PATHOGENESIS OF AORTIC STENOSIS: IMPLICATIONS REGARDING IMPAIRMENT OF NITRIC OXIDE SIGNALLING

Aaron Leonid Sverdlov
MBBS, FRACP, FCSANZ

Department of Medicine, Faculty of Health Sciences
University of Adelaide

&
Department of Cardiology, The Queen Elizabeth Hospital
South Australia, Australia

April 2012
A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy
Dedicated to my beautiful wife Doan and son Joshua
TABLE OF CONTENTS

TABLE OF CONTENTS .................................................................................................................. III
ABSTRACT...................................................................................................................................... VI
DECLARATION ............................................................................................................................. VIII
PUBLISHED WORKS IN WHOLE OR IN PART CONTAINED WITHIN THIS THESIS ................ IX
SCHOLARSHIPS, AWARDS AND GRANTS HELD CURRENTLY ................................................... X
SCHOLARSHIPS, AWARDS AND GRANTS RELATED TO THIS THESIS ..................................... XI
ACKNOWLEDGEMENTS .............................................................................................................. XII
PERSONAL BIBLIOGRAPHY ...................................................................................................... XIV

PEER-REVIEWED FULL TEXT PUBLICATIONS ARISING FROM AND/OR RELATED TO THE WORK CONDUCTED TOWARDS THIS THESIS - PUBLISHED/IN PRESS ................................................................. XIV
PEER-REVIEWED FULL TEXT PUBLICATIONS ARISING FROM AND/OR RELATED TO THE WORK CONDUCTED TOWARDS THIS THESIS - IN SUBMISSION ................................................................. XVI
PUBLICATIONS/PRESENTATIONS IN ABSTRACT FORM RELATED TO THIS THESIS .................. XVI

LIST OF ABBREVIATIONS ........................................................................................................... XX

CHAPTER 1 .................................................................................................................................... 1

1.1 AGING OF THE CARDIOVASCULAR SYSTEM: AN EMERGING EPIDEMIC .................................... 2
1.2 CLINICAL CONSIDERATIONS .................................................................................................... 4
   1.2.1 Spectrum of aortic valve disease .................................................................................... 4
   1.2.2 Epidemiology of AS and ASc ...................................................................................... 5
   1.2.3 Genetics of AS/ASc ...................................................................................................... 5
   1.2.4 Anatomy and histology of the aortic valve in health and disease ................................. 8
   1.2.5 Detection, Experimental and Clinical Assessment of Aortic valve disease ................. 10
      1.2.5.1 Aortic stenosis ........................................................................................................ 10
      1.2.5.1.1 Physical examination .......................................................................................... 10
      1.2.5.1.2 Electrocardiogram ......................................................................................... 10
      1.2.5.1.3 Catheterization ............................................................................................... 11
      1.2.5.1.4 Echocardiography ......................................................................................... 11
      1.2.5.1.5 Computed tomography ............................................................................... 12
      1.2.5.1.6 Magnetic resonance imaging (MRI) ............................................................... 13
      1.2.5.2 Aortic sclerosis .................................................................................................... 14
      1.2.5.2.1 Clinical ........................................................................................................... 14
      1.2.5.2.2 Echocardiography: general ......................................................................... 14
      1.2.5.2.3 Echocardiography: backscatter .................................................................... 15
   1.2.6 Clinical Factors Associated with presence of AS/ASc .................................................... 16
   1.2.7 Left Ventricular Hypertrophy (LVH) in the context of AS/ASc ....................................... 18
   1.2.8 Factors associated with clinical progression of ASc/AS ................................................. 19
   1.2.9 Outcomes of AS ............................................................................................................ 22
   1.2.10 Association of ASc with coronary event risk ................................................................. 24
   1.2.11 Current Treatments in Clinical Practice ...................................................................... 25
      1.2.11.1 Medical therapy ............................................................................................... 25
      1.2.11.2 Aortic valve replacement ............................................................................... 26
      1.2.11.3 Percutaneous aortic valvuloplasty ................................................................. 28
      1.2.11.4 New technologies for AV replacement – percutaneous transcatheter aortic valve implantation (TAVI) ................................................................. 28
   1.3 MECHANISTIC CONSIDERATIONS ..................................................................................... 30
      1.3.1 Aging as a risk factor: cellular and molecular biology ................................................ 30
      1.3.2 Aortic valve mechanics - role of valvular endothelium and valvular interstitial cells (VICs) ........................................................................................................ 32
      1.3.3 Histopathological features of AS ................................................................................. 33
         1.3.3.1 Aortic valve interstitial cells (VICs) .................................................................... 33
         1.3.3.2 Extracellular matrix and fibrosis ...................................................................... 34
         1.3.3.3 Inflammation and lipid deposition .................................................................. 35
         1.3.3.4 Oxidative stress ............................................................................................... 36
         1.3.3.5 Calcification ...................................................................................................... 36
   1.3.4 Possible mechanisms for adverse outcomes associated with AS/ASc ................................ 38
      1.3.4.1 Links with atherosclerosis ................................................................................. 39
Abstract

Aortic valve stenosis (AS) is now the most common valve disease in Western world and its prevalence and incidence are rising. The earliest clinically detectable stage of this process, aortic valve sclerosis (ASc), reflects abnormal aortic valve morphology in the absence of haemodynamic obstruction, but may progress to AS. The prevalence of ASc is as high as 25% in populations over 65 years of age: thus it carries important epidemiological, clinical and pathophysiological implications. Despite the increased interest into studies of ASc/AS, the pathogenesis of this condition remains largely elusive, except to say that rather than the notion of being just a “wear and tear” inevitable process, it is now accepted to be an active pathophysiological process. The relevant literature is reviewed in Chapter 1.

Studies described in this thesis address the determinants of occurrence and progression of ASc in a cohort of aging subjects followed for 4 years. Novel methodology of aortic valve ultrasonic backscatter was utilized to quantitate ASc severity and progression. In the subsequent studies the effects of ASc on left ventricular hypertrophy (LVH) were evaluated in a separate cohort of healthy aging individuals, with no significant cardiovascular risk factors or hypertensive therapy. Finally, effects of aging on integrity of the nitric oxide (NO) signalling cascade were examined in the population cohort recruited for evaluation of progression of ASc.

The key findings from this thesis are:

1. Platelet NO responsiveness is a determinant of both the occurrence and progression of ASc, while age and BMI are determinant of occurrence only. Calcium levels and arterial stiffness correlate only with progression. Categorical assessment of progression reveals that use of inhibitors of the renin-angiotensin system is associated with lack of ASc progression.
(2) Whilst ASc is not correlated with the development of LVH in the absence of treated hypertension, markers of NO generation and of the NO/ cyclic GMP signalling cascade in the peripheral circulation predict both LV mass index and LV diastolic function in a normal, untreated, aging population, irrespective of ASc status.

(3) Aging is associated with both increases in ADP-induced platelet aggregation and plasma asymmetric dimethylarginine (ADMA) concentrations, and with reductions in platelet NO responsiveness. Female gender is associated with more severely impaired platelet NO responsiveness, greater arterial stiffness and a more pronounced fall in platelet NO responsiveness with time, which in turn was also observed in subjects with lower plasma vitamin D concentrations. There is a significant relationship between deterioration in platelet NO responsiveness and increases in ADMA concentrations. Finally, use of angiotensin convertin enzyme inhibitors/angiotensin receptor blockers is associated with preserved platelet NO responsiveness and lower arterial stiffness.

In summary, the aging process is associated with a remarkable degree of attenuation of NO generation and signalling, which constitutes both a correlate of ASc development/progression and of the development of LVH (although the latter is not closely associated with ASc in "normal" populations). Furthermore, the rate of deterioration of NO signalling is greatest in females, in the presence of low vitamin D levels and correlates with rises in ADMA concentrations.
Declaration

I, Aaron Leonid Sverdlov, certify that 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 when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below*) resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Aaron Leonid Sverdlov

April 2012
Published works in whole or in part contained within this thesis


Scholarships, Awards and Grants held currently

National Health and Medical Research Council of Australia Early Career Fellowship

Scholarships, Awards and Grants related to this thesis

1. Heart Foundation SA "E O Myers Trust Fund" Grant awarded in 2009.


5. Faculty of Health Sciences Divisional Scholarship, University of Adelaide.
Acknowledgements

First and foremost I would like to thank my main supervisor Prof John Horowitz. His guidance, encouragement and patience throughout my PhD have been more than I could hope for. He has been more than my supervisor, but my mentor both in work and in life. His enthusiasm for research and ability as a clinician-scientist are remarkable. I feel very privileged to have received such world-class training with him.

I would also like to thank Dr Yuliy Chirkov, my co-supervisor for his expert advice and his help in teaching me platelet aggregometry. Your precise ways and intellectual advice were most welcome. A/Prof Jennifer Kennedy, my other supervisor, provided a laboratory environment rich in translational research experience in aortic valve disease and for that I am grateful.

To my friend and colleague who is “suffering” through her own PhD and still found time to help me with mine: Dr Alicia Chan – thank you for all your help with EPC assay and some other laboratory experiments. Most importantly, thank you for our “soul soothing” chats to vent mutual frustrations! To my other friends and colleagues: Drs Angus Nightingale, Sharmalar Rajendran and Devan Mahadavan and– thank you for your support.

I am grateful to the echocardiography staff at the Queen Elizabeth Hospital for their help in performing detailed echocardiograms to my peculiar specifications at inconvenient hours. In particular my thanks go to Mr Ronald Wuttke, Ms Gina Velissaris and Mr Matthew Chapman.
I would like to thank Dr Ha Nguyen for help with the EPC assays, Ms Irene Stafford and Ms Tamila Heresztyn for running some of the ADMA samples. Furthermore, I would like to acknowledge help from Ms Sue Leslie and Nadine Smith, research nurses in helping me recall some of the subjects for follow up. I would also like to thank the staff of North Western Adelaide Health Study for their help with the initial recruitment of study subjects.

I would like to thank my parents, Irina and Leonid, for the way they brought me up and encouraged me to achieve my best; for their determination to bring the family to Australia so that their children can achieve their full potential in a country where ethnicity would not preclude one from achieving their best. My thanks also go to my grandparents Anna and Ilya for their encouragement, support and instilling me with strong family values. I would also like to thank my parents-in-law, Mai and Duc, for their support and help looking after my son during this journey!

Last, but most importantly, I would like to pay tribute to my wife Doan and son Joshua. Doan, your support, encouragement and advice have been tremendous and valuable especially as you have travelled this road before, getting your PhD 4 years ago. Doan has also paved the way for my research, having been the one who has done most of the work related to the initial evaluation of the patient cohort and helped with the follow-up studies. Your love and understanding have been my source of constant support. I am very lucky to have you by my side. I am particularly proud to have become a father during this journey: Joshua is truly the best thing that has happened to me and the source of endless happiness. This thesis is dedicated to you!
Personal Bibliography

Peer-reviewed full text publications arising from and/or related to the work conducted towards this thesis - published/in press


Peer-reviewed full text publications arising from and/or related to the work conducted towards this thesis - in submission.


* designates joint first authors

Publications/presentations in abstract form related to this thesis


   *Circulation 2011; 124 (21 Suppl): A12974*


   *Eur Heart J 2011; 32 (Suppl 1): 774.*


   *Heart Lung Circ 2011; 20 (suppl 2): S208.*


   *Eur Heart J* 2010; 31 (suppl 1): 121


   *Heart Lung Circ* 2010; 19 (suppl 2): S16


   *Heart Lung Circ* 2010; 19 (suppl 2): S52

8. Sverdlov AL, Ngo DT, Nightingale AK, Rajendran S, Heresztyn T, Horowitz JD. Plasma concentrations of asymmetric dimethylarginine (ADMA) predict LV mass independent of afterload. Oral presentation at the American Heart Association Scientific Sessions 2009, Orlando, USA.
9. **Sverdlov AL**, Ngo DT, McNeil JJ, Horowitz JD. *Diabetes is associated with paradoxically low plasma concentrations of asymmetric dimethylarginine (ADMA)*. Presented at the 4th International Symposium on ADMA 2008, Bregenz, Austria.

    *Heart Lung Circ 2008; 17 (suppl 3): S134*

    *Heart Lung Circ 2007; 16 (suppl 2): S69-70*

    *Heart Lung Circ 2007; 16 (suppl 2): S69*

    *Eur Heart J 2006; 27 (suppl 1): 743*
14. Ngo DT, Sverdlov AL, Willoughby SR, Nightingale AK, Chirkov YY, Horowitz JD.


*Heart Lung Circ 2006; 15 (suppl 1): S77*

15. Nightingale AK, Rajendran S, Mishra K, Sverdlov A, Ngo DT, Horowitz JD.

Vascular responses to GTN but not salbutamol decline with age. Presented at EUROECHO 9 – 2005, Florence, Italy.

*Eur J Echocardiogr 2005; 6 (suppl 1): S86*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACEI</td>
<td>ACE inhibitor</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of co-variance</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>apo</td>
<td>Apolipoproteins</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blockers</td>
</tr>
<tr>
<td>AS</td>
<td>Aortic stenosis</td>
</tr>
<tr>
<td>ASc</td>
<td>Aortic sclerosis</td>
</tr>
<tr>
<td>AVA</td>
<td>Aortic valve area</td>
</tr>
<tr>
<td>AVBS</td>
<td>Aortic valve ultrasonic backscatter score</td>
</tr>
<tr>
<td>AVp</td>
<td>Aortic valve pressure gradient (transvalvular pressure gradient)</td>
</tr>
<tr>
<td>AVR</td>
<td>Aortic valve replacement</td>
</tr>
<tr>
<td>AVv</td>
<td>Aortic valve velocity (transvalvular velocity)</td>
</tr>
<tr>
<td>BAV</td>
<td>Bicuspid aortic valve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CFR</td>
<td>Coronary flow reserve</td>
</tr>
<tr>
<td>CMs</td>
<td>Cardiomyocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CrCl</td>
<td>Creatinine clearance</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTx</td>
<td>C-terminal telopeptide of collagen type 1</td>
</tr>
<tr>
<td>DDAH</td>
<td>Dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow mediated dilatation</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
</tr>
<tr>
<td>HF/HC</td>
<td>High fat/high carbohydrate</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>High sensitivity CRP</td>
</tr>
<tr>
<td>HT</td>
<td>Hypertension</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLr -/-</td>
<td>LDL receptor deficient</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-Nitro-Arginine Methyl Ester</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla-protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide Adenine Dinucleotide (Phosphate) Hydrogen</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>P1NP</td>
<td>N-terminal peptide of procollagen I</td>
</tr>
<tr>
<td>PAI-I</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein arginine methyltransferase</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of Nuclear Factor κ B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor κ B ligand</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>TAVI</td>
<td>Percutaneous transcatheter aortic valve implantation</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin-interacting protein</td>
</tr>
<tr>
<td>VICs</td>
<td>Valvular interstitial cells</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 Aging of the cardiovascular system: an emerging epidemic

The increasing prevalence of aging people in our society represents the culmination of centuries of medical, scientific, and social advances. This population aging brings with it an “epidemic” of cardiovascular disease states, seen rarely in the past, including predominantly diastolic heart failure, atrial fibrillation, systolic hypertension and calcific aortic valve disease (reviewed by Lakatta et al., 2003b). The risk of adverse cardiac events in the elderly patients is further escalated by the fact that they are less likely to receive appropriate therapy, on account of general frailty (Afilalo et al., 2009). The emergence of these clinical disease states has a number of cellular counterparts (for review see Kovacic et al., 2011; Lakatta et al., 2003b; Shih et al., 2011): - increase in redox stress exposure and changes in signalling pathways with age alter the biology/physiology of cardiomyocytes. The progressive accumulation of metabolic waste and damaged organelles in cardiomyocytes increases the cell’s propensity toward apoptosis. Decreased cardiomyocyte renewal capacity in the elderly, due to reduction in cellular division and impaired stem cell function, leads to further cardiac dysfunction and maladaptive responses to disease.

Aging is also accompanied by changes in vascular structure and function, especially in the large arteries, which affect the heart and other organs (for review see Safar, 2010). Essentially, arterial aging can be attributed to two different pathophysiological changes — increase in arterial stiffness and disturbed wave reflections. The capacity of the aorta to absorb the force exerted by the left ventricular ejection and dampen pulsatile flow becomes diminished with advancing age, owing to the progressive hardening of the arterial wall. These changes contribute to increase blood pressure, mainly systolic blood pressure and pulse pressure, which can trigger cardiovascular events.
Furthermore, aging-associated "degenerative" changes occur frequently within the mitral and aortic valves, with particularly severe consequences when they result in aortic valve narrowing (Braunwald et al., 2003). The latter is a major focus of this thesis.
1.2 Clinical considerations

1.2.1 Spectrum of aortic valve disease

As with any valvular heart disease there are 2 main types of aortic valve dysfunction: stenosis and regurgitation. Pure or predominant aortic valve regurgitation is beyond the scope of this thesis: its aetiologies are very diverse and in Western world the most common aetiology is degenerative (Iung et al., 2003). Its incidence, however, is on the decline. On the other hand aortic valve stenosis (AS) is now the most common valve disease in Western world and its incidence is rising (Iung et al., 2003; Lindroos et al., 1993). In general, AS reflects a 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 called aortic valve sclerosis (ASc), which implies the presence of abnormal aortic valve morphology in the absence of haemodynamic 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. Other rare causes are: familial hypercholesterolaemia, hyperuricemia, hyperparathyroidism, Paget's disease, ochronosis, Fabry disease, systemic lupus erythematosus, and drug-induced diseases (Braunwald et al., 2003). Bicuspid aortic valve is a relatively common anomaly, affecting approximately 1% of the general population, with most patients subsequently developing aortic valve calcification by the age of 30 (Yener et al., 2002); however this thesis will not specifically address 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 is of major importance.
1.2.2 Epidemiology of AS and ASc

Calcific aortic valve disease is the commonest form of valvular heart disease in the Western world. Its prevalence increases exponentially with age and varies between studies: generally aortic stenosis is present in 2-7% of all patients over 65 years of age, whilst aortic sclerosis occurs in about 25% of these patients, and in as many as 50% of those over the age of 84 years (Lindroos et al., 1993; Otto et al., 1999; Stewart et al., 1997). Novaro et al (2007) in a follow up of the Cardiovascular Health Study, documented that about 9% of individuals with ASc will progress to AS over a 5 year period.

With recent medical advances resulting in increased longevity, the prevalence of AS is expected to rise significantly in the near future (Cowell et al., 2004). In fact, one study found that 50% of patients admitted to hospital with chest pain had ASc (Chandra et al., 2004). Therefore, the health and socioeconomic burden associated with AS is likely to increase substantially.

1.2.3 Genetics of AS/ASc

Bicuspid aortic valve is a common congenital abnormality, present in 1-2% of the population, with majority of affected individuals progressing to AS over the course of their lifetime (Yener et al., 2002). Subjects with a bicuspid valve are more likely to develop AS during their lifetime, and if so, usually develop AS 1 or 2 decades earlier than those with a tricuspid valve (see Figure 1.1). The exact genetic defect underlying this condition has not been elucidated yet, but it is clearly heritable and a few potential loci have been identified in selected families (for review see (Bosse et al., 2008). Furthermore, a few studies, mainly in France, have found geographical clustering of non-bicuspid cases beyond that could be explained by chance alone, further supporting the hypothesis that many forms of AS are subject to genetic predisposition (reviewed by (Bosse et al., 2008).
Whilst no other inherited AS diseases have been identified, a number of mutations that increase the risk of AS development has been observed. Ortlepp et al. (2001) found a significant association between the B allele of the vitamin D receptor BsmI and AS in a case-control series of the vitamin D receptor polymorphisms in 100 patients with moderate AS. This polymorphism is commonly associated with rapid bone loss and increased osteoporosis, providing one of the few instances of clear dissociation between factors linked to the development of AS and atherosclerosis. Vitamin D also increases expression of thioredoxin-interacting protein (TXNIP) (Han et al., 2003), a fundamental mediator of increased redox stress (Junn et al., 2000), which in turn is associated with activation of apoptosis signalling pathways (Xiang et al., 2005). The implications of this vitamin D–TXNIP relationship will be discussed further in section 1.3.5.

Specific polymorphisms of apolipoprotein (apo) B (X+X+) and E (E2), the genes associated with dyslipidaemia and development of atherosclerosis, were also associated with AS in a small case-control study (Avakian et al., 2001). A larger study of 802 patients found similar association of apo-E 2/4 and 3/4 genotypes and occurrence of AS (Novaro et al., 2003). In the first of these studies patients were matched for lipid profile, and in the Novaro et al (2003) study, association was independent of lipid profile, age, gender and coronary disease on multiple regression analysis. This suggests that the association between AS and polymorphism of these apolipoproteins may not have been due to dyslipidaemia per se.

Another small case-control study (Nordstrom et al., 2003) identified PvuII polymorphism of the estrogen receptor (ER) α gene being independently associated with increased risk of
ASC. This polymorphism was also associated with levels of total cholesterol and low density lipoprotein (LDL), implying potential confounding mechanisms.

Finally, 3 promoter polymorphisms of IL-10, a promoter polymorphism in connective tissue growth factor and a 32-basepair deletion in the chemokine receptor 5 genes were associated with a degree of valvular calcification in an atomic absorption spectroscopy study of 187 surgically excised aortic valves (Ortlepp et al., 2004).

The major limitation of most of these genetic studies, however is their associative nature and lack of pathway identification as well as small number of subjects, which could give false positive results.

A recent major breakthrough was the discovery of NOTCH1 gene and signalling cascade aberration in a family with 5 generations of members affected by aortic valve disease (Garg et al., 2005). The authors identified a specific mutation (R1108X) present in all affected but in none of the unaffected family members or over 1100 unrelated subjects. NOTCH1 is involved in embryonic patterning and osteoblast development and this may provide further insight into mechanisms of valvular calcification.

Thus, there is convincing evidence supporting the hypothesis that, in at least in a subset of individuals, genetic predisposition co-exists with environmental factors to create a biological substrate prone to early calcification of the aortic valve.
1.2.4 Anatomy and histology of the aortic valve in health and disease

Normally, the aortic valve has 3 semilunar cusps attached to the fibrous valve ring. The cusps are named according to their anatomical position as left, right and non- coronary cusps. Aortic valve area in an adult is 2-4cm². Normal leaflets are devoid of blood vessels, covered with endothelium, and consist of 3 layers: ventricularis on the ventricular side; fibrosa on the aortic side of the leaflet and spongiosa in between. The ventricularis is composed of aligned elastin fibers; the spongiosa consists of loose connective tissue, including fibroblasts, mesenchymal cells, and a mucopolysaccharide-rich matrix, and the fibrosa contains collagen fibers, which allows it to cope with greater shear stress (Freeman et al., 2005; Otto et al., 1994). All layers are populated by valve interstitial cells (VICs), maintaining leaflet integrity (reviewed by (Freeman et al., 2005)).

Normally, VICs secrete extracellular matrix (ECM) components such as collagen, fibronectin and glycosoaminoglycans (Schoen, 1997), as well as ECM degrading enzymes such as matrix metalloproteinases (Dreger et al., 2002; Soini et al., 2001). Upon aortic valve injury, VICs have the ability to differentiate to contractile myofibroblasts (Yip et al., 2009) and respond to a variety of biochemical stimuli, including serotonin (5-HT), endothelin (ET)-1 and NO in a manner similar to those of vascular smooth muscle cells (Filip et al., 1986). Myofibroblast activation is regulated tightly by cytokines that control differentiation, proliferation, contraction, ECM secretion, and migration to the site of wound healing or tissue remodeling (Powell et al., 1999). During physiological remodelling, myofibroblasts are eliminated by apoptosis (Desmouliere et al., 1995). However, when the myofibroblast life cycle is dysregulated, myofibroblasts persist, with continued force generation and ECM production, resulting in pathological fibrosis, scarring, and fibrocontractile disease (Desmouliere, 1995).
In diseased aortic valves, studies showed denudation of the endothelial cell layer, with platelets, leukocytes, and scattered erythrocyte adhesions were seen on the exposed subendothelial surface (Harasaki et al., 1978; Riddle et al., 1980). Physiologically, Pompilio et al (1998) showed that intact swine aortic valve significantly contracted to phenylephrine, and responded to acetylcholine-induced endothelium-dependent relaxation. Denudation of the endothelial layer significantly retarded the acetylcholine-induced relaxation. Furthermore, the presence of intact aortic valve endothelium was associated with significantly greater release of prostacyclin compared to those without the endothelium. These effects in swine aortic valves were also seen with canine aortic valves (Ku et al., 1990). In addition, in a porcine ex vivo aortic valve study, El-Hamamsy et al (2009) recently demonstrated the physiological role of the aortic valvular endothelium in regulating aortic valve mechanical properties. This study found that, under physiological conditions, aortic valve contractile responses were predominantly VIC-mediated. Interestingly, the addition of L-NAME or endothelial denudation did not alter valvular tone under basal conditions. Upon stimulation with serotonin, in the presence of intact endothelium, a reduction in tissue stiffness was observed, while the addition of L-NAME or endothelial denudation led to a significant increase in cusp areal strain. Therefore, loss of these adaptive mechanisms due to endothelial dysfunction (or the absence of endothelium in a damaged valve) could result in abnormal biomechanics, structural damage, and exacerbation of disease progression in AS. This study, however, did not examine the effects of shear stress, which may exert additional effects on valve mechanics, especially under basal conditions.

Overall, these studies demonstrated that the aortic valve endothelium is capable of relaxation and contraction to physiological stimuli, and the data suggest a role in the maintenance of valvular homeostasis. Furthermore, the valve endothelium, like
endothelium elsewhere, exerts anti-aggregatory and anti-inflammatory effects in a paracrine manner.

1.2.5 Detection, Experimental and Clinical Assessment of Aortic valve disease

1.2.5.1 Aortic stenosis

1.2.5.1.1 Physical examination

Detection of a typical murmur of AS on physical examination is usually the first step in diagnostic evaluation of this condition. Diagnosis of AS on clinical examination is relatively accurate (Roldan et al., 1996), however assessment of the lesion severity is less accurate (Jaffe et al., 1988). Generally, the signs of severity of AS on physical examination are: murmur peak intensity timing (the later the peak, the more severe the lesion), presence of diastolic murmur, presence of signs of left ventricular decompensation, delay in carotid pulse upstroke, presence of systolic thrill, reverse splitting and audibility of second heart sound (Braunwald et al., 2003). A study by Munt et al. (1999) of 123 patients with AS concluded that no single physical examination finding or combination of findings had both a high sensitivity and specificity for detection of severe valvular aortic stenosis, and combinations of physical examination findings were no better, despite statistically significant (but not clinically useful on a large scale) correlations between some of the physical findings with echocardiographical measures of severity.

1.2.5.1.2 Electrocardiogram

LV hypertrophy by voltage criteria is seen in as many as 80% of patients with severe AS, but is not specific and cannot be used alone for diagnosis.
1.2.5.1.3 Catheterization

With the emergence of echocardiography, AS progression and severity are no longer assessed based on catheter-derived haemodynamic data. Its utility mainly lies in clinical workup of patients for valve replacement surgery as means of confirmation of echocardiographic assessment of severity, where such assessment is equivocal, and as a means of simultaneous imaging of coronary arteries to help with decision-making.

1.2.5.1.4 Echocardiography

Transthoracic echocardiography, including Doppler measurements is the standard imaging modality in the assessment of AS severity and subsequent decision-making regarding its management. Beyond assessment of valve morphology, doppler measurements allow noninvasive assessment of haemodynamic factors predictive of AS severity (reviewed by (Freeman et al., 2005; Mochizuki et al., 2003). The rate of progression, expressed as rate of increase of transvalvular pressure gradient or rate of decrease of aortic valve area (AVA) per year of follow-up, is generally calculated by the Bernoulli and Gorlin equations respectively. The mean annual increase in aortic valve pressure gradient (AVp) is about 5-7mmHg per year in patients with moderate AS, with an associated decrease of 0.1 cm$^2$ per annum in aortic valve area (Bahler et al., 1999; Faggiano et al., 1996; Faggiano et al., 1992; Nassimiha et al., 2001; Otto et al., 1997). 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 be quantitated by Doppler echocardiography. However, the echocardiographic severity does not necessarily translate to symptoms and thus echocardiography cannot predict which patients are most likely to benefit from AVR prior to the development of symptoms.
1.2.5.1.5 Computed tomography

Electron beam computed tomography (EBT), a cross-sectional imaging technique with high temporal resolution, has been used to assess valve calcification in AS (Kaden et al., 2002; Pohle et al., 2001). In particular, the study by Kaden et al (2002) also demonstrated good correlation between EBT calcification area and aortic valve area assessed by echocardiography. However, this study was small with 40 subjects with various degrees of AS severity. In a larger EBT study (Messika-Zeitoun et al., 2004), calcium scoring correlated well with AVA derived from echocardiography, but the relationship was not linear, suggesting that the parameters are complimentary and not interchangeable. In a study of 104 patients, progression of aortic valve calcification, quantitated by EBT, correlated with serum levels of LDLs (Pohle et al., 2001). 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 also possible to use EBT in intervention studies to find treatments that could slow down calcification of the aortic valves.

Computed tomography (CT) with calcium scoring has been widely used as a noninvasive method of screening for atherosclerotic CAD, with estimated 80%-100% sensitivity and 80% specificity (Breen et al., 1992; Haberl et al., 2001; Mautner et al., 1994), although it has now been largely replaced by CT coronary angiography. Aortic valve calcification can also be quantitated with this technique.

More recently, multislice CT (MSCT) has been evaluated for this purpose, as it uses less radiation per scan than EBT and has very good spacial resolution (Feuchtner et al., 2006). MSCT calcium scoring has been shown to correlate well with echocardiographic/haemodynamic measures of AS severity in a few studies (Cueff et al., 2011; Feuchtner et al., 2006; Pouleur et al., 2007), yet assessment of progression of AS
using this technique has not yet been validated. Furthermore, its utility at the early stages of AS is questionable, as some studies combine mild and moderate AS into the same category (Cueff et al., 2011), or include patients with predominantly moderate and severe disease (Ropers et al., 2009), when using MSCT.

The main disadvantage of all CT-based techniques is that they do not allow assessment of functional status of the aortic valve, especially EBT, which does not even allow valve visualization. Thus they are more suitable for following the latter stages of AS, when calcium deposition is the main feature, compared to the ASc stage where inflammation, matrix remodelling, and fibrosis predominate (Freeman et al., 2005). An additional important consideration is the radiation exposure that comes with these techniques, especially if used on multiple occasions for assessment of disease progression. Further limitations of the technique are: need for patients to be in sinus rhythm with heart rates under 80bpm, because of ECG gating; and unsuitability of this contrast-based technique for patients with renal impairment.

1.2.5.1.6 Magnetic resonance imaging (MRI)

In some patients echocardiography utility is limited due to a number of operator and patient-related technical issues as well as inability to accurately estimate transaortic flow due to eccentric jet morphology (Bartunek et al., 1995; Danielsen et al., 1989; Fischer et al., 1995). Cardiac magnetic resonance imaging (MRI) allows planimetry of the aortic valve independent of haemodynamic status and flow turbulence, and even in the presence of aortic valve endocarditis and peri-valvular abscess allows accurate anatomical assessment of the valve (Sverdlov et al., 2008). Several small studies have shown that MRI offers high spatial resolution analysis of cardiac anatomy and myocardial function. AVA measurements, transaortic velocity (AVv) and AVp are in strong agreement with other
methods of quantitating severity of AS (Caruthers et al., 2003; Friedrich et al., 2002; John et al., 2003; Kupfahl et al., 2004; Malyar et al., 2008). Pouleur et al (2007) found that MRI and MSCT were comparable for AVA assessment in a small group of normal subjects and those with AS. The main advantage of MRI over MSCT is lack of radiation exposure. Although MRI is a superior technique in quantitation and visualization of cardiac anatomy (Pennell et al., 2004), echocardiography remains the “gold standard” for quantitating severity of AS, as it allows physiological as well as anatomic assessment. Furthermore, at the current time, MRI cannot be used to quantitate ASc.

1.2.5.2 Aortic sclerosis

1.2.5.2.1 Clinical

Essentially diagnosis is based on the detection of ejection systolic murmur in the aortic area, with minimal or no radiation, and absence of any other feature of severity of AS ((Roldan et al., 1996) and section 1.2.5.1.1). However, serial evaluations have shown that such clinical findings are only moderately reproducible, even in otherwise healthy men (Bodegard et al., 2011).

1.2.5.2.2 Echocardiography: general

The presence of ASc by definition must exclude the presence of haemodynamic changes, such as obstruction to blood flow. The largest study of ASc to date (Otto et al., 1999), 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. In both of these large clinical studies ASc assessment was purely subjective. However the latter study (Cosmi et al., 2002) also
classified ASc severity based on visual detection of calcification of the short axis view on echocardiogram, according to the following 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., 2004b; Shively et al., 1998). This technique of classification of ASc severity is highly operator/assessor, as well as image quality dependent. Furthermore, there has been no study examining the serial operator/assessor reproducibility. Intra-observer reproducibility 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 only semi-quantitatively using this system.

1.2.5.2.3 Echocardiography: backscatter

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 haemodynamic changes occur.
Ngo et al. (2004) developed a quantitative method of assessment of ASc development based on ultrasonic backscatter technique, which 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).

Ngo et al. (2004) demonstrated that the mean levels of aortic valve ultrasonic backscatter (AVBS) in patients with ASc are approximately 60% greater than in healthy young adults (≥16 dB were taken as being diagnostic of ASc) and that AVBS scores in patients with ASc correlate with subjective scoring of sclerosis and with AVp in patients with mild-moderate AS (Figure 1.2). Furthermore the AVBS measurements were reproducible, with mean differences between estimates on the basis of repeated echocardiograms of 2.3 ± 1.7 (9.1% ± 6.2). This method of assessment of aortic valve echogenicity was subsequently used in an animal model of AS (Ngo et al., 2008) and facilitated demonstration of improvements in AVBS with ramipril therapy (Ngo et al., 2011).

1.2.6 Clinical Factors Associated with presence of AS/ASc

Early studies identified a number of clinical factors associated with the presence of calcific aortic valve disease (not always distinguishing between stenosis and sclerosis). These include age, body mass index, smoking, hypertension, dyslipidaemia, diabetes and lipoprotein(a) levels (Aronow et al., 1987; Deutscher et al., 1984; Gotoh et al., 1995; Hoagland et al., 1985; Lindroos et al., 1994; Mohler et al., 1991). In the later, and much larger, Cardiovascular Health Study (Stewart et al., 1997) the risk factors independently associated with calcific aortic valve disease (mainly ASc) were age, male gender,
lipoprotein(a) levels, height, hypertension, smoking and LDL levels, all of which remained as significant correlates of ASc only. More recently presence of metabolic syndrome and diabetes mellitus were found to be associated with increased risk of aortic valve calcification, as assessed by CT calcium scoring (Katz et al., 2006). Furthermore aortic valve calcification prevalence was increased with increasing number of metabolic syndrome components. The limitations of this large study are that it did not distinguish between AS and ASc and that subjects at high cardiovascular risk or known cardiac disease were excluded. Thus this population is not truly representative of "average" Western populations.

Patients with ASc and AS on oral anticoagulants (vitamin K antagonists) were more likely to have high aortic valve calcification as measured by CT scanning (Koos et al., 2005). 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 inhibits matrix Gla protein, a vitamin K-dependent, potent inhibitor of calcification (reviewed by (Proudfoot et al., 2006). This finding is supported by animal studies of warfarin (vitamin K antagonist), inducing rapid and extensive calcification of the elastic lamellae of large arteries in the aorta and aortic valves (Howe et al., 2000; Price et al., 1998).

Incidence of AS is also higher in people with concomitant severe renal disease (Maher et al., 1987; Michel, 1998; Ribeiro et al., 1998). It has been postulated that this is related to abnormal calcium-phosphate metabolism and elevated levels of calcium-phosphate product (Raine, 1994). Abnormal calcium-phosphate product, even in patients with normal renal function, was associated with greater severity of AS (Mills et al., 2004). Subjects with
other diseases, characterized by abnormal calcium metabolism, including Paget's disease and hyperparathyroidism have also been shown to have higher incidence of AS (Stefenelli et al., 1993; Strickberger et al., 1987). However, these data are of course not equivalent to a demonstration of causative mechanism(s).

1.2.7 Left Ventricular Hypertrophy (LVH) in the context of AS/ASc

Ventricular hypertrophy is a major structural adaptive mechanism in response to pressure overload as it normalizes wall stress (Grossman et al., 1975). Yet this adaptation leads to a number of adverse consequences: increased intra-myocardial collagen deposition and reduction in LV compliance, leading to limitation of preload reserve and myocardial ischaemia, in part due to extramural pressure on small coronary arteries, even in the absence of fixed coronary artery disease. It is thought to be caused by a combination of increased myocardial oxygen demand and limitation of coronary blood flow (Strauer, 1979a; Strauer, 1979b). LV systolic performance may be impaired (even if contractility is normal) due to afterload mismatch, leftwards shift of the ventricular preload on the Starling curve, or asynchrony of the temporal sequence of contraction (Braunwald et al., 2003). Late in the course of the disease, the cardiac output declines, whereas the pressures in the left atrium and pulmonary artery rise, leading to dyspnoea.

At the first glance, the relationship between AS and development of LVH is superficial: aortic valve obstruction in AS develops gradually and imposes a pressure overload on the LV, which subsequently causes the development of progressive concentric hypertrophy (Braunwald et al., 2003). However, in practice the occurrence of AS in ageing individuals who have a high prevalence of systolic hypertension renders this subject quite complex.
The relevant biochemistry of LVH development is reviewed in section 1.3.4.4 and chapter 3 of this thesis.

1.2.8 Factors associated with clinical progression of ASc/AS

While clinical progression of AS, as measured by changes in aortic valve area or transvalvular gradient, is non-linear, with apparent acceleration later in the course of disease, the histopathological progression is much more difficult to evaluate in humans and its rate may not be analogous to that of clinically-detectable changes. Table 1.1 summarizes some of the important studies of progression of ASc/AS. One of the first prospective studies of progression of asymptomatic AS reported annual increase in aortic jet velocity of ~0.3m/s and decrease in valve area of 0.1cm² (Otto et al., 1997). This study was not powered to look at clinical factors and found that greater transvalvular velocity and smaller AVA were associated with more rapid progression of AS, as measured by changes in AVA. A large (n > 2000) study of patients with ASc demonstrated 10.5% development of mild AS, 3% of moderate AS and 2.5% of severe AS over a 7 years follow up period (Cosmi et al., 2002). A smaller retrospective study showed a 33% rate of development of AS in 400 patients with ASc over nearly a 4 year period (Faggiano et al., 2003).

Despite similarities in clinical factors associated with AS and atherogenesis, factors underlying progression are not identical. The largest study to date, a follow-up of the Cardiovascular Health Study, involving more than 5600 subjects, of whom 1610 had ASc and 94 had AS, demonstrated 9% progression from ASc to AS, 1% incidence of AS and 44% incidence of ASc over 5 year mean follow-up period (Novaro et al., 2007). The authors found that increasing age, male gender, African-American ethnicity and increases
in low-density lipoprotein cholesterol levels were significant predictors of new
development of AS and ASc (combined). Similarly, progression from ASc to AS was
significantly associated with male gender, advancing age and African-American ethnicity.
Neither history of diabetes, hypertension, or tobacco use, nor the presence of coronary
heart disease or renal insufficiency were independent predictors of progression; CRP levels
were also not associated with development or progression of calcific aortic valve disease
(Novaro et al., 2007). Endothelial and platelet function or other measures of
inflammation/fibrosis were not evaluated in this study. The factors included in multivariate
analysis were age, gender, ethnicity, diabetes mellitus, hypertension, current smoking,
height, CRP, renal insufficiency, and prevalent coronary heart disease.

One of the correlates of rapid progression of AS identified so far is renal dysfunction,
especially end stage renal disease requiring dialysis (Faggiano et al., 1996; Maher et al.,
1987; Perkovic et al., 2003; Wongpraparut et al., 2002); with odds ratio of 2.47 for rapid
progression in the cohort on hemodialysis (Wongpraparut et al., 2002). It is not only
significant renal dysfunction per se that is associated with rapid progression in these
patients - high plasma vitamin D₃ levels in dialysis patients have also been associated with
accelerated progression of AS (Malergue et al., 1997; Urena et al., 1999). However, renal
dysfunction has never emerged as a risk factor in unselected patient populations, and it is
uncertain to what extent this correlation is relevant only in severe renal dysfunction.
Furthermore, the biochemical correlates of this association are incompletely explored (see
future sections).

As regards "conventional" cardiovascular risk factors, results of the studies are less clear
cut. Some have documented that presence of diabetes, hypertension, dyslipidaemia and
coronary artery disease are associated with more rapid progression (Aronow et al., 2001;
Nassimiha et al., 2001; Palta et al., 2000), while others have not (Bahler et al., 1999; Messika-Zeitoun et al., 2007; Novaro et al., 2007). Whilst the history of diabetes, hypertension and obesity were associated with presence of aortic valve calcification, after adjustments for age and gender, only calcification score was predictive of faster progression in a recent electron-beam CT study (Messika-Zeitoun et al., 2007). Yet, dyslipidaemia was related to incidence of new aortic valve calcification in that study. However, renal function, vitamin D, endothelial function or calcium metabolism were not assessed. A small (n=107) retrospective study (Briand et al., 2006) found that presence of metabolic syndrome, as defined by National Cholesterol Education Program-Adult Treatment Panel III criteria (2001), is associated with a faster disease progression and worse outcome in patients with at least moderate AS at the onset of the study.

Thus, the putative risk factor that has attracted the most controversy is that of dyslipidaemia. There is considerable evidence for the involvement (as distinct from pivotal importance) of cholesterol and other lipoproteins in pathogenesis of AS and ASc (reviewed by (Rajamannan, 2009), although not every study reported such an association (Bahler et al., 1999; Messika-Zeitoun et al., 2007; Wongpraparut et al., 2002); yet none of the prospective, randomized, placebo-controlled trials to retard the progression of AS via cholesterol lowering have been successful (Chan et al., 2010; Cowell et al., 2005; Rossebo et al., 2008).
1.2.9 Outcomes of AS

Ross and Braunwald (1968) were amongst the first to describe the natural history of untreated AS, as a slow, progressive disease, spanning decades. They identified the three hallmark symptoms of advanced AS: angina pectoris, syncope and dyspnoea, and linked these to worsened outcome (Figure 1.3). This was further supported in a later study by Turina et al (1987). However, these studies were primarily designed to demonstrate differences in prognosis with valve replacement and were non-randomised. Even more importantly, the average ages of the patient groups in these cohorts at time of clinical presentation were 48 and 45 years respectively (Ross et al., 1968; Turina et al., 1987). Therefore it is likely that many of these cases were of AS superimposed on bicuspid valve, where the natural history is of presentation at a younger age than with tricuspid aortic valves (Yener et al., 2002).

More recently, a study of 123 patients, with a mean follow-up period of 2.5 years, by Otto et al. (1997) stratified the relationship between peak aortic transvalvular jet velocity and emergence of symptoms/cardiac events. The predominant aetiology of AS in this study was calcific, however, 29% of subjects had rheumatic and bicuspid aetiologies. Although the risk factors for progression could not be determined from this study, baseline jet velocity, functional status of the patients and rate of change of jet velocity were predictive of clinical outcome (death or valve replacement) (Figure 1.4) (Otto et al., 1997). However, it is clear that a study of this type carries an obvious inherent bias, unless intervention is based purely on severity of symptoms.

For patients with jet velocities greater than 4 m/s at entry into study the likelihood of remaining alive without valve replacement at 2 years was only 21±18%. Interestingly, age, sex, cause of aortic stenosis, co-morbid disease (hypertension, renal disease, diabetes),
smoking history, or coexisting coronary artery disease were not predictors of this outcome (Otto et al., 1997).

More recently, other studies have also shown that mild to moderate AS is not a completely benign disease, as it is associated with a substantial mortality rate (Rosenhek et al., 2004a). Progression to severe stenosis may be quicker than previously assumed; however, this only partially accounts for the high mortality rate as over 50% of the deaths were not due to advanced aortic stenosis per se. The mechanism of this association is not clear and could possibly be related to the presence of atherosclerosis and endothelial dysfunction in the entire cardiovascular system.

The main predictors of outcome derived from echocardiography are peak aortic jet velocity, rapid increase in peak velocity and moderate to severe calcification (Otto et al., 1997; Rosenhek et al., 2000). In the study by Rosenhek et al (2000), the combination of moderate to severe calcification and rapid increase in peak velocity identified 79% of patients who either underwent surgery or became symptomatic within 2 years. An abnormal exercise capacity was also identified as a strong predictor of poor outcome: the probability of a patient with a positive stress test surviving event free after 24 months was only 19% compared with 85% in those with a negative test (Amato et al., 2001).

In addition to these valve-related outcome evaluations, patients with severe AS undergoing non-cardiac surgery have approximately fivefold increased risk of perioperative mortality and non-fatal myocardial infarction independent of risk factors for coronary artery disease (Kertai et al., 2004). The intriguing finding is that of non-fatal myocardial infarction, which implies some association between AS and the process of atherothrombosis.
Another recognized phenomenon associated with advanced AS is modification and proteolysis of von Willebrand’s factor via shear stress, leading to the loss of the largest multimers of von Willebrand factor - acquired type 2A von Willebrand syndrome (Veyradier et al., 2001; Warkentin et al., 1992). This leads to the increased bleeding risk, particularly that due to gastrointestinal angiodysplasia (so called "Heyde’s syndrome") (King et al., 1987). This anomaly is corrected by AVR, provided prosthesis size is adequate (Vincentelli et al., 2003).

### 1.2.10 Association of ASc with coronary event risk

Historically the presence of ASc, usually diagnosed by incidentally finding an ejection systolic murmur, was thought to be "innocent", apart from the uncertain chance of its progression to aortic stenosis. In 1999, 2 large studies have found that patients with ASc had a higher incidence of cardiovascular events (Aronow et al., 1999; Otto et al., 1999). Specifically, Aronow et al (1999) in a cohort of over 2000 patients found that subjects with ASc had 1.8 times higher chance of developing new coronary events than those without ASc, and in patients with pre-existent symptomatic CAD that chance was 2.8 times higher than in patients with CAD but without ASc. Furthermore, 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).

These results have also been replicated in a very recent study of over 2000 patients with aortic valve calcification: - both all-cause and cardiovascular mortalities were higher over an 8.6 year follow-up period in those with ASc (Volzke et al., 2010). Furthermore, each unit increase in sclerosis score was associated with further increase in mortality. Olsen et al
(2005) in a group of 960 (mean age 55 to 80 years) hypertensive subjects with ASc, demonstrated that they had almost twice the risk for serious CV events during a mean follow-up of 60 months.

It is therefore clear that the presence of ASc is associated with adverse cardiovascular outcomes, due to coronary events. However the mechanism(s) underlying these associations have received little attention to date.

**1.2.11 Current Treatments in Clinical Practice**

**1.2.11.1 Medical therapy**

No medical therapies that have been demonstrably successful in reducing progression of AS in humans. However trials of this type are limited to the 3 interventions with various cholesterol-lowering therapies summarized in Table 1.2. Furthermore, in all cases, these interventions were undertaken when patients already had at least mild-to-moderate AS. Notably, there have been no interventions: -

1. in ASc
2. with other agents in mild/moderate AS
3. in high risk patients with early AS and chronic renal failure

All of these are substantial gaps in current therapeutics and will be discussed further in this thesis.

As regards advanced **AS with symptoms**, some attempts at medical treatment have emerged, but to a very limited extent. Perhexiline, a carnitine palmitoyltransferase inhibitor (Kennedy et al., 1996), which exerts anti-anginal effects without significant negative inotropy, bradycardia, or hypotension (Lee et al., 2005), has been also shown to improve
efficiency of myocardial oxygen utilization (Jeffrey et al., 1995; Unger et al., 2005) and exerts anti-inflammatory effects in aortic valve matrix (Kennedy et al., 2006). A small study (n=15) showed that perhexiline improves symptomatic status in elderly patients with inoperable severe aortic stenosis (Unger et al., 1997). Over 30 months' follow-up symptomatic status improved in 13 of the 15 patients over the first three months of perhexiline therapy (p < 0.01), and five patients became asymptomatic. Twelve month actuarial survival was 80%. Improvement in symptomatic status occurred both in patients with and without concomitant coronary artery disease (Unger et al., 1997). Larger controlled trials are needed to determine whether long-term perhexiline therapy may also alter the natural history of the disease and thereby reduce the need for aortic valve replacement.

Furthermore, a single study with intravenous infusion of sodium nitroprusside suggested that this was useful in pre-operative stabilization of decompensated patients with AS (Khot et al., 2003). However, it must be noted that the mean arterial pressure in this patient cohort was 81 ± 13 mmHg, suggesting that decompensation did not border on onset of cardiogenic shock, but only pulmonary congestion.

Until such time, the mainstay therapy is reduction in general cardiovascular risk profile and then surgical/interventional management of severe and/or symptomatic AS.

1.2.11.2 Aortic valve replacement

Aortic valve replacement surgery is the definitive therapy for severe AS and is now the second most common indication for cardiac surgery after coronary artery grafting (Lloyd-Jones et al., 2009). It uses either a mechanical or a bioprosthetic valve. Data from USA suggests that the mean estimated cost for each surgery is just over $140,000 (Lloyd-Jones
et al., 2009), which seems excessive, although exact costs contributing to this estimation are unclear.

Recent advances in surgical techniques and perioperative care led to substantial improvements in aortic valve replacement outcomes between 1996 and 2007: mortality has decreased by approximately 24%, stroke risk by 27% despite population risk profile being worse, particularly increasing age of patients, undergoing surgery (Brown et al., 2009). In fact, a recent study suggests that surgical outcomes in octogenarians are similar to those in younger patients if major comorbidities (renal failure, previous cardiac surgery, pulmonary hypertension, concomitant coronary artery grafting and female gender) are taken into account (Carnero-Alcazar et al., 2010). Increased operative mortality is associated with older age, multiple comorbidities, female gender, lower functional class, emergency operation, LV dysfunction, pulmonary hypertension, coexisting coronary disease and previous bypass or valve surgery. In patients with severe coronary disease the performance of concomitant bypass surgery approximately doubles operative mortality; however, these figures compare favourably with mortality in patients with coronary disease who did not undergo combined bypass surgery (Brown et al., 2009; Iung, 2000; Iung et al., 2003; Mullany et al., 1987).

After successful valve replacement, long-term survival rates are close to those expected for control populations, symptoms are less marked and quality of life is greatly improved (Kvidal et al., 2000). Risk factors for late death include age, comorbidities, severe functional condition, irreversible myocardial damage (which could also be due to myocardial scarring after infarction or ischaemic cardiomyopathy), ventricular arrhythmias and untreated coexisting coronary artery disease. In addition, poor postoperative outcome
may result from prosthesis-related complications or sub-optimal prosthetic valve haemodynamic performance.

1.2.11.3 Percutaneous aortic valvuloplasty

Percutaneous aortic valvuloplasty was first described by Cribier et al in 1986 (1986). Mortality and morbidity of the procedure are high and its efficacy is limited as final valve area is only between 0.7 and 1.1 cm². Furthermore, it probably does not change the natural course of the disease and it is now predominantly used as a palliative measure (Cribier et al., 2004) or as a bridge to valve replacement.

1.2.11.4 New technologies for AV replacement – percutaneous transcatheter aortic valve implantation (TAVI)

Whilst surgical bioprosthetic AVR offers excellent long-term survival (>95% at 5 years and about 90% at 10 years), these data are largely based on relatively younger patients and/or those without significant comorbidities (Jamieson et al., 1995). Those patients are frequently declined surgery and previously been managed palliatively. Recent development of TAVI offers new hope to such patients. The largest follow-up cohort (n=70) of patients who underwent TAVI and survived past 30 days demonstrated an overall survival rate of 57% at median 3.7 years follow-up (Gurvitch et al., 2010). The most common basis for mortality in this cohort were non-cardiac, such as pneumonia, pancreatitis, gastrointestinal bleeding, ischaemic bowel and neurological decline. Morbidity, however, remained mainly cardiovascular-related.

Given the supposedly dismal prognosis of patients with severe symptomatic aortic stenosis, who do not undergo valve replacement (Ross et al., 1968), TAVI offers an attractive
treatment option, especially in those patients where open thoracotomy and valve replacement is contra-indicated. However, controlled studies with medical management have only recently began to emerge. In a recent study, 358 subjects with severe AS, who were deemed unsuitable for open surgery, were randomized to TAVI or conservative therapy, including balloon valvuloplasty, and then followed up for a median of 1.6 years (Leon et al., 2010). The authors found that TAVI was associated with a significantly lower rate of death at 1 year, fewer hospital readmissions, and a reduction in cardiac symptoms. These improved outcomes were achieved, however, at the cost of a significant increase in the rate of major strokes and vascular events.
1.3 Mechanistic considerations

1.3.1 Aging as a risk factor: cellular and molecular biology

Our society is aging rapidly and cardiovascular diseases are the leading cause of death. Age is a consistent and independent cardiovascular risk factor and disease syndromes affecting the cardiovascular system reach epidemic proportions in the very old (reviewed by (Kovacic et al., 2011)). Molecular links between age and heart syndromes are complex and involve much more than the passage of time.

One of the best studied hypotheses to explain “inevitability” of aging is related to telomere shortening (reviewed by (Kovacic et al., 2011)). Telomeres are regions of repetitive DNA sequences at the end of a chromosome. During cell division, enzymes that duplicate DNA cannot continue their duplication all the way to the end of chromosomes as they require an initial point of attachment on the DNA strand, which then fails to undergo synthesis during each cellular division. As a result of this problem, newly synthesized DNA strands are shorter than the original template and genetic material could be lost each time a cell divides. Over time, due to each cell division, the telomere ends become shorter, despite being replenished by an enzyme, telomerase reverse transcriptase (Minamino et al., 2008). To a point, cells are unaffected by a degree of telomere loss and continue to function normally. However, when telomeres become too short, they signal the arrest of cellular proliferation and senescence ensues.

Leukocyte telomere length shortening has been associated with increased incidence of coronary artery and atherosclerotic disease in a number of studies (Calvert et al., 2011; Farzaneh-Far et al., 2010; Willeit et al., 2010). Furthermore, telomere length has been shown to be a robust predictor of future cardiovascular events (Epel et al., 2009; Farzaneh-Far et al., 2008). Interestingly, treatment of patients with statins was associated with
reduction in telomere attrition in patients with coronary artery disease (Satoh et al., 2009) and those at increased risk of cardiovascular disease (Brouilette et al., 2007).

Another common hypothesis to account for aging-associated morbidity is the oxidative stress hypothesis. Essentially, it is based on the fact that a chronic state of oxidative stress exists in cells of aerobic organisms even under normal physiological conditions because of an imbalance between pro-oxidants and antioxidants. The imbalance leads to a steady-state accumulation of oxidative damage in a variety of macromolecules that increases during aging, resulting in a progressive loss in the functional efficiency of various cellular processes (reviewed by Muller et al., 2007). There is certainly a large body of evidence that age-associated disorders are correlated with elevated oxidative damage (reviewed by Beckman et al., 1998). The extension of that hypothesis - that accumulated oxidative damage determines life span - is more controversial. The issue of course is that there are multiple pro- and anti-oxidant systems in vivo with tens of thousands of downstream targets that can be affected, making dissection of precise mechanisms near impossible at present. Furthermore, these two hypotheses of aging are not mutually exclusive and there is likely interplay between them.

Within the cardiovascular system, aging of cardiomyocytes (CMs) has been studied relatively extensively. They contribute directly to changes in heart function and structure, which display a number of physiological and morphological features with age, including a decrease in the total number of CMs (due to necrosis and apoptosis), alterations in myocyte contraction and relaxation, hypertrophy, and an inability to repair or replace lost cells in sufficient quantities to meet added functional demands (Sheydina et al., 2011). While a number of contributing mechanisms have been proposed within the myocardium (reviewed by Shih et al., 2011), interestingly none of these include primary pivotal roles for
impaired NO signalling. On the other hand, increased oxidative stress, which may affect NO signalling, has been extensively implicated (Muller et al., 2007).

1.3.2 Aortic valve mechanics - role of valvular endothelium and valvular interstitial cells (VICs)

Even very subtle alterations in structure and mechanics of aortic valve leaflets can have profound effects on leaflet opening and coaptation, tensile stress distribution along leaflets, coronary perfusion and left ventricular function (Singh et al., 2008). Until recently, valve mechanics were thought to be determined primarily by shear stress and ventricular contractility with no active input from valve cells. VICs, the predominant cell type in normal valves, have the ability to differentiate to contractile myofibroblasts (Yip et al., 2009) and respond to a variety of biochemical stimuli, including serotonin (5-HT), endothelin (ET)-1 and nitric oxide (NO) in a manner similar to that of vascular smooth muscle cells (Filip et al., 1986).

It is well recognized that vascular smooth muscle tone and vessel compliance are regulated by the vascular endothelium, which releases several mediators, including NO, PGI₂, ET-1, and natriuretic peptides (Simmons, 2009). In a porcine ex-vivo aortic valve study, El-Hamamsy et al. (2009) have recently demonstrated that the valvular endothelium plays a similar role in regulating aortic valve mechanical properties. This study found that in the basal state, VIC-mediated contraction contributed to tissue stiffness, but the endothelium did not. In the presence of 5-HT, however, stiffness and contraction were decreased in intact tissue samples, most likely as a result of endothelial release of NO and its induction of VIC relaxation, as inhibition of NO synthase by L-NAME or denudation of the endothelium reversed the effect of 5-HT, causing an increase in tissue stiffness (and
presumably VIC contractility). ET-1 also caused tissue stiffening, probably due to its induction of VIC contractility (El-Hamamsy et al., 2009). Loss of these adaptive mechanisms due to endothelial dysfunction (or the absence of endothelium in a damaged valve) could result in abnormal biomechanics, structural damage, and exacerbation of disease progression in AS. This study, however, did not examine the effects of shear stress, which may exert additional effects on valve mechanics, especially under basal conditions. Thus it is possible for vasoactive agents to alter valve tissue mechanics analogously to their role in regulating vascular tone and mechanical properties in vascular endothelium: further studies in-vivo and in shear stress models would be required to test this hypothesis.

1.3.3 Histopathological features of AS

Pathological changes in the leaflets of the aortic valve seem to appear primarily at the flexion area of the leaflets where shear stress is the highest (Thubrikar et al., 1986). It has been postulated that such shear stress leads to endothelial disruption and initiates valvular mechanical breakdown (see section 1.3.2). The fibrosa layer is affected first as the aortic side is exposed to turbulent flow with high shear stress (Nicosia et al., 2003). However, the link between shear stress and endothelial dysfunction is complex, including NO cascade and increased expression/activation of NAD(P)H oxidase. (Davignon et al., 2004).

1.3.3.1 Aortic valve interstitial cells (VICs)

VICs are distinct mesenchymal cells within the valve matrix that adapt to the valvular environment with a change in phenotype with specific detectable markers. Embryonic progenitor endothelial/mesenchymal cells give rise to a number of VIC subtypes (Liu et al., 2007), including quiescent VICs. These VICs maintain normal valve structure and inhibit angiogenesis in the leaflets. They can give rise to activated VICs in response to a
number of chemokines and growth factors, one of the most important being TGF-β1 from activated endothelium. Activated VICs (α-smooth-muscle positive VICs) respond to valve injury and mechanical forces by repair processes including proliferation, migration, and matrix remodeling (reviewed by (Liu et al., 2007). Ostoblastic VICs, also derived from quiescent VICs under a number of pathological conditions, participate in calcification, osteogenesis, and chondrogenesis, and secrete a number of pro-fibrotic/pro-calcific factors (Hermans et al., 2010; Liu et al., 2007).

1.3.3.2 Extracellular matrix and fibrosis

Presence of activated and osteoblastic VICs, as well as T-lymphocytes and macrophages leads to activation of pro-fibrotic/pro-calcific cascades and contributes to leaflet remodelling and calcification.

Increases of MMP-1, -3, and -9 have consistently been reported, while changes in their inhibitors TIMP-1 and -2 have varied between studies (Edep et al., 2000; Fondard et al., 2005; Satta et al., 2003). Increased levels of cathepsin G, highly expressed in activated mast cells (Helske et al., 2006a), as well as cathepsins S, K and V have also been detected in stenotic aortic valve leaflets (Helske et al., 2006b). 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 and remodeling, and consequently predispose to calcification. Furthermore, both TGF-β1 and tumour necrosis factor-α (TNFα), another important proinflammatory cytokine commonly responsible for immune regulation, inflammation and tissue remodeling, co-localize with MMP-1 (Kaden et al., 2005).
Angiotensin II (Ang II), an important mediator of inflammation and fibrosis, could be formed by angiotensin converting enzyme (ACE) as well as the mast cell (MC)-derived neutral protease, chymase (Nishimoto et al., 2001). ACE has been identified in stenotic but not in normal aortic valves (O'Brien et al., 2002). 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., 2006a). These findings provide a potential basis for a role of Ang II in aortic valve remodeling along with other profibrotic and proinflammatory mechanisms.

1.3.3.3 Inflammation and lipid deposition

Early AS lesions, characterized by areas of subendothelial thickening containing inflammatory infiltrates including macrophages and T-lymphocytes, lipids and oxidized lipids all of which potentially activate a host of profibrotic and proinflammatory markers have been described since early 90s (O'Brien et al., 1996; Olsson et al., 1994; Otto et al., 1994; Wallby et al., 2002; Warren et al., 1997). Oxidized LDL (Mohty et al., 2008) and apolipoproteins (apo) B, apo (a), apoE (O'Brien et al., 1996) have been shown to be co-localized with macrophages and increased oxidized LDL score was shown to correlate with TNF-α expression in explanted human stenotic valves (Mohty et al., 2008) and with increased tissue remodeling score (Warren et al., 1997). Oxidative modification of LDL have also been demonstrated in early stenotic aortic valve lesions (Olsson et al., 1999).
1.3.3.4 Oxidative stress

A number of recent studies have emphasized increases in intravalvular content of a variety of pro-oxidants in models of AS/clinical samples (Chen et al., 2003; Liberman et al., 2008; Miller et al., 2008; Ngo et al., 2008; Ngo et al., 2011). Importantly, this evidence of increased oxidative stress was not specifically associated with extent of local atherogenesis in any study. However, no comparisons have been undertaken to date of potential pro-oxidative mechanisms in AS versus those in vascular endothelial disease and/or vascular atherogenesis. Table 1.3 summarizes the major evidence available to date regarding the presence of such pro-oxidants in ASc/AS, excluding abovementioned data regarding Ang II.

1.3.3.5 Calcification

Calcific nodules have long been described in stenotic valves (Freeman et al., 2005); this calcific process has been linked to elevated plasma calcium (Ortlepp et al., 2006) and parathyroid hormone (Linhartova et al., 2008) as well as renal disease (Maher et al., 1987). Nodule formation is thought to be initiated by cytokines released from infiltrating inflammatory cells and from apoptosis of VICs (Mohler et al., 2001). Ossification has also been described, at later stages in the disease, characterised by the presence of osteoblastic VICs and upregulation of the pro-calcific markers osteopontin, osteocalcin, alkaline phosphatase, bone sialoprotein, bone morphogenic proteins (BMP) 2- and 4 and osteoblast-specific factor runx2/cbfa-1 (Kaden et al., 2004b; Mohler et al., 2001; O'Brien et al., 1995). Multiple pathways of osteogenesis have been identified, including osteoprotegrin/RANKL/RANK (Kaden et al., 2004a; Steinmetz et al., 2008), Toll-like receptors 2 and 4 (Meng et al., 2008), tenascin-C (Satta et al., 2002), Lrp-5/Wnt/β-catenin (Caira et al., 2006) and angiotensin II (Helske et al., 2004; O'Brien et al., 2002).
Significantly elevated amounts of RANKL, concomitantly with significant reduction of osteoprotegrin-positive cells, were found in calcified valves compared to only a few positive cells in respective control valves (Kaden et al., 2004a); long-term cell culture of stenotic aortic valves with RANKL showed a significant increase in matrix calcium deposition and the formation of cell nodules. Also, mice deficient in osteoprotegrin develop vascular calcification with increased expression of RANKL in calcified areas (Bucay et al., 1998).

Tenascin C, another extracellular matrix glycoprotein implicated in cell proliferation, migration, differentiation and apoptosis and 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).

Fetuin-A is an anti-calcific protein and a member of the cysteine protease inhibitor superfamily. It is a carrier for growth factors and inactivates TGF-β and BMP (Binkert et al., 1999). Low plasma and raised intra-valvular levels of fetuin-A have recently been found in patients with AS (Kaden et al., 2007). Low plasma concentrations of fetuin-A were also found in persons with concomitant coronary artery disease and AS, but without renal disease and diabetes (Ix et al., 2007). In a more recent small study, serum fetuin-A levels were associated with progression of aortic valve calcification (assessed by CT), over an average follow-up period of 12 months (Koos et al., 2009a). Nevertheless, the relative importance of these findings to the overall pathogenesis of aortic stenosis remains uncertain.
Another anti-calcific protein linked to AS is matrix Gla-protein (MGP). It is synthesised in many different tissues and undergoes vitamin K-dependent $\gamma$-carboxylation (reviewed by Schurgers et al., 2008). While its precise mechanisms of action are not well understood, the $\gamma$-carboxylated form of MGP is involved in inhibition of tissue calcification, possibly by preventing differentiation of vascular smooth muscle cells into chondrocyte-like cells or by binding to BMP and blocking its function (Schurgers et al., 2008). Levels of uncarboxylated MGP were lower in patients with aortic valve calcification (assessed by CT) compared with normal controls, while renal dysfunction and oral warfarin therapy were both predictive of low MGP levels (Koos et al., 2009b). However, another study found elevated levels of both uncarboxylated and carboxylated de-phosphorylated forms of MGP in patients with severe AS (Ueland et al., 2010). The biological significance of these findings is still unclear and further mechanistic studies are required.

It is therefore clear that what in the past that was thought to be a simple "wear and tear" process and hence was termed "degenerative", is a complex active process involving interplay of multiple systems.

1.3.4 Possible mechanisms for adverse outcomes associated with AS/ASc

The precise mechanisms underlying increased morbidity and mortality associated with early AS and ASc have not been fully elucidated. It is certainly unlikely to be abnormalities confined to the valve tissue itself. Much more likely is that early AS/ASc are markers for more generalized processes, involving multiple systems and regulatory cascades.
1.3.4.1 Links with atherosclerosis

There is conflicting evidence in the literature as regards mechanistic links between aortic valve disease and atheroma. While atheroma-like histological changes are well documented in aortic valve leaflets even at the early stages of disease (Otto et al., 1994), the evidence that these parallel the findings in vessels is limited. The majority of studies find at most 50% concordance between the presence of AS and CAD (Otto et al., 2001). Furthermore, the risk factors for atherosclerosis and AS/ASC are clearly not identical (Owens et al., 2009). It may be that the link between the two is via inflammatory activation, which has been shown to be a feature of AS/ASC valve lesions (Freeman et al., 2005) and is also known to promote and destabilize coronary atherosclerosis (Burke et al., 2002). This is supported by Chandra et al (2004), in a study suggesting that ASC is not a predictor of cardiovascular outcomes but rather a marker of presence of CAD and inflammation. A more recent study failed to demonstrate a link between CRP and progression or new development of AS/ASC in a large cohort (Novaro et al., 2007).

1.3.4.2 Platelet dysfunction

This critical role of platelets has been well-defined in coronary artery thrombosis (Heeschen et al., 2003; Ruggeri, 2002), and it is possible that the observed excess cardiovascular risk conferred by the presence of ASC/AS is mediated in part by the disturbance of platelet function, namely by increased platelet aggregability.

Both increased platelet aggregability in patients with AS and thrombus formation on severely stenosed aortic valves have been documented in the past (Riddle et al., 1983; Stein et al., 1977). Compared to non-AS, non-ischaemic patients, patients with AS have been found to have increased platelet aggregability and impaired platelet anti-aggregatory responses to the NO donor sodium nitroprusside (SNP) (Chirkov et al., 2002). implying a
dysfunction of both pro-aggregatory cascade and NO-signalling cascade in platelets of these patients. Furthermore, explanted valves from patients with advanced AS have been shown to have impaired valvular endothelial function, unrelated to vascular endothelial function (Chirkov et al., 2006). Thus, there is emerging evidence that AS is associated with platelet hyperaggregability and NO-signalling dysfunction and valvular endothelial dysfunction. The next step would be to investigate at what stage in the disease process these aberrations emerge.

1.3.4.3 Perturbations of NO signalling cascade

Vascular endothelial dysfunction is present at early stages of atherogenesis and is associated with increased cardiovascular risk (Lerman et al., 2005). The relevant issue is whether this interfaces with ASc.

1.3.4.3.1 Endothelial dysfunction in ASc/AS

Poggianti et al (2003) in a study of 102 patients with known or suspected CAD, demonstrated that patients with AS had significantly reduced flow mediated dilatation (FMD) of the brachial artery, a well-accepted reproducible measure of endothelial dysfunction (Celermajer et al., 1992; Corretti et al., 2002), compared with non-AS patients. Furthermore, responses within the forearm to the endothelium-independent vasodilator, glycercyl trinitrate (GTN), were also diminished versus controls (Poggianti et al., 2003). This implies that AS is associated with incremental vascular endothelial dysfunction compared with coronary artery disease patients without AS, and that this physiological disturbance is associated with both impairment of NO generation and vascular NO responsiveness.
Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of NO synthase (eNOS) and a marker and mediator of endothelial dysfunction (Boger, 2003; Boger et al., 1997; Sydow et al., 2003). Our group found that plasma concentrations of ADMA are elevated in patients with AS, compared with controls (Ngo et al., 2007), providing further support to the suggestion that NO generation is impaired in AS.

Coronary flow reserve (CFR), a measure of coronary microvascular function (Saraste et al., 2001), and thus of endothelial function in general, has been shown to be impaired in patients with ASc compared with age- and sex-matched controls (Bozbas et al., 2008). Given that impaired CFR independently predicts long-term cardiovascular outcomes even in subjects with angiographically near-normal coronary arteries (Britten et al., 2004), this observation supports the observation from a pivotal study by Otto et al. (1999) that subjects with ASc, but without evidence of coronary disease, are at an increased risk of cardiovascular events. Coronary flow reserve has also been shown to be impaired in patients with AS (Julius et al., 1997; Rajappan et al., 2002) even in the absence of CAD (Nemes et al., 2002) and to improve after AVR (Hildick-Smith et al., 2000). The limitation of these studies is that CFR is also dependent on left ventricular diastolic function and thus is not a "pure" measure of endothelial function.

1.3.4.3.2 Platelet function

Under normal physiological conditions, platelets adhere to the damaged vessel wall within seconds after vascular injury. This is followed by platelet-to-platelet adherence (aggregation), which culminates in the formation of a ‘platelet plug’ that temporarily seals off the vessel injury. In contrast, in pathological conditions, arterial thrombus formation by
Platelets occur in the absence of vessel injury, and may limit blood supply to nearby tissues, thus causing local ischemia (Holmsen, 1989).

Platelets are anucleate, discoid cells with a circulating life-span of 8 – 10 days. They are formed by fragmentation of megakaryocytes and are released into circulation via regulatory mediators such as interleukins (IL-3, IL-6), thrombopoietin and NO (Battinelli et al., 2001). Platelets have a complex infrastructure, including numerous organelles (mitochondria, lysosomes, peroxisomes and glycogen particles) dispersed in the cytoplasm, as well as two major types of granules, α-granules and dense bodies, which play important roles during platelet activation (reviewed by (Michelson, 2007). α-Granules contain mainly platelet factor-4, β-thromboglobulin, platelet-derived growth factor, fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor-1 (PAI-1), P-selectin and von Willebrand factor (vWf). Dense granules are rich in calcium, serotonin and adenosine diphosphate (ADP).

The phenomenon of platelet adhesion describes the interaction of platelets with the subendothelial bridging molecule vWf, which possesses a binding domain for the GP Ib-IX-V receptor complex, located in the lipid bilayer of the injured vessel. Platelets may also adhere to other proteins in the subendothelium such as collagens I, III and IV as well as fibronectin (reviewed by (Michelson, 2007).

Platelets are activated by a large variety of physiological agonists (thrombin, ADP, adrenalin, collagen, 5-HT) acting on their respective receptors on the platelet surface membrane. Some agonists (thrombin and collagen) induce complete platelet activation with positive feedback and are denoted ‘strong’ agonists. However, other physiological agonists (adrenalin, 5-HT) stimulate platelet shape change and platelet aggregate formation.
without the liberation of the contents of the secretory granules, and are thus known as ‘weak’ agonists. ADP is considered an intermediate agonist, as it can induce platelet activation without and with positive feedback in a concentration dependent manner. (reviewed by (Michelson, 2007).

Several methods are currently available to evaluate platelet function; these usually involve platelet aggregation and/or platelet granule release. Methods include optical platelet aggregometry, impedance platelet aggregometry, rapid platelet function assay and use of the PFA-100 in vitro platelet function analyzer (Zeidan et al., 2007).

One of the fundamental physiological effects of NO is inhibition of platelet aggregation (reviewed by (Tziros et al., 2006). The antiaggregatory activity of NO donors is usually evaluated by their addition to the tested sample before the induction of aggregation (usually with ADP). Inhibition of aggregation is then evaluated as a percentage, comparing the extent of maximal aggregation in the presence and absence of the antiaggregatory agent studied (Chirkov et al., 2007). Hyporesponsiveness of platelets to the effects of NO extends to many disease states including stable and unstable angina (Chirkov et al., 1999; Chirkov et al., 2001), heart failure (Anderson et al., 2004; Chirkov et al., 2010; Chirkov et al., 2004; Ellis et al., 2001), diabetes and obesity (Anderson et al., 2005; Anfossi et al., 1998; Worthley et al., 2007), dyslipidaemia (Stepien et al., 2003), hypertension (Woods et al., 1993) and aortic stenosis (Chirkov et al., 2006). One possible mechanism of a correlation between these disease states and platelet resistance to NO would be incremental oxidative stress, engendered at least in part in association with activation of systemic inflammation, in particular in patients with ischaemia, as this may lead to inactivation of platelet guanylate cyclase and accelerated clearance of NO by $O_2^-$, both events are implicated as probable causes of NO resistance (reviewed by (Chirkov et al., 2007).
1.3.4.3.3 Vascular endothelial function

The endothelium is a monolayer of endothelial cells lining the lumen of macro- and microvascular beds, which separates the vascular wall from the circulating blood components. A healthy endothelium releases a variety of autocrine and paracrine substances, the best characterized being nitric oxide. It acts to prevent atherosclerosis development and its complications through complex modulation of vascular tone, platelet and leukocyte adhesion, as well as inflammation. The maintenance of homeostasis at the vascular level depends on a balance between vasodilatory mediators (NO, prostacyclin) and vasoconstrictors endothelin and Ang II. Thus, 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 et al., 2003a; Griendling et al., 2003b). Thus, endothelial dysfunction has the potential to contribute to key events in the course of human atherosclerosis and possibly the development of AS lesions and LV hypertrophy.

1.3.4.3.4 Endothelial progenitor cells (EPC)

Following a mechanical or chemical injury, the endothelium undergoes a process of repair whereby new endothelial cells can originate from adjacent pre-existing blood vessels or from recruitment of bone marrow-derived circulating endothelial progenitor cells. A relative deficiency of endothelial progenitor cells (Hill et al., 2003) and/or decreased NO activity (Aicher et al., 2003) may impair repair of vascular injury and contribute to
endothelial dysfunction. Furthermore, there is increasingly strong evidence that production of endothelial progenitor cells may be NO-dependent (Thum et al., 2005). EPC number and function have been reported to be reduced in advanced AS (Matsumoto et al., 2009).

1.3.4.4 Left ventricular hypertrophy (LVH)
Both large population-based studies (Otto et al., 1999) and subgroup analyses of cohorts of hypertensive patients (Agno et al., 2005; Olsen et al., 2005) have shown that subjects with ASc have increased left ventricular (LV) wall thickness. This coexistence of LVH with ASc may contribute to the associated increase in cardiovascular risk. If ASc is indeed independently associated with presence of LVH, this is unlikely to be modulated exclusively by increased left ventricular afterload, but potentially by its association with endothelial dysfunction (Poggianti et al., 2003). A number of endothelial autacoids may modulate the development of LVH (Wenzel et al., 2007). In particular, reduction of NO and bradykinin effects, such as might occur in association with endocardial endothelial dysfunction, can stimulate development of LVH independent of haemodynamic factors (Rosenkranz et al., 2000). Thus LVH may be an additional factor accounting for the increased cardiovascular risk observed in ASc.

Irrespective of presence/absence of ASc, the development of LVH is a common manifestation of various cardiac pathologies, including response to chronic pressure overload. Increased LV mass is largely due to increased size of cardiomyocytes, as well as extracellular matrix remodelling (Frey et al., 2003). The initial increase in LV mass in response to pressure overload occurs as a compensatory adaptive mechanism for normalization of ejection performance and tissue perfusion as originally described by Grossman et al (1975). Prolonged and continuous exposure to haemodynamic stress as in hypertension, valvular disease, heart failure, and diabetes mellitus predispose to a
maladaptive LVH state, which is characterized by ventricular dilatation, diastolic and systolic dysfunction, and therefore increased propensity to heart failure and myocardial ischemia (Grossman et al., 1996). Activation of the circulating and tissue renin angiotensin system appears to play a key role in this maladaptive process (Cowan et al., 2009).

Endothelium-derived NO plays an important role in the regulation of cardiovascular function (Gibbons, 1997; Kelly et al., 1996; Spieker et al., 2005). NO is a potent endogenous vasodilator and a critical modulator of regional blood flow, exerting its effect mostly via stimulation of soluble guanylate cyclase (sGC) to produce cyclic GMP (Behrendt et al., 2002; Ritchie et al., 2009). NO is generated from its precursor L-arginine via the enzyme activity of nitric oxide synthase (NOS). All three NOS isoforms, namely eNOS, nNOS, and iNOS, are expressed in the heart, where nNOS and eNOS are constitutively expressed (Shimokawa et al., 2010).

Numerous experimental studies have documented the antihypertrophic actions of NO synthesized by these NOS enzymes. Exogenous administration of NO donors attenuates hypertrophic responses to stimuli including Ang II, endothelin-1, norepinephrine and phenylephrine (Calderone et al., 1998; Irvine et al., 2008; Ritchie, 2009; Ritchie et al., 1998; Wollert et al., 2002). Conversely, the development of left ventricular hypertrophy in experimental models of chronic pressure overload is potentiated in eNOS knockout mice (Ichinose et al., 2004).

ADMA, an endogenous competitive antagonist of eNOS, is a marker and mediator of endothelial dysfunction (Boger et al., 1997; Sydow et al., 2003). ADMA inhibits eNOS-mediated bioconversion of arginine to NO, and thus regulates eNOS activity (Boger, 2003). Some (Ebinc et al., 2008; Zoccali et al., 2002), but not all (Lieb et al., 2009)
previous investigations have suggested that ADMA concentrations may be correlated with LV mass. Potential modulation of the development of myocardial hypertrophy by ADMA might theoretically be mediated via attenuation of peripheral vasomotor and/or of direct myocardial antihypertrophic effects.

### 1.3.5 Animal models of AS

In senile apo-E -/- mice, Tanaka et al (2005) observed the development of marked increases in transaortic valve flow velocity over a period of about 2 years compared with wild type C57BL/6 mice. Apo E -/- mice develop severe hypercholesterolemia (Zhang et al., 1992) and this was postulated to cause aortic valve lesions in this model. Interestingly, this study also noted that wild-type mice also developed significant increases in transaortic valve flow over the 2-year period; and while there was a difference in AV flow velocity between the wild type and Apo E -/- mice, the relationship between age and transaortic valve flow for the wild type group was not significantly different from that in the Apo E -/- mice group. Thus, it is possible that severe hypercholesterolemia from Apo E-deficiency does not contribute entirely to the development of AS in this model.

Another study compared the same type of wild-type mice, the C57BL/6 on a high fat/high carbohydrate (HF/HC) and low-density lipoprotein receptor deficiency (LDLr -/-) on the HF/HC diet with respective controls (Drolet et al., 2006). The study showed that AVA was significantly decreased, similarly in these 2 groups, compared to their respective controls. Although the authors concluded that use of a HF/HC diet resulted in significant changes in haemodynamics in both the wild type and LDLr -/- mice groups compared to their respective controls, the mechanism of this effect on AVA 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 similar
transvalvular velocity increase and AVA decrease as the wild-type mice on the HF/HC diet. One can then conclude that marked increase in total cholesterol, LDL cholesterol, and glucose levels did not affect progression of AS in this study.

The turbulence of blood flow through the stenotic aortic valves has been postulated to be responsible for endothelial damage. It has been suggested that bacteria can adhere to a damaged surface more easily than an intact surface. Induction of bacterial endocarditis in New Zealand White rabbits resulted in calcification of aortic valves developing within 2 weeks (Cohen et al., 2004). Whilst 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, there has been no clinical studies which document the association between recurrent infections and higher prevalence of AS; and injection of bacteria to induce aortic valve calcification does not represent the true pathogenesis of AS.

Other New Zealand White rabbit models of AS were similar to those of atherosclerosis, but also demonstrated similar microscopic changes to those of human aortic valve lesions (Cimini et al., 2005; Rajamannan et al., 2001; Rajamannan et al., 2005; Rajamannan et al., 2002). All these studies utilized high cholesterol diet for 12 weeks to induce valve changes: microscopic changes including macrophages and T-lymphocyte infiltration; cellular proliferation and bone matrix protein expression. However no gross calcification was found on rabbit aortic valves. Of note, none of these studies have utilized echocardiography on these treated rabbits, so haemodynamic 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 high dose vitamin D$_2$ (50,000IU/day), which was given in addition to high cholesterol supplemented diet to New Zealand White rabbits and followed for 12 weeks. There was significantly decreased AVA and increased AVp in the vitamin D$_2$ and cholesterol group as detected by echocardiography, as well as gross calcification of the aortic valve, compared to controls and the cholesterol supplemented group alone. This study provided evidence for the first time that high cholesterol alone may not be sufficient to induce "true" AS, and that the effects of vitamin D and vascular calcification warranted further studies.

Indeed, we demonstrated that AS, with features similar to those of human disease, can be induced in New Zealand White rabbits with moderate dose vitamin D$_2$ alone (25,000 U/day, 4 days/week), without the need for cholesterol supplementation, in as little as 8 weeks (Ngo et al., 2008). Echocardiographically, rabbits in the vitamin D$_2$ alone group had significantly increased transvalvular velocity and pressure gradients compared to those in the control group (normal chow + drinking water); this was consistent for aortic valve ultrasonic backscatter scores. Immunohistochemistry/histology showed marked calcification, neutral lipids, macrophage, and leukocyte infiltrations of the aortic valve in these vitamin D$_2$-treated rabbits - changes consistent with histology of human AS (Otto et al., 1994).

Endothelial dysfunction was demonstrated biochemically with significant elevation of ADMA concentrations in the vitamin D$_2$ group compared to controls over the 8 weeks' treatment period; furthermore, the change in ADMA concentrations correlated significantly with the change in transvalvular pressure gradients for rabbits in the vitamin D$_2$ group. Endothelial dysfunction was also demonstrated via impairment of endothelium-dependent
Chapter 1

Acetylcholine-induced aortic relaxation in vitamin D$_2$-treated rabbits, and this effect was completely abolished by the nitric oxide synthase inhibitor (L-NAME). Immunostaining for thioredoxin-interacting protein (TXNIP) revealed considerably increased immunostaining within valves of vitamin D$_2$-treated animals. TXNIP is a fundamental mediator of increased redox stress (World et al., 2006), which binds to, and inactivates, thioredoxin (Junn et al., 2000). Expression of TXNIP is associated with activation of apoptosis signalling pathways (Xiang et al., 2005), and is suppressed by endothelial NO release (Schulze et al., 2006). This provides further insight into molecular and cellular mechanisms of development of AS. Interestingly, the addition of 0.5% cholesterol-supplemented diet to the vitamin D$_2$ regimen did not accentuate the development of AS in our model.

1.3.6 Is AS/ASc preventable?

1.3.6.1 Animal studies on slowing progression of AS

1.3.6.1.1 Statins

Statins were the first commercially available drugs studied in aortic stenosis, given their established benefit in primary and secondary prevention of cardiovascular diseases, as well as evidence of association of lipid infiltration of aortic valve in AS (O'Brien et al., 1996; Olsson et al., 1999; Otto et al., 1994). Whilst there are many "dyslipidaemic" animal models of AS (see animal model section), only one model so far has shown any evidence of benefit with statin therapy (Rajamannan et al., 2002). In this hypercholesterolaemic rabbit model there was an increase in cholesterol, hsCRP, proliferation cell nuclear antigen, macrophage infiltration, and osteopontin and osteoblast gene markers (alkaline phosphatase, osteopontin, and Cbfa-1) in the cholesterol-fed rabbits compared with control
rabbits. All these markers except hsCRP were reduced by atorvastatin. Given that this was a hypercholesterolemic model to begin with, its results can only be translated to patients with dyslipidaemia as the facilitating factor for AS development. Furthermore, whilst there were favourable histopathological changes, actual haemodynamic changes were not assessed. Perhaps this model corresponds to the occasionally reported cases of AS associated with extreme hypercholesterolaemia in humans (Awan et al., 2008; Tsuchida et al., 2009).

1.3.6.1.2 ACE Inhibitors/Angiotensin receptor-1 blockers (ACEI/ARB)

Potential utility of these agents was suspected on the basis of observations that ACE activity and angiotensin I receptors are present in early AS lesions (Helske et al., 2004; Helske et al., 2006a; O'Brien et al., 2002). Inhibition of the angiotensin pathway with the angiotensin receptor-1 blocker olmesartan in cholesterol-fed rabbits was associated with decreased macrophage infiltration and reductions in osteopontin and ACE in aortic valves (Arishiro et al., 2007). This was also associated with preservation of endothelial integrity and inhibition of the trans-differentiation of valvular fibroblasts into myofibroblasts. Once again, as in the statin animal trial (Rajamannan et al., 2002), no haemodynamic assessments of possible AS progression were performed.

In our New Zealand White rabbit model of AS (Ngo et al., 2008), induced by 8 week course of vitamin D$_2$, we have recently demonstrated that co-treatment with ramipril retarded the development of vitamin D$_2$-induced AS in the rabbit model, as measured by reduction both in transvalvular velocity and AVBS (Ngo et al., 2011). These reductions in AVBS scores correlated with reduction in valvular calcium infiltration (Alazarin red S staining) and macrophage infiltration (RAM-11 staining). This retardation of AS development was associated with a reduction in TXNIP accumulation within the valve
Chapter 1

matrix, and the preservation of vascular endothelial function, as assessed both physiologically (acetylcholine responses) and biochemically (ADMA concentrations). These findings therefore represent the first definitive demonstration that ACE inhibitors may retard the development of AS as measured in any of the following ways: haemodynamically, histologically, physiologically and biochemically.

1.3.6.1.3 Bisphosphonates

Bisphosphonates, a class of medications used for treatment of osteoporosis in humans, bind to hydroxyapatite crystals (Drake et al., 2008). These are a common form of calcium deposition within aortic valves (Mohler et al., 1999) as well as the principal mineral within bone. They inhibit bone resorption as they cause osteoclast apoptosis (Plotkin et al., 2005; Plotkin et al., 1999). Bisphosphonates also work by inhibiting an enzyme in the mevalonate pathway (cholesterol synthesis), which causes abnormalities in the cytoskeleton in the osteoclast, thus reducing bone resorption (Figure 1.5) (Rodan et al., 1996). This inhibition of cholesterol synthesis may also impart some anti-inflammatory properties. Thus, bisphosphonates may directly reduce valvular calcification via their osteoblast action, as well as indirectly via inhibition of inflammation and resultant fibrosis.

A study in a rat model of dialysis suggested that etidronate, now a rarely used bisphosphonate, limited aortic calcification (Tamura et al., 2007).

1.3.6.1.4 Exercise training

Exercise training in patients with coronary artery disease has been shown to modify traditional risk factors, improve myocardial and peripheral perfusion, enhance exercise tolerance, and reduce cardiovascular morbidity and mortality. The mechanisms of these
salutary effects are unclear, but are thought to result from increases in blood flow and shear stress leading to: 1) improvements in endothelial function, secondary to improved nitric oxide bioavailability (via increased expression and activity of endothelial nitric oxide synthase and reduced nitric oxide degradation), reduced endothelial adhesiveness (via diminished expression of cellular adhesion molecules), enhanced endothelial regenerative capacity (via stimulation of endothelial progenitor cells); and 2) reduction of vascular inflammation (via reduction in proinflammatory cytokines and oxidative stress and increase in antiinflammatory cytokines) (Ribeiro et al., 2010).

Given the similarities between pathogenesis of atherosclerotic coronary artery disease and aortic valve disease, Matsumoto et al (2010) evaluated effects of regular exercise training in high cholesterol diet LDL -/- mice model. This mouse develops hypercholesterolemia, atherosclerosis, and aortic valve pathology in an age- and diet-dependent manner. The authors found that regular exercise ameliorated the abnormal responses to the high-fat diet and increases valvular (osteoprotegerin) and circulating (fetuin-A) levels of the inhibitors of calcification. Serum cholesterol remained elevated, which further suggests that the association between lipid levels and aortic valve pathology is not as tight as previously thought. This effect of exercise on the inhibitors of calcification is consistent with a study that found a significant relation between maximal O₂ consumption and fetuin-A levels and a negative correlation with coronary artery calcification in men (Wilund et al., 2008). The finding that an exercise regimen preserved aortic valve endothelial layer/cell integrity is of major importance given recently described interactions of valve mechanics, valvular endothelium and valve interstitial cells (El-Hamamsy et al., 2009).
1.4 Clinical studies on slowing of progression of AS

1.4.1 Retrospective

Many of the initial evaluations concerning possible effects of pharmacotherapy on progression of ASC/AS were purely retrospective analyses of population data. The results of these analyses, in the case of lipid-lowering agents, have proven to be at odds with subsequently obtained trial data.

1.4.1.2 Statins

There were a number of very encouraging retrospective studies of statins in AS, suggesting an average of 50% reduction in the rate of progression of AS with statin use (Aronow et al., 2001; Bellamy et al., 2002; Novaro et al., 2001; Rosenhek et al., 2004b; Shavelle et al., 2002). However, only 2 of these studies (Aronow et al., 2001; Novaro et al., 2001) found an association between cholesterol level and progression, implying that "pleiotropic" effects of statins may be important, rather than their effects on cholesterol homeostasis per se.

1.4.1.3 ACEI/ARB therapy

The observation that ACE activity is increased in stenotic aortic valves has stimulated a number of retrospective data analyses with conflicting results (Table 1.4) (Nadir et al., 2011; O'Brien et al., 2005; Rosenhek et al., 2004b; Wakabayashi et al., 2011). Rosenhek et al (Rosenhek et al., 2004b), utilizing echocardiographic parameters, found no significant effect of ACEI therapy on AS progression, while O'Brien et al (O'Brien et al., 2005), in a study using CT-based calcification assessment found lower rates of AS calcification, after correction for comorbidities. The trials of these agents in humans have been hindered in part by theoretical concerns that the combination of fixed valve obstruction and potent arterial vasodilators could be harmful due to reduction in cardiac output. However, this
does not appear to be a major concern: even in patients with a combination of symptomatic severe AS and left ventricular dysfunction administration of intravenous vasodilators (sodium nitroprusside) has led to clinical and haemodynamic stabilization prior to AVR (Khot et al., 2003). In less severe AS, treatment with ramipril was also not associated with adverse haemodynamic outcomes (O'Brien et al., 2004). Somewhat surprisingly, Wakabayashi et al (2011) found in a retrospective evaluation of a cohort of 194 Japanese patients with mild-to-moderate AS, that whilst therapy with ACEI was an independent predictor of reduced rate of AS progression, that with ARB therapy was not. More recently Nadir et al (2011) in a retrospective population data analysis demonstrated that ACEI/ARB therapy was associated with improved cardiovascular outcomes in subjects with varying degree (but predominantly mild-to-moderate) of AS.

Beneficial effects of ACEI therapy may extend beyond their possible direct effects on the valves, and be mediated via improvements in endothelial function and myocardial remodelling. None of these investigations have correlated utilization of ACEI/ARB, associated effects on NO signalling, and putative effects on ASc/AS progression.

1.4.1.4 Bisphosphonates

In a small retrospective (n=55) study of patients with mild-to-moderate AS bisphosphonate treatment was significantly associated with reduced rate of AS progression (Skolnick et al., 2009). An even more recent retrospective echocardiography-based study of 76 subjects demonstrated slower reduction in AVA in patients treated with bisphosphonates (Innasimuthu et al., 2010). However, the same study failed to demonstrate a significant amelioration of increase in aortic transvalvular gradient, another measure of AS severity/progression. This places doubt on the overall significance of this study. Both human studies suffer from the same major criticisms: their retrospective and observational
nature, small sample size, leading to inability to account for confounders, and small number of subjects on bisphosphonates compared to controls.

1.4.2 Prospective

1.4.2.1 Lipid-lowering (predominantly statin) therapy

Although retrospective studies of statin therapy were encouraging, randomized controlled trials have failed to support the hypothesis that lipid lowering would slow or halt the progression of aortic stenosis. Only one small (n=121) prospective open-label non-randomized observational study of rosuvastatin in patients with moderate AS (RAAVE: Rosuvastatin Affecting Aortic Valve Endothelium) showed slowing of haemodynamic progression of AS (Moura et al., 2007): in this study only patients who otherwise met NCEP/ATPIII criteria (2001) for statin use were started on rosuvastatin 20mg/day, while those who did not meet the criteria were only observed with a mean follow-up of only 73 weeks.

However, 3 large prospective double-blind randomized placebo-controlled trials of lipid-lowering (predominantly statin) therapy in AS failed to show any retardation of AS progression. First of those (SALTIRE: Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression), failed to show any improvement in AS progression with atorvastatin 80mg/day versus placebo in 155 patients followed up for 2 years (Cowell et al., 2005). Major limitations of this study were small sample size and short duration of follow-up. At the time it was suggested that the SALTIRE trial was subject to unacceptably high Type II error.
The next, and the largest, of the prospective double-blind randomized placebo-controlled trials (SEAS: Simvastatin and Ezetimibe in Aortic Stenosis), included almost 1900 subjects with moderate AS randomized to combination of simvastatin and ezetimibe or placebo (Rossebo et al., 2008). Over a median follow-up of 52 months there was no difference in the primary outcome (need for aortic valve replacement) between treatment and placebo groups.

Finally, a recent prospective double-blind randomized placebo control trial (ASTRONOMER: Aortic Stenosis Progression Observation: Measuring the Effects of Rosuvastatin) of 269 subjects randomized to receive either placebo or rosuvastatin 40 mg daily showed no reduction in AS progression over median follow up of 3.5 years (Chan et al., 2010).

Taken together (Teo et al., 2011), these results suggest that the issue of Type II error has effectively been overcome, and that statins should not be used in patients with AS for prevention of progression of valvular disease.

1.4.2.2 ACEI/ARB therapy

There are no prospective human trials assessing effects of angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers on progression of AS. One prospective randomized, double blinded trial of enalapril in 56 patients with severe symptomatic AS for 2 weeks demonstrated improvements in NYHA class and 6 minute walk test (Chockalingam et al., 2004). Currently, a trial is in progress to determine whether candesartan exerts an anti-inflammatory effect within valve matrix in patients with symptomatic AS (Helsinki University, 2012).
1.4.2.3 Aldosterone blockade

Stenotic aortic valve is characterized not only by lipid accumulation, but also by increased fibrosis and inflammation. There is evidence from animal studies that aldosterone contributes to vascular inflammation and myocardial fibrosis – actions that are ameliorated by aldosterone blockade (Rocha et al., 2002). Thus, eplerenone, an aldosterone-receptor antagonist, previously shown to reduce LV mass in hypertension (Pitt et al., 2003a) and improve outcomes in heart failure patients (Pitt et al., 2003b), was trialled in a randomized double-blind placebo-controlled study in patients with moderate-severe AS (Stewart et al., 2008). There was neither reduction in rate of progression of valve stenosis or LV mass nor improvement in LV systolic or diastolic function in this small (n=65) study.

Failure of these therapies is most often ascribed to the severity of valvular disease at the time treatment is initiated, however it is possible that it is due to fundamental differences between atherosclerotic and aortic stenotic lesions.
1.5 After ASTRONOMER: The case that pathogenesis of ASc/AS and atherosclerosis are distinct from one another: epidemiological and biochemical perspectives

Despite significant similarities in factors associated with AS/ASc and atherosclerotic vascular disease, there in fact is marked discordance in their occurrence. Indeed, only 1/3 of subjects in the Euro Heart Study with severe AS, required coronary artery bypass grafting at the time of valve surgery (Iung et al., 2003). In view of the negative results of the three placebo-controlled trials of lipid lowering, it is important to re-examine the case that ASc/AS and atherosclerosis result from fundamentally different pathophysiological processes, despite the commonalities discussed in section 1.3.3). The major distinctions can be categorized as: (1) epidemiological and (2) biochemical.

Upregulation of the calcification signaling pathways, bone morphogenetic protein, Wnt3/Lrp5/β-catenin, and Runx2/Cba 1 (mediated by the bone matrix proteins osteopontin and osteocalcin) is prominent in aortic stenosis, and osteoclastic activity is greater in aortic stenosis than in atherosclerotic plaque (Hakuno et al., 2009). In addition, the clinical sequelae of the 2 lesions (ie, myocardial infarction due to plaque rupture in atherosclerosis and left ventricular outflow obstruction due to stiff leaflets in aortic stenosis) are pathophysiologically distinct. Thus, differences in the relative content of the lesions (ie, lipid-laden, fibrotic, and calcific lesions) may result in variable responses to interventions that are targeted to specific pathological processes. In this regard, normalization of cholesterol levels in a transgenic mouse model of aortic stenosis reversed osteogenic activity and valvular calcification but had no effect on valvular fibrosis (Miller et al., 2009).
Another related but distinct factor to be considered is the timing of therapy relative to the prevailing histopathologic stage of aortic valve disease (ie, inflammation, matrix metalloproteinase–dependent matrix remodeling, myofibroblast transdifferentiation, angiogenesis, osteoblastic/osteoclastic activity, and calcium deposition). Finally, in the case of statins, pleiotropic effects may antagonize their lipid-lowering effect; for example, statins were shown to increase profibrotic signal transduction activating extracellular-regulated kinases (ERK 1/2) in human stenotic aortic valves (Anger et al., 2008).

The "state of the art" regarding pathogenesis of ASc/AS is therefore one of uncertainty: a previously popular "atherogenic" hypothesis can be rejected, and there is evidence of a non-atherogenic inflammatory process, partially but not entirely driven by activation of the renin-angiotensin-aldosterone system. There is clearly a need for considerable mechanistic clarification before intervention studies (with good scientific rationales) can be undertaken.
1.6 Scope of this thesis

As described in this chapter, recent insights into the pathophysiology of AS have included central roles for angiotensin II, for diminished nitric oxide effect at the level of valve endothelium and matrix, and for inflammatory activation/redox stress culminating in activation of pro-calcific stimuli. Despite the presence of atheroma within the stenotic valve, hyperlipidaemia per se does not play a critical role in the development of obstructive disease. However, determinants of the earliest stages of disease occurrence and progression in humans are elusive, making targeted interventional studies difficult.

Primary aims of this thesis were:

1) To elucidate the correlates of occurrence and progression of ASc in an unselected aging Western population.
2) To evaluate the effect of ASc on the development of LVH in “healthy” aging subjects without treated hypertension, and identify determinants of LVH in this cohort irrespective of ASc status.
3) To evaluate the effects of aging on the NO signalling cascade and to establish the correlates of the observed changes.

Chapter 2 describes the evaluation of a cohort of 253 aging subjects, utilizing the novel methodology of AVBS to assess aortic valve echogenicity, over a period of 4 years. The study assesses the correlates of both occurrence and progression of ASc with particular emphasis on measures of NO signalling cascade and importance (or lack thereof) of conventional coronary risk factors.
Chapter 3 describes evaluation of a separate cohort of 79 aging subjects without known cardiovascular disease or treated hypertension, utilizing both AVBS and cardiac MRI, to assess impact of ASc on the development of LVH. Furthermore, determinants of LV mass index are evaluated in this cohort irrespective of ASc presence with emphasis on the markers of NO generation and vascular responsiveness.

Chapter 4 describes evaluation of time course of the parameters of NO generation and effect in the subject cohort evaluated in Chapter 2. Age-related changes are assessed as well as the determinants of such changes.

Chapter 5 addresses considerations for future research contingent on the results of the currently described studies.
1.7 TABLES AND FIGURES for CHAPTER 1
Table 1.1. Summary of clinical studies evaluating factors associated with progression of AS.

<table>
<thead>
<tr>
<th>Reference, Study type, Stage of disease at entry</th>
<th>N</th>
<th>Mean age at entry (years ± SD), Follow-up period</th>
<th>Method of determination of progression rate</th>
<th>Correlates examined</th>
<th>Significant correlates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wongpraparut et al., 2002), Retrospective, Any degree of AS (severity not stated)</td>
<td>58</td>
<td>Age: 75±11, Follow-up: 4 years</td>
<td>Echo “fast progressors” δ AVA ≥ 0.25cm²/yr</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>Study</td>
<td>n</td>
<td>Age: 71±9, Follow-up: 23±11 months</td>
<td>Echo, Percent AVA reduction “fast progressors” δ AVA ≥ 0.1 cm²/yr</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 gender, elevated SeCr, calcium, and cholesterol levels, initial AVA</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----</td>
<td>----------------------------------</td>
<td>-----------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Paltta et al., 2000), Retrospective, Initial AVA 1.17±0.38 cm²</td>
<td>170</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Bahler et al., 1999), Retrospective, Initial AVA ranged from 0.6-2.0 cm²</td>
<td>852</td>
<td>Age: 68±13, Follow-up: 6 to 51 months</td>
<td>Echo, Percent AVA reduction “fast progressors” δ AVA ≥ 0.1 cm²/yr</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, LV mass, and initial AVA</td>
</tr>
<tr>
<td>(Nassimiha et al., 2001), Retrospective,</td>
<td>102</td>
<td>Age: 76±9, Follow-up: 28±21 months</td>
<td>Echo</td>
<td>Smoking, hypertension, hypercholesterolemia, diabetes mellitus, age</td>
<td>Cigarette smoking, hypercholesterolemia</td>
</tr>
<tr>
<td>Study</td>
<td>Sample Size</td>
<td>Age</td>
<td>Follow-up</td>
<td>Imaging Modality</td>
<td>Risk Factors</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
<td>-----</td>
<td>-----------</td>
<td>------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Aronow et al., 2001),</td>
<td>180</td>
<td>82±5</td>
<td>33±12</td>
<td>Echo</td>
<td>Gender, DM, smoking, HT, serum LDL, HDL, triglycerides, obesity, statin therapy</td>
</tr>
<tr>
<td>Retrospective, Mild AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Novaro et al., 2007),</td>
<td>5621</td>
<td>≥ 65</td>
<td>5</td>
<td>Echo</td>
<td>Age, gender, ethnicity, hypertension, smoking, diabetes, renal insufficiency, height, CAD, CRP, LDL cholesterol</td>
</tr>
<tr>
<td>Prospective, 70% - normal,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28.5% - ASc, 1.5% - AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pohle et al., 2001),</td>
<td>104</td>
<td>64.7±8</td>
<td>15.3±5 months</td>
<td>EBCT</td>
<td>LDL cholesterol, age, hypertension, diabetes, smoking</td>
</tr>
<tr>
<td>Retrospective, Any degree of AVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Sample Size</td>
<td>Age</td>
<td>Follow-up</td>
<td>Imaging Modality</td>
<td>Clinical and Demographic Variables</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------</td>
<td>-----</td>
<td>-----------</td>
<td>------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>(Messika-Zeitoun <em>et al.</em>, 2007), Prospective, Any degree of AVC</td>
<td>262</td>
<td>≥60</td>
<td>3.8±0.9 years</td>
<td>EBCT</td>
<td>Age, gender, BMI, systolic and diastolic BP, Smoking, HT, DM, serum glucose, total cholesterol, LDLs, baseline AVC score</td>
</tr>
<tr>
<td>(Wakabayashi <em>et al.</em>, 2011), Retrospective, Majority: mild-to-moderate</td>
<td>194</td>
<td>72±9</td>
<td>406±189 days</td>
<td>Echo</td>
<td>Use of statins, ACEI, ARB, beta blockers, calcium channel blockers, diuretics, history of DM, age, gender, lipid profile, systolic and diastolic blood pressure, renal function, EF and initial AVv</td>
</tr>
</tbody>
</table>

** in absence of baseline AVC only

**ACEI - angiotensin converting enzyme inhibitors; AF – atrial fibrillation; ARB - angiotensin receptor 1 blockers; AVA – aortic valve area; AVC – aortic valve calcification; AVp – transvalvular pressure gradients; AVv – transvalvular velocity; BMI – body mass index; BSA – body surface area;**
CAD – coronary artery disease; Ca₃PO₄ – calcium-phosphate product; DM – diabetes; EBCT – electron beam computed tomography; Echo - echocardiography; EF – ejection fraction; HDL – high density lipoprotein; HT – hypertension; IVSd – interventricular septal dimensions; LAD – left atrial dimension; LVEDD – left ventricular end diastolic dimension; LVESD – left ventricular end systolic dimension; LDL – low density lipoprotein; LVPW – left ventricular posterior wall dimension; LVv – left ventricular outflow tract velocity; PVD – peripheral vascular disease; SeCr – serum creatinine.
Table 1.2. Summary of 3 randomized double-blind placebo-controlled trials with lipid lowering aimed to retard progression of AS.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Number of subjects</th>
<th>Stage of AS</th>
<th>Therapy trialled</th>
<th>Duration of follow-up (median)</th>
<th>Outcome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALTIRE (Cowell et al., 2005)</td>
<td>155 (mean age 68)</td>
<td>Moderate</td>
<td>Atorvastatin 80mg/d (77) vs placebo (78)</td>
<td>25 months</td>
<td>p=0.93 atorvastatin vs placebo effect on progression</td>
<td></td>
</tr>
<tr>
<td>SEAS (Rossebo et al., 2008)</td>
<td>1873 (mean age 67)</td>
<td>Mild-to-moderate</td>
<td>Simvastatin 40mg/d and ezetimibe 10mg/d vs placebo</td>
<td>52 months</td>
<td>P=0.97 active therapy vs placebo effect on need for AVR</td>
<td>Primary outcome was MACE (unrelated to aortic valve disease)</td>
</tr>
<tr>
<td>ASTRONOMER (Chan et al., 2020)</td>
<td>269 (mean age 58)</td>
<td>Mild-to-moderate</td>
<td>Rosuvastatin 40mg/d vs</td>
<td>42 months</td>
<td>P=0.83 rosuvastatin vs</td>
<td>50% had BAV</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td>placebo</td>
<td>placebo effect on progression</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3. Summary of modulators/products of oxidative stress localized in sclerotic/stenotic aortic valve in animal models and clinically. Data regarding angiotensin II are excluded (summarized in section 1.3.3.3).

<table>
<thead>
<tr>
<th>Pro-oxidant/product</th>
<th>Perturbation</th>
<th>Source of evidence</th>
<th>Reference</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Excess</td>
<td>Cell culture</td>
<td>(Kennedy et al., 2009)</td>
<td>Associated with superoxide release</td>
</tr>
</tbody>
</table>
| TXNIP               | Increased levels | Rabbit model of ASC/mild AS | (Ngo et al., 2008; Ngo et al., 2011) | Redox mediator  
Pro-oxidant  
Inflammasome activator |
| Endothelin-1        | Increased levels | Explanted human stenotic aortic valves | (Peltonen et al., 2009) | Pro-oxidant  
Likely reduces NO release  
Pro-inflammatory |
<p>| eNOS                | Downregulation | Explanted human stenotic aortic valves | (Peltonen et al., 2009) | Produces NO, thus anti-oxidant effects |
| eNOS                | Uncoupling   | Explanted human stenotic | (Miller et al., 2008) | Associated with superoxide release |</p>
<table>
<thead>
<tr>
<th>$H_2O_2$</th>
<th>Increased levels</th>
<th>Explanted human stenotic aortic valves</th>
<th>(Miller et al., 2008)</th>
<th>Pro-oxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2O_2$</td>
<td>Increased levels</td>
<td>Rabbit model</td>
<td>(Liberman et al., 2008)</td>
<td>Pro-oxidant</td>
</tr>
<tr>
<td>Superoxide</td>
<td>Increased levels</td>
<td>Explanted human stenotic aortic valves</td>
<td>(Miller et al., 2008)</td>
<td>ROS</td>
</tr>
<tr>
<td>Superoxide</td>
<td>Increased levels</td>
<td>Rabbit model and explanted human stenotic valves</td>
<td>(Liberman et al., 2008)</td>
<td>ROS</td>
</tr>
<tr>
<td>SOD</td>
<td>Downregulated</td>
<td>Explanted human stenotic aortic valves</td>
<td>(Miller et al., 2008)</td>
<td>Anti-oxidant</td>
</tr>
<tr>
<td>3-nitrotyrosine</td>
<td>Increased levels</td>
<td>Rabbit model</td>
<td>(Liberman et al., 2008)</td>
<td>Implies ROS (peroxinitrite) generation</td>
</tr>
</tbody>
</table>

eNOS - endothelial nitric oxide synthase; $H_2O_2$ - hydrogen peroxide; NO - nitric oxide; ROS - reactive oxygen species; SOD - superoxide dismutase; TGF-β1 - transforming growth factor beta 1; TXNIP - thioredoxin-interacting protein
Table 1.4. Summary of retrospective evaluations of effect of ACEI/ARB therapy on progression/outcome of ASc/AS.

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Stage of disease</th>
<th>Method of determination of progression/outcomes</th>
<th>Comments/Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(O'Brien et al., 2005)</td>
<td>123</td>
<td>Only volume of calcification assessed</td>
<td>Serial EBCT</td>
<td>Significant association between ACEI use and a lower rate of AVC accumulation.</td>
</tr>
<tr>
<td>(Rosenhek et al., 2004b)</td>
<td>211</td>
<td>Moderate-to-severe</td>
<td>Serial echocardiography</td>
<td>Significant association between statin use and rate of increase of AVp; Trend towards ACEI therapy benefit</td>
</tr>
<tr>
<td>(Wakabayashi et al., 2011)</td>
<td>194</td>
<td>Mild-to-moderate</td>
<td>Serial echocardiography</td>
<td>ACEI therapy (but not ARB therapy) was associated with lower rates of progression of AS</td>
</tr>
<tr>
<td>(Nadir et al., 2011)</td>
<td>2117</td>
<td>75% mild-to-moderate; 25%</td>
<td>One-off echocardiography. Outcomes derived from Scottish Morbidity</td>
<td>ACEI/ARB therapy is associated with an improved survival and a</td>
</tr>
<tr>
<td></td>
<td>moderate-to severe</td>
<td>Record database.</td>
<td>lower risk of CV events in patients with AS</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------</td>
<td>-----------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>

ACEI - angiotensin converting enzyme inhibitors; ARB - angiotensin receptor 1 blockers; AS - aortic stenosis; ASc - aortic sclerosis; AVC - aortic valve calcification; AVp - aortic valve pressure gradient; CV - cardiovascular; EBCT - electron beam computed tomography.
Figure 1.1 Schematic: probable outcomes in patients with ASc at age 40: - progression, coronary risk and emergence of symptoms. * - Increased prevalence of Heyde's syndrome; # - Increased incidence of emergence of classical symptoms (dyspnoea, angina, syncope)
Figure 1.2 Comparison of calibrated backscatter values between normal control subjects, patients with aortic sclerosis (ASC), and aortic stenosis (AS). *$P < 0.0001$ versus control subjects [Reproduced with permission (Ngo et al., 2004)].
Figure 1.3 Schematic for clinical course of aortic stenosis, as conceived by Ross and Braunwald (1968). Note: - (i) The postulated age of onset of severe disease at around 60; (ii) The short interval between onset of symptoms and death, compared with the long "latent period"; (iii) The differential prognosis according to nature of symptoms. None of these postulates have ever been subject to unbiased natural history study. Furthermore, their relevance to the development of aortic stenosis on tricuspid valve is uncertain (Figure 1.1 and section 1.2.9).
Figure 1.4 Cox regression analysis showing event-free survival in groups defined by aortic jet velocity at entry ($P<.0001$ by log-rank test) [Reproduced with permission from (Otto et al., 1997)].
Figure 1.5 The Mevalonate pathway of cholesterol synthesis with sites of action of statin and bisphosphonates [Reproduced with permission from (Innasimuthu et al., 2010)].
CHAPTER 2

Development and progression of aortic valve sclerosis: clinical, physiological and biochemical insights
2.1 Introduction

Aortic stenosis (AS) is now the most common valve disease in Western world and its prevalence and incidence are rising (Iung et al., 2003; Lindroos et al., 1993). Detailed epidemiology and pathophysiology of ASc and AS is discussed in chapter 1. In general, AS reflects a progressive increase in calcium deposition within the aortic valve, leading to increased stiffness and progressive narrowing of the valve. The earliest clinically detectable stage of this process, ASc, reflects abnormal aortic valve morphology in the absence of haemodynamic obstruction, but may progress to AS (Freeman et al., 2005). A number of population studies have suggested that approximately 25% of individuals aged >65 years have ASc, increasing to approximately 50% at age > 80, despite a far lower prevalence of advanced AS among such populations (Lindroos et al., 1993; Otto et al., 1999; Stewart et al., 1997). Novaro et al (2007), in a follow-up of the Cardiovascular Health Study, documented that about 9% of individuals with ASc progress to AS over a 5 year period.

With increasingly aging populations, especially in the Western world, the prevalence of AS is expected to rise significantly in the near future (Cowell et al., 2004): - one study found that 50% of patients admitted to hospital with chest pain had ASc (Chandra et al., 2004). Therefore, the health and socioeconomic burden associated with AS is likely to increase substantially. It is of clinical relevance for two reasons: firstly, it is the precursor of aortic stenosis and secondly, it is independently associated with a significantly elevated cardiovascular morbidity and mortality (for review see (Nightingale et al., 2005)). This increase in cardiovascular events is unrelated to the progression of aortic sclerosis to stenosis and persists after accounting for prevalence of coronary artery disease (Otto et al.,
This suggests that the pathogenesis of ASc exhibits some commonality with that of acute coronary syndromes, rather than merely atherogenesis.

It has been previously suggested that the pathogenesis of AS involves an “atherogenesis-like process” (Freeman et al., 2005). However there is also evidence that atherogenesis is not the central pathogenic process in AS. Attempts to induce the development of AS via hypercholesteroalemia alone in animal models have generally proved unsuccessful (Drolet et al., 2003; Rajamannan et al., 2001), while none of the 3 randomized double blind placebo controlled trials of lipid lowering demonstrated reduction in rate of progression of moderate AS (Chan et al., 2010; Cowell et al., 2005; Rossebo et al., 2008).

A number of other pathological processes have been described to occur at various stages of ASc and AS. Early AS lesions are characterized by areas of subendothelial thickening containing inflammatory infiltrates including macrophages and T-lymphocytes, atheroma lesions with oxidized lipid content, all of which potentially activate a host of profibrotic and proinflammatory markers (O'Brien et al., 1996; Olsson et al., 1994; Otto et al., 1994; Wallby et al., 2002; Warren et al., 1997) with subsequent calcific nodule formation (Freeman et al., 2005). Angiotensin II (Ang II), an important mediator of inflammation and fibrosis, could be formed by angiotensin converting enzyme (ACE) as well as neutral proteases, chymase and cathepsin G (Nishimoto et al., 2001). ACE has been identified in stenotic but not in normal aortic valves (O'Brien et al., 2002). It has been shown that chymase and cathepsin G are also upregulated in stenotic valves, providing further evidence for local production of Ang II (Helske et al., 2004; Helske et al., 2006a). These findings provide a potential basis for a role of Ang II in aortic valve remodelling along with other profibrotic and proinflammatory mechanisms.
We have recently demonstrated in the rabbit model (Ngo et al., 2008) that ramipril retarded the development of AS and limited intravalvular accumulation of thioredoxin-interacting protein (TXNIP) (Ngo et al., 2011), a marker/mediator of increased redox stress (World et al., 2006). Simultaneously there was preservation of vascular endothelial function (Ngo et al., 2011). These findings therefore represented the first definitive demonstration that ACE inhibitors may retard the development of AS.

The question of the rate of development of ASc has been addressed in few prospective studies to date. A clinical investigation in 50-year old Norwegian males showed that only a small minority of subjects with soft heart murmurs progressed to aortic valve replacement over a 35-year follow-up period (Bodegard et al., 2011). Novaro et al (2007) utilized serial echocardiography in a population aged over 65 years and progression in approximately 44% of subjects over 5 years' follow-up period. While male gender, advancing age and African-American ethnicity were independently related to progression, there was no association with a history of diabetes, hypertension, or tobacco use, nor the presence of coronary heart disease or renal insufficiency. Endothelial and platelet function or other measures of inflammation/fibrosis were not evaluated in this study. Furthermore, it must be emphasized that the assessment of valve disease in this study was categorical rather than quantitative.

It is theoretically possible that a combination of endothelial dysfunction (ie of both valve and vasculature) and redox stress may contribute both to development of ASc and to its associated cardiovascular risk. As regards endothelial dysfunction, it has been recently shown that calcific nodule formation in cell culture of aortic valve fibroblasts is inhibited by nitric oxide (NO) (Kennedy et al., 2009). Clinically, we have also shown that advanced AS is associated with elevation of plasma concentrations of asymmetric dimethylarginine.
(ADMA) (Ngo et al., 2007), a marker of endothelial dysfunction. Therefore, it is probable that endothelial dysfunction develops at some stage in the clinical course of AS, and that NO is important for inhibition of development of valve calcification.

A phenomenon closely related to, but not identical with endothelial dysfunction is nitric oxide resistance (Moncada et al., 1993), which has been described extensively in the vasculature (Adams et al., 1998; Schachinger et al., 2000) and also in platelets (Chirkov et al., 2002). 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 propensity to development of ASc, this would represent both a basis for calcification and a potential link to coronary risk.*

The application to the aortic valve of the technique of ultrasonic backscatter (AVBS), first utilized for the quantification of ASc and mild AS in our previous clinical investigations (Ngo et al., 2004), represents a highly reproducible means for evaluation of valve echogenicity. As such, it has facilitated both clinical (Ngo et al., 2007; Ngo et al., 2009b; Ueland et al., 2011) and basic (Ngo et al., 2008; Ngo et al., 2011; Roosens et al., 2011) studies and represents a technique of choice for ASc quantification (Gharacholou et al., 2011; Nightingale et al., 2005).

In the current study, we have utilized serial estimation of AVBS over a 4 year period to:

1. determine the correlates of the presence of ASc in a cohort of aging ambulatory community dwelling subjects
2. determine the prevalence of detectable progression of aortic valve disease over that period in this cohoort

3. identify correlates of rapid progression, and

4. identify correlates of any progression.

The results therefore provide further insights into the factors modulating the earliest stages of aortic valve disease in the general population.
2.2 Methods

2.2.1 Study subjects

An initial cohort of 253 subjects was recruited prospectively as a substudy of the North Western Adelaide Health Study (NWAHS) (Grant et al., 2006). In brief, NWAHS cohort participants were recruited using the telephone to conduct the interviews and the Electronic White Pages as the sampling frame. Within each household, the person who had their birthday last and was aged 18 years and over, was selected for interview and invited to attend the clinic for a biomedical examination. To minimize potential bias due to differing probabilities of selection in the sample, the data were weighted by region (western and northern health regions), age group and gender. This cohort study did not recruit people residing in institutions, such as nursing homes. However people who had their own telephone number and who were living in individual units attached to a nursing home were eligible to participate. Subjects for the current study were selected from NWAHS cohort randomly. The following inclusion and exclusion criteria applied: men and women aged from 51-77 years, who had not previously undergone aortic valve replacement and did not have any terminal illness.

Study personnel attempted to recall every subject 4 years after initial evaluation to invite them to participate in the follow-up stage. A total of 204 subjects were recalled: of the remaining 49 subjects, 12 were lost to follow-up, 7 were deceased (5 due to cancers and for the remaining 2 the cause of death could not be ascertained), 6 had developed terminal illness or were receiving chemotherapy and 24 declined to participate in follow-up, citing personal reasons. The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital.
2.2.2 Investigations

2.2.2.1 Doppler echocardiography

Comprehensive transthoracic echocardiography was performed for all subjects with a commercially available system (Vivid 7 [GE Vingmed, Horten, Norway], with a 2.5 MHz phased array probe). M-mode and 2-dimensional (2D) echocardiograms with Doppler analysis were obtained for all subjects. Left ventricular (LV) diameters and wall thicknesses were measured from 2D-guided M-mode echocardiography. Mean and peak pressure gradients across the aortic valve were calculated with the modified Bernoulli equation, with continuous-wave Doppler recordings from the highest velocity available from any view. The aortic valve area was computed with the continuity equation with standard methods.

2.2.2.2 Ultrasound backscatter data analysis

Aortic valve backscatter values were obtained for all subjects with methods as previously published (Ngo et al., 2004). Briefly, 2D 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 acquired for each study subject. Backscatter values from the blood pool in the LV 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 (Figure 2.1). ASc was quantitated on the basis of AVBS scores, and AVBS \( \geq 16 \) dB was used as a definition of the presence of ASc (Ngo et al., 2004). Progression of valve disorganization/ASc was defined as any positive change in AVBS scores. Valve morphology was categorized on the basis of visual assessment, as previously described (Cosmi et al., 2002; Otto et al., 1999).
Comparisons between baseline and follow-up echocardiographic data were utilized in order to evaluate changes in valve structure and function.

2.2.2.3 Biochemical and physiological parameters

In all cases, parameters measured at study entry were utilized as potential correlates of subsequent disease progression.

These can be summarized as follows:

1. **Physiological measures**
   a. Resting augmentation index (AIx), a measure of arterial stiffness, was determined by applanation tonometry. Briefly, 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. 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; from which AIx was derived (Figure 2.2). Only high quality recordings with in-device quality index $\geq 90\%$ were used. All augmentation indices were corrected for a standard heart rate of 75 bpm.
   b. Platelet responsiveness to the NO donor sodium nitroprusside (SNP) was assessed using whole blood aggregometry. In brief, blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate); acidified
citrate was used to minimize deterioration of platelet function during the time course of 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). Aggregation was induced with adenosine 5'-diphosphate (ADP) at a 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) (Figure 2.3).

2. **Biochemical measures**

a. Plasma concentrations of asymmetric dimethylarginine (ADMA), a marker and mediator of endothelial dysfunction (Boger, 2003), were determined by high performance liquid chromatography with the derivitization reagent AccQ-Fluor (Waters, Milford, Massachusetts, USA) after solid phase extraction, as previously described (Heresztyn et al., 2004).

b. High-sensitivity C-reactive protein (hs-CRP) concentrations (a marker of systemic inflammatory activation), lipid profile, creatinine, serum calcium levels and 1,25 dihydroxy cholecalciferol (vitamin D levels) were measured by a ^125^I radioimmunoassay (Immunodiagnostic Systems Ltd., Bolden, United Kingdom).

c. C-terminal telopeptide of collagen type 1 (CTx) and N-terminal peptide of procollagen I (P1NP) concentrations were measured as markers of collagen homeostasis.
Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation and indexed for body surface area (BSA) with the Dubois and Dubois formula.

2.2.2.4 Statistical analyses

All data are expressed as mean ± 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 normally distributed data were performed with non-paired t tests and, comparisons for nonparametric data were made with the Mann-Whitney test. Correlations between transformed, continuous nonparametric data were made with linear regression. Stepwise multiple linear regression analyses were performed to assess independent predictors of high AVBS scores. Variables included to predict high AVBS scores were: age, gender, smoking history, previous ischemic events/angina, diabetes mellitus, hypercholesterolemia, hypertension, calculated CrCl, Ca₃PO₄ levels, vitamin D levels, BMI, hs-CRP, AIX, ADMA concentrations, and platelet responses to SNP. Analysis of co-variance (ANCOVA) was utilized to test the relative contribution of presence/absence of ASc on the relationship between BMI and hs-CRP. Binary logistic backward regression analysis was also performed utilizing above variables but using presence of ASc on backscatter as a categorical variable, in order to evaluate whether presence of elevated AVBS scores per se identified similar associations to those seen with stepwise multiple linear regression.

Tests for differential rates of progression and proportions were performed using χ² test. Correlation between baseline AVBS scores and change in scores was established. Determinants of the change in AVBS scores were evaluated utilizing univariate and then multivariate analyses. Variables selected for all multivariate backward regression analyses
were on the basis of univariate significance \((p \leq 0.2)\). Included baseline variables to predict increase in \(AV_{BS}\) scores were: total cholesterol, calcium, ADMA and hs-CRP concentrations; history of dyslipidaemia and platelet NO responsiveness.

Change in AVBS scores were also evaluated as a binary variable in a separate set of univariate and then multivariate analyses. Binary logistic backward regression analysis was performed to assess independent predictors of increase in AVBS scores in the entire cohort. Included baseline variables to predict increase in AVBS scores were: history of hypercholesterolemia; history of use of angiotensin converting enzyme inhibitors/angiotensin II receptor blockers (ACEI/ARB); calcium, LDL, total cholesterol and hs-CRP concentrations; and body mass index (BMI). Furthermore, binary logistic backward regression analysis was also performed to assess independent predictors of increase in AVBS scores in those subjects without ASc at baseline. The baseline variables included in this analysis were: history of statin use, history of use of ACEI/ARBs, history of hypertension, history of coronary events, calcium-phosphate product \((Ca_\times PO_4)\), vitamin D, P1NP and hs-CRP concentrations.

All analyses were performed with SPSS version 17 software (SPSS, Chicago, Illinois), and a p value of < 0.05 was considered to be statistically significant.
2.3 Results

2.3.1 Baseline evaluation

2.3.1.1 Subject characteristics

Mean AVBS score was 12.2 ± 4.4 dB. In 19.4% of subjects, AVBS was ≥ 16 dB, corresponding to the chosen definition of ASc (Ngo et al., 2004). On visual assessment, aortic valve morphology was abnormal in 25.4% of subjects; such subjects had significantly higher (p < 0.001) AVBS scores that those without visual assessment criteria for ASc (Figure 2.4). Mean EF was 68.3 ± 8.6% (only 3 subjects had EF < 40%); mean LV mass index was 113.27 ± 31.1g/m², mean interventricular wall thickness 1.04 ± 0.17cm, mean aortic valve pressure gradient was 7 ± 3.8mmHg. There were no statistically significant echocardiographic differences between those with and without ASc with respect to LV ejection fraction, LV mass index, interventricular wall thickness and aortic valve pressure gradient. There were no subjects with bicuspid aortic valve.

Baseline clinical characteristics are summarized in Table 2.1 for subjects with and without ASc. Overall, there was a high proportion (30.8%) of obese subjects (BMI > 30 kg/m²). 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 angiotensin converting enzyme inhibitors/angiotensin II receptor blockers (ACEI/ARB) respectively. 79% of hypertensive patients were receiving ACEI/ARB, while 55% of hypercholesterolemic patients were treated with statins. There were statistically significant differences between those subjects with ASc compared with those without in respect to age and BMI (p < 0.05 in both cases) only.
2.3.1.2 Biochemical data

Biochemical findings are summarized in Table 2.2. 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 were no patients on dialysis with only 2 subjects with CrCL < 30 mL/min/1.73m². 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). Comparisons between subjects with and without ASc revealed that creatinine clearance was significantly greater in subjects without ASc.

2.3.1.3 Endothelial function and platelet responsiveness to NO

Mean AIx was 27.6 ± 8.5%, substantially greater than values for normal adults (N 15 ± 16%) (McEniery et al., 2006); these findings suggested increased arterial stiffness/wave reflection in this subject cohort. Similarly, mean platelet anti-aggregatory response to SNP was relatively low 33 ± 27% (versus normal 54 ± 24%), implying some degree of platelet resistance to NO (Chirkov et al., 2002). However, mean plasma ADMA concentrations were within the previously described normal range 0.52 ± 0.08 μM (N 0.50 ± 0.08 μM) (Heresztyn et al., 2004; Horowitz et al., 2007).

2.3.1.4 Univariate correlations with AVBS scores

These are summarized in Table 2.3. There was a significant correlation between AVBS scores and age. While total cholesterol, and LDL levels did not correlate with AVBS scores, high HDL levels were significantly associated with high AVBS scores. There was a significant inverse correlation between calculated CrCL (corrected for BSA) and AVBS scores. While there was no correlation between AVBS and calcium levels, there were
borderline correlations between AVBS and phosphate or Ca\textsubscript{3}PO\textsubscript{4} levels. Importantly, there was no significant relationship between vitamin D levels and AVBS scores.

BMI was inversely correlated with AVBS scores. There was also an inverse correlation between AVBS scores and hs-CRP levels. As regards parameters of endothelial function/NO responsiveness in this study population, there was no significant correlation between either AIx or plasma ADMA concentrations and AVBS scores. However, there was a significant inverse correlation between platelet responsiveness to SNP and AVBS scores.

In general, higher BMI is associated with increased inflammatory activation (reviewed by (Hamirani et al., 2008)), which in turn has been linked with ASc (reviewed by (Sverdlov et al., 2011a)). Yet, we observed a superficially paradoxical association of both lower BMI and hs-CRP with presence of ASc. Thus, we utilized ANCOVA to test the impact of the presence or absence of ASc on the BMI : hs-CRP relationship: - whilst in the entire cohort there was a direct correlation between BMI and hs-CRP (Figure 2.5A; r = 0.42; p < 0.0001) and it persisted when this relationship was considered separately for subjects with and without ASc (Figure 2.5B), [ASc: r = 0.42, p = 0.003; no ASc: r = 0.43, p < 0.0001], the difference between the 2 cohorts was not statistically significant (ANCOVA: p for interaction = 0.96).

Of interest, none of the “traditional” major coronary risk factors were significant correlates of high AVBS (p > 0.2 in all cases); nor did treatment with either statins or ACE inhibitors apparently interact with AVBS.
Chapter 2

There was a strong positive correlation between BMI and creatinine clearance indexed for body surface area, which persisted at p = 0.001 level even with calculation of creatinine clearance using the MRDR equation (Levey et al., 1999), where body weight is not a part of the equation (p = 0.001). These data therefore suggest strongly that even after indexing for body surface area, creatinine clearance calculation is not a reliable estimate of renal function for obese subjects. This finding was recognized as having important implications in the multiple regression analyses, as regards interpretation of both BMI and creatinine clearance data.

2.3.1.5 Stepwise multiple linear regression analyses

Table 2.4 documents results of stepwise multiple linear regression analysis. Advanced age was positively associated with high AVBS scores ($\beta = 0.2$, p = 0.004), while platelet anti-aggregatory responsiveness to SNP ($\beta = -0.16$, p = 0.02) and BMI ($\beta = -0.23$, p = 0.001) were negatively associated.

2.3.1.6 Binary logistic backward regression analyses

Binary logistic backward regression analysis, utilizing presence of ASc on backscatter as a categorical variable, produced similar results, however, with weaker statistical power. Advanced age was positively (and significantly) associated with presence of ASc ($\beta = 0.08$, p = 0.008), while platelet anti-aggregatory responsiveness to SNP ($\beta = -0.122$, p = 0.054) and BMI ($\beta = -4$, p = 0.118) tended to be negatively associated.
2.3.2 End-of-study evaluation: data based on 204 subjects who completed follow-up

2.3.2.1 Subject characteristics

Subject characteristics both at baseline and 4 year follow-up are shown in Table 2.5. Of the subjects receiving ACEI/ARB at baseline, 52% were receiving ACEI. Furthermore, during the course of study, in only 3 of the subjects was ACEI/ARB therapy discontinued. Over the study period, there were significant increases in proportion of subjects diagnosed with hypertension, dyslipidaemia and diabetes, as well as a decrease in that of active smokers. More people were treated with ACEI/ARB at the end of the study. At baseline there was no significant difference in any of these parameters amongst subjects with and without ASc.

Table 2.6 summarizes both baseline and end of study biochemical data. Total cholesterol, HDL, CTx and calcium levels as well as creatinine clearance increased marginally over the study period. As regards creatinine, this cohort had well preserved renal function - no subject had CrCl less than 30ml/min. Furthermore, it is possible that the apparent increase in creatinine clearance resulted from a change in methodology of creatinine assay over the study period from Olympus AU5400 Chemistry-Immuno Analyzer (Olympus America, Melville, NY, USA) to Advia 2400 Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL, USA) and associated reagent kits. There were no significant changes in LDL, hs-CRP or vitamin D levels.

Baseline AIx, ADMA and platelet NO responsiveness data have been published previously (Ngo et al., 2009b). In brief, baseline AIx was 27.2 ± 8.3% (normal range 15 ± 16% (McEniery et al., 2006)), ADMA concentrations were 0.52 ± 0.08μM (normal range 0.5 ± 0.08μM (Heresztyn et al., 2004)) and baseline platelet NO responsiveness was 33.9 ± 26.9% (normal range 54 ± 34%).
2.3.2.2 Changes in AVBS

Distribution of AVBS scores at baseline and at follow-up is summarized in Figure 2.6. Overall, AVBS increased markedly over the study period, with mean increase from 12.2 ± 4.4 dB to 14.2 ± 4.9 dB (p < 0.001). The implication of this change was that 68% of subjects had some disease progression, and while at baseline 17.6% of subjects had AVBS ≥ 16dB, constituting the equivalent criterion of presence of ASc (Ngo et al., 2004), 34.8% had ASc at follow-up (p < 0.001). No subject developed haemodynamically significant AS.

Our previous studies have suggested that there is finite maximum for AVBS: for example, even in advanced AS, AVBS scores do not exceed 28dB. In the current data set, extent of increase in AVBS varied inversely with baseline AVBS (Figure 2.7; p < 0.001), proving further evidence of the existence of maximum values as well as the presence of the phenomenon know as regression to the mean (Bland et al., 1994). For these reasons, the baseline AVBS scores were not entered in the multivariate model and progression data were also analysed as a dichotomous variable.

2.3.2.3 Progression of AVBS within the entire cohort

2.3.2.3.1 AVBS change as a continuous variable

Univariate correlates of increasing AVBS scores are presented in Table 2.7. These analyses raised the possibility of associations between dyslipidaemia, high hs-CRP, impaired NO responsiveness and elevated plasma calcium concentrations with increasing AVBS.
On stepwise backward multiple linear regression analysis (Table 2.8), the independent predictors of increasing AVBS scores were lower platelet NO responsiveness, higher A1x and plasma calcium concentrations.

### 2.3.2.3.2 AVBS increase as a dichotomous variable

Univariate correlates of increase in AVBS scores are summarized in Table 2.9. None of the parameters tested were significantly related to disease progression on univariate analysis. However, the subjects in whom no progression occurred tended to be on ACEI/ARBs throughout the period of the study, had lower concentrations of total cholesterol, LDL, hs-CRP and calcium, but higher BMI; and were less likely to have history of dyslipidaemia.

However, on multivariate (backwards binary logistic regression - Table 2.10A) analysis, there was a negative relationship between disease progression and therapy with ACEI/ARB (p = 0.025; \( \beta = 0.8 \)).

In view of the recently published results of Wakabayashi et al (Wakabayashi et al., 2011), suggesting that ACEI, but not ARBs, might retard progression of ASc, post-hoc comparison between effects of ACEI versus ARB on progression rates was performed, and revealed no difference between these agents.

#### 2.3.2.3.3.1 Progression of AVBS in subjects without ASc at baseline

In order to identify factors responsible for very early development of valvular disease, the cohort of subjects (n = 160) in whom baseline AVBS was less than 16dB was analysed separately.
Univariate correlates of increases in AVBS scores in this cohort are listed in Table 2.11. Of note, there was a significant negative correlation between disease progression and therapy with ACEI/ARB ($p = 0.008$). None of the other parameters tested were significantly related to disease progression on univariate analysis.

However, on multivariate (backwards binary logistic regression - Table 2.10B) analysis, there were negative relationships between disease progression and therapy with ACEI/ARB ($p = 0.001; \beta = 1.3$) and lower hs-CRP ($p = 0.02; \beta = 0.97$) as well as a borderline negative relationship with higher vitamin D levels ($p = 0.053; \beta = 0.17$).
2.4 Discussion

There are a number of important conclusions that can be drawn from these investigations. Firstly, as regards occurrence of ASc: - its determinants were advancing age, low BMI and platelet NO resistance. On the other hand, progression of ASc was correlated with increased plasma calcium concentrations, platelet NO resistance and arterial stiffness. Assessed categorically, progression was mainly related to the absence of ACEI/ARB therapy. Furthermore, the current results indicate that there is substantial progression of aortic valve disease in this cohort over a mean period of 4 years: increases in AVBS occurred in 68% of the subjects. The only comparable study in the literature (Novaro et al., 2007) utilized categorical rather than quantitative methodology: over a mean period of 5 years, new ASc developed in 44% of subjects. Importantly, neither occurrence nor progression of ASc was significantly related to conventional coronary risk factors.

Previous prospective evaluations of the epidemiology and progression of ASc have been performed in a substantially larger population of aging individuals as components of the Cardiovascular Health Study (Aronow et al., 1999; Novaro et al., 2007; Otto et al., 1999). These investigations have focused on putative correlations between conventional coronary risk factors and presence/development of ASc (Aronow et al., 1999; Novaro et al., 2007; Otto et al., 1999): in general significant correlations have been documented in the case of some, but not all, coronary risk factors. On the other hand, the current study evaluated a substantially smaller subject cohort, but had the advantage of quantitative, rather than qualitative, assessment of valve thickening, thus increasing the power of the study. Furthermore, the current study focused additionally on the premise that diminished tissue NO responsiveness within the valve might predispose to development and/or progression of ASc in humans, just as NO negatively modulates valve calcification in vitro (Kennedy
et al., 2009): - NO responses in platelets were utilized as a surrogate for valvular responsiveness in this regard.

The current investigation represents the first evaluation of disease occurrence and progression in humans utilizing the highly reproducible technique of serial evaluation of AVBS, with the definition of ASc chosen to be AVBS ≥ 16 dB (Ngo et al., 2004). We have previously demonstrated utility of this technique to quantitate disease occurrence and progression in a rabbit model (Ngo et al., 2008; Ngo et al., 2011). AVBS has strong concordance with visual assessment of valve morphology (Figure 2.4), but had the advantage of substantially increasing the power of the study. For example, at baseline, the association between platelet NO resistance and ASc was of borderline significance only on categorical analysis of AVBS values (Table 2.2) but with quantitative analysis demonstrated statistically significant association both on univariate (Table 2.3) and multivariate (Table 2.4) analyses.

Apart from the Cardiovascular Health Study (Aronow et al., 1999; Novaro et al., 2007; Otto et al., 1999), other investigations have examined determinants of progression of AS, summarized in Table 1.1. In general, these investigations have considered patients in whom moderate AS was present at initial evaluation. Such studies tend to identify coronary risk factors, including male gender, as correlates of rapid progression (Aronow et al., 2001; Nassimiha et al., 2001; Palta et al., 2000; Perkovic et al., 2003; Stewart et al., 1997) - apart from the finding that AS “progression” is seen to accelerate as the disease advances. In contrast, the study of Novaro et al. (2007), the only previously reported investigation concerning correlates of ASc, found a weak correlation with elevation of LDL cholesterol and no association between ASc and other coronary risk factors. Furthermore, evaluation of aortic valve calcification within the MESA (Multi-Ethnic Study of Atherosclerosis)
cohort![](http://example.com/katz2009) indicated that both diabetes and metabolic syndrome were correlates of de novo valve calcification but not of its progression.

The most consistent finding in the current study is that ASc occurrence and progression was associated with platelet resistance to NO, assessed via platelet response to SNP. This finding is in accordance with the known physiological role of NO in valve homeostasis (Pompilio et al., 1998) and the findings of our previous studies both in the rabbit model (Ngo et al., 2008; Ngo et al., 2011). In summary, NO is produced by both valve endothelium and matrix and inhibits calcific nodule formation in a cell culture model (Kennedy et al., 2009) and prevention of AS development in a rabbit model by ramipril was associated with preservation of NO signalling (Ngo et al., 2011). The current data therefore provide further confirmation that ASc development is impeded by NO. Furthermore, if ASc is correlated with NO resistance, an independent marker of coronary event risk (Schachinger et al., 2000; Willoughby et al., 2005), this association might explain the status of ASc itself as a marker of coronary risk (Aronow et al., 1999; Otto et al., 1999).

The major biochemical mechanism underlying NO resistance is “scavenging” of NO by $\text{O}_2^-$ anion (Chirkov et al., 1999), suggesting in turn incremental redox stress in association with ASc. On the other hand, plasma concentrations of ADMA, which potentially can limit endogenous NO formation, were not associated with occurrence or progression of ASc. This suggests that at the early stages of AS development, endothelial dysfunction may be limited to accelerated NO clearance by superoxide anion; the previously observed elevation of ADMA levels (Ngo et al., 2007) presumably develops only in patients with more advanced disease.
It is also of some interest that while the presence of ASc was not associated with increased AIx, a marker of arterial stiffness (as well as endothelial function) (Wilkinson et al., 1998; Wilkinson et al., 2002b), AIx was a weak determinant of ASc progression. AIx represents a measure of apparent arterial stiffness which has both fixed and variable components, the latter modulated largely by NO (Wilkinson et al., 2002b). It is possible that development of ASc is dissociated from the arterial degenerative changes underlying systolic hypertension (Cameron et al., 1998), yet its progression is potentiated by such changes. The current data do not permit delineation of the bases for the association, which might include commonality of biochemical processes (eg including calcium uptake and/or NO signalling) but also the physical effect of increased wave reflection, associated with vascular rigidity, on the aortic valve.

Furthermore, despite inflammatory activation being present as a component of histological appearances in ASc (O'Brien et al., 1996; Wallby et al., 2002), plasma hs-CRP levels were not correlated with occurrence of ASc in this study. These results are similar to those in a related recent evaluation (Nassimiha et al., 2001; Novaro et al., 2007). Yet, disease progression, if assessed categorically, in this study demonstrates such an association, with a trend also evident utilizing progression as a continuous variable. Ours is the first prospective population study to record such an association. Indeed, Novaro et al (2007) found no significant association between these. From a theoretical point of view, a potential role for inflammatory activation in progression of ASc is hardly surprising: - ASc lesions include inflammatory infiltrates (Freeman et al., 2005; O'Brien et al., 1996; Otto et al., 1994), and TXNIP, a key inflammasomal activator (Zhou et al., 2010), has now been implicated in an animal model of AS (Ngo et al., 2008; Ngo et al., 2011). Skowasch et al (2006) have demonstrated significant correlations between intravalvular and circulation hs-
CRP in patients with advanced AS. Furthermore, the nexus between ASc and vascular
events might reflect systemic inflammatory activation.

The equivocal association between ASc, an "inflammatory" condition, given the results
(notably) of Miller et al (2008), and hs-CRP, a marker of systemic inflammation, begs the
obvious question of whether inflammation in ASc is purely localized or part of a
generalized process. The current data support the concept that inflammation in ASc should
be considered first and foremost as a localized process, with limited systemic echoes. It of
course remains possible that ASc might be associated with elevation of levels of other
inflammatory markers.

Advanced age was found to be an independent correlate of baseline AVBS values,
consistent with the findings in most recent study (Nassimiha et al., 2001), and with
previous reports in AS patients (Lindroos et al., 1993; Otto et al., 1999; Stewart et al.,
1997). The implication of this finding is that the biochemical nexus between advanced age
and development of ASc remains to be identified.

Specifically addressing the issue of ASc progression, it was found that there was an inverse
relationship between extent of AVBS progression and baseline AVBS. While this would
reflect in part "regression to the mean" (Bland et al., 1994), it also is consistent with the
presence of a maximal value for AVBS, as suggested by our previous publication in AS
(Ngo et al., 2004). Therefore we analysed our data both as regards extent of increase of
AVBS and also categorically: as presence/absence of AVBS increase, utilizing the extent of
progression as the primary form of data analysis. Finally, in view of differing results, we
explored the issue of de novo ASc development.
Increased calcium levels have been previously linked with increased vascular and extravascular calcification (Rodriguez et al., 2011). Furthermore, there have been a number of reports of aortic valve disease in association with hyperparathyroidism (Iwata et al., 2011). Given that calcium deposition in arteries and valves may potentially be driven by similar process (Bostrom et al., 2011), these results should therefore stimulate interest in potential limitation of ASc/AS progression via modulation of calcium uptake into extra-osseous tissues.

As a secondary analysis, the progression data were analysed on a categorical basis. The only significant finding was that such progression was less frequent in subjects receiving ACEI/ARB therapy. Furthermore, evaluation of data from subjects without ASc at baseline showed that this correlation was particularly prominent in this subgroup. The idea that progression of aortic valve disease might be angiotensin-dependent is consistent with a number of pieces of experimental and clinical evidence. Experimentally, olmesartan retarded macrophage infiltration in valves of cholesterol fed rabbits (Arishiro et al., 2007), while ramipril retarded AS development in rabbits treated with high doses of vitamin D (Ngo et al., 2011). There is also substantial evidence of potentially increased angiotensin II formation in the presence of both ASc and AS (Helske et al., 2004; Nishimoto et al., 2001; O’Brien et al., 2002). As regards clinical data, no prospective trials have been undertaken to date. However, in human retrospective studies Rosenhek et al (2004b), utilizing echocardiographic parameters, found a non-significant trend towards retardation of AS progression with ACEI therapy, while O'Brien et al (2005), in a study using CT-based calcification assessment, found lower rates of AS calcification among ACEI treated subjects, after correction for comorbidities. In a more recent large study of aging subjects with various degrees of AS conducted in Scotland, Nadir et al (2011) demonstrated that use of ACEI/ARBs was associated with improved survival and cardiovascular outcomes.
The emergence of ACEI/ARB therapy as a correlate of AVBS progression in categorical analyses may reflect a disproportionate impact in very early disease, as suggested by the subsidiary analysis (Table 2.10B), but may also reflect the interdependence of NO signalling and angiotensin II-related $O_2^-$ production (Forstermann, 2008). For example, we have previously shown that ACEI therapy potentiates platelet NO responsiveness (Chirkov et al., 2004). Furthermore, we have recently demonstrated that ramipril sensitizes platelets to NO, with a predominant effect in NO resistant individuals (Willoughby et al., 2012). These observations suggest that the two “separate” correlates may be different aspects of the same phenomenon.

It is important to emphasise that there was no evident association between hyperlipidaemia and ASc development or progression. These studies provide complementary information to that implicit in the results of interventional trials performed in patients with more advanced disease (Chan et al., 2010; Cowell et al., 2005; Rossebo et al., 2008).

It is likely that the development and progression of early aortic valve disease relies on intra-valvular biochemical/physiological aberrations and may not precisely be the same as systemic markers of these parameters. In particular, as previously stated in this discussion, markers of inflammatory activation (plasma hs-CRP concentrations) and endothelial dysfunction (plasma ADMA concentrations) utilized in this study may not be sufficiently influenced by intravalvular pathophysiology to change significantly systemically. Furthermore, this non-invasive human study precluded direct assessment of intra-valvular redox stress (eg. TXNIP, ACE, Ang II or ROS concentrations), mediators of calcification (eg TGF-β, osteoprotegrin, osteopontin, fetuin-A, matrix GLA protein) or endothelial dysfunction (eg. intra-valvular ADMA concentration and eNOS activity). All these could
be (and likely are) determinants of early disease development and progression (for reviews see (Akat et al., 2009; Miller et al., 2011)).

The unique methodology utilized in this study – determination of AVBS, has the advantage of considerable reproducibility (Gharacholou et al., 2011; Ngo et al., 2004; Nightingale et al., 2005; Roosens et al., 2011) and is therefore preferable to subjective scoring for ASc. AVBS values correlate well with both calcified volume on histology of aortic valve and calcium scoring on micro-CT in a rat model (Roosens et al., 2011). While this has not been evaluated in human studies in the valves, ultrasonic backscatter scores obtained from atherosclerotic vascular lesions correlate with histologic features (volume of calcium) in a human study (Kawasaki et al., 2001). Nevertheless, the utility of AVBS for quantitation of all but the earliest stages of ASc progression is more doubtful in humans.

The results of the study as regards renal dysfunction are somewhat equivocal. In the subject population examined, renal function was generally well preserved, but low creatinine clearance was a univariate correlate of high baseline AVBS values. Creatinine clearance was “corrected” for body surface area via the Dubois and Dubois formula, but a direct association between BMI and “corrected” creatinine clearance at baseline persisted. Furthermore, creatinine clearance was a strong inverse univariate correlate of AVBS. Therefore it is possible that some relationship between renal dysfunction and AVBS has been obscured by inadequate correction for obesity. However, the overall conclusion from the current findings is that creatinine clearance does not markedly affect development of ASc in a population with overall well-preserved renal function. The finding that BMI was inversely correlated with AVBS scores may therefore also be partially confounded by this interaction with renal function estimates. However, it remains possible, as suggested by
previous studies in advanced AS (Lindroos et al., 1994), that there is a link between low BMI and propensity towards development of ASc.

The other caveat related to this study is that few patients were diabetic and none had severe renal impairment, conditions which may well predispose to early development and/or rapid progression of ASc (Aronow et al., 2001; Palta et al., 2000). Additionally, as most of hypertensive patients were treated with ACEI/ARB, it is impossible to dissect the relative contribution of hypertension to aortic valve disease progression in this population cohort.

Finally, it must be considered that this study has identified associations with ASc (and its progression) but not necessarily causes of that process.

2.4.1 Conclusions

ASc progresses in the majority of an aging cohort over a 4 year period. The presence and progression of ASc in this aging population was associated with platelet NO resistance, a finding which provides a potential mechanism for the propensity to acute coronary syndromes in this condition. On the other hand, neither LDL elevation, nor hypertension or any of the other “conventional” coronary risk factors examined, were predictive of presence of either ASc presence or progression. This further emphasizes differences in risk factors for atheroma and for ASc.

The current study has identified elevation of plasma calcium concentrations and impairment of tissue NO responsiveness as the main predictors of rapid ASc progression, and utilization of ACEI/ARB therapy as a predictor of lack of any progression, especially in very early stages of the disease. Furthermore, increased arterial stiffness and inflammatory activation were borderline correlates of progression, but hyperlipidaemia
was not associated with progression in any way. Both these positive and negative findings should be taken into account in planning interventions to improve outcomes and retard progression in patients with early aortic valve disease.
2.5 TABLES AND FIGURES for CHAPTER 2
Table 2.1 Baseline patient characteristics: clinical data.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No ASc (AVBS score &lt; 16 dB) (n=204)</th>
<th>ASc (AVBS score ≥ 16 dB) (n=49)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (yrs)</td>
<td>63 ± 6.0</td>
<td>64.9 ± 9</td>
<td>0.045</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.5 ± 5.1</td>
<td>26.7 ± 4.2</td>
<td>0.019</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>42%</td>
<td>49%</td>
<td>0.372</td>
</tr>
<tr>
<td>History of hypercholesterolemia</td>
<td>61.8%</td>
<td>53.1%</td>
<td>0.565</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>30.5%</td>
<td>38.8%</td>
<td>0.268</td>
</tr>
<tr>
<td>Previous angina/MI</td>
<td>11.9%</td>
<td>20.4%</td>
<td>0.114</td>
</tr>
<tr>
<td>ACEI/ARB therapy</td>
<td>34.8%</td>
<td>25%</td>
<td>0.193</td>
</tr>
<tr>
<td>Hypertension</td>
<td>44.1%</td>
<td>32.7%</td>
<td>0.146</td>
</tr>
<tr>
<td>Family history of CVD</td>
<td>51%</td>
<td>57.1%</td>
<td>0.439</td>
</tr>
<tr>
<td>Smoking</td>
<td>14.8%</td>
<td>12.2%</td>
<td>0.649</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>10.8%</td>
<td>10.2%</td>
<td>0.898</td>
</tr>
<tr>
<td>Subjects with &gt; 2 cardiovascular risk factors</td>
<td>31.5%</td>
<td>32.7%</td>
<td>0.879</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>History of CVA</td>
<td>3.5%</td>
<td>4.1%</td>
<td>0.835</td>
</tr>
<tr>
<td>Calcium supplementation</td>
<td>16.7%</td>
<td>16.7%</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin D supplementation</td>
<td>2%</td>
<td>0%</td>
<td>0.327</td>
</tr>
</tbody>
</table>

ACEI – Angiotensin converting enzyme inhibitor; ARB – Angiotensin II receptor blocker; BMI – Body mass index; CVA – Cerebrovascular accident; MI – Myocardial infarction; CVD – Cardiovascular disease.
Table 2.2 Baseline patient characteristics: baseline biochemical data (expressed as mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>No ASc (AVBS score &lt; 16 dB)</th>
<th>ASc (AVBS score ≥ 16 dB)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5 ± 1</td>
<td>4.9 ± 1</td>
<td>0.774</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.9 ± 0.9</td>
<td>2.8 ± 0.7</td>
<td>0.549</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>0.126</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
<td>0.129</td>
</tr>
<tr>
<td>Ca₃PO₄</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.5</td>
<td>0.236</td>
</tr>
<tr>
<td>Vitamin D (mmol/L)</td>
<td>71.5 ± 22</td>
<td>79 ± 32</td>
<td>0.138</td>
</tr>
<tr>
<td>CrCL (indexed for BSA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ml/min/1.73m2)</td>
<td>84.2 ± 22.7</td>
<td>76 ± 20</td>
<td>0.022</td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>3.5 ± 3.6</td>
<td>4 ± 5.6</td>
<td>0.576</td>
</tr>
<tr>
<td>AIx (%)</td>
<td>25.7 ± 8.1</td>
<td>24.3 ± 9.4</td>
<td>0.355</td>
</tr>
<tr>
<td>% platelet responsiveness to SNP</td>
<td>34.8 ± 27.5</td>
<td>26.6 ± 25.4</td>
<td>0.084</td>
</tr>
<tr>
<td>ADMA (μM)</td>
<td>0.52 ± 0.08</td>
<td>0.52 ± 0.07</td>
<td>0.746</td>
</tr>
</tbody>
</table>
LDL – low density lipoprotein; HDL – high density lipoprotein; Ca\textsubscript{x}PO\textsubscript{4} – calcium-phosphate product; CrCL – creatinine clearance; hs-CRP – high sensitivity C-reactive protein; AIx – augmentation index; SNP – sodium nitroprusside; ADMA - asymmetric dimethylarginine
Table 2.3 Univariate correlates of baseline AVBS scores. Only parameters with p<0.1 for correlation with AVBS scores are shown.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p</th>
<th>β coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.005</td>
<td>0.18</td>
</tr>
<tr>
<td>HDL levels</td>
<td>0.016</td>
<td>0.154</td>
</tr>
<tr>
<td>CrCL indexed for BSA</td>
<td>0.001</td>
<td>-0.204</td>
</tr>
<tr>
<td>Phosphate levels</td>
<td>0.068</td>
<td>0.116</td>
</tr>
<tr>
<td>Ca\textsubscript{3}PO\textsubscript{4}</td>
<td>0.084</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI</td>
<td>&lt; 0.001</td>
<td>-0.245</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.028</td>
<td>-0.14</td>
</tr>
<tr>
<td>% platelet responsiveness to SNP</td>
<td>0.017</td>
<td>-0.162</td>
</tr>
</tbody>
</table>

HDL – high density lipoprotein; CrCL – creatinine clearance; BSA – body surface area; Ca\textsubscript{3}PO\textsubscript{4} – calcium-phosphate product; BMI – body mass index; hs-CRP – high sensitivity C-reactive protein; SNP – sodium nitroprusside.

Data for HDL levels, BMI, hs-CRP and % platelet responsiveness to SNP were transformed via log transformation in order to achieve normal distributions.
Table 2.4 Variables independently associated with high AVBS scores after stepwise multiple linear regression analysis.

<table>
<thead>
<tr>
<th>Parameter</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>

BMI – body mass index; SNP – sodium nitroprusside.
Table 2.5 Patient characteristics at follow-up.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (%)</th>
<th>End of study (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63 ± 6 years</td>
<td>67 ± 6 years</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>42.4% male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>24 (12%)</td>
<td>33 (16%)</td>
<td>0.013</td>
</tr>
<tr>
<td>Hypertension</td>
<td>85 (42%)</td>
<td>106 (52%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>118 (58%)</td>
<td>137 (67.5%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Smoking</td>
<td>28 (14%)</td>
<td>13 (6.4%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Coronary disease</td>
<td>24 (12%)</td>
<td>28 (14%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>65 (32%)</td>
<td>71 (35%)</td>
<td>0.24</td>
</tr>
<tr>
<td>ACEI/ARB therapy</td>
<td>69 (34%)</td>
<td>83 (41%)</td>
<td>0.008</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.2 ± 5.2</td>
<td>28.2 ± 5.2</td>
<td>0.37</td>
</tr>
</tbody>
</table>

ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; BMI - body mass index
**Table 2.6** Biochemical profile at follow-up

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>End of study</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.9 ± 0.9</td>
<td>5 ± 1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.8 ± 0.8</td>
<td>2.9 ± 1</td>
<td>0.75</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium level (mmol/L)</td>
<td>2.2 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin D level (mmol/L)</td>
<td>72 ± 23.1</td>
<td>74.7 ± 26.6</td>
<td>0.29</td>
</tr>
<tr>
<td>CrCl (ml/min/1.73m²)</td>
<td>92 ± 21.6</td>
<td>98 ± 28.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>3.5 ± 3.7</td>
<td>3.1 ± 3.8</td>
<td>0.14</td>
</tr>
<tr>
<td>CTx (median) (pg/ml)</td>
<td>242 ± 143</td>
<td>283 ± 145</td>
<td>0.001</td>
</tr>
<tr>
<td>P1NP (median) (mcg/L)</td>
<td>40.5 ± 19.8</td>
<td>43.4 ± 50.6</td>
<td>0.45</td>
</tr>
</tbody>
</table>

LDL - low density lipoprotein; HDL - high density lipoprotein; CrCl - creatinine clearance; hs-CRP - high sensitivity C-reactive protein; CTx - C-terminal telopeptide of collagen type 1; P1NP - N-terminal peptide of procollagen I.
Table 2.7 Univariate correlates of increasing AVBS (continuous) in the entire cohort (β coefficients shown for continuous variable with $p \leq 0.2$).

<table>
<thead>
<tr>
<th>Baseline parameter</th>
<th>β coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol concentration</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>LDL concentration</td>
<td>0.12</td>
<td>0.103</td>
</tr>
<tr>
<td>HDL concentration</td>
<td>0.1</td>
<td>0.175</td>
</tr>
<tr>
<td>CrCl</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>hs-CRP concentration</td>
<td>0.14</td>
<td>0.051</td>
</tr>
<tr>
<td>Vitamin D concentration</td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>Alx</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>Calcium concentration</td>
<td>0.14</td>
<td>0.045</td>
</tr>
<tr>
<td>ADMA concentrations</td>
<td>0.1</td>
<td>0.17</td>
</tr>
<tr>
<td>Platelet SNP responsiveness</td>
<td>-0.179</td>
<td>0.018</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>CTx concentration</td>
<td></td>
<td>0.97</td>
</tr>
<tr>
<td>P1NP concentration</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>Statin therapy</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>History of dyslipidaemia</td>
<td></td>
<td>0.053</td>
</tr>
<tr>
<td>ACEI/ARB therapy</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>History of diabetes mellitus</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>History of hypertension</td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>History of coronary disease</td>
<td></td>
<td>0.7</td>
</tr>
</tbody>
</table>
LDL - low density lipoprotein; HDL - high density lipoprotein; CrCl - creatinine clearance; hs-CRP - high sensitivity C-reactive protein; BMI - body mass index; AIx - augmentation index; ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; ADMA - asymmetric dimethylarginine; SNP - sodium nitroprusside; CTx - C-terminal telopeptide of collagen type 1; P1NP - N-terminal peptide of procollagen I.
**Table 2.8** Multivariate (backward stepwise multiple linear regression) correlates of increasing AVBS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet NO responsiveness</td>
<td>-0.179</td>
<td>0.018</td>
</tr>
<tr>
<td>Plasma calcium concentration</td>
<td>0.216</td>
<td>0.004</td>
</tr>
<tr>
<td>AIx</td>
<td>0.152</td>
<td>0.044</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>-0.141</td>
<td>0.062</td>
</tr>
</tbody>
</table>
Table 2.9 Univariate correlates of increase in AVBS (dichotomous) in the entire cohort

<table>
<thead>
<tr>
<th>Baseline parameter</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.39</td>
</tr>
<tr>
<td>Total cholesterol concentration</td>
<td>0.12</td>
</tr>
<tr>
<td>LDL concentration</td>
<td>0.17</td>
</tr>
<tr>
<td>HDL concentration</td>
<td>0.39</td>
</tr>
<tr>
<td>CrCl</td>
<td>0.62</td>
</tr>
<tr>
<td>hs-CRP concentration</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin D concentration</td>
<td>0.93</td>
</tr>
<tr>
<td>BMI</td>
<td>0.19</td>
</tr>
<tr>
<td>AIx</td>
<td>0.69</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>0.69</td>
</tr>
<tr>
<td>History of dyslipidaemia</td>
<td>0.2</td>
</tr>
<tr>
<td>ACEI/ARB therapy</td>
<td>0.09</td>
</tr>
<tr>
<td>History of diabetes mellitus</td>
<td>0.71</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>0.28</td>
</tr>
<tr>
<td>Calcium concentration</td>
<td>0.2</td>
</tr>
<tr>
<td>ADMA concentrations</td>
<td>0.72</td>
</tr>
<tr>
<td>Platelet SNP responsiveness</td>
<td>0.48</td>
</tr>
<tr>
<td>CTx concentration</td>
<td>0.8</td>
</tr>
<tr>
<td>P1NP concentration</td>
<td>0.87</td>
</tr>
</tbody>
</table>

LDL - low density lipoprotein; HDL - high density lipoprotein; CrCl - creatinine clearance; hs-CRP - high sensitivity C-reactive protein; BMI - body mass index; AIx - augmentation index; ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor
blockers; ADMA - asymmetric dimethylarginine; SNP - sodium nitroprusside; CTx - C-terminal telopeptide of collagen type I; P1NP - N-terminal peptide of procollagen I.
Table 2.10 Multivariate (binary multiple logistic regression analysis) correlates of increase in AVBS in A) entire cohort; B) subjects without ASc at baseline.

A)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEI/ARB treatment</td>
<td>-0.77</td>
<td>0.025</td>
</tr>
<tr>
<td>History of hypercholesterolemia</td>
<td>0.487</td>
<td>0.154</td>
</tr>
<tr>
<td>Baseline calcium levels</td>
<td>1.2</td>
<td>0.29</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEI/ARB treatment</td>
<td>-1.3</td>
<td>0.001</td>
</tr>
<tr>
<td>hs-CRP level</td>
<td>0.97</td>
<td>0.02</td>
</tr>
<tr>
<td>Vitamin D level</td>
<td>-0.02</td>
<td>0.053</td>
</tr>
</tbody>
</table>

ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; hs-CRP - high sensitivity C-reactive protein.
Table 2.11 Univariate correlates of increase in AV_{BS} in the subjects without ASc at baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.32</td>
</tr>
<tr>
<td>Gender</td>
<td>0.63</td>
</tr>
<tr>
<td>Baseline total cholesterol</td>
<td>0.48</td>
</tr>
<tr>
<td>Baseline HDL</td>
<td>0.41</td>
</tr>
<tr>
<td>Baseline LDL</td>
<td>0.8</td>
</tr>
<tr>
<td>Baseline CrCl</td>
<td>0.58</td>
</tr>
<tr>
<td>Baseline hs-CRP</td>
<td>0.1</td>
</tr>
<tr>
<td>Baseline vitamin D level</td>
<td>0.19</td>
</tr>
<tr>
<td>Baseline BMI</td>
<td>0.83</td>
</tr>
<tr>
<td>Baseline AIx</td>
<td>0.76</td>
</tr>
<tr>
<td>Baseline statin use</td>
<td>0.09</td>
</tr>
<tr>
<td>Baseline history of dyslipidaemia</td>
<td>0.87</td>
</tr>
<tr>
<td>Baseline history of ACEI/ARB</td>
<td>0.008</td>
</tr>
<tr>
<td>Baseline history of diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>Baseline history of hypertension</td>
<td>0.2</td>
</tr>
<tr>
<td>Baseline history of coronary disease</td>
<td>0.08</td>
</tr>
<tr>
<td>Baseline Ca \times PO_{4}</td>
<td>0.12</td>
</tr>
<tr>
<td>Baseline ADMA</td>
<td>0.51</td>
</tr>
<tr>
<td>Baseline platelet SNP responsiveness</td>
<td>0.63</td>
</tr>
<tr>
<td>Baseline CTx</td>
<td>0.98</td>
</tr>
<tr>
<td>Baseline P1NP</td>
<td>0.09</td>
</tr>
</tbody>
</table>
LDL - low density lipoprotein; HDL - high density lipoprotein; CrCl - creatinine clearance; hs-CRP - high sensitivity C-reactive protein; BMI - body mass index; AIx - augmentation index; ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; Ca₃PO₄ - calcium-phosphate product; ADMA - asymmetric dimethylarginine; SNP - sodium nitroprusside; CTx - C-terminal telopeptide of collagen type 1; P1NP - N-terminal peptide of procollagen I.
Figure 2.1 Methodology for assessing aortic sclerosis by ultrasound backscatter. Regions of interest are placed in the aortic valve and the backscatter from the blood pool is subtracted. 3 values from the more anterior and more posterior leaflets are taken from the zoom view of the aortic valve to obtain and average for the valve.
Figure 2.2 Graphic representation of PWA and AIX derivation. Peripheral pressure waveforms recorded electronically and are converted to a central waveform via a transfer function to derive a rate-corrected augmentation index. Augmentation index is the difference ($\Delta P$) between the first (P1) and second systolic (P2) pressure peaks, divided by pulse pressure (PP), expressed as a percentage of the pulse pressure. (Adapted from (Crilly et al., 2007)).
Figure 2.3 Graphic representation of inhibition of ADP-induced platelet aggregation in whole blood by SNP: demonstration of NO resistance. (A) Normal subject. (B) Study subject, showing both hyperaggregability and reduced responsiveness to antiaggregatory effect of SNP (reproduced with permission (Chirkov et al., 2007)).
Figure 2.4: Comparison of aortic valve backscatter scores ($AV_{BS}$) with visual assessment scores in subjects without and with ASc. There was a significant difference in $AV_{BS}$ between groups (11.2 ± 3.9dB vs 14.9 ± 4.5dB respectively, $p<0.001$). Means and interquartile ranges are depicted.
Figure 2.5 A) Correlation of BMI with normalized plasma hs-CRP concentrations for the entire cohort ($r=0.42; p<0.0001$); B) ANCOVA: relationship between BMI and normalized plasma hs-CRP concentrations according to presence/absence of ASc ($p=0.96$).
Figure 2.6 Mean change in AVBS over a 4 year period (p<0.001)
Figure 2.7 Relationship of change in $AV_{BS}$ to baseline AVBS values ($p<0.001$).
CHAPTER 3

Aortic sclerosis and nitric oxide signalling: implications regarding myocardial hypertrophy and dysfunction
3.1 Introduction

Aortic sclerosis (ASc), defined as thickening of the aortic valve leaflets without any obstruction to flow, is a common condition seen increasingly in the elderly population (Aronow et al., 1999; Otto et al., 1999). In fact, in chapter 2 we have demonstrated that the prevalence of ASc is almost 20% in an unselected aging population. Furthermore, there was significant disease progression over a short period of time: over 60% of subjects had detectable disease progression over 4 year follow-up period.

ASc is important for two main reasons: it is the precursor of aortic stenosis (AS) and it is independently associated with a significantly elevated cardiovascular morbidity and mortality (Nightingale et al., 2005). This increase in cardiovascular events is unrelated to the progression of aortic valve disease and cannot be accounted by the increased prevalence of coronary artery disease (Aronow et al., 1999; Otto et al., 1999).

The reason why aortic sclerosis is associated with incremental cardiovascular risk is not clear, but most hypotheses centre on concepts of overlapping pathophysiology. The key issue, of course, is which precise factors overlap? A number of studies have identified systemic factors that are associated with the presence of ASc. These include inflammation (Agmon et al., 2004; Chandra et al., 2004), lipoprotein abnormalities (O'Brien, 2006) and endothelial dysfunction (Poggianti et al., 2003). In Chapter 2, we demonstrate another important cardiovascular risk factor, reduced platelet responsiveness to anti-aggregatory effects of nitric oxide (NO), is an independent predictor of both presence and progression of ASc. Given that tissue resistance to NO is a widespread phenomenon, with essentially identical biochemical bases in platelets and vessels (Chirkov et al., 2007) it is probable that the presence of NO resistance in association...
with ASc provides at least part of the mechanism underlying the ASc : coronary risk relationship. Indeed it is also worth noting that, although unproven, this association may arise because ASc itself is more likely in theory to develop with impaired NO signalling (Kennedy et al., 2009).

Furthermore, both large population-based studies (Otto et al., 1999) and subgroup analyses of cohorts of hypertensive patients (Agno et al., 2005; Olsen et al., 2005) have shown that subjects with ASc have increased left ventricular (LV) wall thickness. In theory, coexistence of LV hypertrophy (LVH) with ASc may contribute to the increase in cardiovascular risk and therefore it is important to understand the mechanism for the increase in LV mass. If ASc is indeed independently associated with presence of LVH, this is not necessarily mediated purely by increased left ventricular afterload, but potentially by its association with endothelial dysfunction (Poggianti et al., 2003). A number of endothelial autacoids may modulate the development of LVH (Wenzel et al., 2007), and it has been shown that attenuation of effects of NO and bradykinin, such as might occur with in association with (endocardial) endothelial dysfunction, can stimulate development of LVH independent of haemodynamic factors (Rosenkranz et al., 2000). Thus LVH may be an additional factor accounting for the increased cardiovascular risk observed in ASc.

Yet, irrespective of presence/absence of ASc, the development of LVH is a common manifestation of various cardiac pathologies, including response to chronic pressure overload. Increased LV mass is largely due to increased size of cardiomyocytes, as well as extracellular matrix remodelling (Frey et al., 2003). The initial increase in LV mass in response to pressure overload occurs as a compensatory adaptive mechanism for normalization of ejection performance and tissue perfusion as originally described by Grossman et al (1975). Prolonged
and continuous exposure to haemodynamic stress as in hypertension, valvular disease, heart failure, and diabetes mellitus predispose to a maladaptive LVH state, which is characterized by ventricular dilatation, diastolic and systolic dysfunction, and therefore increased propensity to heart failure and myocardial ischemia (Grossman et al., 1996). Activation of the circulating and tissue renin angiotensin system appears to play a key role in this maladaptive process (Cowan et al., 2009).

Endothelium-derived NO plays an important role in the regulation of cardiovascular function (Gibbons, 1997; Kelly et al., 1996; Spieker et al., 2005). NO is a potent endogenous vasodilator and a critical modulator of regional blood flow, exerting its effect mostly via stimulation of soluble guanylate cyclase (sGC) to produce cyclic GMP (Behrendt et al., 2002; Ritchie et al., 2009). NO is generated from its precursor L-arginine via the enzyme activity of nitric oxide synthase (NOS). All three NOS isoforms, namely eNOS, nNOS, and iNOS, are expressed in the heart, where nNOS and eNOS are constitutively expressed (Shimokawa et al., 2010).

Numerous experimental studies have documented the antihypertrophic actions of NO synthesized by the NOS enzymes. Exogenous administration of NO donors attenuates hypertrophic responses to stimuli including angiotensin II, endothelin-1, norepinephrine and phenylephrine (Calderone et al., 1998; Irvine et al., 2008; Ritchie, 2009; Ritchie et al., 1998; Wollert et al., 2002). Conversely, the development of left ventricular hypertrophy in experimental models of chronic pressure overload is potentiated in eNOS knockout mice (Ichinose et al., 2004).
Asymmetric dimethylarginine (ADMA), an endogenous competitive antagonist of eNOS, is a marker and mediator of endothelial dysfunction (Boger et al., 1997; Sydow et al., 2003). ADMA inhibits eNOS-mediated bioconversion of arginine to NO, and thus regulates eNOS activity (Boger, 2003). Some (Ebinc et al., 2008; Zoccali et al., 2002), but not all (Lieb et al., 2009) previous investigations have suggested that ADMA concentrations may be correlated with LV mass. Potential modulation of the development of myocardial hypertrophy by ADMA might theoretically be mediated via attenuation of peripheral vasomotor and/or of direct myocardial antihypertrophic effects.

Furthermore nitric oxide has positive lusitopic effects. Inhibition of NO synthesis reduces the rate of LV active relaxation (Silberman et al., 2010). Therefore improved NO synthesis may play a role in LV diastolic dysfunction.

The investigations described in this chapter have 2 main objectives:

1. Since the association between aortic sclerosis and increased LV mass is well established in hypertensive populations, but not in normotensives, we sought to establish whether this relationship also applies in an elderly population without treated hypertension. We also evaluated whether the presence of increased LV mass in patients with aortic sclerosis might indeed reflect the presence of endothelial dysfunction, rather than purely increased afterload.

2. The underlying hypothesis to be tested is that impaired NO generation/signaling is reflected diffusely within the body, including “parallel” effects in vessels, platelets, valves and myocardium. We therefore chose to utilize plasma concentrations of ADMA as a measure of impaired NO generation, and to establish in a cohort of
subjects free of significant cardiovascular disease or risk factors whether these are correlated with:

a. LV mass independent of afterload, and

b. LV active relaxation rate independent of LV mass.

We also sought to determine whether LV mass is a correlate of vascular tissue responsiveness to NO, which is an ADMA-independent marker of integrity of the NO-soluble guanylate cyclase cascade (Chirkov et al., 2007).
3.2 Methods

Subjects aged ≥ 55 years were recruited through newspaper advertisements. Subjects were excluded if there was a history of hypertension requiring medication, any previous history suggestive of ischemic heart disease or any medication that might influence blood pressure. All subjects underwent an echocardiogram and a cardiac MRI. Blood was taken for total cholesterol, creatinine, 25-OH vitamin D3, high sensitivity CRP and asymmetric dimethylarginine (ADMA) in view of previous data linking these parameters with the presence of aortic valve disease (Ngo et al., 2007; O'Brien, 2006; Ortlepp et al., 2001). Body surface area was calculated by the Dubois formula. Creatinine clearance was calculated using the Cockcroft-Gault formula. C-terminal telopeptide of collagen type 1 (CTx), N-terminal peptide of procollagen I (P1NP), N-terminal pro BNP were also measured as part of evaluation of objective 2 only. The institution’s ethics committee approved the study and all subjects gave informed written consent.

3.2.1 Echocardiographic diagnosis of Aortic Sclerosis (for evaluation of objective 1)

The presence of aortic sclerosis was assessed by echocardiography (Vivid 5, Vingmed, GE Norway) using a 2.5 MHz probe. The aortic valve was zoomed in the parasternal long axis view to 8 cm. Three loops of 3 cardiac cycles each were recorded ensuring that the aortic valve leaflets are visible in diastole. Aortic valve ultrasonic backscatter score (AVBS) was measured as described previously (Ngo et al., 2004). Briefly, 3 regions of interest are placed on the anterior leaflet and 3 on the posterior leaflet in the tissue quantification off-line analysis package (EchoPac). The backscatter from the blood pool (left ventricular outflow tract (LVOT) and aortic root) was then subtracted from the backscatter of the valve leaflets to give
an absolute value of backscatter from the aortic valve. Visual assessment of the valve was then performed by an operator blinded to the backscatter scores. A visual sclerosis score (Ngo et al., 2004) was assigned to each valve on the basis of its 2D appearance in the parasternal short axis view:

0 = normal
1 = slight increase in reflectance of the cusp bodies or margins
2 = mild increase in overall reflectance and cusp thickness
3 = generalized hyper-reflectance, cusps markedly thickened with markedly hyper-reflective masses.

For the purpose of subsequent analysis, the presence of aortic sclerosis was defined as a sclerosis score of $\geq 1$. Doppler echocardiography was used to assess velocity in the LV outflow tract and through the aortic valve.

### 3.2.2 MRI measurement of LV volumes

Cardiac MRI was performed using a Philips 1.5T Gyroscan Intera MRI magnet with a cardiovascular analysis package (ViewForum, Philips) and 5 channel cardiac coil. Cardiac and respiratory gating were applied. A scout image in 3 planes with a field of view of 450°, a slice thickness of 8.0mm and a flip angle of 50° was performed. A series of black blood axial, $T_1$ weighted turbo spin echo (TSE) with a field view of 400°, turbo echo (TE) 25 and TSE factor 15 with a matrix 256 x 512 were performed. 12 slices, 8.0mm thick with no gap and flip angle of $<90°$ were obtained and from these the 2 chamber, 4 chamber, LVOT views and short axis stack were planned. The short axis stack was generated using the 2 chamber and 4 chamber images to orientate the plane. 14 slices 8.0mm thick with no gap were acquired in order to cover the LV from apex to mitral valve in diastole.
Using the cardiac analysis software, cardiac volumes were measured from the short axis stack. The most basal image used was one in which ≥ 75% of the left ventricular wall was seen. The endocardial and epicardial contours were drawn in every slice in diastole and systole (defined as a phase with the smallest LV cavity volume). From these volumes LV mass, LV end diastolic volume, LV end systolic volume and stroke volume were calculated. Inter and intra-observer coefficients of variation in LV volumes were 5% and 8% respectively in our study. LV mass was then indexed to height$^{2.7}$ (LVMI) as recommended by de Simone et al (1995) (Figure 3.1).

### 3.2.3 Endothelial function

Both biochemical and functional indices of endothelial function were measured. From a biochemical standpoint, we determined plasma concentrations of ADMA, which is both a marker and a mediator of endothelial dysfunction, largely via its action as a competitive inhibitor of nitric oxide synthase (Horowitz et al., 2007). ADMA concentrations in plasma were assayed utilizing a high pressure liquid chromatographic assay as previously described (Heresztyn et al., 2004).

Pulse waveform analysis (PWA) and derivation of AIX was performed non-invasively with a commercially available SphygmoCor system (AtCor Medical, Sydney, Australia), as previously described in Chapter 2 and by Wilkinson et al (1998).

As a physiological index of endothelial function, we measured the effects of salbutamol on large artery stiffness. Salbutamol, although a β-adrenoreceptor agonist, induces arterial
vasodilator responses which are largely mediated via release of nitric oxide. The reduction in AIx, a measure of apparent arterial stiffness, following inhalation of salbutamol has been shown to be mediated by vascular endothelium (Wilkinson et al., 2002c) and is impaired in subjects with hypercholesterolaemia (Wilkinson et al., 2002a) and coronary artery disease (Hayward et al., 2002). Applanation tonometry (SphygmoCor, AtCor, Australia) was performed on the right radial artery after 20 minutes of supine rest. 3 consecutive waveforms were obtained. 400 μg of salbutamol was then administered through a spacer device. Waveforms were recorded at 5, 10, 15 and 20 minutes, and central aortic waveform was derived utilizing a validated transfer function. The time point with the largest change was used as previously described (Wilkinson et al., 2002c). Reduction in AIx with sublingual glyceryl trinitrate (50μg) was utilized as an index of endothelium-independent vascular function as described previously (Oliver et al., 2005; Wilkinson et al., 2002a).

3.2.4 Statistics

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.

Statistical analysis was performed using SPSS (version 17, SPSS Inc, USA). Results are presented as mean ± SD, unless otherwise stated, and p values of < 0.05 were considered statistically significant.
3.2.4.1 Objective 1

The primary (null) hypothesis of the objective 1 of the study was that the relationship between afterload and LV mass was independent of the presence of aortic sclerosis. This was tested by ANCOVA. For this purpose, systolic blood pressure was utilized as an index of afterload and LV mass was indexed to height$^{2.7}$ (LVMI). We powered the study on the basis that the mean LVMI would be 30 g/m$^{2.7}$ with a SD of 5 g/m$^{2.7}$. 17 subjects with ASc were needed in order to have an 80% power to detect a difference of 10 g/m$^{2.7}$ between the control and ASc groups at a significance level of 5%. Given that the prevalence of of aortic sclerosis is between 20% and 30% in this age group we aimed to recruit 80 subjects in total. All normally distributed data were compared using t tests; Chi-square tests were utilized for categorical data. Skewed data were analysed via Wilcoxon test. The relationship between sclerosis and AVBS was examined via Spearman’s rank correlation test.

An important component of the study was identification of clinical and biochemical correlates of ASc and LV mass. Following evaluation of univariate comparisons, multivariate analyses were performed utilizing multiple linear regression. When examining the relationship between aortic sclerosis and other variables, AVBS was used as a continuous variable to maximise the power of this component of the study.

3.2.4.2 Objective 2

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 LVMI derived from MRI. Parameters examined as putative
correlates of LVMI were: age, gender, resting systolic blood pressure, calculated CrCL, BMI, normalized hs-CRP, change in AIX 5 minutes post administration of GTN, and ADMA concentrations. These variables were included either due to statistical significance on univariate analyses or as suspected clinical correlates.

Furthermore, ANCOVA was utilized to test the hypotheses that i) the relationship between ADMA and LV mass is independent of systolic blood pressure; ii) the relationship of LVMI and systolic blood pressure is modulated by vascular NO responsiveness; and iii) the relationship of E/E' and LVMI is modulated by vascular NO responsiveness and/or ADMA concentrations.
3.3 Results

3.3.1 Objective 1

3.3.1.1 Subject Characteristics

81 Caucasian subjects with a mean age of 68 ± 6 years (range 55 to 87), including 42 males and 37 females, were recruited. One subject had very poor quality echo windows and one subject refused to have the MRI leaving 79 subjects as the study group. As intended, no subject had a history of prior hypertension and none were on antihypertensive treatment. However, the mean resting systolic blood pressure was 137.8 mmHg with a range from 177 to 98 mmHg and 35 subjects had a systolic blood pressure of > 140 mmHg.

Aortic valve thickening and echogenicity was assessed primarily via AVBS, with AVBS ≥ 16 db being utilized as the primary index of ASc, in view of the reproducibility of this index (Ngo et al., 2004). On this basis, fifteen subjects (19%) had ASc. On visual assessment, 28 subjects (35%) had some degree of aortic valve thickening, but in almost all cases with sclerosis score of only 1 or 2 on visual assessment. AVBS and visual sclerosis scores were closely correlated (p<0.01; Figure 3.2).

3.3.1.2 Comparison of normal aortic valve and aortic sclerosis groups

Demographic and biochemical data for the subgroups with normal aortic valve and ASc are summarized in Table 3.1. The two groups exhibited no significant differences in age or conventional coronary risk factors. When potential baseline correlates of the presence of ASc (total plasma cholesterol, high sensitivity CRP, plasma vitamin D and creatinine clearance)
were considered, only creatinine clearance varied significantly between subgroups, with significantly lower BMI, and creatinine clearance (CrCL) (p < 0.05) in subjects with ASc.

When haemodynamic parameters, LV volume and mass indices were compared (Table 3.2), there were no differences between the groups on the basis of LV volumes, systolic and diastolic blood pressures.

3.3.1.3 Relationship between aortic sclerosis, LVMI and afterload

There was a statistically significant direct correlation (r = 0.29, p < 0.01) between the measure of afterload (systolic blood pressure) and LVMI in the whole cohort. However, this relationship did not vary significantly according to the presence or absence of ASc, as shown in Figure 3.3.

3.3.1.4 Endothelial function

Plasma ADMA concentrations did not vary significantly between the subjects with or without ASc. Neither baseline AIx nor the change in AIx in response to salbutamol varied in the presence of ASc (AIx 23.9 ± 7.6 % vs AIx 26.3 ± 7.9 %, for normal and sclerotic groups respectively), suggesting that endothelial function was similar in both groups.

3.3.1.5 Determinants of LVMI

Results of univariate and multivariate correlations are shown in Table 3.3. Significant univariate correlates of increased LVMI were male gender, reduced creatinine clearance,
increased systolic blood pressure, history of smoking, elevation of ADMA levels, low resting AIx, and reduction in AIx with GTN, while vitamin D level was of borderline significance. Peak velocity through the aortic valve was not correlated with LVMI.

On multiple linear regression analysis male gender (p < 0.001), systolic blood pressure (p < 0.01), and BMI (p < 0.001) remained significant correlates of LVMI. Age, smoking history, CrCL, ADMA, cholesterol (or statin use), salbutamol vascular response and most notably, AVBS scores, were not significant predictors of LV mass index in the model.

3.3.2 Objective 2

3.3.2.1 Subject characteristics

For the purposes of testing objective 2, subjects with history of diabetes mellitus were excluded, leaving 74 subjects in this study cohort. Table 3.4 summarizes the subject characteristics of this modified cohort. Although LV mass index values from MRI were utilized for physiological correlations, there was a significant relationship (r = 0.53, p < 0.001) between MRI- and echocardiographically-derived estimates.

3.3.2.2 Univariate analyses

On linear regression analysis, there was a significant positive correlation between LV mass index and resting systolic blood pressure (r = 0.29, p = 0.01; Figure 3.4A), and plasma ADMA concentrations (r = 0.26, p = 0.025; Figure 3.4B). ANCOVA (Figure 3.4C) revealed that at any systolic blood pressure, LV mass index was significantly greater (F = 6.7; p = 0.01) for
subjects whose ADMA concentrations were in the highest quartile (>0.58 μM) as compared with the remainder of the study group.

In addition, there was a negative relationship between LV mass index and vascular tissue responsiveness to NO, as measured by changes in AIx 5 minutes post GTN administration (r = -0.27, p = 0.02; Figure 3.5A). Analogous to the data shown in Figure 3.4C, ANCOVA (Figure 3.5B) revealed that at any systolic blood pressure, LV mass index was significantly greater (F = 5.0; p = 0.03) for subjects whose responsiveness to GTN was in the lowest quartile (δ AIx < 8%), as compared with the remainder of the study group. Salbutamol-induced changes in AIx were not significantly associated with LV mass index (p = 0.9).

There was a significant relationship between LV mass index and E/E' ratio, (r = 0.38, p = 0.001; Figure 3.6A). On ANCOVA (Figure 3.6B) this relationship was independent of plasma ADMA concentrations. However, E/E' ratio was significantly increased at any LV mass index (ANCOVA: F = 4.2, p = 0.04; Figure 3.6C) among subjects whose responsiveness to GTN was in the lowest quartile (δ AIx < 8%). There was also a significant relationship between LV mass index and measures of LV relaxation, including IVRT (p < 0.01) and E' (p = 0.02).

Other univariate correlates of LV mass index are shown in Table 3.5. Male gender, low CrCL, smoking history, and high BMI were significantly associated with high LV mass index; while age, hs-CRP, total cholesterol, CTx, P1NP, and NT-proBNP were not. On backward multiple linear regression analysis, male gender, high BMI, systolic blood pressure, and plasma ADMA concentrations were significant independent predictors of high LV mass index (Table 3.6).
3.4 Discussion

Although the two studies which constitute this chapter have been published separately (Nightingale et al., 2011; Sverdlov et al., 2011b), they arise from an overlapping pathophysiological hypothesis, which could be categorised as one of multi-organ impact of impaired NO signalling. The rationale for these studies is summarized in Figure 3.7. Since all illustrated effects are impacted by impaired NO generation/signalling: -

1. they should be correlated with each other, and in particular anti-fibrotic/calcific effects should correlate with anti-hypertrophic effects
2. they should also be inversely correlated with ADMA concentrations

The overall results of the two investigations undertaken tend to refute the hypothesis that the “downstream” effects of impaired NO signalling are equally represented in different target tissues (in this case valve and myocardium). On the other hand, when we examined the relationship between ADMA concentrations, LV mass, LV relaxation and vascular endothelial function significant correlations were evident.

The results of the current study reveal no association between early ASc and LV mass index in an asymptomatic, relatively normotensive, low risk elderly subject cohort. The results are therefore discordant with some of the previous literature. It is appropriate both to re-examine the previous data and evaluate the potential causes and significance of this discrepancy.

The major pieces of evidence for association of ASc with LVH arise from general population studies (Otto et al., 1999) but more extensively from sub-analyses in hypertensive populations (Agno et al., 2005; Olsen et al., 2005) and are summarized in Table 3.7. One of the major methodological concerns about these and other similar (Palmiero et al., 2007) previous studies
was that both LV wall thickness and ASc were assessed simultaneously and only subjectively; hence blinding was impossible. This is a particular problem for early ASc where most subjects have no significant transvalvular pressure gradient and only localised increases in valve thickness. Furthermore, in echocardiographic studies, LV mass is extrapolated from one or two-dimensional measurements. On the other hand, in the current study, utilization of MRI provided an independent, highly reproducible and unbiased measure of LV mass, while quantification of ASc by backscatter score was used in parallel with qualitative assessment of the aortic valve (as well as showing a close correlation between these two methods).

This distinction is of great importance because of the independent association between ASc and incremental cardiovascular risk. Our results imply that within the general population, the mechanism(s) underlying this increased risk do not include acceleration of development of LVH as previously suggested.

We hypothesised that in the presence of ASc there might be a shift in the relationship between afterload and LV mass index. Underlying this hypothesis were the known effects of NO (Wenzel et al., 2007) and bradykinin (Rosenkranz et al., 2000) as inhibitors of LVH in cellular models of endothelial dysfunction. Indeed, on a multivariate analysis, both conventional coronary risk factors and plasma ADMA levels were directly associated with increased LV mass index. However, ASc was not associated with either biochemical (ADMA) or physiological (salbutamol response) evidence of endothelial dysfunction. Thus the endothelial dysfunction previously reported in ASc and AS may develop later in the course of the disease process.
On the other hand, Ruetten et al (2005) have examined the relationship between increased afterload (imposed by aortic banding) and NO bioavailability, utilizing a comparison between wild-type and eNOS knockout mice. LVH did not develop in sham-operated eNOS knockout mice, but was accentuated in the presence of aortic banding. This suggests that reduced formation of nitric oxide and/or impairment of NO effect (both of which may be associated with ASc (Ngo et al., 2009b) are more likely to potentiate LVH in established hypertension rather than induce its development de novo. The results of the current study therefore cannot be extrapolated to patients with established hypertension and may partially explain the discrepancy between this and other studies.

The finding that there was no detectable correlation between presence of ASc and that of increased LV mass, although both are known potential consequences of impaired NO signalling, should stimulate evaluation of the nexus between various other NO effects. Indeed, simultaneous evaluation of salbutamol-induced vasodilatation and platelet responsiveness to NO in aging subjects (Sverdlov et al., 2010) also revealed no significant association. The overall implications of these findings are that similar pathophysiology does not equate to similar extent of effect: - the reasons for this may include differing time course and/or concentration-response relationship.

The current data should also stimulate re-examination of the mechanisms underlying the association between aortic sclerosis and cardiovascular events in the aging population. Stimulation of LVH can now be excluded as a primary component in the absence of associated hypertension. As regards the role of activation of systemic inflammation and of impairment of endothelial function, the current study suggests that these also are not associated with the presence of ASc in otherwise healthy individuals. On the other hand, we have demonstrated
that both ASc and stenosis are associated with impairment of anti-aggregatory response to NO as a consequence of increased redox stress (Chirkov et al., 2002; Ngo et al., 2009b), which has been shown to predict incremental cardiovascular morbidity and mortality (Willoughby et al., 2005). Thus the overall thrust of the current findings is that the link between ASc and increased cardiovascular risk is essentially independent of the development of LVH and further emphasises the importance of our findings in chapter 2.

As regards objective 2 of this set of investigations, whilst numerous experimental data suggest a pivotal role of NO in reducing the development of LVH (Ritchie et al., 2009), the evidence in human disease is limited. We demonstrate that plasma concentrations of the endogenous NOS inhibitor ADMA are independently associated with LV mass, in a normal aging population free from overt heart disease and significant cardiovascular risk factors. Specifically, at any particular systolic blood pressure, LV mass index was significantly greater in subjects with elevated ADMA concentrations. Furthermore, ADMA concentrations remained significant predictors of LV mass index on multivariate analysis.

Analogously, impaired tissue responsiveness to the nitrovasodilator GTN predicted increased LV mass per unit systolic blood pressure. Therefore, our data suggest that not only NO generation/bioavailability but also NO effector pathways are important in development of increased LV mass. This is consistent with experimental data showing that the effects of NO in limiting LVH are dependent on soluble guanylate cyclase (sGC) activation (for review see Ritchie et al., 2009). Systolic blood pressure is one of the major correlates of left ventricular afterload, although it is also influenced by the presence/absence of left ventricular systolic dysfunction (Segers et al., 2000). Thus in the current cohort by virtue of correction for systolic
blood pressure, both the ADMA and GTN data also suggest the existence of important intramyocardial components of this NO effect.

We have also observed a direct relationship between LV mass and E/E' derived from echocardiography. Although this parameter is ultimately a measure of LV filling pressures (Mottram et al., 2005), in the absence of fluid overload it represents one of the most robust, and least afterload dependent (Borlaug et al., 2007), measures of diastolic function. There was also a significant relationship between LV mass index and E', which is less preload-dependent than E/E' (Borlaug et al., 2007). While LVH has long been associated with impaired diastolic function (Grossman et al., 1996; Kaplinsky, 1994), this relationship has not previously been demonstrated in subjects whose LV mass lies within the normal range. The relationship between LV mass index and E/E' was independent of ADMA concentrations, but significantly modulated by tissue responsiveness to GTN. This suggests that the NO "cascade" may exert some influence on diastolic function at any particular LV mass and is in keeping with previous data (Bronzwaer et al., 2008).

Given that NO exerts both antihypertrophic and antifibrotic effects in myocardium (Paulus et al., 1999), it might have been expected that plasma concentrations of CTx and P1NP, peripheral markers of fibrosis, would be correlated with elevation of ADMA concentrations and impairment of GTN response. However, there was also no significant relationship between LV mass index and either CTx or P1NP concentrations. There are a number of possible interpretations of these data. First, it is possible that fibrosis was not a prominent component of myocardial structure in this study population. However, it is also possible that the relative stimuli for myocyte hypertrophy and fibrotic infiltration vary. For example, Brilla et al have provided evidence that the renin-angiotensin system may differentially modulate
myocardial fibrosis (Brilla et al., 2000). Analogously, NO exhibiting greater effect on myocyte hypertrophy.

The results of the current investigation should also be viewed relative to the limited data available from previous investigations. In end-stage renal disease patients on haemodialysis (Zoccali et al., 2002) and those on ambulatory peritoneal dialysis (Ebinc et al., 2008), plasma ADMA concentrations correlated with LV mass index assessed by echocardiography. Furthermore, in the latter study plasma ADMA concentrations correlated with measures of diastolic dysfunction, which is similar to our findings. This relationship is further supported by Tang et al (2008), who found a direct correlation of plasma ADMA concentrations with E/E’ in a cohort of 138 patients with chronic systolic heart failure. Our work now confirms that this relationship applies in aging healthy patients, before cardiac pathology becomes evident.

The significant relationship between ADMA concentrations and LV mass emerged in the analyses for objective 2, notably with the exclusion of AVBS values in comparison with objective 1. As previously stated, the relationship between ADMA concentrations and LV mass has not been evaluated widely in more generalized cohorts. Lieb et al (2009), evaluating a large community-based sample, failed to demonstrate a statistically significant association of plasma ADMA with LV mass derived by echocardiography. However, ADMA concentrations were directly correlated with left atrial dimensions and there was a trend for increased LV mass to occur more frequently in subjects with elevated ADMA concentrations. This population consisted predominantly of women, and included patients treated for diabetes mellitus, hypertension, heart failure, valvular heart disease and hypercholesterolaemia, as well as with hormone replacement therapy. Any of these differences together with utilization of echocardiography rather than MRI, might have tended to obscure the relationship between LV
mass and ADMA. Also, given the high prevalence of diabetes and heart failure in this group, it is possible that sGC dysfunction/NO scavenging might have represented more important limitations to NO effect.

3.4.1 Limitations

It is unlikely that these negative results for objective 1 of these investigations reflect a type II error. The use of cardiac MRI to determine LV mass provides the most accurate and reproducible measure of this parameter currently available. The values of LV mass index and the relationship between systolic blood pressure and LV mass were virtually identical in the groups with and without aortic sclerosis. Nevertheless, we cannot exclude very small differences between the groups. More importantly, our data do not apply to hypertensive subjects. It is possible that the presence of aortic sclerosis is a marker of incremental release of factors that potentiate, rather than initiate, the development of left ventricular hypertrophy.

Similarly, no relationship was found between LV mass and response to salbutamol. This may reflect type II error rather than any physiological paradox: the mean response to salbutamol was 4.4%, as against 10.7% for GTN, and the standard deviation of salbutamol responses was 88% of mean values, as against 45% for GTN.

Even within the normal range of renal function, there is a clear relationship between creatinine clearance and the presence of ASc in this study. Whilst this has been shown previously in subjects with established renal disease, this has not previously been shown in a cohort with relatively normal renal function (Schonenberger et al., 2004). In the current study, as in previous investigations, low BMI was independently correlated with the presence of ASc (Ngo
et al., 2009b), whereas presence of increased LV mass was associated with elevated systolic blood pressure (Meijs et al., 2010). We therefore cannot totally exclude the possibility that this bidirectional interaction with BMI may have partially obscured an ASc : LV mass relationship.

The major limitation of the results regarding objective 2 of the current investigations is that its conclusions are based purely on associative data: this problem cannot easily be circumvented in human subjects. Furthermore, the methodology does not lend itself to determination of the proportional role of local factors to the development of increased myocardial mass: it is possible that this role is quite limited. It is also possible that the current results might have been different in populations of different ages or in the presence of pathological increases in LV mass. The surrogates for integrity of the myocardial NO/cyclic GMP system may not be precise: - ADMA concentrations in plasma may not precisely represent the major limitation to NO generation in myocardium. Similarly, vascular response to NO (as measured via GTN) may not be proportional to myocardial response, either regarding antihypertrophic/antifibrotic effects, or impact on myocardial relaxation. Additionally, the moderate size of the current study may have limited detection of all relevant correlates of increased LV mass on backward multiple linear regression analysis. Furthermore, the relationship between NO responsiveness and LV diastolic function could be mediated directly via NO lusinotropy and/or indirectly via LVH induction (Ritchie et al., 2009) or improved titin phosphorylation (Ahmed et al., 2009). These deficiencies are inherent in any in vivo study, and could only be addressed by analogously designed animal studies, none of which appear to have been reported to date.
3.4.2 Conclusions

The current study demonstrates that:

1. There is no association between presence/absence of early aortic sclerosis and left ventricular hypertrophy, if systolic blood pressure is taken into account. These findings suggest that development of left ventricular hypertrophy does not occur in normotensive subjects with aortic sclerosis. It remains possible that abnormalities of endocardial endothelial autacoid function might potentiate development of left ventricular hypertrophy in hypertensive patients with aortic sclerosis.

2. Markers of NO generation (plasma ADMA concentrations) and of the NO/cyclic GMP signalling cascade (vascular responsiveness to GTN) in the peripheral circulation predict both LV mass index and LV diastolic function in a normal, untreated, aging population. These findings add to previous in vitro data concerning the pivotal importance of intramyocardial eNOS in cardiovascular homeostasis, and provide an additional theoretical basis for maintenance of its integrity as a component of primary prevention of left ventricular dysfunction.
3.5 TABLES AND FIGURES for CHAPTER 3
Table 3.1 Baseline characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Normal aortic valve N = 64</th>
<th>Aortic sclerosis N = 15</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67.1 ± 5.9</td>
<td>69.4 ± 6.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Gender (male / female) %</td>
<td>35 / 29 (55 / 45 %)</td>
<td>7 / 8 (47 / 53 %)</td>
<td>0.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 ± 4.4</td>
<td>23.9 ± 3.5*</td>
<td>0.003</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.2*</td>
<td>0.007</td>
</tr>
<tr>
<td>Smoker (non / ex / current) %</td>
<td>40 / 20 / 4 (62.5/ 31.3/ 6.3)</td>
<td>12 / 2 / 1 (80 / 13.3/ 6.7)</td>
<td>0.3</td>
</tr>
<tr>
<td>Family Hx of IHD %</td>
<td>13 (20.3%)</td>
<td>5 (33.3)</td>
<td>0.3</td>
</tr>
<tr>
<td>Hx of hypercholesterolemia %</td>
<td>24 (37.5%)</td>
<td>3 (20%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Statin treatment %</td>
<td>15 (23.4%)</td>
<td>3 (20%)</td>
<td>1</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.2 ± 0.80</td>
<td>5.2 ± 0.81</td>
<td>0.7</td>
</tr>
<tr>
<td>Vit D (nmol/l)</td>
<td>77.4 ± 22.3</td>
<td>78.9 ± 17.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>71.8 ± 15.6</td>
<td>56.4 ± 10.2 *</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hs CRP (mg/l)</td>
<td>2.4 ± 2.5</td>
<td>2.5 ± 3.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Nitric oxide status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADMA (µM)</td>
<td>0.53 ± 0.08</td>
<td>0.53 ± 0.57</td>
<td>0.9</td>
</tr>
<tr>
<td>Resting AIx (%)</td>
<td>23.9 ± 7.6</td>
<td>26.3 ± 7.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Max change in AIx with</td>
<td>5.6 ± 4.0</td>
<td>4.8 ± 4.7</td>
<td>0.5</td>
</tr>
<tr>
<td>salbutamol (%)</td>
<td>Change in AIx with GTN (5 min) (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.7 ± 4.9</td>
<td>9.5 ± 4.5</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* p < 0.05 on unpaired t test

BMI – body mass index; BSA – body surface area; Hx – history; IHD – ischemic heart disease; hs CRP – high sensitivity C-reactive protein; Vit D – vitamin D concentration; ADMA – asymmetric dimethylarginine; AIx – augmentation index
Table 3.2 LV and Haemodynamic parameters (LV mass/volume parameters were derived from MRI data)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal aortic valve</th>
<th>Aortic sclerosis</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 64</td>
<td></td>
<td>N = 15</td>
<td></td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>111.8 ± 31.6</td>
<td>104.9 ± 24.9</td>
<td>0.4</td>
</tr>
<tr>
<td>LV mass indexed to BSA (g/m²)</td>
<td>58.5 ± 12.0</td>
<td>59.5 ± 10.1</td>
<td>0.7</td>
</tr>
<tr>
<td>LV mass indexed to ht (g/m)</td>
<td>65.5 ± 16.0</td>
<td>62.2 ± 13.8</td>
<td>0.7</td>
</tr>
<tr>
<td>LV mass indexed to ht2.7 (g/m²)</td>
<td>26.6 ± 5.4</td>
<td>26.1 ± 5.4</td>
<td>0.5</td>
</tr>
<tr>
<td>LVEDV (ml)</td>
<td>126.2 ± 28.5</td>
<td>120.6 ± 22.4</td>
<td>0.4</td>
</tr>
<tr>
<td>LVESD (ml)</td>
<td>41.9 ± 13.8</td>
<td>40.7 ± 14.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Stroke Volume (ml)</td>
<td>85.2 ± 21.7</td>
<td>79.9 ± 14.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>136 ± 18</td>
<td>139 ± 16</td>
<td>0.2</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79 ± 10</td>
<td>81 ± 8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

LV – left ventricular; BSA – body surface area; ht – height; LVEDV – left ventricular end diastolic volume; LVESD – left ventricular end systolic volume; BP – blood pressure.
Table 3.3 Univariate and Multivariate correlates of LV mass index

A. Univariate analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.17</td>
<td>0.1</td>
</tr>
<tr>
<td>Male Gender</td>
<td>0.498</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cr Clearance</td>
<td>-0.373</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>0.291</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Smoking history</td>
<td>0.270</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.245</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>BMI</td>
<td>0.507</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Resting AIx</td>
<td>-0.257</td>
<td>0.02</td>
</tr>
<tr>
<td>Change in AIx with GTN (5 min)</td>
<td>0.273</td>
<td>0.015</td>
</tr>
<tr>
<td>Vit D</td>
<td>0.222</td>
<td>0.053</td>
</tr>
</tbody>
</table>

B. Multivariate analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Gender</td>
<td>0.438</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>0.227</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BMI</td>
<td>0.461</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Cr – creatinine; BP – blood pressure; ADMA – asymmetric dimethylarginine; Vit D – vitamin D concentration.
Parameters which were not significantly correlated with LV mass index on univariate analyses (p>0.1) were: patient age, presence of hypercholesterolemia, effect of salbutamol on AIx, hs-CRP, aortic valve backscatter score and peak aortic velocity.
Table 3.4 Characteristics of the study cohort utilized for investigation of objective 2.

### A. Clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>67.7 ± 5.8</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>39, (52.7)</td>
</tr>
<tr>
<td>Resting systolic BP (mmHg)</td>
<td>137.2 ± 17.2</td>
</tr>
<tr>
<td>Resting diastolic BP (mmHg)</td>
<td>79.3 ± 9.5</td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>65.2 ± 9.5</td>
</tr>
<tr>
<td>Resting systolic BP &gt; 140mmHg, n, (%)</td>
<td>32, (43.2)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.9 ± 9.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.4 ± 15.8</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>24, (32.4)</td>
</tr>
<tr>
<td>Statin treatment, n (%)</td>
<td>17, (23)</td>
</tr>
<tr>
<td>Family history of CV diseases, n (%)</td>
<td>18, (24.3)</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td>24, (32.4)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.7 ± 4.6</td>
</tr>
</tbody>
</table>

### B. Cardiac mass/function

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass derived by MRI (g)</td>
<td>110.1 ± 30.9</td>
</tr>
<tr>
<td>LV mass derived by MRI indexed for height²/³ (g/m²/³)</td>
<td>26.4 ± 5.5</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>67.4 ± 7</td>
</tr>
<tr>
<td>Isovolumic relaxation time (ms)</td>
<td>115.2 ± 20.3</td>
</tr>
<tr>
<td>E' (septal; cm/s)</td>
<td>6.5 ± 2</td>
</tr>
<tr>
<td>E/E' ratio</td>
<td>9.9 ± 3.4</td>
</tr>
</tbody>
</table>
### C. Biochemistry

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>68.7 ± 16.3</td>
</tr>
<tr>
<td>hs-CRP, (median) (mmol/L)</td>
<td>1.1</td>
</tr>
<tr>
<td>C-terminal telopeptide of collagen type 1 (CTx) (median) (pg/ml)</td>
<td>80.7</td>
</tr>
<tr>
<td>N-terminal peptide of procollagen I (median) (mcg/L)</td>
<td>37.7</td>
</tr>
<tr>
<td>N-terminal pro-BNP (median) (ng/L)</td>
<td>77.5</td>
</tr>
</tbody>
</table>

### D. Endothelial function

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (μM)</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>ADMA &gt; 0.58 μM, n (%)</td>
<td>19, (25.7)</td>
</tr>
<tr>
<td>SDMA (μM)</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>ADMA/SDMA ratio</td>
<td>1.05 ± 0.18</td>
</tr>
<tr>
<td>Resting Alx (%)</td>
<td>24.4 ± 7.7</td>
</tr>
<tr>
<td>Change in Alx 5 min post GTN (%)</td>
<td>10.7 ± 4.9</td>
</tr>
<tr>
<td>Change in Alx 5 min post GTN &lt; 8%, n, (%)</td>
<td>26, (35.1)</td>
</tr>
<tr>
<td>Maximum change in Alx post salbutamol (%)</td>
<td>4.4 ± 3.9</td>
</tr>
</tbody>
</table>
**Table 3.5** Univariate analyses of LV mass index

<table>
<thead>
<tr>
<th></th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.17</td>
<td>0.1</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CrCL (mL/min)</td>
<td>-0.37</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking history</td>
<td>0.27</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>0.06</td>
<td>0.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>C-terminal telopeptide collagen type 1 (pg/ml)</td>
<td>-0.2</td>
<td>0.08</td>
</tr>
<tr>
<td>N-terminal procollagen I (mcg/L)</td>
<td>-0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>NT-proBNP (pg/L)</td>
<td>-0.07</td>
<td>0.5</td>
</tr>
<tr>
<td>History of hypercholesteroalemia</td>
<td></td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 3.6 Multivariate analysis of predictors of increased LVMI

<table>
<thead>
<tr>
<th></th>
<th>β coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>0.45</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.46</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.23</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ADMA (µM)</td>
<td>0.18</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
Table 3.7 Differences in LV mass in subjects with and without aortic sclerosis. Comparison with previously published cohorts.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Methodology for measuring LV mass</th>
<th>Mean LV mass in Aortic sclerosis group</th>
<th>Mean LV mass in normal group</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otto et al (Otto et al., 1999)</td>
<td>Population study in elderly (&gt; 65 years) (44% had hypertension) N = 5621</td>
<td>M-mode echo volumes derived from end diastolic measurements</td>
<td>157.5 g</td>
<td>148.6 g</td>
<td>P &lt; 0.01 for comparison between aortic stenosis, aortic sclerosis and normal groups</td>
</tr>
<tr>
<td>Agno et al (Agno et al., 2005)</td>
<td>Hypertensive population from HyperGen study N = 1624</td>
<td>M-mode and 2D echo volumes derived from end diastolic measurements</td>
<td>178.8 ± 43.5 g</td>
<td>170.0 ± 41.7 g</td>
<td>P = 0.019 comparing LV mass P = NS when LV mass indexed to height&lt;sup&gt;2.7&lt;/sup&gt;. LV mass not significant when adjusted for other factors. LV geometry remains significantly different even after adjustment</td>
</tr>
<tr>
<td>Olsen et al (Olsen et al., 2005)</td>
<td>Hypertensive population from LIFE study N = 960</td>
<td>M-mode and 2D echo volumes derived from end diastolic measurements</td>
<td>244 g</td>
<td>227 g</td>
<td>P &lt; 0.001 for both mass and indexed mass</td>
</tr>
<tr>
<td>Current study</td>
<td>Elderly population without</td>
<td>Cardiac MRI</td>
<td>107.1 ± 25.5 g</td>
<td>112.4 ± 32.9 g</td>
<td>No significant difference in LV</td>
</tr>
<tr>
<td>cardiovascular disease</td>
<td>N = 79</td>
<td>mass between aortic sclerosis and normal subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>--------</td>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25.8 ± 5.0 g/m²</td>
<td>26.9 ± 5.6 g/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1 Cardiac MRI analysis: representative short axis stack images with epicardial and endocardial contours drawn in diastole and systole respectively.
Figure 3.2 Correlation between the two methodologies for assessing severity of aortic sclerosis: aortic valve backscatter (dB) and visual sclerosis scores; *p* < 0.05 (Kruskall-Wallis test).
Figure 3.3 Relationship between LV mass index and systolic blood pressure.

There is a significant correlation between LV mass index and systolic blood pressure; R = 0.29, p < 0.01. However this relationship is not altered by the presence of aortic sclerosis (ANCOVA). Regression lines for normal (continuous line) and subjects with aortic sclerosis (dashed line) are shown.
Figure 3.4: A) Relationship between systolic blood pressure and LV mass index (R=0.29; p=0.01); B) Relationship between ADMA and LV mass index (R=0.26; p=0.025); C) ANCOVA: relationship between systolic blood pressure and LV mass index according to ADMA concentration quartile (F=6.7; p=0.01)
Figure 3.5A

Figure 3.5B

**Figure 3.5:** **A)** Relationship between vascular responsiveness to NO (Δ Alx, 5 minutes post GTN administration) \((R= -0.27; p=0.02)\); **B)** ANCOVA: relationship between vascular responsiveness to NO and LV mass index according to ADMA concentration quartiles \((F=5.0; p=0.03)\) [dashed line – highest ADMA concentration quartile; continuous line – lower 3 ADMA concentration quartiles].
Figure 3.6: A) Relationship between LV mass and E/E' ratio (R=0.38, p=0.001); B) ANCOVA: relationship between LV mass and E/E' ratio according to ADMA concentration quartiles (p=NS); C) ANCOVA: relationship between LV mass and E/E' ratio according to vascular NO responsiveness quartiles (F=4.2, p=0.04).
Figure 3.7 Schematic representation of NO signaling effects and key sites of modulating potential impairment of NO formation/effects.
CHAPTER 4

Aging of the nitric oxide system:

Are we as old as our NO?
4.1 Introduction

Population ageing is one of the most distinctive demographic events of the twentieth century. Increases in the proportions of older persons (60 years or older) are being accompanied by declines in the proportions of the young (under age 15). By 2050, the number of older persons in the world will exceed the number of young for the first time in history. Moreover, by 1998 this historic reversal in relative proportions of young and old had already taken place in the more developed regions (United Nations. Dept. of Economic and Social Affairs. Population Division., 2002). Those trends have clear social and economic implications, including healthcare costs. Furthermore, advancing age itself has long been an independent risk factor for a wide variety of disease states.

Epidemiological studies have delineated the status of lipid levels, systolic hypertension, diabetes, sedentary lifestyle, smoking, obesity and genetic factors as components of risk for coronary disease, congestive heart failure, and stroke within our society (Kovacic et al., 2011). However, advancing age represents a substantial and independent basis for incremental risk: - the incidence and prevalence of these diseases increase steeply with advancing age. Not only does clinically overt cardiovascular disease increase dramatically with aging, but so do subclinical or occult diseases, such as silent coronary atherosclerosis (Fleg et al., 1990).

Some theories postulate that increasing age contributes to the increased exposure time to various other age-dependent risk factors or that cardiovascular structure and function change with time because of an “aging process”, and that over time this process alters the substrate on which specific pathophysiological disease mechanisms become superimposed (Lakatta et al., 2003a). Whether these theories are correct or not, it is clear that whilst
advancing age is an independent risk for cardiovascular disease and adverse cardiac events, there are likely to be multiple underlying biochemical and genetic determinants of this phenomenon.

Among age-associated changes are increased intimal thickening and arterial stiffness, aberrations of vascular tone, left ventricular hypertrophy, reduced threshold for cell calcium overload, reduced cardiovascular reserve, reduced heart rate variability and reduced myocardial contractility (Lakatta, 2003; Lakatta et al., 2003a; Lakatta et al., 2003b). Whilst precise mechanisms underlying these changes are not fully elucidated, many of these pathophysiological processes are regulated by the nitric oxide (NO) system, especially arterial stiffness, vascular tone, platelet function, myocardial hypertrophy and contractility (Chirkov et al., 2007; Gao, 2010; Sverdlov et al., 2011b).

The NO signalling cascade has a number of critical steps, including generation of NO from arginine by nitric oxide synthase (NOS), its stimulation of soluble guanylate cyclase (sGC), catalysing the production of cyclic GMP, which in turn acts as a second messenger (Gao, 2010). Major downstream effects include vascular smooth muscle relaxation, angiogenesis and inhibition of platelet aggregation (Hirst et al., 2011). Some major biochemical and physiological measures of the integrity of the NO signalling cascade can be readily assessed in humans, - notably, plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, which modulates NO production. Secondly, there are a number of ways of assessing vascular endothelial function and NO responsiveness: flow mediated dilatation (FMD), venous occlusion plethysmography or applanation tonometry. Finally, platelet NO responsiveness can be evaluated via NO-dependant inhibition of platelet aggregation.
Increased plasma concentrations of ADMA have been repeatedly shown to be an independent cardiovascular risk factors (Horowitz et al., 2007). Furthermore, impaired platelet NO responsiveness has been shown to be an independent marker of adverse outcomes in patients with coronary disease (Willoughby et al., 2005). Although ADMA levels are thought to increase in older individuals (Marliss et al., 2006), this has not been extensively studied, while age-related changes in vascular or platelet NO responsiveness have never been reported. It is possible that some of the effects of aging are mediated via impairment of the NO system. The impact of aging on vascular function has been evaluated largely in males (Safar, 2010). However, incidence of cardiovascular disease increases sharply in post-menopausal females (Sieveking et al., 2010): the basis for this remains uncertain.

Given the critical role of the NO system in cardiac and vascular function, we have sought to establish the impact of aging on the platelet and vascular NO system in an aging but otherwise unselected population. Evaluation of parameters at baseline and after 4-year follow-up included: (a) ADMA, (b) ADP-induced platelet aggregation, (c) platelet NO responsiveness and (d) augmentation index. Further analyses, performed at follow-up, were directed at the function of the vascular NO system and vascular repair.
4.2 Methods

4.2.1 Patient selection

An initial cohort of 253 subjects was recruited prospectively as a substudy of the North Western Adelaide Health Study (NWAHS) (Grant et al., 2006). This cohort of ambulant but aging individuals was initially evaluated to identify risk factors for aortic valve calcification. We have previously published the selection criteria and baseline patient characteristics (Ngo et al., 2009b). Four years after initial evaluation, study personnel attempted to recall every subject to invite them to participate in the follow-up stage. A total of 204 subjects were recalled: - of the remaining 49 subjects, 12 were lost to follow-up, 7 were deceased (5 due to cancers and for the remaining 2 for uncertain reasons), 6 had developed terminal illness or were receiving chemotherapy and 24 declined to participate in follow-up, citing personal reasons. Only the 204 subjects who attended the follow-up visit are included in the current report. The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital.

4.2.2 Patient data

All patients’ cardiovascular risk factors were delineated at interview. Hypertension was defined on the basis of treatment with antihypertensive drugs or blood pressure greater than 140/80 mm Hg. Hypercholesterolemia was defined by current treatment with cholesterol-lowering drugs or a total cholesterol greater than 5.5 mmol/L. Diabetes mellitus was defined as current treatment for diabetes or a fasting blood glucose greater than 7.8 mmol/L. Known coronary artery disease was defined on the basis of patient history of coronary revascularization, history of myocardial infarction or known significant coronary disease from previous angiogram if available.
4.2.3 Biochemical and physiological parameters

These can be summarized as follows:

**Physiological measures**

a. Resting augmentation index (AIx), a measure of arterial stiffness, was determined by applanation tonometry using a commercially available SphygmoCor system (AtCor Medical, Sydney, Australia), as previously described in Chapter 2 and by Wilkinson et al (1998).

b. Extent of ADP-induced platelet aggregation and platelet responsiveness to the nitric oxide (NO) donor sodium nitroprusside (SNP) were assessed using whole blood aggregometry with a dual channel impedance aggregometer (model 560, Chrono-Log, Havertown, Pennsylvania, USA) as previously described in Chapter 2 and by Chirkov et al (2002).

**Biochemical measures**

a. Plasma concentrations of ADMA, a marker and mediator of endothelial dysfunction (Boger, 2003), were determined by high performance liquid chromatography with the derivatization reagent AccQ-Fluor (Waters, Milford, Massachusetts, USA) after solid phase extraction, as previously described (Heresztyn et al., 2004).

b. Circulating endothelial progenitor cell (EPC) counts were performed utilizing flow cytometric analysis (FACScan, Becton Dickinson, USA) of cells positive for both cell surface antigens, CD34 and CD133 as previously described (Vasa et al., 2001). These measurements were performed only at the 4 year follow-up visit and in randomly selected (n=112) subjects.

c. High-sensitivity C-reactive protein (hs-CRP) concentrations lipid profile, creatinine, serum calcium levels and 1,25 dihydroxy cholecalciferol
(vitamin D levels) were measured by a $^{125}$I radioimmunoassay (Immunodiagnostic Systems Ltd., Bolden, United Kingdom).

d. C-terminal telopeptide of collagen type 1 (CTx) and N-terminal peptide of procollagen I (P1NP) concentrations were measured as markers of collagen homeostasis.

e. Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation and indexed for body surface area (BSA) with the Dubois and Dubois formula.

**4.2.4 Statistical analyses.**

All data are expressed as mean ± 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 normally distributed data were performed with non-paired $t$ tests and, comparisons for nonparametric data were made with the Mann-Whitney test. Comparisons between baseline and end-of-study (4 year follow up) parameters were made with paired $t$ tests or Wilcoxon matched-pairs signed rank test for non-parametric data. Correlations between transformed, continuous nonparametric data were made with linear regression.

Baseline determinants of platelet ADP-induced aggregation and NO responsiveness, plasma ADMA concentrations and AIx as well as determinants of EPC counts at the end of study were evaluated utilizing univariate and then multivariate analyses. Variables selected for all multivariate backward regression analyses were on the basis of univariate significance ($p < 0.2$). Parameters included in these multivariate models are summarized in Table 4.1.
Changes in platelet ADP-induced aggregation, NO responsiveness and plasma ADMA concentrations with time were used as dependent variables for the purpose of further linear regression analyses. Variables selected for all multivariate backward regression analyses were on the basis of univariate significance (p < 0.2). Parameters included in these multivariate models are summarized in Table 4.2.

End-of-study EPC counts were correlated with other measures of integrity of the NO system (follow-up measures of platelet ADP-induced aggregation, NO responsiveness and ADMA concentrations) utilizing linear regression analyses.

All analyses were performed with SPSS version 17 software (SPSS, Chicago, Illinois), and a p value of < 0.05 was considered to be statistically significant.
4.3 Results

4.3.1 Patient characteristics
Table 4.3 summarizes baseline and end-of-study patient characteristics. Over the study period, there were significant increases in the proportion of subjects diagnosed with hypertension, dyslipidaemia and diabetes, as well as a decrease in that of active smokers. More people were treated with ACEI/ARBs by the end of the study.

Table 4.4 summarizes biochemical data at baseline and follow-up. This cohort had well preserved renal function - no subject had CrCl less than 30ml/min. An apparent increase in mean creatinine clearance probably resulted from a change in methodology of creatinine assay over the study period from Olympus AU5400 Chemistry-Immuno Analyzer (Olympus America, Melville, NY, USA) to Advia 2400 Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL, USA) and associated reagent kits.

4.3.2 Change in parameters of NO system with time
Table 4.5 summarizes baseline and follow-up data as regards integrity of the NO system. Results can be categorized as follows:

4.3.2.1 Platelet data
Given that platelet aggregation in response to ADP increased and that the anti-aggregatory response to the NO donor SNP decreased markedly (individual data shown in Figure 4.1 and 4.2) an issue of physiological antagonism was raised (Geiger, 2001). Whilst there was a strong correlation between baseline and follow-up measurements of ADP induced aggregation and platelet NO responsiveness (p < 0.001 for both) (Figure 4.3), there was no
relationship between changes in ADP induced aggregation and those in platelet NO responsiveness (p = 0.42).

4.3.2.2 ADMA

Mean plasma ADMA concentrations at baseline were within the previously described normal range for the methodology used (Heresztyn et al., 2004; Horowitz et al., 2007). However, there was a significant rise in plasma ADMA concentration over 4 year follow-up period: 0.52 ± 0.08 to 0.60 ± 0.09 μM (p < 0.0001), consistent with deterioration of endothelial function with time (Figure 4.4).

4.3.2.3 Arterial stiffness (AIx)

Mean AIx of 27.2 ± 8.3% (normal range: 15 ± 16%) at baseline was substantially greater than values for normal young adults (McEniery et al., 2006). There was no significant change in AIx with time (mean 27.2 ± 8.3% versus 27 ± 6.9%; p = NS).

4.3.2.4 EPCs

As these were determined only at follow-up, the data have been utilized entirely for the purpose of correlation with other end-of-study parameters.

4.3.3 Univariate analyses

4.3.3.1 Correlates of baseline data

Univariate correlates of increased baseline ADP induced aggregation were: female gender (p < 0.001), higher total cholesterol concentrations (p < 0.001), higher Ca₃PO₄ product (p = 0.005), higher AIx (p = 0.017), CAD (p = 0.023), history of hypertension (p =
0.034), higher Framingham 10 year CAD risk score (p = 0.11) and use of ACEI/ARBs (p = 0.16).

Univariate correlates of **impaired baseline platelet NO responsiveness** were: female gender (p = 0.001), lack of use of ACEI/ARBs (p = 0.013), higher Framingham 10 year coronary heart disease risk (p = 0.038), higher CTx concentrations (p = 0.052), higher systolic blood pressure (SBP) (p = 0.09) and higher total cholesterol concentrations (p = 0.16).

Univariate correlates of **high baseline ADMA concentrations** were: higher P1NP concentrations (p < 0.001), lower vitamin D concentrations (p = 0.002), higher CTx concentrations (p = 0.008), no history of DM (p = 0.017), lack of use of ACEI/ARBs (p = 0.025), female gender (p = 0.027), increasing age (p = 0.035), higher total cholesterol concentrations (p = 0.069), AIx (p = 0.078), lower CrCl (p = 0.15) and higher Ca₉PO₄ product (p = 0.16).

Univariate correlates of **higher AIx** at baseline were: female gender (p < 0.001), lower CrCl (p = 0.001), higher Framingham 10 year CAD score (p = 0.01), higher diastolic blood pressure (p = 0.01), greater ADP induced platelet aggregation (p = 0.017), history of DM (p = 0.024), lack of use of ACE/ARB (p = 0.027), higher total cholesterol concentrations (p = 0.039), history of CAD (p = 0.06), higher ADMA concentrations (p = 0.078), increasing age (p = 0.085), higher Ca₉PO₄ product (p = 0.12) and higher SBP (p = 0.12).

### 4.3.3.2 Correlates of changes in parameters over time

The only correlate of **increasing ADP induced platelet aggregation** on univariate analyses was higher diastolic blood pressure (p = 0.11).
Univariate correlates of **worsening platelet NO responsiveness** with time were: higher Ca₃PO₄ product (p = 0.005), higher total cholesterol concentrations (p = 0.042), lower vitamin D concentrations (p = 0.057), female gender (p = 0.11), higher SBP (p = 0.11), history of hypertension (p = 0.16) and higher CTx concentrations (p = 0.17).

Univariate correlates of **rise in ADMA concentrations** were: lack of use of ACEI/ARBs (p = 0.002), higher Ca₃PO₄ product (p = 0.002), history of HT (p = 0.007), impaired CrCl (p = 0.01), higher hs-CRP concentrations (p = 0.017), higher CTx concentrations (p = 0.018), higher total cholesterol concentrations (p = 0.045), P1NP concentrations (p = 0.061), presence of DM (p = 0.076), higher BMI (p = 0.15) and higher AIx (p = 0.18).

**EPC counts** correlated on univariate analyses with the following end-of-study parameters: higher SBP (p = 0.09), increasing age (p = 0.12), higher calcium concentrations (p = 0.125) and lower CrCl (p = 0.13). In particular, there were no significant correlations between EPC count and platelet ADP-induced aggregation (p = 0.2), NO responsiveness (p = 0.77), AIx (p = 0.75) or ADMA concentrations (p = 0.86).

Furthermore, increasing ADMA concentrations correlated strongly with worsening platelet NO responsiveness (p = 0.013) (Figure 4.5). But change in ADP does not correlate with change in ADMA (p = 0.9) nor with change in SNP responsiveness (p = 0.43).
4.3.4 Multivariate analyses

4.3.4.1 Baseline

Multivariate predictors of higher baseline ADP-induced aggregation were: female gender (p < 0.001; β = 0.223); higher total cholesterol concentrations (p = 0.002, β = 0.254).

Multivariate predictors of higher baseline platelet NO responsiveness were: male gender (p = 0.003; β = 0.226), lower SBP (p = 0.035; β = 0.161), use of ACEI/ARB (p = 0.058; β = 0.146).

Multivariate correlates of higher baseline ADMA concentrations were: higher P1NP concentrations (p = 0.001, β = 0.241), lower vitamin D concentrations (p = 0.01; β = 0.18), increasing age (p = 0.045; β = 0.14).

Multivariate predictors of high baseline AIx were: female gender (p < 0.001; β = 0.5), higher diastolic blood pressure (p = 0.004; β = 0.184), lack of use of ACEI/ARB use (p = 0.015; β = 0.153) and increasing age (p = 0.02; β = 0.147).

4.3.4.2 Changes over time

Multivariate correlates of deterioration of platelet NO responsiveness were: female gender (p = 0.034; β = 0.17) and lower vitamin D concentrations (p = 0.04; β = 0.16).

Multivariate correlates of increasing ADMA concentrations were: increased Ca₄PO₄ (p = 0.001, β = 0.226), lower CrCl (p = 0.004, β = 0.202) and presence of diabetes mellitus (p = 0.03, β = 0.158).
4.3.4.3 Correlations of EPC counts at the end of study

There were no significant correlates of EPC counts on multivariate analysis.
4.4 Discussion

This study examined the determinants of a number of parameters of the NO signalling cascade, effect of aging on these parameters and predictors of adverse change in those with time in a cohort of aging Western population.

The main findings of this study are:

1. Aging is associated with both increases in ADP-induced platelet aggregation and plasma ADMA concentrations, and with reductions in platelet NO responsiveness – all important risk factors for cardiovascular events.

2. Female gender is associated with higher ADP-induced platelet aggregation, lower platelet NO responsiveness, greater arterial stiffness and more pronounced fall in platelet NO responsiveness with time.

3. There is a significant relationship between deterioration in platelet NO responsiveness and increases in ADMA concentrations.

4. Use of ACEI/ARBs is associated with better platelet NO responsiveness and lower arterial stiffness.

Previously postulated hypotheses regarding mechanisms of aging process per se, and of cardiovascular aging specifically, have been discussed in section 1.3.1. Of particular interest to the studies described in this chapter, it is important to note that postulates include increased oxidative stress (Muller et al., 2007), which may impact upon NO signalling. However, previous studies have not focused on a central role for NO/sGC signalling cascade in cardiovascular aging.
Our most central finding, that there is significant impairment of platelet NO responsiveness with aging, is indeed consistent with effects of incremental oxidative stress. The phenomenon of NO resistance appears to reflect, from a biochemical point of view, the combined effect of "scavenging" of NO, primarily by superoxide anion, and oxidative dysfunction/inactivation of soluble guanylate cyclase (for review see (Chirkov et al., 2007)). It is also possible that thrombospondin 1, which is released from platelet α granules, may contribute to inhibition of soluble guanylate cyclase (Miller et al., 2010). The current experiments did not permit delineation of the extent of NO "scavenging" versus soluble guanylate cyclase dysfunction: this would have been of potential therapeutic interest, given the development of agents such as nitroxyl donors which circumvent "scavenging" (Kemp-Harper, 2011) and selective soluble guanylate cyclase activators which are effective despite oxidation and/or heme depletion of soluble guanylate cyclase (Pankey et al., 2011). Interestingly, a number of agents such as ACE inhibitors, perhexiline and possibly statins ameliorate platelet NO resistance (Chirkov et al., 2007). The design of the current study did not permit comparison of aging of NO responsiveness in blood vessels versus platelets. However, it has been established that NO resistance, whether at vascular (Schachinger et al., 2000) or platelet (Willoughby et al., 2005) level, is an independent marker of risk of coronary events.

Furthermore we identified two factors associated with accelerated decline of platelet NO responsiveness: - female gender and lower vitamin D levels. Females are generally thought to be “protected” from coronary disease prior to menopause, while post-menopausally they rapidly "catch-up" with their male counterparts and indeed, generally have worse cardiovascular outcomes compared to males (Lee et al., 2011). The finding of more significant impairment of platelet NO responsiveness could offer a partial explanation for this epidemiologically observed risk. Indeed, as previously reported (Yee et al., 2005),
female gender was also a correlate of higher baseline ADP-induced platelet aggregation lending further support towards an increased pro-thrombotic diathesis. The mechanism(s) underlying these phenomena are worthy of specific investigation.

Vitamin D deficiency has also been associated with a number of cardiovascular disease states. The mechanism for this association is not absolutely clear, but our observed association between low vitamin D levels and higher ADMA concentrations in this cohort (Ngo et al., 2010) may provide a partial explanation.

Whilst ADMA concentrations have previously been shown to increase with age in the normal population (Kielstein et al., 2003), longitudinal evaluation of ADMA concentrations in relatively unselected patient cohorts has not been undertaken. Importantly, higher ADMA concentrations are found in renal insufficiency (Vallance et al., 1992) or in patients with coronary risk factors (Abbasi et al., 2001; Achan et al., 2003; Boger et al., 1998). Indeed, consistent with the previous literature, in our population renal function was a determinant of increasing ADMA concentrations, as was the presence of diabetes, yet no other coronary risk factors appear to predict increases in ADMA concentrations with age in our population cohort. The increase in ADMA concentrations was not primarily driven by deterioration in renal function as there was no reduction in renal function over the follow-up period. This suggests that other factors, namely enzymatic generation of ADMA by protein arginine methyltransferase-1 (PRMT-1) and/or metabolic clearance of ADMA by dimethylarginine dimethylaminohydrolase (DDAH) -1 and -2 (Blackwell, 2010) may have contributed to the observed changes.

In this regard, an intriguing finding of the current experiments was the inverse correlation between changes in platelet NO responsiveness and those in plasma ADMA
concentrations. Two possible explanations are available for this correlation. First, DDAH is known to be susceptible to oxidative stress (Tain et al., 2007) and appears to be activated in part by NO (Sakurada et al., 2008). Thus it is possible that oxidative stress might similarly impact on DDAH activity and platelet NO signalling, as well as on an NO-based feedback mechanism at the level of DDAH. A second explanation would relate to the precipitation of NOS "uncoupling" by ADMA (Antoniades et al., 2009). While NO responsiveness is superficially NOS-independent, increased superoxide production within platelets as a result of NOS "uncoupling" would contribute to NO resistance. A schematic of potential interactions between ADMA and NO signalling is depicted in Figure 4.6.

One potential effect of impaired NO signalling would be reduced number and function of EPCs (Fleissner et al., 2011). In the current study, only EPC counts were determined. There was no significant correlation between platelet NO responsiveness and EPC counts. Assessment of EPC function might have more completely delineated the relationship between aging of NO signalling and impairment of vascular regeneration.

The finding that ACEI/ARB therapy at baseline was associated with greater NO responses and lower arterial stiffness is consistent with the previous literature in both platelets (Okrucka et al., 1998) and vasculature (Ngo et al., 2009a).

The current study has a number of potential limitations. The central issue is that the study was predominantly phenomenological, without detailed parallel mechanistic evaluation at the molecular level. In particular, the mechanism(s) underlying changes in ADMA kinetics, the potential impact of these changes on NOS function, and the integrity of soluble guanylate cyclase would be important areas for detailed evaluation. The potential
gain from such an evaluation would be primarily to delineate the optimal approach for amelioration and/or circumvention of the aging-associated changes in NO signalling.

Secondly, the study was not designed to evaluate the impact of the observed changes in incidence of cardiovascular events in this population. To achieve such an objective, a much larger population would have had to be followed up for a longer period of time. While there is abundant evidence that elevation of ADMA concentrations (Horowitz et al., 2007) and presence of NO resistance (Willoughby et al., 2005) are prognostic markers in various populations, it would be worthwhile to identify the extent of association in this "normal" cohort.

It should also be noted that all platelet aggregometry studies in this chapter were performed in whole blood. One implication of this is that “aging” of anti-aggregatory effects of NO cannot be considered to be purely a platelet-derived phenomenon. For example, it is possible that increases in neutrophil-derived superoxide release, whether from NAD(P)H oxidase (Griendling et al., 2000) or from “incoupled” NOS (Satoh et al., 2005) might have contributed to the phenomenon.

Finally, we did not evaluate either changes in telomere length or quantitate oxidative stress, despite the fact that both of the above are clearly relevant to aging-induced pathology in general.

Our observations of aging of the NO system in platelets and the vasculature may have important parallels in other organs. Notably, the phenomenon of impairment of LV diastolic function becomes increasingly frequent with aging (Kane et al., 2011), and there is increasing evidence that impaired NO signalling is important as a modulator of this form
of heart failure (Silberman et al., 2010; Sverdlov et al., 2011b). Both this issue, and also that of impairment of the anti-inflammatory/anti-apoptotic effects of NO (Ritchie et al., 2009) would represent technically more challenging areas of investigation, which are nevertheless increasingly relevant, given our current results.
4.5 TABLES AND FIGURES for CHAPTER 4
Table 4.1 Variables included in multivariate models as potential predictors of baseline parameters related to NO generation/effect.

<table>
<thead>
<tr>
<th>Extent of ADP-induced platelet aggregation</th>
<th>Platelet NO responsiveness</th>
<th>Plasma ADMA concentrations</th>
<th>AIx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Gender</td>
<td>Gender</td>
<td>Gender</td>
</tr>
<tr>
<td>Ca₅PO₄ product</td>
<td>Total cholesterol concentration</td>
<td>Age</td>
<td>Age</td>
</tr>
<tr>
<td>Total cholesterol concentration</td>
<td>Systolic blood pressure</td>
<td>Use of ACEI/ARB</td>
<td>Use of ACEI/ARB</td>
</tr>
<tr>
<td>AIX</td>
<td>10 year Framingham risk score for coronary heart disease</td>
<td>Total cholesterol concentration</td>
<td>Total cholesterol concentration</td>
</tr>
<tr>
<td>History of HT</td>
<td>CTx concentration</td>
<td>CTx concentration</td>
<td>History of DM</td>
</tr>
<tr>
<td>ACEI/ARB use</td>
<td>Use of ACEI/ARB</td>
<td>P1NP concentration</td>
<td>History of CAD</td>
</tr>
<tr>
<td>History of CAD</td>
<td>Vitamin D concentration</td>
<td>ADMA concentration</td>
<td>CrCl</td>
</tr>
<tr>
<td>10 year Framingham risk score for CAD</td>
<td>AIx</td>
<td>ADP-induced platelet aggregation</td>
<td>Ca₅PO₄ product</td>
</tr>
<tr>
<td></td>
<td>History of DM</td>
<td>Diastolic blood pressure</td>
<td>ADMA</td>
</tr>
<tr>
<td></td>
<td>CrCl</td>
<td>10 year Framingham risk score for CAD</td>
<td>ADP-induced platelet aggregation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vitamin D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AIx</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>History of DM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CrCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca₅PO₄ product</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ADMA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ADP-induced platelet aggregation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 year Framingham risk score for CAD</td>
</tr>
</tbody>
</table>
ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; ADP – adenosine diphosphate; ADMA – asymmetric dimethylarginine; AIx - augmentation index; CAD - coronary artery disease; Ca₃PO₄ - calcium-phosphate; CrCl - creatinine clearance; CTx - C-terminal telopeptide of collagen type 1; DM - diabetes mellitus; hs-CRP - high sensitivity C-reactive protein; HT - hypertension; NO – nitric oxide; P1NP - N-terminal peptide of procollagen I.
Table 4.2 Variables included in multivariate models as potential predictors of change in parameters related to NO generation/effect.

<table>
<thead>
<tr>
<th>Extent of ADP-induced platelet aggregation</th>
<th>Platelet NO responsiveness</th>
<th>Plasma ADMA concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (see text)</td>
<td>• Gender</td>
<td>• Ca₃PO₄ product</td>
</tr>
<tr>
<td></td>
<td>• Total cholesterol</td>
<td>• Use of ACEI /ARB</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td>• Total cholesterol</td>
</tr>
<tr>
<td></td>
<td>• History of HT</td>
<td>concentration</td>
</tr>
<tr>
<td></td>
<td>• Vitamin D concentration</td>
<td>• CTx concentration</td>
</tr>
<tr>
<td></td>
<td>• CTx concentration</td>
<td>• P1NP concentration</td>
</tr>
</tbody>
</table>

ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; ADP – adenosine diphosphate; ADMA – asymmetric dimethylarginine; Ca₃PO₄ - calcium-phosphate; CrCl - creatinine clearance; CTx - C-terminal telopeptide of collagen type I; DM - diabetes mellitus; hs-CRP - high sensitivity C-reactive protein; HT - hypertension; NO – nitric oxide.
Table 4.3 Patient characteristics (n = 204)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (%)</th>
<th>End of study (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63 ± 6 years</td>
<td>67 ± 6 years</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>42.4% male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>24 (12%)</td>
<td>33 (16%)</td>
<td>0.013</td>
</tr>
<tr>
<td>Hypertension</td>
<td>85 (42%)</td>
<td>106 (52%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>118 (58%)</td>
<td>137 (67.5%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Smoking</td>
<td>28 (14%)</td>
<td>13 (6.4%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Coronary disease</td>
<td>24 (12%)</td>
<td>28 (14%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Statin use</td>
<td>65 (32%)</td>
<td>71 (35%)</td>
<td>0.24</td>
</tr>
<tr>
<td>ACEI/ARB use</td>
<td>69 (34%)</td>
<td>83 (41%)</td>
<td>0.008</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.2 ± 5.2</td>
<td>28.2 ± 5.2</td>
<td>0.37</td>
</tr>
</tbody>
</table>

ACEI/ARB - angiotensin converting enzyme inhibitors/angiotensin receptor blockers; BMI - body mass index
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>End of study</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.9 ± 0.9</td>
<td>5 ± 1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.8 ± 0.8</td>
<td>2.9 ± 1.1</td>
<td>0.75</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium level (mmol/L)</td>
<td>2.2 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin D level (mmol/L)</td>
<td>72 ± 23.1</td>
<td>74.7 ± 26.6</td>
<td>0.29</td>
</tr>
<tr>
<td>CrCl (ml/min/1.73m²)</td>
<td>92 ± 21.6</td>
<td>98 ± 28.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>3.5 ± 3.7</td>
<td>3.1 ± 3.8</td>
<td>0.14</td>
</tr>
<tr>
<td>CTx (median) (pg/ml)</td>
<td>242 ± 143</td>
<td>283 ± 145</td>
<td>0.001</td>
</tr>
<tr>
<td>P1NP (median) (mcg/L)</td>
<td>40.5 ± 19.8</td>
<td>43.4 ± 50.6</td>
<td>0.45</td>
</tr>
</tbody>
</table>

LDL - low density lipoprotein; HDL - high density lipoprotein; CrCl - creatinine clearance; hs-CRP - high sensitivity C-reactive protein; CTx - C-terminal telopeptide of collagen type I; P1NP - N-terminal peptide of procollagen I.
Table 4.5 Parameters relevant to NO generation/responsiveness at baseline and end of study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>End of study</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-induced platelet aggregation</td>
<td>7.8 (5.05 – 10.2)</td>
<td>8.8 (6.8 – 10.4)</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Platelet NO responsiveness</td>
<td>28.4 (13.8 – 49.5)</td>
<td>15.6 (3.7 – 38.6)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ADMA concentrations [mean ± SD] - μM</td>
<td>0.52 ± 0.08</td>
<td>0.60 ± 0.09</td>
<td>&lt;0.0001#</td>
</tr>
<tr>
<td>AIx [mean ± SD] - %</td>
<td>27.2 ± 8.3</td>
<td>27 ± 6.9</td>
<td>NS#</td>
</tr>
<tr>
<td>EPC count§ [median (25-75%)] – x10^6</td>
<td>**</td>
<td>45.7 (29 – 61.2)</td>
<td>n/a</td>
</tr>
</tbody>
</table>


* Wilcoxon matched pairs signed rank test

# Paired t test

§ n = 112

** not determined
Figure 4.1 Extent of ADP-induced platelet aggregation over the 4 year study period.
Figure 4.2 Platelet NO responsiveness over the 4 year study period.
Figure 4.3 Correlation ($r = 0.49; p < 0.001$) between extent of ADP-induced platelet aggregation and of inhibition of aggregation (platelet NO responsiveness) at baseline. Data were normalized via square root transformation (see statistical methods).
**Figure 4.4** Plasma ADMA concentrations over the 4 year study period.
Figure 4.5 Correlation between changes in platelet NO responsiveness and plasma ADMA concentrations (r = 0.2; p = 0.013).
Figure 4.6 Interactions between integrity of NO signalling and increased ADMA clearance: potential pathways. ADMA can directly inhibit and uncouple eNOS resulting in less NO and more superoxide (O$_2^-$) generation. Furthermore, ADMA can activate NAD(P)H oxidase resulting in increased superoxide production, which in turn scavenges available NO, with end result being reduced NO availability and signalling. Finally, increased superoxide can inactivate DDAH-1 and -2, which in turn reduces ADMA clearance, completing a "vicious cycle" of high ADMA begetting more ADMA.
CHAPTER 5

DE CALCIFICATIONE VALVULARUM

SUMMARY AND FUTURE PROSPECTIVES
Deatiled descriptions of the anatomy of the human aortic valve date back to Vesalius [De *humani corporis fabrica libri septem* 1543] but understanding of the pathology pathophysiology of the valve has lagged considerably, even relative to the technical advances necessary for such evaluations. However, direct access to human valve tissue during early stages of the disease remains an obvious problem. The current thesis has approached this issue indirectly, but a number of important conclusions can be reached.

The epidemiological and clinical data in Chapter 1 summarize evidence that "degenerative" AS represents both an increasing problem in aging populations and an area for which the only established therapy is replacement of the stenotic valve. The nexus between the early stages of AS (ASc) and coronary event risk is well established, but its mechanism uncertain. On the other hand, from a pathophysiological point of view, both ASc and AS represent a challenge, with inflammatory infiltration of the valve, extensive conversion of fibroblasts into reactive myofibroblasts, and impairment of valvular endothelial integrity and function as relevant aspects of the process leading to eventual valve calcification and narrowing.

As regards the key processes driving these pathophysiological changes, the case is made that two inter-related but critical aspects are impairment of local NO signalling and local activation of the renin-angiotensin-aldosterone system (RAAS). On the other hand, there is insufficient evidence to exclude other important influences, for example perturbations in matrix Gla protein kinetics. Furthermore, the precise association between the RAAS and NO effect in the context of AS/ASc is uncertain: critically, little information is available about modulation of AS development by bradykinin, which would provide one potential link with NO generation. Nevertheless the other presumptive link of NAD(P)H oxidase activation by the RAAS, with resultant oxidative stress and impairment of NO signalling,
is supported by our recent studies in a rabbit model of AS (Ngo et al., 2008; Ngo et al., 2011).

The three experimental studies described in this thesis all address various aspects of the consequences of impairment of NO signalling in an aging population at risk of ASc development. Chapter 2 describes the determinants of ASc occurrence and early progression, Chapter 3 the impact of ASc and of impaired NO generation/effect on LVH, while Chapter 4 explores the concept that one aspect of aging per se is progressive impairment of NO signalling.

In summary, the key findings of these experimental studies are as follows: -

**Chapter 2**

- ASc progresses in the majority of an aging cohort over a 4 year period.
- The presence and progression of ASc in this aging population was associated with platelet NO resistance.
- None of the “conventional” coronary risk factors examined were predictive of presence of either ASc presence or progression.
- Elevation of plasma calcium concentrations also predicted rapid ASc progression.
- Utilization of ACEI/ARB therapy was a predictor of lack of any progression, especially in very early stages of the disease.

**Chapter 3**

- The link between aortic sclerosis and increased cardiovascular risk is essentially independent of the development of left ventricular hypertrophy.
- Markers of NO generation (plasma ADMA concentrations) and of the NO/ cyclic GMP signalling cascade (vascular responses to GTN) in the peripheral circulation
predict both LV mass index and LV diastolic function in a normal, untreated, aging population.

Chapter 4

- Aging is associated with both increases in ADP-induced platelet aggregation and plasma ADMA concentrations, and with reductions in platelet NO responsiveness.
- Female gender is associated with greater ADP-induced platelet aggregation, lower platelet NO responsiveness, greater arterial stiffness and more pronounced fall in platelet NO responsiveness with time.
- Additionally, more pronounced fall in platelet NO responsiveness with time was observed in those with lower plasma vitamin D concentrations.
- There is a significant relationship between deterioration in platelet NO responsiveness and increases in ADMA concentrations.
- Use of ACEI/ARBs is associated with preserved platelet NO responsiveness and lower arterial stiffness.

Therefore, an integration of these findings includes the over-riding conclusion that the aging process is associated with a remarkable degree of attenuation of NO generation and signalling, which constitutes both a correlate of ASc development/progression and of the development of LVH (although the latter is not closely associated with ASc in "normal" populations). Furthermore, the rate of deterioration of NO signalling is greatest in females and in the presence of low vitamin D levels.

The main conclusion from Chapter 3, that both LV mass and LV relaxation are modulated by processes similar to these affecting NO generation and signalling outside the heart, is worthy of separate comment. To the best of our knowledge, this is the first study to
indicate in humans the physiological implications of disordered NO effects within the myocardium. However, the results beg a number of important questions: -

- Does the interaction between NO and LV mass reflect equally inhibition of cardiomyocyte and myocardial fibroblast hypertrophy?
- What is the physiology of the implied effects of NO on myocardial relaxation? For example, this might reflect improved myocardial energetics, decreased (fibrocyte) production of superoxide and/or improved titin phosphorylation (Kruger et al., 2009).

What is missing from this overall evaluation is a precise understanding of the relationship between these changes and activation of RAAS. In Chapter 2, it was found that therapy with ACEI/ARB was a categorical correlate of lack of ASc progression, particularly in its earliest stages, but no other information relevant to the NO/RAAS nexus is available from this thesis. The reasons for this are technical: the vascular biology studies performed cannot include those of valve tissue or myocardium. Potentially, correlations between NAD(P)H oxidase expression in platelets and platelet NO signalling might have provided further information.

One important conclusion from all of these findings is that impaired NO signalling may constitute at least part of the basis for the association between ASc and coronary event risk. The putative mechanism is supported by the considerable evidence that both tissue NO resistance (Chirkov et al., 2007; Willoughby et al., 2005) and vascular endothelial dysfunction (Landmesser et al., 2005) represent risk factors for coronary events. It remains for animal studies to establish whether the rates of decline of valvular endothelial function in AS parallel the associated changes (Ngo et al., 2008; Ngo et al., 2011) in vascular endothelial function.
There are two further important conclusions from Chapter 2 (ASC development/progression). While both of these are not entirely novel, they provide important insights. The finding that no aspect of hyperlipidaemia was correlated with either occurrence or progression of ASC extends inferences from clinical studies examining later stages of AS development (Chan et al., 2010; Cowell et al., 2005; Rossebo et al., 2008). Specifically, from the current findings it is unlikely that lipid-lowering interventions would retard ASC development/progression. The other finding concerns elevation of calcium levels, previously examined only in context of AS (Iwata et al., 2011). The findings in Chapter 2 are consistent with both genetic and epidemiological data (Iwata et al., 2011; Linhartova et al., 2008; Ortlepp et al., 2006; Rodriguez et al., 2011; Schurgers et al., 2008) correlating calcium homeostasis with the development of AS.

It must be emphasized that the results of these clinical investigations should be examined in parallel with appropriately designed isolated tissue/whole animal studies, which are able to identify cellular mechanisms underlying changes. Fortunately, many of the conclusions of the current study are consonant with those of recent isolated tissue/whole animal experiments. One key aspect emerging from our recent studies in a rabbit model is the potential role of TXNIP generation within the aortic valve: this might be critically important in humans, but cannot easily be studied, especially in early disease.

What are the potential therapeutic implications of the findings in this thesis? Overall, the data in Chapter 2 support the concept that neither ASC nor AS are "inevitable" processes, but clearly an intervention is required to test the Koch's postulates concerned. Logically speaking, randomized studies with either ACEI or ARB would be the greatest priority. However, such a trial would be difficult, because of lack of commercial impetus, the
widespread use of such agents in population at risk, and the necessity for long duration of follow-up. To date, only one small study is being undertaken, with surrogate end-points and in patients with advanced AS (Helsinki University, 2012), not really the relevant treatment group. However, if the results of this study are positive, they may provide a further impetus towards appropriate clinical investigations.

The data on “aging” of the NO system are of substantial importance, which perhaps transcends the issue of AS alone. In the studies in Chapter 4, alterations in ADMA concentrations are documented without definite identification of the precise biochemical perturbations (eg DDAH inhibition) underlying these changes in peripheral blood, which modulate attenuation of SNP response in whole blood, have not been dissected out. For example, the potential role of neutrophil-platelet interactions may be important. As is so often the case, therefore, the translational experiments undertaken in this thesis should inspire not only a search for clinical applicability of the findings, but a return to basics to delineate their wider implications.
Bibliography


Bibliography


correlation with haemodynamic severity of aortic stenosis and clinical implication for patients with low ejection fraction. *Heart* 97(9): 721-726.


Bibliography


Bibliography


Wollert KC, Drexler H (2002). Regulation of cardiac remodeling by nitric oxide: focus on cardiac myocyte hypertrophy and apoptosis. *Heart Fail Rev* 7(4): 317-325.


Appendix: Published works in whole or in part contained within this thesis

**NOTE:**
This publication is included on pages 251-259 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1016/j.jcmg.2009.03.016](http://dx.doi.org/10.1016/j.jcmg.2009.03.016)
Introduction

Aortic stenosis (AS) may be defined as narrowing of the aortic valve, due primarily to a combination of progressive fibrosis and calcification of the matrix, with consequent increase in valve stiffness, progressive reductions in valve area and concomitant increases in left ventricular afterload and work. The earliest stages of AS have been designated aortic valve sclerosis (ASc), implying disordered valve morphology (including potential calcification as well as fibrosis) in the absence of marked obstruction to left ventricular outflow.

AS is currently the most common form of valvular heart disease in the Western world [1], in large part because the most frequently occurring form of AS develops predominantly in individuals of advancing age. For example, in the Helsinki Ageing Study [2], the proportion of individuals with detectable valve calcification increased from approximately 40% to 75% between ages of 65 and 85 years, while approximately 3% of subjects over 75 years of age had severe AS.

Given that the only proven therapy for severe AS is aortic valve replacement, and that there is currently no definitive evidence that any treatment can retard progression of the disease, the development of severe symptomatic AS in elderly individuals presents an increasing medical and health economic dilemma. On the one hand, severe AS rarely remains asymptomatic for any great length of time: patients classically develop variable components of exertional dyspnoea, angina (irrespective of the presence or
absence of epicardial coronary artery disease) and atrial or ventricular arrhythmias, resulting in poor quality of life, increased rates of hospitalization and increased mortality rates [3]. Although the precise natural history of severe AS in the elderly is uncertain, a number of studies suggested that mortality is high in the somewhat selected subgroup of individuals who do not undergo aortic valve replacement [4-7]. Additionally, both ASC and AS may be associated with cardiovascular problems which are apparently remote from the valve itself. For example, there is considerable evidence that ASC is an independent marker of increased risk of cardiovascular events [8, 9], while severe AS is associated with increased risk of haemorrhage, especially into the gastrointestinal tract, primarily because of the development of acquired von Willebrand's factor deficiency [10, 11].

It should also be stated at the outset that AS is a substantially heterogeneous disease, but with only two "common causes". These are AS developing in a previously normal trileaflet valve, and AS associated with congenitally bicuspid aortic valves (BAV). These and other, rarer, causes of AS are summarized in Table 1.

Given that AS frequently develops in otherwise normal valves in aged individuals, this has been regarded as degenerative AS, implying a relationship to the normal process of "wear and tear" within the valve [12]. The purpose of this review is to demonstrate that AS is not primarily a "degenerative" process, but rather the result of a progressive inflammatory process within the valve matrix. Furthermore, we will present evidence that the development and progression of AS, rather than occurring with the implied inevitability of a purely degenerative disease, should theoretically be amenable to pharmacotherapy.

### Aortic valve anatomy and physiology: what are the homeostatic determinants?

The normal aortic valve consists of several layers of fibroblast-rich tissue, containing both collagen and elastin fibres, covered by a monolayer of endothelial cells [13]. There are also minute intravalvular blood vessels [14], consistent with a substantial oxygen demand by the other matrix cells.

The normal aortic valve interstitial cells are probably mainly fibroblasts, but some smooth muscle cells have been identified [15], raising the issue of potential variability in tone of the valve, an area which has been pursued in a number of experimental studies. For example, Pompilio et al [16] showed that intact porcine aortic valve contracted in response to phenylephrine, and also exhibited (endothelium-dependent) relaxation with acetylcholine. Furthermore, normal aortic valve interstitial cells (presumably including smooth muscle cells) have been shown to contract in response to 5-hydroxytryptamine, endothelin-1 and norepinephrine [17]. An important, but incompletely resolved issue relates to the prevalence and function of myofibroblasts, which exhibit some contractile properties, within normal aortic valves. The role of the myofibroblasts will be discussed more extensively in the context of valve pathology.

The physiological role of the aortic valve endothelium has attracted considerable attention over the past 15 years, although its role is still incompletely understood. A number of studies suggest that valve endothelium behaves in a qualitatively similar manner to vascular endothelium, for example releasing nitric oxide (NO) in response to stimuli such as acetylcholine [16], or 5-hydroxytryptamine [18].
A critical but unresolved issue is what is the physiological response of the valve endothelium to shear stress, a normal stimulus for NO release, but also potentially for activation of endothelial NAD(P)H oxidase [19, 20], which in turn would lead to release of superoxide anion (O$_2^-$) and "scavenging" of NO. Despite ongoing uncertainties, the differences between normal aortic valve and vascular endothelial cells regarding responses to shear stress were reviewed by Butcher et al [21], who demonstrated that the changes in gene expression in response to shear stress varied substantially.

Finally, little work has been done regarding other endothelial autoco ids within the valve. Notably, little is known about the physiology of prostacyclin release from the valve, although it has been detected [22]. No studies have properly addressed the issue of tissue penetration of autoco ids released from valvular endothelium, other than the obvious implications of studies measuring valve contraction. It is therefore uncertain to what extent endothelial NO and prostacyclin might modulate the function of valve interstitial cells, a physiologically important issue given evidence that in AS valve endothelium is dysfunctional or absent, as discussed below.

**Cellular histopathology of AS**

**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 has been suggested that aortic valve lesions begin with disruption of valve endothelium predominantly on the aortic side due to high shear stress [23-25].

**Inflammation and lipid deposition**

Aortic valve lesions typically present with areas of subendothelial thickening, which represents the early stage of aortic stenosis. Increased thickening of aortic valve leaflets is characterized by accumulation of inflammatory infiltrates of predominantly macrophages and T-lymphocytes, lipids, oxidized lipids, (summarized in Figure 1) [25-28] all of which potentially activate a host of pro-fibrotic and pro-inflammatory markers. Macrophages and T-lymphocytes have been detected and tend to be located near the surface of the lesion [25-28]. Immunohistochemical studies have found co-localization of apolipoproteins (apo) B, apo (a), apoE with lipid laden foam cells and macrophages [29] as well as oxidative modification of residential low density lipoproteins (LDLs) in early stenotic aortic valve lesions [30].

**Valvular matrix remodelling and fibrosis**

The presence of macrophages and T-lymphocytes, along with oxidized LDL and apolipoprotein accumulation activate several pro-fibrotic and pro-inflammatory cytokines which may modulate aortic valve remodelling and subsequent calcification. Transforming growth factor β1 (TGF-β1) [31] and interleukin-1β [32] have been found in valve matrix and are associated with increased local production of matrix metalloproteinases I and II (MMP-1 and MMP-2). All of these contribute to cell apoptosis, extracellular matrix formation, remodelling and consequently predispose to calcification. In addition to TGF-β1 and interleukin-1β, tumour necrosis factor-α (TNFα), another pro-inflammatory cytokine commonly responsible for immune regulation, inflammation and tissue remodelling, is co-localized with MMP-1 [33]. 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, is co-localized with MMP-2 in calcified aortic valve leaflets [34], and is associated with progression of AS [35].

Angiotensin II (Ang II), an important mediator of inflammation and fibrosis, could be formed by angiotensin converting enzyme (ACE) as well as the mast cell (MC)-derived neutral protease, chymase [36]. ACE has been identified in stenotic but not in normal aortic valves [37]. It has been shown that MC-derived chymase is also upregulated in stenotic valves, providing further evidence for local production of Ang II [38]. 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 [39]. These findings provide a potential basis for a role of angiotensin II in aortic valve remodelling along with other pro-fibrotic and pro-inflammatory mechanisms.

As summarized in Figure 1, current concepts of the pathogenesis of AS centre histologically on
Pathogenesis of aortic stenosis

**Activated inflammation**
- Lipid accumulation

**Valve matrix remodelling**
- Fibrosis
- Mineralization

**Aortic valve calcification**

**Disrupted aortic valve endothelium/valvular endothelial dysfunction**

**Lymphocyte/macrophage infiltration**
- Accumulation of Apo B, apo(a), apoE

**Release of chemokines, interleukins, TNF-α, TGF-β1**
- Activation of mast cells, chymase, cathepsin G, ACE
- Local angiotensin II production
- Increased TXNIP

**Valve fibroblasts**
- **Valve myofibroblasts**

**Production of reactive oxygen species**
- **Oxidative stress**

**Extracellular matrix proteins secretion**

**Activation of osteoblasts, osteoclasts**
- Production of osteopontin, osteocalcin, osteonectin, BMP-2
- Inhibition of fetuin-A, matrix Gla protein

**Figure 1.** Schema of postulated mechanisms underlying aortic valve lesion formation. Inflammatory infiltrations of T-lymphocytes and macrophages, along with lipid accumulation, is primarily responsible for early thickening of aortic valves. Interactions between chemical stimuli and disruption of valvular homeostasis: pro- and anti-fibrotic mechanisms. Later stages of aortic stenosis: 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 largely at the end stage of aortic stenosis where aortic valves mobility is significantly reduced due to a build up of bone-like calcific nodules.

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.

**Calcification**

Calcification of aortic valve leaflets tends to occur more predominantly in the later stages of AS, and is located deeper in the lesion [25]. 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).
Pathogenesis of aortic stenosis

Ossification of aortic valve leaflets resembles that associated with 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 thought to be involved in extracellular matrix production in human stenotic aortic valves [40, 41]. Furthermore, Mohler et al [42] 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, this and other investigators [43] detected bone morphogenic proteins 2- and 4- (BMP-2, BMP-4). BMPs stimulate osteoblastic differentiation with subsequent calcification. In agreement with previous studies [25, 41], Mohler et al [42] also found that macrophages and lymphocytes accumulate in areas of calcification.

There is also evidence of angiogenesis, which is essential for longitudinal bone growth in stenotic valves. T-lymphocytes aggregates tend to co-localize with sites of neoangiogenesis within ossified valves [42]. This and the presence of heat shock protein 60 (hsp60) [44], commonly expressed by cells under stress conditions, together indicate a highly active, and chronic immunomediarded 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 κB, its ligand (RANK, and RANKL), and the soluble receptor osteoprotegerin (OPG) [45]. The study detected increased concentrations of RANKL in calcified compared to 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 was associated with increased expression of RANKL [46]. Additionally, in Kaden et al [45] shown that long-term cell culture of stenotic aortic valves in the presence of RANKL induced significant increase in matrix calcium deposition and the formation of cell nodules compared to controls. Thus, the RANKL/OPG pathway may also be involved in the calcific process of aortic valve stenosis.

A recent study [47] also 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.

Matrix Gla-protein (MGP), another anti-calcific protein implicated in development of AS, is synthesised in different tissues and undergoes vitamin K dependent γ-carboxylation (reviewed by [48]). The γ-carboxylated form of MGP is involved in inhibition of tissue calcification possibly by preventing differentiation of vascular smooth muscle cells into chondrocyte-like cells or by blocking BMP function [48]. Patients with aortic valve calcification were found to have lower concentrations of uncarboxylated MGP compared with normal controls [49]; additionally both renal dysfunction and oral warfarin therapy were predictive of low MGP levels. Interestingly, another study found elevated levels of both uncarboxylated and carboxylated de-phosphorylated forms of MGP in patients with severe AS [50]. The exact biological significance of these findings is still unclear.

Oxidative stress

A number of studies have documented increased intravalvular content of a variety of pro-oxidants in models of AS and in clinical samples [23, 51-54]. Importantly, this evidence of increased oxidative stress was not specifically associated with extent of local atherogenesis in any study.

Thioredoxin, thioredoxin reductase, NADPH oxidase and thioredoxin binding protein (TXNIP) are components of a ubiquitous system, which regulates intracellular redox stress [55]. TXNIP in particular is a fundamental mediator of increased redox stress as it binds to, and inactivates thioredoxin [56]. Increased expression of TXNIP is associated with activation of apoptosis signalling pathways [57], and is suppressed by endothelial NO release [58]. We have recently demonstrated in a rabbit model of mild AS with histological features similar to that of human disease, increased intravalvular concentration...
of TXNIP compared to control animals [53]. Furthermore, co-treatment with ramipril retarded the development of AS in this model, as measured by reduction both in transvalvular velocity and valve echogenicity on echocardiography [59]. This retardation further correlated with reduction in valvular calcium and macrophage infiltration and a reduction in TXNIP accumulation within the valve matrix. This finding further underscores the importance of ACE - Ang II system in pathogenesis of AS.

Evidence of the role of nitric oxide in the pathogenesis of aortic stenosis

AS, even in its early phases is associated with the pathogenesis of acute coronary syndromes (ACS) [8, 60, 61], which are paralleled by platelet hyper-aggregability [62]. Stenotic aortic valves constitute a pro-aggregatory milieu, potentially contributing to thromboembolism [24, 63]. It has been shown that patients with AS exhibit increased platelet reactivity [64], and thrombus formation has been documented on severely stenosed valves [65]. AS has recently been linked to the phenomenon of nitric oxide (NO) resistance, even at its earliest stages [66, 67].

NO is a physiological modulator of both vasomotor tone and platelet aggregation. These effects of NO are predominantly mediated by cyclic guanosine-3',5'-monophosphate (cGMP), via activation of soluble guanylate cyclase (sGC). However, in patients with ischemic heart disease, platelets and coronary/peripheral arteries respond poorly to the anti-aggregatory and vasodilator effects of NO donors (e.g. nitroglycerin) [68]. This “NO resistance” represents a multifaceted disorder at sites of abnormal NO-driven physiology, and as such may contribute to the increased risk of ischemic events. NO resistance results from a combination of “scavenging” of NO by superoxide radical (O₂⁻) and of inactivation of sGC. The haem moiety of sGC is a “receptor” for NO and a mediator of NO-dependent activation. However, reactive oxygen species, and O₂⁻ in particular, diminish sGC sensitivity to NO because of the oxidation of the enzyme-bound haem and its subsequent loss (haem-deficient sGC).

We have investigated [66] whether AS, either with or without concomitant coronary artery disease, is associated with impaired NO responsiveness. Two major abnormalities of platelet function were documented in patients with AS. First, platelets manifested increased aggregability in response to ADP. Second, the anti-aggregating effects of NO donor SNP were significantly reduced, thus representing NO resistance at the platelet level. However, presence of the former abnormality did not account for the occurrence of the latter.

The severity of NO resistance was independent of presence/absence of hemodynamically significant coronary artery disease. These results indicate therefore that AS represents an additional "marker" of platelet NO resistance. Interestingly, there was no correlation between AVS severity and the extent of platelet NO resistance. This suggests that platelet abnormalities and impairment of NO-related mechanisms may appear early in the clinical course of AVS.

Indeed, in our recent cross-sectional population study evaluating biochemical and physiological correlates of the presence of aortic valve sclerosis (Asc), a precursor to AS, in aging individuals [67] we have documented that Asc was also associated with impaired platelet responsiveness to NO. In that study, the extent of Asc manifestation was strongly associated with the extent of platelet resistance to NO. This finding is of potential relevance to the association between Asc and thrombotic events [8]. Furthermore, tissue resistance to NO per se might also contribute to the calcification process [69].

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NO synthase (eNOS) and a marker and mediator of endothelial dysfunction [70-72]. We have also demonstrated that plasma concentrations of ADMA are elevated in patients with AS, compared with controls [73]. Thus, this finding provides further support to the suggestion that NO generation is impaired in AS.

A critical question which arises is whether the pathogenesis of AS is fundamentally related to dysfunction of the valve endothelium. NO resistance in AS may be paralleled by impairment of aortic valve endothelial function. Previous studies [63, 74, 75] have demonstrated that even
Pathogenesis of aortic stenosis

**Table 2.** Subtypes of BAV by anatomical structure and associated features [118].

<table>
<thead>
<tr>
<th>Subtype of BAV</th>
<th>Valvular anatomical features</th>
<th>Associated features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I (70-75%)</td>
<td>Fusion of the right and left coronary cusp resulting in anterior – posterior commissural orientation</td>
<td>More common in males. Aortic root dilatation and higher prevalence of aortic coarctation.</td>
</tr>
<tr>
<td>Type II (25-30%)</td>
<td>Fusion of the right and non-coronary cusp result in right and left commissural orientation.</td>
<td>More common in females. Higher association with progression to aortic stenosis and regurgitation</td>
</tr>
<tr>
<td>Type III (~1%)</td>
<td>Fusion of the non-and left coronary cusp.</td>
<td></td>
</tr>
</tbody>
</table>

early aortic valve calcification is associated with some decrease in endothelial function and even loss of valvular endothelium. Narrowing of the aortic valve orifice in AS, together with deformation of leaflets and increasingly rough surface of the valve, contributes to local turbulence in blood flow which creates shear stress, affecting both valve endothelium and passing platelets [76]. Furthermore, loss of aortic valve endothelium may predispose towards calcification of the aortic valve leaflets [69, 77, 78]. Potentially, aortic valve endothelium plays a critical role in maintaining normal aortic valve function.

In isolated porcine and canine aortic valves, it was shown that the aortic valve endothelium releases NO and prostacyclin, which both exert local anti-thrombotic effects and reduce leaflet tone [16, 18, 63]. The main "luminal" implication of these studies was that the stenotic aortic valve constitutes a pro-aggregatory milieu: - the clinical consequences of a loss of valve anti-aggregatory function must be considered together with platelet hyperaggregability and platelet resistance to NO occurring in AVS [66]. Furthermore, loss of aortic valve endothelium may predispose towards calcification of the aortic valve leaflets [69, 77, 78]. Potentially, aortic valve endothelium plays a critical role in maintaining normal aortic valve function.

As regards available data on the pathogenesis of BAV, despite a very large number of recent investigations, both basic and clinical, no coherent picture has emerged. Rather, it needs to be emphasised that there is increasing evidence of heterogeneity of pathogenesis, notably between Type I and Type II BAV, with increasing evidence of redox stress and inflammatory activation for Type I, and of predominant endothelial dysfunction for Type II [82]. Furthermore, there appears to be a substantial genetic component to BAV.

**Bicuspid aortic valve (BAV): a special case?**

BAV represents the most common congenital cardiac abnormality, with estimates of its prevalence ranging from 0.5 to 2% of the general population [79]. Structurally, BAV is heterogeneous, depending on the pattern of valve leaflet fusion, and its association with extravalvular disease, as summarized in Table 2. However, the most common pattern, accounting for about 75% of BAV cases, is fusion of the right and left (R-L: Type I) valve leaflets, while Type II BAV (fusion of the right and non-coronary cusps) accounts for virtually all other cases.

A fundamental and unique issue with BAV is the association with dilatation of the aortic root and arch, which is a particularly prominent feature of Type I BAV, and which is associated with risk of aortic dissection and rupture. On the other hand, progression of AS appears to be more rapid in Type II [79], where most patients require eventual aortic valve replacement.

From a pathophysiological point of view, BAV is unique and intriguing because it presents as a basis for a rapidly progressive form of AS and/or aortopathy, and because of the possible insight it provides regarding the pathogenesis of AS.

As regards the rapid progression, in a retrospective study of 156 adult patients with BAV, Thanassoulis et al [80] have demonstrated a mean increase in diameter of ascending aorta of 0.37mm/year and of aortic sinus of Valsava of 0.17mm/year. Tzemos et al [81] documented increase in aortic valve gradient of 0.7 mmHg per annum in a prospective cohort of 642 subjects with BAV.

As regards available data on the pathogenesis of BAV, despite a very large number of recent investigations, both basic and clinical, no coherent picture has emerged. Rather, it needs to be emphasised that there is increasing evidence of heterogeneity of pathogenesis, notably between Type I and Type II BAV, with increasing evidence of redox stress and inflammatory activation for Type I, and of predominant endothelial dysfunction for Type II [82]. Furthermore, there appears to be a substantial genetic component to BAV.
As regards the issue of inheritance of BAV, although all series show a predominance of male patients, the overall thrust of genetic investigations is to suggest autosomal dominant inheritance in most cases. A number of studies reveal multiple cases within kindred [83-85]. The most extensively documented mutations underlying BAV occur in the NOTCH1 gene [86], which would also predispose towards valve calcification, given that NOTCH signalling has been linked to expression of osteogenic genes osteopontin and osteocalcin [87]. However the relevant signal transduction pathways for AS development in BAV remain uncertain [88] and there is also evidence of multiple mutations associated with BAV [85].

The issue of association of BAV with endothelial dysfunction and possibly with impaired eNOS signalling has attracted considerable attention since it was observed that mice lacking the eNOS gene frequently also had BAV (but not other congenital cardiovascular abnormalities) [89]. However, Fernandez et al [82] have more recently clarified this observation: the abnormality is actually Type II BAV (right - non-coronary cusp fusion). Interestingly, these investigators documented that Type I BAV is present in inbred Syrian hamsters, without any known association with eNOS deficiency.

While no other endothelial function studies have specified BAV subtype, there is substantial additional evidence that BAV may indeed be associated with eNOS deficiency and/or endothelial dysfunction. Aicher et al [90] quantitated eNOS protein expression in the aortic wall (but not the valve) at the time of surgical valve replacement and observed lower levels of eNOS in BAV versus tricuspid (“normal”) valves in the presence of AS. Furthermore, eNOS expression was negatively correlated with aortic diameter. A further study has examined brachial flow-mediated dilatation (FMD): - in a group of men with BAV, there was an association between aortic dilatation and impaired FMD [91], consistent with findings of Archer et al [90]. A number of other studies have evaluated the association between AS and NO deficiency, but not always in the context of BAV: these findings will be discussed later.

The concept of inflammatory change and oxidative stress is a relevant component of the pathogenesis of BAV, and is in a sense linked to that of NO deficiency. For example, Tzemos et al [91] observed that aortic dilatation in BAV was associated not only with impaired FMD, but also with increased plasma levels of matrix metalloproteinase 2 (MMP-2), which might have contributed to the dilatation process. Furthermore, Phillippi et al [92] demonstrated that susceptibility to oxidative stress was increased, and responsive expression of metallothionein decreased in BAV aortae. While these findings implicate oxidative stress and inflammatory activation, there is also evidence that endothelial progenitor cell mobilization in response to these changes may be abnormal [93], contributing to perturbation of aortic and valvular endothelial function.

**Intervention studies to retard the progression of AS**

**Statins**

Statins were the first commercially available drugs studied in aortic stenosis given their established benefit in primary and secondary prevention of cardiovascular diseases, as well as evidence of association of lipid infiltration of aortic valve in AS [25, 29, 30]. Whilst there are many “dyslipidemic” animal models of AS (see animal model section), only one model so far has shown benefit of statin therapy [94].

There were a number of very encouraging human retrospective studies of statins in AS, suggesting significant reduction in the rate of progression of AS with statin use [95-99]. Yet, only one small prospective open-label non-randomized observational study of rosuvastatin in patients with moderate AS showed slowing of haemodynamic progression of AS [100]. Three large prospective double-blind randomized placebo control trials of statins in AS failed to show any retardation of AS progression [101-103].

**ACE-inhibitors (ACEI)/Angiotensin receptor blockers (ARB)**

Numerous studies have demonstrated increased ACE activity/expression and Ang II presence in stenotic valves [37-39], providing a rationale for investigation of benefits of ACEI/ARB therapy in AS. Treatment of cholesterol-fed rabbits with angiotensin receptor-1 blocker olme-
sartan was associated with decreased macrophage infiltration and reductions in osteopontin and ACE in aortic valves [104]. Unfortunately, no hemodynamic valvular measures of AS progression were performed. We have demonstrated hemodynamic retardation of AS, concomitantly with reduction in calcification, macrophage infiltration, redox stress and improvement in endothelial function, with ACEI ramipril treatment in a rabbit model of AS [59].

As regards human studies, no prospective trials of either class of agents have been published to date. Two main retrospective evaluations of population studies have provided conflicting data: - Rosenhek et al [98], utilizing echocardiographic parameters, found no significant effect of ACE-I therapy on AS progression (albeit with a trend to slower progression), while O’Brien et al [105], in a study using CT-based calcification assessment, found lower rates of AS calcification, after correction for comorbidities.

**Bisphosphonates**

Bisphosphonates, usually used for treatment of osteoporosis in humans, inhibit bone resorption as they cause osteoclast apoptosis [106, 107]. They also inhibit an enzyme in the cholesterol synthesis pathway, which causes abnormalities in the cytoskeleton in the osteoclast, thus reducing bone resorption [108]. Thus, bisphosphonates may directly reduce valvular calcification via their osteoblast action, as well as indirectly via inhibition of inflammation and resultant fibrosis. A study in a rat model of dialysis suggested that etidronate, now rarely used bisphosphonate, limited aortic calcification [109].

Two small retrospective human studies of bisphophonates demonstrated reduction in the AS progression rate [110, 111]. However the small size and observational nature of these studies make the results hypothesis generating at best.

**Aldosterone blockade**

Aldosterone has been implicated in animal studies in vascular inflammation and myocardial fibrosis, and these were ameliorated by aldosterone blockade [112]. Eplerenone, an aldosterone-receptor antagonist, shown to improve outcomes in heart failure patients [113], was trialled in a randomized double-blind placebo-controlled study in patients with moderate-severe AS [114]. This small trial failed to show reduction in rate of progression of valve stenosis.

**Conclusion**

The major objective of this review is to present the case that pathogenesis of AS is an active process that involves a combination of inflammatory activation, increased oxidative stress, fibrosis and calcification, which should be amenable to therapeutic intervention.

AS is the commonest form of valvular heart disease and its prevalence is rising due to increasing longevity, especially in Western world. We present evidence that AS, previously thought to be "degenerative" disorder of aging, is a complex active process, involving valvular endothelium, fibroblasts and extracellular matrix. The process is characterized by inflammatory activation and lipid deposition within valve lesions. There is extensive valvular matrix remodelling and fibrosis with increased production of MMP-1 and -2, TGF-β1, interleukin-1β and TNFα. There is extensive evidence for increased production of Ang II, a major pro-inflammatory and pro-fibrotic mediator, within stenotic valves. This would lead to further fibrosis and calcification. Impaired activation of anti-calcific modulators, such as fetuin-A and MGP, is also important in AS. There is concurrent increased in oxidative stress and evidence of impairment of the nitric oxide system as well as associated systemic endothelial dysfunction.

In BAV, representing the most common congenital cardiac abnormality, valvular inflammation is combined with aortopathy. BAV illustrates unique interplay of genetically driven inflammatory activation and, in some cases, deficiency of nitric oxide formation.

The most promising targets for pharmacological interventions have been thought to be lipid infiltration and atheroma formation. However, results of all randomized double-blind prospective studies of statins have been disappointing [101-103]. ACE-I/ARB therapy, on the other hand, shows promising results. Although post-hoc clinical data are limited and inconclusive, we have recently demonstrated in an animal model that ACE-I ramipril retards progression of mild
AS [59]. In a separate study, olmesartan treatment was also associated with reductions in macrophage infiltration and ACE in aortic valves in a rabbit model [104].

Therefore AS represents the result of a prolonged inflammatory process leading to valve calcification and ossification, which represents a potential target for therapeutic interventions.

Acknowledgements

This work is supported in part by research grants from the National Health and Medical Research Council of Australia (NHMRC) and Cardiovascular Lipid Research Grants (Australia).

Address correspondence to: John D Horowitz, Cardiology Unit, The Queen Elizabeth Hospital, University of Adelaide, 28 Woodville Road, Woodville, SA 5011, Australia. Tel: +61 8 82226000; Fax: +61 8 82227201, E-mail: john.horowitz@adelaide.edu.au

References

[22] Ku DD, Nelson JM, Caulfield JB and Winn MJ.
Pathogenesis of aortic stenosis


Pathogenesis of aortic stenosis


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


Pathogenesis of aortic stenosis


Pathogenesis of aortic stenosis


**NOTE:**
This publication is included on pages 281-286 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1016/j.niox.2011.04.009](http://dx.doi.org/10.1016/j.niox.2011.04.009)
www.AJCD.us /ISSN:2160-200X/AJCD1110003

Original Article
Pathogenesis of aortic sclerosis: association with low BMI, tissue nitric oxide resistance, but not systemic inflammatory activation

Aaron L Sverdlov, Doan TM Ngo, John D Horowitz
University of Adelaide, The Queen Elizabeth Hospital, Adelaide, Australia

Received October 31, 2011; accepted November 11, 2011; Epub December 15, 2011; Published January 1, 2012

Abstract: Aortic sclerosis (ASc) represents the earliest stage of development of aortic valve thickening, and may eventually progress to aortic valve stenosis (AS). ASc is associated with intra-valvular inflammatory activation, and potentially with attenuation of the anti-inflammatory effect of nitric oxide (NO). We have shown that ASc occurs less frequently in obese individuals, in whom systemic inflammatory activity is generally increased. We explored these relationships further by stratifying a population of 253 ageing individuals according to BMI. Increasing BMI was associated with increased hs-CRP concentrations (r=0.43; p<0.001). However, presence/absence of ASc did not significantly modify this relationship. Furthermore, increasing BMI was independent of tissue responsiveness to NO, as measured via inhibition of platelet aggregation by the NO donor sodium nitroprusside. Therefore the association of low BMI with increased risk of ASc appears to interact neither with systemic inflammatory activation in such individuals, nor with any “paradoxical” occurrence of NO resistance.

Keywords: Aortic valve sclerosis, nitric oxide, BMI, inflammation

Introduction
Aortic stenosis (AS) is a result of a progressive increase in calcium deposition within the aortic valve, leading to increased stiffness and progressive narrowing of the valve. Whilst in the past the pathogenesis of AS was thought to be due to “wear and tear”, it is now recognized that it is a complex process, involving inflammatory infiltration, endothelial disruption and dysfunction, fibrosis and calcification. Aortic sclerosis (ASc), the earliest stage of this process, is characterized by abnormal valve morphology in the absence of haemodynamically significant obstruction of valve orifice [1].

Even the ASc stage of the disease is associated with the considerable cardiovascular morbidity and mortality [2, 3]. Early studies identified a number of clinical factors associated with the presence of calcific aortic valve disease (not always distinguishing between stenosis and sclerosis). These include age, body mass index, smoking, hypertension, dyslipidaemia, diabetes and lipoprotein (a) levels [4-9]. In the more recent, and much larger, Cardiovascular Health Study [1] the risk factors independently associated with calcific aortic valve disease were age, male gender, lipoprotein (a) levels, height, hypertension, smoking and LDL levels, all of which remained significant when only ASc was considered. We have demonstrated that reduced platelet responsiveness to nitric oxide, advanced age and low BMI, but not conventional coronary risk factors, are independent correlates of presence of ASc in a randomly selected ageing Western population cohort [10].

Thus the investigations have been directed towards better understanding of pathogenesis of ASc, with the ultimate objective of developing strategies to retard its progression. Whilst a number of studies, mainly epidemiological, have examined the factors associated with the presence of ASc, very few have looked at the determinants of progression of ASc. Despite similarities in clinical factors associated with AS and atherogenesis, factors underlying progression
Relationship between aortic sclerosis and BMI

are not identical. The putative risk factor that has attracted the most controversy is that of dyslipidemia. There is considerable evidence for the involvement (as distinct from pivotal importance) of cholesterol and other lipoproteins in pathogenesis of AS and ASc (reviewed by [11]), although not every study reported such an association [12-14]; yet none of the prospective, randomized, place-controlled trials to retard the progression of AS via cholesterol lowering have been successful [15-17].

Presence of inflammatory activation and infiltration has been demonstrated in AS/ASc lesions histologically [18, 19], yet the clinical relevance of the markers of inflammation has only been assessed in one prospective study to date. In a follow-up of the Cardiovascular Health Study, involving more than 5600 subjects, of whom 1610 had ASc and 94 had AS, the authors found that increasing age, male gender, African-American ethnicity and increases in low-density lipoprotein cholesterol levels were significant predictors of new development of AS and ASc (combined). Similarly, progression from ASc to AS was significantly associated with male gender, advancing age and African-American ethnicity. CRP levels were not associated with development or progression of calcific aortic valve disease [20].

Previous studies have widely reported the association between increasing BMI and systemic inflammatory activation (reviewed by [21]). Yet we have found a “paradoxical” association between low BMI and reduced risk of presence of ASc [10]. In fact this “paradoxical” relationship has also been documented as regards survival in patients with chronic kidney disease [22], where it is thought to be that malnourishment leads to even greater atherogenic risk than obesity.

Nitric oxide (NO) has been shown to exert a wide variety of physiologic effects in cardiovascular system and specifically related to aortic valve structure and function. NO has important anti-inflammatory and vasodilator properties; it limits calcification in cell culture of aortic valve fibroblasts [23] and impairment of NO responsiveness has been demonstrated in patients with AS and ASc [10, 24]. Impairment of platelet NO responsiveness in itself has been shown to be an independent marker of adverse cardiovascular events [25].

In the current investigation, we have therefore sought to resolve the paradox inherent in the inverse relationship between BMI and risk of ASc, given that increasing BMI is associated with systemic inflammatory activation, and yet with reduced prevalence of ASc [10]. We hypothesised that the BMI : hsCRP relationship would be attenuated or reversed in the presence of ASc. In view of the association of platelet NO resistance with prevalence of ASc [10], but also with obesity [26], we also evaluated possible dissociation of the BMI : platelet NO responsiveness relationship in ASc.

Materials and methods

Study population

The study cohort (n=253) represented a subset of the North Western Adelaide Health Study aged 51 to 77 years, who had not undergone aortic valve surgery. This cohort of ambulant but aging individuals was initially evaluated to identify risk factors for aortic valve calcification [10]. Subject characteristics are summarized in Table 1. All volunteers gave informed consent before the study. The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital.

Doppler echocardiography

Complete transthoracic echocardiographic studies were performed in all subjects with a commercially available system (Vivid 5 [GE Vingmed, Horten, Norway], with a 2.5 MHz phased array probe). M-mode and 2-dimensional (2D) echocardiograms with Doppler analysis were obtained for all subjects. Mean and peak pressure gradients across the aortic valve were calculated with the modified Bernoulli equation, with continuous-wave Doppler recordings from the highest velocity available from any view. The aortic valve area was computed with the continuity equation with standard methods. Valve morphology was categorized on the basis of visual assessment, as previously described [27].

Ultrasound backscatter data analysis

Aortic valve backscatter values (AVBS) were obtained for all subjects with methods as previously published [28]. Briefly, 2D ultrasonic backscatter images of the aortic valves were ob-
Relationship between aortic sclerosis and BMI

Table 1. Patient characteristics: clinical data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No ASc (AVBS score &lt; 16 dB) (n=204)</th>
<th>ASc (AVBS score ≥ 16 dB) (n=49)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (yrs)</td>
<td>63 ± 6.0</td>
<td>64.9 ± 9</td>
<td>0.045</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.5 ± 5.1</td>
<td>26.7 ± 4.2</td>
<td>0.019</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>42%</td>
<td>49%</td>
<td>0.372</td>
</tr>
<tr>
<td>History of hypercholesterolemia</td>
<td>61.8%</td>
<td>53.1%</td>
<td>0.565</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>30.5%</td>
<td>38.8%</td>
<td>0.268</td>
</tr>
<tr>
<td>Previous angina/MI</td>
<td>11.9%</td>
<td>20.4%</td>
<td>0.114</td>
</tr>
<tr>
<td>ACEI/AIIIB therapy</td>
<td>34.8%</td>
<td>25%</td>
<td>0.193</td>
</tr>
<tr>
<td>Hypertension</td>
<td>44.1%</td>
<td>32.7%</td>
<td>0.146</td>
</tr>
<tr>
<td>Family history of CVD</td>
<td>51%</td>
<td>57.1%</td>
<td>0.439</td>
</tr>
<tr>
<td>Smoking</td>
<td>14.8%</td>
<td>12.2%</td>
<td>0.649</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>10.8%</td>
<td>10.2%</td>
<td>0.898</td>
</tr>
</tbody>
</table>

BMI – Body mass index; MI – Myocardial infarction; ACEI – Angiotensin converting enzyme inhibitor; AIIIB – Angiotensin II receptor blocker; CVD – Cardiovascular disease.

Biochemical measurements

Lipid profile, high-sensitivity C-reactive protein (hs-CRP), serum creatinine, calcium, phosphate, and 1,25 dihydroxy cholecalciferol (vitamin D levels) were measured by a 125I radioimmunoassay (Immunodiagnostic Systems Ltd., Bolden, United Kingdom). Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation and indexed for body surface area (BSA) with the Dubois and Dubois formula.

Statistical analyses

All data are expressed as mean ± 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 normally distributed data were performed with nonpaired t tests and, comparisons for nonparametric data were made with the Mann-Whitney test. Correlations between transformed, continuous nonparametric data were made with linear regression. The impact of the presence or absence of ASc on the BMI : hs-CRP and BMI : platelet NO responsiveness relationships were tested by ANCOVA. All analyses were performed using GraphPad Prism 5 software (GraphPad, USA), and a p value of less than .05 was considered to be statistically significant.

Results

Subject characteristics

Baseline patient characteristics are shown in Table 1. There was a high proportion of obese subjects (31% of subjects had BMI ≥ 30), multiple coronary risk factors were frequently present, and there was extensive therapy with statins and angiotensin-converting enzyme inhibitor/angiotensin receptor blockers. ASc was present in 19% (n=49) of subjects based on AVBS scores. Subjects with ASc were generally older and leaner than those without ASc.

Biochemistry

Biochemical findings are summarized in Table 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 were no patients on dialysis, with only 2 subjects with CrCl < 30 ml/min/1.73 m². Comparisons between subjects with and without ASc revealed that CrCl was significantly greater in subjects without ASc. Levels of hs-CRP were not significantly different...
Relationship between aortic sclerosis and BMI

**Table 2.** Patient characteristics: baseline biochemical data (expressed as mean ± standard deviation)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No ASc (AVBS score &lt; 16 dB)</th>
<th>ASc (AVBS score ≥ 16 dB)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP (mmol/L)</td>
<td>3.5 ± 3.6</td>
<td>4 ± 5.6</td>
<td>0.576</td>
</tr>
<tr>
<td>% platelet responsiveness to SNP</td>
<td>34.8 ± 27.5</td>
<td>26.6 ± 25.4</td>
<td>0.084</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5 ± 1</td>
<td>4.9 ± 1</td>
<td>0.774</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.9 ± 0.9</td>
<td>2.8 ± 0.7</td>
<td>0.549</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>0.126</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
<td>0.129</td>
</tr>
<tr>
<td>CrCL (indexed for BSA) (ml/min/1.73m2)</td>
<td>84.2 ±22.7</td>
<td>76 ± 20</td>
<td>0.022</td>
</tr>
</tbody>
</table>

hs-CRP – high sensitivity C-reactive protein; SNP – sodium nitroprusside; LDL – low density lipoprotein; HDL – high density lipoprotein; CrCL – creatinine clearance.

for those with and without ASc.

**BMI vs hs-CRP; impact of ASc**

In the entire cohort there was a direct correlation between BMI and hs-CRP (Figure 1A; $r=0.42; p<0.0001$). When this relationship was considered separately for subjects with and without ASc (Figure 1B), the relationship was significant for each cohort [ASc: $r=0.42$, $p=0.003$; no ASc: $r=0.43, p<0.0001$], but the difference between gradients was not statistically significant (ANCOVA: $p$ for interaction = 0.96).

**BMI vs platelet NO responsiveness: impact of ASc**

Surprisingly, there was no significant relationship between BMI and platelet responsiveness to NO in the entire population (Figure 2A). When subjects with and without ASc were considered separately (Figure 2B), there was a trend (ANCOVA: $p=0.08$) for subjects with ASc to have greater reductions in NO responsiveness with increasing BMI than those without ASc.

**Discussion**

This study demonstrates that the presence of ASc is not associated with significant perturbation of the BMI : hs-CRP relationship. Furthermore, in this population obesity was not significantly associated with platelet NO resistance, nor did a different relationship emerge in the presence of ASc.

The rationale for this investigation is the emerging evidence for inflammatory activation and impairment of NO signaling cascade in subjects with ASc as discussed below.

**ASc is associated with intra-valvular inflammatory activation**

This includes evidence of local secretion of angiotensin II [29], presence of inflammatory infla-
Relationship between aortic sclerosis and BMI

Thermore, on multivariate analysis, presence of aSp is associated with impaired platelet NO responsiveness in humans [10]. Therefore it has been postulated that impaired NO generation/effect might (a) result from increased oxidative stress in aSp, (b) account for the increased risk of cardiovascular events in aSp patients [3]. However, aSp (and indeed AS) occurs predominantly in lean individuals, with a negative relationship on multivariate analysis between BMI and AVBS [10], a measure of valve echogenicity [28]. This relationship is puzzling, as increasing BMI is associated with systemic inflammatory activation, largely reflecting underlying changes in adipocytes [35]. In order to reconcile these superficially paradoxical findings, we hypothesized that the BMI : hs-CRP and BMI : platelet NO responsiveness relationships would be “reversed” in the presence of aSp.

In the case of hs-CRP, there was non-significant attenuation of the BMI : hs-CRP relationship in the presence of aSp (Figure 1B), such that there was no significant increase in hs-CRP with BMI increases in aSp subjects. It is possible that the lack of significant differences reflects Type II error. However, it is more likely that the result suggests that aSp per se is not associated with marked systemic inflammatory activation, at least as measured by hs-CRP.

The results with platelet NO responsiveness are surprising, in view of the previous data of Anfossi et al [26], who demonstrated NO resistance in an obese insulin-resistant cohort. While insulin responsiveness was not measured, there was no evidence of a relationship between BMI and platelet NO responsiveness overall. In subjects with aSp, there was a borderline significant (p=0.08) decline in platelet NO responsiveness with increasing BMI. Thus, there was no evidence to suggest that in subjects with aSp NO resistance might emerge in lean individuals. These data therefore suggest that the bases of

Figure 2. A. Correlation of BMI with normalized platelet SNP responsiveness for the entire cohort (p=0.6); B. ANCOVA: relationship between BMI and normalized platelet SNP responsiveness according to presence/absence of aSp (F= 3.1; p=0.08).
Relationship between aortic sclerosis and BMI

platelet NO resistance and systemic inflammatory activation can be dissociated.

Conclusion

The inverse relationship between obesity and prevalence of ASc does not appear to be reflected in substantial alteration of obesity-related inflammatory activation or of BMI-selective disturbances of platelet NO reponsiveness. Although ASc is clearly associated with intra-valvular inflammatory activation, those data suggest that it is not the product of a primary systemic process.

Acknowledgements

This work is supported in part by the grant from the National Health and Medical Research Council of Australia and Cardiovascular Lipid Research Grants (Australia). Dr. AL Sverdlov is a recipient of CardioVascular Lipid (CVL) research Grant. We would also like to thank the staff of the North Western Adelaide Health Study (NWAHS) for their help in patient recruitment.

Address correspondence to: Dr. John D Horowitz, Cardiology Unit, The Queen Elizabeth Hospital, University of Adelaide, 28 Woodville Road, Woodville, SA 5011, Australia Tel: +61 8 82226000; Fax: +61 8 82227201; E-mail: john.horowitz@adelaide.edu.au

References


Miller JD, Chu Y, Brooks RM, Richenbacher WE, Pena-Silva R and Heistad DD. Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. J Am Coll Cardiol 2008; 52: 843-850.


Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J and Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw 2006; 17: 4-12.
*Journal of Internal Medicine, v. 271(6), pp. 569-572*
Prevention of aortic valve stenosis: A realistic therapeutic target?

D.T. Ngo a,b,1, A.L. Sverdlov a,1, J.D. Horowitz a,*

a University of Adelaide, Basil Hetzel Research Institute, Department of Cardiology, The Queen Elizabeth Hospital, Australia
b University of South Australia, School of Pharmacy and Medical Sciences, Australia

ABSTRACT

Aortic valve stenosis (AS) is the most common form of valvular heart disease in the Western world, affecting ~40% of the population over the age of 80; to date the only established treatment is valve replacement. However, AS progression occurs over many years, and is associated from its earliest stages with increased risk of coronary events.

Recent insight into the pathophysiology of AS has included central roles for angiotensin II, for diminished nitric oxide effect at the level of valve endothelium and matrix, and for inflammatory activation/redox stress culminating in activation of pro-calcific stimuli. Despite the presence of atheroma within the stenotic valve, hyperlipidemia per se does not play a critical role in the development of obstructive disease.

We review emerging options for pharmacotherapy of AS, including in particular retardation of disease progression. The various clinical evaluations of lipid-reducing therapy have been uniformly unsuccessful in slowing AS progression. However, recent studies in animal models and retrospective evaluations in humans suggest that ACE inhibitors and/or angiotensin receptor blockers may be effective in this regard. Furthermore, agents normally utilized to treat osteoporosis also offer promise in retarding AS. Given the considerable morbidity, mortality and health care costs associated with AS, such therapeutic developments should be expedited.

© 2012 Published by Elsevier Inc.

1 Drs Ngo and Sverdlov contributed equally to this manuscript.
1. Aortic stenosis: causes, epidemiology, health care implications

Aortic stenosis (AS), or progressive narrowing of the aortic valve, is the most common valve disease and the primary indication for aortic valve replacement (AVR) in the Western world (Carabello & Paulus, 2009). AS occurs as a result of progressive calcium deposition within the aortic valve, leading to increased stiffness and progressive narrowing of the valve. The earlier stages of AS are designated as aortic valve sclerosis (ASC), which implies the presence of abnormal aortic valve morphology in the absence of marked obstruction.

ASC can present at any age, and causes of ASC in adolescents and adults under the age of 60 years include congenital stenosis, developmental superimposed on congenitally bicuspid aortic valves, and post-rheumatic stenosis. Other rare causes are: familial hypercholesterolemia, hyperuricemia, hyperparathyroidism, Paget’s disease, ochronosis, Fabry disease, and systemic lupus erythematosus (Braunwald & Goldman, 2003). 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 (Yener et al., 2002); however, this review will not specifically address this form of ASC, as the pathophysiology is potentially different. With the decline of incidence of rheumatic fever, and increasing duration of survival in Western populations, the occurrence of progressive ASC on previously normal aortic valves is of major importance.

The prevalence of ASC increases exponentially with age and varies between studies: generally ASC is present in 2–7% of all patients over 65 years of age, while ASC occurs in about 25% of these patients, and in as many as 50% of those over the age of 84 years (Lindroos et al., 1993; Stewart et al., 1997; Otto et al., 1999). Novaro et al. (2007), in a follow-up of the Cardiovascular Health Study, documented that about 9% of individuals with ASC progress to ASC over a 5-year period.

With recent medical advances resulting in increased longevity, the prevalence of ASC is expected to rise significantly in the near future (Cowell et al., 2004). In fact, one study found that 50% of patients admitted to hospital with chest pain had ASC (Chandra et al., 2004).

Therefore, the health and socioeconomic burden associated with ASC is likely to increase substantially.

The presence of ASC, previously thought of as a “benign” finding, is actually independent associated with a significant increase in cardiovascular mortality and morbidity (Otto et al., 1999). In the Cardiovascular Health Study (Novaro et al., 2007), it was also found that patients with ASC had approximately twice the chance of developing new coronary events than those without ASC (Aronow et al., 1999).

Progression to significant valve narrowing can result in development of left ventricular hypertrophy, angina, heart failure, and sudden death (Braunwald & Goldman, 2003). A more recently recognized aspect of the clinical consequences of advanced ASC is modification of von Willebrand’s factor via shear stress on stenosed valves, leading to increased bleeding risk (Vincentelli et al., 2003).

However, currently, the only effective treatment, usually reserved for critical or symptomatic cases of ASC is still prompt AVR whether via open-chested operation or transcatheter implantation, with poor prognosis (via the development of increasing heart failure and arrhythmias) if surgery is contraindicated, and medical symptomatic management is prescribed (Ross & Braunwald, 1968; Braunwald & Goldman, 2003; Pai et al., 2006; Varadarajan et al., 2006; Bakaee et al., 2010). Among elderly patients with severe ASC 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. There is evidence of under-referral of elderly patients who would potentially benefit from AVR (Iung et al., 2005).

Thus therapeutic options with regard to modifying the “natural history” of ASC can be categorized according to primary objectives such as:

- a) limitation of associated risk for coronary events
- b) retardation of valve narrowing, or
- c) facilitation of surgical access/symptomatic palliation for advanced cases as summarized in Fig. 1.

Over the past 15 years, there has been significant increase in understanding of the pathophysiology of ASC, and it is now understood that ASC involves an active disease process, not the previously suggested “wear and tear” theory (Freeman & Otto, 2005). Clinical and experimental studies have been performed to assess pathological changes of aortic valve fibrosis/calcification; to examine factors associated with development and progression of ASC; and numerous animal models of the disease have been developed: all aimed at finding medical therapy that could prevent the development as well as retard progression of ASC.

To date, no medical therapies have been demonstrably successful in reducing progression of ASC in humans. However, clinical trials of this type are limited to the 3 interventions with the use of various statins (Cowell et al., 2005; Rossebo et al., 2008; Chan et al., 2010). In all cases, these interventions were undertaken when patients already had moderate ASC. Notably, there have been no interventions in ASC with other agents in mild/moderate ASC, or in high risk patients with early ASC and chronic renal failure. All of these are substantial gaps in current therapeutics and will be discussed further in this review.

2. Current treatment strategies

2.1. Surgical/percutaneous transcatheter aortic valve implantation (TAVI)

Aortic valve replacement surgery, and for inoperable cases the more recently developed percutaneous transcatheter aortic valve implantation (TAVI) represent definitive therapy for severe/symptomatic ASC. Recent advances in surgical techniques and periprocedural care have led to substantial improvements in aortic valve replacement outcomes: mortality has decreased by ~24%, stroke risk by 27% despite population risk profile being worse, particularly increasing age of patients, undergoing surgery (Brown et al., 2009). After successful valve replacement, long-term survival rates are close to those in age-adjusted control subjects, symptoms are less marked and quality of life is greatly improved (Kvídal et al., 2000). However, it is clear that symptomatic status frequently remains impaired, due primarily to the presence of substantial pre-existing intramyocardial fibrosis in many patients (Dweck et al., 2011).

For those elderly patients and those with multiple co-morbidities, who are frequently declined surgery and previously been managed palliatively, recent development of TAVI offers new hope. TAVI has been shown to be associated with sustained clinical and functional cardiovascular benefits in high-risk patients with symptomatic aortic stenosis up to a 3-year follow-up (Ussia et al., 2012). Finally, percutaneous aortic valvuloplasty, first described by Cribier et al. (1986) probably does not change the natural course of the disease and it is
now predominantly used as a palliative measure (Cribier et al., 2004) or as a bridge to valve replacement.

Advanced AS is associated with increased risk of gastrointestinal bleeding as a consequence of acquired von Willebrand factor deficiency (Warkentin et al., 1992). This bleeding tendency is reduced by successful AVR (Vincentelli et al., 2003).

2.2. Medical options for symptomatic disease

Most medical treatment options for symptomatic AS cases utilized thus far have been directed either to expedite the approach towards valve replacement, for example by improving preoperative hemodynamic status, or to improve symptoms and exercise tolerance in patients in whom valve replacement deemed impracticable. However, one prospective randomized, short-term double blinded trial of enalapril in 56 patients with severe symptomatic AS demonstrated improvements in New York Heart Association class and 6 minute walk test (ChocKalingam et al., 2004).

Perioperative morbidity and mortality risk with AS patients increases with age, severity of AS and with extent of impairment of left ventricular systolic function. At least two studies of medical preoperative “stabilization” therapy have been reported to date, utilizing completely different forms of pharmacotherapy. Short term infusion of the nitric oxide (NO) donor sodium nitroprusside (SNP) has been reported to stabilize patients with severe AS and associated persistent pulmonary congestion. However, this only practicable in the absence of systemic hypotension (Khot et al., 2003). Furthermore, it is possible that the benefits of SNP infusion extended beyond dilatation: NO may improve left ventricular relaxation and thus reduce myocardial oxygen demand while improving diastolic coronary blood flow. In this regard, it is important that there have been no studies to date evaluating either impaired NO response as a potential contributor to hemodynamic decompensation in severe AS, nor the potential long-term benefits of pharmacotherapies which specifically increase NO availability in AS.

An alternative approach would involve utilization of perhexiline for perioperative cardioprotection in advanced AS. Perhexiline has been used primarily as an anti-anginal agent which is devoid of hypotensive or negative inotropic effects (Phan et al., 2009). However, more recently it has been shown to have beneficial effects on symptomatic status in patients with inoperable AS (albeit in a non-controlled study) (Unger et al., 1997), in systolic heart failure (Lee et al., 2005), and hypertrophic cardiomyopathy (Abozguia et al., 2010). The theoretical bases for potential cardioprotective effects of perhexiline in AS are improvements in efficiency of myocardial oxygen utilization (Jeffrey et al., 1995; Kennedy et al., 2006) resulting in improvements in cellular energetics (Abozguia et al., 2010), as well as known NO-potentiating and anti-inflammatory effects (Willoughby et al., 2002; Liberts et al., 2007).

One of the effects of perhexiline on cardiac biochemistry is inhibition of the “carnitine shuffle” via carnitine plamitoyl transferase 1 and 2 inhibition (Horowitz et al., 2010). This would result in some, but not all, of the energetic advantages observed with the drug. Given the potential efficacy of perhexiline in both short- and long-term myocardial protection, it can be postulated that the growing classes of “metabolic” anti-ischemic/cytoprotective agents may exert similar effects. Such agents include partial fatty acid oxidation inhibitors, agents which potentiate malonyl CoA effect and also AMPK stimulants, as summarized in Fig. 2 (Horowitz et al., 2010).

This very limited role of current pharmacotherapy for patients with advanced symptomatic AS is likely to remain under-researched, due to the considerable difficulties inherent in evaluation of drug effects in aged patients with multiple co-morbidities.

On the other hand, this review is concerned primarily with the development of therapeutic options earlier in the course of AS, where the following represent desirable end-points: a) retardation of AS progression, throughout the course of the disease; and b) diminution of cardiovascular risk associated with the presence of even early AS (ASC).

In order to explore the growing potential for such interventions to become realistic, it is appropriate that we briefly review: 1) the structure and normal physiology of the aortic valve; 2) our understanding of the pathological processes which underlie the development and progression of AS, from structural, biochemical and physiological standpoints; 3) the “natural history” of progression of AS with both biochemical and clinical determinants of slow vs rapid progression; and 4) the pathophysiological “commonalities” between early AS and coronary risk.

3. Structure and normal physiology of the aortic valve

3.1. Structure of the aortic valve

The normal aortic valve has 3 cusps. Each cusp consists of several components: the ventricularis, on the ventricular side of the
leaflet, the fibroa, which is on the aortic side of the leaflet, and the spongiosa, 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 a high tensile strength and is subjected to less shear stress (Warren & Yong, 1997; Freeman & Otto, 2005).

Valvar interstitial cells (VICs), the predominant cell type in normal valves, are spindle-shaped mesenchymal cells, and actively participate in response to injury to maintain structural integrity of the valve leaflet (Mulholland & Gottlieb, 1996). VICs are precursors of contractile myofibroblasts (Yip et al., 2009), which have been histochemically characterized by expression of α-smooth muscle actin (Skalli et al., 1989; Sappino et al., 1990) and other contractile proteins, such as the striated-muscle isoforms of myosin heavy chain (Mayer & Leinwand, 1997; Rice & Leinwand, 2003). Normally, VICs secrete extracellular matrix (ECM) components such as collagen, fibronectin and glycosaminoglycans (Schoen, 1997), as well as ECM degrading enzymes such as matrix metalloproteinases (Soini et al., 2001; Dreger et al., 2002). Upon aortic valve injury, VICs have the ability to differentiate to contractile myofibroblasts (Yip et al., 2009) and respond to a variety of biochemical stimuli, including serotonin (5-HT), endothelin (ET)-1 and NO in a manner similar to those of vascular smooth muscle cells (Filip et al., 1986). Myofibroblast activation is regulated tightly by cytokines that control differentiation, proliferation, contraction, ECM secretion, and migration to the site of wound healing or tissue remodeling (Powell et al., 1999). During physiological remodeling, myofibroblasts are eliminated by apoptosis (Desmouliere & Gabbiani, 1995). However, when the myofibroblast life cycle is dysregulated, myofibroblasts persist, with continued force generation and ECM production, resulting in pathological fibrosis, scarring, and fibrocontractile disease (Desmouliere, 1995).

3.2. 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, leucocytes, and scattered erythrocyte adhesions seen on the exposed subendothelial surface. Physiologically, Pompilio et al. (1998) showed that intact swine aortic valve significantly contracted to phenylephrine, and responded to acetylcholine-induced endothelium-dependent relaxation. Denudation of the endothelial layer significantly retarded the acetylcholine-induced relaxation. Furthermore, the presence of intact aortic valve endothelium was associated with significantly greater release of prostacyclin compared to those without the endothelium. These effects in swine aortic valves were also seen with canine aortic valves (Ku et al., 1999). In addition, in a porcine ex-vivo aortic valve study, El-Hamamsy et al. (2009) recently demonstrated the physiological role of the aortic valvar endothelium in regulating aortic valve mechanical properties. This study found that, under physiological conditions, aortic valve contractile responses were predominantly VIC-mediated. Interestingly, the addition of L-NAME or endothelium-dependent denudation did not alter valvar tone under basal conditions. Upon stimulation with serotonin, in the presence of intact endothelium, a reduction in tissue stiffness was observed, while the addition of L-NAME or endothelium denudation led to a significant increase in cusp aeral strain. Therefore, loss of these adaptive
mechanisms due to endothelial dysfunction (or the absence of endothelium in a damaged valve) could potentially result in abnormal biomechanics, structural damage, and exacerbation of disease progression in AS. This study, however, did not examine the effects of shear stress, which may exert additional effects on valve mechanics, especially under basal conditions.

Overall, these studies demonstrated that the aortic valve endothelium is capable of relaxation and contraction to physiological stimuli, and the data suggests a role in the maintenance of valvular homeostasis. Furthermore, the valve endothelium, like endothelium elsewhere, exerts anti-aggregatory and anti-inflammatory effects in a paracrine manner. However, there is also some evidence of heterogeneity between aortic valve and vascular endothelial physiology. Specifically, in a study comparing aortic and aortic valve endothelial cells, Butcher et al. (2006) described differential expression of over 400 genes.

Key questions which remain largely unresolved at this stage about the physiology of the valve endothelium concern:

1. Its specific adaptations to the conditions of high shear stress which are associated with normal valve function, and
2. The extent to which autacoids such as NO, PGs, and ET-1 released from valve endothelium penetrate valve matrix and affect valve contraction and resistance to inflammatory change.

4. Pathological processes underlying aortic valve stenosis development and progression: structural changes and biochemical mechanisms

A schematic of the relationship between endothelium and matrix in pathophysiology of AS is provided in Fig. 3.

4.1. Inflammatory infiltration

The early lesions of AS represent an active inflammatory process, which begins with disruption of valve endothelium, predominantly on the aortic side, followed by inflammatory infiltrations which include mainly macrophages and T-lymphocytes (Otto et al., 1994; Chen et al., 2003). C-reactive protein (CRP) was also found in the fibrosa of human stenotic valves (Skowasch et al., 2006), and it has been suggested that the presence of CRP in aortic stenotic valves could activate the classical complement system (Helske et al., 2008). Indeed, these investigators have shown that complement activation occurs in stenotic aortic valves, with marked increase of the terminal complement complex C5b-9 and C3a in both sclerotic and stenotic vs control valves (Helske et al., 2008). Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, has also been found in the extracellular matrix of stenotic aortic valves, predominantly in areas of leukocyte infiltration, and localized mainly to macrophages. The expression of matrix metalloproteinase-1 (MMP-1) was also significantly enhanced in the lesions containing leukocyte infiltration and TNF-α (Kaden et al., 2005a). Cultured human aortic myofibroblasts with TNF-α showed significant induction of MMP-1 expression (Kaden et al., 2005a). The presence of TNF-α has also been linked to activation of the “receptor activator of nuclear factor kappa B ligand” (RANKL), a key regulator of bone turnover and lymphocyte differentiation (Kaden et al., 2005b). The addition of TNF-α to cultured stenotic human aortic valve interstitial cells showed markedly increased calcification, activity and expressions of calciogenic markers (Yu et al., 2011). These results suggest that TNF-α is an active regulator of aortic valve calcification, and thus a potential therapeutic target.

4.2. Lipid infiltration

In conjunction with leukocyte and macrophage accumulation, lipids and oxidized lipids are also found in the early aortic valve lesions. Apolipoproteins (apo) B, apo (a), apoE (O’Brien et al., 1996) have been shown to be co-localized with macrophages and increased oxidized low density lipoproteins (LDL) score was shown to correlate with TNF-α expression in explanted human stenotic valves (Molty et al., 2008) and with increased tissue remodeling score (Warren & Yong, 1997). Oxidative modification of residual LDL has also been demonstrated in early stenotic aortic valve lesions (Olsson et al., 1999).

4.3. Extracellular matrix remodeling and fibrosis

The presence of activated and osteoblastic VICs, as well as T-lymphocytes and macrophages leads to activation of pro-fibrotic/pro-calcific cascades and contributes to leaflet remodeling and calcification. Increases of MMP-1, -3, and -9 have consistently been reported, while documented changes in their inhibitors, TIMP-1 and -2, have varied between studies (Edip et al., 2000; Satta et al., 2003; Fondard et al., 2005). 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 metalloproteinase II (MMP-2), all of which contribute to cell apoptosis, extracellular matrix formation, remodeling and consequently predispose to calcification. Furthermore, both TGF-β1 and TNF-α, other important proinflammatory cytokines commonly responsible for immune regulation, inflammation and tissue remodeling, co-localize with MMP-1 (Kaden et al., 2005a).

4.4. Calcification

Calcific nodules have long been described in stenotic valves (Freeman & Otto, 2005); that has been linked to plasma calcium (Ortlepp et al., 2006) and parathyroid hormone (Linhartova et al., 2008) as well as renal disease (Maher et al., 1987). Nodule formation is thought to be initiated by cytokines released from infiltrating inflammatory cells and from apoptosis of VICs (Mohler et al., 2001). Osteogenesis has also been described, at later stages in the disease, characterized by the presence of osteoblastic VICs and upregulation of pro-calcific markers osteopontin, osteocalcin, alkaline phosphatase, bone sialoprotein, bone morphogenic proteins 2- and 4 and osteoblast-specific factor runx2/cbfα-1 (O’Brien et al., 1995; Mohler et al., 2001; Kaden et al., 2004). Multiple pathways of osteogenesis have been identified (as summarized in Fig. 3), including osteoprotein-granulocyte colony stimulating factor (Kaden et al., 2004; Steinmetz et al., 2008), Toll-like receptors 2 and 4 (Meng et al., 2008), tenascin-C (Satta et al., 2002), Lrp-5/Wnt/Wnt5a (Cheng et al., 2006) and angiotensin II (O’Brien et al., 2002; Helske et al., 2004).

These changes in expression of markers of bone turnover raised the issue of a nexus between the pathophysiology of AS in the elderly and that of osteoporosis. Furthermore, correlations have been documented between polymorphisms of vitamin D receptors (Ortlepp et al., 2001), variations in genotype for parathyroid hormone (Schmitz et al., 2009) and probability of development of AS, again suggesting potential relationships between control of bone turnover and of valvular calcification.

Significantly elevated content of RANKL, concomitant with significant reduction of osteoprotegrin-positive cells, was found in calcified valves compared to only few positive cells in respective control valves (Kaden et al., 2004). Long-term cell culture of stenotic aortic valves with RANKL showed a significant increase in matrix calcium deposition and the formation of cell nodules. Also, mice deficient in osteoprotegrin develop vascular calcification with increased expression of RANKL in calcified areas (Bucay et al., 1998).

Tenascin C, another extracellular matrix glycoprotein implicated in cell proliferation, migration, differentiation and apoptosis, and which is involved in stimulation of bone formation and mineralization, has been found co-localized with MMP-2 in calcified aortic valves.
Fetuin-A is an anti-calciotic protein and a member of the cysteine protease inhibitor superfamily. It is a carrier for growth factors and inactivates TGF-β and BMP (Binkert et al., 1999). Low plasma and raised intra-valvular levels of fetuin-A have recently been found in patients with AS (Kaden et al., 2007). Low plasma concentrations of fetuin-A were also found in persons with concomitant coronary artery disease and AS, but without renal disease and diabetes (Ix et al., 2007). In a more recent small study, serum fetuin-A levels were directly associated with progression of aortic valve calcification (assessed by CT), over an average follow-up period of 12 months (Koos et al., 2009). Nevertheless, the relative importance of these findings to the overall pathogenesis of aortic stenosis remains uncertain.

Another anti-calciotic protein linked to AS is matrix Gla-protein (MGP). It is synthesized in many different tissues and undergoes vitamin K dependent γ-carboxylation (Schurgers et al., 2008). While its precise mechanisms of action are not well understood, the active, γ-carboxylated form of MGP is involved in inhibition of tissue calcification, possibly by preventing differentiation of vascular smooth muscle cells into chondrocyte-like cells or by binding to bone morphogenic protein and blocking its function (Schurgers et al., 2008). Levels of uncarboxylated MGP were lower in patients with aortic valve leaflets (Jian et al., 2001), and is associated with progression of AS (Satta et al., 2002).
valve calcification (assessed by CT) compared with normal controls, while renal dysfunction and oral warfarin therapy were both predictive of low MGP levels (Koo et al., 2009). However, another study found elevated levels of both uncarooxylated and carboxylated desphosphorylated forms of MGP in patients with severe AS (Ueland et al., 2010). Fig. 4 summarizes the major osteogenic mechanisms postulated to be critical to AS development.

5. Critical roles of the renin–angiotensin and nitric oxide/soluble guanylate cyclase systems in the pathogenesis of aortic valve stenosis

5.1. Contributions of the renin–angiotensin system to the pathogenesis of aortic valve stenosis

O’Brien et al. (2002) were able to identify angiotensin-converting enzyme (ACE-1) in stenotic, but not normal aortic valves. 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 III, 2004). Although ACE-1 represents a major mechanism for generation of the proinflammatory and profibrotic peptide angiotensin II (Ang II), there are other alternative generation pathways for Ang II. For example, Ang II 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 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 also is 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 provides a potential basis for a role of Ang II in aortic valve remodeling along with other profibrotic and proinflammatory mechanisms. However, until recently there were no data implicating ACE effect in the pathogenesis of AS.

In addition to the formation of Ang II, ACE-1 could also exert profibrotic effects via increased bradykinin (BK) degradation. Bradykinin exerts its fibrotic mechanisms by binding to BK-type 2 receptor (BK-2R), which stimulates production of nitric oxide (NO) (Liesmaa et al., 2009). Helske et al. (2007) have demonstrated net anti-fibrotic effects of BK in aortic valve: addition of BK to cultured aortic valve myofibroblasts resulted in a reduction of TNF-α induced collagen expression, which was reversed by the addition of icatibant, an inhibitor of the BK type-2 receptor (BK-2R). On the other hand, they found that the proinflammatory BK-type 1 receptor (BK-1R), was markedly upregulated compared to the anti-fibrotic, bradykinin-2 receptor (BK-2R) in stenotic valves vs controls (Helske et al., 2007). Finally, AS was associated with increased activity and expressions of neutral endopeptidase (NEP), an enzyme responsible for BK degradation.

Thus it might be hypothesized that AS is associated with reduced activity of BK, resulting in loss of a net anti-fibrotic effect, via a combination of:

1. Degradation of BK via ACE-1 and NEP, and
2. Upregulation of BK-1R, with resultant emergence of proinflammatory effects of BK.

Furthermore, it is entirely possible that either activation of the BK systems, or inhibition of both ACE-1 and NEP could delay aortic valve fibrosis.

5.2. Abnormalities of nitric oxide signaling and pathogenesis of aortic valve stenosis

The NO-soluble guanylate cyclase (sGC) system is fundamentally involved in the pathogenesis of AS: abnormalities of the NO/sGC axis are present not only within the valve but also in the vasculature and platelets from the earliest stages of the disease, and are relevant both to disease progression and to associated risk of cardiovascular events (Fig. 5).

5.2.1. Abnormalities of the nitric oxide system within the vasculature

Poggianti et al. (2003) in a study of 102 patients with known or suspected CAD, demonstrated that patients with AS had significantly reduced flow mediated dilatation (FMD) of brachial artery, a reproducible measure of endothelial function (Celermajer et al., 1992; Corretti et al., 2002), compared with non-AS patients. Furthermore, administration of endothelium-independent vasodilator, glyceryl trinitrate (GTN), did not show any improvement in FMD (Poggianti et al., 2003). In areas with prominent inflammatory mechanisms. However, until recently there were no data implicating ACE effect in the pathogenesis of AS.

Fig. 4. Procalcific/osteogenic mechanisms in aortic stenosis.

Recently demonstrated that impairment of platelet responsiveness to signaling cascade in platelets of these patients. Furthermore, we have ascribed a dysfunction of both pro-aggregatory cascade and NO-aggregatory responses to the NO donor SNP (Chirkov et al., 2002), impaired von Willebrand factor deactivation has been documented on severely stenosed valves (Stein et al., 1997). Furthermore, advanced AS is frequently associated with increased platelet reactivity (Riddle et al., 1983), and thrombus formation induced by TGF-β. The importance of potential attenuation of NO-based signaling within the valve matrix should be seen in parallel with evidence that thioredoxin-interacting protein (TXNIP), a physiological antagonist of NO (Schulze et al., 2006), may accumulate in valve matrix in AS (Ngo et al., 2008). Thus, there is emerging evidence that both ASC and heterogeneity, independent of other cardiovascular risk factors (Ngo et al., 2009). However, it must be noted that development of AS is associated with impaired NO signaling, which includes, and may be primarily based upon impaired NO effect.

Fig. 5. Nitric oxide/oxidative stress interactions: relationship to pathogenesis of aortic stenosis and associated risk of cardiovascular events.

5.2.2. Abnormalities within the valve

As previously mentioned, there is substantial evidence from animal models that NO produced by valve endothelium can induce relaxation of myofibroblasts in valve matrix (Pompilio et al., 1998; El-Hamamsy et al., 2009). However, the role of putative valvular endothelial dysfunction as a basis for development of fibrotic and calcific changes within the valve matrix has been studied less extensively, while little is known of the role of the NO/sGC system within the valve matrix itself.

However, it is clear that NO exerts an anti-calcific effect in models of AS. For example, in cell culture of porcine (myo)fibroblasts, Kennedy et al. (2009) observed that NO inhibited calcific nodule formation induced by TGF-β. The importance of potential attenuation of NO-based signaling within the valve matrix should be seen in parallel with evidence that thioredoxin-interacting protein (TXNIP), a physiological antagonist of NO (Schulze et al., 2006), may accumulate in valve matrix in AS (Ngo et al., 2008).

On the other hand, to date there have been no specifically directed evaluations of the relationship between the development of vascular endothelial dysfunction and changes in valvular NO signaling; nor have there been any studies to determine whether NOS inhibition accelerates development of AS in animal models. Therefore evidence regarding the "pivotal" role of impaired NO effect within the valve matrix remains far from complete.

Additionally, it must be noted that development of AS is associated with partial denudation of valve surface endothelium (Chirkov et al., 2006). The implications of such loss of endothelium include a loss of an anti-aggregatory effect on the surrounding blood, and may contribute to reports of thrombo-embolism associated with AS (Otto et al., 1999).
6. Sources of oxidative and redox stress

A number of recent studies have emphasized increases in intravascular content of a variety of pro-oxidants in models of AS/c clinical samples. In explanted human AS valves, Miller et al. (2008) demonstrated significant increases in superoxide and hydrogen peroxide vs control valves, inhibitable by L-NAME (NOS inhibitor), which suggested that eNOS uncoupling had occurred. Furthermore, concomitant reduction in expressions of antioxidants such as superoxide dismutase, and catalase was also found in aortic stenotic valves vs controls. The same group has also found that AS development in low-density lipoprotein receptor-deficient apolipoprotein B-100-only (LDLr−/−) ApoB (100/100) mice was associated with significantly elevated intra-vascular superoxide levels (Weiss et al., 2008).

In a hypercholesterolemic/calcific model of AS in rabbits, increased production of hydrogen peroxide was demonstrated in the calcific regions of aortic stenotic valves vs controls (Liberman et al., 2008).

We have found that aortic valve stenotic lesions in a rabbit model had significantly elevated TXNIP vs controls (Ngo et al., 2008). More importantly, retardation of stenosis development by ramipril was associated with falls in aortic valve TXNIP (Chen et al., 2003; Liberman et al., 2008; Miller et al., 2008; Ngo et al., 2008; Ngo et al., 2010).

TXNIP is the endogenous inhibitor of thioredoxin (Trx), a major antioxidant. The main function of the Trx/TXNIP balance is to maintain the intracellular protein thiol redox state. Trx requires thioredoxin reductase and electron transfer from NADPH, to sustain the generation of reduced Trx, the active antioxidative state. NO induces S-nitrosylation of Trx at cysteine 69 in endothelial cells, and this S-nitrosylation is required for the redox-regulating activity of Trx and its ability to reduce intracellular ROS in endothelial cells (Patwari & Lee, 2007).

Recently, it was shown that in resting conditions, TXNIP binds to Trx and regulates cellular redox state. However, in conditions of high oxidative stress, TXNIP is released from oxidized Trx, binds to and activates the Nod-like receptor protein 3 inflammasome, which in turn facilitates the production of mature interleukin-1β (Zhou et al., 2010). Furthermore, the addition of a NO donor was found to suppress TXNIP, and increased Trx activity (Schulze et al., 2006).

Importantly, the above evidence of increased oxidative stress was not specifically associated with extent of local atherogenesis in any study. However, no comparison has been undertaken to date of potential pro-oxidative mechanisms in AS versus vascular endothelial disease and/or vascular atherogenesis.

7. The natural history of aortic stenosis (AS)

7.1. Progression of aortic valve sclerosis/aortic valve stenosis: determinants and details

While clinical progression of AS, as measured at echocardiography by changes in aortic valve area or transvalvular gradient, may be non-linear, with apparent acceleration later in the course of disease, histopathological progression is much more difficult to evaluate in humans and its rate may not be analogous to that of clinically-detectable changes. One of the first prospective studies of progression of asymptomatic AS reported an annual increase in aortic jet velocity of −0.3 m/s and a decrease in aortic valve area (AVA) of 0.1 cm² (Otto et al., 1997). This study was not powered to evaluate clinical factors and found that greater transvalvular velocity and smaller AVA were associated with more rapid progression of AS, as measured by changes inAVA. A large (n>2000) study of patients with ASc demonstrated that 10.5% developed mild AS, 3% moderate AS and 2.5% severe AS over a 7 year follow up period (Costanzo et al., 2002). A smaller retrospective study showed a 33% rate of development of AS in 400 patients with ASc over a nearly 4 year period (Faggiano et al., 2003).

Despite similarities in clinical factors associated with AS and atherogenesis, factors underlying progression are not identical. The longest study to date, a follow-up of Cardiovascular Health Study, involving more than 5600 subjects, of whom 1610 had ASC and 94 had AS, demonstrated 9% progression from ASC to AS, 1% incidence of AS and 44% incidence of ASC over a 5 year mean follow-up period (Novaro et al., 2007). The authors found that increasing age, male gender, African-American ethnicity and increases in low-density lipoprotein cholesterol levels were significant predictors of new development of AS and ASC (combined). Similarly, progression from ASC to AS was significantly associated with male gender, advancing age and African-American ethnicity. Neither history of diabetes, hypertension, or tobacco use, nor the presence of coronary heart disease or renal insufficiency was an independent predictor of progression; CRP levels were also not associated with development or progression of calcific aortic valve disease (Novaro et al., 2007).

One of the correlates of rapid progression of AS identified so far is renal dysfunction, especially end stage renal disease requiring dialysis (Maher et al., 1987; Faggiano et al., 1996; Wongpraparut et al., 2002; Perkovic et al., 2003); with odds ratio of ~2.5 for rapid progression in the cohort on hemodialysis (Wongpraparut et al., 2002). It is not only significant renal dysfunction per se that is associated with rapid progression in these patients—high vitamin D₃ levels in dialysis patients have also been associated with accelerated progression of AS (Malergue et al., 1997; Urena et al., 1999).

However, renal dysfunction has never emerged as a risk factor in unselected patient populations, and it is uncertain to what extent this correlation is relevant only for severe renal dysfunction. Furthermore, the biochemical correlates of this association are incompletely explored, but include substantial elevation of plasma ADMA concentrations (Schwedhelm & Boger, 2011) and thus presumptive diminution of NO effect.

As regards “conventional” cardiovascular risk factors, results of the studies are less clear cut. Some have documented that presence of diabetes, hypertension, dyslipidemia and coronary artery disease is associated with more rapid progression (Paltta et al., 2000; Aronow et al., 2001; Nissimihia et al., 2001), while others have not (Bahler et al., 1999; Messika-Zeitoun et al., 2007; Novaro et al., 2007). While history of diabetes, hypertension and obesity was associated with the presence of aortic valve calcification, after adjustments for age and gender; only calcification score was predictive of faster progression in a recent electron-beam CT study (Messika-Zeitoun et al., 2007). Nevertheless, dyslipidemia was related to incidence of new aortic valve calcification in that study, while renal function, vitamin D levels, endothelial function or calcium metabolism were not assessed. A small (n = 107) retrospective study (Briand et al., 2006) found that the presence of metabolic syndrome, as defined by the National Cholesterol Education Program-Adult Treatment Panel III criteria (Executive Summary of The Third Report Of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults; Adult Treatment Panel III, 2001), is associated with a faster disease progression and worse outcome in patients with at least moderate AS at the onset of the study.

Thus, the putative risk factor that has attracted the most controversy is that of dyslipidemia. There is considerable evidence for the involvement (as distinct from pivotal importance) of cholesterol and other lipoproteins in pathogenesis of AS and ASC (reviewed by Rajamannan, 2009), although not every study reported such an association (Bahler et al., 1999; Wongpraparut et al., 2002; Messika-Zeitoun et al., 2007); yet none of the prospective, randomized, placebo-controlled trials to retard the progression of AS via cholesterol lowering has been successful (Cowell et al., 2005; Rossebo et al., 2008; Chan et al., 2010). Importantly, none of these studies investigated the early stages of AS development, so the role of statins in this circumstance remains unclear.

7.2. Severe/symptomatic aortic valve stenosis

Ross and Braunwald (1968) were among the first to describe the natural history of untreated AS, as a slow, progressive disease, spanning decades. They identified the appearance of the three hallmark symptoms of advanced AS: angina pectoris, syncope and dyspnoea, and linked these to worsened outcome. This was further supported in a later study by Turina et al. (1987). However, these studies were primarily designed to demonstrate prognostic impact of valve replacement and were non-randomized. Even more importantly, the average ages of the patients in these cohorts at time of clinical presentation were 48 and 45 years respectively (Ross & Braunwald, 1968; Turina et al., 1987). Therefore it is likely that many of these cases were of AS superimposed on bicuspid valve, where the natural history is of presentation at a younger age than with tricuspid aortic valves, secondary to relatively rapid disease progression (Siu & Silversides, 2010).

More recently, a study of 123 patients, with a mean follow-up period of 2.5 years (Otto et al., 1997) stratified the relationship between peak aortic transvalvular jet velocity and evidence of symptoms/coronary events. The predominant etiology of AS in this study was calcified. However, 29% of subjects had rheumatic and bicuspid etiologies. Although risk factors for progression could not be determined from this study, baseline jet velocity, functional status of the patients and rate of change of jet velocity were predictive of clinical outcome (death or valve replacement) (Otto et al., 1997). However, it is clear that a study of this type carries an obvious inherent bias, unless intervention is based purely on severity of symptoms.

For patients with transvalvular jet velocities on Doppler echocardiography greater than 4 m/s at entry into the study, the likelihood of remaining alive without valve replacement at 2 years was only 21 ± 18%. Interestingly, age, gender, cause of aortic stenosis, morbid disease (hypertension, renal disease, diabetes), smoking history, or coexisting coronary artery disease were not correlates of this outcome (Otto et al., 1997).

The main predictors of outcome derived from echocardiography are peak aortic jet velocity, rapid increase in peak velocity and moderate to severe calcification (Otto et al., 1997; Rosenhek et al., 2000). In the study by Rosenhek et al. (2000), the combination of moderate to severe calcification and rapid increase in peak velocity identified 79% of patients who either underwent surgery or became symptomatic within 2 years. An abnormal exercise capacity was also identified as a strong predictor of poor outcome: the probability of a patient with a positive stress test surviving event free after 24 months was only 19% compared with 85% in those with a negative test (Amato et al., 2001).

From a pharmacological/therapeutic point of view, therefore, these observations provide little in the way of incremental understanding: severe valve obstruction predicts emergence of symptoms, which in turn predict adverse outcomes in the absence of relief of obstruction. Pharmacological strategies for preoperative stabilization of high-risk patients, and for management of inoperable patients have been described earlier (Section 2.2).

Another phenomenon associated with advanced AS is modification and proteolysis of von Willebrand’s factor via shear stress, leading to the loss of the largest multimers of von Willebrand factor acquired type 2A von Willebrand syndrome (Warkentin et al., 1992; Veyeradier et al., 2001). This leads to increased bleeding risk, particularly that due to gastrointestinal angiodysplasia designated Heyde’s syndrome (King et al., 1987). This anomaly is corrected by AVR, provided prosthesis size is adequate (Vincentelli et al., 2003).

8. Pathophysiological “commonalities” between early aortic valve stenosis (aortic valve sclerosis) and coronary risk

Historically the presence of ASC, usually diagnosed by incidentally finding an ejection systolic murmur, was thought to be “innocent”, apart from the uncertain chance of its progression to AS. In 1999, 2 large studies have found that patients with ASC had higher incidence of cardiovascular events (Aronow et al., 1999; Otto et al., 1999). Specifically, Aronow et al. (1999) in a cohort of over 2000 patients, found that subjects with ASC had 1.8 times higher rates of developing new coronary events than those without ASC, and in patients with pre-existent symptomatic coronary artery disease (CAD) the presence of ASC implied almost a 3-fold increase in risk. Furthermore, 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).

These results have also been replicated in a very recent study of over 2000 patients with aortic valve calcification: both all-cause and cardiovascular mortalities were higher in those with ASC over an 8.6 year follow-up period (Volzke et al., 2010). Furthermore, each unit increase in sclerosis score was associated with further increase in mortality. Olsen et al. (2005) in a group of 960 (mean age 55 to 80 years) hypertensive subjects with ASC, demonstrated that they had almost twice the risk for serious CV events during a mean follow-up of 60 months.

The precise mechanisms underlying increased morbidity and mortality associated with early AS and ASC have not been fully elucidated. It is certainly unlikely to be abnormalities confined to the valve tissue itself. Much more likely is that early AS/ASC are markers for more generalized process, involving multiple systems and regulatory cascades.

As previously described (Sections 5.2 and 6), both ASC and AS are associated with impairment of vascular endothelial function and of platelet NO signaling, as well as with inflammatory activation/generalized redox stress. We postulate that the predominant basis for the observed link between ASC and coronary event risk is its role as a marker of these physiological anomalies, not via any “direct” relationship. As regards the therapeutic implications of the presence of ASC, no studies to date have evaluated whether the presence of ASC per se might be utilized as a specific basis for interventions, such as with ACE inhibitors, to reduce the risk of coronary events.  

8.1. Links with atherosclerosis

Another possibility is that ASC is simply a marker of vascular atherogenesis. While atheroma-like histological changes are well documented in aortic valve leaflets even at the early stages of disease (Otto et al., 1994), the evidence that these parallel findings in vessels is limited. The majority of studies find at most 50% concordance between the presence of AS and CAD (Otto & O’Brien, 2001). Furthermore, risk factors for atherosclerosis and for AS/ASC are clearly not identical (Owens & Otto, 2009). It may be that the link between the two is via inflammatory activation, which has been shown to be present in AS/ASC valve lesions (Freeman & Otto, 2005) and also known to promote and destabilize coronary atherosclerosis (Burke et al., 2009). This is supported by Chandra et al. (2004), in a study suggesting that ASC is not a predictor of cardiovascular outcomes but rather a marker of the presence of CAD and inflammation. However, a more recent study failed to demonstrate any link between CRP and progression or new development of AS/ASC in a large cohort (Novaro et al., 2007).


9.1. Statins

Statins were the first commercially available drugs studied in AS given their established benefit in primary and secondary prevention of cardiovascular diseases, as well as evidence of association of lipid
infiltration of aortic valve in AS (Otto et al., 1994; O’Brien et al., 1996; Olsson et al., 1999). Indeed, histopathologic studies have demonstrated that development and progression of calcific AS are based on an active process that shares some similarities with atherosclerosis. It has been 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; Chen et al., 2003). Inflammation is a prominent feature of early aortic valve lesions. Macrophages and T-lymphocytes have been detected predominantly near the surface of the lesion, in areas of subendothelial thickening (Olsson et al., 1994).

9.1.1. Animal models
The anti-inflammatory and anti-calcific effects of statins have also been studied in cell culture and animal models of AS. Osman et al. (2006a, 2006b) demonstrated that in a human interstitial cell culture model of aortic valve mineralization, atorvastatin reduced activity and expression, not only of TGF-β1, but also of TGF-β3 and two bone morphogenenic proteins. Additionally, in a similar cell culture model, Osman et al. (2006a, 2006b) 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 decreased activation of P2Y receptors. Therefore, these findings suggested several potentially different anti-calcific mechanisms for atorvastatin in vitro.

Dietary cholesterol supplementation and genetic modification to induce severe hypercholesterolemia in animals both result in abnormal aortic valve lesions (Rajamannan et al., 2001; Cimini et al., 2005; Tanaka et al., 2005; Drolet et al., 2006). Many of these models recapitulate a number of histological features of early AS (for example, Rajamannan et al., 2005), and indeed in one model, statin therapy recapitulate a number of histological features of early AS (Novaro et al., 2001; Wallby et al., 2002; Kaden et al., 2005a). In conjunction with leukocyte and macrophage accumulation, lipids and oxidized lipids are also found in the early aortic valve lesions. Apolipoproteins (apo) B, apo (a), apoE (O’Brien et al., 1996) have been shown to be co-localized with macrophages and increased oxidized LDL score was shown to correlate with TNF-α expression in explanted human stenotic valves (Mohy et al., 2008) and with increased tissue remodeling score (Warren & Yong, 1997).

Oxidative modification of resident LDLS has also been demonstrated in early stenotic aortic valve lesions (Olsson et al., 1999).

9.1.2. Clinical studies
There have been a number of encouraging retrospective evaluations of statin use in AS, suggesting an average of 50% reduction in the rate of progression of AS with statin use (Aronow et al., 2001; Novaro et al., 2001; Bélamy et al., 2002; Shavelle et al., 2002; Rosenhek et al., 2004). However, only 2 of these studies (Aronow et al., 2001; Novaro et al., 2001) found an association between cholesterol level and progression, implying that pleiotropic effects of statins may be important, rather their effects on cholesterol homeostasis per se.

Although retrospective studies of statin therapy were encouraging, randomized controlled trials have failed to support the hypothesis that lipid lowering would slow or halt the progression of aortic stenosis. Only one small (n=121) prospective open-label non-randomized observational study of rosuvastatin in patients with moderate AS (RAAVE: Rosuvastatin Affecting Aortic Valve Endothelium) showed slowing of hemodynamic progression of AS (Moura et al., 2007): in this study only patients who otherwise met the NCEP/ATPIII criteria (Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults; Adult Treatment Panel III, 2001) for statin use were started on rosuvastatin 20 mg/day, while those who did not meet the criteria were only observed with a mean follow-up of only 73 weeks.

However, 3 large prospective double-blind randomized placebo-controlled trials of statins in AS failed to show any retardation of AS progression. The first of these (SALTIRE: Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression), failed to show any changes in AS progression with atorvastatin 80 mg/day versus placebo in 155 patients followed up for 2 years (Cowell et al., 2005). Although SALTIRE study was relatively small, potential for major type II error was quite limited. Nevertheless further studies followed.

The next, and the largest, of the prospective double-blind randomized placebo control trials (SEAS: Simvastatin and Ezetimibe in Aortic Stenosis), included almost 1900 subjects with moderate AS randomized to a combination of simvastatin and ezetimibe or placebo (Rössebo et al., 2008). Over a median follow-up of 52 months there was no difference in the primary outcome (need for aortic valve replacement) between treatment and placebo groups.

Finally, a recent prospective double-blind randomized placebo control trial (ASTRONOMER: Aortic Stenosis Progression Observation: Measuring the Effects of Rosuvastatin) of 269 subjects randomized to receive either placebo or rosuvastatin 40 mg daily showed no reduction in AS progression over a median follow up of 3.5 years (Chan et al., 2010). It should also be stated that neither SALTIRE, SEAS nor ASTRONOMER data showed any trend towards treatment benefit (Table 1).

It should therefore be argued that lowering total cholesterol, although beneficial in patients with CAD, does not influence progression of AS. These results are therefore substantially concordant with the available animal data. The question that therefore arises is whether any attractive alternative treatments are available.

9.2. Angiotensin II inhibition
9.2.1. Animal models
Inhibition of the renin–angiotensin pathway with the angiotensin receptor-1 blocker olmesartan in cholesterol-fed rabbits was
associated with decreased macrophage infiltration and reductions in osteopontin and ACE in aortic valves (Arishiro et al., 2007). This was also associated with preservation of endothelial integrity and inhibition of the trans-differentiation of valvular fibroblasts into myofibroblasts. However, no hemodynamic measures of AS progression were performed.

In our New Zealand White rabbit model of AS (Ngo et al., 2008), induced by an 8 week course of vitamin D₃, we have recently demonstrated that co-treatment with ramipril retarded the development of vitamin D₃-induced AS in the rabbit model, as measured by reduction both in transvalvular velocity and aortic valve backscatter (AVBS), a measure of aortic valve structural heterogeneity (Ngo et al., 2010).

These reductions in AVBS scores correlated with reduction in valvular calcium infiltration (Alizarin red S staining) and macrophage infiltration (RAM-11 staining). This retardation of AS development was associated with a reduction in TXNIP accumulation within the valve matrix, and preservation of vascular endothelial function, as assessed both physiologically (acetylcholine responses) and biochemically (ADMA concentrations). These findings therefore represent the first definitive demonstration that ACE inhibitors may retard the development of AS, whether measured hemodynamically, histologically, physiologically or biochemically.

9.3.2. Bisphosphonates

The potential nexus between determinants of bone formation and development of AS has been discussed in Section 4.4. This has led to evaluation of the possibility of overlapping therapeutic options. Bisphosphonates, a class of medications used for treatment of osteoporosis in humans, bind to hydroxyapatite crystals (Drake et al., 2008). These are a common form of calcium deposition within aortic valves (Mohler et al., 1999) as well as the principal mineral within bone. They inhibit bone resorption as they cause osteoclast apoptosis (Plotkin et al., 1999; Plotkin et al., 2005). Bisphosphonates also work by inhibiting an enzyme in the mevalonate pathway (cholesterol synthesis), which causes abnormalities in the cytoskeleton in the osteoclast, thus reducing bone resorption (Rodan & Fleisch, 1996). This inhibition of cholesterol synthesis may also impart some anti-inflammatory properties. Thus, bisphosphonates may directly reduce valvular calcification via their osteoblast action, as well as indirectly via inhibition of inflammation and resultant fibrosis. A study in a rat model of dialysis suggested that etidronate, a now rarely used bisphosphate, limited aortic calcification (Tamura et al., 2007).

In a small retrospective (n=55) study of patients with mild-to-moderate AS, bisphosphonate treatment was significantly associated with a reduced rate of AS progression (Skolnick et al., 2009). Even more recently, a retrospective echocardiography-based study of 76 subjects demonstrated slower reduction in AVA in patients treated with bisphosphonates (Innasimuthu & Katz, 2010). However, the same study failed to demonstrate a significant amelioration of increase in aortic transvalvular gradient, another measure of AS severity/progression. This places some doubt on the overall significance of this study. Nevertheless, evaluation of the effects of bisphosphonates on a prospective basis seems worthwhile.

9.3.3. Vitamin K cycle “preservation”

As previously mentioned, vitamin K antagonists such as warfarin have been associated with a procalcific effect, including acceleration of AS progression (Ing et al., 2009; Yamamoto et al., 2010), which is mediated by failure of γ-carboxylative activation of matrix GLA protein via the vitamin K cycle (Schurgers et al., 2008). The therapeutic implications of this finding as regards AS are substantial:

1. A number of new oral anticoagulants, such as prothrombin inhibitor dabigatran and various factor Xa inhibitors, do not affect the vitamin K cycle and therefore may be preferable for patients with early AS.
2. Apart from warfarin, a number of other agents may affect the vitamin K cycle (Lopes et al., 2011): notable among these is paracetamol, which induces expression of cytochrome P450 2E1, with resultant impairment of vitamin K reductive reactivation. The concept of avoiding “vitamin K cycle toxins” other than warfarin also needs to be evaluated with regard to AS.

10. Summary

Potential areas for pharmacotherapeutics of AS include agents to stabilize patients with advanced disease, therapy to address the increased risk of ischemic events associated with both early and advanced disease and, most intriguingly, drugs to retard the progression of valve calcification and narrowing.

Greater understanding of the pathogenesis of AS has led to a focus on the relationship between impairment of the NO signaling cascade, and a pivotal role of angiotensin II and TXNIP in modulation of inflammatory activation and associated redox stress.

To date, the majority of interventional studies related to AS progression have tested the hypothesis that hypercholesterolemia is central to disease progression. Neither recent epidemiological studies, experimental work nor clinical trials support this hypothesis: atherogenesis appears to be relatively independent of valve calcification.

On the other hand, recent evidence in animal models, together with retrospective clinical analyses, offers hope that interventions to limit effects of angiotensin II on valve endothelium and matrix, restore NO signaling and limit TNXP expression, together with suppression of other osteogenic stimuli, will finally reduce the morbidity and mortality currently imposed by AS.

Conflict of interest statement

The authors declare that they have no conflict of interest as defined by the policy.

References


Veresh, Z., Racz, A., Lotz, G., & Koller, A. (2008). ADMA impairs nitric oxide-mediated arteriolar function due to increased superoxide production by angiotensin II-140


Vezzetti, A., Racz, A., Lotz, G., & Koller, A. (2008). ADMA impairs nitric oxide-mediated arteriolar function due to increased superoxide production by angiotensin II-1647


