Pathogenesis of aortic valve stenosis:
bench to bedside approach

Doan Thi Minh Ngo
B.Pharm; B.Health Sci. (Hons)

A Thesis submitted to The University of
Adelaide as the requirement for the degree of
Doctor of Philosophy

Cardiology Unit, North Western Adelaide
Health Service
Department of Medicine
University of Adelaide
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THESIS SUMMARY

Experiments described in this thesis address the pathogenesis of aortic valve sclerosis/stenosis using a bench to bedside approach. In particular, the thesis begins with development of a technique using ultrasonic backscatter analyses to quantitate the early stages of aortic stenosis. Subsequent chapters utilized this methodology to quantitate aortic valve structural changes in a model and intervention study of aortic stenosis in rabbits. The last chapters are human studies designed to identify factors associated with presence of aortic sclerosis/stenosis; with particular interest in potential association of endothelial dysfunction/inflammation/platelet aggregation with abnormal aortic valve structure quantitated by ultrasonic backscatter. In Chapter 1 (Introduction) the relevant literature is reviewed.

Development of ultrasonic backscatter to quantitate aortic sclerosis (Chapter 2)

Aortic valve sclerosis (ASc) is detected when there is visual assessment of focal increases in echogenicity of the aortic valve most commonly assessed by echocardiography. However, there is no previously described method to quantitate degree of aortic valve structural abnormality as ASc is not associated with marked hemodynamic obstruction quantifiable by Doppler echocardiography. The current study used ultrasonic backscatter to quantitate aortic valve structural abnormality in patients assessed as having ASc based on valve appearances, compared to young healthy volunteers with normal aortic valves.
The results of the study indicate: 1) that the mean levels of aortic valve backscatter in ASc patients are approximately 60% greater than in young healthy volunteers (ie aortic valve backscatter scores ≥ 16dB are not consistent with normal aortic valve structure), 2) ultrasonic backscatter scores in ASc patients are directly correlated with subjective scoring of sclerosis and with a positive trend with transvalvular pressure gradients in patients with mild-moderate aortic stenosis, and most importantly, 3) ultrasonic backscatter is a reproducible technique, with mean differences between estimates based on repeat echocardiograms of 2.3 ± 1.7 (9.1%). These results indicate that ultrasonic backscatter could be used as a quantitative measure of aortic valve structural abnormality in epidemiology and for examination of interventions.

In vivo studies

Development of an animal model of aortic stenosis with vitamin D$_2$ (Chapter 3)

The aim of the study was to develop an appropriate animal model for AS. The study used vitamin D$_2$ alone at 25,000IU/4 days weekly (vit-D$_2$) for 8 weeks to induce AS in rabbits. Results showed that: 1) rabbits in the vit-D$_2$ group had significantly increased in transvalvular velocity and pressure gradients compared to rabbits in the control group (normal chow + drinking water); this was consistent for aortic valve ultrasonic backscatter scores; 2) aortic valve immunohistochemistry/histology showed marked calcification, neutral lipids, macrophage, and leukocyte infiltrations for rabbits in the vit-D$_2$ group (ie consistent with histology of human AS); 3) significant elevation of asymmetric dimethylarginine (ADMA) concentrations in the vit-D$_2$ group occurred.
compared to controls over the 8 weeks treatment period; the change in ADMA concentrations correlated significantly with the change in transvalvular pressure gradients for rabbits in the vit-D$_2$ group; 4) rabbits in the vit-D$_2$ group had significantly impaired endothelium-dependent acetylcholine-induced aortic relaxation, and this effect was completely abolished by the nitric oxide synthase inhibitor (L-NAME); 5) the addition of 0.5% cholesterol-supplemented diet to the vitamin D$_2$ regimen did not accentuate the development of AS. Thus, treatment with vitamin D$_2$ at 25,000IU/4 days weekly for 8 weeks significantly induced AS with similar aortic valve pathology to that of human AS; therefore, the model is suitable for use in examining potential therapeutic interventions in AS.

**Effects of ramipril on development of AS in rabbits (Chapter 4)**

Using this animal model, this study aimed to examine the effects of the angiotensin-converting enzyme inhibitor (ACEi) ramipril on development of AS. Rabbits (n=28) treated for 8 weeks were divided into 2 groups: (a) vitamin D$_2$ alone (n=10) (normal chow + 25,000IU vitamin D$_2$ in drinking water); (b) vitamin D$_2$/Ramipril (n=12) (normal chow+25,000IU vitamin D$_2$/Ramipril (0.5mg/kg) in drinking water). Six further rabbits constituted a normal reference group (no treatment was given). The results for comparisons between vitamin D$_2$/ramipril vs vitamin D$_2$ alone were as follows: 1) ramipril-treated rabbits had significantly less severe hemodynamic obstructions (p<0.05, for both) as assessed by transvalvular velocity, and aortic valve area; with borderline reduction in aortic valve backscatter (p=0.08); 2) ramipril significantly reduced plasma
ADMA concentrations; 3) there was improvement in acetylcholine-induced aortic relaxation (p=0.056), with significant improvement in sodium nitroprusside-induced relaxation (p<0.05); 4) there was a strong inverse correlation between acetylcholine-induced aortic relaxation and aortic valve backscatter score (0<0.001), thus providing further evidence of the potential role of nitric oxide in retarding the development of AS in this model.

These data provide a strong rationale for the inception of a randomized trial of ACE inhibition as a strategy for limitation of AS progression in humans.

**Human studies**

Aortic stenosis is associated with elevated plasma levels of asymmetric dimethylarginine (ADMA) concentrations in humans (Chapter 5).

Given the findings that aortic stenosis induced by vitamin D₂ in rabbits also caused elevation of plasma ADMA concentrations, a physiological inhibitor of nitric oxide synthase, a mediator and marker of endothelial dysfunction and an indicator of incremental cardiovascular risk. The study sought to determine whether plasma ADMA concentrations are elevated independently of pre-existing coronary risk factors in subjects with at least moderate aortic stenosis (n=42) compared to age-matched patients with normal aortic valves (n=42): as determined both by visual assessment and with aortic valve backscatter scores < 16dB. Results for this study were as follows: 1) plasma ADMA concentrations were not statistically different between the AS and non-AS group
(median 0.59 vs 0.54 µmol/L, p=0.13, Mann-Whitney test) on univariate analysis; 2) backward stepwise multiple linear regression showed the presence of AS was a significant predictor of elevated ADMA concentrations (p=0.04, 95% CI =0.001, 0.072).

3) in addition, elevated plasma ADMA concentrations were also associated with history of atrial fibrillation (p=0.009, 95% CI=0.015, 0.100), and negatively associated with creatinine clearance (p=0.01, 95% CI=-0.002, 0.000), and the use of statin therapy (p=0.01, 95% CI=-0.081, -0.011). Therefore, in conclusion, this study found that AS is independently associated with elevation of ADMA concentrations, beyond that implied by “conventional” risk factors for endothelial dysfunction. The clinical status of AS as an incremental marker of cardiovascular risk may reflect ADMA-mediated endothelial dysfunction.

Assessment of factors associated with ASc in a random ageing population study

(Chapter 6).

There have been few clinical studies of factors associated with ASc. Previous population studies have established that ASc is an independent correlate of incremental risk of coronary events. Having established that patients with AS have increased plasma ADMA concentrations (Chapter 5), it was now aimed to determine whether subjects with increased aortic valve backscatter scores (ASc) also have other markers of endothelial dysfunction/NO effects, independent of preexisting coronary risk factors. The study was designed to identify such anomalies, if they existed, on an incremental basis to other
putative correlates of ASc, including coronary risk factors, renal dysfunction and vitamin D levels.

Random selected subjects (n=253) aged between 51 to 77 years were evaluated. All patients underwent transthoracic echocardiography examination; aortic valve ultrasonic backscatter score (AVBS), was used to quantitate echogenicity of the aortic valve. Conventional coronary risk factors were identified on history. Integrity of NO generation/response was assessed via (i) plasma ADMA concentrations; (ii) inhibition of platelet aggregation by the NO donor sodium nitroprusside (SNP); (iii) aortic augmentation index (AIx), a measure of arterial stiffness/wave reflection. All putative correlations with AVBS were examined by univariate and stepwise multiple linear regression analyses.

On the basis of echocardiographic appearances, ASc was present in 63 subjects (25.4%); mean AVBS scores was 14.9±4.6dB (SD) vs 11.2±3.9dB (SD) in the presence vs absence of ASc (p<0.001). Univariate analyses revealed that platelet responsiveness to NO was inversely correlated with AVBS (β=-0.16, p=0.02); but [ADMA] and AIx were not. On multiple linear regression, significant correlates of increased AVBS were: (i) advanced age (β=0.21, p=0.003), (ii) low body mass index (β=-0.23, p=0.001); and (iii) impaired platelet responsiveness to NO (β=-0.16, p=0.02).

In Chapter 7, the implications of the overall findings in this thesis are discussed in relation to future perspective.
DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University Library.

Doan Thi Minh Ngo
(December 2007)
ACKNOWLEDGEMENTS

I am most grateful to my supervisors Dr Jennifer Kennedy and Professor John Horowitz and for their guidance, encouragements, and patience throughout the course of my PhD.

I am indebted to Professor John Horowitz for always believing in me. His enthusiasm, passion, and encouragements have inspired me to achieve more than I could hope for. Thank you for giving me the freedom to express my ideas and for guiding my ideas throughout the 4 years. It has been an absolute pleasure and a fantastic opportunity to work with someone like him at such an early stage of my research career.

I would like to sincerely thank the echocardiography staff: Mr Ronald Wuttke, without whom I could not have achieved so much; thankyou for believing in me, for constantly supporting my demands and putting up with the late hours; Ms Gina Vellisaris and Linda Passfield for their support and friendship.

Thank you to Irene Stafford, Tamila Heresztyn, Geraldine Murphy, and Helen Weedon for teaching me various techniques, and for instilling in me the value of honest research. To my friends Dr Angus Nightingale, Dr Sharmalar Rajendran, Dr Anke Rosenkranz, Dr Kumaril Mishra, and Dr Alicia Chan for their support and understanding my frustrations.
My sincere thanks to all the staff of The Queen Elizabeth Hospital Cardiology Unit for their cooperation, patience and understanding throughout the years. I am very proud to have started my research career there.

Thank you to Dr Aaron Sverdlov for traveling this journey with me. For the love, inspiration, patience, understanding, and tremendous support I received throughout my PhD, I could not ask for a better partner.

Finally, thank you to my parents: my father whose quest for knowledge never ceases, for instilling in me the passion for learning, and for teaching me invaluable life skills; my mother whose determination, enthusiasm, and optimism have taught and carried me through some of the most difficult stages of my life.

Throughout my PhD, I have been a recipient of postgraduate scholarships from Division of Health Science, University of Adelaide; and The Queen Elizabeth Hospital Research Foundation.
PUBLICATIONS/PRESENTATIONS

Peer reviewed articles relating to this thesis


Accepted presentations at national/international meetings:


iii) Ngo DT, Wuttke RD, Weedon H, Nightingale AK, Sverdlrov AL, Kennedy JA, Horowitz JD. Vitamin D3 supplementation induces aortic stenosis in rabbits. *Accepted oral abstract at World Congress of Cardiology 2006, European Society of Cardiology.* Abstract number 83197.


ix) Ngo DT, Herestyzn T, Mishra K, Marwick TH, Horowitz JD. Aortic stenosis is associated with elevated plasma levels of asymmetric dimethylarginine (ADMA). Accepted abstract at Scientific Sessions American Heart Meeting 2005. Session number APS.56.1M, presentation number 3227.
CHAPTER 1

Introduction
1.1 Anatomy of normal aortic valve

The normal aortic valve has 3 cusps. Each cusp or leaflet has a ventricularis, on the ventricular side of the leaflet, and the fibrosa, which is on the aortic side of the leaflet. The spongiosa is a layer of loose connective tissue at the base of the leaflet, between the fibrosa and ventricularis, composed of fibroblasts, mesenchymal cells, and a mucopolysaccharide-rich matrix. An endothelial layer covers the entire external surface of the valve. Normally, the aortic valve leaflet is avascular and derives nourishment from the blood that flows through. The fibrosa because of its location on the aortic side, is subjected to high shear stress, and therefore consists primarily of fibroblasts and closely packed collagen fibers, whereas the ventricularis is composed of elastin-rich fibers, does not have as high tensile strength and is subjected to less shear stress (reviewed by Freeman and Otto 2005, Warren and Yong 1997).

1.2 Aortic stenosis (AS)

Aortic stenosis (AS) occurs as a result of progressive increase in calcium deposition within the aortic valve, leading to increased stiffness and progressive narrowing of the valve. The earliest stages of this process are designated as aortic valve sclerosis (ASc), which implies the presence of abnormal aortic valve morphology in the absence of marked obstruction. AS can present at any age, and causes of AS in adolescents and adults under the age of 60 years include congenital stenosis, history of rheumatic fever,
and congenitally bicuspid aortic valves. Although bicuspid aortic valve is a relatively common anomaly, affecting 1% of the general population, with most patients subsequently develop aortic valve calcification by the age of 30 (reviewed by Yener et al 2002); this thesis will not address specifically this form of AS. However, with the decline of rheumatic fever, and increasing duration of survival in the Western populations, the occurrence of progressive AS on previously normal aortic valves are of major interest.

1.3 Features of stenotic aortic valve lesions

Histopathologic studies have demonstrated that development and progression of calcific AS are based on an active process that shares some similarities with atherosclerosis. It is suggested that aortic valve lesions begin with disruption of valve endothelium predominantly on the aortic side due to high shear stress (Otto et al 1994, reviewed by Mohler 2004, Chan 2003).

1.3.1 Inflammation and lipid deposition

Aortic valve lesions typically present with areas of subendothelial thickening, which is suggested to represent the early stage of aortic stenosis. Increased thickening of aortic valve leaflets are identified to be characterized by accumulation of inflammatory infiltrates of predominantly macrophages and T-lymphocytes, lipids, oxidized lipids, (Summarized in Figure 1.1A) (Otto et al 1994, Warren and Yong 1997, Olsson et al 1994, Wallby et al 2002) all of which potentially activate a host of profibrotic and
proinflammatory markers. Macrophages and T-lymphocytes have been detected and tend to be located near the surface of the lesion (Otto et al 1994, Warren and Yong 1997, Olsson et al 1994, Wallby et al 2002). Immunohistochemical studies have found co-localization of apolipoproteins (apo) B, apo (a), apoE with lipid laden foam cells and macrophages (O’Brien et al 1996) as well as oxidative modification of residential low density lipoproteins (LDLs) in early stenotic aortic valve lesions (Olsson et al 1999).

1.3.2 Valvular matrix remodeling and fibrosis

The presence of macrophages and T-lymphocytes along with oxidized LDLs, and apolipoproteins accumulations activate several profibrotic and proinflammatory cytokines may modulate aortic valve remodeling, and subsequent calcification. Transforming growth factor β1 (TGF-β1) (Jian et al 2003) and interleukin-1β (Kaden et al 2003) have been found in valve matrix and are associated with increased local production of matrix metalloproteinases II (MMP-2), all of which contribute to cell apoptosis, extracellular matrix formation, remodeling and consequently predispose to calcification. In addition to TGF-β1 and interleukin-1β; tumour necrosis factor-α (TNFα), another proinflammatory cytokine commonly responsible for immune regulation, inflammation and tissue remodeling, and is found co-localized with MMP-1 (Kaden et al 2005). Furthermore, tenascin C, an extracellular matrix glycoprotein implicated in cell proliferation, migration, differentiation and apoptosis which is involved in stimulation of bone formation, and mineralization has been found co-localized with MMP-2 in calcified
aortic valve leaflets (Jian et al 2001), and is associated with progression of AS (Satta et al 2002),

Interestingly, the presence of angiotensin-converting enzyme (ACE) was identified in stenotic but not in normal aortic valves (O’Brien et al 2002). It was initially postulated that ACE enters the stenotic valve lesions from the circulation bound to or “carried” by LDL cholesterol particles (O’Brien et al 2002, Mohler 2004). One major product of ACE, angiotensin II (Ang II), an important mediator of inflammation and fibrosis, could also be formed by the mast cell (MC)-derived neutral protease, chymase (Nishimoto et al 2001). It has been shown that MC-derived chymase is also upregulated in stenotic valves, providing further evidence for local production of Ang II (Helske et al 2004). In addition, cathepsin G, another neutral protease also capable of generating Ang II, is present in increased concentrations throughout human stenotic aortic valves compared to normal valves (Helske et al 2006). Cathepsin G was also seen to be activated by mast cells, and colocalized with TGF-β1. In areas with prominent elastin degradation, there was elevation of cathepsin G expression and accumulation of cathepsin G-positive cells, notably, mast cells (Helske et al 2006). Thus, the presence of ACE, MC-derived chymase, and cathepsin G provide a potential basis for a role of angiotensin II in aortic valve remodeling along with other profibrotic and proinflammatory mechanisms.

As summarized in Figure 1.1B, current concepts of the pathogenesis of AS centre histologically on inflammation and lipid deposition, and biochemically on activation of cytokines and matrix metalloproteinases, together with generation of angiotensin II.
These processes are postulated to induce injury of all valve components, leading to fibrosis and calcification.

1.3.3 Calcification

Calcification of aortic valve leaflets tend to occur more predominantly in the later stages of AS, and located deeper in the lesion (Otto et al 1994). Active calcification is a major factor in reducing valvular mobility in severe AS. Early lesions of aortic valves show fine stippled mineralization, progressing to active bone formation resulting in gross calcification at later stages of the disease (Figure 1.1C).

The process of calcification (and sometimes ossification) of aortic valve leaflets resembles that in atheroma formation. The presence of inflammation, fatty streak formation from lipid depositions, cytokine release, metalloproteinases, ACE, Ang II, all possibly contribute to the production of an extracellular matrix, and matrix vesicles that initiate mineralization.

Co-localization of macrophages and oxidized LDLs with osteopontin, a protein needed in bone formation, has been found and is involved in the release of matrix vesicles in human stenotic aortic valves (O’Brien et al 1995, Mohler et al 1997). Furthermore, Mohler et al (2001) described an active process of calcification using immunohistochemistry in an extensive study of 347 human stenotic aortic valves. In addition to active osteoblasts and osteoclasts, the study in agreement with others (Kaden et al 2004 (Journal of Heart Valve
Disease)) found presence of bone morphogenic proteins 2- and 4- (BMP-2, BMP-4). BMPs stimulate osteoblastic differentiation with subsequent calcification. In agreement with previous studies (Otto et al 1994, O’Brien et al 1995), Mohler et al (2001) also found that macrophages and lymphocytes accumulate in areas of calcification.

There has been evidence of angiogenesis which is essential for longitudinal bone growth in stenotic valves. The presence of neoangiogenesis in ossified valves has been found co-aggregates with T-lymphocytes (Mohler et al 2001); and the presence of heat shock protein 60 (hsp60) (Mazzone et al 2004), commonly expressed by cells under stress conditions together indicate a highly active, and chronic immunomediated process from stress to inflammation to calcification.

It has also been shown that the calcification process of aortic valves may also be regulated by receptor activator of nuclear factor \( \kappa \)B, its ligand (RANK, and RANKL), and the soluble receptor osteoprotegerin (OPN) (Kaden et al 2004 (J Mol Cell Cardiol)). The study showed significant presence of RANKL in calcified valves compared to only few positive cells in respective control valves. Furthermore, there was a significant reduction of OPG positive cells in aortic stenotic valves compared to controls. It has been shown that in mice deficient for OPG, vascular calcification developed with high expression of RANKL in calcified areas (Bucay et al 1998). Additionally, in Kaden et al (2004, J Mol Cell Cardiol), long-term cell culture of stenotic aortic valves with RANKL showed a significant increase in matrix calcium deposition and the formation of cell
nodules compared to controls. Thus, RANKL/OPN pathway may also be involved in the calcific process of aortic valve stenosis.

A recent study (Kaden et al 2007) revealed an association between presence of aortic stenosis and low serum levels of the anti-calcific protein fetuin-A. Furthermore, there was evidence that fetuin-A was deposited in calcific aortic valves. Nevertheless, the relative importance of these findings to the overall pathogenesis of aortic stenosis remains uncertain.

1.4 Epidemiology

There is now increasing evidence from studies in Western subjects that the prevalence of ASc and AS increases with age, affecting about 25% of adults over 65 years of age, and up to 50% over 80 years, with development of severe AS occurring in 4% of patients by the age of 80 years (Stewart et al 1997, Otto et al 1995, Lindroos et al 1993). In addition to age, the prevalence of ASc rises further in patients with higher cardiovascular risk factors. For example, in patients who were admitted to hospital with chest pain, 50% were found to have ASc (Chandra et al 2004).
1.5 Clinical factors associated with presence of ASc/AS

1.5.1 Conventional cardiovascular risk factors

Clinical risk factors found to be associated with development of AS although vary between studies, these factors are similar to those associated with the development of atherosclerosis. Aronow et al (1987) studied a population of over 500 patients with mean age of 82 years found that hypercholesterolemia, history of hypertension (HT), diabetes mellitus (DM), low high density lipoproteins (HDL) were significantly associated with development of AS, while serum calcium, phosphorus, creatinine, and alkaline phosphatase levels were not. In the Helsinki Aging Study (Lindroos et al 1994), hypertension, age, and a low body mass index were independent predictors of aortic valve calcification, while hypercholesterolemia, smoking, and DM were not. Renal function, calcium and phosphorus profiles were not measured. Gotoh et al (1995) identified an association between lipoprotein (a) levels and development of ASc. The Cardiovascular Health Study (Stewart et al 1997), studied a combination of ASc and AS patients, the largest study to date, found age, male gender, HT, smoking, Lp(a), low height, and high LDLs to be associated with AS development.

1.5.2 Other risk factors

Patients with chronic renal disease have been found to be at increased risk of development of AS (Maher et al 1987, Straumann et al 1992, Michel 1998). It has been postulated that the association may be due to abnormal calcium-phosphate products
(Raine 1994). Other disease states that induce abnormal calcium metabolism such as hyperparathyroidism, and Paget’s disease have also been linked with higher incidence of AS (Stefenelli et al 1993, Strickberger et al 1987). In addition, Mills et al (2004) found that abnormal calcium-phosphate products in patients with normal renal function were associated with severity of AS.

Genetic factors may also play a role in the development of AS. The influence of genetic polymorphism of the vitamin D receptor on bone metabolism was investigated in a case-control study (Ortlepp et al 2001). There was a significant association between the B allele of the vitamin D receptor BsmI and AS. This polymorphism is commonly associated with rapid bone loss and progressive osteoporosis, providing for the first time a genetic association and perhaps provided a separation between factors associated with development of AS and atherosclerosis. Polymorphisms of apolipoprotein (apo) AI, B, and E, the genes associated with dyslipidemia and development of atherosclerosis, were also associated with AS (Avakian et al 2001, Novaro et al 2003). However, lipid profiles were similar between patients with AS and the control group, indicating that the association between AS and polymorphism of these apolipoproteins may not have been due to dyslipidemia. Furthermore, an epidemiological study in France found a familial aggregation of AS, suggesting evidence for the role of genetic factors and development of AS (Probst et al 2000). However, this study did not find an association between AS and polymorphisms of the vitamin D receptor or apoliproteins, thus could not provide an explanation for this familial aggregation of AS.
Interestingly, in an observational study, Koos et al (2005) using multislice spiral computed tomography, assessed whether patients with ASc and AS on oral anticoagulants (vitamin K antagonists) were more likely to have high aortic valve calcification. The study found that subjects on oral anticoagulants had two-fold increases of valvular calcium as well as coronary calcium scores on univariate analysis. On multiple regression, oral anticoagulant treatment was the only correlate of valvular calcium score; while male gender, hypertension, and oral anticoagulant treatment were correlates of coronary calcium scores. The study postulated that the use of vitamin K antagonists inhibit matrix Gla protein, a vitamin K-dependent, potent inhibitor of calcification (reviewed by Proudfoot and Shanahan 2006). Although it has been shown in animal studies, that warfarin, a vitamin K antagonist at high doses induced rapid and extensive calcification of the elastic lamellae of large arteries in the aorta and aortic valves (Price et al 1998, Howe et al 2000); the study by Koos et al (2005) was not prospectively randomized (a currently difficult task in the absence of effective alternatives to warfarin) and therefore the results, while strongly suggestive, are not completely definitive.

1.6 Consequences of ASc

ASc was previously thought to be a benign finding despite the risk of progressing to AS over decades. In a prospective study of over 2,000 patients, Aronow et al (1999) found that patients with ASc had 1.8 times higher chance of developing new coronary events than those without ASc, and patients with prior CAD had a 2.8 times higher chance of
developing new coronary events in the presence of ASc. The Cardiovascular Health Study of over 5000 patients found that the presence of ASc, as identified by echocardiography was associated with 50% increase in cardiovascular risk and myocardial infarction over an average follow-up of 5.5 years (Otto et al 1999). Furthermore, in a group hypertensive patients (n=960), with a mean follow-up of approximately 60 months (mean age 55 to 80 years), 40% of these study patients were found to have ASc; and subjects with ASc had almost twice the risk for serious CV events (Olsen et al 2005). However, these studies did not take renal function into account. Nonetheless, the presence of ASc is now generally agreed to be associated with adverse outcomes, although the mechanism(s) underlying these associations have received little attention.

1.7 Consequences of AS

Patients with ASc typically remain asymptomatic for many years, progressing to AS and deteriorate rapidly when symptoms of severe AS develop with the expected average survival of 2 to 3 years without aortic valve replacement (AVR) (Ross and Braunwald 1968). Pathologically, progressive AS due to hemodynamic changes may result in left ventricular hypertrophy (LVH), left ventricular diastolic and systolic dysfunction, angina, arrhythmias, and syncope, with subsequent congestive heart failure (Ross and Braunwald 1968, Turina et al 1986). In addition, patients with AS undergoing non-cardiac surgery have approximately fivefold increased risk of perioperative mortality and nonfatal
myocardial infarction independent of risk factors for coronary artery disease (CAD) (Kertai et al 2004).

The relationship between emergence of symptoms and outcomes in AS has never been studied in detail. Ross and Braunwald (1968) made initial observations suggesting that following development of any components of the classical triad of symptoms (angina, dyspnoea, syncope) there was a “natural history” of 2-3 years’ survival (Ross and Braunwald 1968, Turina et al 1987). However, these studies were fundamentally designed to demonstrate differences in prognosis with valve replacement. Even as such, they were not randomized studies; nevertheless the prognostic advantage of surgery was obvious.

More importantly, the average age of the patient groups concerned at time of clinical presentation were 48 and 45 years respectively (Ross and Braunwald 1968, Turina et al 1987). Therefore it is likely that many of these cases were of AS involving bicuspid aortic valves, where the natural history is of presentation at a younger age than with tricuspid aortic valves (reviewed by Yener et al 2002). It is not certain whether prognostic aspects of AS are (a) related to valve structure; (b) related to age. Therefore, the economically/medically crucial issues of prognosis of AS in patients with tricuspid aortic valves remain incompletely evaluated.
A more recently recognized aspect of the clinical consequences of advanced AS is modification of von Willebrand’s factor via shear stress on stenosed valves, leading to increased bleeding risk. This anomaly is corrected by AVR (Vincentelli et al 2003).

1.8 Factors associated with progression of ASc and AS

In the largest study to date, > 2000 patients with ASc were studied regarding progression to AS over mean follow-up period of approximately 7 years. Overall, 10.5% of this population developed mild AS, 3% moderate AS, and 2.5% with severe AS (Cosmi et al 2002). A retrospective study of 400 patients with ASc with a mean follow-up period of 44 months found about 33% of this population developed some degree of AS defined as any increase in velocity and pressure gradient measured (Faggiano et al 2003). However, to date, no clinical study has been performed assessing factors associated with ASc progression.

Although identified clinical factors associated with development of AS are similar to those predisposing to atherogenesis, the factors underlying progression of AS are superficially different. The major factor for rapid progression of AS is coexisting chronic renal failure, in particular dialysis patients (Wongpraparut et al 2002, Maher et al 1987, Faggiano et al 1996, Perkovic et al 2003). The estimated odds ratio for rapid progression (> 0.25cm²/year) was 2.47 in the presence of hemodialysis (Wongpraparut et al 2002). It has also been documented that there was an association between higher 25-
hydroxyvitamin D levels (vitamin D$_3$) and AS in patients on dialysis and rapid progression (Malergue et al 1997, Urena et al 1999). These findings make it clear that the determinants of progression of AS in patients on chronic dialysis can be distinguished from plasma creatinine levels per se, and presumably reflect anomalies which are not corrected by dialysis.

It is of course inconceivable that factors underlying occurrence and rapid progression of AS could be totally different. Results are inconsistent between studies of AS progression as some studies have documented presence of HT, DM, hypercholesterolemia, presence of CAD as factors associated with more rapid progressions (Aronow et al 2001, Palta et al 2000, Nassimiha et al 2001) while others have not (Bahler et al 1999, Messika-Zetouin et al 2007). To date, there is therefore some discrepancy as to which cardiovascular risk factor is more important in the progression of AS. While some clinical studies documented association with elevated cholesterol levels (Yilmaz et al 2002, Aronow et al 2001, Pohle et al 2001, Palta et al 2001), others have demonstrated no such association (Wongpraparut et al 2002, Messika-Zetouin et al 2007, Bahler et al 1999). Overall, the most consistent risk factor for rapid development/progression of AS is chronic renal disease, particularly patients on dialysis (reviewed by London et al 2000, Wang et al 2005). However, most clinical studies do not examine the possible importance of elevated serum creatinine in non-dialysed patients, patients with end-stage renal disease and/or on hemodialysis. It may be possible that the presence of cardiovascular risk factors predisposes the aortic valves to initial injury, hence development of aortic valve lesions; with worsening of factors associated with calcification eg renal dysfunction, abnormal
Ca₃PO₄ are responsible for progression of aortic valve disease. Clinical studies of factors of progression of AS are summarized in table 1.1.

Recently, Messika-Zetouin et al (2007) performed the first study to document the differences in factors associated vs progression of aortic valve calcification. This study found that presence of diabetes, high BMI, systolic BP, serum glucose were associated with aortic valve calcification (AVC) upon adjustment for age and gender. However, after 3.8 ± 0.9 years follow-up, the only factor associated with faster progression of AVC was the initial AVC score, which further confirmed that factors associated with presence of AS vs progression of AS are different. Subjects with no initial AVC who subsequently developed AVC had significantly higher total cholesterol levels. This in turns, suggests that the role of cholesterol in development of AVC may be at the early stages. However, this study did not look into the role of renal dysfunction, Ca₃PO₄, vitamin D, and serum calcium levels in progression of AVC.

A number of clinical studies, analogous to the calcification-based study of Messika-Zetouin et al (2007), have suggested that progression of AS may “accelerate” late in the disease process. Specifically, they have obtained data suggesting that increases in transvalvular pressure gradient and reductions in valve area progress most rapidly once moderate AS is already present (Palta et al 2000, Bahler et al 1999).

This is a finding of potentially considerable significance, but is not necessarily correct. While findings of Messika-Zetoun et al (2007) are consistent with “late” acceleration of
calcification in AS, the basis for changes in valve area may be subject to non-linear relationship, with increases in valve infiltration/calcification. For example, it is possible that valve mobility decreases in an asymptotic manner as disease progresses. It is unlikely that this issue will ever be resolved completely, but it should also be noted that even if it were true that progression of AS “accelerates” in disease “mid-course”, this would not necessarily imply that the optimal therapeutic window begins at that time.

1.9 Methods of quantitating severity of AS

1.9.1 Catheterization

In the older studies, (Davies et al 1991, Wagner and Selzer 1982) AS progression was usually assessed based on catheter-derived hemodynamic data, but this technique is invasive and not without risk (Omron et al 2003). In practice, this technique is not appropriate for experimental purposes.

1.9.2 Echocardiography

1.9.2.1 Quantitating severity of AS

Currently, conventional echocardiography, including Doppler echocardiography is the standard imaging modality in the assessment of AS severity and plays a major part in determining the management of AS. Doppler echocardiography permits noninvasive assessment of hemodynamic factors, valve morphology predictive of AS severity (reviewed by Mochizuki and Pandan 2003, Stout and Otto 2003). The rate of progression,
expressed as rate of increase of transvalvular pressure gradients or rate of decrease of AVA per year of follow-up, is calculated by the Bernoulli and Gorlin equations respectively (reviewed by Stout and Otto 2003). The mean annual increase in aortic valve pressure gradient (AVp) appears to be averaging about 5-7mmHg per year in patients with initially moderate AS, with an associated decrease of 0.1 cm² for aortic valve area (Bahler et al 1999, Otto et al 1995, Faggiano et al 1996, Faggiano et al 1992, Nassimiha et al 2001) in patients with AS. However, detection of increased AVp and reduction of AVA can only occur once significant obstruction of the aortic valves is present. Initial valve distortion and thickening of the aortic valves in ASc cannot generally be quantitated by Doppler echocardiography. Furthermore, there is significant overlap in hemodynamic severity between symptomatic and asymptomatic adults. This led to some confusion as to why some patients became symptomatic at “moderate stenosis”, but asymptomatic at “severe stenosis” as classified based on hemodynamic parameters quantitated by Doppler echocardiography (reviewed by Otto 1999). Thus, even though echocardiographic parameters can provide some estimation as to the progression of mild to severe AS, it is still not the gold standard for predicting which patients are most likely to benefit from AVR prior to the developments of symptoms.

1.9.2.2 Quantitating severity of ASc

The presence of ASc by definition must exclude the presence of hemodynamic changes, with minor obstruction to blood flow. In the largest study of ASc to date (Otto et al 1999), the Cardiovascular Health Study (CHS) defined ASc as: “focal areas of increased
echogenicity, and thickening of the aortic valve leaflets without restriction of leaflet motion”. Cosmi et al (2002), in a progression study, defined ASc as: “focal or diffuse leaflet thickening or calcification, normal valve excursion, and peak Doppler flow velocity of < 2m/s). Both of these large clinical studies relied upon purely subjective visual assessment to identify ASc. Cosmi et al also classified ASc severity based on visual detection of calcification of the short axis view on echocardiogram, and classify ASc severity on a 0-3 scoring system:

- 0 = normal
- 1 = mild (minor involvement of one leaflet)
- 2 = moderate (minor involvement of two leaflets or extensive involvement of one leaflet)
- 3 = severe (extensive involvement of two leaflets or involvements of all three leaflets)

Other studies score the aortic valves using a similar scoring system of 0-4 based on visual assessment of the increased reflectivity of the aortic valves (Rosenhek et al 2004, Shively et al 1998). This technique of classification of ASc severity is highly operator/assessor, as well as image quality dependent. Furthermore, as regards reproducibility of such methodology, there has been no study examining the serial operator/assessor reproducibility. Reproducibility (between observers) has been reported, but not between operators and in serial examinations. Furthermore, there is no assumption of linearity of progression on the basis of changes in gradings: hence ASc progression can be assessed
semi-quantitatively. (Figure 1.2 gives examples of visual assessment of normal, sclerotic, and stenotic aortic valves).

1.9.3 Computed tomography

Computed tomography (CT) is a widely used noninvasive method of screening for atherosclerotic CAD, with estimated 80%-100% sensitivity and 80% specificity (Breen et al 1992, Mautner et al 1994, Haberl et al 2001). Incidental findings of aortic valve calcification during CAD screenings using CT have initiated the use of this technique to quantitate calcium on aortic valves in AS. In addition to multislice CT, electron beam tomography (EBT), a cross-sectional imaging technique with high temporal resolution, which used mainly to sensitively detect and quantify coronary calcifications, has also been used to assess valve calcification in AS (Pohle et al 2001, Kaden et al 2002, Budoff et al 2000). Pohle et al (2001) in a study of 104 patients, it was found that progression of aortic valve calcification, quantitated by EBT was correlated with serum levels of LDLs. This study was the first to investigate the association between progression status of patients with AS and degree of calcification of aortic valve leaflets. It is possible to use EBT in medical intervention studies to find treatments that could slow down calcification of the aortic valves.

Although EBT can quantitate calcium deposition, it is not possible for this technique to visualize or assess functional status of the aortic valve. In addition, there may be some calcification during ASc, but inflammation, matrix remodeling, and thus fibrosis are
likely to precede calcium deposition. Thus, EBT is more suitable for following the latter stages of AS.

1.9.4 Magnetic resonance imaging (MRI)

Assessment of AS severity is currently done by echocardiography by measuring AVv, AVp and AVA. However, in some patients this technique is limited due to poor acoustic windows, operator/angle-dependent, and inaccurate estimate of transaortic flow due to eccentric jet morphology (Fischer et al 1995, Bartunek et al 1995, Danielsen et al 1989).

Direct planimetry of the aortic valve by cardiac magnetic resonance imaging (MRI) is a possible new method independent of hemodynamic status, flow turbulence and quality of images. Several small studies have shown that MRI offer high spatial resolution analysis of cardiac anatomy and myocardial function, with AVA measurements, transaortic velocity (AVv) and AVp are in strong agreement with other methods of quantitating severity of AS (Caruthers et al 2003, John et al 2003, Friedrich et al 2002, Kupfahl et al 2004). However, although it is generally accepted that MRI is a more superior technique in quantitating and visualization of cardiac anatomy and myocardial function (Pennell et al 2004), cardiac ultrasound remains the gold standard for quantitating severity of AS. There is still lack of studies which can show conclusively that the cost and benefit of MRI in patients with AS outweighs the cost and benefit of using cardiac ultrasound. Furthermore, at the current time, MRI cannot be used to quantitate ASc.
Clinical strategies to retard the progression of ASc demand the development of an optimal methodology for quantitation of the degree of sclerosis as a measure of the rate of progression, and the effectiveness of clinical interventions. The recent emergence of techniques utilizing changes in ultrasonic backscatter to detect and characterize pathological changes in tissues may offer a potential means for assessment of aortic valve diseases, possibly before hemodynamic changes occur.

1.10 Treatment of AS

Therapeutic priorities in ASc/AS may be classified, on the basis of potential adverse outcomes, and according to natural history is depicted in Figure 1.3.

1.10.1 Possible therapy for ASc

To date, there has been no study to retard progression or for cardiovascular risk reduction therapy for ASc, possibly due to lack of quantitative measure of early AS, and the long course of disease. It is possible that reduction in CV morbidity and mortality for example with statins eg. the WOSCOPS study (Shepherd et al 1995); or with ACE inhibition, eg. perindopril (EUROPA, the EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease Investigators 2003) and ramipril (HOPE, Yusuf et al 2000), may have been particularly evident in that subset of individuals with ASc. Furthermore, it is also possible, but equally non-evaluated, that these treatments may have retarded progression of ASc (See Figure 1.3). There are emerging prospective
randomized clinical studies using statins; with much fewer retrospective clinical studies suggesting a glimpse of evidence with the use of ACE inhibitors in AS progression.

1.10.2 Treatment to slow progression of AS

1.10.2.1 Statin therapy

It is generally agreed that the presence of hypercholesterolemia is associated with higher incidence and progression of AS in clinical studies (Aronow et al 2001, Stewart et al 1997). Dietary cholesterol supplementation, or genetic modification to induce severe hypercholesterolemia in animals result in abnormal aortic valve lesions (Rajamannan et al 2001, Cimini et al 2004, Drolet et al 2006, Tanaka et al 2005). The combination of vitamin D2 supplementation and a high cholesterol diet resulted induced AS development in rabbits (Drolet et al 2003). Both clinical and experimental evidence showed a strong trend that cholesterol deposition play an important role in development and progression of AS.

In animal studies, using atherogenic diets (1% cholesterol-supplemented) (Rajamannan et al 2001, Cimini et al 2004), it was found that atorvastatin significantly reduced osteopontin expression in rabbits. Furthermore, atorvastatin significantly increased aortic valve eNOS expression (Rajamannan et al 2005, Heart). Rajamannan et al (2005, Circulation) also showed in rabbits that treatment with high cholesterol diet, marked upregulation of the LDL coreceptor (Lrp5), important in skeletal formation (reviewed by
Ferrari SL et al 205); and that addition of atorvastatin normalized aortic valve Lrp5 receptor expression compared to rabbits treated with cholesterol alone. The authors postulated that atorvastatin could possibly function via the Lrp5 receptor pathway in reducing aortic valve calcification.

Furthermore, the interaction between statin therapy and progression of AS could in theory be modulated by suppression of expression of a variety of bone morphogenic proteins. Osman and Chester et al (2006) demonstrated that in a human interstitial cell culture model, atorvastatin reduced activity and expression, not only of TGF-β1, but also of TGF-β3 and two bone morphogenic proteins. Additionally, in a similar cell culture model Osman and Yacoub et al (2006) have demonstrated that osteoblast activation is also under the control of purinergic receptors with P2Y receptor activation by ATP associated with osteoblast activation. These studies suggested that the anti-calcific effects of statins could also be modulated in part by decreases in activation of P2Y receptors. Therefore, these findings suggested several different anti-calcific mechanisms for atorvastatin both in vitro and in vivo models of aortic valve mineralization.

A number of retrospective clinical studies showed promising results for patients who were on statin therapy, by documenting significantly slower progression of AS for this group (Aronow et al 2001, Novaro et al 2001, Bellamy et al 2002, Rosenhek and Rader et al 2004). Duration of these studies ranged from 21 to 44 months, with patient population characteristics relatively similar to each other: typically patients had moderate AS at initial evaluation.
However, the first prospective, randomized, placebo-controlled trial of high dose atorvastatin therapy for 25 months showed no trend towards improving progression rate of patients moderate AS, despite significantly lowering cholesterol levels (Cowell et al 2005). It remains possible that the study was negative because statins are potentially effective only in relatively early AS. Recently, another prospective clinical study using rosuvastatin found that over a mean follow-up of 73 ± 24 weeks in patients with at least moderate AS, subjects in the rosuvastatin group had significantly slower rate of progression compared to those who were not. However, the study was not a blinded randomized study and thus was subjected to selection biases. In turns, the question whether statin therapy is truly beneficial in AS progression remains largely elusive (Moura et al 2007).

It could be therefore argued that by lowering total cholesterol per se, although beneficial in patients with CAD, does not influence progression of AS. Perhaps the effects of statin therapy seen in retrospective studies, where patients received statins for cholesterol lowering over a long period time, reflected beneficial “pleiotropic” effects of statin therapy. For example, in severely hypercholesterolemic rabbits, atorvastatin inhibits hypercholesterolemia-induced cellular proliferation, bone matrix production, reduces inflammatory markers, enhances nitric oxide synthase expression, and calcification of aortic valve lesions (Rajamannan et al 2001, Rajamannan et al 2002, Rajamannan et al 2005 Heart, Rajamannan et al Circulation 2005). However, due to the fundamental hypercholesterolemic nature of the model, it cannot be excluded that atorvastatin exerted
some beneficial effects on aortic valve lesions simply by lowering cholesterol levels ie by restoring the status quo. The use of statin therapy for slowing down progression of AS requires further evidence from more prospective clinical trials, which are currently in progress.

1.10.2.2 Angiotensin-converting enzyme inhibitors (ACEi)

Presence of angiotensin-converting enzymes (ACE), angiotensin II (Ang II) found on stenotic aortic valve lesions (O’Brien et al 2002), together with absence on normal valves raised the possibility for the use of angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blocker to delay progression of AS. Furthermore, there is also evidence of local production of Ang II by chymase, tryptase and cathepsin G from activated mast cells identified on stenotic aortic valves (Helske et al 2004, Helske et al 200). Activation of the renin-angiotensin system has been shown to be in parallel with increased matrix remodeling, collagen, fibronectin expression, and tissue fibrosis (see section 1.3.2, reviewed by Mehta and Griendling 2007). ACEi have been proven effective in reducing fibrosis and matrix remodeling of the myocardium in left ventricular hypertrophy, as well as proven beneficial in atherosclerotic complications (Ferrari R et al 2006, Greenberg et al 2005), it is thus possible that ACEi could also reduce aortic valve matrix remodeling and perhaps delay progression of valve fibrosis.

Recently, utilizing a hypercholesterolemic rabbit model to induce aortic valve lesions, Arishiro et al (2007) found that treatment with olmesartan, an angiotensin receptor
blocker, significantly reduced macrophage, lipid, and osteopontin accumulation. Furthermore, this study found that rabbits in the olmesartan group had preserved endothelial integrity of the aortic valves as seen with CD31 immunohistochemistry, as well as increased eNOS expression. While the functional connotations of these findings were not explored by the investigators, these results provide incremental evidence for a pivotal role of the renin-angiotensin system in the pathogenesis of aortic valve infiltration.

There has not been as much interest in the use of ACEi compared to statin therapy for AS progression. In a retrospective, observational study of 123 patients, aortic valve calcium was significantly lower in patients who received ACEi compared to those who did not, as assessed by EBT (O’Brien et al 2002). However, in another retrospective study of 211 patients, effects of ACEi and statins on the rate of AS progression were compared (Rosenhek and Rader et al 2004). Patients on statin therapy had significantly slower progression rate compared those who were not; although there was a trend towards slower progression for patients on ACEi, this was not statistically significant. If the postulated mechanisms of ACEi in delaying AS progression were to prevent valve matrix remodeling and fibrosis, which are upstream to calcification and occur at the sclerosis stage, it may be more beneficial to study ACEi in patients with ASc rather than AS. Thus, there must be methods which can quantitate the degree of change in valve morphology at the earlier stage of the disease. There is no current method for quantititating valve thickness at ASc stage. In turns, this limits the studies of ACEi for delaying AS progression.
1.10.3 Treatment of end-stage disease

1.10.3.1 Surgical intervention

In severe cases of calcific AS, the onset of symptoms of congestive heart failure, syncope or angina indicates a poor prognosis if medical therapy alone is utilized (Chizner et al 1982, Horstkotte and Loogen 1988, Turina et al 1987). If prompt aortic valve replacement is not received at the onset of symptoms, the average survival in these patients could be as early as 2 years (Turina et al 1987), with few as 12% of patients surviving for up to 5 years (Horstkotte and Loogen 1988).

Operative mortality rates within 30 days after the operation is as low as 1%, but can be up to 6% (Lindblom et al 1990, Culliford et al 1991), with 3 year survival of 80% (Bouma et al 1999). The risk of postoperative morbidity such as stroke, hemorrhagic complications, prosthetic valve dysfunction and endocarditis vary between 2% to 5% (Freeman and Otto 2005, Thourani et al 2000). However, surgical intervention for symptomatic patients is only generally offered to patients < 80 years (Bouma et al 1999), since the aging population in the Western world is on the rise, there is a significant push to delay progression of AS with medical therapies.

As regards alternatives to open-heart surgery, aortic valvuloplasty has been to all extent abandoned as a clinically useful technique, due to very high recurrence rates (reviewed by Dauterman et al 2003). However, the possibility of catheter-laboratory-based “non-
operative” valve replacement has now become a reality, although thus far only a small number of patients have had the procedure (Lichtenstein et al 2006).

1.10.3.2 Palliative symptomatic relief

Among elderly patients with severe AS and concomitant major non-cardiac morbidity, only a minority ever receive AVR. However, these patients are at serious risk of ongoing disability and multiple hospital admissions prior to death.

No treatment for non-surgical management of AS have been subjected to clinical trial to date. Short-term infusion of sodium nitroprusside has been used to stabilize decompensated patients with AS prior to valve replacement (Khot et al 2003), but the implications of long-term treatment with other NO donors have not been explored.

The anti-anginal agent perhexiline, which improves efficiency of myocardial oxygen utilization (Unger et al 2005, Jeffrey et al 1995) and exerts anti-inflammatory effects in aortic valve matrix (Kennedy et al 2006), has been shown to improve symptomatic status in some “inoperable” patients with AS (Unger et al 1997) but no blinded randomized data have been reported to date.
1.11 Pathogenesis of aortic stenosis: methodological constraints

With increased aging population of the Western world, the prevalence of AS is on the rise, along with the costs associated with complications and hospitalizations from AVR, symptoms of severe AS, gastrointestinal bleeding, and from adverse cardiovascular events. Despite increased knowledge in the morphology of stenotic valves, consequences of ASc/AS, clinical factors associated with increased prevalence of ASc/AS; the pathogenesis of ASc/AS remain largely elusive.

While it is easier to obtain stenotic aortic valves for pathogenetic studies from patients undergoing aortic valve replacement (AVR); patients with ASc generally do not undergo AVR, and therefore ASc in humans can be studied directly only in post-mortem specimens or in recipient hearts after cardiac transplantation. Therefore, characterization of aortic valve lesions has mainly been performed at a single point in time and of stenotic aortic valves rather than sclerotic valves (Otto et al 1994, Warren and Yong 1997). This makes it difficult to study the course of the disease as well as for intervention studies where serial examinations of aortic valves are required to assess changes in valve morphology. Therefore, there is a need to develop animal models of AS to assess periodic changes of aortic valve lesions for interventional studies.
1.12 Current animal models of AS

Stenotic aortic valves and bicuspid aortic valves cause turbulent transvalvular blood flow. The turbulence of flow has been postulated to be responsible for endothelial damage and it is postulated that bacteria can adhere to a damaged surface more easily than an intact surface. Cohen et al (2004) induced bacterial endocarditis in New Zealand White rabbits with calcification of aortic valves developing within 2 weeks. Although, there is evidence showing presence of *Chlamydia pneumoniae* on nonrheumatic human aortic stenotic valves (Juvonen et al 1997, Juvonen et al 1998), there has been no clinical studies which documented the association between recurrent infections and higher prevalence of AS. It is possible that the presence of bacteria and chronic infection might result in systemic chronic inflammation which contributes to valvular inflammation and development of aortic valve lesions. However, injection of bacteria to induce aortic valve calcification lesions do not represent the true pathogenesis of AS and would only represent a small number of human cases.

In mice model of AS, Tanaka et al (2005) observed that apolipoprotein E-deficient (Apo E -/-) mice developed marked increases in transaortic valve flow velocity over a period of about 2 years. Apo E -/- mice develops severe hypercholesterolemia (Zhang et al 1992) and this is postulated to cause aortic valve lesions in this model. Interestingly, this study also noted that wild-type C57BL/6 mice also developed significantly increases in transaortic valve flow over the 2-year period. Although there was a difference in AV flow velocity between the wild type C57BL/6 and Apo E -/- mice, the regression lines between
age and transaortic valve flow for the wild type C57BL/6 group were not significantly different compared to the Apo E -/- mice group. This indicates that some mice with Apo E-deficiency developed similar increases of transaortic valve flow compared to naturally aging wild type C57BL/6 mice. Thus, it is possible that severe hypercholesterolemia from Apo E-deficiency do not contribute entirely to the development of AS in mice.

In addition, another study used the same type of wild-type mice, the C57BL/6, which the authors acknowledge are genetically prone to develop diet-induced obesity and atherosclerotic lesions, in an attempt to induce AS. Drolet et al (2006) compared wild type C57BL/6 mice on a high fat/high carbohydrate (HF/HC) and low-density lipoprotein receptor deficiency (LDLr-/-) on the HF/HC diet, compared to their respective controls where a normal diet was given, as regards the development of AS. The study showed that AVA was significantly decreased at an approximately similar extent for these 2 groups compared to their respective controls. Although the authors concluded that use of a HF/HC diet resulted in significant changes in echocardiography hemodynamics in both the wild type and LDL -/- mice groups compared to their respective controls, the mechanism of this difference was not clear. Despite a 9-fold increase in LDL cholesterol levels, an almost 5-fold increase in total cholesterol levels, and a 2-fold increase in glucose levels, mice on a HF/HC diet in the LDL -/- group had a 12.2 mm²/mg decrease in AVA, and a 24.5 cm/s increase in maximum transvalvular velocity; compared to wild-type mice on the HF/HC diet who had decreased AVA by 17.5 mm²/mg, and increase of 20.8 cm/s in velocity over 4 months of treatment. Therefore, marked increase in total
cholesterol, LDL cholesterol, and glucose levels did not affect progression of AS in this study.

Other animal models of AS, although generally resembling those of atherosclerosis, showed similar microscopic changes to those of human aortic valve lesions (Rajamannan et al 2001, Rajamannan et al 2002, Cimini et al 2004). In these studies, New Zealand White rabbits were given high cholesterol diet for 12 weeks. Although no gross calcification was found on rabbit aortic valves, microscopic changes such as infiltration with macrophages and T-lymphocytes; cellular proliferation and bone matrix protein expression were seen (Rajamannan et al 2001, Rajamannan et al 2002, Cimini et al 2004). Interestingly, none of these studies have utilized echocardiography on these treated rabbits, so hemodynamic changes associated with changes in aortic valve pathology are unknown.

The “atherosclerotic” model of AS in rabbits was modified by Drolet et al (2003) with the use of vitamin D$_2$. High dose vitamin D$_2$ (50,000IU/day) was given in addition to high cholesterol supplemented diet to New Zealand White rabbits and followed for 12 weeks. Echocardiography was performed, significantly decreased AVA and increased AVp were found in the vitamin D$_2$ and cholesterol group compared to controls and the cholesterol supplemented group alone. Gross calcification was also seen in the vitamin D$_2$ supplemented group but no calcification seen in the other 2 groups. This study therefore provided evidence for the first time that high cholesterol alone may not be
sufficient to induce AS, and that the effects of vitamin D and vascular calcification warrant further studies.

1.13 Postulated mechanisms for adverse outcomes of ASc/AS

1.13.1 Underlying atherosclerosis

The mechanisms of adverse outcomes with ASc and AS remain unclear. The common suggestion is that adverse outcomes of ASc is not due to the primary valvular disorder but rather ASc is a surrogate marker for the underlying atherosclerotic disease (Freeman and Otto 2005; Chandra et al 2003).

Chandra et al (2003), in contrast to other studies (Otto et al 1999, Aronow et al 1999) showed that the presence of ASc did not directly predict cardiovascular outcomes but was rather a marker of the presence of CAD and inflammation. The study was subjected to selection bias as patient population was not truly random, but selected patients with chest pain from the emergency room. Patients with ASc combined with having high hs-CRP levels were at a fivefold increased risk of adverse events. The study postulated that systemic inflammation, which has been shown to promote and destabilize coronary atherosclerosis (Burke et al 2002) may also be involved in the development of aortic valve lesions and could potentially be the link between increased adverse cardiovascular events and ASc.
Further evidence supporting the role of systemic inflammation and ASc was provided by Galante et al 2001 (2001). This study found that hs-CRP levels were elevated two-fold in patients with severe AS, without coronary artery disease compared to healthy control group. However, in adults with severe AS, only about 50% have hemodynamically significant CAD (Otto 2001), and similarly patients with CAD do not necessarily have coexisting AS.

In summary, the available literature on ASc suggests that it is a marker of potential plaque instability, via activation of inflammatory processes rather than of atheroma alone.

### 1.13.2 Presence of renal dysfunction

Although it is clear that the development and progression of AS are significantly accelerated in patients with end-stage renal disease or patients on dialysis, the impact of degree of renal dysfunction has not been evaluated closely. With the exception of 2 studies, which found that elevated serum creatinine is associated with presence and faster progression of AS (Bahler et al 2000, Palta et al 2001), other larger studies did not examine this (Aronow et al 1999, Otto et al 1999). The obvious basis for this failure is technical: a) renal function tends to deteriorate with time; b) the means of evaluating patients on dialysis in this regard would be problematic. The presence of end-stage renal disease and/or dialysis is associated with abnormal mineral metabolism, in particular calcium and phosphorus and this is thought to precipitate vascular/valvular calcification (Giachelli 2004, Wang et al 2003). The nexus between renal dysfunction and
cardiovascular events has been recognized, with worsening of renal function being a strong marker of increased myocardial infarction (Go et al 2004). Notably, none of the studies linking ASc with cardiovascular risk have taken renal dysfunction into account.

1.13.3 Platelet aggregability

Platelets have an increasingly well-defined critical role in coronary artery thrombosis (Ruggeri 2002, Heeschen et al 2003). It is possible that the association between ASc and cardiovascular events are mediated at least some part by nett platelet hyperaggregability. Patients with AS have been found to exhibit increased platelet reactivity to some proaggregants, and thrombus formation has been documented on severely stenosed valves (Riddle et al 1983, Stein et al 1977, Kaul et al 1998).

Furthermore, Chirkov et al (2002) measured platelet aggregatory responses to adenosine 5’- diphosphate (ADP) to determine whether AS, with or without concomitant CAD is associated with abnormalities in platelet aggregation. Although among patients with AS the study found no difference in platelet aggregability between those with and without concomitant CAD, platelets from patients with AS were more aggregable than those from nonischemic patients. In addition, platelet responsiveness to the NO donor, sodium SNP was also assessed in this population. The antiaggregating effects of SNP were significantly impaired in blood samples from AS patients, constituting evidence for platelet resistance to NO in this group of patients.
Additionally, impairment of valvular endothelial function was also present in explanted valves of patients with advanced AS (relative to physiology of non-calcified valves) (Chirkov et al 2006), but these findings were not related to systemic endothelial function. These 2 studies provided for the first time that AS is associated with abnormal platelet aggregability, impaired platelet responsiveness to NO, valvular endothelial dysfunction, and raise the question of whether these forms of vascular and valvular dysfunction emerge at the stage of ASc.

1.13.4 Endothelial dysfunction

Vascular endothelial dysfunction was demonstrated to be present at an early stage of atherogenesis and is associated with increased risk of adverse cardiovascular events (reviewed by Lerman and Zeiher 2005). Poggianti et al (2003) compared systemic endothelial function in patients with suspected or known CAD with and without ASc using flow-mediated dilation (FMD). FMD was significantly impaired in patients with ASc compared to those with normal valves, while administration of the endothelium-independent vasodilator response to sublingual glyceryl trinitrate (GTN) did not show any improvement in FMD for either groups of patients. This study therefore showed that in the presence of CAD, the concomitant finding of ASc is associated with incremental increases of endothelial dysfunction. To some extent, therefore, this study can be seen as a partial physiological correlate of the findings of Otto et al 1999, and Aronow et al 1999: that the presence of ASc implies greater physiological disturbance as well as greater prognostic risk than would otherwise be apparent.
The existence of endothelial dysfunction at systemic and valvular level in cases of ASc is of potential significance (respectively) as regards cardiovascular risk in ASc and potential for progression of ASc to AS. The issue of ASc progression in particular is a central theme of this thesis, and therefore the role of the valve endothelium and its relationship with matrix function is reversed in detail below.

1.14 Endothelial function and dysfunction: vascular and valvular implications

Endothelial dysfunction is a broad term that has come to imply the biological effects of diminished production, availability or effect of NO and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors. The loss of biological activity of endothelium-derived NO is accompanied by other alterations that further promote the increase in propensity for vasoconstriction, thrombosis, inflammation, and cellular proliferation in the vascular wall (reviewed by Griendling and Fitzgerald I and II (2003)). Thus, endothelial dysfunction has the potential to contribute to key events in the course of human atherosclerosis and possibly the development of AS lesions.

Endothelium-derived NO plays an important role in the regulation of cardiovascular function. NO is the most potent endogenous vasodilator known, and a critical modulator of regional blood flow, exerting its effect mostly via stimulation of soluble guanylate cyclase to produce cyclic GMP (reviewed by Behrendt and Ganz 2002). Endothelial
release of NO attenuates the vasoconstrictor effects of noradrenaline, endothelin, angiotensin II, and serotonin. It is formed in endothelial cells from the amino acid L-arginine by endothelial isoform of NO synthase (eNOS). In addition to producing NO constitutively, the enzyme may be stimulated by a variety of physiological agonists, shear stress, and pharmacological agents (reviewed by Searles 2006, Tai et al 2004).

Although initially characterized as a vasodilator, NO has since been discovered to mediate many other “protective” functions of the endothelium. It inhibits vascular smooth muscle proliferation which could have resulted in medial thickening and/or myointimal hyperplasia. NO inhibits the interaction of circulating blood elements with the vessel wall. It limits vascular recruitment of platelet aggregation and leukocyte adherence by inhibiting the expression of proinflammatory cytokines, chemokines, and leukocyte adhesion molecules (reviewed by Yang and Loscalzo 2000). In addition, NO inhibits the production of tissue factor, a molecule that plays a critical role in the propensity of disrupted atherosclerotic plaques to cause intravascular thrombosis (reviewed by Gimbrone 1995).

Of the causes of reduced vasodilator influence, derangements of the nitric oxide synthase (NOS) pathway have been most studied. Mechanisms involved in the derangements of the NOS pathway include reductions in: (1) NO half-life; (2) tissue responsiveness to NO; (3) nitric oxide synthase (NOS) expression; (4) NOS activity. (Summarized in Figure 1.4).
1.14.1 The valve endothelium: normal function

Several early studies using electron micrograph scanning of aortic valves showed that the surface of an intact aortic valve is covered by polygonal endothelial cells (Harasaki et al 1978, Riddle et al 1980). In diseased aortic valves, these studies showed denudation of the endothelial cell layer, with platelets, leukocytes, and scattered erythrocyte adhesions were seen on the exposed subendothelial surface. Physiologically, Pompilio et al (1998) showed that intact porcine aortic valve significantly contracted to phenylephrine, and relaxed with acetylcholine, analogous to endothelium-dependent relaxation. Hence, denudation of the endothelial layer significantly retarded the acetylcholine-induced relaxation. Furthermore, in the presence of intact aortic valve endothelium, there was significantly greater release of prostacyclin compared to those without the endothelium. These effects in porcine aortic valves were also seen with canine aortic valves (Ku et al 1990). These studies therefore demonstrated that the aortic valve endothelium is capable of relaxation and contraction to physiological stimuli. There is abundant evidence that endothelium from valve surfaces is functionally somewhat different from vascular endothelium. For example, in response to shear stress, porcine valve endothelial cells tended to express genes associated with chondrogenesis, rather than osteogenesis (Butcher et al 2006). These data also raise the possibility that valve endothelium plays crucial roles in the maintenance of valvular homeostasis; via anti-aggregatory properties; and provision of protection against adhesion of inflammatory cells.
1.15 Biochemical mechanisms of “endothelial dysfunction”: role of reactive oxygen species (ROS).

Dysfunction of the NO pathway can occur via an increase in oxidative stress (increased free radical activity). Reactive oxygen species (ROS) such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) can combine with NO directly, terminating effects of NO and resulting in the production of the highly reactive peroxynitrite anion (ONOO$^-$) (reviewed by Maxwell 2002).

Superoxide radical is formed as a result of one electron reduction of oxygen. When O$_2^-$ is produced in concert with NO, they react rapidly to form the highly reactive molecule ONOO$^-$. ONOO$^-$ is an important mediator of lipid peroxidation and protein nitration, including oxidation of LDL. In the absence of immediately accessible NO, O$_2^-$ is rapidly dismutated to the more stable ROS, H$_2$O$_2$ by superoxide dismutase, which can then be converted to H$_2$O by either catalase or glutathione peroxidase (reviewed by Griendling and Fitzgerald 2003). The reaction of O$_2^-$ with NO occurs constitutively at a low rate. When formed in low amounts intracellularly, O$_2^-$ and H$_2$O$_2$ can act as intracellular second messengers, modulating the function of biochemical pathways mediating such responses as growth of vascular smooth muscle cells (VSMCs) and fibroblasts. Higher amounts of ROS can cause DNA damage, lipid peroxidation and protein nitration, including oxidation of LDL, producing significant toxicity or even apoptosis in endothelial cells and smooth muscle cells (Li et al 1997, reviewed by Clempus and Griendling 2006).
1.15.1 NAD(P)H oxidase(s)

In mammalian cells, potential major enzymatic sources of ROS include the mitochondrial 
electron transport chain, arachidonic acid pathway enzymes lipoxygenase and 
cyclooxygenase, cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, NO 
synthase, peroxidases, and other hemoproteins (reviewed by Cai and Harrison 2000). 
Although many of these sources could potentially produce ROS that inactivate NO, two 
of the most important sources in cardiovascular system are thought to be 
NADH/NAD(P)H oxidase(s) and NO synthase.

The NAD(P)H oxidases of the cardiovascular system are membrane-associated enzymes 
that catalyze the 1-electron reduction of oxygen using NADH or NADPH as the electron 
donor. The activity of the vascular NADH/NAD(P)H oxidase is regulated by a large 
number of cytokines, hormones, and mechanical forces that are known to be involved in 
the pathogenesis of vascular disease. NAD(P)H oxidase-derived (O$_2$•$^-$) and (H$_2$O$_2$) are 
intimately involved in the growth response and apoptosis signaling of VSMCs and 
fibroblasts. These ROS are thus functioning as signaling molecules to mediate specific 
cellular responses when present at low amounts (review by Cai 2005).

In atherosclerotic lesions, VSMC NAD(P)H oxidase-derived ROS may play a crucial role 
in progression and biological activity. Angiotensin II, platelet-derived growth factor 
(PDGF), and tumour necrosis factor – alpha (TNF-α) may increase ROS production in
the atherosclerotic lesion by stimulating the local vascular myocytes to produce ROS (reviewed by Cai et al 2003). Subsequently, ROS may contribute to LDL oxidation, local monocytic chemoattractant protein -1 (MCP-1) production, upregulation of adhesion molecules and macrophage recruitment, endothelial dysfunction, and extracellular matrix (ECM) remodeling through collagen degradation (reviewed by Griendling et al 2000).

Recently, we have demonstrated that sections of stenotic human aortic valve are capable of substantial \( (O_2^{\cdot-}) \) generation via NAD(P)H oxidase. The stimulation of \( (O_2^{\cdot-}) \) production by the addition of exogenous NADPH suggests that the myofibroblasts of the valve matrix are a likely major source of \( (O_2^{\cdot-}) \) in these valves. It is postulated that this \( (O_2^{\cdot-}) \) may be an important factor in accelerating valve calcification, and therefore that inhibition of \( (O_2^{\cdot-}) \) release may slow the process of calcification (Kennedy et al 2006).

### 1.15.2 Endothelial nitric oxide synthase as a source of ROS

Another potential source of ROS production is eNOS. eNOS is a cytochrome p450 reductase-like enzyme that catalyzes flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. The enzyme requires tetrahydrobiopterin \( (BH_4) \), bound near this heme group, to transfer electrons to a guanidino nitrogen of L-arginine to form nitric oxide. In the absence of either L-arginine or BH\(_4\), eNOS can produce O\(_2^{\cdot-}\) and H\(_2\)O\(_2\). This phenomenon has been referred to as NOS uncoupling (Pou et al 1992, Vasquez-Vicar et al 1998).
Uncoupling of eNOS in the endothelium may contribute to oxidative stress and endothelial dysfunction via 3 postulated mechanisms: (1) the enzymatic production of NO is diminished, allowing the radicals that it normally might react with to attack other cellular targets; (2) the enzyme begins to produce $O_2^{=} \cdot$ contributing to oxidative stress; (3) eNOS can become partially uncoupled, such that both $O_2^{=} \cdot$ and NO are produced simultaneously. Under this circumstance, eNOS may become a peroxynitrite generator.

1.16 Asymmetric dimethylarginine (ADMA)

1.16.1 Formation of ADMA

At least 3 arginine analogues are present physiologically in plasma and tissues: $N^G$-monomethyl-L-arginine (L-NMMA), $N^{G_i}$, $N^{G_i}$-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), and $N^{G_i},N^{G_i'}$-dimethyl-L-arginine (symmetric dimethylarginine, SDMA). The asymmetric methylated arginine residues (L-NMMA and ADMA), but not the symmetrically methylated arginine (SDMA), are competitive inhibitors of the endothelium NOS (eNOS) enzyme.

The free guanidine-methylated ($N^{G_i}$) arginine residues occur endogenously as a result of protein methylation. The protein-arginine methyltransferase (PRMT) enzyme is primarily responsible for the arginine methylation of all substrate proteins (Paik and Kim 1968). Although the existence of protein-arginine methylation has been known for over 30 years, the cellular functions and genetic coding are still poorly understood.
1.16.2 Clearance of ADMA

The early identification of both ADMA and SDMA in human urine led to the assumption that renal excretion was the only route for removal of free methylarginines. However, further investigation into the route of elimination of these amino acids showed that urinary excretion of SDMA was 30 times greater than that of either L-NMMA or ADMA in rabbits (McDermott 1976). It was demonstrated by radiolabeled studies of ADMA and SDMA in rats that the enzyme responsible for the clearance of ADMA was $\text{N}^{\text{G}},\text{N}^{\text{G}}$-dimethylarginine dimethylaminohydrolase (DDAH) (Ogawa et al 1989).

DDAH was shown to hydrolyse ADMA and L-NMMA to yield citrulline and dimethylamine or monomethylamin, respectively. The functional importance of DDAH was tested by McAllister et al. (1996) when a DDAH inhibitor was shown to cause an accumulation of endogenously generated ADMA (but not SDMA). They also found that inhibition of DDAH causes gradual vasoconstriction of vascular segments, which was reversed by L-arginine. Furthermore DDAH activity in cultured endothelial cells decreased upon exposure to oxidized LDL or TNF-α, and this was associated with increased ADMA concentrations. DDAH activity was also decreased in an in vivo model of hypercholesterolaemic rabbits, but there was no change in expression of the enzyme (Akira et al 1999). Given the pleiotropic effects of NO in multiple systems, DDAH has the potential to regulate all of these actions of NO through modulation of ADMA levels reviewed by (Valkonen et al 2005) (Summarized in Figure 1.5)
It is currently postulated that one way to lower ADMA levels is to increase expression or activity of DDAH. Conversely, there is also increasing evidence that DDAH activity is potentially impaired under conditions of incremental redox stress (Ueda et al 2003), providing a potential mechanistic link between redox stress and elevation of ADMA concentrations.

1.16.3 Pathophysiologic importance of ADMA

The competitive NOS inhibitor, L-NMMA was recognized to play an important role in NO formation both in vitro and in vivo before the importance of ADMA was discovered (Palmer et al 1988, Vallance et al 1989). ADMA and L-NMMA exert their NOS inhibition with equal potency. However, only low concentrations of L-NMMA are present in plasma, suggesting that ADMA may be the major endogenous NOS inhibitor. Vallance et al. (1992) first reported elevation of plasma ADMA concentrations, in patients with renal failure, postulating ADMA accumulates as a result of reduced renal clearance. These investigators showed that the concentration of ADMA in plasma of patients with end-stage renal disease is several-fold higher than that in the plasma of healthy human subjects.

Furthermore, plasma ADMA concentrations have also been found to be elevated in hypercholesterolaemia, in elderly patients with peripheral arterial disease and generalized atherosclerosis (Boger et al 1997). Patients with diabetes (Lin et al 2002), systemic hypertension (Takiuchi et al 2004), and advanced age (Horowitz and Hereztyn 2006)
were found to have higher plasma ADMA concentrations than their respective controls. Elevated plasma ADMA concentrations have also been demonstrated in patients with congestive heart failure (Usui et al 1998), pulmonary hypertension (Kielstein et al 2005) and in symptomatic coronary artery disease (Krempl et al 2005, Miyazaki et al 1999).

The postulated pathophysiological role of ADMA is supported by the observed effects of ADMA infusion on endothelial function, blood pressure and cardiac function. Achan et al (2003) found that acute low-dose ADMA challenges in healthy human volunteers induced a decrease in heart rate, an increase in peripheral vascular resistance, a decrease in cardiac output, and an increase in blood pressure. Furthermore, several published studies have demonstrated that increased ADMA concentrations in cultured endothelial cells or in patients with endothelial dysfunction are associated with increased ROS production (Sydow et al 2003, Boger et al 2000, Lin et al 2002).

Prospective clinical trials show increased evidence that ADMA is not only a marker of endothelial dysfunction, but is also associated with significant cardiovascular events and mortality. In a prospective study of ADMA in 225 patients on hemodialysis, the median plasma ADMA concentration in the 75th percentile within this group had a three-fold higher risk of death from any cause than those with ADMA below the median (Zoccali et al 2001). Importantly, even a small elevation of plasma ADMA concentrations is associated with poor prognosis. Lu et al (2003) showed that rising ADMA concentrations were independently associated with cardiovascular events in patients undergoing percutaneous coronary intervention. The difference in median ADMA concentrations
between the highest tertile and the study population was 0.32µmol/L. This small difference in ADMA concentrations was also seen by Mittermayer et al (2006), where a difference of 0.16µmol/L between the lowest and highest quartile was associated with 1.7 times the risk for myocardial infarction, stroke and death in patients with peripheral artery disease. In turns, this suggests that a small elevation of plasma ADMA concentrations is associated with marked adverse outcomes.

This issue has also been addressed by two previous studies Schnabel et al (2005), examining a population with known coronary artery disease, found an increase in ADMA concentrations of 0.21µmol/L was associated with an approximately 2.5 fold increase in cardiovascular mortality and infarction rates. More remarkably, Valkonen et al (1999) found, in a population of asymptomatic middle-aged men, that after logistic regression modeling there was a 27-fold increase in coronary risk associated with a 0.1µmol/L increase in ADMA.

1.17 Scope of the current study

Despite the increased interest into studies of ASc/AS, the pathogenesis of this condition remains largely elusive. This thesis was designed to utilize a “bench to bedside” approach to undertake a series of investigations related to the pathogenesis of AS.

Primary aims of this thesis were:
1) To establish a method for quantitating severity of ASc, for use as a measure of ASc progression.

2) To develop an animal model of ASc/AS for future intervention studies and assess valvular and vascular functions associated with development of aortic valve lesions.

3) To examine the effects of the angiotensin-converting enzyme inhibitor ramipril on development of aortic valve stenosis using the animal model described above.

4) To investigate the relationship between AS and plasma ADMA concentrations.

5) Identify clinical correlates of the presence of ASc in an ageing population.

The following chapters will discuss the study designs, results, discussions, and conclusions which will address the above aims specifically.

Chapter 2 describes the utilization of ultrasonic backscatter to assess aortic valve echogenicity. This is a novel non-invasive technique which has the potential to detect aortic valve abnormalities even before any hemodynamic changes occur. The development of this technique is crucial for the rest of the chapters as it is used continuously for both animal and human studies to assess changes in aortic valve structure.

Chapter 3 describes the development of an animal model of aortic stenosis. The animal model utilizes vitamin D\textsubscript{2} alone, independent of any cholesterol supplementation to
induce aortic stenosis. The chapter also uniquely compares the effects of vitamin D$_2$ on aortic valve and systemic vascular endothelial function.

Chapter 4 describes therapeutic intervention using an angiotensin-converting enzyme inhibitor (ACEi) Ramipril on development of aortic stenosis using the animal model described above. This is the first time anyone has performed therapeutic intervention in a calcific model of aortic stenosis with an ACEi; as well as comparing the effects of Ramipril on both aortic valves and vascular endothelial function.

Chapter 5 describes a human study testing whether the presence of at least moderate aortic stenosis is associated with incremental elevation of asymmetric dimethylarginine (ADMA) levels; a marker of endothelial dysfunction from pre-existing coronary risk factors.

Chapter 6 describes a human population study of 250 randomly selected subjects from the community to assess factors associated with high ultrasonic backscatter signals of the aortic valves. The study addresses 4 main components possibly associated with high backscatter signals: a) “traditional” cardiovascular risk factors; b) “non-traditional” cardiovascular risk factors; c) presence of inflammation and endothelial dysfunction as assessed by measurements of: hs-CRP, ADMA concentrations, augmentation index, and platelet nitric oxide responsiveness; and d) the role of vitamin D levels.
Chapter 7 addresses considerations for future research contingent on the results of the currently described studies.
**Figure 1.1a:** Postulated early process of aortic valve stenosis lesion. Inflammatory infiltrations of T-lymphocytes and macrophages, along with lipid accumulations responsible for early thickening of aortic valves.
Figure 1.1b: Postulated fibrosis process of early aortic valve lesions. Oxidative modification of lipids, activated inflammatory cells, and macrophages promote cytokine release as well as accumulation of ACE and its enzymatic product angiotensin II. Cytokine release and angiotensin II are responsible for initial valve matrix remodeling and thus fibrosis of aortic valve leaflets.
Figure 1.1c: Postulated processes of calcification/nodule formation in later stages of aortic stenotic valves. Cytokine release and angiotensin II promote extracellular matrix proteins secretion at early stages of mineralization which in turns begin the processes of bone formation. This process occurs at the end stage of aortic stenosis where aortic valves mobility is significantly reduced due to a build up of bone-like calcific nodules.
Figure 1.2: Echocardiogram of a parasternal long-axis view: A) normal aortic valve, the leaflets are thin and uniform by visual assessment; B) sclerotic aortic valve, there is increased in echogenicity and thickening of the aortic valve leaflets without restriction of leaflet motion; C) marked increased in echogenicity of the aortic valve leaflets with marked restriction of leaflet motion.
Figure 1.3: Schematic of natural history of ASc/AS in patients with tricuspid valves, with established or postulated potential therapeutic options.
Figure 1.4: State of homeostasis in the vasculature

Vasodilatation
Anti-inflammatory
Anti-atherogenic
Anti-aggregatory

\[ \text{EDRF} \quad \text{NO (most potent)} \]

Vasoconstriction
Pro-inflammatory
Pro-atherogenic
Pro-aggregatory

\[ \text{EDCF} \quad \text{ET, NA, Ang II, 5-HT} \]

Vascular homeostasis

Endothelium
**Figure 1.5:** Mechanisms of endothelial dysfunction via oxidative stress mechanisms.

NAD(P)H oxidase, Xanthine oxidase (XO), the major source of superoxide radicals (O$_2^-$), under normal physiological state, production of (O$_2^-$) are important as second messenger in cells. Under oxidative stress state, excess (O$_2^-$) can combine with NO and produce peroxynitrite (ONOO$^-$). Production of the very reactive (ONOO$^-$) and increase in (O$_2^-$) can lead to uncoupling of eNOS, leading to a further decrease of NO production. In combination, this leads to a vicious cycle that results ultimately in reduction of NO, and thus causing endothelial dysfunction.

Normal physiological state

<table>
<thead>
<tr>
<th>NAD(P)H oxidase, Xanthine oxidase (XO)</th>
<th>O$_2^-$</th>
<th>H$_2$O$_2$</th>
<th>H$_2$O</th>
</tr>
</thead>
</table>

Oxidative stress state

Impaired relaxation
Angiogenesis
Apoptosis
Monocyte adhesion
Hypertrophy
Cell proliferation
Cell migration
Matrix regulation
Cytokine production

Uncoupling
Endothelial dysfunction
**Figure 1.6:** Postulated mechanisms for elevation of plasma ADMA concentrations in disease states.
### Table 1.1: Summary of clinical studies into factors associated with progression of AS.

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Mean age at entry (years ± SD)</th>
<th>Follow-up period</th>
<th>Study type</th>
<th>Method of determination of progression rate</th>
<th>Stage of disease at entry</th>
<th>Correlates examined</th>
<th>Significant correlates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wongpraparut et al 2002</td>
<td>58</td>
<td>75±11</td>
<td>about 4 years</td>
<td>Retrospective analysis</td>
<td>Echo fast progressors AVA ≥ 0.25 cm²/yr</td>
<td>Any degree of AS (severity) not stated</td>
<td>Age, gender, PVD, CAD, DM, HT, hypercholesterolemia renal insufficiency, dialysis, diuretics use, Digoxin, Phosphate binder, calcitriol, statin, ACEi, Systolic and diastolic BP, mean arterial pressure, serum urea nitrogen, serum calcium, serum phosphate, serum Ca₃PO₄</td>
<td>SeCr, dialysis, calcium supplementation,</td>
</tr>
<tr>
<td>Palta et al 2000</td>
<td>170</td>
<td>71±9</td>
<td>23±11 months</td>
<td>Retrospective analysis</td>
<td>Echo, Percent AVA reduction fast progressors AVA ≥ 0.1 cm²/yr</td>
<td>Initial AVA 1.17±0.38 cm²</td>
<td>Age, gender, smoking, HT, DM, dialysis, initial AVA, mean AVp, EF, LVEDD, LVESD, IVSd, LVPW, LAD, LVv, AVv, serum cholesterol, uric acid, SeCr, phosphate, serum calcium, Ca₃PO₄</td>
<td>Smoking, male sex, elevated SeCr, calcium, and cholesterol levels, initial AVA</td>
</tr>
<tr>
<td>Study</td>
<td>N</td>
<td>Age (±SD)</td>
<td>Duration (±SD)</td>
<td>Study Design</td>
<td>Echocardiography</td>
<td>Imaging Modality</td>
<td>Independent Factors</td>
<td>Dependent Factors</td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>-----------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bahler et al 1999</td>
<td>852</td>
<td>68±13</td>
<td>6 to 51 mths</td>
<td>Retrospective analysis included 21% of population with bicuspid valve and rheumatic fever</td>
<td>Echo, Percent AVA reduction fast progressors AVA ≥ 0.1 cm²/yr</td>
<td>Initial AVA ranged from 0.6-2.0 cm²</td>
<td>Age, BSA, blood urea nitrogen, SeCr, cholesterol, hematocrit, systolic and diastolic BP, gender, smoking, DM, HT, CAD, AF, hypothyroidism, initial AVA, LVmass</td>
<td>Male sex, SeCr, LVmass, and initial AVA</td>
</tr>
<tr>
<td>Nassimiha et al 2001</td>
<td>102</td>
<td>76±9</td>
<td>28±21 mths</td>
<td>Retrospective analysis</td>
<td>Echo</td>
<td>Any degree of AS</td>
<td>Smoking, hypertension, hypercholesterolemia, diabetes mellitus, age</td>
<td>Cigarette smoking, hypercholesterolemia</td>
</tr>
<tr>
<td>Aronow et al 2001</td>
<td>180</td>
<td>82±5</td>
<td>33±12 mths</td>
<td>Retrospective analysis</td>
<td>Echo</td>
<td>Mild AS, AVp (10 to 25 mmHg)</td>
<td>Gender, DM, smoking, HT, serum LDL, HDL, triglycerides, obesity, statin therapy</td>
<td>Male gender, smoking, HT, DM, increased LDL levels, low HDLs</td>
</tr>
<tr>
<td>Pohle et al 2001</td>
<td>104</td>
<td>64.7±8</td>
<td>15.3±5</td>
<td>Retrospective Echo</td>
<td>Any degree of AVC</td>
<td>Any degree of AVC</td>
<td>LDL cholesterol, age, hypertension, diabetes, smoking progression of coronary calcification</td>
<td>LDL cholesterol, fast progression of coronary calcification</td>
</tr>
<tr>
<td>Messika-Zeitoun et al 2007</td>
<td>262</td>
<td>≥ 60</td>
<td>3.8±0.9</td>
<td>Prospective Echocardiography</td>
<td>Any degree of AVC</td>
<td>Any degree of AVC</td>
<td>Age, gender, BMI, systolic and diastolic BP, Smoking, HT, DM, serum glucose, total cholesterol, LDLs, baseline AVC score</td>
<td>Baseline AVC score Cholesterol levels**</td>
</tr>
</tbody>
</table>

** in absence of baseline AVC only
Abbreviations: EBT = electron computed tomography, AVC = aortic valve calcification, PVD = peripheral vascular disease, CAD = coronary artery disease, AVp = transvalvular pressure gradients, AVv = transvalvular velocity, EF = ejection fraction, LVEDD = left ventricular end diastolic dimension, LVESD = left ventricular end systolic dimension, IVSd = interventricular septal dimensions, LVPW = left ventricular posterior wall dimension, LAD = left atrial dimension, LVv = left ventricular outflow tract velocity,
CHAPTER 2

Quantitative assessment of aortic valve sclerosis using ultrasonic backscatter
2.1 Background

Aortic sclerosis (ASc), characterized by aortic valve thickening or calcification without restriction of motion, affects approximately 30% of adults over 65 years of age, and 50% older than 85 years (Stewart et al. 1997; Lindroos et al. 1993). Patients with ASc have increased risk of cardiovascular mortality and myocardial infarction (Otto, et al. 1999; Aronow et al. 2001). However, the major concern with ASc is the risk of progression to symptomatic aortic stenosis (AS). This progression has been studied mainly in patients with mild or moderate AS already at initial assessment with transvalvular pressure gradients of 15 mmHg and above (Otto et al. 1995; Palta et al. 2000; Bahler et al. 1999). Once ASc has progressed to this stage, development of symptomatic stenosis may occur over a few years, with a predicted rate of progression at about 5-7 mmHg of mean pressure gradient annually (Otto et al. 1995; Palta et al. 2000; Novaro et al. 2001). The use of patient cohorts of this type in previous progression studies probably reflects the fact that changes in transvalvular pressure gradients were employed to measure progression, rather than a measure of changes to the valve matrix. Recent studies of the pathology, clinical and biochemical correlates of AS have suggested that it may be a “preventable” disease (Rajamannan et al. 2003; Mitka 2003). However, the development of clinical strategies to retard the progression of ASc requires a technique to measure the degree of sclerosis as a marker of the rate of progression, and the effectiveness of clinical interventions.

While the conventional Doppler echocardiography examination is a standard approach for the assessment and management of AS, Doppler gradients are not generally present in ASc and visual evaluation of the valve thickness, mobility and calcification are subjective.
Improvements in the feasibility of ultrasonic backscatter to detect and characterize pathological changes in tissues offer a potential means for assessment of aortic valve diseases, possibly before hemodynamic changes occur.

Ultrasonic backscatter has been used to detect preclinical changes within the myocardium, even at a stage when conventional echocardiographic indices are within the normal range in myocardial ischemia (Picano et al. 1993), acute cardiac transplant rejection (Stempfle et al. 1993), and diabetes mellitus (Di Bello et al. 1995; Fang et al. 2003). Although increased myocardial echodensity has also been identified in patients with AS without overt systolic dysfunction (Di Bello et al. 1997), changes in ultrasonic backscatter of the aortic valve have not been assessed. Nonetheless, histological findings of diseased aortic valve in AS have shown a range of abnormalities starting with small areas of fibrosis and thickening and extending marked structural degeneration, nodular thickening, and dystrophic calcification (Otto et al 1999, Warren and Yong 1997). In this study, we sought to determine whether the extent of alterations of the aortic valves could be quantified by ultrasonic backscatter in patients with ASc.

2.2 Methods

2.2.1 Study subjects:

The study population consisted of 31 consecutive ASc (13 men, mean age 76.4, range 57 to 91 years), 10 aortic stenosis patients (5 males/5 females; mean age 73.7, range 55 to 89 years) who were identified while being referred for non-emergency echocardiographic evaluation. Additionally, to establish a normal range of aortic valve backscatter scores
(AV<sub>BS</sub>), we quantitated AV<sub>BS</sub> in a group of 38 healthy volunteers (17 males/21 females; mean age 31.6, range 22 to 41 years), had no left ventricular dysfunction, valvular heart disease, history of rheumatic fever or thickening of the aortic valves detected by echocardiography. Furthermore, AV<sub>BS</sub> was measured in a group of 46 consecutive elderly age-matched subjects (25 males, mean age 72.9, range 62 to 89) referred for an echocardiogram, with normal aortic valves upon visual assessment. The AV<sub>BS</sub> scores obtained for these patients represented a range of AV<sub>BS</sub> scores for “normal” aging aortic valves with multiple coronary risk factors characterizing a cross-section of subjects more closely resembling those with ASc (elderly controls). Patients with “normal” aortic valves were defined on the basis of no focal increase in aortic valve echogenicity or thickening with no restriction of motion, irrespective of other cardiac structural/functional anomalies, had normal aortic valve structure (as previously shown in Figure 1.2A). All elderly controls, ASc and AS patients had undergone complete baseline echocardiographic examination and were excluded if image quality was not suitable for backscatter analysis. The study was approved by the Ethics of Human Research of The Queen Elizabeth Hospital.

A full clinical history and clinical examination was performed in all patients. Hypertension was defined by treatment with anti-hypertensive drugs or a blood pressure >140/80 mmHg. Hypercholesterolemia was defined by treatment with cholesterol-lowering drugs or a total cholesterol >4.5 mmol/l. Diabetes mellitus was defined by treatment for diabetes or a fasting blood glucose >7.8 mmol/l. Plasma serum creatinine levels (a risk factor for rapid progression of aortic stenosis (reviewed by London 2000) and hemoglobin concentrations were measured in all patients.
2.2.2 Evaluation of ASc.

ASc was defined according to criteria described by Otto et al (1999), as focal areas of increased echogenicity and thickening of the aortic valve leaflets without restriction of leaflet motion; and AS as thickened leaflets with reduced systolic opening and increased velocity across the aortic valve > 2.5m/s (an example as shown in Figure 1.2B). Subjective assessment of the degree of ASc was also performed as described by Shively et al (1998). Briefly, sclerotic valves were classified on the basis of extent of increase in degree of reflectance of cusp bodies and margins, as well as valve thickness. The grading of sclerosis was performed by a cardiologist blinded to all other echocardiographic results.

2.2.3 Doppler echocardiography.

M-mode and two-dimensional (2D) echocardiograms with Doppler analysis were obtained for all subjects by means of a commercially available ultrasound machine (Vivid 5, GE Vingmed, Horten, Norway with a 2.5 MHz phased array probe). Left ventricular (LV) diameters and wall thicknesses were measured from 2D-guided M-mode echocardiography. Mean and peak transaortic pressure gradients were calculated with the modified Bernoulli equation, using continuous-wave Doppler recordings from the highest velocity available from any view. The aortic valve area was computed with the continuity equation using standard methods (Perkovic et al. 2003).
2.2.4 Ultrasound backscatter data analysis.

Two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal long-axis view over 3 cardiac cycles with a zoom of 8 cm. Three consecutive scans were done for each study subject. A total of 6 square-shaped regions of interest (ROI) (3 on the anterior leaflet and 3 on the posterior leaflet) were obtained by placing sample areas on the valves. Different region of interest size were used to minimize variability of backscatter in a normal subject. Backscatter values from the blood pool adjacent to the left ventricular outflow tract (LVOT) and the aortic (AO) blood pool were used as reference values (Figure 2.1). Calibrated backscatter values were obtained by subtracting the regions of interest in the LVOT blood pool, and the AO blood pool from the averaged backscatter values obtained on the aortic valves (Figure 2.1, Equation 1). The final calibrated backscatter values (dB) were averaged over the 3 consecutive scans (Figure 2.1, Equation 2).

2.2.5 Reproducibility

Reproducibility of backscatter values was evaluated in 12 randomly selected subjects (6 healthy volunteers, and 6 patients with ASc). To determine variability in backscatter measures between different operators, the 12 participants were assessed for aortic valve backscatter by two different sonographers. To evaluate variability of backscatter values on different occasions, in the absence of disease progression, the 12 participants also had repeated estimations of backscatter 9 ± 6 weeks apart. Reproducibility of backscatter values by two different evaluators of backscatter (i.e. in placing regions of interests on the aortic
valve and in the blood pools) blinded to the results was also tested on 11 subjects (5 healthy volunteers, and 6 ASc patients). To test reproducibility of calibrated backscatter scores between 2 different echocardiography machines: (Vivid 7, GE Vingmed, Horten, Norway) vs (Vivid 5, GE Vingmed, Horten, Norway); the same techniques as discussed above were used for 14 randomly selected subjects (7 elderly controls, and 7 patients with ASc).

### 2.2.6 Statistics.

The Student’s non-paired t-test was used to compare continuous (normally distributed) variables. One-way ANOVA with Dunnett’s post hoc tests were used to compare means between multiple groups. Correlations between backscatter and two-dimensional echocardiographic measurements as well as continuous patient characteristics were examined via terms of linear regression analysis. Correlations between backscatter and subjective grading of ASc were assessed by Spearman’s rank correlation test. A value of p<0.05 was considered significant. Unless otherwise stated, results for normally distributed parameters are expressed as mean ± SD. The principal hypothesis to be tested related to the presence/absence of incremental backscatter in patients with ASc, compared to normal subjects.

### 2.3 Results

#### 2.3.1 Subject/patient characteristics.

Table 2.1 summarizes the clinical characteristics of study patients. Many of the age-matched controls group had multiple cardiovascular diseases, in similar proportions compared to
subjects with ASc. Many of the ASc subjects had multiple coronary risk factors for ischemic heart disease (85%). Approximately 50% of patients had known symptomatic ischemic heart disease. There were 10 patients with renal dysfunction (serum creatinine >0.12 mmol/L), and 5 patients with mild anemia (Hb < 115 g/L). In general, ASc patient population was older, presented with higher incidence of coronary risk factors, and myocardial ischemia compared to the elderly control population.

2.3.2 Aortic valve findings.

Echocardiographic parameters for ASc patients are summarized in Table 2.2. There were 6 ASc patients with impaired LV systolic function (LVEF < 55%). A total of 14 patients had (generally mild) left ventricular hypertrophy (IVSd > 1.2cm). All patients had evidence of minimal or mild narrowing of the aortic valve. None of these patients had a calculated mean transvalvular pressure gradient greater than 15 mmHg. Among the 10 patients with AS, mean transvalvular pressure gradient ranged from 22 mmHg to 77 mmHg, and mean aortic valve area was 0.9 ± 0.2 cm², consistent with mild to moderate stenosis (Mochizuki et al. 2003).

2.3.3 Relationship between backscatter data and conventional echocardiographic measurements.

The healthy volunteers group had mean AVBS scores of 10.0 ± 3.3dB, ranging from 4.5 to 16.5. Backscatter comparison between the healthy volunteers and elderly control group are shown in Figure 2.2. The mean backscatter value for the elderly control group (AVBS scores
ranged from 5.1 to 19.1) tended to be higher than for the healthy volunteers, although this
difference was not statistically significant (11.2 ± 3.5dB vs 10.0 ± 3.3dB, respectively,
p=0.12).

Backscatter results for the 3 cohorts examined (elderly control, ASc, and AS) are shown in
Figure 2.3. While backscatter values in the ASc population overlapped considerably with the
elderly control population, the mean level was significantly greater (16.7 ± 4.4 dB vs. 11.2 ±
3.5, respectively, p< 0.01. The small AS group also had the highest AVBS scores (23.0 ± 3.9
dB).

Within the ASc group, a number of correlations were sought between measures of severity
of valve disease and calculated backscatter. Correlations of backscatter with mean
transvalvular pressure gradient and with aortic valve area are depicted in Figures 2.4A and
2.4B respectively. There was a positive trend in the correlation between mean pressure
gradient and backscatter (r²=0.11, p=0.07), and a negative trend between aortic valve area
and backscatter (r²=0.09, p=0.1); however neither trend were statistically significant.
Furthermore, there was no correlation between AVA and backscatter scores in the AS
patients shown in Figure 2.5 (r²=0.06, p=0.5). Upon Spearman’s correlation of subjective
severity of valve disruption by visual scoring showed a direct relation to backscatter
calculations (Figure 2.6; p< 0.05).
2.3.4 Relationship between backscatter data and patient characteristics.

There was no correlation between AV\textsubscript{BS} and body mass index in the healthy volunteers group shown in Figure 2.7 ($r^2=0.0006$, $p=0.9$). Additionally, there was no correlation between AV\textsubscript{BS} in the blood pools, and hemoglobin concentrations in patients with ASc and AS shown in Figure 2.8A ($r^2=0.02$, $p=0.3$). This finding was consistent between hemoglobin concentrations and final calibrated backscatter scores ($r^2=0.016$, $p=0.4$) (Figure 2.8B). Upon analyses between AV\textsubscript{BS} and age in all patient populations; Figure 2.9 showed no correlation between these 2 parameters in all patient populations. Furthermore, patients in the ASc group with evidence of left ventricular hypertrophy as assessed by echocardiography did not have significantly higher mean AV\textsubscript{BS} compared to those without left ventricular hypertrophy showed in Figure 2.10A (16.6 ± 4.1dB vs 17.7 ± 4.9, respectively, $p=0.5$); this finding was consistent for the elderly control population with/without hypertension (10.6 ± 2.8dB vs 11.8 ± 3.9dB, respectively, $p=0.3$) (Figure 2.10B).

2.3.5 Reproducibility of backscatter values:

Reproducibility of backscatter findings was examined: (a) between sonographers; (b) between serial examinations for both normal and ASc individuals; (c) between different observers; (d) between different echocardiography machines. Results are shown in Figure 2.11A, 2.11B, 2.11C and 2.11D respectively. The mean difference in backscatter values between sonographers is 2.4 ± 1.8dB (9.3%). For serial examinations of both normal and aortic sclerotic individuals, the mean difference in backscatter values was 2.3 ± 1.7dB (9.1%). Between 2 blinded observers (i.e. in placing of the regions of interests), the mean
difference in backscatter values was $1.1 \pm 1.0$ dB (4.2%). The mean difference in backscatter values between echocardiography machines was $2.2 \pm 1.6$ dB (8.2%).

2.4 Discussion

The results of the study indicate: 1) that the mean levels of $\text{AV}_{\text{BS}}$ in patients with ASc are approximately 60% greater than in healthy young adults, 2) ultrasonic backscatter scores in ASc patients are directly correlated with subjective scoring of sclerosis and a positive trend with transvalvular pressure gradients in patients with mild-moderate aortic stenosis, and most importantly, 3) ultrasonic backscatter is a reproducible technique, with mean differences between estimates based on repeat echocardiograms of $2.3 \pm 1.7$ (9.1%).

2.4.1 Tissue characterization with ultrasonic backscatter.

Measurement of backscatter has been used for many years to detect changes in pathologic tissues based on the analysis of the interactions between ultrasound waves and tissues. Variations of the ultrasound backscatter signals and their relationship to tissue structure forms the basis of tissue characterization principles (Miller et al. 1985). There have been several approaches using ultrasonic backscatter to quantitatively define changes, mainly in myocardial ultrasonic reflectivity. An increase in backscatter has been correlated with collagen content and the development of fibrosis in the myocardium (Hoyt et al. 1984; Picano et al. 1990; Naito et al. 1996). Advances in computing power and the ability to save ultrasound studies in raw data format have made this technique more feasible for clinical application.
Changes in backscatter may be used in theory to characterize the structural and biochemical composition of atherosclerotic plaque, thereby differentiating between fibrofatty lesions, fatty lesions, and calcification of the arterial walls (Barziliai et al 1987; Urbani et al 1993; Picano et al 1990). Kawasaki et al (2001) measured backscatter values of carotid and femoral arteries in 12 patients before and after death, and compared the backscatter values with their histopathologic features. They found that ultrasonic backscatter values differed significantly for thrombus, fibrosis, calcification and lipid pool, intimal and medial hyperplasia. These differences in backscatter values correlated well with histological classification.

However, the exact mechanisms underlying changes in $AV_{BS}$ in ASc subjects are not clear. To our knowledge, there has been no study that measured changes in backscatter signals on aortic valves. In the current study, specific structural determinants of aortic valve backscatter could not be assessed. However, histopathological lesions of ASc have been shown to resemble arterial atherosclerotic plaque (Chan 2003). Characterisation of the “early” degenerative lesions of aortic stenosis by Otto et al (1994) demonstrated subendothelial thickening with disruption of basement membrane, accumulation of inflammatory infiltrates such as T-lymphocytes, and macrophages; presence of intracellular and extracellular lipids; mineralization, and regions with protein, lipid, and calcium accumulation. Severely stenosed aortic valves have been described as grossly deformed and distorted, with marked fibrosis and nodular calcification, eventually involving the full thickness of the aortic cusp (Warren and Yong, 1997).
Scoring of valvular calcium content using electron beam or multi-slice computed tomography (CT) scanning (Kaden et al 2002) offers potential quantitation of the “end-stage” component of the pathogenesis of AS, but may not provide insight into the atheromatous and fibrotic changes which characterize early ASc. Indeed, it is possible that serial combined use of backscatter and quantitation of valve calcium content might provide greater insights into the time course of calcification relative to non-calcific valve thickening.

2.4.2 Comparison of AV_{BS} and Doppler echocardiography.

Previous methods of quantifying ASc involved direct estimation of hemodynamic parameters using Doppler echocardiography and visualization of aortic valve structure. Doppler echocardiography (mean AVp and AVA) did not show significant correlations with AV_{BS} in this study. This perhaps indicates that minor obstruction to blood flow (quantifiable by Doppler echocardiography) does not significantly reflect the structural abnormalities quantifiable by aortic valve backscatter. Nonetheless, Doppler echocardiography is not the “gold standard” for quantifying severity of ASc.

Although visualization of aortic valve structure can detect marked alterations in valve appearance and mobility, such methods are subjective and may not be reproducible. Furthermore, the subjective assessment emphasizes difficulty in distinguishing between “normal” aortic valves, particularly in elderly subjects, and minimal ASc. In this study, ultrasonic backscatter could quantitatively categorise ASc.
Presence of LVH was not associated with elevation of AV$_{BS}$ in both patients with ASc or in the elderly control group in this study. These patient populations had numerous cardiovascular risk factors (e.g., hypertension), and are perhaps more significant contributors to LVH than presence of minor structural abnormalities quantified by AV$_{BS}$.

### 2.4.3 Limitations.

The current study has several limitations. The considerable overlap in backscatter values between the ASc, healthy volunteers, and elderly control populations (Figure 2.3) suggests that backscatter values cannot be utilized for diagnosis of ASc; rather, they should be used as an index of progression. Nevertheless, backscatter values of $>16$ dB are not consistent with normal valve structure (Figure 2.3).

A further theoretical limitation on the technique utilized for estimating valvular backscatter is the use of blood pool backscatter as a reference value. It is possible that anaemia, common in patients with advanced aortic valve disease (Vincentelli et al. 2003), might affect blood pool backscatter. However, within the study population, there was no significant correlation between backscatter of blood pool and hemoglobin concentrations (Figure 2.8).

The use of subjective optimization of valve image quality via adjustments of gain and time-gain compensation is theoretically inferior to the use of pre-defined settings, but is difficult to avoid in a study in which subjective scoring of valve appearance was a predefined comparison (see data in Figure 2.5). Furthermore, there is no correlation between body mass index and aortic valve backscatter (Figure 2.6) which indicates that inter-individual
variability in chest wall thickness does not interfere with quantitation of aortic valve heterogeneity. The applicability of the current technique to population group data is evident despite this theoretical limitation.

Finally, the current study does not address in detail the extent of progression of backscatter associated with the transition from ASc to moderate/severe AS, other than to demonstrate that further increases in backscatter do occur. The main conclusion that can be drawn from this small component of the study is that the considerable increases in calcification (and associated ossification) within the valve matrix in advanced AS do not cause remarkable accentuation of backscatter changes. However, it is not the intention of this study to develop backscatter as a means for quantitating advanced aortic valve disease; rather it is postulated as an ideal method for assessing ASc/early AS and measuring rates of disease progression in pre-symptomatic individuals.

2.4.4 Clinical implications.

It is planned that ultrasonic backscatter score be utilized primarily as a measure of progression of ASc. Reproducibility data permit calculation of sample sizes of studies required to detect specific extents of backscatter progression. For example, assuming baseline values of backscatter in a study population of ASc patients of 16.7 ± 4.4 dB, the study size required to detect mean progression to 18.9 units (ie 0.5 SD) progression at a β value of 0.8 would be approximately 44 patients. Assuming an intervention, study size to detect heterogeneity of progression of a mean of 2.2 units (ie 0.5 SD) between two populations at β value of 0.8 would be approximately 170 patients in each group.
To date, there have been no definitive intervention studies in patients with ASc, or indeed with moderate AS. However, there has been a focus in the literature on the possible relationship between aortic valve calcification and statin therapy (Aronow et al. 2001). In view of these observational data, at least 2 studies are in progress addressing the possible beneficial effects of cholesterol reduction on progress of aortic valve calcification. The currently described methodology would theoretically have the advantage of facilitating examination of disease progression without relying exclusively upon valve calcification, a relatively late component of the pathophysiology of AS (Otto et al 1994).

There are many other areas of potential applicability of this technology, both for epidemiological studies and for examination of interventions. For example, case-control study design might be utilized to examine the implications of various degrees of renal dysfunction, or of the presence of various coronary risk factors, or of the activation of inflammatory mediators such as C-reactive protein, on occurrence/progression of ASc. Physiologically orientated studies might be utilized to delineate the relationship between vascular endothelial dysfunction (and its biochemical markers) and occurrence of ASc. Finally, there are intriguing data suggesting other potential forms of pharmacotherapy of ASc. For example, early lesions in ASc exhibit colocalization of atheromatous/inflammatory change and high concentrations of angiotensin-converting enzyme (ACE) (O’Brien et al. 2002). Generation of angiotensin II might lead to increased superoxide formation (Cai et al. 2003), and also contribute to platelet dysfunction (Chirkov et al 2004). Thus, ACE inhibitors/angiotensin II receptor blocker therapy might limit progression of ASc. The main
direction of the following chapters in this thesis is a series of investigations related to the pathogenesis of ASc/AS utilizing:

a) a model of the development of AS in rabbits; and utilization of this model to investigate pharmacological therapeutic interventions.

b) evaluation of the factors associated with presence/absence of ASc/AS in an ageing population.

In both of these areas, assessment of valve echogenicity with echocardiographic backscatter methodology, as described in this chapter, represents pivotal technology.
Table 2.1: Clinical characteristics of age-matched controls (n=46), patients with aortic sclerosis (n=31); and aortic stenosis (n=10)

<table>
<thead>
<tr>
<th></th>
<th>Age-matched controls (n=46)</th>
<th>Aortic sclerosis (n=31)</th>
<th>Aortic stenosis (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n (%)</strong></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Age (yrs ± SD)</strong></td>
<td>72.7 ± 6.7</td>
<td>76.4 ± 7.2</td>
<td>73.7 ± 11.0</td>
</tr>
<tr>
<td><strong>Gender (male)</strong></td>
<td>25 54.3</td>
<td>13 41.9</td>
<td>10 50</td>
</tr>
<tr>
<td><strong>Coronary risk factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current/previous smoking</td>
<td>14 30.4</td>
<td>15 48.4</td>
<td>3 30</td>
</tr>
<tr>
<td>Hypertension</td>
<td>29 63</td>
<td>22 71.0</td>
<td>2 20</td>
</tr>
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<td>Hypercholesterolaemia</td>
<td>23 50</td>
<td>19 61.3</td>
<td>6 60</td>
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<td>Diabetes mellitus</td>
<td>10 21.7</td>
<td>3 9.7</td>
<td>1 10</td>
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<td><strong>Renal insufficiency</strong></td>
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<tr>
<td>Serum creatinine &gt; 0.20 mmol/L</td>
<td>4 8.7</td>
<td>4 12.9</td>
<td>2 20</td>
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<td><strong>Known myocardial ischemia</strong></td>
<td>15 32.6</td>
<td>15 48</td>
<td>7 70</td>
</tr>
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</table>
Table 2.2: Individual echocardiographic parameters for aortic sclerosis and stenosis patients with respective ultrasonic backscatter values

<table>
<thead>
<tr>
<th>Aortic sclerosis patients</th>
<th>IVSd (cm)</th>
<th>LVEF (%)</th>
<th>FS (%)</th>
<th>Sclerosis grading</th>
<th>AV (m/s)</th>
<th>AVp (mmHg)</th>
<th>AVpmn (mmHg)</th>
<th>AVA (cm²)</th>
<th>BS values (dB)</th>
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<tr>
<td>1</td>
<td>1.00</td>
<td>70.3</td>
<td>44.0</td>
<td>moderate</td>
<td>1.62</td>
<td>10.56</td>
<td>5.05</td>
<td>2.10</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>1.11</td>
<td>51.0</td>
<td>26.3</td>
<td>moderate</td>
<td>1.64</td>
<td>10.71</td>
<td>4.94</td>
<td>2.16</td>
<td>18.9</td>
</tr>
<tr>
<td>3</td>
<td>1.26</td>
<td>65.5</td>
<td>36.4</td>
<td>moderate</td>
<td>1.58</td>
<td>10.03</td>
<td>5.01</td>
<td>3.04</td>
<td>13.1</td>
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<tr>
<td>4</td>
<td>1.14</td>
<td>56.8</td>
<td>29.4</td>
<td>severe</td>
<td>2.01</td>
<td>16.18</td>
<td>9.14</td>
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<td>18.3</td>
</tr>
<tr>
<td>5</td>
<td>1.02</td>
<td>74.5</td>
<td>43.2</td>
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<td>1.11</td>
<td>4.97</td>
<td>2.52</td>
<td>2.30</td>
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<td>6</td>
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<td>71.6</td>
<td>41.3</td>
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<td>mild</td>
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<td>40.9</td>
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<td>11</td>
<td>1.25</td>
<td>57.9</td>
<td>30.8</td>
<td>severe</td>
<td>1.87</td>
<td>14.00</td>
<td>6.97</td>
<td>2.70</td>
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<td>12</td>
<td>1.08</td>
<td>64.8</td>
<td>34.9</td>
<td>moderate</td>
<td>1.09</td>
<td>4.76</td>
<td>2.19</td>
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<tr>
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<td>1.51</td>
<td>58.7</td>
<td>31.0</td>
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<td>13.88</td>
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IVSd = interventricular septum thickness

AV = peak aortic valve velocity

AVA = aortic valve area

LEVF = left ventricular ejection fraction

AVp = peak transvalvular pressure gradient

BS = backscatter

FS = fractional shortening

Avpmn = mean transvalvular pressure gradient

LV hypertrophy is indicated when IVSd > 1.2cm.

LV systolic impairment was when EF < 55%
Figure 2.1: Zoomed view of the parasternal long-axis view of the aortic valves. Regions of interests were placed on blood pool and on the anterior, posterior leaflets of the aortic valves to obtain backscatter measurements.

Equation 1: Calculation of backscatter for a single beat.

\[
BSc_1 = \text{Average BSbp} - \frac{1}{2} [(\text{ROIant})\text{ave} + (\text{ROIpost})\text{ave}]
\]

\(BSc_1, BSc_2, BSc_3,\) = corrected backscatter value for beat 1, beat 2, and beat 3

\(\text{BSbp}\) = backscatter values obtained in the blood pool adjacent to the aortic valves.

\((\text{ROIant})\) = average of the regions of interests on the anterior leaflet of the aortic valve

\((\text{ROIpost})\) = average of the regions of interests on the posterior leaflet of the aortic valve

\(BScf\) = final corrected backscatter after 3 consecutive scans for each individual

Equation 2: Calculation of final backscatter value after 3 consecutive scans
\[ B_{Scf} = \frac{1}{3} (B_{Sc1} + B_{Sc2} + B_{Sc3}) \]

The transmit power and compression setting will be kept constant at each backscatter study for standardization purpose. Gray scale transfer function will be adjusted to be linear for the entire signal range. The gain and depth settings will also be adjusted for all study subjects to obtain apparently uniform brightness throughout the echocardiogram to achieve a precise and reproducible sampling backscatter values.
**Figure 2.2:** Comparison of calibrated backscatter values A) range of $AV_{BS}$ in young and healthy volunteers; and B) range of $AV_{BS}$ in elderly control populations who had no visual thickening of aortic valves. Mean backscatter values for A) the healthy volunteers and B) elderly controls were: $10.0 \pm 3.3$dB vs $11.2 \pm 3.4$dB, respectively $p=0.12$. 
**Figure 2.3:** Comparison of calibrated backscatter values between age-match controls vs patients with ASc and AS. There was a statistically significant difference in backscatter values between the elderly controls (11.2 ± 3.4dB) vs ASc (16.7 ± 4.4dB), p<0.01, and AS (23.0 ± 3.9dB), p<0.01, by Dunnett’s post hoc test.
Figure 2.4: (A) Scattergram showing a non-significant relationship between backscatter values and mean transvalvular pressure gradient ($r^2 = 0.11; p = 0.07$), and a non-significant inverse correlation ($r^2 = 0.09; p = 0.1$) between backscatter and the aortic valve area (AVA) for ASc patients in (B).
**Figure 2.5:** Scattergram showing a non-significant relationship between backscatter values the aortic valve area (AVA) for AS patients in ($r^2=0.06$, $p=0.5$).
Figure 2.6: Relationship between ultrasonic backscatter values and subjective assessment of ASc severity (p = 0.02; Spearman’s correlation). ASc grading was performed according to the method of Shively et al 1998: (+1) = slight increase in reflectance of the cusp bodies or margins; (+2) = mild increase in overall reflectance and cusp thickness; (+3/4) = when hyperreflectance was generalized and aortic valve cusps appear markedly thick/valve cusps were essentially replaced by markedly hyperreflectant masses.
Figure 2.7: Scattergram showing no correlation between calibrated backscatter values and BMI in the healthy normal group ($r^2=0.0006$, p=0.9).
Figure 2.8: Scattergram showing no correlation between (A) average backscatter from blood pools ($r^2=0.02$, $p=0.35$) (B) calibrated backscatter values ($r^2=0.015$, $p=0.44$) and hemoglobin levels in patients with ASc and AS.
Figure 2.9: Scattergram showing no correlation between aortic valve backscatter and age in all groups. (A) There was no correlation between age and calibrated backscatter values in the healthy control group ($r^2=0.01$, $p=0.5$), and the elderly control group ($r^2=0.01$, $p=0.5$). (B) There was no correlation between age and backscatter values for patients with ASc ($r^2=0.03$, $p=0.3$), and AS ($r^2=0.05$, $p=0.5$).
Figure 2.10: Comparison of calibrated backscatter values in patients who have/have not left ventricular hypertrophy (LVH) in (A) ASc, and (B) patients with hypertension in the elderly control group. (A) There was no significant difference in backscatter values in patients with LVH vs patients with no LVH in the ASc group (16.6 ± 4.1dB vs 17.7 ± 4.9, p=0.5). (B) There was no significant difference in backscatter values in patients with LVH vs no LVH in the age-match control group (10.6 ± 2.8dB vs 11.8 ± 3.9dB, p=0.3).
Figure 2.11: (A) Reproducibility of backscatter values between echocardiographic technicians for 12 subjects, 2.4 ± 1.8 dB (B) Reproducibility of backscatter for serial examinations of 9 ± 6 weeks apart by the same echocardiographic technician 2.3 ± 1.7 dB. (C) Reproducibility of backscatter values between 2 different observers 1.1 ± 1 dB (D) Reproducibility of backscatter values using 2 different echocardiographic machines 2.2 ± 1.6.
CHAPTER 3

Vitamin D$_2$ induces development of aortic valve stenosis in rabbits
3.1 Background

The pathogenesis of AS is increasingly recognized to reflect a more complex process than the originally postulated “wear and tear”. In view of the considerable cardiovascular morbidity and mortality associated not only with AS, but its earlier stage, aortic sclerosis (ASc) (Aronow et al 1999, Otto et al 1999); investigations have been directed towards better understanding of its pathogenesis, with the ultimate objective of developing strategies to retard its progression.


In 2003, Drolet et al demonstrated in a rabbit model that although cholesterol dietary supplementation alone did not induce development of AS, concomitant administration of vitamin D$_2$ and cholesterol supplementation induced development of AS within 12 weeks. As these studies did not evaluate the effects of vitamin D$_2$ alone, it remained
uncertain whether development of AS reflected an additive/synergistic interaction between vitamin D₂ and cholesterol.

The potential role of vitamin D as a modulator of development of AS has been evident both from in vitro experiments and epidemiological studies. Jono et al (1998) observed that calcitriol (10⁻⁹ to 10⁻⁷ mol/L) induced a dose-dependent increase in calcification of bovine vascular smooth muscle cells (VSMCs) in vitro. The B allele of the vitamin D receptor BsmI has been shown to be more common in patients with AS, perhaps reflecting the pivotal importance of calcium kinetics (Ortlepp et al 2001). Malurgue et al (1998) demonstrated that plasma levels of vitamin D were directly and independently correlated with the development of AS in a group of patients with chronic renal failure. Elevated calcium-phosphate products (Ca₃(PO₄)₂) in patients with normal renal function were also associated with the severity of AS after adjusting for age, gender, and creatinine clearance (CrCL) (Mills et al 2004), in a study that did not examine concomitant vitamin D levels.

The principal objective of the current study therefore was to determine the potential effect of vitamin D₂ alone as a precipitant of development of AS in the rabbit model. We also sought to determine:

1) Whether the histopathology of AS in this model resembled that of human AS.
2) Whether vitamin D₂ also affected vascular function.
3) Whether additional cholesterol supplementation accentuated the effects of vitamin D₂.
3.2 Methods.

3.2.1 Experimental protocol:

Male New Zealand White rabbits (2-2.5 kg) were treated for 8 weeks according to the schema shown in Figure 1. The principal hypothesis was examined in 8 animals each in the control group, and the high dose vitamin D₂ (HD vit-D₂: 25,000IU / 4 days weekly). HD vit-D₂ plus 0.5% cholesterol-fed chow (Cholesterol/HD Vit-D₂ group) and the low dose vitamin D₂ (LD vit-D₂: 5,000IU / 4 days weekly) animals (n=4 each group) were utilized to examine the secondary issues of interactions with cholesterol supplementation and effects of lower doses of vitamin D₂ respectively. Vitamin D₂ solutions were prepared fresh daily.

At baseline and 8 weeks post treatment, echocardiography was performed under sedation with ketamine (3.5mg/kg) / xylazine (5mg/kg) and blood samples were taken every 4 weeks were taken for the measurement of serum creatinine (SeCr), calcium, phosphate, total cholesterol, and plasma asymmetric dimethylarginine (ADMA).

At the end of protocol, animals were sacrificed under anaesthesia with ketamine/xylazine, the heart and thoracic aortae rapidly removed and placed in ice cold Krebs solution and aortic valve leaflets were excised as soon as possible.

The experimental protocol was approved by the North Western Adelaide Health Service and University of Adelaide animal ethics committee and conforms to the National Health
and Medical Research Council of Australia guidelines of animal usage for experimentation.

3.2.2 Echocardiographic measurements:

Ultrasound images were obtained with a 5-MHz sector phased-array probe, operated at 6.7MHz, connected to a Vivid 5, GE Vingmed, (Horten, Norway). Aortic valve backscatter (AV$_{BS}$) was utilized as the primary measure of increased valve echogenicity (Ngo et al 2004, reviewed by Nightingale and Horowitz 2005). Peak left ventricular outflow tract flow (LVOTv) and transaortic valve flow (AVv) were measured utilizing continuous-wave Doppler recordings from the highest velocity available were used as measures of aortic valve stenosis. Ratio of AVv/LVOTv was used to compare changes in hemodynamics between the 2 groups. Left ventricular wall thicknesses were measured from 2D-guided M-mode echocardiographic tracings. Echocardiographic imaging and analyses were performed by observers blinded to rabbit treatments.

3.2.3 Calculation of ultrasound backscatter data analysis:

Determination of AV$_{BS}$ in rabbits was analogous to methodology used in humans (Ngo et al 2004, reviewed by Nightingale and Horowitz 2005). Two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal-like long-axis view over 3 cardiac cycles with a zoom of 2 cm. Three consecutive scans were performed per animal. A total of 10 square-shaped regions of interest (5 on the anterior leaflet and 5 on the posterior leaflets) were obtained by placing 7x7 pixel sample volumes on the valves. Backscatter values from the blood pool adjacent to the left ventricular outflow tract and the aortic blood pool were used as reference values. Calibrated
backscatter values were obtained by subtracting the averaged regions of interests in the left ventricular outflow tract blood pool (n=3), and the aortic blood pool (n=3) from the averaged backscatter values obtained on the aortic valves. The final calibrated backscatter values (decibel (dB)) were averaged over 3 consecutive scans.

3.2.4 Histology and Immunohistochemistry:

Immediately after dissection from the heart, one leaflet from each aortic valve was embedded in Tissue-Tek O.C.T compound, snap-frozen in liquid nitrogen, and stored at -80°C until serially sectioned. Serial sections of aortic valve leaflets were stained with Oil Red O for neutral lipid deposition, and Alizarin Red S to detect calcification.

Sections (6µm) were subjected to double-labeled immunohistochemistry using the following antibodies; anti-rabbit macrophage RAM-11 (1:100) (Dako); and anti-rabbit CD45 (1:5) (Dako). For control sections, the primary antibodies were omitted or irrelevant isotype-matched rabbit antibodies were applied. Staining was performed according to a 3-step immunoperoxidase method (Kraan et al 2000). Before use, the slides were warmed to room temperature and air-dried. The sections were washed between steps with phosphate-buffered saline (PBS). All incubations were carried out at room temperature. The primary antibodies were diluted in PBS-1% bovine serum albumin (BSA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted in PBS-1% BSA with 10% normal human serum as blocking serum. Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in PBS. The primary antibody was added for 30 minutes, followed by incubation with HRP-conjugated goat anti-mouse (Dakocytomation) was added for 30
minutes, followed by incubation with HRP-conjugated swine anti-goat antibody (Dakocyтомation) for another 30 minutes. HRP activity was detected using hydrogen peroxide as substrate and amino ethylarbazole (AEC) (Sigma) as dye. Immunostaining with RAM-11 identifies the presence of macrophages, a feature present on human aortic valve lesions (Otto et al 1994), which indicates that AS is an active process. Anti-CD45 is a marker for leukocyte deposition, which also typified inflammatory cell deposition on aortic valve stenosis lesions (Warren and Yong 1997).

3.2.5 Endothelial function:

1) Plasma ADMA concentrations: Plasma concentrations of ADMA, an endogenous inhibitor of nitric oxide (NO) synthase (reviewed by Boger 2003) were measured by high performance liquid chromatography using AccQ-Fluor as the derivatizing reagent using methods as previously published (Heresztyn et al 2004).

2) Isolated aortic preparations: The aortae were cut into 2-3mm rings and mounted under 2g tension in organ baths as previously described for rat aortic rings (De la Lande et al 1999, De la Lande et al 2004). The vascular segments were suspended under tension in 15mL organ bath in Krebs bicarbonate solution and gassed with 5% CO₂ in O₂ at 37°C. Isometric tension was recorded via two stainless steel wires through the lumen, one of which was fixed and the other attached to a Grass FT03 transducer.

Each preparation was allowed to equilibrate for 120 minutes before exposure to potassium physiological saline solution (KPSS; 122mM); segments which contracted < 1g were discarded. After a further 30 minute equilibration period, contractile responses
were assessed via a cumulative concentration response curve to phenylephrine (PE; 0.001 - 100µmol/L, 0.5 log unit increments). Contractile responses were expressed as a percentage of the KPSS response. In aortic rings pre-contracted with PE to 70% maximal contraction, relaxant responses were then assessed between groups. Effects of the endothelium-dependent vasodilator, acetylcholine (ACh) (0.001 - 30µmol/L, 0.5 log units increment), the endothelium-independent NO-mediated vasodilator sodium nitroprusside (SNP) (0.001 - 100µmol/L, 0.5 log units increments) and the NO-independent vasodilator isoproterenol (ISO) (0.001 - 10µmol/L, 0.5 log unit increments) were assessed utilizing 30 to 40 minute equilibration periods between each concentration-response curve. Aortic rings that responded to ACh were co-incubated with N(G)-nitro-arginine methyl ester (L-NAME) (a nitric oxide synthase inhibitor) (300µM), indomethacin (cyclo-oxygenase inhibitor) (10µM) or charybdotoxin (10nM)/apamin (for endothelium-derived hyperpolarization factor inhibition) (1 µM) was used to delineate mechanism(s) of endothelium-dependent vasodilatation. Vasodilator responses were calculated as % inhibition of PE response.

3.2.6 Valvular function:

1) Anti-aggregatory effects of valvular autacoid release: In order to detect physiological active materials, presumably NO and PGI\textsubscript{2}, released from AV, we used a previously described bio-assay system, where fragments of AV tissue were co-incubated with human whole blood or platelet-rich plasma obtained from healthy subjects (Chirkov et al 2006). In this system, we assessed effects of AV fragments on adenosine 5\textsuperscript{'}-diphosphate (ADP)-induced platelet aggregation and intra-platelet content of cGMP and cAMP. Platelet aggregation in whole blood was examined utilizing a dual-channel impedance
aggregometer (Model 560, Chrono-Log, Haverstown, PA, USA). Tests were performed at 37°C and stirring speed of 900rpm. Samples of whole blood were diluted twofold with normal saline (final volume 1ml) and prewarmed for 5 min at 37°C. Aggregation was induced with ADP (sodium salt, obtained from Sigma, St.Louis, MO, USA), final concentration 2.5 µmol/L). Aggregation was monitored continually for 7 min, and responses were recorded as electrical impedance, in Ohms. Fragments (1mm²) of AV tissue, excised from rabbit hearts, were co-incubated (for 5 min before the induction of aggregation) with whole blood obtained from healthy male subjects, and were present during the aggregation test, in order to assess the inhibitory effects of diffusible materials released from AV tissue on ADP-induced platelet aggregation. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the added material. Intraplatelet cGMP and cAMP content was determined by radioimmunoassay, as described previously (Chirkov et al 2006).

2) Basal PGI₂ release: One AV leaflet from each rabbit was incubated for 1 hour in Krebs-Hepes solution at 37°C. Basal PGI₂ released into the incubation medium was analyzed by measuring its stable hydrolysis product 6-keto-PGF₁α. Commercially available enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI) were used. 6-keto-PGF₁α production was expressed as pg/µg of tissue protein of AV leaflet estimated by Lowry’s method.

3.2.7 Study analysis/ statistics:

The primary (null) hypothesis tested in this study was that vitamin D₂ alone would not affect AS as measured by AVBS after 8 weeks; this hypothesis was evaluated by non-
paired t-test. This study therefore had 80% power to detect $\geq 2.25$ SD difference in $AV_{BS}$ between groups. Two-way ANOVA was used to compare other echocardiographic parameters and biochemistry results between the control group and HD vit-D$_2$ group, as well as comparison of ADMA levels at baseline and after 8 weeks of treatment, to assess time-treatment interaction. Correlations between $AV_{BS}$ and change in ADMA, and biochemistry from baseline and after 8 weeks of treatment were performed using linear regression analyses. Neither the cholesterol/HD vit-D$_2$ group nor the LD vit-D$_2$ was subjected to formal statistical analysis as examinations of these groups were subsidiary objectives of the study. Results were expressed as mean ± SEM, unless otherwise stated. A level of probability of p<0.05 was accepted as statistically significant. All statistics were performed using Prism 4.0

3.3 Results.

3.3.1 Effects of vitamin D$_2$ on aortic valves assessed by echocardiography:

After 8 weeks of treatment, visual assessment of rabbit AV showed marked structural differences between groups (Figure 3.2). Aortic valve backscatter for all rabbits at baseline was minimal; therefore $AV_{BS}$ scores were only obtained after 8 weeks of treatment. Aortic valve backscatter was significantly higher in HD vit-D$_2$ compared to control group (p<0.0001) (Figure 3.3). Due to the difference in weight of rabbits in the control and HD vit-D$_2$ group with time, ratio of $AV_v/LVOT_v$ (independent of changes of size of left ventricular outflow tract) was used. Two-way ANOVA demonstrated significant treatment differences for the HD vit-D$_2$ vs control both at the level of $AV_v/LVOT_v$ (F=5.75, p=0.02) and $AV_p$ (F=9.9, p<0.01), consistent with more extensive increases in both parameters within the HD vit-D$_2$ (Figure 3.4). Table 3.1 records mean
values of AVv/LVOTv and AVp for all groups of treatments. In addition, interventricular septal dimensions (IVSd) as measures of left ventricular thickness indexed for body weight were significantly different between treatments (F=10.6, p<0.01) (Figure 3.5). Additionally, AVBS values for the cholesterol/HD vit-D2 group were only marginally elevated vs control, while no changes in AVBS were seen in the LD vit-D2 group (Figure 3.3).

3.3.2 Histology and immunohistochemistry:

Histological staining with Oil Red O, and Alizarin Red S were positive for the HD vit-D2, and the cholesterol/HD vit-D2 group, while remaining negative for the control group, and LD vit-D2 group (Figure 3.6a).

CD45 positive cell infiltration was present in all 4 groups, but more pronounced in the HD vit-D2 group and the cholesterol/HD vit-D2 group. Macrophage infiltrate on immunohistochemical analysis was detected only in the HD vit-D2 group and the cholesterol/HD vit-D2 group (Figure 3.6b).

3.3.3 Biochemistry:

Figure 3.7 shows graphical representations of biochemistry profile for (A) calcium levels, (B) calcium-phosphate levels, (C) serum creatinine, (D) total cholesterol levels for the 4 study groups. There were significant elevations of all of the abovementioned parameters for the HD vit-D2 group compared to the control group over 8 weeks of treatment (p<0.01 for all). Table 3.2 shows mean levels of these parameters at 4-weekly intervals. While
there were some elevations of calcium levels and calcium-phosphate products in the cholesterol/HD vit-D₂, rabbits in the LD vit-D₂ group had similar levels to the control. Interestingly, SeCr levels in the cholesterol/HD vit-D₂ group were higher than the HD vit-D₂ group after 4 weeks of treatment, but subsequently dropped to almost baseline after 8 weeks of treatment (Figure 3.7C). As expected, total cholesterol levels were much higher in the cholesterol/HD vit-D₂ group. However, rabbits in the HD vit-D₂ group alone had significantly elevated total cholesterol levels compared to the control group, despite being fed the same diet (Figure 3.7D). Rabbits in the LD vit-D₂ group had no consistent increase in either SeCr or total cholesterol levels compared to the control. Furthermore, there was no significant correlation between AVBS and change in Ca₃PO₄, total cholesterol, and SeCr levels in the HD vit-D₂ group (Figure 3.8).

3.3.4 Endothelial function:

1) Plasma ADMA concentrations: Plasma concentrations of ADMA tended to decrease with time (Figure 3.9). Decreases in ADMA concentrations in the HD vit-D₂ group were significantly attenuated compared to the control group over 8 weeks of treatment (F=4.7, p=0.04). Additionally, 2 out of 4 of the rabbits in the cholesterol/HD vit-D₂ group had attenuated falls in ADMA concentrations, compared to consistent fall in ADMA concentrations in all of the rabbits in the LD vit-D₂ group (Figure 3.9). Furthermore, there was a statistically significant negative correlation between change in plasma ADMA concentrations and AVBS for the HD vit-D₂ group (r²=0.5, p<0.05) (ie the larger the fall in ADMA concentrations, the lower the AVBS scores) (Figure 3.10A). The result was consistent also for other echocardiographic parameter such as AVp (r²=0.8, p=0.002) and AVp (r²=0.85, p=0.001) (Figure 3.10B). Furthermore, there was no significant
relationship between change in ADMA concentrations and SeCr ($r^2=0.04$, p=0.63) (Figure 3.11).

2) Isolated aortic ring preparations: There was no significant difference in contractile responses of the aortic rings to PE in all rabbit groups (Figure 3.12A). Maximum ACh-induced relaxation (Emax) was significantly impaired for aortic rings in the HD vit-D$_2$ group (17.3 ± 5.4%) compared to the control group (70.6 ± 1.1%) (p<0.0001). ACh-induced maximal relaxation in the cholesterol/HD vit-D$_2$ group tended to be reduced (48.1 ± 6.1%), whereas the LD vit-D$_2$ group results were similar to the control group (71.6 ± 1.7%) (Figure 3.12B). Neither SNP- or ISO-induced maximal relaxations were significantly different between the control group and HD vit-D$_2$ group (Figure 3.12C and 3.13D) (raw data Table 3.3A). Furthermore, ACh, SNP, and ISO concentrations inducing 50% relaxation were not significantly different between the control group and HD vit-D$_2$ group (Table 3.3B). In all groups the relaxant effect of ACh was totally abolished in the presence of L-NAME and unaffected by either indomethacin or charybdotoxin/apamin (Figure 3.13).

3.3.5 Valvular function:

1) Anti-aggregatory effects of valvular autacoid release: Table 3.4 summarises the results of co-incubation of rabbit AV fragments with human blood samples. AV fragments obtained from all groups of animals significantly inhibited platelet aggregation in whole blood. While in the control group, inhibition of aggregation was 48 ± 4% (p<0.01), this effect was even more marked in the HD vit-D$_2$ (64 ± 4%, p<0.03). In order to determine whether the observed anti-aggregatory effects can be attributed to the valvular release of
NO or PGI₂, we assessed intraplatelet cGMP and cAMP formation respectively. While there were no significant changes in cGMP content, cAMP content was increased in the presence of AV tissue (145 ± 17%, p<0.03 with control AV) and did not significantly differ when compared to HD vitamin D₂ group.

2) Basal production of PGI₂: Mean 6-keto-PGF₁α release in the control group was 134.1 ± 49.8 pg/μg tissue protein (n=6) compared to the HD vit-D₂ group 192.4 ± 90.4 pg/μg tissue protein (n=5) (p=NS). 6-keto-PGF1α content for the cholesterol/HD vit-D₂ group was 238.8 ± 71.3 pg/μg tissue protein, and the LD vit-D₂ group was 148.4 ± 41.7 pg/μg tissue protein.

3.3.6 Other effects of vitamin D₂:

Weight gain in the HD vit-D₂ group was attenuated: mean weight at 8 weeks was 2.5kg ± 0.06 for HD vit-D₂ vs 3.1kg ± 0.1 for the control group (p<0.05); for the cholesterol/HD vit-D₂: 2.65 ± 0.37kg; and the LD vit-D₂ group: 2.95 ± 0.12kg (Figure 3.2).

3.4 Discussion

The current series of experiments demonstrate that the regimen of dietary supplementation with HD vit-D₂ leads to the development of AS over an 8-week period. Furthermore, AS in this model is histologically similar to human disease, with fibrosis/calcification, inflammatory activation and the presence of atheromatous changes.
The functional studies performed revealed that HD vit-D$_2$, while markedly impairing aortic endothelial function (as assessed by ACh-induced relaxation), did not reduce either PGI$_2$ production from the AV or indeed overall valvular release of anti-aggregatory autacoids.

The study also explored the possible impact of treatment with LD vit-D$_2$ and with cholesterol/HD vit-D$_2$ supplementation. The latter investigations each comprised only 4 rabbits: therefore the results must be treated with caution. Nevertheless it can be said that (1) LD vit-D$_2$ did not induce any detectable degree of AS development at 8 weeks, and (2) cholesterol supplementation did not accentuate the effects of HD vit-D$_2$.

The main end-point of the study was backscatter from the AV, validated as a means of quantitation of early AS (“ASc”) in humans (Ngo et al 2004, reviewed by Nightingale and Horowitz 2005) but not previously examined in rabbits. AV$_{BS}$ values were very low in all rabbits at entry. However, by the end of 8 weeks, AV$_{BS}$ values were far greater for HD vit-D$_2$ treated rabbits than for controls (Figure 3.3), corresponding to the increased AVv/LVOTv ratio consistent with mild AS. Furthermore, HD vit-D$_2$-treated rabbits showed increases in IVSd suggestive of the development of left ventricular hypertrophy. All of these changes were apparent on the basis of blinded assessment. Thus, from a structural point of view, the HD vit-D$_2$-treated rabbits developed AS.

Although vitamin D has only been peripherally implicated to date in the pathogenesis of AS, it was important, even in an animal model, to determine whether this form of AS also exhibited the histological/histochemical markers of human AS. To this end, we examined the presence of atheromatous changes, all of which have been associated with human AS.
(Otto et al 1994, Warren and Yong et al 1997). Again, the development of AS in the rabbit model was associated with all of these markers.

A possible component of the pathogenesis of AS is the development of endothelial dysfunction affecting the valve. In fact, no studies have examined valvular endothelial function in early human AS; we have recently reported selective attenuation of valvular autacoid release in advanced AS (Chirkov et al 2006). High dose vitamin D$_2$ treatment attenuated the reduction in ADMA concentrations over 8 weeks, consistent with our recent observation that the presence of AS is associated with elevated plasma ADMA concentrations in patients (Ngo et al 2006). The reduction in ADMA levels, seen in control animals, most likely signifies the physiological changes as the animals mature. In addition, there was a significant negative correlation between changes in ADMA concentrations and AV$_{BS}$ at week 8. While it is also known that ADMA concentrations tend to increase with the development of renal insufficiency (Vallance et al 1992), there was no correlation between ADMA and SeCr in the current study (Figure 3.11). Although these data are consistent with NO modulation of the development of AS, causality is not proven. Nonetheless, we have recently observed in a tissue culture model of AV calcification that high concentrations of NO attenuate formation of calcific nodules (Kennedy et al 2006 abstract). Furthermore, aortic segments from HD vit-D$_2$ treated rabbits also exhibited evidence of severe systemic endothelial dysfunction, mediated entirely by diminution of NO release. On the other hand, it appeared that PGI$_2$ release from the valve was intact, or even somewhat stimulated in the presence of this model of AS. However, it has been shown that production of PGI$_2$ by VSMCs is stimulated upon addition of vitamin D$_3$, or its synthetic analogue 22-oxa-1,25-dihydroxy-vitamin D$_1$ (Inoue et al 1992, Wakasugi et al 1991). Thus, it is possible that elevation of
PGI₂ production seen in the vitamin D₂ treated groups may relate to this effect. The exact implication of this elevation of PGI₂ production, however, is uncertain.

A number of epidemiological studies have suggested that hypercholesterolemia is a risk factor for development and progression of AS (Aronow et al 1987, Stewart et al 1997, Palta et al 2000); furthermore experimental studies have demonstrated valve calcification in cholesterol-supplemented rabbits utilizing micro-CT (Rajamannan et al 2005). Retrospective clinical studies initially suggested that statin therapy was associated with reduced rates of progression of AS (Novaro et al 2001, Bellamy et al 2002). However, the only prospective clinical study reported to date showed no difference in progression of AS in patients treated with high dose atorvastatin (Cowell et al 2005). On the other hand, Drolet et al (2003) found that atherogenic diet alone was not sufficient to induce changes in AV hemodynamics in rabbits. One possible interpretation of these data is that redox stress associated with HD vit-D₂ administration leads, partially via alteration of ADMA kinetics (Jia et al 2006), to development of AS. We would postulate that cholesterol supplementation, while potentially relevant in the long term, did not significantly accentuate the impact of HD vit-D₂ alone. As in a previous study (Drolet et al 2003), the HD vit-D₂ treated group had elevated total cholesterol levels compared to controls despite being on the same diet. However, we found that there was no correlation between total cholesterol levels and AV₉₅ scores, or with SeCr (Figure 3.8B). Additionally, cholesterol supplementation did not worsen AS; nonetheless, this finding was supplemental and requires further investigations.

The primary role of vitamin D and its active metabolites is to maintain calcium homeostasis by increasing intestinal calcium absorption, and thus influences the balance
between bone resorption and formation. The effect of excess vitamin D on arteries has been studied in many animal models. Short courses of vitamin D as well as chronic less toxic treatments have all induced some metastatic calcification and deteriorating renal function (Mortensen et al 1993, Niederhoffer et al 1997). Although these studies did not consider valvular function, they all documented arterial calcification. In addition to inducing hypercalcemia, vitamin D is also important in influencing migration, proliferation, and gene expression of VSMCs (Rebsamen et al 2002, Cardus et al 2006) which are important in the pathogenesis of atherosclerosis. The potential significance of effects of this type in the context of myofibroblast development within the AV matrix was not explored in the current study.

Mechanistically, it is possible that vitamin D induced AS via several ways:

1) Vitamin D$_3$ stimulates expression of the intracellular thioredoxin inhibitor vitamin D$_3$ upregulated protein-1 (VDUP1; or otherwise known as Thioredoxin-interacting protein (Txnip), or Thioredoxin-binding protein (TBP2)) (Junn et al 2000). The thioredoxin and glutathione systems are the two major thiol-reducing pathways by which living cells regulate redox responses to stress signals (reviewed by Holmgren 1985). In turn, VDUP1 has been found to be of pivotal importance in the regulation of cellular redox balance, apoptosis, proliferation, and differentiation (Junn et al 2000, Yoshida et al 2005, Schulze et al 2006, Schulze et al 2002). Using a sequence-specific DNA enzyme to down-regulate VDUP1 mRNA, Xiang et al (2005) found that down-regulation of VDUP1 expression in cardiomyocyte significantly reduced myocardial collagen scarring, and cardiomyocyte apoptosis following acute ischemia in rats. Furthermore, it has
been shown that increased thioredoxin-1-expression regulates redox status in RAW cells (a macrophagic cell line that can be induced to form osteoclast) and stimulates osteoclast formation (Lean et al 2004). Additionally, overexpression of VDUP1 inhibited osteoclastogenesis and thioredoxin-1 expression, as well as soluble RANKL in human ostoclastogenesis cultures (Aitken et al 2004). Given the fact that osteoclast formation is responsible for bone resorption, and bone resorption has been proposed to link with arterial calcification; it is possible, that the addition of vitamin D stimulates VDUP1, which alters aortic valve cellular redox status, leading to increased in apoptosis, collagen deposition, and differentiation of osteoclast, potentially precipating aortic valve calcification.

2) Matrix Gla protein is a vitamin K-dependent protein that has been shown to be essential in preventing vascular calcification (Murshed et al 2004). In a rat model warfarin (a vitamin K antagonist) induced vascular calcification, calcification of the aortic heart valve was also seen (Price et al 1998). This study showed that the effects of impaired γ-carboxylation of matrix Gla protein induced by warfarin not only contributed to the development of arterial calcification, but also aortic valve calcification.

On the contrary, vitamin D₃ has been shown to increase matrix Gla protein expression in human osteoclasts, rat chondrocytes, osteoblasts and osteosarcoma cells (reviewed by Proudfoot and Shanahan 2006); and high matrix Gla protein expression has been associated with vascular calcification (Proudfoot et al 1998, Herrmann et al 2000). The combination of vitamin D₃ and warfarin in growing rats induced significant rapid arterial calcification compared to rats treated with
vitamin D$_3$ alone (Price et al 2000). Vitamin D$_3$ and warfarin combination lead to significant elevation of matrix Gla protein, where the rise in matrix Gla protein is associated with increased aortae mineralization.

Thus, it is possible, that supra physiological doses of vitamin D used to induce AS in this model, could have induced a rise in aortic valve matrix Gla protein expression. Overexpression of matrix Gla protein could have contributed to vitamin D toxicity and thus vascular/valvular calcification by depleting the vitamin K reserves and thereby induced an absolute deficiency of vitamin K (reviewed by Masterjohn 2007, Koshihara and Hoshi 1997)

3) The Lrp5, a co-receptor of low-density lipoprotein receptor family, has been discovered to be an important receptor in the activation of bone formation (reviewed by Ferrari SL 2005). It has been shown that in hypercholesterolemic rabbits, mineralization of the aortic valve was associated with upregulation of the Lrp5 receptors (Rajamannan et al 2005). Furthermore, the Lrp5 receptor was also increased in stenotic aortic valves compared to control (Caira et al 2006). These studies suggested that Lrp5 could play a role in mediating osteoblast differentiation and thus bone formation of stenotic valves. It has been recently demonstrated that 1,25 dihydroxyvitamin D$_3$ induces the expression of the signaling mechanisms of the Lrp5 receptor in the maintenance of calcium and phosphorus homeostasis (Fretz et al 2007). Thus, it is possible that in this model of AS, the use of vitamin D could have stimulated signaling cascades associated with increased Lrp5 receptor and leading to osteogenesis of the aortic valve.
However, despite multiple possible mechanisms which could explain vitamin D induced AS in this model, a major limitation of the study was the lack of exploration of the signal transduction pathway related to vitamin D2 in this case; and the failure to determine whether the impairment of endothelial function with vitamin D2 treatment was of central importance. It is unclear whether renal insufficiency contributed in any way to the development of AS. Nonetheless, there was no correlation between AV BS and rising SeCr. The fact that LD vit-D2 did not induce any change in the aortic valves compared to controls indicates that perhaps only high doses are effective in inducing development of AS; thus these findings may have no human analog. However, it is possible that LD vit-D2 supplementation over a longer period of treatment might induce AS in this model.

It is emphasized that the data on the LD vit-D2 and cholesterol/HD vit-D2 groups are limited, and were not subjected to formal statistical analysis. Nevertheless, the results are sufficient to exclude an additive effect of cholesterol supplementation to those of HD vit-D2 alone as well as to demonstrate that the effects of vitamin D2 were dose-dependent over the 2 doses examined.

Finally, ASc/AS are disorders affecting the ageing human population, and it could be argued that ageing rabbits might have responded differently to the treatments utilized. That possibility must remain speculative at present.

In conclusion, we report for the first time the rapid development of AS in rabbits induced by vitamin D2 supplementation alone. This animal model of AS showed AV lesions with features resembling those of human AS. The addition of cholesterol supplementation did not worsen AS. In view of current trends towards the widespread utilization of vitamin
D_2 in many subsets of the ageing human population (eg renal impairment, osteoporosis) these findings have potential implications beyond the animal model.
Figure 3.1: Schematic of study design: The principal hypothesis involved comparison of the control group and HD vit-D$_2$ treated group.
**Figure 3.2:** Echocardiographic views of rabbit aortic valves at baseline and after 8 weeks of treatment. Zoom pictures (2cm) of parasternal-like long axis view of AV leaflets. Typical examples for control group, HD vit-D$_2$ group, cholesterol/HD vit-D$_2$ group, and LD vit-D$_2$ group are shown at baseline and after 8 weeks. Arrows indicate position of valve in the 8-week views. Note increased echogenicity in the HD vit-D$_2$ group (in comparison with control valves at 8 weeks. Cholesterol/HD vit-D$_2$ animals also showed some increase in valve echogenicity, which was not apparent in the LD vit-D$_2$ group.
**Figure 3.3:** \( AV_{BS} \) values for the 4 treatment groups. There was a significant increase in \( AV_{BS} \) in HD vit-D\(_2\) group compared to control group (17.6 ± 1.4 dB vs 6.7 ± 0.8 dB, respectively, \( p<0.0001 \)). Mean \( AV_{BS} \) value for the cholesterol/HD vit-D\(_2\) group was increased (9.6 ± 3.6 dB); while \( AV_{BS} \) for the LD vit-D\(_2\) group (6.0 ± 0.7 dB) remained similar to the control group. *\( p<0.0001 \) compared to control. (Statistical comparisons were limited to HD vit-D\(_2\) vs control: see section 3.2.6).
Figure 3.4: (A) Flow ratio profiles comparisons (AV/LVOTv), and (B) pressure gradient (AVp) comparisons from at baseline and at end of treatment (8 weeks) for different groups of treatment. There was a statistically significant difference in both AV/LVOTv (F=5.75, p=0.02) and AVp (F=9.9, p<0.01) between the control and HD vit-D2 group indicating relative increases in AV/LVOTv and AVp associated with HD vit-D2 treatment over time. There were no consistent changes in either the HD vit-D2/cholesterol or LD vit-D2 groups.
**Table 3.1**: Data corresponding to Figure 3.4: mean values of (A) AVv/LVOTv and (B) AVp comparison for all groups of treatment. p<0.05 on 2-way ANOVA between control and HD vit-D$_2$ group, for both (A) and (B).

(A) AVv/LVOTv

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>HD vit-D$_2$</th>
<th>Cholesterol/HD vit-D$_2$</th>
<th>LD vit-D$_2$</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>1.17 ± 0.05</td>
<td>1.23 ± 0.05</td>
<td>1.23 ± 0.05</td>
<td>1.13 ± 0.02</td>
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<tr>
<td>8-week</td>
<td>1.20 ± 0.03</td>
<td>1.34 ± 0.04</td>
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(B) AVp (mmHg)

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<th>Group</th>
<th>Control</th>
<th>HD vit-D$_2$</th>
<th>Cholesterol/HD vit-D$_2$</th>
<th>LD vit-D$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3.75 ± 0.43</td>
<td>4.93 ± 0.40</td>
<td>4.08 ± 0.54</td>
<td>4.70 ± 0.33</td>
</tr>
<tr>
<td>8-week</td>
<td>4.98 ± 0.38</td>
<td>6.73 ± 0.62</td>
<td>4.30 ± 1.11</td>
<td>4.88 ± 0.32</td>
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</table>
Figure 3.5: Comparison of interventricular septal dimensions (IVSd) (indexed for body weight between groups). There was a significant increase in IVSd (indexed for body weight) in the HD vit-D₂ group compared to the control over time by 2-way ANOVA (F=10.6, p<0.01).

<table>
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<tr>
<th></th>
<th>Control</th>
<th>HD vit-D₂</th>
<th>Cholesterol/HD vit-D₂</th>
<th>LD vit-D₂</th>
</tr>
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<tr>
<td>Baseline</td>
<td>0.09 ± 0.007</td>
<td>0.09 ± 0.005</td>
<td>0.08 ± 0.003</td>
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<td>8-week</td>
<td>0.08 ± 0.005</td>
<td>0.13 ± 0.008</td>
<td>0.09 ± 0.014</td>
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Figure 3.6a: Histological staining after 8 weeks of treatment (i) Alizarin Red S – to detect calcification; (ii) Oil Red O – to detect neutral lipids (with region of interest marked)
Figure 3.6b: Immunohistochemistry with RAM-11 (for macrophage) and CD45 (for leukocytes (with region of interest marked). All views were taken at x100 magnification.
**Figure 3.7**: Biochemistry comparisons between groups of treatment over time. There was significant change in (A) calcium levels (Ca) \((F=17.8; \ p<0.001)\); (B) calcium-phosphate (Ca₃PO₄) product \((F=14.9; \ p<0.001)\); (C) serum creatinine (SeCr) \((F=9.66; \ p<0.001)\); and (D) total cholesterol levels \((F=6.3; \ p<0.01)\) between the HD vit-D₂ and control group with time upon 2-way ANOVA comparisons.
**Table 3.2:** Data corresponding to Figure 3.7: mean values of (A) Calcium levels; (B) Ca$_3$PO$_4$ levels; (C) SeCr; (D) total cholesterol at 4-weekly intervals for all treatment groups.

(A) Calcium levels (mmol/L)

<table>
<thead>
<tr>
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<th>HD vit-D$_2$</th>
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<th>LD vit-D$_2$</th>
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<tr>
<td>Baseline</td>
<td>3.68 ± 0.06</td>
<td>3.59 ± 0.05</td>
<td>3.61 ± 0.11</td>
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<td>4-week</td>
<td>3.64 ± 0.04</td>
<td>3.97 ± 0.04</td>
<td>4.18 ± 0.12</td>
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<td>8-week</td>
<td>3.41 ± 0.11</td>
<td>4.61 ± 0.21</td>
<td>4.13 ± 0.28</td>
<td>3.47 ± 0.03</td>
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(B) Calcium-phosphate product

<table>
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<th>Cholesterol/HD vit-D$_2$</th>
<th>LD vit-D$_2$</th>
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<tr>
<td>Baseline</td>
<td>6.08 ± 0.12</td>
<td>6.55 ± 0.25</td>
<td>5.57 ± 0.29</td>
<td>6.83 ± 0.30</td>
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<tr>
<td>4-week</td>
<td>5.18 ± 0.38</td>
<td>9.26 ± 0.27</td>
<td>6.78 ± 0.60</td>
<td>4.77 ± 0.12</td>
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<tr>
<td>8-week</td>
<td>4.94 ± 0.46</td>
<td>9.81 ± 0.69</td>
<td>7.32 ± 1.19</td>
<td>4.63 ± 0.38</td>
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Table 3.2 (continued)

(C) SeCr levels (mmol/L)

<table>
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<th>Control</th>
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<th>Cholesterol/HD vit-D₂</th>
<th>LD vit-D₂</th>
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<td>Baseline</td>
<td>0.07 ± 0.004</td>
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<td>0.06 ± 0.002</td>
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<td>4-week</td>
<td>0.08 ± 0.003</td>
<td>0.13 ± 0.007</td>
<td>0.23 ± 0.016</td>
<td>0.09 ± 0.001</td>
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<td>8-week</td>
<td>0.09 ± 0.006</td>
<td>0.15 ± 0.014</td>
<td>0.09 ± 0.030</td>
<td>0.10 ± 0.011</td>
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(D) Total cholesterol levels (mmol/L)

<table>
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<th>Control</th>
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<th>Cholesterol/HD vit-D₂</th>
<th>LD vit-D₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.26 ± 0.13</td>
<td>1.20 ± 0.09</td>
<td>1.23 ± 0.13</td>
<td>1.18 ± 0.11</td>
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<tr>
<td>4-week</td>
<td>1.38 ± 0.34</td>
<td>5.96 ± 1.61</td>
<td>33.5 ± 2.97</td>
<td>3.40 ± 1.29</td>
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<td>8-week</td>
<td>0.63 ± 0.10</td>
<td>5.10 ± 0.78</td>
<td>47.3 ± 15.31</td>
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**Figure 3.8:** Correlations of $AV_{BS}$ in the HD vit-D$_2$ group and biochemistry results. There were no statistical significant correlations between $AV_{BS}$ and change in (a) $Ca_3PO_4$ product ($r^2=0.08$, $p=0.5$); (b) total cholesterol levels ($r^2=0.02$, $p=0.75$); and (c) $SeCr$ levels ($r^2=0.0009$, $p=0.9$) in the HD vit-D$_2$ group.
**Figure 3.9:** Change in plasma ADMA concentrations over 8 weeks: comparison between the control group and the HD vit-D$_2$ group. Difference in time-treatment interaction was significant (2-way ANOVA: $F=4.7$, $p<0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD vit-D$_2$</th>
<th>Cholesterol/HD vit-D$_2$</th>
<th>LD vit-D$_2$</th>
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<tr>
<td><strong>Baseline</strong></td>
<td>1.01 ± 0.04</td>
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<td><strong>8-week</strong></td>
<td>0.74 ± 0.04</td>
<td>0.84 ± 0.03</td>
<td>0.77 ± 0.08</td>
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**Figure 3.10:** Correlations between change in ADMA levels in the HD vit-D$_2$ group and various echocardiographic parameters. There were statistically significant relationships between change in ADMA levels and (A) rabbit AV$_{BS}$ ($r^2=0.5$, $p<0.05$); (B) transaortic pressure gradients ($r^2=0.85$, $p=0.001$).
Figure 3.11: Correlations between change in ADMA concentrations and SeCr. There was no significant relationship between delta ADMA concentrations and SeCr ($r^2=0.04$; $p=0.63$) in the HD vit-D$_2$ group.
Figure 3.12: Contractile and relaxation responses of aortic rings from rabbits in the control group (○); HD vit-D2 group (∙); Cholesterol/HD vit-D2 group (X); and the LD vit-D2 group (◊). (7A) There was no significant difference in PE contractile responses between the control and HD vit-D2 group. (7B) ACh-induced relaxation was significantly impaired in the HD vit-D2 group compared to control (Emax: 17.3 ± 5.4% vs 70.6 ± 1.1% respectively, p<0.0001). While LD vit-D2 group had no impairment in ACh-induced relaxation (Emax: 71.6 ± 1.7%), there was some impairment in cholesterol/HD vit-D2 group (Emax: 48.1 ± 6.1%) (7B). (7C and 7D) Neither SNP- or ISO-induced maximum relaxation was reduced in the HD vit-D2 group compared to control.
Figure 3.13: Relaxant responses to ACh upon addition of indomethacin, L-NAME and charybdo-toxin/apamin for (A) Control group; (B) HD vit-D₂ group; (C) Cholesterol/HD vit-D₂; (D) LD vit-D₂ group.
Table 3.3: (A) Maximal contraction/relaxation percentages induced by ACh, SNP, and ISO for all rabbit groups. (B) ACh, SNP, and ISO concentrations inducing 50% relaxation values for all rabbit groups. * = p<0.001.

(A)

<table>
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<tr>
<th></th>
<th>Control</th>
<th>HD vit-D₂</th>
<th>Cholesterol/HD vit-D₂</th>
<th>LD vit-D₂</th>
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<tr>
<td>PE/KPSS</td>
<td>116.6 ± 2.0</td>
<td>121.6 ± 2.0</td>
<td>113.0 ±2.2</td>
<td>120.3 ± 4.1</td>
</tr>
<tr>
<td>ACh</td>
<td>70.6 ± 1.1</td>
<td>17.3 ± 5.4*</td>
<td>48.1 ± 6.1</td>
<td>71.6 ± 1.7</td>
</tr>
<tr>
<td>SNP</td>
<td>91.6 ± 2.2</td>
<td>77.8 ± 4.1</td>
<td>86.4 ± 2.1</td>
<td>90.2 ± 3.5</td>
</tr>
<tr>
<td>ISO</td>
<td>60.2 ± 2.1</td>
<td>49.7 ± 6.9</td>
<td>68.4 ± 2.0</td>
<td>56.4 ± 6.0</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD vit-D₂</th>
<th>Cholesterol/HD vit-D₂</th>
<th>LD vit-D₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE/KPSS</td>
<td>-6.5 ± 0.04</td>
<td>-6.5 ± 0.04</td>
<td>-6.1 ± 0.04</td>
<td>-6.5 ± 0.08</td>
</tr>
<tr>
<td>ACh</td>
<td>-7.2 ± 0.03</td>
<td>-7.2 ± 0.7</td>
<td>-7.0 ± 0.3</td>
<td>-7.3 ± 0.05</td>
</tr>
<tr>
<td>SNP</td>
<td>-6.7 ± 2.2</td>
<td>-6.4 ± 4.1</td>
<td>-6.7 ± 2.1</td>
<td>-6.6 ± 3.5</td>
</tr>
<tr>
<td>ISO</td>
<td>-7.5 ± 2.1</td>
<td>-7.5 ± 6.9</td>
<td>-7.1 ± 2.0</td>
<td>-7.5 ± 6.0</td>
</tr>
</tbody>
</table>
Table 3.4: Co-incubation of rabbit aortic valve fragments with human blood samples. Effects of (% of control, i.e. in the absence of aortic valve fragment) on ADP-induced platelet aggregation and intraplatelet content of cGMP and cAMP.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Extent of aggregation (%)</th>
<th>cGMP (% of control)</th>
<th>cAMP (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52 ± 4*</td>
<td>107 ± 11</td>
<td>145 ± 17#</td>
</tr>
<tr>
<td>HD vit-D₂</td>
<td>36 ± 7**</td>
<td>98 ± 9</td>
<td>140 ± 12#</td>
</tr>
<tr>
<td>Chol/HD vit-D₂</td>
<td>27 ± 12</td>
<td>117 ± 6</td>
<td>148 ± 14</td>
</tr>
<tr>
<td>LD vit-D₂</td>
<td>36 ± 5</td>
<td>114 ± 8</td>
<td>132 ± 13</td>
</tr>
</tbody>
</table>

For aggregation: * p<0.01 vs control (ie without AV fragments); ** p<0.03 vs group A
For cAMP: # p<0.03 vs control (ie without AV fragments)
Figure 3.14: Rabbit weight comparisons at baseline and after 8 weeks of treatment for:
(A) the control group (2.4 ± 0.1 kg; 3.1 ± 0.1 kg, respectively); (B) the HD vit-D2 group (2.6 ± 0.06; 2.4 ± 0.06 kg, respectively); (C) Cholesterol/HD vit-D2 group (2.56 ± 0.06 kg; 2.65 ± 0.4 kg, respectively); (D) the LD vit-D2 group (2.6 ± 0.03 kg; 2.95 ± 0.1 kg, respectively).
CHAPTER 4

Effects of ramipril on development of aortic valve stenosis in rabbits
4.1 Background

It is now generally accepted that the presence of AS is associated with significant adverse outcomes (Otto et al 1999, Aronow et al 2002), and imparting considerable health care costs on the Western community. Studies of AS pathogenesis in humans are often inherently restricted by the lack of serial examinations of aortic valves. There are now several postulated animal models of AS, mostly concentrating on the induction of atherosclerotic lesions on aortic valve (Rajamannan et al 2001, Rajamannan et al 2002, Weiss et al 2006). However, given the recent negative findings of the SALTIRE study, which showed high dose atorvastatin did not affect AS progression (Cowell et al 2005); it is likely that hypercholesterolemia alone may not be solely responsible for the development and progression of AS. Furthermore, in the previous chapter, we found that the addition of high cholesterol supplementation did not accelerate development of vitamin D₂-induced AS in rabbits.

The circulating/tissue peptidyl-dipeptidase angiotensin-converting enzyme (ACE), and angiotensin II (Ang II) have also been detected on stenotic valve lesions (O’Brien et al 2002), but not on normal valves. Furthermore, there is also evidence of local production of Ang II by chymase, tryptase and cathepsin G from activated mast cells identified on stenotic aortic valves (Helske et al 2004, Helske et al 2006). From a theoretical point of view, there are a number of reasons why ACE inhibitor therapy might decelerate progression of AS. Essentially, these relate to the putative procalcific role of angiotensin II (reviewed in section 1.3.2, chapter 1) and the potential for increased tissue concentrations
of bradykinin (normally inactivated by ACE) to exert direct and indirect (via nitric oxide release) effects in suppressing fibrosis and apoptosis possibly within the valve (reviewed by McLean et al 2002). However, it could equally be argued that it is unknown to what extent angiotensin II generation within the valve matrix is ACE-dependent, given the presence of chymase, tryptase, and cathepsin G (Helske et al 2004, Helske et al 2006).

Nonetheless, presence of ACE and Ang II in stenotic aortic valves do not necessarily imply role in the pathogenesis, and only limited data are available to suggest such a role. In a retrospective clinical study, O’Brien et al (2005) found that the use of ACE inhibitors was significantly associated with lower aortic valve calcium scores. However, Rosenhek and Rader et al (2004) found a (non-significant) trend for ACE inhibitors to be associated with slower rate of progression of AS in another retrospective study. To date, no prospective clinical study has been undertaken to study effects of ACE inhibitors on progression of AS, compared to at least 3 large studies investigating statins effects.

The principal objective of the current study is to determine whether ramipril could slow the development of AS in the animal model described in the previous chapter. We also planned to examine:

1) Effects of ramipril on valvular histology and immunohistochemistry

2) Effects of ramipril on systemic inflammation and vascular endothelial function.
4.2 Methods

4.2.1 Experimental protocol:

Male New Zealand White rabbits (2-2.5 kg) were treated for 8 weeks according to the schema shown in Figure 1. The principal hypothesis was examined via comparison of vitamin D$_2$ alone at 25,000IU/4 days weekly (n=10) (Vitamin D$_2$); and the vitamin D$_2$ at 25,000IU/4 days weekly and ramipril daily using a quarter of the Tritace® 5mg (Aventis Pharmaceutical) tablet per rabbit (n=12) (approximately 0.5mg/kg/rabbit) (ramipril/vitamin D$_2$). Additionally, n=6 rabbits in the untreated rabbit group which received normal chow and drinking water.

At baseline and after 8 weeks of treatment, echocardiography was performed under sedation with ketamine (3.5mg/kg) / xylazine (5mg/kg) and blood samples were taken for the measurement of serum creatinine (SeCr), calcium, phosphate, total cholesterol, and plasma asymmetric dimethylarginine (ADMA).

At the end of the protocol, animals were sacrificed under anesthesia, hearts and thoracic aortae rapidly removed and placed in ice cold Krebs solution and aortic valve leaflets were excised as soon as possible.

The experimental protocol was approved by the North Western Adelaide Health Service and University of Adelaide animal ethics committee and conforms to the National Health
and Medical Research Council of Australia guidelines of animal usage for experimentation.

4.2.2 Preparation of solutions:

Vitamin D$_2$ (Sigma) was prepared fresh daily in rabbit drinking water. First, vitamin D$_2$ powder stored under nitrogen gas was weighed and dissolved in ethanol (100µL), and then tap water was added to make up 55mL of tap water for each rabbit. For the control water, equal amount of ethanol was added.

Tritace® (ramipril) 5mg tablets were used in this study. First, appropriate numbers of Tritace® 5mg tablets were crushed in a mortar and pestle. Powdered ramipril was suspended in a small amount of ethanol (100µL). Then appropriate volumes of vitamin D$_2$ solutions were mixed with the suspended ramipril solution to make a final volume of 55mL per rabbit.

Rabbits in each group received 55mL of appropriate solutions every morning, (about 9am), and ad lib tap water was added at about 4pm every day. All rabbits received ad lib normal rabbit chow.
4.2.3 Echocardiographic measurements:

Ultrasound images were obtained with a 5-MHz sector phased-array probe, operated at 6.7MHz, connected to a Vivid 5, GE Vingmed, (Horten, Norway). Aortic valve backscatter (AVBS) was utilized as the primary measure of increased valve echogenicity (Ngo DT et al 2004). Peak left ventricular outflow tract flow (LVOTv) and transaortic valve flow (AVv) were measured utilizing continuous-wave Doppler recordings from the highest velocity available were used as measures of aortic valve stenosis. Ratio of AVv/LVOTv was used to compare changes in hemodynamics between the 2 groups. Reproducibility between different observers of AVv/LVOTv was 3.3%. Left ventricular wall thicknesses (indexed for body weight) were measured from 2D-guided M-mode echocardiographic tracings. Echocardiographic imaging and analyses were performed by observers blinded to rabbit treatments. All aortic valve area (AVA) measurements were indexed for body weights.

4.2.4 Calculation of ultrasound backscatter data analysis:

Determination of AVBS in rabbits was analogous to methodology used in humans (Ngo et al 2004, Ngo et al 2006). Two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal-like long-axis view over 3 cardiac cycles with a zoom of 2 cm. Three consecutive scans were performed per animal. A total of 10 square-shaped regions of interest (5 on the anterior leaflet and 5 on the posterior leaflets)
were obtained by placing 7x7 pixel sample volumes on the valves. Backscatter values from the blood pool adjacent to the left ventricular outflow tract and the aortic blood pool were used as reference values. Calibrated backscatter values were obtained by subtracting the averaged regions of interests in the left ventricular outflow tract blood pool (n=3), and the aortic blood pool (n=3) from the averaged backscatter values obtained on the aortic valves. The final calibrated backscatter values (decibel (dB)) were averaged over 3 consecutive scans.

### 4.2.5 Histology and Immunohistochemistry:

Immediately after dissection from the heart, one leaflet from each aortic valve was embedded in Tissue-Tek O.C.T compound, snap-frozen in liquid nitrogen, and stored at -80°C until serially sectioned. Serial sections of aortic valve leaflets were stained with Alizarin red S for calcification.

Sections (6µm) were subjected to double-labeled immunohistochemistry using the following antibodies; anti-rabbit macrophage RAM-11 (1:100) (Dako); monoclonal anti-collagen type I clone COL-1 mouse ascites fluid (1:4000) (Sigma); and anti-nitrotyrosine, clone 1A6 (Upstate) (1:50). For control sections, the primary antibodies were omitted or irrelevant isotype-matched mouse antibodies were applied, for RAM-11 and collagen stain. According to manufacturing instructions, a negative and positive control for nitrotyrosine was prepared with the run for nitrotyrosine stain. Staining was performed according to a 3-step immunoperoxidase method (Kraan et al 2000, and according to Section 3.2.4,
Chapter 3). Immunostaining with RAM-11 identifies the presence of macrophages, a feature present on human aortic valve lesions (Otto et al 1994), which indicates that AS is an active process. Collagen I staining was performed as it is the main component of fibrotic tissues. The presence of nitrotyrosine has been used as a marker of several pathological disease processes and oxidative stress (reviewed by Turko and Murad 2002).

4.2.6 Quantification of staining:

All sections were coded and analysed in a random order by independent observers (DTN, MDS) who were blinded for the clinical data. Semi-quantitative assessments were done for all stains in triplicates. Staining scores for the different stains are depicted below:

<table>
<thead>
<tr>
<th>Alizarin red S:</th>
<th>Collagen:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = no calcification</td>
<td>1 = &lt;75% valve covered</td>
</tr>
<tr>
<td>1 = &lt; 10% of valve with calcium</td>
<td>2 = 75-80%</td>
</tr>
<tr>
<td>2 = 10-25% calcification</td>
<td>3 = 80-90%</td>
</tr>
<tr>
<td>3 = 25-50% calcification</td>
<td>4 = 90-100%</td>
</tr>
<tr>
<td>4 = &gt;50% calcification</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrotyrosine:</th>
<th>RAM 11 (macrophage):</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = negative staining</td>
<td>0 = negative staining</td>
</tr>
<tr>
<td>1 = focal staining &lt; 5% of total cells</td>
<td>1 = focal staining &lt; 5% of total cells</td>
</tr>
<tr>
<td>2 = 5-10% of total cells</td>
<td>2 = 5-10% of total cells</td>
</tr>
<tr>
<td>3 = 10-25% of total cells</td>
<td>3 = 10-25% of total cells</td>
</tr>
</tbody>
</table>
4.2.7 Endothelial function:

1) Plasma asymmetric dimethylarginine (ADMA) concentrations: Plasma concentrations of ADMA, an endogenous inhibitor of nitric oxide (NO) synthase were measured by high performance liquid chromatography with fluorescence detection using AccQ-Fluor as the derivatizing reagent using methods as previously published (Heresztyn et al 2004).

2) Isolated aortic preparations: were performed as previously described in Section 3.2.5, Chapter 3. Experiments with L-NAME, indomethacin, charybdotoxin/apamin were not performed in this study.

4.2.8 Study analysis/ statistics:

All statistical data involved comparisons between the vitamin D2 and vitamin D2/ramipril groups. The primary (null) hypothesis tested in this study was that ramipril would not affect AS induced by vitamin D2 alone, as measured by AVBS; this analysis was performed using unpaired t-test. A power calculation, performed on the basis of results outlined in Chapter 3. For a study with 12 animals in each group, and p<0.05, there was 67% power to detect 1.50 SD difference between group means (approximately 50% reduction of AVBS), corresponding to 6dB of AVBS. Furthermore, the study needed 80% power to detect 1.75
SD and 89% power to detect 2.0 SD differences between groups. Two-way ANOVA was used to compare other echocardiographic parameters; biochemistry; as well as comparison of plasma ADMA concentrations over the 8 weeks of treatment. All other differences between groups were expressed as changes between baseline and after 8 weeks of treatment and examined using unpaired t-test. Comparisons between non-parametric data were performed using Mann-Whitney U test. Spearman’s rank correlation was performed for correlations between staining and AV_BS scores. The untreated reference normal rabbit group was not subjected to formal statistical analysis as examinations of this group were subsidiary objectives of the study. Results were expressed as mean ± SEM, unless otherwise stated. A level of probability of p<0.05 was accepted as statistically significant. All analyses were performed using SPSS version 13.

4.3 Results:

4.3.1 Effects of ramipril on aortic valves assessed by echocardiography:

As previously shown, weight gain was attenuated in the vitamin D2 alone group compared to the reference untreated group; the addition of ramipril was associated with increase in weight compared to the vitamin D2 alone group over the 8 weeks of treatment (Figure 4.2).

After 8 weeks of treatment, there was a non-significant reduction in AV_BS in the vitamin D2/ramipril group compared to vitamin D2 alone, (p=0.083, Figure 4.3). There was a statistically significant difference in the time-treatment interaction for AVv/LVOTv ratios between the vitamin D2 and vitamin D2/ramipril group (p=0.045) (Figure 4.4) indicating
significant retardation of the development of AS. Similarly, rabbits in the vitamin D2/ramipril group had significantly higher AVA over 8 weeks of treatment compared to the vitamin D2 group alone (p=0.048) (Figure 4.5). In addition, rabbits in the vitamin D2/ramipril group had significantly lower interventricular septal dimensions (IVSd) over time compared to the vitamin D2 alone group (F=10.1, p=0.042) (Figure 4.6). Furthermore, assessment of association between IVSd and AVBS showed no significant correlation in the vitamin D2/ramipril (r²=0.06, p=0.4) group compared to a significant positive correlation in the vitamin D2 treated group alone (r²=0.51, p=0.02) (Figure 4.7), which suggests that the addition of ramipril did not affect IVSd by lowering of AVBS scores.

4.3.2 Histology and immunohistochemistry:

Histological stain for calcium with Alizarin Red S was negative for aortic valves in the normal group (data not shown) similar to results shown in the previous chapter. However, there was no difference in Alizarin Red S staining scores between the vitamin D2 and vitamin D2/ramipril groups (Figure 4.8A). This lack of effect was also seen for collagen, nitrotyrosine, and macrophage scores (Figure 4.8B, 4.8C, 4.8D).

Further analyses were performed to evaluate possible correlations between histochemical and structural changes within the valve. There was a significant direct correlation between calcium and backscatter scores (p<0.001), and also a trend for a similar relationship regarding RAM-11 and AVBS (p=0.065). There was no interaction between nitrotyrosine and collagen scores with AVBS (Figure 4.9).
Figure 4.10 depicts distribution of RAM-11, nitrotyrosine, and collagen staining with calcium scores. Upon chi-square testing, the only significant association with high calcification was high RAM-11 scores (p=0.001); whilst there was a trend in high nitrotyrosine and collagen vs calcium scores, these interactions were not statistically significant.

4.3.3 Biochemistry:

There was a trend in improved SeCr levels in the vitamin D2/ramipril group after 4 weeks of treatment; this improvement was not statistically significant (Figure 4.11). Furthermore, there was a direct positive correlation between SeCr levels and AVBS in both groups of treatment (Figure 4.12). It appears that the addition of ramipril did not alter calcium levels after 8 weeks of treatment; there was no significant difference in calcium levels between the vitamin D2 and vitamin D2/ramipril group (Figure 4.13). Mean time-treatment difference in calcium levels was not significantly different between the vitamin D2 and vitamin D2/ramipril group; however, calcium-phosphate product was significantly lower in the vitamin D2/ramipril group compared to the vitamin D2 alone group (Figure 4.14). Furthermore, there was no significant correlation between delta Ca₃PO₄ and AVBS scores for both vitamin D₂ and vitamin D₂/ramipril group (Figure 4.15), which suggested that Ca₃PO₄ may not have played a crucial role in development of AVBS in this model.
4.3.4 Vascular endothelial function:

1) Plasma ADMA concentrations: Plasma concentrations of ADMA tended to decrease with time as previously observed (see Figure 3.9, Chapter 3). However, decreases in ADMA concentrations in the vitamin D2 group were significantly less pronounced compared to the vitamin D2/ramipril (Figure 4.16). Furthermore, change in plasma ADMA concentrations in the vitamin D2 group showed a significant inverse correlation with AVBS score at 8 weeks ($r^2=0.44$, $p=0.04$), with an even stronger inverse correlation between change in ADMA concentrations and AVBS score in the vitamin D2/ramipril group ($r^2=0.66$, $p=0.001$) (Figure 4.17). As regards the relationship between renal function and plasma ADMA accumulations, Figure 4.18 showed a non-significant correlation between change in SeCr and ADMA concentrations for rabbits in the vitamin D2 group, with a strong statistically significant inverse correlation in the vitamin D2/ramipril group. The addition of ramipril appeared to shift the relationship between SeCr and ADMA accumulations. These data therefore establish a close nexus between renoprotective effects of ramipril and preservation of endothelial function.

2) Isolated aortic ring preparations: There was no significant variability between groups in contractile responses of the aortic rings to PE (Figure 4.19A). Rabbits in the vitamin D2/ramipril group had improvement in ACh-induced aortic relaxation (Mann Whitney test, $p=0.056$) (Figure 4.19B). There was however, a significant improvement of approximately 10% in SNP-induced maximal relaxation in the vitamin D2/ramipril group compared to the vitamin D2 alone group ($p=0.036$) (Figure 4.19C). There was non-significant improvement.
in ISO-induced maximal relaxation in the ramipril treated group compared to the vitamin D$_2$ alone group (Figure 4.19D). Table 1 shows mean values ± SEM of maximal contractile/relaxation responses as well as concentrations required for 50% relaxation comparisons between groups. There was no significant difference in concentrations of PE, ACh, SNP, and ISO in inducing 50% contraction/relaxation responses between the 2 treatment groups. Interestingly, there was a highly significant inverse correlation between AV$_{BS}$ and maximum ACh-induced relaxation in the vitamin D$_2$/ramipril group ($r^2$=p<0.001) (Figure 4.20).

4.4 Discussion

As discussed in Chapter 3, vitamin D$_2$ induces AS in rabbits with significant increases in AV$_{BS}$, and echocardiographic hemodynamic profiles consistent with AS relative to a normal control group. Furthermore, this model showed histological/immunohistochemical changes of aortic valves that resemble those in human AS. In the current study, using the model above, treatment with ramipril induced borderline reduction in AV$_{BS}$, significant reduction in transvalvular flow, and improvement in AVA relative to vitamin D$_2$ alone group; all measures indicative of retardation of development of AS. Additionally, ramipril-treated rabbits showed borderline protection of aortic endothelial function as assessed by response to ACh (Figure 4.19B), and this salvage correlated inversely with AV$_{BS}$ (Figure 4.20). Interestingly, the addition of ramipril in this model normalized ADMA concentrations after 8 weeks of treatment (Figure 4.16) suggesting improvement of systemic endothelial function.
In this model of mild AS, there was a 16% increase in transvalvular velocity, and a 20% reduction in AVA with vitamin D₂; the addition of ramipril for 8 weeks approximately halved the increase in transvalvular velocity (Figure 4.3), and improved AVA by a total of 15% (Figure 4.4). Furthermore, development of left ventricular hypertrophy with vitamin D₂ was significantly attenuated by the addition of ramipril (Figure 4.6).

There was a marginal reduction (p=0.08) in AVₜs with ramipril. Aortic valve backscatter has been shown in humans to increase with the presence of ASc/AS (Ngo et al 2004) compared to aortic valves from normal healthy subjects. However, no previous studies have correlated AVₜs, whether in humans or rabbits, with actual structural changes within the valve tissue.

In the current studies, changes in intensity of valve matrix staining for calcification, collagen deposition, macrophage infiltration (indicative of one component of inflammatory change), and accumulation of nitrotyrosine (reflecting the product of nitric oxide scavenging by superoxide anion, and thus one component of oxidative stress) were determined using semi-quantitative histochemical methodology. Relative to untreated rabbits, there was no clearcut increase in either collagen or nitrotyrosine staining with vitamin D₂. Calcification was increased to a similar extent in both vitamin D₂ and vitamin D₂/ramipril treated rabbits, as was macrophage infiltration. However, it must be emphasized that the semi-quantitative nature of the methodology does not preclude a small difference in either calcification or inflammatory change between these two groups. An
alternative approach utilizing CT scanning of valves might have proved more sensitive in this regard (Rajamannan et al 2005).

The histochemical data, applied to the entire group of rabbits studied, also shed some light on the structural basis for backscatter data, not previously explored in the aortic valve. As shown in Figure 4.9A, there was a highly significant correlation between calcification and AVBS scores. This suggests that, while some AVBS occurs in the absence of calcification (as in untreated rabbits), increases in AVBS largely reflect calcium deposition in this model. Notably, there was no correlation with collagen staining. The weak correlation with macrophage staining (Figure 4.9D) may imply a causative relationship between inflammation and calcification, as shown in (Figure 4.10A), and previously postulated by Otto et al (1994).

Clinically, there is a close relationship between chronic renal failure and prevalence and rapid progression of AS (reviewed by London 2000). In this series of experiments, the use of vitamin D2 elevated SeCr (Figure 4.11), and change in SeCr over the 8 weeks of treatment directly correlated with AVBS (Figure 4.12) indicating that worsening of renal function was associated with rate of progression of AS. The question therefore arises: did renal dysfunction per se contribute to the development of AS, and if so, how? Furthermore, given that there is now evidence that ramipril decelerates the progression of AS, was this effect due to salvage of renal function?

The second question (re ramipril effects on renal function) is easily addressed because:
a) ramipril did not significantly affect renal function deterioration (albeit with a hint of late stabilization) (Figure 4.11); and also

b) ramipril did not significantly shift the delta SeCr : delta AVBS relationship on ANCOVA (Figure 4.12).

As regards the issue of whether renal function deterioration “caused” development of AS, this cannot be addressed completely, but there are 3 potential mechanisms:

i) increases in calcium-phosphate product (Figure 4.14)

ii) increases in ADMA concentrations

iii) decreases in vascular repair mechanisms all associated with renal insufficiency (Varma et al 2005).

As regards calcium-phosphate product, serum calcium levels were elevated over 8 weeks of treatment with vitamin D2 alone treatment producing changes similar to those in the previous chapter; the addition of ramipril did not alter this. However, there was a significant attenuation in the rise in calcium-phosphate products in rabbits treated with vitamin D2/ramipril compared to vitamin D2 alone (Figure 4.14). It has been documented that calcium-phosphate product is associated with severity of AS in patients with normal renal function (Mills et al 2004); however, there was no significant correlation between change in Ca₈PO₄ and AVBS scores in either groups of treated rabbits in this study, which suggests that Ca₈PO₄ (Figure 4.15) may not perhaps play a pivotal role in the development of AS in this model.
In relation to the role of ADMA in development of AS, in agreement with data previously shown, rabbits with AS in the vitamin D$_2$ alone group had significantly elevated plasma ADMA concentrations, which correlated with AV$_{BS}$, indicating that more severe AS is associated with potential reduction in NO formation. The addition of ramipril in these series of experiments significantly reduced ADMA concentrations compared to the vitamin D$_2$ alone group (Figure 4.16). Furthermore, reduction in ADMA concentrations also significantly correlated to AV$_{BS}$ in both treatment groups (Figure 4.17). While it is known that ADMA concentration is elevated in the presence of renal impairment (Vallance et al 1992); it is uncertain to what extent ADMA concentrations were modulated via (i) rates of ADMA formation, (ii) metabolic ADMA clearance vs (iii) renal ADMA clearance in this study. It is therefore not possible to state with certainty whether renal dysfunction contributed to the observed changes in ADMA concentrations.

The next issue to consider is whether changes in ADMA kinetics might have played a central role in deceleration of AS development under the influence of ramipril. First, there is no doubt that ACE inhibitors/Ang II receptor antagonists can reduce ADMA concentrations. For example, it has been shown that the addition of an ACE inhibitor could reduce plasma concentrations of ADMA in patients with essential hypertension (Ito et al 2001), and syndrome X (Chen JW et al 2002). After 8 weeks of treatment with enalapril, significant reduction of von Willebrand factor, a marker of endothelial injury, ADMA concentrations, and improved coronary flow reserve occurred in patients with syndrome X (Chen JW et al 2002).
Additionally, there is a link between ADMA and angiotensin II: Suda et al (Suda et al 2004) showed that infusion of ADMA to wild-type mice induced significant coronary microvascular lesions; and this was not due to eNOS inhibition alone, but due to up-regulation of ACE and a resultant of angiotensin II mediated superoxide production. They also showed that addition of an ACE inhibitor or AT1 receptor blocker suppressed this O2-vascular formation. Thus, it is possible that aortic valve lesions in rabbits treated with vitamin D2 may be due to elevation of ADMA concentrations.

As regards whether ADMA might play a central role in AS development, there is considerable evidence that NO might act as a physiological inhibitor of inflammatory change and calcification within the aortic valve. It has been demonstrated that the addition of NO donors significantly inhibited TGF-β1-induced calcific nodule formation in pig aortic valve interstitial cells (Kennedy et al 2006). It is therefore possible that elevation of ADMA concentrations induces upregulation of ACE and superoxide production; and the addition of ramipril abolished these effects and thus resulted in improvement of AS in this study.

In this study, we also aimed to examine the effects of ramipril on aortic endothelial function, based on the concept that development of AS might reflect endothelial dysfunction and/or hyporesponsiveness to nitric oxide. Rabbits in the vitamin D2/ramipril group had a trend towards improvement of endothelium-dependent ACh-induced relaxation compared to the vitamin D2 group alone (Figure 4.19B). There was a strong negative correlation between ACh-induced relaxation and AVBS in individual rabbits in the
ramipril treated group. This suggests that there was considerable heterogeneity in salvage of endothelial function with ramipril treatment. In the absence of measurement of inhibition of plasma and tissue ACE activity, we cannot exclude a pharmacokinetic basis for this heterogeneity. Another possibility (also impossible to evaluate on the available data) is that there was variability in either ACE-dependent or ACE-independent generation of angiotensin II in individual rabbits.

As regards the close correlation between ACh response and AVBS, this provides further evidence of the potential role of NO in retarding the development of AVS in this model. Direct measurement of NO production within the valves, technically a difficult undertaking (reviewed by Tarpey and Fridovich 2001) would potentially have established this association in the same, rather than different tissues, but not proved a causal association. Additionally, whilst there have been numerous large clinical trials showing the benefits of some ACE inhibitors in prevention of cardiovascular death and myocardial infarction in patients with coronary heart disease (Yusuf et al 2000, EUROPA), none of these studies looked at incidence and progression of AS with ACE inhibitors. It would be interesting, given the current findings, to see if ACE inhibitor therapy could have affected AS development also.

Additionally, rabbits in the ramipril treated group had significant salvage of approximately 10% SNP-induced relaxation. Sodium nitroprusside is a nitric oxide donor, which induces endothelium-independent, NO-dependent relaxation. It has been shown that Ang II, increases synthesis of some subunits of NAD(P)H oxidase, a major superoxide anion-
generating enzyme. Increased levels of superoxide lead to decreased activity, 
bioavailability, and synthesis of NO (reviewed by Griendling et al 1994). The addition of 
ramipril potentially blocks the generation of Ang II, and thus decreases NAD(P)H oxidase 
activity and superoxide generation, and therefore increases NO bioavailability. 
Presumably, through these mechanisms, rabbits in the ramipril group had significantly 
improved SNP-, (and borderline ACh-induced) relaxation. There was also borderline 
improvement in isoproterenol response for rabbits in the vitamin D2/ramipril group 
compared to vitamin D2 alone. Vasorelaxation induced by isoproterenol through 
stimulation of β-adrenoceptors, could somewhat be potentiated by the presence of 
endothelium (Brawley et al 2000, Gray and Marshall 1992; Delpy et al 1996). For example, 
removal of the endothelium or addition of the NOS inhibitor L-NAME significantly 
attenuated isoproterenol-induced relaxation (Brawley et al 2000; Gray and Marshall 1992; 
Delpy et al 1996). Thus, it is possible that the trend towards improvement in isoproterenol-
induced vasorelaxation is due to the variable effects of vitamin D2-induced aortic 
endothelial dysfunction as seen with ACh-induced relaxation results (Table 1).

4.1 Clinical implications and study limitations:

The importance of this study is therefore that it establishes, for the first time some 
evidence that ACE inhibitors may retard progression of AS. However, it leaves a number 
of questions unanswered. Critical among these are:

1) What were the humoral mediators of this effect? ACE inhibitory effects may reflect:
a) decreased formation of angiotensin II

b) increased formation of bradykinin

c) changes in clearance of other ACE substrates (eg enkephalins, substance P, Ang 1-7) all of which are potent vasodilators (Fuch et al 2004).

d) changes in “secondary” effectors of ACE effect, eg NO production via BK2 receptors (reviewed by McLean et al 2000), or decreased NAD(P)H oxidase activity (Li JM et al 2004).

e) “direct” effects of ACE (reviewed by Fleming I et al 2006).

2) The potential role of bradykinin is of particular interest because it has been shown to be pivotal in some models of myocardial protection by ACE inhibitors (Koike et al 2005, Wollert et al 1997). Indeed, recent reports by Helske et al (Helske et al 2006 abstract) suggest upregulation of BK1 and BK2 receptors in human AS. Thus, experiments with coadministration of ramipril and specific or non-specific BK receptor antagonists might clarify this issue (Helske and Laine et al 2006)

3) On the other hand, if angiotensin II is a pivotal effector, then angiotensin II antagonists would be at least equi-effective as ramipril (potentially more so, because not all angiotensin II generation is via ACE) (reviewed by Urata et al 1994).
4) Lastly, cardioprotection may not be equally exhibited by all ACE inhibitors, as shown by the results of the PEACE trial with trandolapril (Solomon et al 2006). It is theoretically possible that this might also be true of AS.

Irrespective of these limitations, the current data provide a strong rationale for the inception of a randomized trial of ACE inhibition as a strategy for limitation of AS progression in humans.
**Figure 4.1:** Schema of study design.

New Zealand White rabbits (2.0-2.5kg) (n=28)

- **Normal group** (n=6)
  - Normal chow/drinking water

- **Vitamin D\textsubscript{2} alone** (n=10)
  - Normal chow + 25,000 units Vitamin D\textsubscript{2}/4 days weekly in drinking water

- **Vitamin D\textsubscript{2}/ Ramipril** (n=12)
  - Normal chow + 25,000 units Vitamin D\textsubscript{2}/4 days weekly + 0.5 mg/kg Ramipril in drinking water

Statistical comparison
Figure 4.2: Weight profile for the 3 rabbit groups. Rabbits in the reference normal group experienced weight gain over the 8 weeks of treatment (2.64±0.1 vs 3.56±0.1kg). Weight gain was attenuated in the vitamin D₂ group (2.58±0.1 vs 2.43±0.1kg) with slight improvement in weight after 8 weeks of treatment in the vitamin D₂/ramipril group (2.53±0.06 vs 2.56±0.09kg). The difference in weight change profile between untreated and vitamin D₂-treated rabbits was discussed in Chapter 3. There was no significant difference in weight change profile associated with addition of ramipril.
Figure 4.3: Aortic valve backscatter score (AV\textsubscript{BS}) comparison between groups. Mean AV\textsubscript{BS} in the vitamin D\textsubscript{2} alone group was higher compared to the vitamin D\textsubscript{2}/ramipril group (17.8 ± 2.1 dB vs 11.9 ± 2.4 dB, respectively). Mean AV\textsubscript{BS} for the normal group was 4.7 ± 0.7 dB.
**Figure 4.4:** Comparison of transvalvular flow between groups. 2-way ANOVA showed statistically significant difference in time-treatment interaction between vitamin D$_2$ and vitamin D$_2$/ramipril group (F=4.4; p=0.045).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D$_2$</th>
<th>Vitamin D$_2$/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.12 ± 0.04</td>
<td>1.12 ± 0.02</td>
<td>1.17 ± 0.02</td>
</tr>
<tr>
<td>8-week</td>
<td>1.17 ± 0.04</td>
<td>1.30 ± 0.05</td>
<td>1.22 ± 0.04</td>
</tr>
</tbody>
</table>

p<0.05
Figure 4.5: Comparison of aortic valve area (AVA) indexed for body weight between groups. 2-way ANOVA showed statistically significant difference in time-treatment interaction between vitamin D$_2$ and vitamin D$_2$/ramipril group ($F=4.4; p=0.048$).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D$_2$</th>
<th>Vitamin D$_2$/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.69 ± 0.04</td>
<td>0.66 ± 0.03</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>8-week</td>
<td>0.93 ± 0.05</td>
<td>0.53 ± 0.04</td>
<td>0.56 ± 0.04</td>
</tr>
</tbody>
</table>
Figure 4.6: Comparison of interventricular septal dimensions (IVSd) between groups. There was a statistically significant time-treatment difference in IVSd between rabbits treated with ramipril compared to those on vitamin D2 alone (F=4.7; p=0.042).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D2</th>
<th>Vitamin D2/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.09 ± 0.004</td>
<td>0.090 ± 0.005</td>
<td>0.093 ± 0.004</td>
</tr>
<tr>
<td>8-week</td>
<td>0.073 ± 0.002</td>
<td>0.117 ± 0.007</td>
<td>0.096 ± 0.006</td>
</tr>
</tbody>
</table>
**Figure 4.7:** Correlation between delta IVSd indexed for body weight and aortic valve backscatter scores (AVBS). There was a statistically significant relationship between delta IVSd and AVBS scores for the vitamin D2 group ($r^2=0.51$, $p=0.02$); with no correlation for the vitamin D2/ramipril group ($r^2=0.06$, $p=0.4$). Furthermore, there was no significant difference in slopes and intercepts for both lines.
**Figure 4.8:** Scatterplots showing median staining scores for all stains with range. There was no significant difference between the 2 groups in all the stains performed by Mann-Whitney test: (A) Alizarin Red S (p=0.41); (B) Collagen (p=0.55); (C) Nitrotyrosine (p=0.96); (C) RAM-11 (p=0.36).
Figure 4.9: Comparison between staining scores and AV$_{BS}$. There was a statistically significant correlation between AV$_{BS}$ and (A) calcium scores of the aortic valves by Spearman’s correlation (R=0.76; p<0.001). Data for correlation of (B) collagen scores (R=0.02; p=0.94) and (C) Nitrotyrosine scores (R=0.095; p=0.69) indicated no significant correlations; while (D) RAM-11 scores revealed borderline association with AV$_{BS}$ scores (R=0.42; p=0.07).
**Figure 4.10:** Cross-tabulations of distribution of RAM-11, nitrotyrosine, and collagen with calcium scores. Upon chi-square testing: (A) correlation between macrophage and calcium scores ($p=0.001$); (B) correlation between collagen and calcium scores ($p=0.075$); and (C) correlation between nitrotyrosine and calcium scores ($p=0.105$).
Figure 4.11: Comparison of SeCr levels between vitamin D$_2$ and vitamin D$_2$/ramipril over the 8 weeks of treatment. Upon 2-way ANOVA analysis, although there was a trend in improving renal function after 8 weeks of treatment with ramipril, there was no statistical significance in SeCr between the 2 groups (F=1.9; p=0.19).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D$_2$</th>
<th>Vitamin D$_2$/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>0.062 ± 0.006</td>
<td>0.062 ± 0.003</td>
<td>0.056 ± 0.002</td>
</tr>
<tr>
<td><strong>4-week</strong></td>
<td>0.066 ± 0.004</td>
<td>0.119 ± 0.013</td>
<td>0.123 ± 0.012</td>
</tr>
<tr>
<td><strong>8-week</strong></td>
<td>0.080 ± 0.007</td>
<td>0.147 ± 0.016</td>
<td>0.112 ± 0.014</td>
</tr>
</tbody>
</table>
**Figure 4.12:** Relationship between AVBS and SeCr for both vitamin D2 and vitamin D2/ramipril groups. There was a statistically significant correlation between AVBS and SeCr for both vitamin D2 and vitamin D2/ramipril groups ($r^2=0.57$, $p=0.01$ and $r^2=0.65$, $p=0.002$, respectively). There was no significant difference in slopes ($p=0.9$) and intercepts ($p=0.3$) between the 2 lines.
**Figure 4.13**: Comparison of calcium levels between vitamin D₂ and vitamin D₂/ramipril over the 8 weeks of treatment. Upon 2-way ANOVA analysis, there was no statistical difference in calcium levels between the 2 groups over time (F=0.003; p=0.9).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D₂</th>
<th>Vitamin D₂/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3.5 ± 0.08</td>
<td>3.5 ± 0.04</td>
<td>3.5 ± 0.03</td>
</tr>
<tr>
<td>4-week</td>
<td>3.5 ± 0.06</td>
<td>3.9 ± 0.11</td>
<td>4.1 ± 0.10</td>
</tr>
<tr>
<td>8-week</td>
<td>3.4 ± 0.04</td>
<td>4.2 ± 0.29</td>
<td>4.2 ± 0.26</td>
</tr>
</tbody>
</table>
**Figure 4.14:** Comparison of calcium-phosphate (Ca₃PO₄) levels between vitamin D₂ and vitamin D₂/ramipril over the 8 weeks of treatment. Upon 2-way ANOVA analysis, there was a statistically significant difference in Ca₃PO₄ between treatment groups (F=10.6; p=0.004).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D₂</th>
<th>Vitamin D₂/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>6.33 ± 0.31</td>
<td>6.04 ± 0.22</td>
<td>5.53 ± 0.20</td>
</tr>
<tr>
<td><strong>4-week</strong></td>
<td>5.50 ± 0.31</td>
<td>9.01 ± 0.38</td>
<td>8.25 ± 0.35</td>
</tr>
<tr>
<td><strong>8-week</strong></td>
<td>5.96 ± 0.37</td>
<td>9.91 ± 0.61</td>
<td>7.78 ± 0.55</td>
</tr>
</tbody>
</table>
Figure 4.15: Correlation between change in Ca₉PO₄ levels and AV₉S scores. There was no significant correlation between delta Ca₉PO₄ levels and AV₉S scores for both the vitamin D₂ group alone ($r^2=0.03$, $p=0.63$); and the vitamin D₂/ramipril group ($r^2=0.18$, $p=0.17$).
Figure 4.16: Comparison of ADMA concentrations between vitamin D$_2$ and vitamin D$_2$/ramipril over the 8 weeks of treatment. Upon 2-way ANOVA analysis, there was statistical difference in ADMA concentrations between the 2 groups over time (F=9.5; p=0.006).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rabbits</th>
<th>Vitamin D$_2$</th>
<th>Vitamin D$_2$/ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.99 ± 0.05</td>
<td>1.03 ± 0.03</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>8-week</td>
<td>0.78 ± 0.04</td>
<td>0.93 ± 0.04</td>
<td>0.80 ± 0.04</td>
</tr>
</tbody>
</table>
Figure 4.17: Relationship between AV$_{BS}$ and ADMA concentrations for both vitamin D$_2$ and vitamin D$_2$/ramipril groups. There was a statistically significant correlation between AV$_{BS}$ and SeCr for both vitamin D$_2$ and vitamin D$_2$/ramipril groups ($r^2=0.44$, $p=0.04$ and $r^2=0.66$, $p=0.001$, respectively). There were no significant differences in slopes ($p=0.6$), and intercepts between the 2 lines ($p=0.5$).
Figure 4.18: Correlation between change in ADMA concentrations and SeCr levels for rabbits in the vitamin D₂ and vitamin D₂/ramipril.

Delta ADMA concentrations and SeCr were not significantly correlated for the vitamin D₂ alone ($r^2=0.23$, $p=0.16$) but significantly correlated for the vitamin D₂/ramipril treated rabbits ($r^2=0.74$, $p<0.001$). There was no significant difference in slopes ($p=0.09$), but a significant difference in intercepts between the 2 lines ($p=0.008$).
Figure 4.19: Contractile and relaxation responses of aortic rings from rabbits in the normal group (■); vitamin D2 group (□); and vitamin D2/ramipril group (●). (A) There was no difference in contractile responses comparison between the vitamin D2 and vitamin D2/ramipril group; (B) Maximal ACh-induced relaxation was improved in the vitamin D2/ramipril group, however this was not significantly different compared to the vitamin D2 alone group (Mann Whitney t-test, p=0.056); (C) Relaxation responses to sodium nitroprusside (SNP) was significantly higher in the vitamin D2/ramipril group compared to the vitamin D2 group alone *(p=0.036); (D) Relaxation responses to isoproterenol (ISO) were not significantly different between the two treatment groups (p=0.077).
Table 1: (A) Maximum contractile and relaxation responses between treatments (Emax).
(B) Concentrations inducing 50% contractile/relaxation responses (EC50).

(A)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PE (%)</th>
<th>ACh (%)</th>
<th>SNP (%)</th>
<th>ISO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>114.2 ± 1.1</td>
<td>76.0 ± 2.8</td>
<td>94.0 ± 1.4</td>
<td>56.3 ± 3.5</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>113.7 ± 3.7</td>
<td>21.4 ± 9.4</td>
<td>83.1 ± 3.4</td>
<td>46.9 ± 5.4</td>
</tr>
<tr>
<td>Vitamin D2/ramipril</td>
<td>112.1 ± 2.4</td>
<td>47.0 ± 9.7</td>
<td>91.6 ± 2.0*</td>
<td>61.2 ± 5.3</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PE (µM)</th>
<th>ACh (µM)</th>
<th>SNP (µM)</th>
<th>ISO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-6.2 ± 0.04</td>
<td>-7.2 ± 0.05</td>
<td>-6.6 ± 0.03</td>
<td>-7.3 ± 0.08</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>-6.3 ± 0.08</td>
<td>-7.3 ± 0.46</td>
<td>-6.4 ± 0.08</td>
<td>-7.4 ± 0.17</td>
</tr>
<tr>
<td>Vitamin D2/ramipril</td>
<td>-6.4 ± 0.06</td>
<td>-7.2 ± 0.19</td>
<td>-6.7 ± 0.06</td>
<td>-7.4 ± 0.12</td>
</tr>
</tbody>
</table>
**Figure 4.20:** Correlation between ACh-induced maximal relaxation (%) and AV$_{BS}$ scores in the vitamin D$_2$/ramipril group. There was a highly significant inverse correlation between maximum ACh-induced relaxation and AV$_{BS}$ scores ($r^2=0.93$, $p<0.001$).
CHAPTER 5

Association of aortic stenosis with elevation of plasma concentrations of asymmetric dimethylarginine (ADMA)
5.1 Background

As summarized in Chapter 1, aortic stenosis (AS) now represents the most frequently occurring form of hemodynamically significant valvular heart disease in most Western populations. The incidence of AS increases with age; its precursor, disorganization of valve structure without marked obstruction (aortic sclerosis: ASc) is present in approximately 40% of individuals > 80 years (Lindroos et al 1993; Stewart et al 1997).

In view of the considerable morbidity and mortality associated with AS, investigations have been directed towards better understanding of its pathogenesis, with the ultimate objective of developing strategies to retard its progression. Poggianti et al ((2003) have observed that ASc (and therefore presumably AS) is associated with systemic endothelial dysfunction, while other investigators have demonstrated activation of inflammatory processes and evidence of associated atherogenesis (O’Brien et al 1996; Otto et al 1994). Consistent with these findings, ASc has been identified as a marker of increased risk of acute myocardial infarction (Otto et al 1999).

A number of recent investigations have indicated that asymmetric dimethylarginine (ADMA), a physiologically occurring competitive antagonist of endothelial nitric oxide synthase (eNOS) is both a strong marker and also a mediator of many aspects of endothelial dysfunction (Sydow et al 2003; Boger et al 1997; Boger 2003). ADMA appears not only to inhibit the bioconversion of arginine by eNOS to release nitric oxide (NO), but also to regulate eNOS activity under certain conditions (Boger 2003). While ASc has been reported to be associated with endothelial dysfunction (Poggianti et al
2003), endothelial function has not been investigated in patients with advanced AS. The possibility that ADMA contributes to the development of endothelial dysfunction in patients with ASc and AS is relevant because the pathogenesis of AS appears to involve inflammatory infiltration of the valvular matrix, consistent with loss of protective endothelial function. Furthermore renal dysfunction, which is characterized by marked elevation of ADMA levels (Vallance et al 1992; Zoccali et al 2001; MacAllister et al 1996) appears to be a major risk factor for rapid progression of AS (Perkovic et al 2003; Palta et al 2000).

To date, no studies have evaluated ADMA levels in patients with AS. The current investigation was performed to test the hypothesis that the presence of AS (as distinct from normal valve structure) is independently associated with elevation of ADMA levels, even in a patient population with a high prevalence of other coronary risk factors.

5.2 Methods:

5.2.1 Study patients.

The study population consisted of 42 consecutive patients with moderate to severe AS (AS group) (mean aortic valve area $0.81 \pm 0.23$ SD) identified while being referred for an echocardiogram, and 42 referred patients with normal aortic valves (non AS group) identified by echocardiography. In the case of 34 of the referred patients with normal aortic valves, the main indication was evaluation of left ventricular function. Patients in both study groups were matched for age. Informed consent was obtained prior to study
entry. The study was approved by the Ethics of Human Research of The Queen Elizabeth Hospital.

5.2.2 Doppler echocardiography.

Complete transthoracic studies were performed in all patients, by use of a commercially available system (Vivid 5, GE Vingmed, Horten, Norway with a 2.5 MHz phased array probe). M-mode and two-dimensional (2D) echocardiograms with Doppler analysis were obtained for all patients. Left ventricular diameters and wall thicknesses were measured from 2D-guided M-mode echocardiography. Mean, peak transaortic pressure gradients, and aortic valve area were quantitated using standard methods. Patients with AS were excluded if their calculated aortic valve area (AVA) was > 1.4 cm², consistent with mild AS.

5.2.3 Ultrasound backscatter data analysis

Aortic valve backscatter values were obtained for all patients with normal aortic valves, using methods as described in Chapter 2. Patients with “normal” aortic valves were defined on the basis of no focal increase in aortic valve echogenicity or thickening with no restriction of motion, irrespective of other cardiac structural/functional anomalies, had normal aortic valve structure including normal aortic valve backscatter of < 16dB. Patients (n=4) with backscatter values ≥ 16dB but otherwise normal valves were excluded, given that previous data suggested that such valvues are consistent with visually normal aortic valve structure (Chapter 2).
5.2.4 Risk factors

All patients’ cardiovascular risk factors were determined by chart review. Hypertension was defined by treatment with antihypertensive drugs or blood pressure > 140/80 mmHg. Hypercholesterolaemia was defined by treatment with cholesterol-lowering drugs and/or a total cholesterol > 5.5 mmol/L. Diabetes mellitus was defined by treatment for diabetes or a fasting blood glucose > 7.8 mmol/L. Known coronary artery disease (CAD) in AS patients was defined on the basis of the presence of at least one of the following: > 50% luminal diameter narrowing of ≥ 1 epicardial coronary artery on angiography, history of coronary revascularization, history of myocardial infarction, regional left ventricular wall akinesia and/or dyskinesia on echocardiogram.

5.2.5 Biochemical measurements

Blood was collected from all patients into heparinised tubes, centrifuged at 4°C at 2700 x g for 20 minutes, and plasma was stored at -80°C until assay. Concentrations of ADMA and L-arginine in plasma were measured by high-performance liquid chromatography (HPLC) using the derivatisation reagent AccQ-Fluor after solid phase extraction as previously described (Heresztyn et al 2004). The recovery rate for ADMA was 92 ± 2%, and the detection limit of the assay was 0.1µM. Concentrations of total cholesterol, and serum creatinine was measured by standard laboratory assays. Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation. High sensitivity CRP
(hs-CRP) was determined by the Beckman Coulter High Sensitivity C-reactive protein kit measured by the IMMAGE® Immunochemistry system.

5.2.6 Hypotheses and statistical methods:

It was recognized that impairment of renal function was likely to be associated with elevation of ADMA levels (Vallance et al 1992). However, the primary hypothesis to be tested in this study was that AS (as distinct from normal valve structure) was independently associated with elevation of ADMA levels. Therefore the primary method of analysis utilized for hypothesis testing was backward stepwise multiple linear regression analysis, utilizing ADMA as the dependent variable. Independent variables utilized in the final model were: presence/absence of AS, creatinine clearance (CrCL), known IHD, previous or current hypercholesterolaemia, statin therapy, diabetes mellitus (DM), atrial fibrillation (AF), systemic hypertension (HT), and left ventricular ejection fraction (EF) with p>0.1 as model exit criteria, using SPSS 12 software. Fisher’s exact test was used to evaluate differences in patients’ baseline characteristics if the variables were categorical, and Mann-Whitney test was used if the variables were continuous. Plasma ADMA concentrations were log-transformed for linear regressions and for the multiple linear regression. Univariate comparisons of data sets were performed using SPSS 12 software. Data are expressed as mean ± SD unless otherwise stated; the level of statistical significance was set at p<0.05.
5.3 Results:

5.3.1 Characteristics of the study group:

Clinical characteristics of patients are listed in Table 5.1. As planned, there was no difference in age between the AS and non-AS group (Figure 5.1). The mean transaortic pressure gradients and AVA for AS patients were $68.7 \pm 27.4\, \text{mmHg}$, and $0.81 \pm 0.23\, \text{cm}^2$ respectively. The mean $AV_{BS}$ for the non-AS group was $10.5 \pm 2.8\, \text{dB}$ approximating to the previously described mean for normal values (Ngo et al 2003; Nightingale and Horowitz 2005). Patients with AS had a greater prevalence of known IHD, as well as increased IVSd. Other parameters associated with endothelial dysfunction such as history of HT, DM, hypercholesterolemia, AF, low EF, and reduced CrCL occurred at similar frequencies among the 2 groups. A total of 95.5% of the patients with hypercholesterolemia were receiving statin treatment.

5.3.2 Association of clinical characteristics with ADMA:

Figure 5.2 compares median plasma ADMA levels in the AS and non-AS group. Median plasma ADMA concentrations were marginally greater in the AS group ($0.59\, \mu\text{M}$ vs $0.55\, \mu\text{M}$), ($p=0.13$). There was also no significant difference in mean L-Arginine concentrations between the AS vs non AS group (Figure 5.3) ($77.6 \pm 18.2$ vs $76.6 \pm 23.7\, \mu\text{M}$, respectively, $p=0.8$). Within the AS group, there was no correlation between peak transaortic pressure gradients and log plasma ADMA concentrations (Figure 5.4A); the effect was similar with aortic valve area (Figure 5.4B). As expected, mean IVSd were
significantly higher in the AS group compared to the non-AS group (1.33 ± 0.23 vs 1.20 ± 0.16cm, respectively; p=0.004) (Figure 5.5).

The results of univariate analyses are summarized in Table 2. Univariate analysis showed no significant correlation between log ADMA concentrations and age (Figure 5.6). There was a significant inverse correlation between creatinine clearance (CrCL) and log ADMA concentrations (r²=0.054, p=0.03) (Figure 5.7). Interestingly, there was significantly higher median ADMA concentrations in patients with a history of atrial fibrillation (AF), compared to those without (p=0.02) (Figure 5.8). Patients with a history of ischemic heart disease did not have significantly higher ADMA concentrations compared to those who did not (p=0.23) (Figure 5.9). Although, patients with a history of hypercholesterolemia had a trend towards lower ADMA concentrations compared to those without hypercholesterolemia, this relationship was not statistically significant (p=0.13) (Figure 5.10A). Nonetheless, patients who were taking statin therapy had significantly lower ADMA concentrations compared to those who were not (p=0.03) (Figure 5.10B). Furthermore, patients with AS tended to have lower median plasma hs-CRP levels compared to those without AS, however, this difference was not statistically significant (Figure 5.11). In this study population, there was no significant correlation between log ADMA concentrations and hs-CRP (Figure 12).

In accordance with the hypothesis tested, the primary method of data analysis was via backward stepwise multiple linear regression, performed to determine whether the presence of AS was a predictor of elevated ADMA levels after adjustment for other cardiovascular risk factors. The results are summarized in Table 3. The presence of AS
was independently associated with elevated plasma ADMA concentrations. Other significant determinants of elevated ADMA concentrations were low creatinine clearance, and history of AF. In addition, the use of statin therapy was significantly associated with low ADMA levels.

5.4 Discussion

The presence of ASc has been recognized as a marker of greater propensity to cardiovascular risk than is apparent from conventional risk factors (Otto et al 1999). Pathological examinations of diseased aortic valves show areas of subendothelial thickening with disruption of basement membrane, accumulation of inflammatory infiltrates; presence of lipids, mineralization and calcium deposits have also been described. The presence of these lesions is consistent with a cascade of cellular changes that have been postulated to begin with endothelial disruption/dysfunction (O’Brien et al 1996; Otto et al 1994; Olsson et al 1996). To date, only the study of Poggianti et al (Poggianti et al 2003) has examined systemic endothelial function in patients with aortic valve disease. These investigators demonstrated impairment of flow-mediated dilatation within the brachial artery in patients with ASc, but did not measure ADMA or any other circulating markers of endothelial function. More recently, we have demonstrated impairment of valvular endothelial function in explanted valves of patients with advanced AS (Chirkov et al 2006), but these findings were not related to systemic endothelial function.
The current study was designed to test the hypothesis that the presence of AS is independently associated with elevation of plasma ADMA concentrations relative to the absence of any aortic valve disease. Evaluation of this hypothesis therefore was contingent upon a multivariate analysis method (in this case a stepwise multiple regression) which demonstrated this association to be present \( p=0.04 \). It is now appropriate to examine the reasons why this hypothesis was tested, the implications of this central finding, and the possible impact of secondary findings of this study.

The presence of ASc has been recognized as a marker of greater propensity to cardiovascular risk than apparent from conventional risk factors (Otto et al 1999). Pathological examinations of diseased aortic valves show areas of subendothelial thickening with disruption of basement membrane, accumulation of inflammatory infiltrates, presence of lipids, mineralization and calcium deposits have also been described. The presence of these lesions is consistent with a cascade of cellular changes that have been postulated to begin with endothelial disruption/dysfunction (Otto et al 1994).

In the current study, results of multiple regression analysis suggest that AS is associated independently with an elevation of ADMA concentrations (Table 5.2) of approximately 0.05\( \mu \text{mol/L} \), or 10\% above values in the absence of AS in a cohort of patients with multiple “conventional” risk factors for endothelial dysfunction (and ADMA elevation). The question arises as to whether such an elevation in ADMA concentrations is likely to be of biological/clinical importance. This issue has been addressed by two previous studies Schnabel et al (Schnabel et al 2006), examining a population with known
coronary artery disease, found an increase in ADMA concentrations of 0.21\(\mu\)mol/L was associated with an approximately 2.5 fold increase in cardiovascular mortality and infarction rates. More remarkably, Valkonen et al (2001) found, in a population of asymptomatic middle-aged men, that after logistic regression modeling there was a 27-fold increase in coronary risk associated with a 0.1\(\mu\)mol/L increase in ADMA.

Consistent with other studies, presence of renal dysfunction is associated with ADMA elevations. We showed a correlation between plasma ADMA concentrations and impaired creatinine clearance. The degree of renal dysfunction was the most significant predictor of ADMA elevations, not just the presence of end-stage renal failure. This possibly implies that endothelial dysfunction occurs gradually with worsening of renal function. This is in agreement with previous studies which showed that levels of ADMA are increased in patients with chronic renal failure (MacAllister et al 1996; Perkovic et al 2003; Palta et al 2000). There is also increased evidence that elevation of ADMA levels is predictive of cardiovascular and all-cause mortality in chronic renal failure patients (Zocalli et al 2001). Despite the strong association between elevated ADMA concentrations and presence of AS with chronic renal failure, the presence of AS was a significant predictor of elevated ADMA concentrations independent of creatinine clearance. This implies that the presence of AS is an incremental marker of endothelial dysfunction beyond known impairment of renal function.

The inverse association between statin therapy and ADMA concentrations was unexpected, as the majority of previously published studies suggest that statins do not lower ADMA levels (reviewed by Maas 2005). No patients were receiving rosuvastatin,
the only member of this class which has been reported to lower ADMA concentrations. Interestingly, a history of AF was also a significant predictor of elevated ADMA levels. However, AF has been linked to endothelial dysfunction (Conway et al 2004). Angiotensin converting enzyme inhibitors (ACEi) and angiotensin II (ATII) receptor antagonists have been shown to decrease the occurrence of AF (Vermes et al 2003; L’Allier et al 2004) possibly via improved atrial endocardial endothelial function. However, it must be emphasized that these findings are largely hypothesis-generating, as none of these were a part of the principal hypothesis to be tested.

5.4.1 Study limitations

The study is dependent on referral bias for echocardiogram. However, matching was good in general. The major difference was the frequency of known coronary disease. Furthermore, the study relates to well established AS. While it demonstrates an association with ADMA at that stage, the important question really relates to ADMA in early “sclerotic” disease. In view of the current results, a prospective evaluation in patients with ASc is now of great interest. Although the results of this study found an association between elevated ADMA levels and the presence of AS, we have yet to establish the cause of ADMA elevation in these patients. Specifically, it is unclear from this study whether the observed elevation of ADMA concentrations results primarily from incremental ADMA generation or from impaired clearance of ADMA.

In summary, the presence of AS is predictive of elevated ADMA concentrations independent of creatinine clearance, and other cardiovascular risk factors. Elevated
ADMA concentrations appear to contribute to endothelial dysfunction (Sydow and Munzel 2003), as well as recognized as a marker of cardiovascular and all-cause mortality risk (Valkonen et al 2001). Thus, the association of AS and elevated ADMA concentrations could possibly explain the increased cardiovascular events that occur in patients with AS and even ASc. It is also possible that elevation of ADMA concentrations is relevant to the pathogenesis of AS, largely via a decrease in protective role of endothelial NO against oxidative stress in the valve matrix. Figure 5.13 depicts the postulated pivotal role of ADMA in vascular endothelial and platelet dysfunction (hence in risk of coronary events) but also potentially in the pathogenesis of AS.
**Figure 5.1:** Mean age comparison between AS vs non AS group. The 2 groups were matched for age, and there was no statistical significance difference in mean age between the AS vs non AS group (mean: $73.1 \pm 7.7$ vs $72.7 \pm 6.6$ yrs, respectively).
Figure 5.2: Median ADMA concentration comparisons between the AS and non-AS group. ADMA concentrations for the AS group are slightly higher (0.59μM) but not statistically different from these in the non AS group (0.54μM), p=0.13.
Figure 5.3: Mean L-arginine concentration comparisons between the AS and non-AS group. There was no statistically significant difference for the AS group vs non-AS group (77.6 ± 18.2 vs 76.6 ± 23.7 μM, respectively).
Figure 5.4: Correlations between log plasma ADMA concentrations and (A) peak pressure gradients ($r^2 < 0.001$, $p=0.87$) and (B) AVA in the AS group ($r^2=0.02$, $p=0.34$).
Figure 5.5: Comparison of interventricular septal dimensions (IVSd) between the AS vs non-AS group. Patients in the AS group had significantly higher IVSd compared to the non-AS group (1.33 ± 0.23 vs 1.20 ± 0.16cm, respectively; p=0.004).
Figure 5.6: Correlation between log plasma ADMA concentrations and age. Despite a weak trend between log plasma ADMA concentrations and age, the correlation was not statistically significant ($r^2=0.01$, $p=0.28$).
**Figure 5.7:** Correlation between log plasma ADMA concentrations and creatinine clearance (CrCL). There was a significant inverse correlation between log plasma ADMA concentrations and CrCL ($r^2=0.06$, $p=0.03$)
Figure 5.8: Plasma ADMA concentrations in patients with and without a history of atrial fibrillation (AF). Median values are: 0.62µM vs. 0.54µM, respectively; p=0.02.
Figure 5.9: Comparison of plasma ADMA concentrations between patients with IHD vs no IHD. There was no significant difference in median plasma ADMA concentrations between these patients with IHD vs no IHD (0.57 μM vs 0.54 μM, respectively, p=0.24).
**Figure 5.10:** Median plasma ADMA concentrations for (A) cohorts of patients with history of hypercholesterolaemia (HC) and without (non HC) (0.53 μM vs 0.58 μM, respectively; p=0.08), and (B) patients receiving vs. not receiving statin therapy, (0.52 μM vs 0.58 μM, respectively; p=0.02)
**Figure 5.11:** Median plasma high-sensitivity CRP (hs-CRP) concentration comparison for patients with AS vs non-AS. Patients in the non-AS group had slightly higher hs-CRP concentrations vs those in the AS group, however this difference was not statistically significant (4.6 mmol/L vs. 4.0 mmol/L, respectively; p=0.3).
**Figure 5.12**: Correlations between log ADMA concentrations and log hs-CRP concentrations. There was no significant relationship between ADMA concentrations and the systemic inflammatory marker hs-CRP ($r^2=0.004; p=0.6$).
**Table 5.1:** Clinical characteristic comparisons between AS and non-AS group (n=42 for both groups).

<table>
<thead>
<tr>
<th></th>
<th>AS group</th>
<th>non-AS group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>73.1±7.7</td>
<td>72.7±6.7</td>
</tr>
<tr>
<td>Presence of known IHD</td>
<td>28 (67%)</td>
<td>13 (31%) **</td>
</tr>
<tr>
<td>HT</td>
<td>28 (67%)</td>
<td>26 (62%)</td>
</tr>
<tr>
<td>DM</td>
<td>17 (40%)</td>
<td>10 (24%)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>27 (64%)</td>
<td>22 (52%)</td>
</tr>
<tr>
<td>AF</td>
<td>7 (17%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>CrCL (ml/min)</td>
<td>43.6±18.7</td>
<td>39.4±17.4</td>
</tr>
<tr>
<td>EF</td>
<td>63.06±15.3</td>
<td>61.22±12.8</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>1.33</td>
<td>1.2 **</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>20 (48%)</td>
<td>22 (52%)</td>
</tr>
<tr>
<td>ACE/AngII antagonist</td>
<td>26 (62%)</td>
<td>24 (57%)</td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>4.0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

IHD=Ischemic heart disease, HT=hypertension, DM=diabetes mellitus, AF=atrial fibrillation, CrCL=creatinine clearance, EF=ejection fraction, IVSd=interventricular septal dimension, ACE/AngII antagonist= Angiotensin converting enzyme/Angiotensin II receptor antagonists.

**p<0.005 vs. AS group**
**Table 5.2:** Univariate analyses: relationship between plasma ADMA levels and clinical characteristics. Linear regressions were performed between log-plasma ADMA levels and continuous variables such as age, CrCL, hs-CRP and EF. Mann-Whitney tests were used to compare median plasma ADMA levels for all other categorical variables.

<table>
<thead>
<tr>
<th></th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.29</td>
</tr>
<tr>
<td>Presence of AS</td>
<td>0.13</td>
</tr>
<tr>
<td>Presence of known IHD</td>
<td>0.24</td>
</tr>
<tr>
<td>HT</td>
<td>0.99</td>
</tr>
<tr>
<td>DM</td>
<td>0.81</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>0.08</td>
</tr>
<tr>
<td>AF</td>
<td>0.02*</td>
</tr>
<tr>
<td>CrCL (ml/min)</td>
<td>0.03*</td>
</tr>
<tr>
<td>EF</td>
<td>0.37</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>0.02*</td>
</tr>
<tr>
<td>ACE/AngII antagonist</td>
<td>0.33</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>0.60</td>
</tr>
</tbody>
</table>

AS = Aortic stenosis; IHD = ischemic heart disease; HT = hypertension; DM = diabetes mellitus; AF = atrial fibrillation; CrCL = Creatinine clearance; EF = ejection fraction; ACE/AngII antagonist = Angiotensin converting enzyme/ Angiotensin II receptor antagonist; hs-CRP = high-sensitivity C-reactive protein.
Table 5.3: Variables independently associated with ADMA elevation after backward stepwise multiple regression, with p>0.1 as model exit criteria.

<table>
<thead>
<tr>
<th></th>
<th>β coefficient</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of AS</td>
<td>0.21</td>
<td>0.001, 0.072</td>
<td>0.04</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>-0.26</td>
<td>-0.081, -0.011</td>
<td>0.01</td>
</tr>
<tr>
<td>CrCL</td>
<td>-0.26</td>
<td>-0.002, 0.000</td>
<td>0.01</td>
</tr>
<tr>
<td>AF</td>
<td>0.27</td>
<td>0.015, 0.100</td>
<td>0.009</td>
</tr>
</tbody>
</table>

AS = Aortic stenosis; CrCL = Creatinine clearance; AF = Atrial fibrillation
Figure 5.13: Schematic representation of postulated interaction of ADMA with function of vascular and valvular endothelium and of platelets with particular reference to the pathogenesis of aortic stenosis.
CHAPTER 6

Relationship between aortic valve sclerosis, endothelial function and platelet responsiveness to nitric oxide
6.1 Background

As mentioned in the introductory chapter 1, AS affects approximately 2% of adults over the age of 65 years (Lindroos et al 1993, Stewart et al 1997). Its earlier stage, aortic sclerosis (ASc) which reflects initial disorganization of the aortic valve result without evident hemodynamic changes is even more common, affecting approximately 30% of adults over the age of 65 years (Lindroos et al 1993).

The pathogenesis of ASc, like that of advanced AS, is subject to current debate, but is thought to reflect an inflammatory process rather than purely “wear and tear”. Furthermore, at least two studies (Otto et al 1999, Aronow et al 1999) have demonstrated that ASc is an independent, incremental marker of risk of cardiac events and cardiovascular mortality. This suggests that the pathogenesis of ASc exhibits some commonality with that of acute coronary syndromes, rather than merely atherogenesis.

Previous investigations have established that a number of “conventional” coronary risk factors, as well as chronic renal failure, are associated with incremental risk of the development/progression of AS (as discussed in section 1.5). However, the studies of Otto et al (1999) and Aronow et al (1999) suggested that there is a closer nexus between ASc and risk of acute coronary events than that which would be implicit from “conventional” coronary risk.

*The question therefore arises: what is/are the biochemical mechanism(s) underlying the relationship between ASc and risk of acute coronary events?*
No previous investigations of the relationship between ASc and coronary risk have taken into account any of the recently identified markers which extend prediction of coronary events beyond Framingham risk scores. Such markers include those of inflammatory activation (eg hs-CRP, myeloperoxidase, and interleukin-6), of endothelial dysfunction (eg ADMA, von Willebrand factor) and of pro-coagulant activity (platelet aggregability, plasma fibrinogen levels). It is possible that the biochemical/physiological nexus between ASc and coronary thrombosis may be explained by one or more of these factors.

In particular, it is theoretically possible that the combination of endothelial dysfunction (ie of both valve and vasculature) and redox stress may contribute to cardiovascular risk in ASc. As regards endothelial dysfunction, it has been recently shown that calcific nodule formation in cell culture of aortic valve fibroblasts is inhibited by NO (Kennedy et al 2006). Furthermore, in chapters 3 and 4, addition of vitamin D₂ caused AS in rabbits; and extent of AS was significantly associated with elevation of ADMA concentrations. The addition of ramipril (chapter 4) significantly reduced plasma ADMA concentrations, which correlated strongly with AVBS scores. Clinically, we have also shown that advanced AS is associated with elevation of plasma ADMA concentrations (Chapter 5) (Ngo et al 2007). Therefore, it is probable that endothelial dysfunction develops at some stage in the clinical course of AS, and that NO is important for suppression of valve calcification.

Although originally, endothelial dysfunction was defined on the basis of impairment of release of NO-like materials from the endothelium (Moncada and Higgs 1993), it is now
clear that in many circumstances there is associated impairment of tissue responsiveness to NO. This has been described extensively in the vasculature (Schachinger et al 2000, Adams et al 1998) and also in platelets (Chirkov et al 1999). In both coronary arteries and platelets, this phenomenon of NO resistance is an independent marker of coronary event risk (Schachinger et al 2000, Willoughby et al 2005). While the basis for NO resistance remains uncertain in the vasculature, the major underlying mechanism in platelets is incremental oxidative stress, with “scavenging” of NO by O$_2^-$ anion (Chirkov et al 1999). If NO resistance were associated with ASc, this would represent a potential link to coronary risk.

Indeed, the development of AS induced by high dose vitamin D$_2$ (see chapters 3 and 4) may also reflect, at least in part, increased oxidative stress and decreased NO release and/or effect. Vitamin D$_2$ stimulates release of vitamin D-upregulated protein (VDUP1; also known as thioredoxin-interacting protein (Txnip); or thioredoxin-binding protein 2 (TBP2), a physiological antagonist of thioredoxin, which therefore increases intracellular redox stress (Junn et al 2000, Schulze et al 2006, Schulze et al 2002). Increases in redox stress are frequently associated with endothelial dysfunction, as indeed was demonstrated in the current model.

*It must be emphasized that the importance of ASc is dual: as the earliest stage of AS (and therefore as a marker of AS risk) and as a vascular “warning sign”. The biochemical/physiological factors underlying ASc progression may be completely different from those mediating thrombotic risk.*
In order to facilitate population-based studies of both implications and progression of ASc, it is desirable that an objective, quantitative measure of ASc severity be available. Visual scoring of aortic valve thickening and immobility has been commonly employed in these studies (Shiveli et al 1998, Cosmi et al 2002), with limited information on reproducibility and accuracy to detect serial changes of the aortic valves. Recently, the emergence of techniques utilizing changes in ultrasonic backscatter to detect and characterize pathological changes in tissues has been used to quantitate sclerotic aortic valves (Ngo et al 2004). In chapter 2, it has been shown that patients with sclerotic aortic valves have significantly higher aortic valve backscatter scores ($AV_{BS}$) compared to normal valves from healthy subjects. Patients with $AV_{BS}$ scores $\geq$ 16dB were consistent with ASc.

The purpose of the experiments described in the current study was to identify novel biochemical/physiological correlates of the presence of ASc in an ageing component of the general population, utilizing quantitation of aortic valve backscatter. A number of specific hypotheses were to be tested within the structure of this population study. It was proposed that beyond “traditional” coronary risk factors and renal dysfunction, presence of ASc was associated with:

1) **Markers of endothelial dysfunction/NO resistance:** these included plasma ADMA concentrations, augmentation index (AIx: a marker of vascular stiffness/wave reflection) and platelet responsiveness to NO donors.

2) **Activation of inflammation:** plasma concentrations of hs-CRP were utilized for this purpose.
3) Elevated plasma vitamin D levels

Because it was recognized that the pathogenesis of ASc was likely to be multifactorial, the study utilized multiple regression analysis of a population cohort in whom a moderate prevalence of ASc was anticipated.

6.2 Methods

6.2.1 Study subjects

The study population consisted of 255 consecutive, randomly selected subjects from the North Western Adelaide Health area of South Australia aged from 51-77 years (mean 63.4 ± 6 years). Of 255 patients recruited, 2 patients were subsequently excluded due to presence of terminal cancer (n=1), and dextrocardia on echocardiography (n=1). All patients received informed consent prior to study. The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital.

6.2.2 Doppler echocardiography

Complete TTE studies were performed in all patients, by use of a commercially available system (Vivid 5, GE Vingmed, Horten, Norway with a 2.5 MHz phased array probe). M-mode and two-dimensional (2D) echocardiograms with Doppler analysis were obtained for all patients. Left ventricular diameters and wall thicknesses were measured from 2D-guided M-mode echocardiography. Mean and peak transaortic pressure gradients were calculated with the modified Bernoulli equation, using continuous-wave Doppler
recordings from the highest velocity available from any view. The aortic valve area was computed with the continuity equation using standard methods.

6.2.3 Ultrasound backscatter data analysis

Aortic valve backscatter values were obtained for all patients with normal aortic valves, using methods as previously published. Briefly, two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal long-axis views over 3 cardiac cycles with a zoom of 8 cm. Three consecutive scans were done for each study subject. Backscatter values from the blood pool in the left ventricular outflow tract and aortic root were used as reference values. Calibrated backscatter values were obtained by subtracting the average blood pool value from the averaged backscatter values obtained from the aortic valves.

6.2.4 Risk factors

All patients’ cardiovascular risk factors were determined by interview. Hypertension was defined by treatment with antihypertensive drugs or blood pressure > 140/80 mmHg. Hypercholesterolaemia was defined by current treatment with cholesterol-lowering drugs and/or a total cholesterol > 5.5 mmol/L. Diabetes mellitus was defined by current treatment for diabetes and/or a fasting blood glucose > 7.8 mmol/L. Known coronary artery disease (CAD) in AS patients was defined on the basis of the presence of at least one of the following: > 50% luminal diameter narrowing of ≥ 1 epicardial coronary artery on angiography, history of coronary revascularization, history of myocardial infarction,
regional left ventricular wall akinesia and/or dyskinesia on echocardiogram. Furthermore, patients with exertional angina pectoris without AS were categorized as having CAD irrespective of performance of coronary angiography.

6.2.5 Biochemical measurements

Blood was collected from all patients into heparinised tubes, centrifuged at 4°C at 2700 x g for 20 minutes, and plasma was stored at -80°C until assay. Concentrations of ADMA in plasma were measured by high-performance liquid chromatography (HPLC) using the derivatisation reagent AccQ-Fluor after solid phase extraction as previously described (Heretzyn et al 2004). The recovery rate for ADMA was 92 ± 2%, and the detection limit of the assay was 0.1µM. Lipid profile, hs-CRP, serum creatinine, calcium, phosphate, and 1,25 dihydroxy cholecalciferol (vitamin D levels) were measured by a 125I radioimmunoassay (RIA) (Immunodiagnostic Systems Ltd, Bolden UK). Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation, and indexed for body surface area using the Dubois and Dubois formula.

6.2.6 Platelet aggregation

Blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate); acidified citrate was used to minimize deterioration of platelet function during experiments. Platelet aggregation in whole blood was examined using a dual-channel impedance aggregometer (model 560, Chrono-Log, Haverstown, Pennsylvania) as previously described (Chirkov et
al 2002). In brief, aggregation was induced with adenosine 5’-diphosphate (ADP) at final concentration of 2.5 μmol/L. Aggregation was monitored continually for 7 minutes, and responses were recorded for electrical impedance (Ohms). In control tests, physiologic saline was added in appropriate volumes. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of SNP (10μmol/L).

6.2.7 Measurement of augmentation index (AIx)

Pulse waveform analysis (PWA) was performed non-invasively with a commercially available SphygmoCor system (AtCor Medical, Sydney, Australia), as previously described (Wilkinson et al 1998). All subjects were asked to lie down in a quiet room for 15 minutes prior to procedure. Briefly, PWA was computed from the radial artery at the wrist, and recorded by applanation tonometry using a high fidelity micromanometer. Three recordings of 10 sequential waveforms were acquired for each subject; a validated, generalized transfer function was used to generate the corresponding central aortic pressure waveform; which augmentation index (AIx) was derived. Only high quality recordings with in-device quality index ≥ 90% were used. All augmentation indices were corrected for a standard heart rate of 75 bpm.

6.2.8 Statistical analyses

All data are expressed as mean ± standard deviation (SD) unless otherwise stated. Normal distribution was tested for all continuous variables, and skewed data were normalized
either by log or square root transformation. Comparisons between groups for non-parametric data were made using the Mann-Whitney test. Correlations between transformed, continuous non-parametric data were made using linear regression. Stepwise multiple linear regression analyses were performed to assess independent predictors of high AV<sub>BS</sub> scores. Variables included to predict high AV<sub>BS</sub> scores were: age, gender, smoking history, previous ischemic events/angina, diabetes mellitus, hypercholesterolemia, hypertension, calculated CrCL, Ca<sub>4</sub>PO<sub>4</sub> levels, vitamin D levels, BMI, hs-CRP, AIx, ADMA concentrations, and % platelet responses to SNP. All analyses were done using SPSS 13 software, and a p value of < 0.05 was considered to be statistically significant.

6.3 Results

6.3.1 Subject characteristics:

Table 6.1 shows baseline characteristics of the population examined. Mean age for study subjects was 63.4 ± 6 years. There was a high proportion (30.8%) of obese subjects (BMI > 30 kg/m<sup>2</sup>). Multiple (≥ 3) coronary risk factors were present in 32% of subjects, and approximately one third of all subjects were receiving treatment with statins and/or ACEi/Ang II antagonists respectively. Only a very small minority of subjects were receiving vitamin D supplementations.

Routine biochemical findings are summarized in Table 6.2. Plasma cholesterol concentrations were elevated beyond normal (> 5.5 mmol/L) in 26.4% of subjects at
entry. In general, renal function was well preserved: there was no patient on dialysis with only 2 subjects with CrCL < 30 mL/min/1.73m².

Table 6.3 shows echocardiographic parameters. Based on visual assessment criteria (Otto et al 1999), 25.4% of subjects were categorized as ASc. Such subjects had significantly higher AV$_{BS}$ scores than those without visual assessment criteria for ASc (p<0.001) (Figure 6.1). There were 19.4% of subjects with AV$_{BS}$ scores ≥ 16dB, the previously described mean value for AV$_{BS}$ in subjects with ASc (Ngo et al 2004). Approximately 31% of subjects had evidence of left ventricular hypertrophy (LV mass index ≥ 120g/m²) (Levy et al 1987).

Of the various investigations performed specifically related to hypotheses assessed in this study:

1) Vitamin D levels were generally towards the lower end of the reference normal range for the laboratory assay (normal range 50-160 pmol/L) (Nordin et al 2004)

2) Augmentation index (AIX) was consistent with moderate elevation of arterial stiffness/wave reflection (McEniery et al 2006).

3) Mean platelet anti-aggregatory response to SNP was relatively low, consistent with some degree of platelet resistance to NO (Chirkov et al 2001).

4) Mean plasma ADMA concentrations were within the previously described normal range for the methodology used (Heresztyn et al 2004, reviewed by Horowitz and Heresztyn 2006).
6.3.2 Correlations between univariate parameters: Correlates of univariates with AV$_{BS}$ scores.

Univariate analyses between AV$_{BS}$ scores and continuous variables are documented in Table 6.4. There was a significant correlation between AV$_{BS}$ scores and age ($p=0.005$) (Figure 6.2). While total cholesterol, and LDL levels did not correlate with AV$_{BS}$ scores, high HDL levels were significantly associated with high AV$_{BS}$ scores ($\beta=0.15$, $p=0.016$). Biochemistry results showed a significant inverse correlation between calculated CrCL (corrected for BSA) and AV$_{BS}$ scores ($\beta=-0.2$, $p=0.001$) (Figure 6.3). While there was no correlation between AV$_{BS}$ vs calcium levels, there were borderline correlations between AV$_{BS}$ vs phosphate, and Ca$_4$PO$_4$ levels (Figure 6.4). Importantly, there was no significant relationship between vitamin D levels and AV$_{BS}$ scores (Figure 6.5).

Low BMI was strongly inversely correlated with AV$_{BS}$ scores ($\beta=-0.25$, $p<0.001$) (Figure 6.6). There was also an inverse correlation between AV$_{BS}$ scores and hs-CRP levels ($\beta=-0.14$, $p=0.03$) (Figure 6.7). As regards parameters of endothelial function/NO response in this study population, there was no significant correlation between either AIx or plasma ADMA concentrations and AV$_{BS}$ scores (Figure 6.8A, 6.8B). However, there was a significant inverse correlation between platelet responsiveness to SNP and AV$_{BS}$ scores (Figure 6.9).

Univariate comparisons of AV$_{BS}$ scores for the categorical variables are documented in Table 6.5. AV$_{BS}$ scores were not significantly higher in subjects with any “traditional” cardiovascular risk factors. None of the “traditional” major coronary risk factors were
significant correlates of high $AV_{BS}$ and nor did treatment with either statins or ACE inhibitors apparently interact with $AV_{BS}$.

### 6.3.3 Interactions between univariate parameters.

In summary, examination of the univariate parameters yielded a number of unexpected results, for example:

1) The absence of correlation between $AV_{BS}$ and the major coronary risk factors (eg smoking, hypertension, hypercholesterolemia, diabetes, family history).

2) The strongly negative univariate correlation between BMI and $AV_{BS}$, superficially suggests that obesity is “protective” against development of ASc.

3) The absence of correlation between plasma ADMA concentrations and AIx, both parameters related to endothelial function, and $AV_{BS}$ (despite the observed correlation with SNP response).

4) The absence of correlation between plasma vitamin D concentrations and $AV_{BS}$.

A number of exploratory cross-correlation studies were therefore undertaken, in order to determine whether the various univariate parameters might be interdependent. The following results were of potential significance:

1) Vitamin D levels:
a) There was a strong ($\beta=-0.23, p=0.001$) inverse correlation between vitamin D levels and plasma ADMA concentrations (Figure 6.11A).

b) Furthermore, a similar negative correlation was found with hs-CRP levels ($\beta=-0.17, p=0.009$) (Figure 6.11B).

c) There was a strong inverse correlation between vitamin D levels and BMI ($\beta=-0.15, p=0.02$) (Figure 6.12).

d) Hypertension was associated with low vitamin D levels ($p=0.008$) (Figure 6.13).

2) BMI correlations:

a) There was a very strong positive correlation between BMI and creatinine clearance indexed for body surface area (Figure 6.14A). The strong relationship between BMI and creatinine clearance persisted upon calculation of creatinine clearance using the MRDR equation, where body weight is not a part of the equation (Figure 6.14B) ($p=0.001$). These data therefore suggest strongly that even after indexing for body surface area, creatinine clearance calculation is not a reliable estimation for obesed subjects.

b) This finding was recognized as having important implications in the multiple regression analyses.

The implications of these cross-correlations are discussed below; nevertheless these data demonstrated the interdependence of a number of parameters utilized in the multiple linear regression analyses.
### 6.3.4 Stepwise multiple linear regression analyses

Table 6.6 documents results of a stepwise multiple linear regression analysis. Independent variables predictive of high $AV_{BS}$ scores were: BMI ($\beta=-0.23$, $p=0.001$); advanced age ($\beta=0.2$, $p=0.004$); and platelet anti-aggregatory responsiveness to SNP ($\beta=-0.16$, $p=0.02$).

### 6.4 Discussion

This is the first population study to examine biochemical/physiological correlates of ASc. Among 253 ageing subjects randomly selected from the community, factors associated with increased aortic valve echogenicity as quantitated by $AV_{BS}$ scores were: advanced age, low BMI, and impaired tissue responsiveness to NO, at the level of platelet aggregation.

These multiple regression predictors of high $AV_{BS}$ were also seen in univariate comparisons. This is a very important result, in that it is statistically robust, and suggests that, despite the overlapping histopathological characteristics of early atheromatous plaques and sclerotic aortic valves, their occurrence reflects considerably different underlying mechanisms. One possible explanation for these findings would be that the various cardiovascular disease states in this subject population were extensively treated; however, this was not necessarily the case, with many subjects being on no treatment for known hypertension, and with frequently incomplete treatment of hypercholesterolemia.
In particular, these results are important because they raise the question of whether hypercholesterolemia is a primary precipitant of the development of AS, despite numerous previous studies focusing on this “lipidogenic” hypothesis (Liebe et al 2005). These negative findings, of course, consistent with the results of the animal study by Drolet et al (2003) and the results outlined in Chapters 3 and 4. Nevertheless, it remains possible that hypercholesterolemia might be a risk factor for rapid progression from ASe to AS: follow-up of the subject cohort would be desirable to address this issue.

The finding on univariate analyses that HDL concentrations were inversely related to AV_{BS} was also unexpected. However, recent clinical data utilizing torcetrapib (Nissen et al 2007), an agent which markedly increases HDL levels, have cast doubt upon previous views that increases in HDL uniformly lead to anti-atherogenic effects; the current finding therefore merits further examination.

It is generally accepted that severe renal impairment and hemodialysis are strong risk factors for development, and progression of AS (reviewed by London et al 2000). Abnormal Ca\textsubscript{3}PO\textsubscript{4} products have also been previously documented to be associated with higher incidence of AS (Mills et al 2004), even in subjects with normal renal function. In this study, neither of these parameters remained significant after a stepwise multiple linear regression analysis. It is possible that the negative correlation between creatinine clearance and age ($\beta=-0.44$, $p<0.001$); and creatinine clearance and Ca\textsubscript{3}PO\textsubscript{4} ($\beta=-0.14$, $p=0.03$) (data not shown); as well as creatinine clearance and BMI ($\beta=0.53$, $p<0.001$) partially obscured the relationship between creatinine clearance and AV\textsubscript{BS} scores upon multiple linear regression. However, it is more likely that the main problem in this regard
is that the chosen measure of renal function did not adequately correct for obesity, as seen from Figure 6.14. The relationship between creatinine clearance and BMI was not corrected even if creatinine clearance was calculated using the MDRD equation (Modification of Diet in Renal Disease) ($\beta=0.21$, $p=0.001$, Figure 6.14B). Thus, the identification of obese patients with impaired renal function was impeded by current methods of “corrected” creatinine clearance calculations, as suggested by Verhave et al (2005).

These findings have the implication that subjects characterized in the current model with “low BMI” should be regarded effectively as having “low BMI/under-estimated renal function”. The importance of low BMI in the final multivariate model is thus overstated, and that of renal dysfunction understated. This unintended interaction between the parameters occurred despite the use of a conventional method for “correcting” renal function for body surface area.

The question therefore arises: what was the actual importance of renal dysfunction and low BMI as determinants of presence of ASc? As regards renal dysfunction, one possible way of addressing the data would be to replace the continuous variable of “BSA-corrected creatinine clearance” with categorical impairment of creatinine clearance in the multiple regression model. If this was done, utilizing $< 60\text{ml/min/1.73m}^2$ as the cut-off for “impairment”, the univariate relationship between low creatinine clearance and high $AV_{BS}$ scores was no longer statistically significant ($p=0.26$); and the variables that remained after the multiple regression analysis were still advanced age, low BMI, and impaired platelet responsiveness to SNP.
As regards low BMI, it might be suggested that this result was an artifact of the process of determination of $A_{\text{VBS}}$ scores, via selective attenuation of signal. However, as shown earlier (Figure 2.7, Chapter 2), there was no correlation between $A_{\text{VBS}}$ and BMI in health volunteers with normal aortic valve on visual assessment.

If BMI is indeed inversely related to $A_{\text{VBS}}$, beyond the “artefactual” effect of renal dysfunction as described above, the question arises: what are the mechanisms underlying this association? Interestingly, Lindroos et al (1994) also observed that low BMI appeared to predispose to developmental of AS, without demonstrating any mechanism. It is possible that various adipokines now identified as originating primarily from adipocytes may have modulating effects on inflammatory change in aortic valve interstitium: this possibility needs to be examined prospectively in cell culture models. Importantly, the current results separate out “cause vs effect”: it cannot be argued that ASc induces weight loss/cachexia, a possibility that might have been valid for the more advanced cases of AS described by Lindroos et al (1994).

While it has been previously documented that high vitamin D levels were associated with AS in subjects with renal impairment (Malergue et al 1998. Urena et al 1999), vitamin D levels were not significantly associated with increased valvular echogenicity in this study population. Despite the fact that in chapter 3 and 4 it was successfully shown that high dose vitamin D supplementation induced development of AS; vitamin D levels are not correlates of ASc/AS development in this population. This could be explained by several reasons: 1) it is the combination of high dose vitamin D inducing constant elevation of
Ca₃PO₄ products, and renal impairment that induced AS in rabbits (eventhough there was no correlation between vitamin D levels and creatinine clearance in this population study (p=0.4, data not shown); 2) these study subjects had predominantly low-normal vitamin D levels, and thus are not at risk of inducing hypercalcemia; 3) vitamin D levels are not predictive of development of ASc/AS, but the effects of vitamin D may play a more important role. Patients with Paget’s disease have been documented to have increased risk of AS development (Strickberger et a 1987). It is known that Paget’s disease is associated with normal vitamin D levels, but abnormal vitamin D effects (reviewed by Reddy 2004, Kurihara et al 2004). Furthermore, vitamin D is a negative regulator of renin synthesis, and has been suggested to lower blood pressure (Li YC et al 2004). This effect of vitamin D was also seen with this study population (Figure 6.13). Additionally, vitamin D upregulates vitamin-D upregulated protein 1 (VDUP1), which is an endogenous inhibitor of the antioxidant thioredoxin (Junn et al 2000). It is thus difficult to determine if vitamin D levels precisely reflect vitamin D effects.

Furthermore, evaluation of the interaction between plasma vitamin D levels and prevalence of ASc in this population is complicated by a totally unexpected finding: there was a strong inverse correlation between vitamin D levels and plasma ADMA concentrations, ie a high vitamin D levels corresponded (p=0.001) to a low ADMA concentration. This interaction might have led to obscuring of any relationship between either of these parameters alone and AVBS score. There is no current literature to suggest that vitamin D metabolism is NO-dependent, but on the other hand, some of the effects of vitamin D, such as those on local regulation of bone remodeling, do indeed appear to be NO-dependent (Riancho et al 1995).
A critically important aspect of the current experiments concerns the markers of NO formation (ADMA), effect of (AIx) and impaired platelet responsiveness to SNP. Of these, only impaired platelet responsiveness to SNP was significantly elevated AV_{BS} scores. This finding is of obviously great interest, providing further mechanistic insight into the association between ASc and risk of cardiac events: as shown by Willoughby et al (2005), platelet NO resistance is an independent prognostic marker, which it now can be said appears early in the course of AS. However, AIx is also related, at least in part, to endothelial function (Wilkinson et al 1998). Since neither ADMA nor AIx were clearly related to ASc in this population, it is likely that any marked degree of endothelial dysfunction appears relatively later in the course of the disease. Indeed, in the ramipril experiments outlined in Chapter 4, there was a more marked differential in vascular NO responsiveness (to SNP) than in markers of NO release (acetylcholine response). The precise contribution of activation of O_2^- release to these findings and the potential for accentuated O_2^- release differentially affecting platelet function (as distinct from vascular function on DDAH, the redox-sensitive clearance mechanism for ADMA) (MacAllister et al 1996). Furthermore, follow-up of these subjects may serve to demonstrate whether ADMA is increasingly important in this population as the disease progresses (Chapter 5).

The final correlate of ASc in this population remained increasing age. Clearly the implication of this finding was that study design had not fully accounted for biochemical events associated with ageing. It is possible that this finding, which recalls the “wear and tear” hypothesis of AS, should above all serve to remind researchers how much remains to be learnt about the pathogenesis of this condition.
Table 6.1: Patient characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (yrs)</td>
<td>63.4 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.2 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>110/253 43.5</td>
<td></td>
</tr>
<tr>
<td>History of hypercholesterolemia</td>
<td>148/252* 58.7</td>
<td></td>
</tr>
<tr>
<td>Statin therapy</td>
<td>81/251* 32.1</td>
<td></td>
</tr>
<tr>
<td>Previous angina/MI</td>
<td>34/252* 13.5</td>
<td></td>
</tr>
<tr>
<td>ACE/Ang II therapy</td>
<td>83/251* 33.1</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>105/251* 41.8</td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td>131/252* 52.2</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>36/252* 14.3</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>28/251* 11.2</td>
<td></td>
</tr>
<tr>
<td>Subjects with &gt; 2 cardiovascular risk factors</td>
<td>80/253 31.7</td>
<td></td>
</tr>
<tr>
<td>History of CVA</td>
<td>9/251* 3.6</td>
<td></td>
</tr>
<tr>
<td>Calcium supplementation</td>
<td>42/251* 16.7</td>
<td></td>
</tr>
<tr>
<td>Vitamin D supplementation</td>
<td>4/250* 1.6</td>
<td></td>
</tr>
</tbody>
</table>

* Denominators supplied in cases of uncertain data.
Table 6.2: Patient characteristics: baseline characteristics/biochemistry data

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>Skewness</th>
<th>Std error of skewness</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>250</td>
<td>4.96</td>
<td>5.0</td>
<td>1.0</td>
<td>0.27</td>
<td>0.15</td>
<td>2.4</td>
<td>8.9</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>248</td>
<td>2.9</td>
<td>2.9</td>
<td>0.9</td>
<td>0.14</td>
<td>0.16</td>
<td>0.5</td>
<td>5.5</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>250</td>
<td>1.3</td>
<td>1.2</td>
<td>0.3</td>
<td>0.79</td>
<td>0.15</td>
<td>0.6</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Other biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium levels</td>
<td>252</td>
<td>2.24</td>
<td>2.26</td>
<td>0.15</td>
<td>-0.23</td>
<td>0.15</td>
<td>1.61</td>
<td>2.75</td>
</tr>
<tr>
<td>Phosphate levels</td>
<td>251</td>
<td>1</td>
<td>0.99</td>
<td>0.17</td>
<td>0.26</td>
<td>0.15</td>
<td>0.52</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca₃PO₄</td>
<td>251</td>
<td>2.3</td>
<td>2.2</td>
<td>0.5</td>
<td>0.23</td>
<td>0.15</td>
<td>1.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Vitamin D (mmol/L)</td>
<td>249</td>
<td>72.2</td>
<td>70.0</td>
<td>24.3</td>
<td>0.62</td>
<td>0.15</td>
<td>22</td>
<td>159</td>
</tr>
<tr>
<td><strong>Creatinine clearance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrCL (indexed for BSA)</td>
<td>246</td>
<td>90.8</td>
<td>87.1</td>
<td>30.3</td>
<td>0.35</td>
<td>0.16</td>
<td>22.9</td>
<td>176.8</td>
</tr>
<tr>
<td>(ml/min/1.73m2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inflammatory/Arterial stiffness/endothelial function/platelet function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>250</td>
<td>3.6</td>
<td>2.2</td>
<td>4.0</td>
<td>2.9</td>
<td>0.2</td>
<td>0.2</td>
<td>29.0</td>
</tr>
<tr>
<td>AIx (%)</td>
<td>250</td>
<td>27.6</td>
<td>27.9</td>
<td>8.5</td>
<td>-0.6</td>
<td>0.15</td>
<td>1.0</td>
<td>50.7</td>
</tr>
<tr>
<td>% ADP inhibition by SNP</td>
<td>222</td>
<td>33.1</td>
<td>27.4</td>
<td>27.3</td>
<td>0.76</td>
<td>0.16</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ADMA (μM)</td>
<td>249</td>
<td>0.52</td>
<td>0.51</td>
<td>0.08</td>
<td>0.9</td>
<td>0.15</td>
<td>0.34</td>
<td>0.91</td>
</tr>
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</table>
Table 6.3: Echocardiographic parameters

<table>
<thead>
<tr>
<th>Ultrasonic backscatter parameters (UBS)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$AV_{BS}$ score (mean ± SD)</td>
<td>12.2 ± 4.4 dB</td>
</tr>
<tr>
<td>$AV_{BS}$ score ≥ 16dB (%)</td>
<td>19.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other echocardiographic parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ASc on visual assessment (%)</td>
<td>25.4</td>
</tr>
<tr>
<td>IVSd (cm) (mean ± SD)</td>
<td>1.04 ± 0.17</td>
</tr>
<tr>
<td>LVmass index (g/m²) (mean ± SD)</td>
<td>113.27 ± 31.1</td>
</tr>
<tr>
<td>LVEF (%) (mean ± SD)</td>
<td>68.3 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Lipid profiles</strong></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol levels</td>
<td>0.925</td>
</tr>
<tr>
<td>LDL levels</td>
<td>0.677</td>
</tr>
<tr>
<td>Normalized HDL levels</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
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</tr>
<tr>
<td>CrCL indexed for BSA</td>
<td>0.001</td>
</tr>
<tr>
<td>Calcium levels</td>
<td>0.58</td>
</tr>
<tr>
<td>Phosphate levels</td>
<td>0.068</td>
</tr>
<tr>
<td>Ca\textsubscript{3}PO\textsubscript{4}</td>
<td>0.084</td>
</tr>
<tr>
<td>Vitamin D levels</td>
<td>0.351</td>
</tr>
<tr>
<td><strong>Body mass index</strong></td>
<td></td>
</tr>
<tr>
<td>Normalized BMI</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Inflammation/Integrity of NO production/effects</strong></td>
<td></td>
</tr>
<tr>
<td>Normalized hs-CRP</td>
<td>0.028</td>
</tr>
<tr>
<td>A1x</td>
<td>0.458</td>
</tr>
<tr>
<td>Normalized ADMA concentrations</td>
<td>0.425</td>
</tr>
<tr>
<td>Normalized % ADP inhibition by SNP</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Echocardiographic parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Normalized LV mass index</td>
<td>0.106</td>
</tr>
<tr>
<td>Normalized ejection fraction</td>
<td>0.82</td>
</tr>
<tr>
<td>Interventricular septal dimensions</td>
<td>0.46</td>
</tr>
</tbody>
</table>
Table 6.5: Univariate analyses – categorical variables.

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>Mean values ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No vs Yes</td>
</tr>
<tr>
<td>Gender</td>
<td>0.66</td>
<td>12.0±4.2 vs 12.3±4.7</td>
</tr>
<tr>
<td>Calcium supplementation</td>
<td>0.58</td>
<td>12.1±4.5 vs 12.5±4.0</td>
</tr>
<tr>
<td>ACE/Ang II therapy</td>
<td>0.78</td>
<td>12.2±4.6 vs 12.0±4.0</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.36</td>
<td>12.3±4.4 vs 11.6±4.6</td>
</tr>
<tr>
<td>Previous angina/MI</td>
<td>0.74</td>
<td>12.1±4.3 vs 12.4±4.7</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.54</td>
<td>12.2±4.4 vs 11.7±4.7</td>
</tr>
<tr>
<td>Statins therapy</td>
<td>0.33</td>
<td>12.0±4.5 vs 12.6±4.2</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0.30</td>
<td>12.5±4.6 vs 11.9±4.3</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.52</td>
<td>12.3±4.7 vs 12.0±4.0</td>
</tr>
<tr>
<td>Family history</td>
<td>0.92</td>
<td>12.2±4.3 vs 12.2±4.5</td>
</tr>
</tbody>
</table>
Table 6.6: Variables independently associated with high AV$_{BS}$ scores after a stepwise multiple linear regression analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>-0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.20</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelet responsiveness to SNP</td>
<td>-0.16</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figure 6.1: Comparison of aortic valve backscatter scores (AV$_{BS}$) by visual assessment of ASc.

There was a significant difference in AV$_{BS}$ between subjects with and without ASc as assessed by visual assessment (14.9 ± 4.5dB vs 11.2 ± 3.9dB respectively, p<0.001).
Figure 6.2: Correlation between AV$_{BS}$ scores and age. There was a significant correlation between AV$_{BS}$ scores and age ($\beta=0.18$, $p=0.005$).
**Figure 6.3:** Correlation between aortic valve backscatter scores (AV$_{BS}$) and creatinine clearance indexed for body surface area. There was a significant inverse correlation between AV$_{BS}$ and CrCL ($\beta$=-0.20, p=0.001).
Figure 6.4: Correlations between AVBS scores vs a) phosphate levels, and b) calcium-phosphate products (Ca₃PO₄). There were non-significant trends between AVBS scores vs phosphate levels ($\beta=0.12$, $p=0.07$), and Ca₃PO₄ ($\beta=0.11$, $p=0.08$).
**Figure 6.5:** Correlation between vitamin D levels and AV\textsubscript{BS} scores. There was no significant correlation between vitamin D levels and AV\textsubscript{BS} scores ($\beta=0.05$, $p=0.47$).
Figure 6.6: Correlation between AV$_{BS}$ and BMI. There was a strong inverse correlation between AV$_{BS}$ and BMI ($\beta=0.25$, $p<0.001$).
Figure 6.7: Correlation between $AV_{BS}$ and hs-CRP. There was a significant inverse correlation between $AV_{BS}$ and hs-CRP ($\beta=-0.14$, $p=0.03$).
**Figure 6.8:** Correlations between AVBS scores and A) plasma ADMA concentrations (μmol/L), and B) augmentation index (AIx). There was no correlation between AVBS vs A) plasma ADMA concentrations ($\beta$=-0.05, $p=0.43$); and B) augmentation index ($\beta$=-0.05, $p=0.46$).
Figure 6.9: Correlation between AV_{BS} scores and platelet responsiveness to SNP. There was a statistically significant inverse correlation between AV_{BS} scores and platelet responsiveness to SNP (β=-0.16, p=0.02).
Figure 6.10: Correlation between AVBS scores and left ventricular mass index. There was a positive trend between AVBS scores and left ventricular mass index ($\beta=0.1$, $p=0.1$).
Figure 6.11: Correlation between vitamin D levels (pmol/L) vs A) ADMA concentrations (μmol/L); and B) hs-CRP levels (mmol/L).

There were significant inverse correlations between vitamin D levels and A) ADMA concentrations ($\beta=-0.023$, $p=0.001$); and B) hs-CRP levels ($\beta=-0.17$, $p=0.009$).
Figure 6.12: Correlation between vitamin D levels and BMI. There was a significant inverse correlation between vitamin D levels and BMI ($\beta=-0.15$, $p=0.02$)
Figure 6.13: Comparison in vitamin D levels between subjects with hyperension vs non-hypertensives. There was significantly higher vitamin D levels in subjects with a hypertension vs non-hypertensives (p=0.012).
**Figure 6.14:** Correlation between A) BMI and creatinine clearance indexed for body weight calculated by the Cock-croft Gault equation, and B) BMI and creatinine clearance calculated using the MDRD (Modified Diet in Renal Disease) formula. There was a strong positive correlation between BMI and creatinine clearance for A) ($\beta=0.54$, $p<0.001$), and B) ($\beta=0.21$, $p=0.001$).
CHAPTER 7

Implications and future directions.
The findings of this thesis carry a number of implications regarding the pathogenesis and potential therapeutics of ASc/AS; these can be summarized as follows:

a. Both ASc and AS are associated with anomalies of NO production/response, as demonstrated by the results of the experiments in Chapters 3, 4, 5, and 6. From the results of the human experiments, it appears that impaired tissue responsiveness to NO is an early feature of AS, not confined to the later stages of the disease which were the subject of previous evaluations (Chirkov et al 2002). This may well be important as a contributing mechanism to the vascular risk associated with ASc. However, the biochemical mechanism(s) underlying NO resistance in ASc were not evaluated in this study. Assuming, as in the majority of previous analogous investigations (Kennedy et al 2006), that elevated $\mathrm{O}_2^-$ production was causally involved, it follows that treatment with ACE inhibitors would be likely to reduce cardiovascular risk in ASc patients. Potentially, re-analysis of patient data from HOPE and EUROPA studies to take into account echocardiographic information could be used to test this specific hypothesis.

Equally important is the possibility that impaired NO effect is a modulator of ASc progression rates, via loss of the inhibitory effect of NO on calcification. Thus, it would be potentially useful to evaluate whether specific NO-based manipulations (eg L-NAME, or treatment with nitro-vasodilators) modulates the development of AS in the rabbit model. If NO is indeed a modulator of AS development, this in turns casts further attention on:

1. The potential role of NO in the beneficial effects of ramipril in this model.
2. The lack of beneficial effects of statin therapy in advanced AS (SALTIRE), despite the known “pleiotropic” effect of increased expression of eNOS (Rajamannan et al 2005, Heart).

Therefore it would be worthwhile to examine whether the effects of ramipril are indeed NOS-dependent.

b. The effects of ramipril are potentially of pivotal importance, and extend beyond the putative role of NO. Specifically, the other critical mechanistic issues include:
   i. The role of angiotensin II vs bradykinin.
   ii. The potential role of angiotensin (1-7) as a modulator of ASc development, given that ACE inhibitors may increase angiotensin (1-7) (Ferrario et al 2005)
   iii. The importance of NAD(P)H oxidase, which is activated by angiotensin II (reviewed by Cai 2005) in the pathogenesis of AS.

However, the critically important issue from Chapter 4 is that these data greatly strengthen the case for a prospective trial of ramipril in the treatment of early AS.

c. The development of AS in rabbits treated with vitamin D is a pivotal finding in this thesis. There are a number of key issues meriting follow-up:
   i. These rabbits developed “endothelial dysfunction”, as measured by ADMA and by vascular responses to acetylcholine. Thus, it is important to know whether this was critical to the development of AS.
ii. Vitamin D also increases intracellular redox stress, via vitamin D-upregulated protein 1 (VDUP-1) (Junn et al 2000). This increase in redox stress might have been incidental or fundamental to AS development. Furthermore, interactions between vitamin D and other potential pathogenic factors in AS (eg matrix Gla protein) remains to be explored.

iii. Clinically, the main question which arises is whether development of AS is a “hidden risk” of vitamin D supplementation in the elderly. Prospective studies to test this hypothesis seem warranted.

d. The results of this thesis did not support the idea that hypercholesterolemia predisposes to the development of AS. However, particularly in view of the NO-related findings, these results to not exclude beneficial effect of statin therapy. The major thrust of the current findings is that in AS, as distinct from coronary atheroma, the presence of intraleision cholesterol deposits may be of only minor importance, relative to the ongoing processes of fibrosis, calcification and ossification.

e. The utility of AV$_{BS}$ scoring has been demonstrated throughout this thesis, but this technique is likely to be most advantageous in future studies of ASc progression. To date, there have been no such studies, but the lack of such information is very regrettable, given the long “pre-stenotic” course of AS. It is of the greatest importance that the subject cohort studied in Chapter 6 be followed-up, and the determinants of rapid progression of ASc characterized.
Unexpected findings of the experiments described in this thesis, the majority produced in this thesis, the majority produced results that were, more or less, “anticipated”. It is true that the ramipril data in Chapter 4 was just statistical significance, but that reflects the disparities between standard deviations for AV$_{BS}$ values in vitamin D-treated rabbits in Chapter 3 and 4, which affected sizing calculations to same extent. However, the major “surprises” in this thesis were confined to Chapter 6. Specifically:

1. Obesity appeared to “protect” against AS. This finding was not an artifact of AV$_{BS}$ determination, as exemplified by the BMI data in Chapter 2. Therefore the basis for this association needs further evaluation. By far the most likely explanation is that the formula utilized for measuring renal function in this study does not adequately correct for BMI (see Discussion, Chapter 6). Hence, there is considerable need to address the BMI findings, potentially as a contributor to understatement of the impact(s) of renal and endothelial dysfunction in the current epidemiological data. However, the possibility that obesity is indeed protective against AS cannot be excluded (Lindroos et al 1994).

2. Increasing age remained a correlate of ASc on multivariate analysis. This finding suggests that the current investigations to not include a complete evaluation of the effector pathways by which ageing predisposes to ASc development.

The experiments described in this thesis therefore, as is common, raise as many questions as they answer, and establish that AS/ASc is indeed a disease with similarities and important differences
from atheromatous states. There remains much to understand before we have a comprehensive approach towards minimizing the morbidity of this condition.


Aronow WS, Schwartz KS & Koenigsberg M: Correlation of serum lipids, calcium, and phosphorus, diabetes mellitus and history of systemic hypertension with presence or absence
of calcified or thickened aortic cusps or root in elderly patients. Am J Cardiol 1987, 59, 998-9.


Behrendt D & Ganz P: Endothelial function: from vascular biology to clinical applications. Am J Cardiol 2002, 90, 40L-48L.


Keidar S, Kaplan M & Aviram M: Angiotensin II-modified LDL is taken up by macrophages ia the scavenger receptor, leading to cellular cholesterol accumulation. *Arterioscler Thromb Vasc Biol* 1996, **16**, 97-105.

Kennedy JA, Hua X, Rosenkranz AC, Murphy GA, Mishra K & Horowitz JD: Nitric oxide donors and PGE1 inhibit calcification of aortic valve interstitial cells. *As part of the World Congress of Cardiology*, Abstract number 3461.


McDermott JR: Studies on the catabolism of Ng-methylarginine, Ng, Ng-dimethylarginine and Ng, Ng-dimethylarginine in the rabbit. *Biochem J* 1976, 154, 179-184.


Nassimiha D, Aronow WS, Ahn C & Goldman ME: Rate of progression of valvular aortic stenosis in patients $\geq 60$ years of age. *Am J Cardiol* 2001, **87**, 807-809.

Ngo DT, Heresztyn T, Mishra K, Marwick TH & Horowitz JD: Aortic stenosis is associated with elevated plasma levels of asymmetric dimethylarginine (ADMA). *Nitric Oxide: Biology and Biochemistry* 2007, **16**, 197-201.


protein 1 mRNA enhances cardiomyocyte survival and prevents left ventricular remodeling after myocardial ischemia. *J Biol Chem* 2005, **280**, 39394-39402.


Quantitative Assessment of Aortic Sclerosis Using Ultrasonic Backscatter

Doan T. M. Ngo, BPharm, Ronald D. Wuttke, BSci, Stuart Turner, BMed, FRACP, Thomas H. Marwick, MBBS, PhD, FACC, and John D. Horowitz, MBBS, FRACP, PhD,
Adelaide and Brisbane, Australia

Background: The development of therapeutic interventions to prevent progressive valve damage is more likely to limit the progression of structural damage to the aortic valve with normal function (aortic sclerosis [ASC]) than clinically apparent aortic stenosis. Currently, the ability to appreciate the progression of ASC is compromised by the subjective and qualitative evaluation of sclerosis severity.

Methods: We sought to reveal whether the intensity of ultrasonic backscatter could be used to quantify sclerosis severity in 26 patients with ASC and 23 healthy young adults. Images of the aortic valve were obtained in the parasternal long-axis view and saved in raw data format. Six square-shaped 11 × 11 pixel regions of interest were placed on the anterior and posterior leaflets, and calibrated backscatter values were obtained by subtracting the regions of interest in the blood pool from the averaged backscatter values obtained from the leaflets.

Results: Mean ultrasonic backscatter values for sclerotic valves exceeded the results in normal valve tissue (16.3 ± 4.4 dB vs 9.8 ± 3.3 dB, P < .0001). Backscatter values were greater (22.0 ± 3.5 dB) in a group of 6 patients with aortic stenosis. Within the sclerosis group, the magnitude of backscatter was directly correlated (P < .05) with a subjective sclerosis score, and with transvalvular pressure gradient. Mean reproducibility was 2.4 ± 1.8 dB (SD) between observers, and 2.3 ± 1.7 dB (SD) between examinations.

Conclusion: Measurement of backscatter from the valve leaflets of patients with ASC may be a feasible means of following the progression and treatment response of aortic sclerosis. (J Am Soc Echocardiogr 2004;17:1123-30.)

Aortic sclerosis (ASC), characterized by aortic valve thickening or calcification without restriction of motion, affects approximately 30% of adults older than 65 years, and 50% older than 85 years.1,2 Patients with ASC have increased risk of cardiovascular mortality and myocardial infarction.3,4 The major concern with ASC is the risk of progression to symptomatic aortic stenosis (AS). This progression has been studied mainly for patients with mild or moderate AS and transvalvular pressure gradients of 15 mm Hg and above.5,7 Once ASC has progressed to this stage, development of symptomatic stenosis may occur over a few years, with a predicted rate of progression at about 5 to 7 mm Hg of mean pressure gradient annually.5,6,8

Recent studies of the pathologic, clinical, and biochemical correlates of AS have suggested that it may be a preventable disease.9,10 However, the development of clinical strategies to retard the progression of ASC requires a technique to measure the degree of sclerosis as a marker of the rate of progression, and the effectiveness of clinical interventions.

Although the conventional Doppler echocardiography examination is a standard approach for the assessment and management of AS, Doppler gradients are not present in ASC, and visual evaluation of the valve thickness, mobility, and calcification are subjective. Improvements in the feasibility of ultrasonic backscatter to detect and characterize pathologic changes in tissues offer a potential means for assessment of aortic valve diseases, possibly before hemodynamic changes occur. Ultrasonic backscatter has been used to detect preclinical changes within the myocardium, even at a stage when conventional echocardiographic indices are within the normal range in myocardial ischemia,11 acute cardiac transplant rejection,12 and diabetes mellitus.13,14 Although increased myocardial echodensity has also been identified in patients with AS without overt systolic dysfunction,15 changes in ultrasonic backscatter of the aortic valve have not been assessed. Nonetheless, histologic findings of diseased
Aortic valve in AS have shown a range of abnormalities starting with small areas of fibrosis, and thickening and extending marked structural degeneration, nodular thickening, and dystrophic calcification. In this study, we sought whether the extent of alterations of the aortic valves could be quantified by ultrasonic backscatter for patients with ASC.

**METHODS**

**Patients**

The study population consisted of 26 consecutive patients with ASC (14 women, mean age 76 years, range 57-91 years), 6 patients with AS (3 women, mean age 79 years, range 69-89 years) who were identified while being referred for nonemergency echocardiographic evaluation, and a control group of 23 healthy volunteers (11 women, mean age 30 years, range 21-39 years), none of whom had left ventricular (LV) dysfunction, valvular heart disease, or thickening of the aortic valves detected by echocardiography. All patients with ASC and AS had undergone complete baseline echocardiographic examination and were excluded if image quality was not suitable for backscatter analysis. The study was approved by the ethics of human research of Queen Elizabeth Hospital, Adelaide, Australia.

A full clinical history and clinical examination was performed in all patients. Hypertension was defined by treatment with antihypertensive drugs or a blood pressure > 140/80 mm Hg. Hypercholesterolemia was defined by treatment with cholesterol-lowering drugs or a total cholesterol > 4.5 mmol/L. Diabetes mellitus was defined by treatment for diabetes or a fasting blood glucose > 7.0 mmol/L. Plasma serum creatinine levels (a risk factor for rapid progression of AS) and hemoglobin concentrations were measured in all patients.

**Evaluation of ASC**

ASC was defined according to Otto et al as focal areas of increased echogenicity and thickening of the aortic valve leaflets without restriction of leaflet motion, and AS was defined as thickened leaflets with reduced systolic opening and increased velocity across the aortic valve > 2.5 m/s. Subjective assessment of the degree of ASC was also performed as described by Shively et al. Briefly, sclerotic valves were classified on the basis of extent of increase in degree of reflectance of cusp bodies and margins, and valve thickness. The grading of sclerosis was performed by a cardiologist blinded to all other echocardiographic results.

**Doppler Echocardiography**

M-mode and 2-dimensional echocardiograms with Doppler analysis were obtained for all participants by means of a commercially available ultrasound machine (Vivid 5, GE Vingmed, Horten, Norway) with a 2.5-MHz phased-array probe. LV diameters and wall thicknesses were measured from 2-dimensionally guided M-mode echocardiography. Mean and peak transaortic pressure gradients were calculated with the modified Bernoulli equation, using continuous wave Doppler recordings from the highest velocity available from any view. The aortic valve area was computed with the continuity equation using standard methods.

**Ultrasound Backscatter Data Analysis**

Two-dimensional ultrasonic backscatter images of the aortic valves were obtained from standard parasternal long-axis view over 3 cardiac cycles with a zoom of 8 cm. Gain and time-gain compensation settings were adjusted for all study participants to obtain apparently uniform brightness throughout the echocardiogram, to facilitate reproducible sampling of backscatter values. Transmit power and compression settings were kept constant throughout each backscatter study.

Three consecutive scans were done for each study participant. A total of 6 square-shaped regions of interest (3 on the anterior leaflet and 3 on the posterior leaflet) were obtained by placing 11 × 11 pixel sample volumes on the valves. Backscatter values from the blood pool in the LV outflow tract and aortic root were used as reference values (Figure 1). Calibrated backscatter values were obtained by subtracting the average blood pool value from the averaged backscatter values obtained from the aortic valves. The final calibrated backscatter values (decibel) were averaged over the 3 consecutive scans.

**Reproducibility**

Reproducibility of backscatter values was evaluated in 12 randomly selected participants (6 control subjects and 6
patients with ASC). To determine variability in backscatter measures between different operators, the 12 participants were assessed for aortic valve backscatter by two different sonographers. To evaluate variability of backscatter values on different occasions, in the absence of disease progression, the 12 participants also had repeated estimations of backscatter 9/11006/6 weeks after the initial estimation.

Statistics

The Student nonpaired t test was used to compare continuous (normally distributed) variables. Correlation between backscatter and 2-dimensional echocardiographic measurements was examined by terms of linear regression analysis. Correlation between backscatter and subjective grading of ASC was assessed by Spearman rank correlation test. A value of P < .05 was considered significant. Unless otherwise stated, results for normally distributed parameters are expressed as mean ± SD.

The principal hypothesis to be tested related to the presence/absence of incremental backscatter for patients with ASC compared with control subjects. Therefore, although some data were obtained for patients with AS, this small group was not compared statistically with the other two cohorts.

RESULTS

Patient Characteristics

Table 1 summarizes the clinical characteristics of patients with aortic sclerosis (n = 26) and AS aortic stenosis (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Aortic sclerosis</th>
<th>Aortic stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients</td>
<td>(%)</td>
</tr>
<tr>
<td>Male sex</td>
<td>11</td>
<td>42.3</td>
</tr>
<tr>
<td>Coronary risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current/previous smoking</td>
<td>13</td>
<td>50</td>
</tr>
<tr>
<td>Hypertension</td>
<td>20</td>
<td>76.9</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>17</td>
<td>65.4</td>
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<tr>
<td>Diabetes mellitus</td>
<td>3</td>
<td>11.5</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine &gt; 0.12 mmol/L</td>
<td>10</td>
<td>38.4</td>
</tr>
<tr>
<td>Anemia (hemoglobin &lt; 115 g/L)</td>
<td>4</td>
<td>15.4</td>
</tr>
<tr>
<td>Known myocardial ischemia</td>
<td>14</td>
<td>53.8</td>
</tr>
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</table>

Aortic Valve Findings

Echocardiographic parameters for patients with ASC are summarized in Table 2. There were 5 patients with ASC with impaired LV systolic function. A total of 12 patients had (generally mild) LV hypertrophy. All patients had evidence of minimal or mild narrowing of the aortic valve. None of these patients had a calculated mean transvalvular pressure gradient greater than 15 mm Hg. Among the 6 patients with AS, mean transvalvular pressure gradient ranged from 22 mm Hg to 44 mm Hg and mean aortic valve area was 0.9 ± 0.2 cm², consistent with mild to moderate stenosis.

Relationship Between Backscatter Data and Conventional Echocardiographic Measurements

Backscatter results for the 3 cohorts examined (control, ASC, and AS) are shown in Figure 2. The mean backscatter value for control subjects was 9.8 ± 3.3 dB. Although backscatter values in the ASC population overlapped considerably with the control population, the mean level was significantly greater (16.3 ± 4.4 dB vs 9.8 ± 3.5 dB, P < .0001). The small AS group had the greatest backscatter value (22.0 ± 3.5 dB).

Within the ASC group, a number of correlations were sought between measures of severity of valve disease and calculated backscatter. Correlation of backscatter with mean transvalvular pressure gradient and with aortic valve area is depicted in Figure 3. There was a direct correlation between mean pressure gradient and backscatter (P < .05) and a nonsignificant trend toward an inverse correlation between aortic valve area and backscatter. In addition, subjective severity of valve disruption was also directly related to backscatter calculations (Figure 4) (P < .05).
Reproducibility of Backscatter Values

Reproducibility of backscatter findings was examined: (1) between observers; and (2) between serial examinations for both control subjects and patients with ASC. Results are shown in Figure 5. The mean difference in backscatter values between observers is 2.4 ± 1.8 (9.1% ± 6.9). For serial examinations of both control subjects and patients with ASC, the mean difference in backscatter values was 2.3 ± 1.7 (9.1% ± 6.2).

Tissue Characterization with Ultrasonic Backscatter

Measurement of backscatter has been used for many years to detect changes in pathologic tissues on the basis of the analysis of the interactions between ultrasound waves and tissue.21 Variations of the

Table 2 Individual echocardiographic parameters for patients with aortic sclerosis and stenosis with respective ultrasonic backscatter values

<table>
<thead>
<tr>
<th>Patients with aortic sclerosis</th>
<th>IVSd (cm)</th>
<th>LVEF (%)</th>
<th>FS (%)</th>
<th>Sclerosis grading</th>
<th>AV (m/s)</th>
<th>AVp (mm Hg)</th>
<th>AVp mean (mm Hg)</th>
<th>AV (cm²)</th>
<th>BS values (dB)</th>
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<tr>
<td>1</td>
<td>1.00</td>
<td>70.3</td>
<td>44.0</td>
<td>Moderate</td>
<td>1.62</td>
<td>10.56</td>
<td>5.05</td>
<td>2.10</td>
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<td>2</td>
<td>1.11</td>
<td>51.0</td>
<td>26.3</td>
<td>Moderate</td>
<td>1.64</td>
<td>10.71</td>
<td>4.94</td>
<td>2.16</td>
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<td>1.35</td>
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<td>Mild</td>
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<td>4.97</td>
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<td>2.30</td>
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<td>20.77</td>
<td>11.40</td>
<td>1.74</td>
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</table>

AV: Peak aortic valve velocity; AVA: aortic valve area; AVp: peak transvalvular pressure gradient; AVp mean: mean transvalvular pressure gradient; BS: backscatter; FS: fractional shortening; IVSd: interventricular septum thickness; LVEF: left ventricular (LV) ejection fraction (EF); LV hypertrophy is indicated when IVSd > 1.2 cm. LV systolic impairment was when EF < 55%.

**DISCUSSION**

The results of the study indicate that: (1) the mean levels of ultrasonic backscatter in patients with ASC are approximately 60% greater than in healthy young adults; (2) ultrasonic backscatter scores in patients with ASC are directly correlated with subjective scoring of sclerosis and with transvalvular pressure gradients in patients with mild-moderate AS; and, most importantly, (3) ultrasonic backscatter is a reproducible technique, with mean differences between estimates on the basis of repeated echocardiograms of 2.3 ± 1.7 (9.1% ± 6.2).
ultrasound backscatter signals and their relationship to tissue structure forms the basis of tissue characterization principles. There have been several approaches using ultrasonic backscatter to quantitatively define changes, mainly in myocardial ultrasonic reflectivity. An increase in backscatter has been correlated with collagen content and the development of fibrosis in the myocardium. Advances in computing power and the ability to save ultrasound studies in raw data format have made this technique more feasible for clinical application.

Changes in backscatter may be used to characterize the structural and biochemical composition of atherosclerotic plaque, thereby differentiating among fibrofatty lesions, fatty lesions, and calcification of the arterial walls. Kawasaki et al measured backscatter values of carotid and femoral arteries in 12 patients before and after death, and compared the backscatter values with their histopathologic features. They found that ultrasonic backscatter values differed significantly for thrombus, fibrosis, calcification and lipid pool, and intimal and medial hyperplasia. These differences in backscatter values correlated well with histologic classification.

The exact mechanisms underlying changes in ultrasonic backscatter for patients with ASC are not clear. To our knowledge, there has been no study that measured changes in backscatter signals on aortic valves. In this study, specific structural determinants of aortic valve backscatter could not be assessed. However, histopathologic lesions of ASC have been shown to resemble arterial atherosclerotic...
Characterization of the early degenerative lesions of AS by Otto et al demonstrated subendothelial thickening with disruption of basement membrane, accumulation of inflammatory infiltrates such as T-lymphocytes and macrophages; presence of intracellular and extracellular lipids; mineralization; and regions with protein, lipid, and calcium accumulation. Severely stenosed aortic valves have been described as grossly deformed and distorted, with marked fibrosis and nodular calcification, eventually involving the full thickness of the aortic cusp. Scoring of valvular calcium content using electron beam or multislice computed tomography scanning offers potential quantitation of the end-stage component of the pathogenesis of AS, but may not provide insight into the atheromatous and fibrotic changes that characterize early ASC.

Previous methods of quantifying ASC involved direct visualization of aortic valve structure and estimation of hemodynamic parameters using Doppler echocardiography. Although visualization of aortic valve structure can detect marked alterations in valve appearance and mobility, such methods are subjective and may not be reproducible. Furthermore, the subjective assessment emphasizes difficulty in distinguishing between normal aortic valves, particularly in elderly people, and minimal ASC. In this study, ultrasonic backscatter could quantitatively categorize ASC.

Limitations

The current study has several limitations. First, it lacked age-matched control subjects. Although in both the ASC and the control group there was no significant correlation between backscatter values and patient age (data not shown), it remains probable that a minor increase in backscatter does indeed occur with advancing age. Furthermore, patients with very mild ASC are differentiated only arbitrarily from age-adjusted control subjects. It is, therefore, likely that the difference between control subjects and patients with ASC would be reduced if an age-matched group of control subjects was considered. Nonetheless, this in no way invalidates the use of ultrasonic backscatter as a measure of ASC.

A further theoretic limitation on the technique used for estimating valvular backscatter is the use of blood pool backscatter as a reference value. It is possible that anemia, common for patients with aortic valve disease, might affect blood pool backscatter. However, within the study population, there was no significant correlation between backscatter of blood pool and hemoglobin concentrations.

The use of subjective optimization of valve image quality by adjustments of gain and time-gain compensation is theoretically inferior to the use of predefined settings, but is difficult to avoid in a study in which subjective scoring of valve appearance was a predefined comparison. The applicability of the current technique to population group data is evident despite this theoretic limitation.

Clinical Implications

It is planned that ultrasonic backscatter score be used primarily as a measure of progression of ASC. Reproducibility data permit calculation of sample sizes of studies required to detect specific extents of backscatter progression. For example, assuming baseline values of backscatter in a study population of patients with ASC of 16.3 ± 4.4 dB, the study size required to detect mean progression to 18.5 U (ie, 0.5 SD) progression at a β value of 0.8 would be approximately 44 patients. Assuming an intervention, study size to detect heterogeneity of progres-
sion of a mean of 2.2 U (ie, 0.5 SD) between two populations at β value of 0.8 would be approximately 170 patients in each group.

To date, there have been no definitive intervention studies for patients with ASC, or indeed with moderate AS. However, there has been a focus in the literature on the possible relationship between aortic valve calcification and statin therapy. In view of these observational data, at least two studies are in progress addressing the possible beneficial effects of cholesterol reduction on progress of aortic valve calcification. The currently described methodology would theoretically have the advantage of facilitating examination of disease progression without relying exclusively on valve calcification, a relatively late component of the pathophysiology of AS.

There are many other areas of potential applicability of this technology, both for epidemiologic studies and for examination of interventions. For example, case-control study design might be used to examine the implications of various degrees of renal dysfunction, of the presence of various coronary risk factors, or of the activation of inflammatory mediators such as C-reactive protein on occurrence/progression of ASC. Physiologically oriented studies might be used to delineate the relationship between vascular endothelial dysfunction (and its biochemical markers) and occurrence of ASC. Finally, there are intriguing data suggesting other potential forms of pharmacotherapy of ASC. For example, early intervention studies for patients with ASC, or indeed with moderate AS. However, there has been a focus in the literature on the possible relationship between aortic valve calcification and statin therapy. In view of these observational data, at least two studies are in progress addressing the possible beneficial effects of cholesterol reduction on progress of aortic valve calcification. The currently described methodology would theoretically have the advantage of facilitating examination of disease progression without relying exclusively on valve calcification, a relatively late component of the pathophysiology of AS.

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We gratefully acknowledge the technical assistance of Ms Linda Passfield in this publication.

REFERENCES


Aortic stenosis is associated with elevated plasma levels of asymmetric dimethylarginine (ADMA)

Doan T.M. Ngo a, Tamila Heresztyn a, Kumaril Mishra a, Thomas H. Marwick b, John D. Horowitz a, *

a Cardiology Unit, The Queen Elizabeth Hospital, University of Adelaide, 28 Woodville Rd, Woodville South, SA 5011, Australia
b Department of Medicine, Princess Alexandra Hospital, University of Queensland, Qld, Australia

Received 5 April 2006; revised 15 July 2006
Available online 14 October 2006

Abstract

Objectives: We tested the hypothesis that the presence of aortic stenosis (AS) is associated with elevation of plasma levels of asymmetric dimethylarginine (ADMA), a physiological inhibitor of nitric oxide synthase, a mediator and marker of endothelial dysfunction and an indicator of incremental cardiovascular risk.

Background: The presence of aortic sclerosis (ASC), the precursor of AS is independently associated both with endothelial dysfunction, and with incremental coronary event risk. It remains uncertain whether elevations of ADMA levels might mediate endothelial dysfunction in these conditions.

Methods: Forty two consecutive patients referred for echocardiography for evaluation of AS, who had calculated aortic valve areas of <1.4 cm² (AS group) were evaluated together with 42 consecutive age-matched referred patients (non-AS group). Plasma ADMA levels were measured by high-performance liquid chromatography (HPLC). Determinants of elevation of plasma ADMA levels were identified via stepwise multiple linear regression analysis.

Results: Plasma ADMA levels were not statistically different between the AS and non-AS group (median 0.59 vs 0.54 μmol/L, p = 0.13, Mann-Whitney test) on univariate analysis. However, in backward stepwise multiple linear regression, the presence of AS was a significant predictor of elevated ADMA levels (p = 0.04, 95% CI = 0.001, 0.072). In addition, elevated plasma ADMA levels were also associated with history of atrial fibrillation (p = 0.009, 95% CI = 0.015, 0.100), and negatively associated with creatinine clearance (p = 0.01, 95% CI = −0.002, 0.000), and the use of statin therapy (p = 0.01, 95% CI = −0.081, −0.011).

Conclusions: AS is independently associated with elevation of ADMA levels, beyond that implied by “conventional” risk factors for endothelial dysfunction. The clinical status of AS as an incremental marker of cardiovascular risk may reflect ADMA-mediated endothelial dysfunction.

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Keywords: Asymmetric dimethylarginine (ADMA); Aortic stenosis (AS); Endothelial dysfunction

Aortic stenosis (AS) now represents the most frequently occurring form of hemodynamically significant valvular heart disease in most Western populations. The incidence of AS increases with age; its precursor, disorganization of valve structure without marked obstruction (aortic sclerosis: ASc) is present in approximately 40% of individuals >80 years [1,2].

In view of the considerable morbidity and mortality associated with AS, investigations have been directed towards better understanding of its pathogenesis, with the ultimate objective of developing strategies to retard its progression. Poggianti et al. [3] have observed that ASc (and therefore presumably AS) is associated with systemic endothelial dysfunction, while other investigators have
demonstrated activation of inflammatory processes and evidence of associated atherogenesis [4–6]. Consistent with these findings, ASC has been identified as a marker of increased risk of acute myocardial infarction [7].

A number of recent investigations have indicated that asymmetric dimethylarginine (ADMA), a physiologically occurring competitive antagonist of endothelial nitric oxide synthase (eNOS) is both a strong marker and also a mediator of many aspects of endothelial dysfunction [8–10]. ADMA appears not only to inhibit the bioconversion of arginine by eNOS to release nitric oxide (NO), but also to regulate eNOS activity under certain conditions [10]. While ASCs have been reported to be associated with endothelial dysfunction [3], endothelial function has not been investigated in patients with advanced AS. The possibility that ADMA contributes to the development of endothelial dysfunction irregardless of other cardiac structural/functional anomalies, had normal aortic valve structure including normal aortic valve backscatter of <16 dB. Patients (n = 4) with backscatter values ≥ 16 dB but otherwise normal valves were excluded.

Risk factors

All patients’ cardiovascular risk factors were determined by chart review. Hypertension was defined with antihypertensive drugs or blood pressure > 140/80 mm Hg. Hypercholesterolaemia was defined with treatment with cholesterol-lowering drugs and/or a total cholesterol > 5.5 mmol/L. Diabetes mellitus was defined with treatment for diabetes or a fasting blood glucose > 7.8 mmol/L. Known coronary artery disease (CAD) in AS patients was defined on the basis of the presence of at least one of the following: > 50% luminal diameter narrowing of ≥1 epicardial coronary artery on angiography, history of coronary revascularization, history of myocardial infarction, regional left ventricular wall akinesia and/or dyskinesia on echocardiogram.

Biochemical measurements

Blood was collected from all patients into heparinised tubes, centrifuged at 4°C at 2700g for 20 min, and plasma was stored at −80°C until assay. Concentrations of ADMA and L-arginine in plasma were measured by high-performance liquid chromatography (HPLC) using the derivatisation reagent AccQ-Fluor after solid phase extraction as previously described [17]. The recovery rate for ADMA was 92 ± 2%, and the detection limit of the assay was 0.1 μM. Concentrations of total cholesterol, and serum creatinine were measured by standard laboratory assays. Creatinine clearance (CrCl) was calculated according to the Cockcroft–Gault equation. High sensitivity CRP (hs-CRP) was determined by the Beckman Coulter High Sensitivity C-reactive protein kit measured by the IMMAGE® Immunochemistry system.

Hypotheses and statistical methods

It was recognized that impairment of renal function was likely to be associated with elevation of ADMA levels [11,12]. However, the primary hypothesis to be tested in this study was that AS (as distinct from normal valve structure) is independently associated with elevation of ADMA levels, even in a patient population with a high prevalence of other coronary risk factors.
was independently associated with elevation of ADMA levels. Therefore the primary method of analysis utilized for hypothesis testing was backward stepwise multiple linear regression analysis, utilizing ADMA as the dependent variable. Independent variables utilized in the final model were: presence/absence of AS, creatinine clearance (CrCL), known IHD, previous or current hypercholesterolaemia, statin therapy, diabetes mellitus (DM), atrial fibrillation (AF), systemic hypertension (HT), and left ventricular ejection fraction (EF) with \( p > 0.1 \) as model exit criteria, using SPSS 12 software. Fisher’s exact test was used to evaluate differences in patients’ baseline characteristics if the variables were categorical, and Mann-Whitney test was used if the variables were continuous. Plasma ADMA concentrations were log-transformed for linear regressions and for the multiple linear regression. Univariate comparisons of data sets were performed using SPSS 12 software. Data are expressed as mean \( \pm \) SD unless otherwise stated; the level of statistical significance was set at \( p < 0.05 \).

Results

Characteristics of the study group

Clinical characteristics of patients are listed in Table 1. As planned, there was no difference in age between the AS and non-AS group. None of the patients had bicuspid aortic valves or history of rheumatic fever. The mean transaortic pressure gradients and AVA for AS patients were 68.7 \( \pm \) 27.4 mmHg and 0.81 \( \pm \) 0.23 cm\(^2\), respectively. The mean ultrasonic backscatter (UBS) for the non-AS group was 10.5 \( \pm \) 2.8 dB approximating to the previously described mean for normal values [16]. Patients with AS had a greater prevalence of known IHD (reflecting differential rates of cardiac catheterization), as well as increased interventricular septal thickness. Other parameters associated with endothelial dysfunction such as history of HT, DM, hypercholesterolemia, AF, low EF, and reduced CrCL occurred at similar frequencies among the two groups. Median plasma concentrations of hs-CRP did not differ in the non-AS group and AS (4.6 mg/L vs 4.0 mg/L, respectively \( p = 0.3 \)). A total of 95.5% of the patients with hypercholesterolemia were receiving statin treatment.

Association of clinical characteristics with ADMA

Plasma ADMA levels for all patients were skewed and therefore this parameter was log-transformed for subsequent analyses. Median plasma ADMA concentrations were marginally but not significant greater \( (p = 0.13) \) in the AS group \( (0.59 \mu M \text{ vs } 0.54 \mu M) \). Plasma L-arginine concentrations were not significantly different between groups \( (76.5 \pm 23.7 \mu M \text{ in the non-AS group vs } 77.6 \pm 18.2 \mu M \text{ in the AS group, } p = 0.8) \). Within the AS group, there was no correlation between peak transaortic pressure gradients or aortic valve areas and ADMA levels. Similarly in the non-AS group, there was no correlation between UBS values and ADMA (data not shown).

The results of the univariate analyses are summarized in Table 2. Univariate analysis showed significantly greater ADMA concentrations in patients with a history of AF, compared to those without AF \( (p = 0.01) \). There was also a significant inverse relationship between ADMA concentrations and CrCL \( (p = 0.03) \), as well as with statin therapy \( (p = 0.04) \). No other factors were associated with elevated ADMA levels.

In accordance with the hypothesis tested, the primary method of data analysis was via backward stepwise multiple linear regression, performed to determine whether the presence of AS was a predictor of elevated ADMA levels after adjustment for other cardiovascular risk factors. The results are summarized in Table 3. The presence of AS was independently associated with elevated plasma ADMA

| Table 1 Clinical characteristic comparisons between AS and non-AS group (\( n = 42 \) for both groups) |
|----------------------------------|----------------------------------|
|                                 | AS group                        | Non-AS group                   |
| Age (years)                     | 73.1 \( \pm \) 7.7              | 72.7 \( \pm \) 6.7              |
| Presence of known IHD           | 28 (67%)                        | 26 (62%)                       |
| HT                              | 28 (67%)                        | 26 (62%)                       |
| DM                              | 17 (40%)                        | 10 (24%)                       |
| Hypercholesterolaemia           | 27 (64%)                        | 22 (52%)                       |
| AF                              | 7 (17%)                         | 11 (26%)                       |
| CrCL (ml/min)                   | 43.6 \( \pm \) 18.7             | 39.4 \( \pm \) 17.4             |
| EF                              | 63.06 \( \pm \) 15.3            | 61.22 \( \pm \) 12.8            |
| IVSd (cm)                       | 1.33                             | 1.2                             |
| Statin therapy                  | 20 (48%)                        | 22 (52%)                       |
| ACE/AngII antagonist            | 26 (62%)                        | 24 (57%)                       |

IHD, ischemic heart disease; HT, hypertension; DM, diabetes mellitus; AF, atrial fibrillation; CrCL, creatinine clearance; EF, ejection fraction; IVSd, interventricular septal dimension; ACE/AngII antagonist, angiotoxin converting enzyme/angiotensin II receptor antagonists.

* \( p < 0.005 \) vs. AS group.

Table 2 Univariate analyses: relationship between plasma ADMA levels and clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.29</td>
</tr>
<tr>
<td>Presence of AS</td>
<td>0.13</td>
</tr>
<tr>
<td>Presence of known IHD</td>
<td>0.11</td>
</tr>
<tr>
<td>HT</td>
<td>0.99</td>
</tr>
<tr>
<td>DM</td>
<td>0.81</td>
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<td>Hypercholesterolaemia</td>
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</tr>
<tr>
<td>AF</td>
<td>0.02*</td>
</tr>
<tr>
<td>CrCL (ml/min)</td>
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</tr>
<tr>
<td>EF</td>
<td>0.37</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>0.02*</td>
</tr>
<tr>
<td>ACE/AngII antagonist</td>
<td>0.33</td>
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</tbody>
</table>

Linear regressions were performed between log-plasma ADMA levels and continuous variables such as age, CrCL, and EF. Mann-Whitney tests were used to compare median plasma ADMA levels for all other categorical variables. AS, aortic stenosis; IHD, ischemic heart disease; HT, hypertension; DM, diabetes mellitus; AF, atrial fibrillation; CrCL, creatinine clearance; EF, ejection fraction; ACE/AngII antagonist, angiotensin converting enzyme/angiotensin II receptor antagonist.

* \( p < 0.05 \).
concentrations. Other significant determinants of elevated ADMA concentrations were low creatinine clearance, and history of AF. In addition, the use of statin therapy was significantly associated with low ADMA levels.

Discussion

The presence of AS has been recognized as a marker of greater propensity to cardiovascular risk than is apparent from conventional risk factors [7]. Pathological examinations of diseased aortic valves show areas of subendothelial thickening with disruption of basement membrane, accumulation of inflammatory infiltrates; presence of lipids, mineralization and calcium deposits have also been described. The presence of these lesions is consistent with a cascade of cellular changes that have been postulated to begin with endothelial disruption/dysfunction [4–6]. To date, only the study of Poggianti et al. [3] has examined systemic endothelial function in patients with aortic valve disease. These investigators demonstrated impairment of flow-mediated dilatation within the brachial artery in patients with AS, but did not measure ADMA or any other circulating markers of endothelial function. More recently, we have demonstrated impairment of valvular endothelial function in explanted valves of patients with advanced AS [18], but these findings were not related to systemic endothelial function.

The current study was designed to test the hypothesis that the presence of AS is independently associated with elevation of plasma ADMA levels relative to the absence of any aortic valve disease. Evaluation of this hypothesis therefore was contingent upon a multivariate analysis method (in this case a stepwise multiple regression), which demonstrated this association to be present (p = 0.04).

Although the corrected increase in ADMA concentrations in patients with AS is only approximately 7% of normal mean values, even such small increases are associated with significantly increased cardiovascular risks [19, 20]. It is also possible that elevation of ADMA levels is relevant to the pathogenesis of AS, largely via a decrease in protective role of endothelial NO against oxidative stress in the valve matrix.

Consistent with other studies, presence of renal dysfunction is associated with ADMA elevations [11–14]. We showed a correlation between plasma ADMA concentrations and impaired creatinine clearance. The degree of renal dysfunction was also correlated with plasma ADMA levels, not just the presence of end-stage renal failure. This possibly implies that endothelial dysfunction occurs gradually with worsening of renal dysfunction. This is in agreement with previous studies which showed that levels of ADMA are increased in patients with chronic renal failure [14, 15].

The inverse association between statin therapy and ADMA concentrations was unexpected, as the majority of previously published studies suggest that statins do not lower ADMA levels (reviewed by Maas [21]). No patients were receiving rosuvastatin, the only member of this class which has been reported to lower ADMA concentrations. Interestingly, a history of AF was also a significant predictor of elevated ADMA levels. However, AF has been linked to endothelial dysfunction [22]. Angiotensin converting enzyme inhibitors (ACEi) and angiotensin II (ATII) receptor antagonists have been shown to decrease the occurrence of AF [23, 24] possibly via improved atrial endocardial endothelial function. However, it must be emphasized that these findings are largely hypothesis-generating, as none of these were a part of the principal hypothesis to be tested.

Study limitations

The study is dependent on referral bias for echocardiogram. However, matching was good in general. The major difference was the frequency of known coronary disease. Furthermore, the study relates to well established AS. While it demonstrates an association with ADMA at that stage, the important question really relates to ADMA in early “sclerotic” disease. In view of the current results, a prospective evaluation in patients with ASC is now of great interest. Although the results of this study found an association between elevated ADMA levels and the presence of AS, we have yet to establish the cause of ADMA elevation in these patients.

In summary, the presence of AS is predictive of elevated ADMA levels independent of creatinine clearance, and other cardiovascular risk factors. Elevated ADMA levels appear to contribute to endothelial dysfunction [25], as well as being recognized as a marker of cardiovascular and all-cause mortality risk [19, 20]. Thus, the association of AS and elevated ADMA levels could possibly explain the increased cardiovascular events that occur in patients with AS and even ASC.

Acknowledgments

We wish to express our gratitude to Mr. Ronald D. Wuttke, Ms. Georgina Velissaris, Ms. Linda Passfield and other staff of the Queen Elizabeth Hospital Echocardiography department, as well as staff of the cardiac catheterization laboratory. We are indebted to Dr. Angus Nightingale for his suggestions in manuscript preparation.

References


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_Nitric Oxide_ v.16 (2) pp. 197-201, March 2007

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http://dx.doi.org/10.1016/j.niox.2006.10.003