, smoking and type 2 diabetes mellitus.[38, 41-43] AVC seems to be not only part of generalized atherosclerosis,[44] but also a complex phenomenon that is related to other non-infectious and non-inflammatory processes.[45, 46] The process of AVC is even related to bone formation in human embryos.[47-49] Cell-mediated processes may influence calcium deposition, which is

more extensive and has an earlier onset in AVC compared with atherosclerosis.[43, 50] A spectrum of cell types is involved in the process of valvular calcium deposition, including not only valvular endothelial and interstitial cells, but also cardiac chondrocytes and circulating osteoprogenitor cells.[35, 51] AVC is composed of calcium phosphate, mainly carbapatite, in both BAV and TAV, irrespective of concomitant coronary disease. [52] Genetics play a role in the pathophysiology of AVC, as exemplified by BAV, which is considered a risk factor for the early onset and progression of AVC.[17, 53]

Calcific aortic valve disease progresses slowly through a series of different stages, from valve thickening to calcification with or without impaired leaflet function. AVC may lead to restricted leaflet opening, i.e., aortic stenosis and/or inadequate leaflet closure, as observed in aortic regurgitation (AR). Some terms that describe the stages of sclerotic aortic valve disease are defined in Table 1. A summary of the risk factors for AVC was presented by Mohler; they include dyslipidaemia, hypertension, diabetes mellitus, smoking, bicuspid valve, hyperparathyroidism, end-stage renal disease and Paget's disease.[54]

Table 1. Definitions of aortic valve sclerosis,[55] aortic valve calcification and aortic valve stenosis.[56]

Aortic valve sclerosis	Echocardiographically defined as focal areas of increased echogenicity and thickening of aortic valve leaflets with no evidence of aortic stenosis (V_{max} < 2 - 2.5 m/s).
Aortic valve calcification (AVC)	Defined as calcium deposition on aortic valve leaflets, as tiny spots or up to extensive calcification greatly affecting the mobility of the valve leaflets. On echocardiographic images, it presents as high echogenic (white) spots found on the valve leaflets.
Aortic valve stenosis (AS)	Subcategorized, according to the AHA/ACC 2014 guidelines, into stage A (patients at risk of AS), stage B (progressive AS with V_{max} = 2–4 m/s) and stages C and D (severe AS with V_{max} > 4 m/s; stage D patients are also symptomatic).

1.3.1 **Atherosclerosis and aortic valve calcification**

Atherosclerosis is a disease of elastic and large muscular arteries, in which atheroma is the characteristic lesion. In the process of atherosclerosis, the arterial intima is enlarged by the deposition of variable amounts and types of lipids, connective tissue, inflammatory cells and extra-cellular components, including proteins, enzymes and calcium deposits.[57-59] Atherosclerosis is the number one killer in the industrialized world. Extensive studies have

contributed to great progress in understanding its pathogenesis, risk factors, natural history, treatment and prevention.[60]

Thus, the development of aortic valve thickening and calcification is complex, and clearly related to,[44, 61] but not fully explained by, atherosclerosis, rather than a simple wear and tear aging process.[41, 43] As mentioned above, clinical studies that showed a significant association between risk factors of atherosclerosis and AVC support the hypothesis of its atherosclerotic nature.[62, 63] Pathological studies have also proven that certain similarities exist between vascular atherosclerosis and AVC.[43, 64, 65] AVC and atherosclerosis seem to have common initiating events.[66, 67] Nitric oxide release by the valvular endothelium is crucial for the maintenance of normal aortic valve elasticity.[68] The renin–angiotensin system also contributes to the development of AVC, as angiotensin II, which is known to be involved in atherosclerosis development, was identified in diseased valves together with the angiotensin-converting enzyme (ACE), but not in normal aortic valve leaflets.[69] The atherosclerotic process seems to continue even in advanced AVC, with accumulation of inflammatory cells, complement activation and expression of metalloproteinases.[70-73] An ongoing active process characterized by the presence of neovascularization,[74, 75] deposition of calcium[76, 77] and lipid accumulation[67, 75, 78] further proves the contribution of atherosclerosis to AVC development (Figure 4).

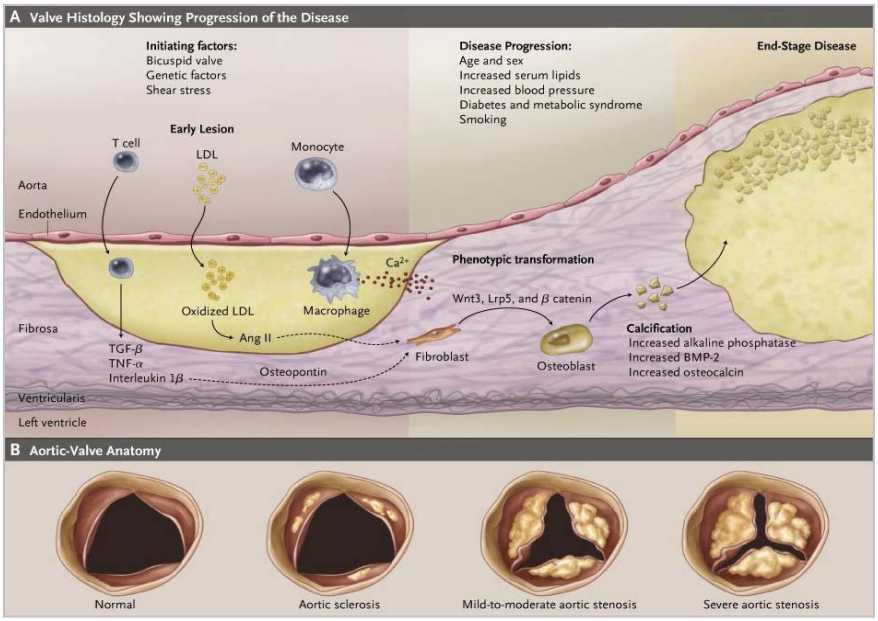

Figure 4. Disease progression in calcific aortic stenosis showing changes in aortic valve histologic features, leaflet opening in systole. *Reproduced with permission from The New England Journal of Medicine 2008, Copyright Massachusetts Medical Society.*[48]

1.3.2 Biomarkers and aortic valve calcification

Several biomarkers of AVC have been addressed in the literature. The presence of AVC was associated inversely with platelet nitric oxide, which is an intrinsic vasodilator, and its responsiveness, which is a marker of endothelial dysfunction.[79] An elevated blood level of cholesterol is a well-known risk factor for atherosclerosis.[80] Studies aiming to associate AVC with blood lipids reported mixed results, as some proved the association between AVC and hypercholesterolaemia [38, 81-83] and elevated levels of lipoprotein(a);[84] conversely, other studies failed to show this correlation.[85-87] Inflammatory markers such as C-reactive protein levels seem to be more closely associated with AS than with measures of AVC.[88, 89] However, C-reactive protein is related to AVC and is a prognostic marker of AVC,[90, 91] with mixed results of AVC being present without significant AS.[45, 92] A small study confirmed the relationship between AVC and the levels of plasma osteopontin,[93] a glycophosphoprotein that is involved in regulating bone remodelling.[94] Osteoprotegerin may counteract valve calcification, as shown in mice and humans,[95, 96] and is a prognostic marker of AS.[97]

1.3.3 Clinical importance of aortic valve calcification

Echocardiography is the main technique that is used for the evaluation of valvular disease. Echocardiographically, aortic valve sclerosis is defined as focal thickening of aortic valve leaflets with areas of increased echogenicity (calcium deposition) without restriction of leaflet motion and an antegrade Doppler velocity across the aortic valve < 2–2.5 m/s[55] (see Table 1).

Several studies showed that AVC has a strong predictive value for cardiovascular morbidity and mortality in the general population. In one study, AVC independently predicted coronary artery disease (CAD), to a greater extent than sex, hypertension, family history and hypercholesterolaemia.[98] An association was found between AVC and a higher prevalence of left ventricular hypertrophy, ventricular arrhythmias, myocardial infarction and systolic heart failure in the general population.[99] AVC is associated with cardiovascular events independent of Framingham risk factors, but does add to prediction in addition to Framingham risk factors and coronary artery calcification.[100] In another sample from the general population, AVC predicted cardiovascular mortality beyond information on risk factors, including coronary calcification.[101]

AVC predicted poor outcome in severe asymptomatic AS patients,[102] and even in patients with mild and moderate AS.[103] AVC was associated with reduced-flow-mediated

dilatation of the brachial artery,[104] and the AVC score was correlated with early atherosclerotic markers, such as carotid intima–media thickness (IMT) and dispensability.[105] AVC was associated with increased incidence of stroke and cerebral emboli.[106]

In patients with renal disease, AVC seems to be clinically and prognostically important. The prevalence of valvular calcification (including mitral annular calcification and aortic calcification) is increased among patients with chronic kidney disease undergoing haemodialysis. [37, 107-109]Although AVC is not directly associated with the degree of renal dysfunction,[110] it is associated with carotid IMT in patients on haemodialysis.[111] Further, AVC is a powerful predictor of cardiovascular morbidity and mortality in patients with long-term haemodialysis,[107, 112] and a predictor of restenosis of drug-eluting stents in patients with CAD and chronic haemodialysis.[113]

1.3.4 **Management of calcific aortic valve disease**

No specific treatment is currently available to prevent or reduce the rate of progression of the calcification of aortic valves. The involvement of cholesterol and other lipoproteins in the pathogenesis of AVC and AS found in experimental and clinical studies motivated the performance of lipid-lowering studies.[114] An excellent review of lipid lowering in AS summarized five retrospective studies and one prospective non-randomized study, which were positive, and discussed in detail two randomized studies (SALTIRE, n = 155; and SEAS n = 1873), with neutral results.[115] Another randomized study, the ASTRONOMER trial, showed that lowering LDL-cholesterol blood levels did not stop the progression of AS or AVC in patients with mild-to-moderate aortic valve disease.[116] Based on the involvement of the renin–angiotensin system in the development of AVC, it was hypothesized that the use of ACE inhibitors may halt or lead to the regression of calcium accumulation within aortic valve leaflets.[117] Ongoing controlled randomized trials including ACE inhibitors, statins and biphosphonates are addressing this issue; however, to date, there is no conclusive evidence that medical therapy slows the progression of AS.[118] Thus, although guidance for the medical prevention of calcific AS progression may be obtained within the next few years, the current treatment strategies deal with the haemodynamic consequences of severe AVC, including aortic valve stenosis and regurgitation.

1.3.4.1 *Diagnosis and management of aortic stenosis*

Based on the 2014 American College of Cardiology (ACC)/American Heart Association (AHA) Guideline for the Management of Patients with Valvular Heart Disease, a new approach has been adopted, not only regarding the way of writing and presenting

evidence, but also in describing the disease stage and management of patients with AS. A new grading system has been introduced based not only on the presence of symptoms and haemodynamic data, but also taking into account valve anatomy and calcium deposition, ranging from stage A (patients at risk of developing AS) reaching up to stage D (patients with severe symptomatic disease).[56, 119] These stages constitute an important basis for treatment strategies and recommendations, and support the recommendations of the Joint Task Force on the Management of Valvular Heart Disease of the European Society of Cardiology (ESC) and the Guidelines on the management of valvular heart disease of the European Association for Cardio-Thoracic Surgery (EACTS).[56, 119, 120]

Based on different levels of evidence, treatment and procedure recommendations mentioned in guidelines are classified into: class I (recommended treatment/procedure), class IIa (reasonably used), class IIb (might be used) and class III (may cause harm). According to the 2014 AHA/ACC Guideline,[56] medical therapy is mainly directed towards treating hypertension in all patients at risk of developing AS and in patients with AS stages B and C (Class I). Aortic valve replacement (AVR), either surgical or transcatheter aortic valve replacement (TAVR), is recommended for all patients with severe symptomatic AS (Class I). Patients with severe asymptomatic AS with left ventricular (LV) dysfunction or who are undergoing cardiac surgery for other indications are also recommended for aortic valve intervention (Class I). Aortic valve replacement is reasonable in patients with severe asymptomatic AS, patients with symptomatic low-flow/low-gradient severe AS with LV dysfunction (or normal LV function with severely reduced leaflet motion due to AVC) or patients with moderate AS undergoing cardiac surgery for other indications (Class IIa).

Surgical aortic valve replacement (AVR) is recommended in all patients who are indicated for AVR with low or moderate surgical risk (Class I). When surgical risk is high and the expected post-TAVR survival is longer than 12 months (as evaluated by a team including cardiologists, cardiac surgeons and cardiac imaging and anaesthesia professionals with good expertise in valvular heart disease), TAVR is indicated (Class I). In the absence of the professional team and uncertain 12-month survival after intervention, TAVR is a reasonable (Class IIa) indication.[56]

1.3.4.2 *Diagnosis and management of aortic regurgitation (AR)*

According to the 2014 AHA/ACC Guideline, AR is categorized into four stages, from stage A (patients at risk of developing AR), through to stages B and C and reaching stage D, which includes patients with severe symptomatic AR. The management of AR follows the stage of the disease and is further divided into medical therapy and intervention.

Medical therapy of AR is directed mainly towards lowering blood pressure (systolic > 140 mm Hg) using calcium-channel blockers (dihydropyridine), ACE inhibitors or angiotensin receptor blockers, and is recommended for all patients with chronic AR and hypertension (Class I). ACE inhibitors/angiotensin receptor blockers and beta-blockers are the drugs of choice (Class IIa) for patients with severe AR in addition to LV dysfunction and/or symptoms who are not fit for surgery. Intervention (AVR) is indicated for all patients with severe symptomatic AR, regardless of LV function, patients with severe asymptomatic AR with LV dysfunction and patients with severe AR undergoing cardiac surgery for other indications (Class I). Furthermore, AVR is recommended for patients with severe AR and dilated LV dimensions with no symptoms or LV dysfunction (Class IIa). Patients with moderate AR can have AVR while undergoing surgery of the ascending aorta, mitral valve or coronary artery by-pass graft (Class IIa).[56]

1.3.4.3 *Management of bicuspid aortic valve*

Thus, BAV is a congenital disease of the aortic valve, which is often associated with aortopathy with development of ascending aortic aneurysm and sometimes dissection. Its presence heralds complications that occur a decade earlier in life compared with TAV, such as AS, AR, infective endocarditis and thrombus formation.[121] Treatment of BAV can be divided into either treatment of the aortic valve diseases that developed or treatment directed at the control or treatment of the accompanying aortopathy. Currently, no drug therapies have been shown to reduce the rate of progression of aortic dilation in patients with BAV. Medical treatment is merited in patients with BAV and hypertension in whom the control of blood pressure can reduce further stress on the aortic wall.[56] Recommended therapies focusing on the aortopathy that is often associated with BAV include beta-blockers, ACE inhibitors, angiotensin receptor blockers and adrenergic receptor antagonists.[122, 123] Statins do not have any effect on mortality in patients with thoracic aortic aneurysm, despite the fact that they reduce it in patients with abdominal aortic aneurysm.[124] Despite the theoretical advantages of beta-blockers and angiotensin receptor blockers in reducing the progression of thoracic aortic aneurysm disease, no randomized clinical study has proven their beneficial effects.[56]

1.3.5 **Imaging of aortic valve calcification**

Several diagnostic modalities have been used for the assessment of AVC and its complications in clinical practice and in research. CT scanning and TTE are the most frequently used imaging techniques.

1.3.5.1 *Role of echocardiography*

Transthoracic echocardiography (TTE) is currently the most frequently used tool for aortic valve assessment, including morphology, the presence of AR, LV function, aortic pathology and concomitant abnormalities of other valves.[125] TTE is useful in identifying aortic valve morphology and type, although in the case of severely calcified valves, distinguishing BAV from TAV may be difficult.[35] TTE is well suited to determine the degree of stenosis and whether an increased velocity is caused by valvular or by sub- or supra-valvular stenosis. In cases with AR, TTE is useful in identifying the magnitude and cause of the regurgitation. In some cases, transoesophageal echocardiography (TOE) may be needed to assess aortic valve morphology, valvular dimensions prior to procedures, valvular and para-valvular lesions in addition to suspected complications, such as infective endocarditis.[125]

The guidelines for aortic valve assessment include the measurement of jet velocity and mean pressure gradient and the application of the continuity equation to calculate valve area. These measurements are exposed to several possible errors, because of variable measurement experience, the haemodynamic situation, machine settings and measurement standardization.[48] The assessment of the aortic valve by echocardiography should comprise several echo windows and views. These include parasternal long axis and zoom for measurement of the LVOT, the aortic sinus, dimensions at the sinotubular junction (STJ) and ascending aorta and short axis view of the aortic leaflets (Figure 5), as well as an apical long axis view of the aortic leaflets and an apical 5-chamber view of the aortic leaflets. The different view should include colour Doppler, and the 5-chamber view should include pulsed-wave and continuous-wave Doppler. In addition, suprasternal and right parasternal views may be required to assess maximum aortic flow velocity.

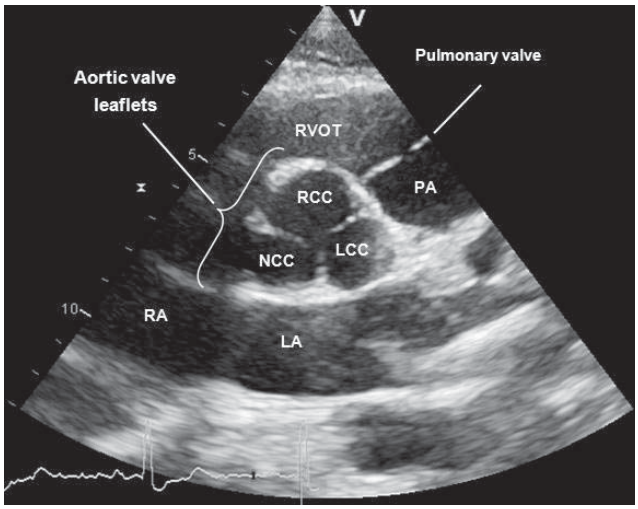

Figure 5.

Transthoracic echocardiography (TTE) short axis view at the level of the aortic valve showing a fully closed normal tricuspid aortic valve. LA, left atrium; RA, right atrium; RVOT, right ventricular outflow tract; NCC, non-coronary cusp; RCC, right coronary cusp; LCC, left coronary cusp. *From our local TTE image archive.*

1.3.5.2 *Role of computed tomography*

Echocardiography excels in haemodynamic measurements and for following up the development of AVC, especially at a late stage when it has developed to AS and/or AR. At an early stage, before haemodynamic alterations occur, CT can detect morphological changes and calcium depositions. The superior spatial resolution of CT enables it to provide the most accurate images and data regarding aortic valve anatomy and calcium content. CT calcium-scoring capabilities were developed especially for the quantification of coronary calcium content, and the presence of the aortic valve in the same plane facilitated the use of these capabilities to quantify AVC.

For the quantification of calcium using CT machines, the scanner detects calcium deposits and displays them as bright spots or areas in the image. AVC is then quantified using the method originally introduced by Agatston. The method can be summarized as follows: each attenuation of 130 Hounsfield units or above with an area ≥3 pixels is considered a calcium deposit. The Agatston calcium score is then calculated by multiplying the measured area by an attenuation coefficient that depends on the maximum attenuation value in the region of interest and is expressed in Agatston units.[126] Other measurements can be performed using CT, including 3-dimensional volumetric measurement[127] and mineral mass measurement. The latter measurement was used in several studies because of its high accuracy and reproducibility.[128-132] The introduction of multi-slice computed tomography (MSCT) improved image quality and provided shorter rotation times and thinner slices.[133] Currently, MSCT scanners are the most commonly used because of their lower radiation dose and lower cost.[134, 135]

CT provides the only non-invasive method of quantification of AVC. AVC CT scoring has gained additional importance recently with the increasing implementation of TAVR procedures, because of its high precision in detecting the position and amount of AVC, in addition to aortic valve dimensions, which are crucial for the selection of proper valve size.[136]

1.3.5.3 *Other in vivo techniques*

Because of its advantages of avoiding radiation exposure and providing good image quality, magnetic resonance imaging (MRI) has been used to assess the geometry of the aortic valve, determine effective and geometrical orifice areas and perform haemodynamic measurements. These measurements are particularly useful at a later stage of the AVC process, at which significant valve lesions have developed. Because of its inability to

diagnose calcification, its lower spatial resolution compared with CT and its higher cost, MRI cannot be used to assess AVC in its earlier stages.[125] It is worth mentioning here the presence of some efforts aimed at quantifying calcification in tissues using MRI ultra-short echo time imaging,[137] although without notable success to date.

A recent study showed good results for the evaluation of AVC and aortic valve inflammation using positron emission tomography (PET) in patients with AS, in whom the increased activity of the tracer used correlated with disease severity.[138]

Similar to MRI, cardiac catheterization is of value at the later stage of the disease, because of its superiority in providing reliable haemodynamic measurements. Despite the increasing use of cardiac catheterization in the past few years because of the increase in the use of TAVR procedures, its application is confined to later stages of AVC, for assessing haemodynamic valvular disorders; however, it is not as useful in the early stages of the disease because of its weakness in identifying and quantifying calcification.[56, 125]

1.3.5.4 *Ex vivo computed tomography*

Ex vivo CT scanning of different body tissue specimens to quantify calcium content is a growing field of research. It has been used to quantify calcium in carotid specimens[139] and in excised aortic artery leaflets.[133] Ex vivo CT AVC scoring is an appealing field of research, as it does not encounter some of the difficulties inherent to in vivo scanning, such as coronary ostial calcification, motion artifacts and calcification overestimation in contrast examinations.[140] In a single study using electron beam CT (EBCT), a good correlation was found between in vivo and ex vivo AVC.[140] In a micro CT study, AVC volume correlated well with AS severity.[141] Inter-scanner variability was further reduced and image quality improved after the introduction of MSCT, as it provided shorter rotation times and thinner slices.[133] The calcium-scoring accuracy and reproducibility of CT scanners were also improved by the introduction of new measurement units and calculation methods, such as mineral mass (MM) and calibration phantoms using calcium hydroxyapatite (CaHA).[129-133] Despite all these efforts in the field, there is currently no scanning and reconstruction protocol that is widely used or agreed upon, as the detection threshold, calibration factor, slice thicknesses and reconstruction protocols vary between different studies, which renders comparison tricky and not very reliable.

2 AIMS

The overall aim of the thesis was to evaluate imaging methods in vivo and ex vivo to estimate aortic valve calcification. The specific aims were as follows.

1. To investigate the accuracy of AVC estimation using a 5-degree visual scoring system on echocardiographic images (Studies I and II).
2. To compare TTE and TOE for AVC scoring (Study II).
3. To assess the value of GSM for the quantification of AVC (Studies I and II).
4. To develop a protocol for ex vivo CT calcium scoring (Study III).
5. To compare in vivo echocardiography to ex vivo AVC evaluation (Study IV).

3 METHODS

3.1 PATIENTS

The patients included in these studies were recruited from a large prospective study (Advanced Study of Aortic Pathology (ASAP)) that included 600 patients undergoing cardiac surgery because of aortic valve lesions and/or aortic root or ascending aortic pathology. ASAP is a unicentre study conducted at the Karolinska University Hospital, Stockholm. All patients underwent open-heart surgery at the Department of Thoracic Surgery, ultrasound investigations were performed at the Department of Clinical Physiology and blood tests were performed at the Department of Clinical Chemistry.

The exclusion criteria of the ASAP study were age <18 years, inability to give an informed consent or patient refusal to take part in the study, significant CAD, blood-borne infections, prior cardiac surgery and indication for other concomitant valve surgery.

The patients who were enrolled in the study were evaluated preoperatively by questionnaire regarding medical history, cardiovascular risk profile, concomitant diseases and medications, further laboratory tests, echocardiography and diagnostic coronary angiography. Intra-operatively, the surgeon determined the type of aortic valve by direct inspection (number of leaflets and commissures), in addition to scoring visually the calcification of the valve via the AVC scoring system used in the study (Table 2).

In this thesis, we performed four different studies. The first two studies (Studies I and II) included 185 and 169 patients; all patients included in Study II were part of Study I, and both studies included patients from the first 207 patients enrolled in ASAP. The first two studies concentrated on assessing AVC using echocardiography and testing a greyscale (GSM) computer software against visual scoring using the intra-operative score (IOS) as a gold standard. Study III was methodological in nature and did not include any patients. This study aimed at identifying the most suitable conditions for ex vivo CT scanning of small tissues for calcium scoring. In Study IV, 155 patients were included from among the later 300 patients of the ASAP (none of whom were included in the previous studies). In this study, we applied the results obtained in Study III for ex vivo CT scanning of the surgically excised aortic valves.

3.2 ECHOCARDIOGRAPHY

3.2.1 Transthoracic echocardiography

All patients underwent comprehensive TTE using a Philips IE33 ultrasound scanner with an S5-1 transducer (1–5 MHz) (Philips Healthcare, Best, the Netherlands). Aortic valve morphology and function were assessed by an experienced echocardiographer. Studies were performed within 1 week of surgery, and all images were stored digitally for offline analysis (Figure 6).

3.2.2 Transoesophageal echocardiography

A comprehensive TOE study was performed in the operating room immediately before the heart surgery using a Sequoia c512 ultrasound scanner (Siemens Medical Systems, Mountain View, CA, USA) with a V5Ms TOE transducer at a frequency of 6 or 7 MHz. All TOE studies were performed by experienced investigators, with focus on the aortic valve and aortic root morphology and function. Ultrasound images were digitally stored and analysed offline on dedicated work stations (Figure 6).

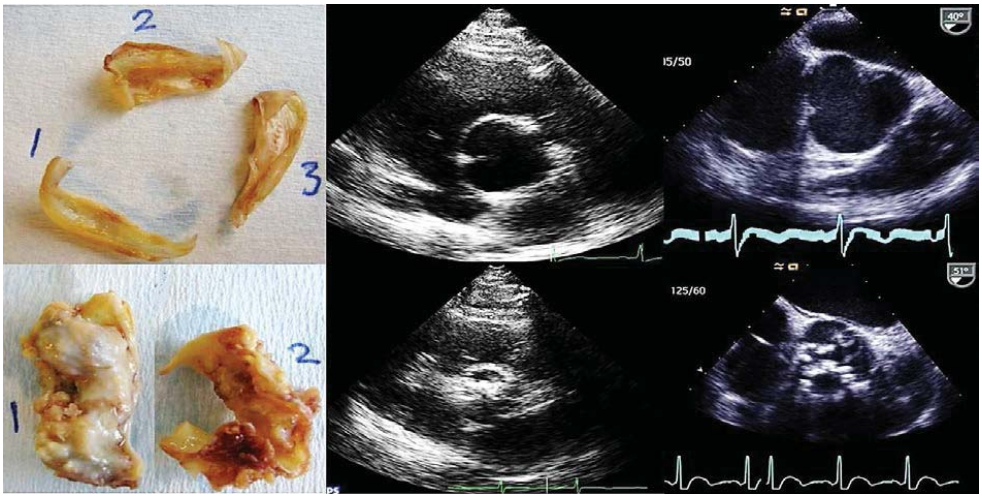

Figure 6. Sample valves from the study showing explanted valves, transthoracic and transoesophageal echocardiographic images (left to right). **Top**, mildly thickened tricuspid aortic valve. **Bottom**, heavily calcified bicuspid aortic valve.

3.3 AORTIC VALVE CALCIFICATION SCORING SYSTEM

A 5-grade AVC scoring system was used, starting at grade 1 (normal aortic valve leaflet(s)) and up to grade 5 (heavily calcified aortic valve leaflet(s)). Description of the five grades is summarized in Table 2.

Table 2. Aortic valve calcification scoring system.

Grade	Description
1	Normal leaflets with no evidence of thickening or calcification
2	Evidence of thickening (sclerosis), but no evidence of calcification
3	Calcification (small calcium spot(s) not exceeding one-third of the leaflet area)
4	Moderate calcification (calcification not exceeding two-thirds of the leaflet area)
5	Heavily calcified (calcification covering more than two-thirds of the leaflet area)

The scoring system was used in all visual assessments of echocardiography images, both TTE and TOE, image examples shown in Figure 6. Scoring was performed using real-time images from the short axis view at the level of the aortic valve at the base of the heart, where each leaflet was given a single score and the average of the valve AVC score was calculated. All AVC scoring was performed by two experienced echocardiographers (MY and AR) at different occasions. These individuals were unaware of the other's results. This applies to real-time and still image evaluation using either TTE or TOE images.

A still frame of the aortic valve in the parasternal short axis view at the end-diastolic phase with fully closed aortic valve leaflets was used to score AVC by assigning a single score to the whole valve.

3.4 GREYSCALE MEASUREMENT SOFTWARE

For the computerized evaluation of ultrasound reflection from the aortic valve, a software that was developed for atherosclerotic plaque analysis (Artery Measurement System, AMS-II) was used. It was developed by collaborative work between the Physiology Group at the Wallenberg Laboratory (www.wlab.gu.se), Gothenburg University, Sweden and

the Department of Signals and Systems at Chalmers University of Technology, Gothenburg to quantify intima–media thickness (IMT) and for greyscale evaluation of carotid plaque.[142]

A parasternal short axis view still frame with the aortic valve leaflets fully closed at end-diastole was used for AVC quantification using the AMS II (GSM) software (investigator MY), Figure 7. For each patient, a greyscale calibration was performed using a sample of the intravascular blood pool (avoiding areas of noise) as the black reference (greyscale value of 0), and the brightest part of the pericardium (or occasionally a calcium spot within the leaflets, if the pericardium was not visualized in the image used) as the white reference (greyscale value of 255).[143] A region of interest was manually drawn around the aortic valve leaflets, excluding the annulus, and the greyscale mean (GSMn) of the valve was automatically calculated by the AMS program. We used GSMn rather than the median, as valvular calcification may be unevenly distributed.

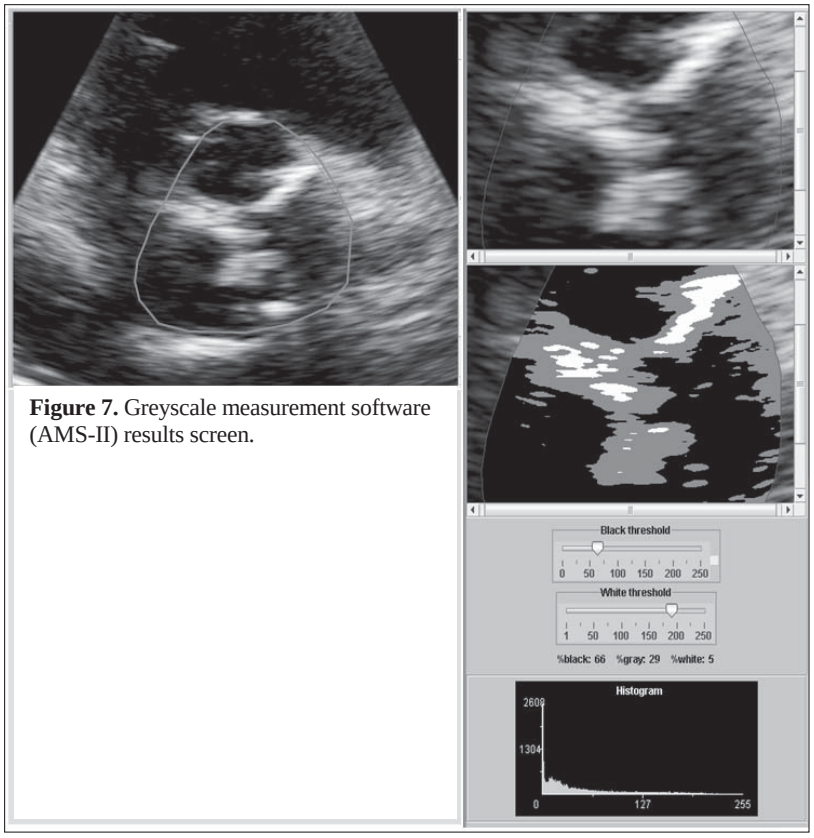

Figure 7. Greyscale measurement software (AMS-II) results screen.

3.5 INTRA-OPERATIVE SCORE

Intra-operative AVC scoring (IOS) was performed by the operating surgeon at the time of the operation via gross inspection and palpation of the aortic valve leaflets (Studies I, II and IV). The surgeon gave a single score for the whole aortic valve using the same 5-degree scoring system as that used for AVC scoring on echocardiographic images.

3.6 EX VIVO COMPUTED TOMOGRAPHY

Computed tomography was performed using a Siemens Symbia T16 True Point hybrid single-photon emission computed tomography/computed tomography (SPECT-CT) (Siemens Medical Solutions USA, Inc., Knoxville, TN) scanner (Studies III and IV). Raw data were processed on the main CT work station and stored digitally for further analysis using different reconstruction parameters. Calcium scoring was performed using the default Siemens calcium-scoring module found as part of the Symbia analysis software using a Siemens work station. The default program settings for in vivo coronary calcium scoring were used in addition to other modified protocols. The calcium-scoring program was used to detect calcification by applying the default or the pre-specified parameters. The detected calcifications were given a magenta colour by default, and were then manually selected by the user to score the calcium found in the selected area. The software provided calcium scoring in volume (mm^3), MM (mg CaHA) and calcium score. See Table 3 for definitions.

Table 3. Definitions of factors used in calcium scoring by computed tomography (CT).

Calibration factor	A reconstruction factor used by the CT calcium-scoring work station assuming the calcium density in a lesion/phantom. Measured in mg $CaHA/cm^3$.
Threshold value	A reconstruction factor used by the CT calcium-scoring work station to assign the lower threshold for the detection of calcium presence in a lesion/phantom. Measured in HU.
Slice thickness	A scanning and/or reconstruction parameter used by a CT scanner denoting the number of images taken/reconstructed per mm. Measured in number of images/mm
Mineral mass (MM)	A calcium measurement value used by the CT calcium-scoring work station to detect the amount of calcium found in a lesion/phantom. Measured in mg CaHA.

CaHA, calcium hydroxyapatite; HU, Hounsfild Units.

3.6.1 Computed tomography scanning of phantoms

In our methodological study (Study III), different types of phantoms containing CaHA at different concentrations in different types of surrounding media were used. Therefore, we used different CT scanning and reconstruction parameters to identify the most suitable conditions for ex vivo tissue CT scanning and calcium scoring. The commercially available Cardiac CT Calibration Insert Phantom for the Quantification of Coronary Calcium (QRM, Möhrendorf, Germany) contained nine cylindrical CaHA inserts with different size, density and mass, plus two large calibration inserts made of a water-equivalent material and 200 mg/cm^3 CaHA (QRM Phantom product manual; http://www.qrm.de/content/pdf/QRM-Cardio-Phantom.pdf) (Figure 8).

Figure 8. Cardiac CT Calibration Insert Phantom for the Quantification of Coronary Calcium (QRM, Möhrendorf, Germany).

Further, we used a series of "in-house-produced" test-tubes containing different concentrations of CaHA (50, 150, 250, 500, 1000, 1500 and 2000 mg CaHA) immersed in different types of surrounding media (air, 0.9% saline and 70% ethanol), Figure 9. We used 4.5 × 1 cm plastic tubes with a screw plastic cap. Scanning was always performed in the median plane of the scanner. We also put the tubes in a larger container that had the same type of surrounding media as the small tube, to test the effect of increasing the amount of surrounding media on the results obtained.

Figure 9. "In-house-produced" test-tube phantoms containing known masses of calcium hydroxyapatite (CaHA).

3.6.2 Computed tomography of explanted aortic valve leaflets

In Study IV, all valves that were explanted during open-heart surgery were immediately stored either in RNA-later (ribonucleic acid stabilization and storage reagent solution) or in formaldehyde solution for 24 h, followed by ethanol treatment, until analysis. Each valve was scanned in a plastic culture dish immersed in normal saline solution (sodium chloride, 0.9%). Before CT scanning, the valve fragments were dried from the medium, carefully put in the plastic dish and then introduced into the CT machine. The actual scanning was performed at a slice thickness of 0.6 mm, and reconstruction of the offline-saved raw data file was done using the B50 reconstruction protocol, 0.75 mm slice thickness, a threshold level of 130 HU and a calibration factor of 0.600 mg CaHA/cm^3, which were the settings that proved to be most reliable and reproducible, as described in our phantom investigation (Study III). For the offline analysis of calcium content, we used the Siemens CT work station calcium-scoring software, as described previously. The CaHA equivalent MM of each valve was then calculated and used for later comparisons.

3.7 EX VIVO VALVE WEIGHT

The weight of each valve was obtained using a Sartorius Extend ED153-CW scale (Sartorius AG, Göttingen, Germany) with a sensitivity of 0.001 grams. The scale was calibrated with an atmosphere seal. Prior to weighing, all valve fragments were carefully dried from any excess medium using soft study tissue. The total valve weight was recorded. The valve was then returned to the tube together with the medium for later studies.

3.8 LABORATORY INVESTIGATIONS

All blood samples were obtained in the fasting state and were analysed for plasma concentrations of creatinine, high-sensitivity C-reactive protein (hsCRP) RP, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and serum concentrations of cholesterol and triglycerides at the Laboratory of Clinical Chemistry at Karolinska University Hospital using standard methods (Unicel DXC 800 Synchron, Beckman Coulter, Brea, CA, USA).

3.9 STATISTICAL ANALYSIS

For all statistical analyses, IBM SPSS (Statistics Chicago, IL, USA) was used. We used the latest versions of the program that were available at the time of the study (versions 17.0.2 and 22.0). Descriptive statistics and frequencies, mean values and standard deviations are presented for normally distributed data. Pearson's product moment correlation coefficient (r) was computed to evaluate the relationship between two variables. We used Wilcoxon's test for paired samples, and the Mann–Whitney U test for unpaired samples in Study I.

Student's *t* test and the chi-squared test were used as applicable to compare means and determine significant differences between variables and characteristics in Studies II and IV. Inter- and intra-observer variability were analysed using the intra-class correlation coefficient (ICC), with values above 0.75 representing good correlation and values between 0.4 and 0.75 indicating fair reproducibility. A linear regression analysis was used to create the regression formula used to predict valve weight using CT MM measurement. Coefficients of variation (CV) were computed to illustrate reproducibility in Study III. A *p*-value of < 0.05 was considered significant in all studies of the thesis.

3.10 ETHICAL CONSIDERATIONS

The Regional Ethics Review Board at Karolinska Institutet, Stockholm, Sweden approved the study protocol and all patients gave their informed consent to participate.

4 RESULTS

4.1 IN VIVO ASSESSMENT OF AORTIC VALVE CALCIFICATION (STUDIES I, II AND IV)

As echocardiography is the dominant method for evaluation of cardiac valves, a major goal was to determine the possibility to estimate AVC by this technique, using both TTE and TOE approaches. We compared the different methods to interpret the echocardiographic recordings with an intra-operative 5-degree score (Table 2) of a similar construction (Studies I and II), and to valve weight and ex vivo CT (Study IV).

In Study I, we used the TTE investigations from 185 patients (104 with BAV) to compare visual AVC scoring and greyscale assessment with the surgeons intra-operative score (IOS). In Study II, in all patients of Study I who had an available TOE investigation (n = 169), we evaluated if a higher resolution or better image with TOE would increase the diagnostic accuracy compared with the IOS. Finally, in Study IV, we evaluated, in 155 patients, the accuracy of the echocardiographic evaluations of AVC, as well as the intra-operative scoring against the actual weight of the valve, and the CT scoring. The relation between the latter two was also analysed.

Baseline characteristics and echocardiographic measurements of the 185 patients and subgroups studied here are summarized in Tables 4 and 5. The demographic data used in Study II did not differ from that of Study I. Thus, for Studies I and II, the average age was 63.9 ± 11.6 and 63.7 ± 11.6 years, and the proportion of females was 31% and 30.2%, respectively. A higher percentage of dilated aortic roots (52%) was observed in the BAV group, up to 72% in patients with AR, compared with only 30% in the TAV group, and as low as 7% in TAV with AS.

The higher-age group of BAV and TAV (≥ 65years) included more females and more AS cases than did the younger-age group (< 65years). Lesion type, haemodynamics and GSMn according to subgroup are shown in Tables 4 and 5. For comparison between studies, some demographics used in the in vivo investigations (Studies I, II and IV) are shown in Table 6.

The observer variation for AVC scoring (Table 2 and Figure 10) was low, with high correlations. In Study I, the inter- and intra-observer variability measured by ICC for real-time TTE scoring were 0.93 and 0.93, respectively, whereas those for still frames were 0.90 and 0.85, respectively. In Study II, where we explored whether TOE images would improve

the correlations to IOS, the ICC between two observers was 0.93 for real-time TTE and 0.94 for TOE.

Table 4. Patient characteristics according to valve type (Study I).

	All patients (n = 185) n (%)	BAV patients (n = 104)		TAV patients (n = 81)	
		< 65years (n = 66) n (%)	≥ 65years (n = 38) n (%)	< 65years (n = 25) n (%)	≥ 65years (n = 56) n (%)
Mean age (years)	63.9 ± 11.6	54.6 ± 8.9	72.1 ± 4.8	54.4 ± 8.2	73.6 ± 5.1
Females, n (%)	57 (31)	15 (23)	14 (37)	4 (16)	24 (43)
Weight (kg)	82 ± 15	85 ± 14	77 ± 12	88 ± 11	80 ± 17
Height (cm)	175 ± 10	178 ± 9	173 ± 8	178 ± 9	171 ± 11
BSA (m^2)	1.97 ± 0.2	2.03 ± 0.18	1.9 ± 0.17	2.06 ± 0.15	1.92 ± 0.23
AS, n (%)	109 (59)	34 (52)	34 (89)	4 (16)	37 (66)
AR, n (%)	61 (33)	24 (36)	1 (3)	20 (80)	16 (28)
AS/AR, n (%)	8 (4)	5 (8)	2 (5)	0	1 (2)
AAD, n (%)	7 (4)	3 (5)	1 (3)	1 (4)	2 (4)

AAD, aortic aneurysm or dissection; AS, aortic stenosis; AR, aortic regurgitation; BSA, body surface area; BAV, bicuspid aortic valve; TAV, tricuspid aortic valve.

In group comparisons the ultrasound reflectivity, objectively measured in terms of the GSMn of end-diastolic still frames of the fully closed aortic valve, was higher in AS than in AR, for both BAV and TAV (Table 5). The GSMn evaluated in still frames was clearly higher in AS than in AR, and somewhat higher values for AS were obtained in TTE compared with TOE (Figure 11). The AVC difference between BAV and TAV was not more obvious in GSMn than in the visual scoring of still frames (Table 7). In contrast, both the IOS and real-time evaluation by both TTE and TOE showed highly significant differences between BAV and TAV. Furthermore, the overall score was numerically higher and essentially similar using IOS and real-time evaluation than using still frame interpretation, which yielded lower scores (Table 7 and Figure 12). The differences between BAV and TAV were essentially noted in the AR groups (Figure 12).

Table 5. Echocardiography data and study results according to aortic valve phenotype and primary lesion (Ascending aortic aneurysm and mixed lesion valves not shown) (Study I).

Variables	Bicuspid valve (n = 93)		Tricuspid valve (n = 77)	
	AS (n = 68)	AR (n = 25)	AS (n = 41)	AR (n = 36)
Aortic root, ED diameter (mm)	36.1 ± 5.8	42.0 ± 6.7	32.1 ± 4.3	42 ± 6.9
Ascending aorta, ED diameter (mm)	40.4 ± 7.6	44.6 ± 7.6	33.8 ± 4.6	43.4 ± 9.4
Ascending aorta, ED diameter > 40 mm, n (%)	29 (43)	18 (72)	3 (7)	18 (50)
LVOT, ES diameter (mm)	22.8 ± 2.8	27.4 ± 3.9	20.8 ± 1.8	24.4 ± 3.4
Aortic valve area (cm^2)	0.9 ± 0.4	3.0 ± 1.0	0.8 ± 0.3	3.2 ± 1.1
Aortic valve peak gradient (mmHg)	80.1 ± 31.4	20.4 ± 14.4	77.6 ± 19.9	15.4 ± 8.0
Aortic valve mean gradient (mmHg)	51.1 ± 19.7	12.0 ± 8.7	48.9 ± 13.5	8.0 ± 3.9
Surgical AVC score	4.5 ± 0.7	2.5 ± 1.1	4.3 ± 0.9	1.4 ± 0.6
Greyscale mean	78.0 ± 18.2	44.2 ± 18.8	91.3 ± 22.1	34.8 ± 13.5

AR, aortic regurgitation; AS, aortic stenosis; ED, end-diastolic, ES, end-systolic, AVC, aortic valve calcification; LVOT, left ventricular outflow tract.

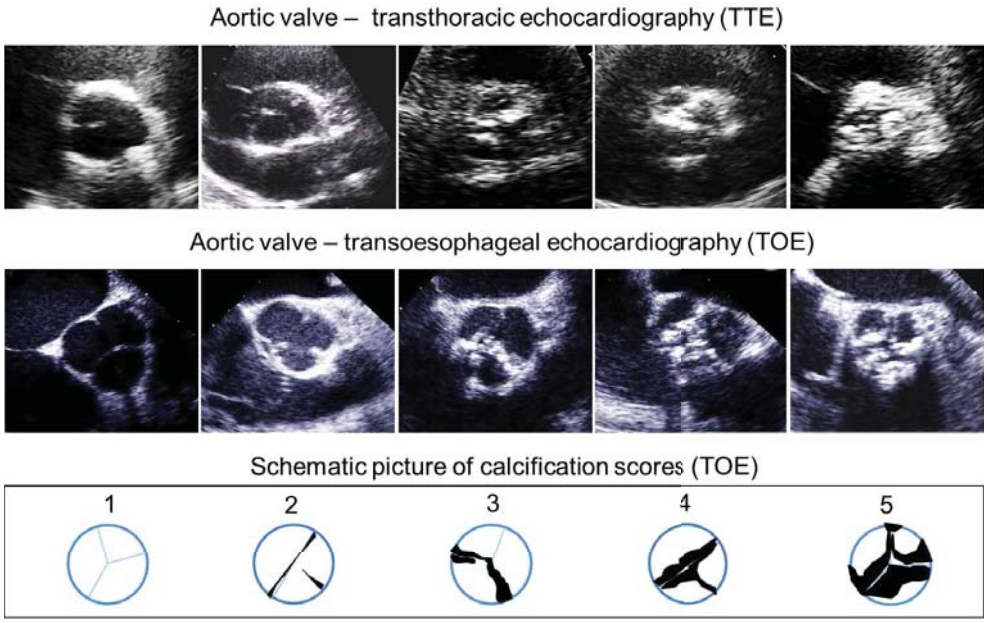

Figure 10. Transthoracic and transoesophageal short axis view images from our studies showing aortic valves representing the 5 grades used in our scoring system and their schematic drawings. [144]

Table 6. Comparison of age, sex, aortic diameter and aortic stenosis in the echocardiographic investigations (Studies I, II and IV) according to valve type.

	n	Age (years)	Female n (%)	AscAo mm	AscAo > 40 mm n (%)	AS n (%)
Study I	185	63.9 ± 11.6	57 (31)	40.7 ± 8.6	78 (42)	109 (59)
BAV	104	61.0 ± 11.4	29 (28)	42.1 ± 8.0	54 (52)	68 (65)
TAV	81	67.7 ± 10.8	28 (35)	38.8 ± 9.0	24 (30)	41 (50.6)
Study II	169 (all in Study I)	63.7 ± 11.6	51 (30)	40.9 ± 8.5	72 (43)	99 (58.6)
BAV	98	61.2 ± 11.7	27 (28)	42.1 ± 7.7	51 (52)	64 (65.3)
TAV	71	67.1 ± 10.8	24 (34)	39.2 ± 9.1	21 (30)	35 (49.3)
Study IV	155 (none in Studies I or II)	64.6 ± 12.6	51 (33)	40.1 ± 8.0	71 (46)	107 (69)
BAV	81	58.9 ± 12.0	19 (23)	41.8 ± 7.4	46 (57)	52 (64)
TAV	74	74.7 ± 10.1	32 (43)	38.3 ± 8.2	25 (34)	55 (74)

AS, Aortic stenosis; AscAo, ascending aorta; BAV, bicuspid aortic valve; TAV, tricuspid aortic valve.

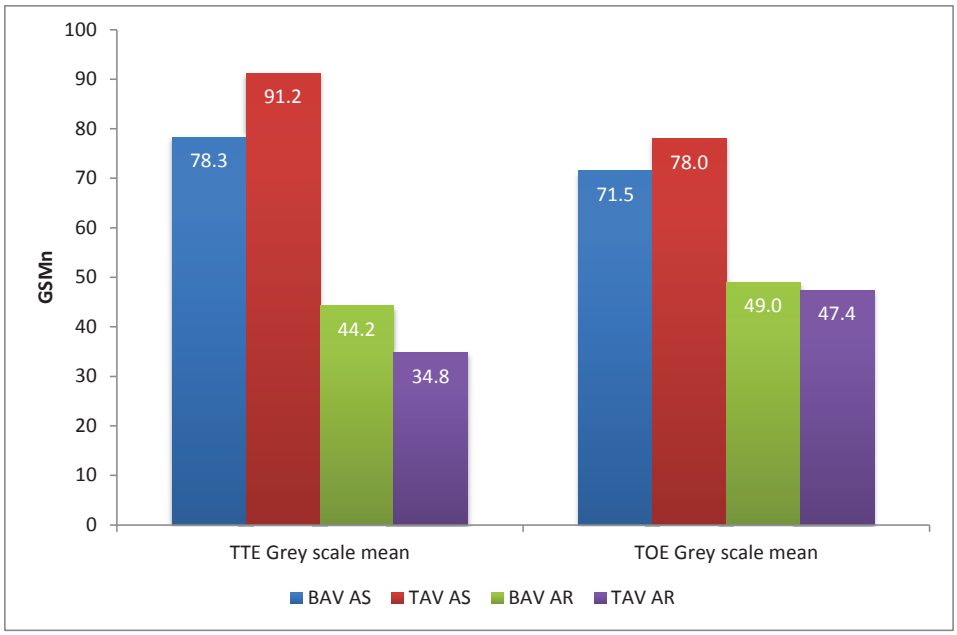

Figure 11. Greyscale mean (GSMn) in relation to the valve type and the aortic valve lesion. BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; AS, aortic stenosis; AR, aortic regurgitation, TTE, transthoracic echocardiography; TOE, transoesophageal echocardiography.[144]

Table 7. Mean values of different AVC scoring methods, BAV vs TAV groups (Study II).

	All patients (n = 169)	BAV group (n = 98)	TAV group (n = 71)	*P*-value
Aortic valve calcification				
Mean intra-operative score, score 1–5	3.5 (1.5)	4.0 (1.2)	2.8 (1.7)	< 0.001
TTE Greyscale mean, grey level 0–255	66.6 (28.8)	68.9 (24.4)	63.4 (33.9)	0.217
TOE Greyscale mean, grey level 0–255	64.1 (21.1)	64.9 (20.2)	62.9 (22.3)	0.529
TTE still frame AVC score, score 1–5	2.5 (1.2)	2.7 (1.1)	2.3 (1.3)	0.037
TOE still frame AVC score, score 1–5	2.0 (1.1)	2.1 (1.0)	1.9 (1.1)	0.118
TTE real-time AVC score, score 1–5	3.6 (1.3)	3.9 (1.1)	3.1(1.5)	< 0.001
TOE real-time AVC score, score 1–5	3.3 (1.3)	3.6 (1.1)	2.8 (1.3)	< 0.001

BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; TTE, transthoracic echocardiography; TOE, transoesophageal echocardiography, AVC, aortic valve calcification.

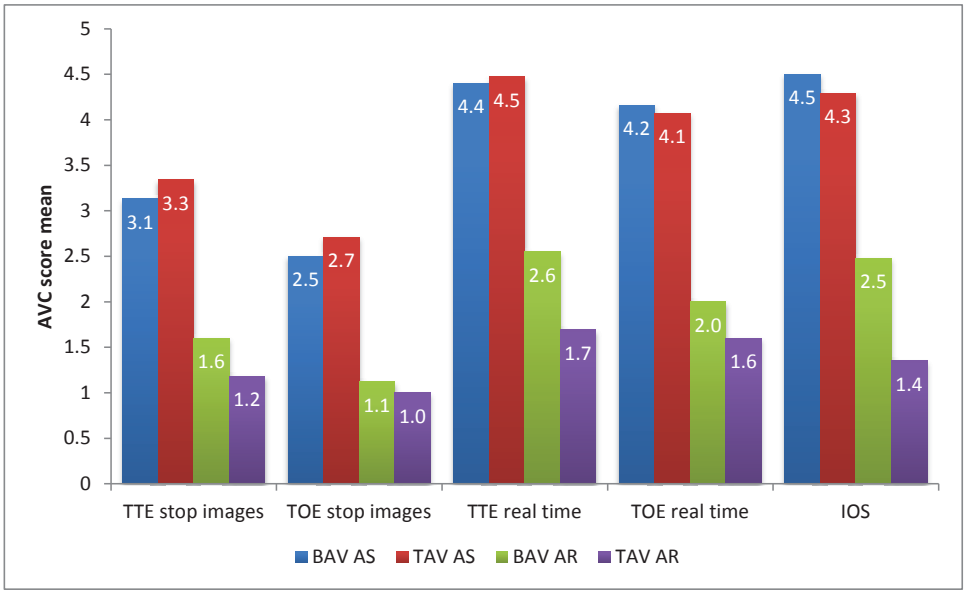

Figure 12. Mean aortic valve calcification (AVC) score according to valve lesion, valve type and type of evaluation (Study II). BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; AS, aortic stenosis; AR, aortic regurgitation; TTE, transthoracic echocardiography; TOE, transoesophageal echocardiography; IOS, intra-operative score.[144]

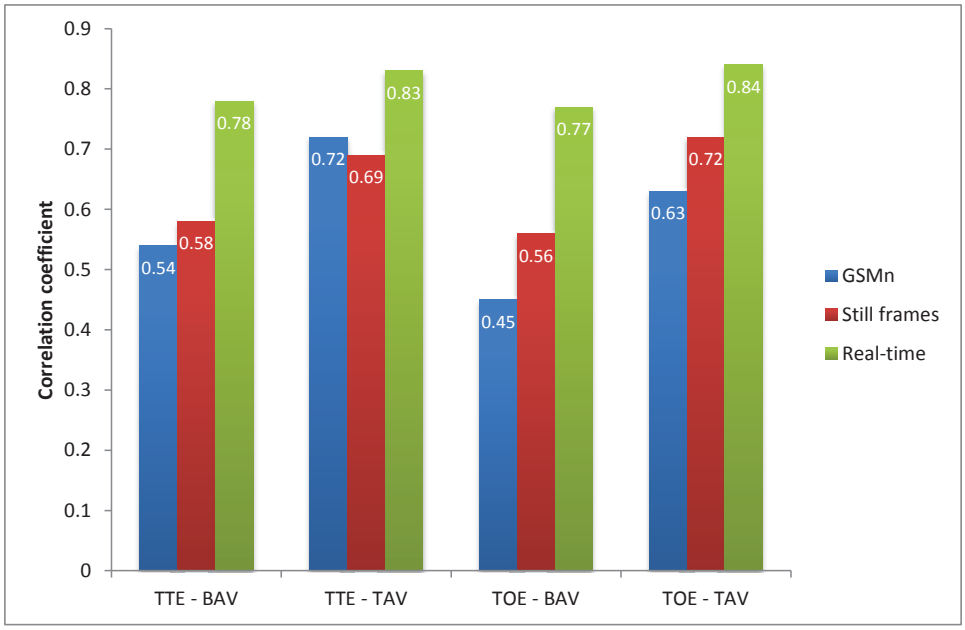

Figure 13. Pearson's correlation coefficients for the comparison with intra-operative score using visual scoring of still frames and real-time loops (Study II). BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; TTE, transthoracic echocardiography; TOE, transoesophageal echocardiography; IOS, intra-operative score.

In TTE images, the GSMn did not improve the correlation with the intra-operative evaluation (r = 0.73 vs. 0.72 for TAV and r = 0.56 vs. 0.57 for BAV; Table 4 in Paper I). The relationships between the ultrasonic evaluations and the IOS in Study II are shown in terms of correlation coefficients (Figure 13). It is obvious that GSMn did not for TTE, neither for TOE, provide better correlations to IOS than did the visual scoring of still frames.

Higher correlations with the intra-operative score were obtained when real-time evaluation of TTE was used (overall correlations to IOS for two independent observers r = 0.83 and r = 0.82; see Table 4 in Paper I). The correlations were somewhat higher for TAV (r = 0.83 and 0.84) than for BAV (r = 0.78 and 0.76). Also in Study II scoring of real-time loops yielded the closest correlations with IOS overall: r = 0.83 for TTE and r = 0.82 for TOE. The real-time score correlations to IOS were somewhat higher numerically within the TAV group than within the BAV group, but less than for visual or GSMn evaluation of still frames (Figure 13). As seen in Figure 13, correlations to IOS were not higher for TOE than for TTE applying GSMn or visual AVC scoring.

Thus, the evaluation of both TTE (Study I) and TOE (Study II) by visual scoring and GSMn showed that real-time evaluation was superior to evaluation using still frames, both

via eyeballing or greyscale measurement. Therefore, we focused on real-time evaluation to determine its accuracy in greater detail by applying both another cohort and another gold-standard approach (Study IV). For demographic comparison, see Table 6. Compared with Studies I and II, the Study IV TAV group was somewhat older, with a slightly higher proportion of women compared with those in Study I. Within the cohort of Study IV, valve type groups (TAV 74 (48%) and BAV 81 (52%)) did not show significant differences regarding clinical data or laboratory results, with the exception of male predominance and lower age in BAV compared with TAV (Table 1 of Paper IV). In addition to the evaluation of real-time loops in the short axis, one leaflet at a time as described in Studies I-II, we added a composite visual evaluation of the aortic valve from different views in the TTE recordings. The intra-operative score was also compared with the gold standard used in Study IV, i.e., the mineral mass measured by ex vivo CT, and with valve weight. In addition to the comparison with the gold standards, we compared the estimation of AVC with aortic valve area and mean pressure; with hsCRP level, cholesterol, serum creatinine, body surface area and mass index; and with medical history of diabetes, hypertension, stroke and other diseases (Table 2 of Paper IV).

All estimations of AVC were related to aortic valve area and mean pressure, but none were significantly related to any of the other mentioned variables. Interestingly, the echocardiographic evaluations and IOS were related more closely to aortic valve area and mean gradient than were the mineral mass and valve weight. We also evaluated separately in the BAV and TAV groups the relationships between all measures of AVC and valve weight, aortic valve area and mean gradient, as well as hsCRP. There were significant relationships between all parameters, with the exception of hsCRP, and the strongest relationship observed was that between mineral mass and aortic valve weight in both BAV and TAV. There was no clear difference between the real-time methods of evaluation of AVC; importantly, TOE was not better than TTE. Moreover, in valve groups, the relationships with valve lesion measures were weaker for mineral mass and valve weight than they were for echocardiographic measures of AVC.

4.2 AORTIC VALVE PHENOTYPE (STUDY II)

An additional goal of Study II was to compare the accuracy of the diagnosis of aortic valve BAV phenotype by TTE and TOE using the intra-operative assessment as the gold standard. We used real-time images from both the TTE and TOE studies to determine aortic valve phenotype. The results (summarized in Table 8) showed that using TOE images enabled the investigator to diagnose the presence of BAV with a sensitivity of 92% and a

specificity of 94%; moreover, the inter-observer agreement between two independent investigators was excellent, as denoted by a kappa value of 0.86. Conversely, using TTE, BAV was recognized with a sensitivity of 77%, a specificity of 82% and a moderate Kappa agreement value of 0.57. When no or mildly calcified valves were considered separately, the specificity and agreement values for diagnosing BAV were slightly better compared with assessments performed considering more calcified valves, both for TTE and TOE. TOE was also superior to TTE in this group of patients.

Table 8. Bicuspid aortic valve diagnosis in relation to AVC degree; agreement with the surgical intra-operative assessment (Study II).

Compared with intra-operative assessment		True positive	True negative	False positive	False negative	Agreement (κ value)	Sensitivity	Specificity
Mild or no AVC (AVC score ≤ 3)	**TTE**	18	30	5	7	0.58	72%	78%
	TOE	22	34	1	3	0.86	88%	97%
Moderate to severe AVC (AVC score > 3)	**TTE**	57	28	8	16	0.53	78%	78%
	TOE	68	33	3	5	0.84	93%	92%
All valves	**TTE**	75	58	13	23	0.57	77%	82%
	TOE	90	67	4	8	0.86	92%	94%

AVC, aortic valve calcification score; κ, kappa value. Inter-observer agreement: κ = 38% and 90% for transthoracic (TTE) and transoesophageal (TOE) echocardiography, respectively.

4.3 CALCIUM CONTENT—PHANTOM CT STUDIES OF SMALL OBJECTS (STUDY III)

To use ex vivo CT as a gold standard for the evaluation of echocardiographic techniques, and to develop a useful protocol for ex vivo CT applications using small tissue materials, we performed this methodological study. As both the settings of equipment and surrounding media may influence the results, we evaluated various alternatives of both parameters. In addition to different settings, we used two types of phantoms. Each step was designed to test different values of a single parameter while keeping the other unchanged.

We found that the calcium scoring software of the CT work station (originally designed for scoring coronary calcium) was not able to quantify calcium masses of more than

2 g as a single mass when scanned in air. This was demonstrated when we used five test-tubes containing 1, 2, 3, 4 and 5 g of CaHA in air, in which the software could detect all CaHA in all tubes, but failed to score masses beyond 2 g, as this value exceeded its maximum measuring capacity, as denoted by the software.

We then aimed at testing the effect of different media surrounding the CaHA mass to be scored. For this, we used two tubes containing 50 and 150 mg of CaHA per each medium to be studied. We tested several media, including air, normal saline, zinc formaldehyde, OCT (optimum cutting temperature imbedding medium), ethanol and RNA-later. Within this step, we also tested two different calibration factors during offline scoring of CaHA, with the machine defaults at 0.772 mg CaHA/cm^3 and 0.600 mg CaHA/cm^3.

Favourable results were obtained when using 0.600 mg CaHA/cm^3 as a calibration factor and saline, which showed values of 51.4 and 149.9 mg for the 50 and 150 mg CaHA tubes. Good results were obtained when measuring the 50 and 150 mg CaHA tubes using zinc formaldehyde (54.9 and 158.8 mg), OCT (51.0 and 150.7 mg), followed by ethanol (39.8 and 121.7 mg); less favourable results were obtained using air as the surrounding medium (19.5 and 68.7 mg, respectively). RNA-later as a surrounding medium was itself detected as calcium, showing the whole tube as a single calcium mass.

When the QRM phantom was evaluated using both 130 HU and 90 HU thresholds, while other parameters were kept fixed, we did not find any significant difference between the 130 HU and 90 HU thresholds regarding the measured values of the true CaHA masses (range, 72.1–96.3% and 72.6–96.9%, respectively). These results were obtained by measuring masses ranging between 4.2 and 78.5 mg CaHA inserts. Tiny inserts (0.2–0.6 mg CaHA) were either not detected or scored at a much lower percentage of their true values.

When the same experimental 130 HU and 90 HU thresholds were applied to the “in-house-produced” phantom tubes containing 50 and 150 mg CaHA in either air or saline, similar results were obtained, with no significant differences.

In the next step, we compared slice thicknesses of 1 and 0.75 mm. We used the 0.75 mm slice thickness as a default, which can be theoretically more accurate for small masses. However, no significant difference was noted when the results were compared with those obtained using a 1 mm slice thickness and a threshold of 130 HU. In contrast, the use of the 1 mm slice thickness was marginally more accurate compared with 0.75 mm when the 90 HU threshold was used; this was only shown at larger insert masses (19.6 mg CaHA and above), and not at smaller masses.

The effect of the surrounding medium was further tested using “in-house-produced” tubes containing known masses of CaHA inserted in air, saline or ethanol. Saline was superior to the other media as a surrounding medium for ex vivo scanning of tissues for calcium scoring (range, 83.8–103.9% of the true CaHA mass). Air was the least favourable medium, with the measured CaHA mass ranging between 31.6% and 45.2% of the true mass.

When evaluating the effect of the amount of the surrounding medium on the measurements, we found that saline was superior to air and ethanol. Larger amounts of the surrounding medium (in a container) resulted in lower measured values compared with lower amounts of the surrounding media, probably because of increased attenuation.

Reproducibility was assessed from eight scanning sessions that were performed more than one week apart both for the QRM phantom with its standardized composition and for the in-house-produced tubes. Using the QRM phantom, we found a low CV (~ 3% or less) for both the 130 HU and 90 HU threshold levels when the smallest inserts (less than 1 mg) were used. When using saline as a surrounding medium and the in-house-produced phantom, CV of less than 1% was obtained, and even when using air as the surrounding medium, the CV was around 3% or less.

4.4 CALCIUM CONTENT—EX VIVO STUDY USING COMPUTED TOMOGRAPHY (STUDY IV)

In this evaluation, we used the weight of the explanted valve as the gold standard. The surgically explanted aortic valves were scanned by CT using the parameters that were determined as being found favourable in Study III. We scanned AV leaflets in saline using a calibration factor of 0.600 mg CaHA/cm^3, a threshold value of 130 HU, a B51 reconstruction protocol and a slice thickness of 0.75 mm.

Ex vivo CT calcium scoring expressed as CaHA equivalent MM was compared with valve weight and with haemodynamic measures of aortic stenosis in terms of valve area and mean gradient.

The mean MM value measured by ex vivo CT was 734.4 mg CaHA for AS valves and 309.6 mg CaHA for AR valves (p-value <0.001), which was expected because of the presence of greater calcification in stenotic valves compared with incompetent ones. The CT CaHA MM measurements showed a very strong correlation with the total weight of the explanted valves (correlation coefficient, 0.91), which also allowed us to generate a regression equation to calculate the weight of the valve from the MM value according to: valve weight (g) = 0.916 + 0.002 × calcium MM (in saline).

Despite the good correlation observed between CT AVC MM and valve weight, none of these parameters showed a high correlation with the haemodynamic importance of the aortic valve lesion (0.39 and 0.48 for MM and 0.37 and 0.43 for weight relation with valve area and mean gradient). Relationships were of the same order in the BAV and TAV groups. The relation between mineral mass and valve weight was equally high (r = 0.91) for both BAV and TAV.

5 DISCUSSION

The prognostic power of AVC regarding different cardiovascular diseases has been addressed in several studies over the last 15 years. In asymptomatic AS patients, AVC is a strong predictor of poor prognosis, with increased risk of death and need for AVR regardless of the severity of AS.[102, 103] Many studies have reported the association between AVC as a disease marker and adverse clinical events in patients with cardiac diseases such as coronary artery disease and left ventricular hypertrophy.[99, 140, 145, 146] Its importance was also addressed when it was shown to be a representative marker of the burden of generalized atherosclerosis in the body. [44, 99, 113, 140, 145, 147, 148] Thus, several studies evaluated the prognostic power of AVC assessment[37, 102] and its ability to predict cardiovascular diseases in different patient populations[145, 149, 150] or complications of cardiovascular procedures.[151] The presence and burden of AVC have been linked to an increased risk of adverse results in cardiovascular interventions, especially in TAVR.[152] In recent years, the increased number of TAVR procedures and the importance of AVC in predicting outcomes, particularly after procedural AR, have intensified the interest to study and quantify AVC in patients with AS.[151, 153] Echocardiography is very useful for the preoperative evaluation and follow-up of valve lesions and their haemodynamic effects, as well as for the evaluation of cardiac murmurs and cardiac function in clinical and epidemiological settings. CT has been important to demonstrate the predictive value of cardiac valve calcifications and their relation to coronary calcium. Recently, it was stated that "it's time to test echocardiography, an incredibly easy, low-cost and radiation-free method, to investigate the potential of ultrasound calcium to risk-reclassify asymptomatic subjects".[154] To pursue such an objective, it is essential to optimize the diagnostic techniques—the goal of the current thesis.

5.1 ECHOCARDIOGRAPHY AND AORTIC VALVE CALCIFICATION

To our knowledge, the comparison of AVC scoring with IOS and ex vivo CT has not been reported. In Studies I, II and IV, we evaluated different ultrasound methods for AVC assessment. To quantify the echogenicity of the aortic valve, we used greyscale measurement software that was developed for tissue characterization of carotid artery plaques.[143, 155, 156] We chose GSMn as a quantitative index, rather than the greyscale median, which is used commonly for vascular plaque characterization, because the mean value also takes small calcifications with high echo reflection into account. Despite the fact that it is an objective measure and a continuous variable, GSMn did not significantly improve the agreement in comparison with visual AVC scoring of the same still image, using the IOS as a gold standard. This was true for TTE as well as TOE images. This partly depended on the fact that

human visual assessment, especially for a trained investigator, has an excellent pattern-recognition ability.[156] When a single still image is used, the evaluation of the calcium burden depends on imaging the adequate section of the aortic leaflets. Unfortunately, the complete visualization of calcium content may not be possible in a single slice or transection, because of the irregular distribution of calcifications. The fact that an echocardiographic two-dimensional image has a certain thickness may only partly help to correct this problem, and viewing the complete thickness of the valve is anticipated to provide a more accurate interpretation. Real-time images should therefore be more optimal, as the valve passes through the image in its full thickness. This should allow a more comprehensive evaluation of AVC throughout the whole valve. In our study, this was clearly shown as a stronger correlation between AVC scores and IOS for real-time images. The reproducibility of TTE scoring was very good ($r = 0.93$, $p < 0.001$) for real-time images, and was comparable to that reported by studies that used CT and Agatston score to assess AVC.[153, 157, 158]

Although the use of different techniques of imaging of AVC has been described in the literature, comparisons of the modalities and scoring systems are scarce. In general, echocardiographic assessment has relied on several scoring systems and indices. Total cardiac calcification scoring by echocardiography compares favourably to CT.[145, 151] In the scoring system developed by Corciu et al., each leaflet receives a score of 0, 1 or 2 for normal, enhanced echogenicity or calcified valves, respectively, yielding a maximum possible score of 6 for a tricuspid valve.[145] Our scoring system was rather similar, although we applied additional grades and calculated an average value for the whole valve, via which the number of leaflets (BAV or TAV) did not in itself influence the result. Another 5-grade scoring system was used by Tolstrup et al., in which a score of 0 denoted a normal valve, 1 denoted slight reflectance, 2 denoted moderate reflectance, 3 denoted increased or generalized reflectance and 4 denoted marked reflectance with a leaflet thickness >6 mm.[159, 160] In their scoring system, the highest score given to any of the leaflets represented the whole valve's sclerosis score. We used the parasternal short axis view in all TTE still-image and real-time evaluations, to be able to visualize all aortic valve cusps in a single view. Short axis view images were used in a simple score by Nucifora et al., in which the aortic valve sclerosis score was either 0 (absent) or 1–3 (mild to severe sclerosis).[149] In our 5-grade scoring system, a score of 1 described a normal valve, 2 indicated a thickened/sclerotic valve with no evidence of calcification and 3–5 denoted mildly, moderately or severely calcified valves, respectively (Table 5). We presumed that the evaluation of the valves by surgeons would allow the estimation not only of calcification, but also of thickened valve tissue without calcification.

We found a better correlation between IOS and AVC in TAV compared with BAV, especially for still frames. This was true for both visual assessment and greyscale quantifications. Although the explanation of this finding is unclear, the more complex three-dimensional structure of BAV may have contributed to the more difficult assessment, especially using still frames. This finding could not be compared with the results of other laboratories because of the lack of studies that used still images for the assessment of AVC, either visually or using GSM. When TOE still images were used for AVC scoring, we recorded results that were similar to those obtained by TTE. Thus, TOE still images did not yield a significantly better correlation with the gold-standard technique (IOS) for AVC visual scoring or for GSMn.

However, the AVC scores used for visual evaluation of real-time images obtained by TTE and TOE showed high correlation with IOS and correlated well with each other. This finding is not surprising because the aortic valve is a three-dimensional structure in which some areas of calcification can only be visualized in one stage of the cardiac cycle and disappear during other stages. As it was obvious from the initial investigations (Studies I and II) that the evaluation of real-time loops gave more accurate results regarding AVC scoring, we focused on real-time evaluations in Study IV for both TTE and TOE; for TTE, we also used a "global" score, meaning that the interpretations used available projections and yielded an overall score, instead of evaluating each leaflet and calculating an average. The comparison with the best easily available gold standards, i.e., weight and CT score of the valve, revealed no major difference between the TTE mean and global scores or the TOE mean score. We did not compare our results with the ashes of valves (which is a more cumbersome technique). Such technique has been shown to compare very well with CT MM in carotid ex vivo comparison (with r^2=0.98) and we relied on ex vivo CT for our study. [139]

5.2 AORTIC VALVE PHENOTYPE

When diagnosing the presence of BAV, TOE showed higher sensitivity and specificity than TTE compared with surgical evaluation as a gold standard. This finding is in line with those of previous reports that confirmed the superiority of TOE for determining aortic valve morphology.[161, 162] This can be attributed to the close proximity of the transducer to the aortic valve and improved image resolution, resulting in the high sensitivity and specificity of TOE.[162] The presence of BAV has a high clinical significance, as it is a genetic disease that leads to increased risk for aortic valve lesions and complications and presentation of the disease almost a decade before TAV patients.[121] The accuracy of the diagnosis of BAV can affect decision-making and treatment plans in many patients.[22, 23] TOE cannot be

performed in all patients, but should be considered in patients with a dilated aorta and in cases of inconclusive TTE studies with suboptimal images in patients with suspected aortic pathology.

Even intra-operative recognition of BAV may be difficult. Roberts et al. noted that TAV is more accurately diagnosed during surgery than is BAV compared with histopathological evaluation. Two explanations were suggested for this finding: BAV is usually more calcified than TAV, and the presence of a calcified raphe can be misdiagnosed as a calcified border between two cusps.[163]

5.3 CALCIUM CONTENT — METHODOLOGICAL ASPECTS OF COMPUTED TOMOGRAPHY

Computed tomography (CT) is an established method for the estimation of calcium content. Because we evaluated patients who had undergone valve replacement, it was possible to perform CT evaluation of the explanted valves. However, the methodological issues involved in such evaluation have not been described in detail. Therefore, we performed extensive phantom studies to determine optimal parameter settings. For this, we used two types of phantoms to develop a standard protocol for ex vivo calcium scoring using multi-slice CT (MSCT). Since its introduction in 1990, the reproducibility and accuracy of the Agatston score have been questioned because of the variable results obtained using different scanners and scanning parameters. This can be explained by its non-linear calculation algorithm and the fact that it is sensitive to noise.[128, 164]Among the several measurement modalities available (such as the modified Agatston score and the estimated calcification volume), here we used the CaHA equivalent mineral mass (MM), as it has been shown to be the most reproducible and accurate, as well as the least affected by changes in threshold, slice thickness and energy, and little affected by type of scanner.[128-132, 139]

The maximum single calcium mass that could be detected by the work station and software used here was between 2 and 3 g. However, a larger total amount of calcium can be estimated when several smaller masses are evaluated separately and summarized, even if in the same image. We found that using normal saline (0.9%) as the surrounding medium yielded the most accurate results compared with the real weight. The other media tested showed a systematic under-estimation of the calcium MM, regardless of slice thickness or calibration factor.

Both the threshold values of 90 and 130 HU yielded good results, with one exception, namely when 1 mm slice thickness was used with the QRM phantom, in which the

90 HU threshold level yielded higher values that were somewhat closer to the actual mass compared with the 130 HU threshold. This was in agreement with the results of previous studies.[165, 166] However, Ferencik et al. recommended the use of the 130 HU threshold value for ex vivo calcium measurement,[165] and a threshold value of 130 HU has been widely used in similar studies with good results.[167, 168] The conclusion from this would be that both 90 and 130 HU can be used.

It has been suggested that, when larger CT slice thickness is used, smaller CaHA inserts may be missed.[165, 166] However, this was not confirmed in our phantom study, in which both 1 mm and 0.75 mm slice thicknesses could detect and measure all three smallest CaHA inserts of the QRM phantom. A smaller slice thickness could be of greater use when scanning ex vivo tissues in which small calcium depositions are expected to be present, such as vessel walls and plaques.

In our study, the use of an increased amount of the surrounding media resulted in a negative effect on the accuracy of MM CaHA measurements, which has also been reported elsewhere.[139] These inferior results can probably be attributed to a higher noise and/or attenuation.

5.4 AORTIC VALVE CALCIFICATION — EX VIVO STUDIES

To our knowledge, this was the first study to explore ex vivo aortic valve weight and estimate AVC by CT MM compared with preoperative echocardiographic and intra-operative AVC assessment.

With the growing number of chest CT scanning events, AVC may be accidentally detected more often; however, it is usually overlooked and not thoroughly studied.[141, 169] We found an excellent correlation between CT evaluation of calcium content and the weight of valves. This result was supported by the findings of a study that evaluated porcine plaque using dual-energy CT.[170] Although we did not evaluate the ability to estimate AVC by in vivo CT scanning, it can be assumed to have an accuracy that is similar to that of the scanning of explanted valves in saline. Despite the better correlation between CT-evaluated calcium content and valve weight, the ultrasound estimation of calcium content actually correlated better with haemodynamic measures of the aortic lesion. In recent years, several studies have used CT scanning, even ex vivo, [141] to evaluate AVC and to correlate valve stenosis with the burden of valve calcification. [171, 172] Different brands and types of CT machines have been used throughout the years of CT development, including micro-CT, electron beam CT and MSCT machines, with up to 64-slice units.[171, 173, 174] AVC scoring has been performed using

different methods, including the Agatston score and the modified Agatston score, volume scores, mass scores and even subjective scores.[172, 175, 176] Aortic valve area or flow parameters were obtained from TTE studies, as is currently standard for AS evaluation,[158, 172, 177] whereas TOE[174] and cardiac catheterization have also been used to calculate AVA.[153, 178] The results of these studies did not yield similar conclusions, with some showing good correlation[141, 172] and others showing modest, [171] like ours, or even no correlation, [177] between AVC and the degree of AS. These conflicting results can be explained by discrepancies in the patient populations and degree of calcification. Additional calcium deposition in an already severely stenotic valve will most probably increase the AVC score, while it could have no or little effect on the AVA; similarly, essentially normal AVA can exist with variable degree of calcification. Good correlations between AVC MM and AVA can probably be explained by the inclusion of all types of valve lesions and calcification scores, including absence of calcification, while uniform study populations, with severe AVC and significant AS in all, would be expected to yield lower correlations.

6 STRENGTHS AND LIMITATIONS

The echocardiographic investigations were performed in a standardized manner by a small number of experienced investigators. TTE investigations were performed within a few days before the operation and the TOE was performed at the time of surgery. The evaluation of AVC from ultrasound images and CT measurements was performed by one investigator on separate occasions; moreover, this investigator was unaware of the results of the other modality. It is a limitation that we did not evaluate AVC by grey scale measurement in real-time loops. The absence of in vivo preoperative CT scanning was also a limitation.

7 FUTURE PERSPECTIVES

AVC has shown prognostic importance in different cardiovascular diseases and as a marker of generalized atherosclerosis. In our project, we aimed to investigate different in vivo and ex vivo methods for quantifying AVC and relate the severity of AVC to haemodynamic variables of aortic valve disease and clinical risk factors. Further research is needed in this field involving new high-resolution imaging modalities, including molecular imaging.

In terms of two-dimensional (2-D) echocardiography future studies should focus on defining the state-of-the-art techniques for quantification of AVC, also at early stages. Continued research to develop and validate new methods for a quantitative volumetric AVC assessment by three-dimensional (3-D) echocardiography is mandatory. Development of contrast agents for molecular and cellular imaging in atherosclerosis may be of great interest also for aortic valve imaging. The optimal role of different imaging modalities to study AVC should also be determined in future studies.

New large scale prospective clinical research is needed to identify relationship between quantitative imaging of AVC in vivo and clinical and genetic factors.

8 CONCLUSIONS

- When assessing AVC, real-time images of the aortic valve were crucial for accurate assessment, either using TTE or TOE.
- Using a predefined scoring system resulted in low inter- and intra-observer variability when assessing AVC on TTE or TOE images.
- TOE was superior to TTE in determining aortic valve phenotype.
- More accurate and reproducible results can be obtained when using normal saline as the surrounding medium for ex vivo CT scanning for calcium scoring compared with air and the other surrounding media studied.
- Thinner slice thicknesses, in addition to a calibration factor of 0.600 mg CaHA/cm^3 and threshold values of 130 HU or 90 HU, were the preferred parameters for ex vivo CT scanning for calcium detection in small tissue specimens.
- CaHA mineral mass measured by ex vivo CT correlated best with the ex vivo valve weight, whereas visual assessment of AVC using echocardiographic real-time images correlated better with the haemodynamic measurements of aortic valve disease.

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