The Role of Oxidant Stress in a Cellular Model of Aortic Valve Cell Calcification

by

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ABSTRACT

INTRODUCTION: Calcific aortic valve stenosis (AS) is associated with a significant increase in morbidity and mortality in affected individuals, especially with advancing age. However, the pathogenesis of AS has not been fully understood, in particular, the role of oxidative stress and its contribution towards the development of AS.

STUDY OBJECTIVE: The aim of the current study was to further delineate the role of redox stress, in particular as modulated by the endogenous anti-oxidant, thioredoxin (TRX) and the pro-oxidant, thioredoxin-interacting protein (TXNIP) following stimulation of cellular calcification / nodule formation induced by transforming growth factor-beta 1 (TGF-β1). In addition, the hypothesis that nitric oxide (NO) suppresses TXNIP expression in this system was also tested.

METHODS: Cultured porcine aortic valve interstitial cells (AVICs) at 90% confluence were treated with TGF-β1 (5ng/ml) or vehicle, +/- 20μM Deta-NONOate (nitric oxide donor) or 10μM SB431542 (TGF-β1 inhibitor). TRX activity was quantified using the insulin disulphide reduction method with absorbance measured at 415 nm. Experiments were conducted in triplicate, and repeated in at least 3 cultures, between cell passages 2 and 4. Nodules were counted by an observer blinded to treatments. Experiments were also conducted in parallel, whereby TXNIP was measured by immunofluorescence and subsequently underwent image analysis. Cell survival quantification was performed in all experiments in response to various treatments as described above. Results were expressed as mean ± SEM. Multiple comparisons between the effects of treatments

relative to respective controls were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. A critical $P<0.05$ was considered statistically significant.

RESULTS: TGF-β1 significantly increased calcific nodule formation compared to controls (37.19±2.67 vs. 0.33±0.12 (nodule count/well), $P<0.001$, n=4), and correspondingly decreased TRX activity (39.94±0.66 vs. 58.96±2.22 (mU/mg protein), $P<0.001$, n=4, figure 3.2) and cell survival/area (11.98±0.74 vs. 20.10±0.56 ($x10^4/cm^2$), $P<0.001$, n=4), and increased TXNIP immunofluorescence (IF) intensity/cell (17059±204 vs. 7984±423 (arbitrary units), $P<0.001$, n=3). Deta-NONOate significantly suppressed TGF-β1-induced nodule formation (9.40±1.28 vs. 37.19±2.67 (nodule count/well), $P<0.001$, n=4), and correspondingly increased TRX activity (59.21±2.49 vs. 39.94±0.66 (mU/mg protein), $P<0.001$, n=4) and cell survival/area (16.93±0.95 vs. 11.98±0.74 ($x10^4/cm^2$), $P<0.01$, n=4), and decreased TXNIP IF intensity/cell (7918±310 vs. 17059±204 (arbitrary units), $P<0.001$, n=3), compared with TGF-β1 treatment alone. SB431542 significantly decreased TGF-β1-induced nodule formation (0.42±0.25 vs. 37.19±2.67 (nodule count/well), $P<0.001$, n=4), and correspondingly increased TRX activity (59.94±1.25 vs. 39.94±0.66 (mU/mg protein), $P<0.001$, n=3) and cell survival/area (20.50±0.78 vs. 11.98±0.74 ($x10^4/cm^2$), $P<0.001$, n=4), and decreased TXNIP IF intensity/cell (7670±798 vs. 17059±204 (arbitrary units), $P<0.001$, n=3), compared with TGF-β1 treatment alone.

CONCLUSION: TGF-β1-induced aortic valve interstitial cell calcific nodule formation is related to an increase in redox stress, involving a decrease in the endogenous anti-oxidant activity of thioredoxin (TRX), with a corresponding increase in the pro-oxidant, thioredoxin-interacting protein (TXNIP). In addition, TGF-B1-induced aortic valve interstitial cell calcific nodule formation results in a decrease in cell survival. These effects are ameliorated by nitric oxide (NO).

GLOSSARY OF COMMON ABBREVIATIONS

Abbreviations	Definition
ACE	**Angiotensin-converting enzyme**
ACEIs	**Angiotensin-converting enzyme inhibitors**
ADMA	**Asymmetric dimethylarginine**
AF	**Atrial fibrillation**
ALP	**Alkaline phosphatase**
Ang	**Angiotensin**
ANOVA	**Analysis of variance**
A2RBs	**Angiotensin-2 receptor blockers**
ARIC study	**Atherosclerosis Risk in Communities study**
AS	**Aortic valve stenosis**
ASc	**Aortic valve sclerosis**
ASK-1	**Apoptosis signalling kinase-1**
ASTRONOMER substudy	**Aortic Stenosis Progression Observation Measuring Effects of Rosuvastatin substudy**
AV	**Aortic valve**
AVA	**Aortic valve area**
AVC	**Aortic valve calcium/calcification**
AVECs	**Aortic valve endothelial cells**
AVICs	**Aortic valve interstitial cells**
AVR	**Aortic valve replacement**
AV-Vel	**Peak aortic jet velocity**
BMI	**Body mass index**
BMP(s)	**Bone morphogenic protein(s)**
BSA	**Bovine serum albumin**
CAC	**Coronary artery calcification**
CAD	**Coronary artery disease**

Cbfa1	**Core-binding factor α1**
cGMP	**Cyclic guanosine monophosphate**
CHD	**Coronary heart disease**
CHS	**Cardiovascular health study**
CRF	**Chronic renal failure**
CT	**Computed tomography**
DETA-NONOate	**(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate**
DM	**Diabetes mellitus**
DMEM	**Dulbecco's modified Eagle's media**
DNA	**Deoxyribonucleic acid**
EBCT	**Electron-beam-computed-tomography**
ECs	**Endothelial cells**
EDRF	**Endothelium-derived relaxing factor**
EDTA	**Ethylenediamine tetra-acetic acid**
EF	**Ejection fraction**
eNOS	**Endothelial nitric oxide synthase**
EPCs	**Endothelial progenitor cells**
ESRD	**End-stage renal disease**
ET-1	**Endothelin-1**
FCS	**Fetal calf serum**
FZD	**Frizzled**
HCL	**Hydrochloric acid**
HDL	**High-density lipoprotein**
HEPES	**N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid**
HMG-CoA	**Hydroxymethylglutaryl coenzyme-A**
HR	**Hazard ratio**
hs CRP	**High sensitivity C-reactive protein**
5-HT	**5-hydroxytryptamine**
IF	**Immunofluorescence**

IL	**Interleukin**
KLF-2	**Kruppel-like factor-2**
LDL	**Low-density lipoprotein**
L-NAME	**N-Nitro-L-arginine-methyl ester**
Lrp	**LDL receptor-related protein**
LV	**Left ventricular**
LVEF	**Left ventricular ejection fraction**
MAP	**Mitogen-activated protein**
MESA	**Multi-Ethnic Study of Atherosclerosis**
MI	**Myocardial infarction**
MMP	**Matrix metalloproteinases**
mRNA	**Messenger Ribonucleic acid**
MS	**Metabolic syndrome**
NADPH	**Nicotinamide adenine dinucleotide phosphate**
NEP	**Neutral endopeptidase**
NFATc1	**Nuclear factor of activated T cells c1**
NF-κB	**Nuclear factor-κB**
NK	**Natural killer**
NO	**Nitric oxide**
OPG	**Osteoprotegerin**
OSP	**Osteopontin**
Osx	**Osterix**
oxLDLs	**Oxidized LDLs**
PAECs	**Porcine aortic endothelial cells**
PAVECs	**Porcine aortic valve endothelial cells**
PBS	**Phosphate-buffered solution**
PPAR-γ	**Peroxisome proliferator-activated receptor-γ**
RAA	**Renin-angiotensin-aldosterone**
RANK	**Receptor activator of nuclear factor-κB**
RANKL	**Receptor activator of nuclear factor-κB ligand**

ROS	**Reactive oxygen species**
SB431542	**4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate, 4-[4-(1,3-Benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide hydrate, 4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide hydrate**
SEAS substudy	**Simvastatin Ezetimibe in Aortic Stenosis substudy**
SEM	**Standard error of the mean**
sGC	**Soluble guanylate cyclase**
siRNA	**Single interrupting ribonucleic acid**
SMCs	**Smooth muscle cells**
SOD	**Superoxide dismutase**
SPARC	**Secreted protein, acidic and rich in cysteine/osteonectin**
TAVR	**Transcatheter aortic-valve replacement**
Tempol	**4-hydroxy-TEMPO**
TF	**Tissue factor**
TGF-β1	**Transforming growth factor-β1**
TIMPS	**Tissue inhibitors of metalloproteinases**
TLR	**Toll-like receptor**
TNF-α	**Tumour necrosis factor-α**
TRX	**Thioredoxin**
TXNIP	**Thioredoxin-interacting protein**
VCAM-1	**Vascular cell adhesion molecule-1**
VDUP1	**Vitamin D3 up-regulated protein 1**
VECs	**Vascular endothelial cells**
VEGF	**Vascular endothelial growth factor**
VICs	**Valve interstitial cells**

DECLARATION

The work within this thesis contains no material which has been accepted in its entirety for the award of any other diploma or degree in any university or 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 also give my consent to this copy of the thesis being made available for viewing, loan and photocopying at the University Library, subject to the provisions of the Copyright Act 1968. In addition, 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.

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PUBLICATIONS, PRESENTATIONS & SCHOLARSHIPS

Published abstracts related to this thesis

- **S Kanagasingam N**, Horowitz JD & Kennedy JA 2009, 'Transforming growth factor- β1 increases thioredoxin-interacting protein (TXNIP) in calcifying aortic valve cells: attenuation by nitric oxide', *Heart Lung and Circulation*, **18**, supp. 3, abstract 436, pp. S193.
- **S Kanagasingam N**, Horowitz JD & Kennedy JA 2009, 'Development of aortic valve cellular calcification is associated with intracellular redox stress: amelioration by nitric oxide', *European Journal of Heart Failure*, **8**, supp. 2, abstract 1596, pp.ii825.

Local, national and international scientific meetings/conferences whereby presentations relating to this thesis were accepted

- *Heart Failure Congress 2009* (Nice, FRANCE)
- *Cardiac Society of Australia and New Zealand (CSANZ) 2009 57th Annual Scientific Meeting* (Sydney, New South Wales, AUS)
- *The Queen Elizabeth Hospital Research Day 2009* (Adelaide, South Australia, AUS)

Scholarships related to this thesis

- The University of Adelaide Divisional Scholarship
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CHAPTER 1

INTRODUCTION

1.1 Introduction

The normal human heart consists of four major heart valves, two on the left and two on the right side of the heart. Each valve has a major role in ensuring that blood flows in the forward direction and backward leakage is prevented. One of the major heart valves is known as the aortic valve, whereby oxygenated blood flows through to the rest of the human body (Moore & Dalley 1999). Unfortunately, in some individuals, this valve may be diseased. A major disease that affects this valve is known as aortic valve stenosis (AS), which also happens to be the most common cardiac valve lesion in the United States (Carabello 2002). However, it is only in the last half century that much research has been undertaken to try and understand, and further delineate the complexities of the various mechanisms involved that result in this severely debilitating disease. In the early stage of the disease, known as aortic valve sclerosis (ASc), there is associated valve thickening and calcification, without obstruction to ventricular outflow (Lindroos et al. 1993). ASc has been found to be present in about 25% of individuals over the age of 65 years, with 16% progressing to AS within 7 years (Cosmi et al. 2002). However, even in early disease, ASc is associated with a 50% increase in risk of myocardial infarction (Stewart et al. 1997). In the case of symptomatic AS, there is an approximately 50% death rate within the first 2 years following onset of symptoms in untreated patients (Ross & Braunwald 1968; Turina et al. 1987; Kelly et al. 1988).

Only recently has it been discovered that the reason for its complexity is not just the results of “simple degenerative ageing processes”, but rather due to a vastly complicated

link between congenital, genetic, environmental (nutrition), temporal, inflammatory and molecular mechanisms (Akat, Borggrefe & Kaden 2009).

At present, aortic valve replacement (AVR) surgery is the only proven effective therapy in patients with severe AS. Individuals who undergo AVR have 10-year age corrected survival rates, which approach those of the normal population (Schwarz et al. 1982; Lindblom et al. 1990). However, about a third of patients with severe symptomatic AS do not undergo AVR due to their advanced age, left ventricular (LV) dysfunction, or associated multiple co-morbidities, that renders them at an increased risk of peri-operative morbidity and mortality (Iung et al. 2005; Bach et al. 2009). For some of these high operative-risk patients with severe AS, trans-catheter aortic valve implantation has been developed in the hope of improving survival. However, this technology is still in its infancy, with a higher mortality rate compared to AVR surgery (7.2% versus 1.4%). Additionally, percutaneously delivered valves are currently expensive, costing about US$ 30,000, compared to US$ 2,500 to US$ 6,000 for a standard aortic valve (Svensson 2008). Currently in the United States, approximately 75,000 heart valve replacements are performed annually, with the cost of each AVR surgery at some centres approximating US$ 60,000 (Wu et al. 2007).

Thus, due to the significant increase in morbidity and mortality associated with this disease, and with treatment costs spiralling in excess of billions of dollars worldwide, more work needs to be done to try and decrease, arrest or even prevent AS from ever developing. Over the next few sections, the normal aortic valve structure and function

will be discussed, then progressing on to the natural history of AS, further expanding on its pathogenesis (including biochemical and molecular mechanisms), risk factors, clinical implications, and culminating in current treatment options; all in the quest to try and understand this complex yet fascinating disease known as aortic valve stenosis.

1.2 The normal aortic valve: structure and function

1.2.1 Gross anatomy and histology of the normal aortic valve

In the vast majority of individuals, the normal aortic valve consists of three leaflets or cusps (tricuspid), which are sometimes called semi-lunar valves owing to their shape (figure 1.1, page 32). Some 1-2 % of the population have bicuspid aortic valves, with an even smaller minority having unicuspid aortic valves (Roberts & Ko 2005). Within the human body, the aortic valve is positioned obliquely and located posterior to the left sternal edge, at the level of the third intercostal space. The aortic valve opens into the ascending aorta, and just superior to it lay the aortic sinuses, from which the coronary arteries originate (Moore & Dalley 1999).

Histologically, the aortic heart valve consists of distinct tissue zones that extend from the valve base towards the distal free edge (Mulholland & Gotlieb 1997), which include the fibrosa, spongiosa and ventricularis. The fibrosa, located on the aortic side of the valve leaflet, is primarily composed of fibroblasts and collagen fibres. The ventricularis, which lies on the ventricular side of the leaflet, is composed mainly of elastic fibres. The spongiosa, which is located between the fibrosa and ventricularis, is a layer of loose connective tissue extending from the annulus to the free edge that contains fibroblasts, mesenchymal cells and has a prominent matrix consisting of proteoglycans, collagen and elastin (Mulholland & Gotlieb 1997; Taylor et al. 2003; Freeman & Otto 2005). A monolayer of endothelial cells that is continuous from the endocardial endothelium covers the entire surface of the leaflet (Andries, Sys & Brutsaert 1995).

It was previously thought that the aortic valve was avascular and receives oxygen and other nutrients by passive diffusion from the surrounding oxygen rich blood that flows through it. Weind, Ellis and Boughner (2000) showed that 71% (32/45) of cusps isolated from 15 normal porcine aortic valves contained vessels, and that these vessels were primarily located in the basal third of the cusps and extended from the commissures almost to the free edge of the valve. Thus, the presence of vessels within the aortic valve cusps provides further support that the metabolic activity of the aortic valve is greater than that allowed for by passive diffusion via the cusps' surfaces alone.

In addition to vasculature, the heart valves also have a complex neuronal network (Steele, Gibbins & Morris 1996). The ventricular endocardial plexus (Marron et al. 1996) and some fibres originating from the aortic adventitial wall that penetrates the leaflet via the raphe (Kawano et al. 1996), give rise to aortic valve innervation, which is primarily found in the ventricularis. Interestingly, nerve fibres were found to be in close proximity to CD31-positive aortic valve endothelial cells (AVECs) (Marron et al. 1996), making feasible the close regulatory interplay between the neurons, AVECs and aortic valve interstitial cells (AVICs). Also, it is now known that aortic valve neuronal modulation plays a significant role in influencing valvular mechanical properties, structure and pathophysiology. It has been suggested that there may be potential pharmacological targets resulting in neuronal modulation, which may contribute to the prevention of aortic valve calcification (El-Hamamsy, Yacoub & Chester 2009a).

1.2.2 Aortic valve interstitial cells

The valve interstitial cells (VICs), which are the most prevalent cells found in heart valves, are thought to play a major role in not just maintaining valvular function, but are also involved in pathological disease. When VICs are cultured, two cell morphologies have been observed. These include small islands of cuboidal cells present in low densities, and elongated spindle shaped cells. The vast majority of cultures were overgrown by elongated cells, few showed a mixed pattern (cobblestone and elongated cells), but in about 20% of cultures, there was spontaneous selection of cobblestone cells that formed confluent monolayers (Mulholland & Gotlieb 1997).

Currently, 4 phenotypes of VICs have been identified. One type are the myofibroblasts that are thought to be involved in proliferation and migration, have prominent stress fibres, and are associated with smooth muscle α-actin expression. Another subtype is characterized by prominent synthetic and secretory organelles important in collagen synthesis, and is thought to be important in matrix regulation. The third phenotype identified constitutes smooth muscle cells (Taylor et al. 2003). In a recent study, Chen et al. (2009) identified high frequencies of mesenchymal progenitors and osteoprogenitors (subpopulation) following primary culture of porcine AVICs. These mesenchymal progenitors were able to differentiate to the osteogenic, adipogenic, chondrogenic, and myofibroblast lineages. As mentioned, these cells contained a large subpopulation of morphologically distinct cells (osteoprogenitors) that were able to self renew, and elaborate bone matrix from single cells. This would suggest a strong possibility of osteoprogenitors contributing towards the calcification of aortic valves.

1.2.3 Important cellular functions of aortic valve interstitial cells

The VICs are important in not just maintaining the structural integrity of the valve (Taylor et al. 2003) but are also involved in extracellular matrix (ECM) metabolism. As mentioned earlier, these spindle-shaped mesenchymal cells secrete a range of ECM components, which include collagen, chondroitin sulphate, fibronectin and prolyl-4-hydroxylase (Messier et al. 1994). These VICs have also been shown to express matrix metalloproteinases (MMPs) which are involved in degrading the ECM, and their tissue inhibitors (TIMPs) (Soini et al. 2001). In addition, the VICs appear to modulate the immune response by being less immunogenic compared to valve endothelial cells (Batten et al. 2001). Along with being involved in secretion of various cytokines, chemokines and growth factors, VICs also respond in a similar fashion to vascular smooth muscle cells when stimulated by various vasoactive molecules. Contraction of VICs is induced by serotonin (5-hydroxytryptamine [5-HT]), catecholamines, endothelin-1 (ET-1) and histamine, whereas nitric oxide (NO) induces VIC relaxation (Filip et al. 1986; Chester et al. 2000; Kershaw et al. 2004). VICs have also been shown to play a significant role in cell-cell communication (Mulholland & Gotlieb 1996), thus likely influencing cellular proliferation, differentiation and migration. In addition, the communication between human VICs has been shown to mediate cell-cell adhesion via N-cadherin, connexin-26 and -43, and desmoglein (Latif et al. 2006). In general, the VICs within the normal adult valves are essentially quiescent, maintaining baseline levels of homeostatic ECM gene expression, and exhibit little or no cell proliferation (Aikawa et al. 2006). As mentioned earlier, the recently identified mesenchymal

progenitors within aortic valves are able to differentiate into a variety of cell lineages, and likely contribute to maintaining valve cellular homeostasis.

Recent evidence has revealed that many of the interstitial cells within native heart leaflets are likely to be myofibroblasts, which display characteristics of both fibroblasts and smooth muscle cells (SMCs). These cells express genes encoding structural components of the cardiac and skeletal contractile apparatus, express smooth muscle α-actin and have features similar to SMCs, are involved in synthesis of matrix proteins and collagen, and are also able to contract when immobilized within a collagen gel matrix (Messier et al. 1994; Roy, Brand & Yacoub 2000; Taylor et al. 2003). Other studies (Powell et al. 1999; Walker, Guerrero & Leinwand 2001; Tomasek et al. 2002) have suggested that myofibroblasts are 'hyper-activated' fibroblasts that are involved in tissue remodelling, wound healing and play an important role in fibrotic disease. In particular, transforming growth factor-β1 (TGF-β1) has been shown to cause myofibroblast activation (Walker et al. 2004), and is a major player in valvular heart disease, especially contributing towards the development of AS. This will be further elaborated in the later parts of this chapter. Selected functions of VICs are summarised in Table 1.1.

Table 1.1 Summary of selected functions of valve interstitial cells (VICs)	
Secretion of cytokines, chemokines and growth factors	Plays an important role in cell-cell adhesion and communication
Important role in maintaining valvular integrity	Regulates other VICs to differentiate, migrate and proliferate
Subpopulations of VICs able to differentiate into various cell lineages	Able to undergo relaxation or contraction following stimulation by various vasoactive compounds
Able to secrete several extracellular matrix components	Involved in tissue remodelling and fibrotic disease

1.2.4 Aortic valve endothelial cells and their contribution towards valvular function

In the developing human heart, cells from the endocardial cushions give rise to the aortic valve endothelium (Moore & Persaud 1998). This endothelium consists of a monolayer of cells that covers the entire surface of all the valve leaflets, and is continuous with the endocardial endothelium of the heart. Unlike the AVICs and other VICs that have been studied quite extensively, there is little known about aortic valve endothelial cells (AVECs), and their relative contributions towards maintaining normal valvular structure and function.

The vascular endothelium has been quite extensively studied with thousands of publications to date, and it has been well described that vascular smooth muscle tone and vessel compliance are closely regulated by the vascular endothelium via its various mediators, such as nitric oxide (NO) and endothelin-1 (ET-1). NO is synthesized by the enzyme endothelial nitric oxide synthase (eNOS), and is released by endothelial cells, resulting in vasodilatation. Thus, it plays a major role in regulating blood flow and pressure (Moncada & Higgs 2006). However, unlike the vascular endothelium, very little is still known about the intrinsic role of the valvular endothelium, and its possible contribution towards maintaining structure and proper functioning of the heart valves. Under shear stress, valvular endothelial cells align perpendicularly to the direction of flow, whereas the vascular endothelial cells align in a parallel fashion (Butcher et al. 2004). A study (Butcher et al. 2006) that compared porcine aortic endothelial cells (PAECs) to porcine aortic valve endothelial cells (PAVECs) under the influence of shear stress revealed that more than 400 genes were significantly differentially expressed in

both groups. In addition, the study also found that PAVECs were less intrinsically inflammatory compared to PAECs, but both expressed similar antioxidant and anti-inflammatory genes under the conditions of shear stress. Of particular interest was the finding that PAVECs expressed more genes associated with chondrogenesis, whereas PAECs expressed osteogenic genes. This adds further evidence that the endothelial cells from these two groups (valvular and vascular) have some unique and distinct features.

Interestingly, a recent study (Weinberg et al. 2010) has revealed that the differences in blood flow between the aortic and ventricular surfaces of the aortic valve have resulted in significantly distinct endothelial phenotypes in vitro. The aortic side is subjected to more turbulent circulatory flow, whereas the ventricular side experiences more unidirectional flow with minimal turbulence (Butcher et al. 2006).

El-Hamamsy et al. (2009b) has revealed that the porcine aortic valve endothelium plays an important role in regulating the mechanical properties of aortic valve cusps. In this study, serotonin induced a significant decrease in areal stiffness of the aortic cusp, which was reversed by the nitric oxide synthase inhibitor, N-Nitro-L-arginine-methyl ester (L-NAME), or endothelial denudation. Serotonin has been shown to induce NO release from the coronary endothelium (Tschudi et al. 1991), resulting in vasodilatation. In the case of this study (El-Hamamsy et al. 2009b), NO release resulted in valve cusp relaxation. However, when the endothelium was removed or eNOS inhibited, the unopposed effects of serotonin on the VICs resulted in valve contraction. Thus, it is very

likely that a healthy valvular endothelium is important in not just maintaining valve cellular homeostasis, but also in ensuring proper functioning of the aortic valve.

1.2.5 Function of the normal aortic valve

The aortic valve opens and closes over 3 billion times during the average life cycle, and has the ability to allow between 1 to over 20 litres of blood per minute to flow through it during times of rest, exercise or pathological disease (Misfeld & Sievers 2007). As discussed previously, the primary function of the aortic valve is to prevent retrograde blood flow back into the left ventricle during ventricular filling or diastole (see figure 1.1). This intrinsically fine-tuned opening of the aortic valve is important in ensuring unobstructed laminar blood flow from the left ventricle with a concurrent decrease in ventricular workload during systole (Higashidate et al. 1995; Yacoub et al. 1999). In addition, it is predominantly during diastole that coronary perfusion occurs, whereby the pressure from the elastic recoil of the aorta is transmitted onto the properly closed aortic valve cusps creating vortices in the sinuses of Valsalva (Davies et al. 2008). Thus, when the valve is affected by severe pathological disease, its function is significantly impaired, often resulting in devastating consequences.

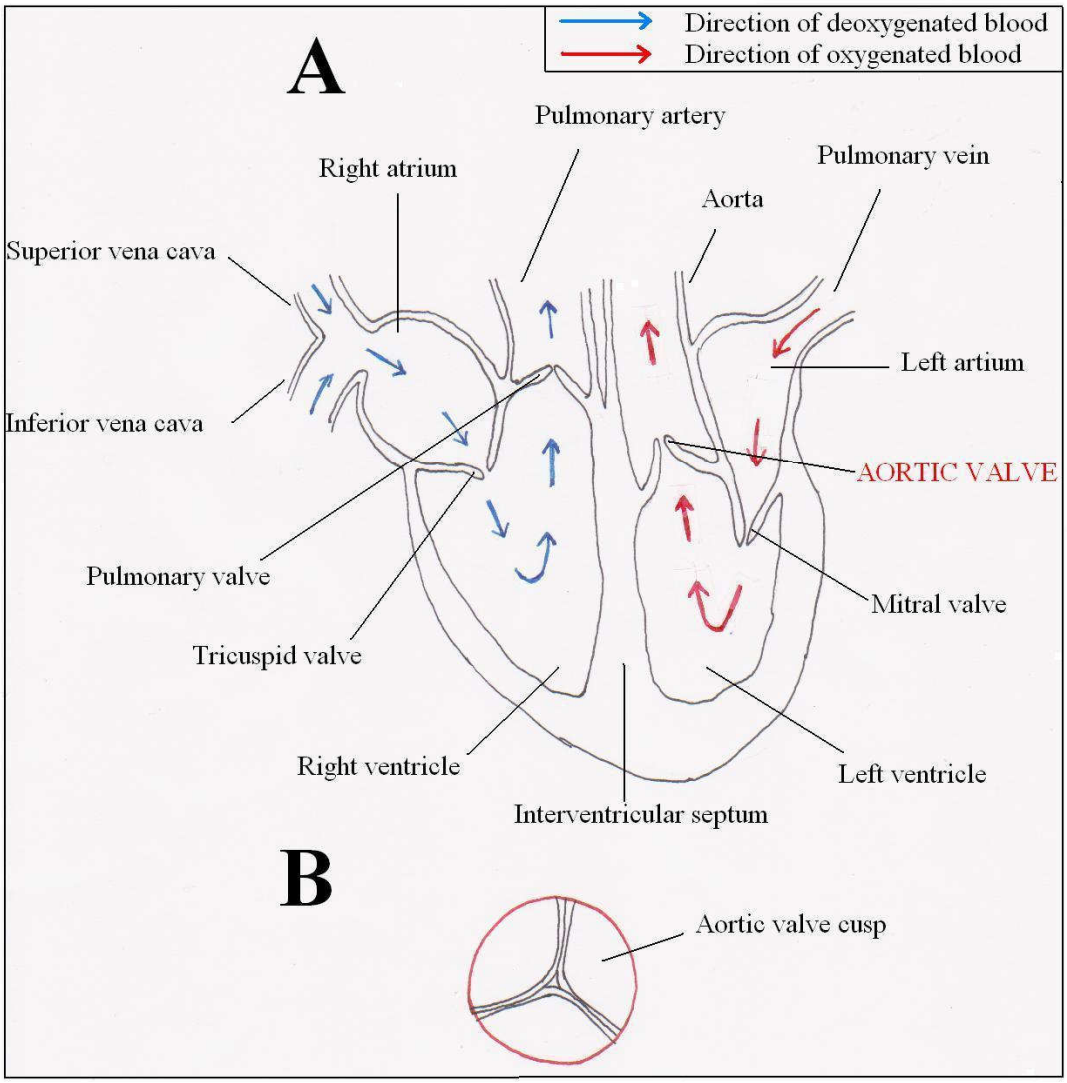

Figure 1.1 (A) Schematic drawing of the heart showing selected anatomical structures, including the aortic valve. The direction of blood flow is also shown in the diagram (see key above).
(B) Drawing of a cross-sectional/superior view of the tricuspid aortic valve

1.3 Aortic valve stenosis: a worldwide problem and growing

1.3.1 Epidemiology and causes of aortic valve stenosis

Aortic valve stenosis (AS) has become the most prevalent valvular heart lesion, not just in the United States (Carabello 2002), but also in the rest of the developed world (Carabello & Paulus 2009). In the early stage of disease, known as aortic valve sclerosis (ASc), there is calcification and thickening of the aortic valve leaflets without any haemodynamically significant ventricular outflow obstruction. A major prospective longitudinal study of 5201 randomly selected individuals, known as the Cardiovascular Health Study (CHS) (Stewart et al. 1997), revealed that ASc was very common in the elderly and increased with age. The study revealed the prevalence of ASc in the different age groups of 65-74, 75-84 and 84+ year olds to be 20%, 35% and 48% respectively. The overall prevalence of ASc and AS in the entire cohort (individuals aged 65 years and above) were 26% and 2% respectively. It was also noted that when subjects were separated into groups of increasing age (65-74, 75-84, 84+), the prevalence of AS increased significantly to 1.3%, 2.4% and 4% respectively. The CHS (Otto et al. 1999) underwent further recruitment increasing its total subjects to 5621, and the overall prevalence of ASc in this cohort rose to 29%. Interestingly, being male was also a major clinical factor associated with ASc and AS. Another major study by Cosmi et al. (2002) involving 2131 patients with ASc revealed that 15.9% of the cohort went on to develop AS within approximately 7-8 years (mild, 10.9%; moderate, 2.9%; and severe, 2.5%). The same study also showed that among individuals with normal aortic valves, only 1% went on to develop mild AS.

An earlier landmark study, The Helsinki Ageing Study (Lindroos et al. 1993), found that some degree of aortic valve calcification was found in 53% of its study population aged 55 years and above, with 40% showing mild calcification and 13% showing severe calcification. More concerning was the finding that 75% of people aged 85 to 86 years had some degree of calcification. The study revealed a 5% prevalence of at least moderate AS, and a 2.9% prevalence of critical AS among the individuals aged between 75 and 86 years. When different age groups were considered, there was a notable increase in the prevalence of critical AS from 1 to 2% in persons aged 75 to 76 years, reaching nearly 6% in those aged 85 to 86 years. Thus, the study clearly illustrated a dramatic increase in calcification and prevalence of critical AS with progressing age. Surprisingly, the study found no cases of AS between the ages of 55 and 71 years. In another study, African-Americans between the ages of 65 to 74 years were found to have an 18.6% prevalence of ASc (Taylor et al. 2005).

There are several known conditions or diseases that predispose to the development of AS, some of which have become less relevant in today's developed world, while others are increasing in significance. Some of these conditions will be discussed in more detail in the paragraphs that follow.

Rheumatic heart disease tends to affect the aortic valve in 40 to 45% of cases, often associated with mitral valve disease. It results in progressive thickening, fusion and calcification of the commissures and cusps of the aortic valve (Zipes et al. 2005).

However, with the decline of rheumatic fever in industrialised nations, rheumatic AS has become a very rare cause of AS (Roberts 1970).

Rarely, AS also tends to develop secondarily to other disease processes or treatments, which include *Paget's bone disease, homozygous familial hypercholesterolemia, renal failure* and *radiotherapy to the chest.*

Congenital aortic stenosis is a rare cause of AS. The congenital malformations of the aortic valve may be unicuspid, bicuspid and sometime tricuspid. The most frequent malformation found in fatal valvular AS in children younger than 1 year of age is a stenosed unicuspid aortic valve, which is often associated with severe obstruction to LV outflow (Zipes et al. 2005; Carabello & Paulus 2009). Congenital bicuspid aortic valves may be stenotic with some associated commissural fusion at birth. However, these stenotic valves rarely cause serious ventricular outflow obstruction until early adulthood, whereby the chronically induced turbulent blood flow resultant from their structure, likely leads to progressive trauma, fibrosis and calcification of the aortic valve leaflets, eventuating in further narrowing of the aortic orifice during adulthood. The congenital tricuspid aortic valves often have cusps of unequal size and thickness, with some commissural fusion, usually causing some degree of mild aortic orificial narrowing. The mechanism of progressive narrowing or stenosis is likely similar to that of congenitally stenotic bicuspid aortic valves, though many of these tricuspid valves tend to have preserved function throughout life (Zipes et al. 2005).

Calcific aortic valve stenosis (which includes calcification and stenosis of bicuspid and tricuspid aortic valves) is by far the most common cause of AS in adults, and the most frequent reason for aortic valve replacement (AVR) (Rajamannan, Gersh & Bonow 2003a). In the past, calcific AS was assumed to be a simple degenerative process that occurred with advancing age. Over recent decades, it is now known that the development of calcific AS is a complex process involving endothelial disruption, oxidative stress and inflammation, and associated active cellular morphological changes leading to calcification, fibrosis and bone formation of the aortic valve leaflets (Chandra et al. 2004; Freeman & Otto 2005; Liberman et al. 2008; Akat, Borggrefe & Kaden 2009; Kennedy et al. 2009). In addition, 'traditional' risk factors (elevated serum low-density lipoprotein (LDL), smoking, history of hypertension, diabetes, age, male gender, body mass index) leading to vascular atherosclerosis have also been found to be involved in the development and progression of calcific AS (Deutscher, Rockette & Krishnaswami 1984; Aronow, Schwartz & Koenigsberg 1987; Lindroos et al. 1994; Stewart et al. 1997; Aronow et al. 2001; Nassimiha et al. 2001; Peltier et al. 2003; Chandra et al. 2004; Messika-Zeitoun et al. 2007; Novaro et al. 2007; Thanassoulis et al. 2010). Importantly, approximately 1-2 % of babies are born with bicuspid aortic valves (Alpert 2003), and interestingly, a major study revealed a prevalence of 7.1 per 1000 in newborn males versus 1.9 per 1000 in newborn females (Tutar et al. 2005). Unlike some congenitally stenotic bicuspid aortic valves that are present at birth, many congenital bicuspid aortic valves are not stenosed at birth, and thus do not exhibit any significant aortic orificial narrowing. However, due to their particular malformation or architecture, there is likely more turbulent blood flow that persists over time, leading to AS

developing sooner. It is important to note that individuals with symptomatic bicuspid AS tend to present around 2 decades earlier (usually in their late 40s to mid 60s), compared to those with trileaflet AS who tend to present in their mid to late 70s (Alpert 2003; Carabello & Paulus 2009; Siu & Silversides 2010). This has been supported in a previous study that showed a rapid progression of ASc and AS in individuals with congenital bicuspid aortic valves (Beppu et al. 1993). An important study by Roberts and Ko (2005) revealed that of 932 patients between the ages of 26 and 91 who underwent AVR surgery for AS, 49% of them had stenotic bicuspid aortic valves excised compared to 46% who had stenotic tricuspid aortic valves. Especially interesting was the finding that 59% of men who underwent isolated AVR for AS had congenitally malformed aortic valves (unicuspid or bicuspid), compared to 40% who had tricuspid aortic valves. The findings however were different for women whereby 46% had congenitally malformed aortic valves (unicuspid or bicuspid) compared to 53% who had tricuspid aortic valves.

1.3.2 Aortic valve sclerosis: Progression and long-term outcome

ASc has often been thought of as an early 'innocent' pathological state, eventually leading to the development of AS. An estimated 26 to 29% of people aged 65 years and above have ASc, with an alarming 48% of those older than 84 years having the disease (Stewart et al. 1997; Otto et al 1999). ASc may not exhibit any significant obstruction to LV outflow, but it is far from being an innocent bystander. As Nightingale and Horowitz (2005) summarized, ASc is not only a potential precursor to clinically significant ventricular outflow obstruction, but carries a significantly increased risk of

cardiovascular events. In this review, it was also noted that investigational techniques used to assess the degree and progression of ASc was suboptimal and subjective at best, and thus they proposed an improved modality to assess for ASc and its progression utilising tissue quantification (backscatter) echocardiography (Ngo et al. 2004).

As previously mentioned, a major echocardiographic study by Cosmi et al. (2002) showed that approximately 16% of individuals with ASc went on to develop AS, with 2.5% developing severe AS. In another study involving 400 patients with ASc, there was a doubling of progression whereby 32.75% (131/400) went on to develop AS (25% mild, 5.25% moderate, 2.5% severe) during a mean follow-up of 4 years (Faggiano et al 2003). However, as was discussed earlier, this is not the only negative consequence of ASc. The Cardiovascular Health Study revealed that individuals with ASc had a 50% increase in their risk of cardiovascular death despite having no pre-existing diagnosis of coronary artery disease at study entry, and a 40% increase in their risk of myocardial infarction (MI) (after adjusting for age, sex and factors associated with ASc). There was also a moderate 28% increase in the risk of developing congestive cardiac failure, and a non-significant trend towards an increased risk of developing angina pectoris and stroke (Otto et al. 1999). One study (Chandra et al. 2004) found that of the 415 (425 subjects were recruited initially but 10 were later excluded) patients who presented to the emergency room with chest pain, 49% of them had ASc. Although on multivariate analysis of the data from this study, ASc was not found to be an independent predictor of adverse cardiovascular outcomes. One study however revealed that there was an 80% increase in the risk of developing a new coronary event in elderly patients with ASc,

compared to those without ASc (Aronow et al. 1999). Another major study (Taylor et al. 2005) involving 2,279 middle-aged African-Americans enrolled in the Jackson Mississippi Atherosclerosis Risk in Communities (ARIC) study cohort had a 3.8-fold increased risk of developing an incident first MI or fatal coronary heart disease when ASc was present, compared to those without ASc (following adjustments for multiple risk factors, including markers of inflammation). Interestingly, it was observed that when ASc was associated with high levels of serum inflammatory markers (fibrinogen and von Willebrand Factor), there was a greater than 5-fold increased risk of incident MI or fatal coronary heart disease.

In a selected group of 960 hypertensive participants (Olsen et al. 2005), 40.4% were found to have ASc, 58% had no aortic valvular disease, and 1.6% were found to have AS. The prevalence of ASc was much higher in this group compared to the 26 to 29% observed in other studies (Stewart et al 1997; Otto et al. 1999). Furthermore, participants with ASc in this study (Olsen et al. 2005) had a thicker ventricular wall, greater LV mass, greater LV index and a greater frequency of LV hypertrophy, compared to participants without ASc. In addition, the study also found ASc to be a significant independent risk factor in predicting the composite cardiovascular endpoint (cardiovascular death, fatal and non-fatal MI, or fatal and non-fatal stroke). Thus, despite the fact that ASc does not cause any clinically or haemodynamically significant obstruction to LV outflow, it is however associated, or possibly results in some pathological changes to the myocardium, albeit modest especially in the early stages.

Messika-Zeitoun et al. (2007) assessed aortic valve calcification (AVC) and coronary artery calcification (CAC) utilising Electron-beam computed tomography (EBCT), and found 27% of their subjects aged 60 and above to have AVC. This was comparable to the rates found in previous studies (Stewart et al. 1997; Otto et al. 1999). However, it must be stated that the authors in this study (Messika-Zeitoun et al. 2007) have taken AVC to be similar to ASc, which may render extrapolation of some results difficult. The study revealed that AVC was frequent with aging, associated with atherosclerotic risk factors and was a marker of sub-clinical coronary artery disease. They also found that after 4 years of follow-up, there was AVC progression in 28% of individuals with baseline AVC, compared to only 10% progression in those without AVC at baseline. The rate of progression was also noted to be faster in those with pre-existing AVC.

A recently published study by Owens et al. (2010) involving 5,880 participants aged 45 to 84 years in the Multi-Ethnic Study of Atherosclerosis (MESA), revealed that aortic valve calcium (AVC) increased significantly with age, as measured by computed tomography. Of the 5,880 participants, 87% (5,142 participants) did not have AVC at baseline. However, during the mean follow-up of 2.4 ± 0.9 years, 4.1% of those 5,142 participants went on to develop AVC. In addition, the study found a marked increase in the AVC incidence rate in older age groups, whereby there was a six-fold greater rate of incident AVC in subjects aged 70 to 79 years, compared to subjects aged 50 to 54 years (3.5% /year versus 0.6% /year). In keeping with previous studies (Chan et al. 2003), the study by Owens et al. (2010) also found that incident AVC risk was associated with many of the ‘traditional’ cardiovascular or atherosclerotic risk factors.

In summary, it is obvious that ASc, which is characterized by calcification and thickening of the aortic valve leaflets, is certainly associated with an increased risk of poor cardiovascular outcomes through uncertain pathological mechanisms. However, the progression to AS has far more detrimental consequences. We should not look at ASc as being a separate disease, but rather look at it as being on a disease spectrum or continuum leading to AS development. From another perspective, a contrary argument can be made: why do most people with ASc not progress to develop AS? After all, Cosmi et al. (2002) had shown that only about 16% of individuals with ASc progressed to develop AS, with most of them being above the age of 70 years, whereas Faggiano et al. (2003) showed that approximately 33% of individuals with ASc went on to develop AS. Also, it was revealed that about 50% of people above 84 years of age had ASc, yet AS was only present in 4% of individuals in that age group (Stewart et al. 1997). For some unknown reason, there appears to be processes or mechanisms that are likely to be involved that just 'halt', or possibly slow the progression of ASc developing into AS. Thus, the story is far from complete, and we do not yet have the full picture. We know that 'traditional' atherosclerotic risk factors (Nassimiha et al. 2001; Chan 2003; Novaro et al. 2007; Thanassoulis et al. 2010; Owens et al. 2010) result in an increased frequency of ASc and progression of AS. Thus, it is probably these risk factors, genetic and environmental factors, and a series of other possible individual characteristics or factors yet to be discovered, that possibly result in ASc developing into AS.

1.3.3 Calcific aortic valve stenosis: Natural history of progression and prognosis

In the early phase of disease, it is probably either due to abnormal valve architecture (such as a congenitally bicuspid aortic valve) (Alpert 2003) or unequal leaflet size or thickness (Choo et al. 1999; Ho 2009), coupled with persistent turbulent blood flow forces notably on the aortic side of the cusps (Butcher & Nerem 2007; El-Hamamsy et al. 2009a; Weinberg et al. 2010), that results in progressive traumatisation of the valve leaflets. Subsequently, progressive injury with concurrent endothelial damage and dysfunction (Otto et al. 1994; Poggianti et al. 2003; El-Hamamsy et al. 2009b), along with associated ongoing oxidative stress and inflammation (Miller et al. 2008; Liberman et al. 2008; Kennedy et al. 2009), likely contributes to progressive calcification, thickening, fibrosis, and ossification of the aortic valve leaflets. This complex process results in progressive narrowing of the aortic valve orifice, and unlike ASc, eventuates in haemodynamically significant LV outflow obstruction.

Before attempting to understand the history and progression of this complex disease, it is important to define certain parameters that are currently utilised to grade AS severity. Table 1.2 overleaf illustrates the various grades of AS and their associated parameters (Bonow et al. 2008).

	Parameters			
AS Grade	**Mean transvalvular pressure gradient (mm Hg)**	**Jet velocity** ***(assuming normal cardiac index)*** **(m/s)**	**Valve area (cm^2)**	**Valve area index (cm^2/m^2)**
Mild	< 25	< 3	> 1.5	-
Moderate	25 – 40	3 – 4	1.0 – 1.5	-
Severe	> 40	> 4	< 1.0	< 0.6

Table 1.2 Stratification of AS severity and associated parameters

Most individuals during the early ***asymptomatic*** phase of AS tend to have a prolonged period whereby they generally remain well. During this phase, the overall morbidity and mortality is comparable to that of the general 'normal' population, and the risk of sudden death tends to be less than 1% per year, especially in the genuinely asymptomatic individuals with AS (Turina et al. 1987; Horstkotte & Loogen 1988; Kelly et al. 1988; Pellikka et al. 1990; Rosenhek et al. 2000). Conversely, Ross and Braunwald (1968) revealed in a major landmark study that individuals with ***symptomatic*** AS had a poor prognosis. AS patients with syncope and angina survived on average for 3 years, those with dyspnoea for 2 years, and patients with associated heart failure survived 1.5 to 2 years on average. As time progresses, so does the severity of AS. However, there is significant variability in the progression of AS and the onset of symptoms in different individuals, and thus it is impossible to predict the severity in a particular individual at any given point in time. In patients affected with AS, selected studies have revealed an average rate of progression of measurable parameters as an increase in the mean aortic

pressure gradient of 7 mm Hg per year, increase in the aortic jet velocity of 0.3 m/s per year, and a decrease in the aortic valve area of 0.1 cm^2 per year (Otto, Pearlman & Gardner 1989; Brener et al. 1995; Faggiano et al. 1996; Otto et al. 1997). A recently published study by Bartschi et al. (2010) showed somewhat different rates of AS progression, whereby they found that the progression of the mean pressure gradient was 2 mm Hg per year for mild AS, and 4 mm Hg per year for moderate AS. A 9 mm Hg per year increase in the mean pressure gradient was found in patients with severe AS, but the group was too small for statistical analysis. Not surprisingly, a study has also found a more rapid rate of increase in the aortic jet velocity of moderate to severely calcified aortic valves, compared to those with none or minimally calcified valves (Rosenhek et al. 2004a).

Recently, valvuloarterial impedance (Hachicha, Dumesnil & Pibarot 2009) has been shown to be useful in predicting adverse outcomes in asymptomatic patients with AS. However, Baumgartner and Otto (2009) argued that this modality had its limitations, and proposed evaluating patients with AS following consideration of AS severity parameters (measured by echocardiography), symptom status, LV function, stroke volume and systemic blood pressure. A novel approach was proposed by Monin et al. (2009) whereby a risk score was utilised to predict outcomes in patients with asymptomatic AS. The study cohort consisted of 104 patients, of which 75 had severe AS and 29 had moderate AS. The score involved the availability of specific parameters, which consisted of the patient's peak aortic velocity, natural logarithm of B-type natriuretic peptide, and an addition of 1.5 to the score if the patient was female. By applying this score, they

were able to predict 80% of patients having an event-free survival after 20 months if they were within the first score quartile, compared to only 7% for the fourth quartile. However, it is likely that this risk score is too simplistic in its current form to be solely utilised in predicting event-free survival, and thus should be used as an adjunct to other already well known patient factors, and currently established predictive markers and models of AS severity and progression. In addition, many studies have also revealed a more rapid progression of aortic valve calcification, ASc or AS when associated with cardiovascular risk factors, ethnicity, height, or other specific factors such as renal failure or patients on dialysis (Perkovic et al. 2003; Wang et al. 2003; Faggiano et al. 2006; Kume et al. 2006). Hence, due to the unpredictability in the rate of progression and to an increased risk of poor cardiovascular outcomes (including death), it is necessary to closely monitor patients with AS, even if they are asymptomatic (Shah, Desai & O'Gara 2010).

A landmark study (Rosenhek et al. 2004a) involving 176 subjects with asymptomatic mild and moderate AS followed for 48±19 months, served to further delineate the natural histories of these less severe forms of AS. Previously, it was assumed that mild and moderate AS were generally 'harmless' requiring less rigorous monitoring. However, this study proved contrary revealing that event-free survival (end-points defined as death or AVR) was approximately 95%, 75% and 60% at 1, 3 and 5 years respectively, and that there was a significant 80% increase in mortality (cardiac and non-cardiac). With regards to moderate or severe aortic valve calcification, the event-free survival rates were approximately 92%, 61% and 42% at 1, 3 and 5 years respectively,

compared to event-free survival rates of 100% at 1 year, 90% at 2 years and 82% at 5 years, for patients with no or mild calcification. Most alarming was the finding that of the patients who underwent a follow-up echocardiogram, 46% were found to have developed severe AS. Pertaining to moderate AS, a study by Kennedy et al. (1991) published almost 2 decades ago revealed that the probability of individuals remaining free of AS related complications (death and AVR) was 90%, 76%, 66% and 59% at 1, 2, 3 and 4 years of follow-up respectively. This study also indicated that decreased LV ejection fraction (EF) in symptomatic patients with moderate AS, conferred an increased risk of developing AS related complications. It is important to note that most of the patients (82%) in this cohort had symptomatic AS.

A prospective study by Otto et al. (1997) involving 123 individuals with asymptomatic valvular AS revealed that jet velocity (and hence stenosis severity) was a strong predictor of long-term survival. They revealed that the chances of being alive at 2 years without AVR was approximately 21% for a jet velocity at study entry of more than 4 m/s, compared to 66% (jet velocity of 3 – 4 m/s) and 84% for a jet velocity of less than 3 m/s at study entry. 7% of their subjects died with a further 39% requiring AVR, of which their primary indication for surgery was decreased exercise tolerance, heart failure, angina, syncope or near syncope, and severe asymptomatic AS. However, an earlier study (Pellikka et al. 1990) revealed somewhat contrary results. This study involved 143 asymptomatic patients with AS: group 1 consisted of 30 patients who underwent early intervention (operation or valvuloplasty) within 3 months, and group 2 had 113 patients who did not undergo early intervention. Of the 113 asymptomatic

patients (group 2), their echocardiographic parameters constituted a mean peak velocity of 4.3 m/s (range 4 to 6), average maximal gradient of 74 mm Hg (range 64 to 145) and an average aortic valve mean gradient of 47 mm Hg (range 35 to 90). Thus, by current standard definitions, all of these patients had severe AS. Surprisingly, the predicted survival rates in this cohort were markedly better, approximating 94% at 1 year and 90% at 2 years. In addition, the predicted probability of being free of cardiac events related to AS (including cardiac death or aortic valve surgery) was approximately 93% at 1 year and 74% at 2 years. This is in stark contrast with the findings of the previous study (Otto et al. 1997), which revealed a survival probability of approximately only 21% (without AVR) at 2 years in individuals with a jet velocity of more than 4 m/s at study entry. Rosenhek et al. (2000) in a study involving 128 asymptomatic patients with severe AS that were prospectively followed-up for approximately 22 months, found that a rapid increase in aortic jet velocity conferred an extremely poor prognosis. However, the authors concluded that it was relatively safe to delay surgery in patients with asymptomatic AS.

Subsequent to this, a recent study by Pellikka et al. (2005) involved a larger cohort consisting of 622 patients with asymptomatic, haemodynamically significant ('severe') AS, in an attempt to resolve the discrepancies shown in previous studies. This population had an average peak velocity of 4.4 m/s (range 4 to 6.6), mean gradient of 46 mm Hg (range 21 to 107), and a mean aortic valve area of 0.9 cm^2 (range 0.38 to 1.80). In addition, the follow-up time was much longer in this study (5.4 ± 4.0 years). 50% of patients in this cohort developed symptoms of angina, dyspnoea or syncope during

follow-up and before any aortic valve surgery was undertaken. The calculated probability of remaining symptom free without surgery was 82% at 1 year, 67% at 2 years, and 33% at 5 years. The calculated Kaplan-Meier probability of remaining free of cardiac events (including cardiac death or AVR) was 80% at 1 year, 63% at 2 years and 25% at 5 years. This study also found that sudden death occurred at a rate of about 1 % per year, and that chronic renal failure, age, inactivity and aortic valve velocity independently predicted all-cause mortality in this population.

A less well understood form of severe AS, characterized by low flow, low gradient and preserved LV ejection fraction (LVEF), has been shown to confer a poorer prognosis and a lower overall 3-year survival compared to patients with normal flow severe AS (76% versus 86% respectively) (Hachicha et al. 2007). Due to the presumed 'normal' parameters (low aortic gradient, preserved EF) associated with this paradoxical form of low-flow severe AS, patients in this category are sometimes missed and often presumed to be have less severe AS, and thus may result in significant delays in life saving surgery. Another study (Schueler et al. 2010) involving octogenarians showed that there was significantly decreased short term survival in those with severe AS (aortic valve area < $1cm^2$) compared to octogenarians with less severe AS. The study also revealed cumulative mortality rates of 77.5% versus 44.4% respectively.

More work has been undertaken recently to further understand the progression and consequences of 'very' severe AS. In a recent study by Rosenhek et al. (2010), 116 asymptomatic patients with very severe AS were followed-up prospectively, and their

event-free (indication for AVR or cardiac death) survival was assessed. Very severe AS in this study was defined by a peak aortic jet velocity (AV-Vel) of ≥5m/s and an aortic valve area (AVA) of 0.63 ± 0.12cm². Dramatic results were obtained whereby the event-free survival at 1, 2, 3, 4, and 6 years was 64%, 36%, 25%, 12%, and 3% respectively. The study also found that peak AV-Vel independently affected event-free survival, and that patients with an AV-Vel ≥5.5m/s had a higher probability of developing severe symptom onset compared to those with an AV-Vel<5.5m/s (52% versus 27%). Recently, an interventional study (Kang et al. 2010a) showed prospectively that patients with very severe AS (defined by an AVA ≤0.75cm², peak AV-Vel ≥4.5m/s and mean trans-aortic pressure gradient ≥50mm Hg) had poorer outcomes when treated conventionally compared to those who underwent early AVR surgery. The findings again were markedly different, whereby estimated actuarial 6-year cardiac and all-cause mortality rates were approximately 0% and 2% in patients who underwent early AVR, and 24% and 32% in the conventionally treated group, respectively.

Thus, all the evidence has conclusively shown that the severity of AS progresses with time, and is associated with a significant increase in the rate of poor cardiovascular outcomes including death. The decreased survival rate is particularly more pronounced when AS is symptomatic. However, even in the setting of asymptomatic AS, there is a conferred poorer prognosis with an increased rate in poor cardiovascular outcomes, especially in the setting of ‘severe’ asymptomatic AS. Thus, until tools are developed that are better able to more accurately predict AS severity, progression and development

of symptoms, close vigilance should be applied to all patients with AS, especially to those with moderate and severe AS, even if they are asymptomatic in the first instance.

1.4 Clinical parameters associated with aortic valve sclerosis / aortic valve stenosis: development, progression and outcome

It is now well known that ASc and AS are associated with traditional cardiovascular or atherosclerotic risk factors, and also with more specific risk factors such as renal failure, dialysis, or pathological bone disease. Most, if not all of these risk factors, confer not just to an increased risk of AS development, but also renders to a more rapid progression of the disease process, thus contributing significantly to an increased risk of poor cardiovascular outcomes (including death). In this section, current evidence will be further evaluated pertaining to these associated risk factors, and their relative contributions towards AS development and progression. As ASc or aortic valve calcification/calcium (AVC) exist on a continuum leading to AS development, the clinical risk factors delineated from various studies will be extrapolated to be associated with AS.

Age: In a multitude of different disease processes, age tends not only to be associated with a higher prevalence of a particular disease, but also to its rate of progression. After all, as the individual is alive for a longer period of time, their bodies (extrinsically and intrinsically) are exposed to a multitude of factors over the course of that time. Some of which are environmentally related (such as due to poor or unhealthy nutrition, chemical exposure, repeated bacterial of viral insults, smoking, exposure to radiation or to a variety of noxious fumes), and many in combination with chronic exposure to ‘inherited’ or ‘acquired’ internal factors such as genetic predisposition to disease, hypertension,

hypercholesterolemia and diabetes mellitus, just to name a few. Thus, the prolonged exposure to these factors over time will inevitably lead to chronic inflammation and oxidative stress, likely eventuating in pathological changes not just at a macroscopic level, but also at a cellular and molecular level, and often affecting deoxyribonucleic acid (DNA) repair and stability. As such, it should not come as a surprise that many malignancies, for example skin, lung, head, neck and bowel cancer, have a much higher incidence and prevalence when associated independently with advancing age, or in combination with other previously discussed factors (Kasper et al. 2006). Lindroos et al. (1993) in a survey involving more than 500 elderly people found that the prevalence of AVC and AS increased significantly with advancing age, similar to that found in other studies (Stewart et al. 1997; Aronow et al. 1999; Otto et al. 1999; Olsen et al. 2005; Taylor et al. 2005; Messika-Zeitoun et al. 2007; Ngo et al. 2009; Owens et al. 2010). With respect to progression, either from AVC or ASc to AS, or progression of mild or moderate AS to severe AS, the evidence is just as compelling with many studies showing age as an independent risk or clinical factor leading to progression of AS (Stewart et. al. 1997; Messika-Zeitoun et al. 2007; Novaro et al. 2007; Thanassoulis et al. 2010). The Cardiovascular Health Study (CHS) (Stewart et al. 1997) showed that there was a two-fold increased risk in developing AS for each 10-year increase in age. Even more interesting in the study was the finding that age carried the highest odds ratio for incident ASc or AS development, compared to other clinical factors (male gender, height, smoking, low density lipoprotein cholesterol, lipoprotein (a), and a history of hypertension). A recent study by Owens et al. (2010) showed that age significantly increased the risk of incident aortic valve calcium, but revealed a non-significant trend

towards risk of aortic valve calcium progression. In support of this, Cosmi et al. (2002) had previously revealed a similar trend, which was also not statistically significant. There also exists another aspect linking age to AS that is often overlooked in many reviews, which commonly involves age being an independent or combined risk factor leading to poor cardiovascular outcomes (death, MI, or requirement for AVR) (Aronow et al. 1999; Chandra et al. 2004; Olsen et al. 2005; Hachicha et al. 2007; Kang et al. 2010a; Kang et al. 2010b). In conclusion, age is not only a major clinical or risk factor leading to the development and progression of AS, but also to poor cardiovascular outcomes.

Dyslipidaemia *(Hypercholesterolemia, high low-density lipoprotein (LDL) levels, high lipoprotein (a) levels)*: Several studies have confirmed the presence of atherosclerosis and LDL within stenosed non-rheumatic human calcified aortic valves (O'Brien et al. 1996; Olsson, Thyberg & Nilsson 1999). This has been further supported by numerous animal studies (involving rats, mice or rabbits) showing the strong association between hypercholesterolemia and development of ASc or AS (Zahor & Czabanova 1977; Fazio et al. 1994; Rajamannan et al. 2002). Many earlier human studies also showed that hypercholesterolemia was a positive risk factor associated with AS (Deutscher, Rockette & Krishnaswami 1984; Aronow, Schwartz & Koenigsberg 1987; Aronow et al. 2001). A landmark study, the CHS (Stewart et al. 1997) found high serum lipoprotein (a) and LDL levels to be strong clinical factors associated with AS. Later, Peltier et al. (2003) found high total serum cholesterol levels to be strongly associated with severe AS. Taylor et al. (2005) also revealed that these findings were similar in non-white

populations, whereby high serum LDL levels found in African-Americans (n=2445) were significantly associated with AS. Conversely, this study also showed that low serum high-density lipoprotein (HDL) levels were more common in patients with ASc and AS, which was also confirmed in the LIFE substudy published in the same year (Olsen et al. 2005). More recently, Katz et al. (2009) in the Multi-ethnic Study of Atherosclerosis (MESA) revealed that in patients with metabolic syndrome free of diabetes, serum LDL was a significant positive risk factor (fully adjusted odds ratio (OR)=1.54) for incident aortic valve calcium (AVC). Similarly, a study (Owens et al. 2010) involving 5,880 participants revealed that higher total cholesterol to HDL ratios significantly increased the risk (univariate analysis) of incident AVC, but failed to show a significant contribution to the risk of AVC progression. Despite many studies revealing dyslipidaemia, encompassing its various permutations and combinations, as being a strong risk factor for AS, there are others that have shown a non-significant association between the two. Indeed, Chandra et al. (2004) found that serum LDL and triglyceride levels were significantly higher in the cohort of patients with normal aortic valves compared to those at any stage of ASc, including those with moderate to severe ASc. With regards to dyslipidaemia contributing to the progression of AS, the evidence is also somewhat controversial. Many studies did not reveal hypercholesterolemia to be a significant independent positive risk factor for progression of AS (Palta et al. 2000; Bellamy et al. 2002; Novaro et al. 2007; Owens et al. 2010), while others showed that there was a significant association. An earlier study by Nassimiha et al. (2001) showed hypercholesterolemia to be a significant independent risk factor for progression of AS (following analysis by multiple linear regression), albeit the sample size was rather

modest. Similarly, Messika-Zeitoun et al. (2007) showed that on multiple regression analysis, LDL cholesterol was independently related with aortic valve calcification acquisition. A recently published study (Thanassoulis et al. 2010) showed that on multivariate analysis, higher values of total cholesterol were associated with development of AVC (OR=1.74), whereas higher values of long-term mean HDL were associated with a decreased risk (OR=0.77) of developing AVC. In terms of poor cardiovascular outcomes (death, MI, requirement for AVR), many studies have not shown dyslipidaemia to be an independent predictor or risk factor leading to poor cardiovascular outcomes in patients with asymptomatic AS, moderate AS, severe AS or very severe AS (Rosenhek et al. 2000; Rosenhek et al. 2004a; Kang et al. 2010a; Rosenhek et al. 2010; Schueler et al. 2010). Even though previous smaller retrospective studies showed a decrease in the progression of AS in patients treated with hydroxymethylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors, or statins (Aronow et al. 2001; Novaro et al. 2001; Bellamy et al. 2002; Rosenhek et al. 2004b), disappointingly, recent major prospective placebo-controlled randomised studies have revealed that statins do not significantly alter the progression of AS once moderate disease is present (Cowell et al. 2005; Rossebo et al. 2008; Chan et al. 2010). The significance of these recent large trials will be discussed in the later parts of this chapter. In conclusion, despite many epidemiological studies revealing a significant association between dyslipidaemia and AS incidence/progression, the negative findings of the recent major intervention trials are conclusive. However, it must be stated that the results pertain to an older cohort with many having well established moderate disease. Thus, it

is possible that therapy targeted towards a younger demographic with mild AS may still prove beneficial in the future.

Smoking: A well known social 'habit' that has been practiced throughout the ages, which has only over the last half century been discovered to be a major 'enemy' contributing to an increased risk towards the development of many human diseases. Smoking contributes to an increased risk of cerebrovascular accidents (or stokes), chronic airways disease (emphysema and bronchitis), various cancers (involving the oral cavity, pharynx, oesophagus, stomach, pancreas, lung, cervix, kidneys and bladder), coronary artery disease, peripheral vascular disease, aortic aneurysm, sudden infant death syndrome and low birth weight at delivery; just to name a few. In the United States alone, over 400,000 people die prematurely every year as a result of cigarette use, representing approximately 20% of total deaths per year (Kasper et al. 2006). Thus, it should not come as a surprise that smoking very likely contributes to the development of AS, with many studies revealing smoking to be a positive risk factor associated with AS (Mohler et al. 1991; Aronow et al. 2001; Peltier et al. 2003; Owens et al. 2010), and contributing to its progression (Palta et al. 2000; Nassimiha et al. 2001; Ngo et al. 2001, Yilmaz et al. 2004). Not surprising, a recent study showed that smoking was significantly prevalent in high-risk patients with severe symptomatic AS with concurrent significant coronary artery disease (CAD), compared to those with normal coronaries or non-significant CAD (Ben-Dor et al. 2010). Briand et al. (2006) showed that a smoking history was significantly more prevalent in patients with concurrent AS and metabolic syndrome, compared to those with AS without metabolic syndrome. The

CHS (Stewart et al. 1997) showed that current smoking conferred a 35% increase in risk of developing AS. Interestingly, the study by Messika-Zeitoun et al. (2007) showed no significant association between current smoking status and risk of incident AVC or progression, measured by electron-beam computed tomography (EBCT). However, Thanassoulis et al. (2010) recently showed smoking to be strongly associated with AVC (multivariate OR=1.23), measured by cardiac multi-detector computed tomography (CT). A recently published study (Owens et al. 2010) showed incident AVC risk to be significantly associated with current smoking, however, it also showed that current smoking was not significantly associated with AVC progression. With respect to smoking contributing to poor cardiovascular outcomes including death, a study by Olsen et al. (2005) involving 960 participants revealed that current smoking conferred a hazard ratio (HR) of 2.47 in predicting the composite cardiovascular end point (cardiovascular death, fatal and non-fatal MI, and fatal and nonfatal stroke). Similarly, Taylor et al. (2005) also revealed that current smokers carried an increased risk (multivariate HR=2.65) in predicting incident coronary heart disease (composed of hospitalized MI or fatal coronary heart disease (CHD)), whereas, a recent study showed smoking to marginally miss significance (p=0.06, HR=2.71 (CI:0.94-7.80) in correlation to cardiac mortality in conventionally treated patients with asymptomatic very severe AS (Kang et al. 2010a). Thus, the evidence overall is very compelling, revealing that smoking contributes to the development and progression of AS, and even resulting in poor cardiovascular outcomes including death.

Hypertension: There are many reasons why hypertension has often been called 'the silent killer'. After all, most of its victims are usually unaware that they have been afflicted by this condition until months or years later, by manifesting itself symptomatically (headache or neck pain), or is often unmasked by its resultant end-organ damage, such as renal failure, retinal haemorrhage, cardiac failure or stroke. Hypertension has also been shown to be a significant major risk or clinical factor associated with incident AS (Aronow, Schwartz & Koenigsberg 1987; Lindroos et al. 1994; Stewart et al. 1997; Aronow et al. 2001; Peltier et al. 2003; Olsen et al. 2005; Taylor et al. 2005; Messika-Zeitoun et al. 2007; Novaro et al. 2007). Interestingly, one study revealed that antihypertensive therapy usage but not hypertension, was shown to be significantly associated with incident AS (Owens et al. 2010). A study by Taylor et al. (2005) showed that hypertension conferred an increased risk to poor cardiovascular outcomes (hospitalized MI or fatal CHD) in patients with AS. The evidence that hypertension is associated with AS is convincing, however, most if not all of the studies to date fail to show hypertension contributing significantly to AS progression. In addition, most of the studies reveal hypertension not being significantly associated with poor cardiovascular outcomes in patients with AS.

Gender: Many philosophers over the ages have claimed that 'men and women are not made equal'. This may also be true with regards to AS. Numerous studies have indeed revealed that male gender is a significant risk factor for AS (Mohler et al. 1991; Stewart et al. 1997; Aronow et al. 2001; Messika-Zeitoun et al. 2007; Novaro et al. 2007; Owens et al. 2010), and leading to its progression (Bahler et al. 1999; Owens et al. 2010). A

recent study by Kang et al. (2010b) involving 492 patients with asymptomatic significant AS, showed that male gender was independently associated with cardiac mortality, and carried the highest hazard ratio (HR) of 3.56 ($p<0.001$), compared to the other independent predictors in the study (aortic jet velocity, HR=2.58; age HR=1.06; diabetes mellitus, HR=2.23). Interestingly but not entirely surprising is the finding that being female may be protective, and has been shown to be significantly associated with reduced incidence of AS (Thanassoulis et al. 2010). Taylor et al. (2005) showed that being female significantly reduced the risk of incident CHD (hospitalized MI or fatal CHD) in patients with ASc (adjusted for age), but failed to reach significance on multivariate analysis. Controversially, a recent study revealed that female sex conferred an increased risk leading to poor cardiovascular outcomes (death or requirement for AVR) in patients with AS (Monin et al. 2009). Also, Rosenhek et al. (2010) recently showed that female sex was not significantly associated in predicting an increase in event-free survival in patients with very severe AS. Thus, even though male gender has been shown to be a significant risk factor associated with AS, and also leading to poor cardiovascular outcomes, the contribution of female gender to this picture remains controversial. It is likely that future studies may indeed shed more light on the issue. However, there are probably a multitude of factors, including social and lifestyle factors that confer gender its unique position and this may vary with successive generations.

Diabetes mellitus: This disease is a major player in many pathological states resulting in significant macro and microvascular complications. Its contribution to CHD, peripheral vascular disease and cerebrovascular disease is well documented. Equally prominent is

its contribution towards retinopathy, neuropathy and nephropathy. Thus, since the strong association between AS and other 'traditional' atherosclerotic risk factors have been established, it is not surprising that diabetes mellitus (DM) would also play a part in its development and progression. DM has indeed been shown in several studies to be a significant risk factor associated with AS (Aronow, Schwartz & Koenigsberg 1987; Aronow et al. 2001; Olsen et al. 2005; Messika-Zeitoun et al. 2007; Owens et al. 2010), with one study showing DM achieving borderline significance (p=0.0581 on multiple liner regression analysis) in terms of contributing to AS progression (Nassimiha et al. 2001). Not surprising, DM has been found to be independently associated with poor cardiovascular outcomes (death, fatal and non-fatal MI, requirement for AVR) in patients with AS (Rosenhek et al. 2000 (univariate analysis); Olsen et al. 2005; Taylor et al. 2005 (age adjusted model); Kang et al. 2010b; Rosenhek et al. 2010; Schueler et al. 2010). In conclusion, there is sufficient evidence that DM is significantly associated with AS, and contributes to poor cardiovascular outcomes.

Body mass index or obesity: Body mass index (BMI) is a measure used to categorize an individuals weight. It is the product of weight (in kilograms) over the height (metre) squared (m^2). The prescribed categories are a BMI<18.5 is underweight, between 18.5 and 25 is ideal or target weight, between 25 and 30 is overweight, between 30 and 40 is obese, and finally an individual with a BMI>40 is considered to be morbidly obese. Many studies have managed to associate an increased BMI or obesity, to be a significant risk factor for AS (Peltier et al. 2003; Messika-Zeitoun et al. 2007; Owens et al. 2010; Thanassoulis et al. 2010), and also showing its contribution towards AS progression

(Ngo et al. 2001). The Simvastatin Ezetimibe in Aortic Stenosis (SEAS) substudy by Lund et al. (2010) involving 1,703 patients with asymptomatic AS showed that an increased BMI was significantly associated with the presence of LV hypertrophy in patients with asymptomatic AS, independent of hypertension and AS severity. It is worth mentioning an older study, the Helsinki Aging Study (Lindroos et al. 1994), which surprisingly found some contradictory results whereby a low BMI independently predicted aortic valve calcification. Furthermore, a study conducted in South Australia involving 253 randomly selected patients has also revealed a low BMI to be significantly associated with ASc (Ngo et al. 2009). However, we now know that some individuals with a low BMI have abnormal calcium or bone metabolism resulting in osteopenia, and this may probably help explain the contradictory results obtained in these studies.

Metabolic syndrome: This has been a more recent risk factor that has been associated with AS. Many have considered this association rather obvious due to the fact that metabolic syndrome (MS) constitutes a collection of several cardiovascular risk factors. Kasper et al. (2006) has defined MS as constituting any of these 3 risk factors, which include abdominal obesity measured by waist circumference (men>102cm, women>88cm), triglycerides>1.7mmol/L, HDL cholesterol (men<1.0mmol/L, women<1.3mmol/L), fasting glucose >6.1 mmol/L or type 2 diabetes, or blood pressure readings of ≥130/≥85 mmHg. In particular, it has only been over the last decade that studies have specifically investigated the contribution of MS towards AS development and progression. The Multi-ethnic Study of Atherosclerosis (MESA) (Katz et al. 2006) involved 6,780 participants (1,550 with MS, 1,016 with diabetes mellitus (DM), and

4,024 with neither condition) and was performed to assess the prevalence of aortic valve calcium (AVC) in the different subgroups. It found that MS and DM were significantly associated with an increased risk of AVC, and as the number of MS components increased, so did the prevalence of AVC. In a retrospective study by Briand et al. (2006) involving 106 patients with at least moderate AS, MS was found to be a strong independent factor in predicting AS progression and outcome (death or requirement for AVR) (OR=3.85, $p<0.001$). Later, Katz et al. (2009) went on to confirm that MS was significantly associated with an increase in incident AVC. More recently, the ASTRONOMER (Aortic Stenosis Progression Observation Measuring Effects of Rosuvastatin) substudy revealed that MS was independently associated with worse cardiac function and more pronounced LV concentric hypertrophy in patients with AS (Page et al. 2010). Thus, with the passage of time and more future studies, it is likely that MS will be found not just to be independently associated with AS incidence and progression, but also in predicting poor cardiovascular outcomes in AS patients.

Coronary artery disease: There is a variable association between the presence of coronary artery disease (CAD) and ASc or AS. Many studies have shown CAD to be a significant correlate with AS (Aronow et al. 1999; Peltier et al. 2003), progression of AS (Peter et al. 1993; Rosenhek et al. 2004a), and leading to poor cardiovascular outcomes (non-cardiac death or sudden cardiac death, fatal and non-fatal MI, requirement for AVR) (Aronow et al. 1999; Rosenhek et al. 2000 (on univariate analysis); Rosenhek et al. 2004a) in patients with AS. However, Rosenhek et al. (2010) recently found a non-

significant association between CAD and event-free survival in patients with very severe AS, whereby an event was defined as cardiac death or indication for AVR.

Ethnicity: It had long been assumed that different ethnic groups had similar risks of developing major diseases including CHD and diabetes mellitus, when identical diagnostic parameters were utilised between all the different ethnic groups. However, we now know that different ethnic groups develop some diseases at different rates and under different circumstances. For example, a given BMI or abdominal circumference may render an Asian male to a higher risk of developing CHD, compared to his European counterpart with similar numerical characteristics. Novaro et al. (2007) found that African-American ethnicity resulted in a decreased risk of AS progression (OR=0.49, p=0.035). This finding was in stark contrast to other studies that revealed African-Americans at high risk for acute coronary syndrome were in fact younger and more likely to have hypertension, diabetes mellitus, renal dysfunction and heart failure, compared to Caucasian patients (Sonel et al. 2005). On univariate analysis, Owens et al. (2010) found that Chinese ethnicity conferred a decreased risk (RR=0.43, p=0.006) to incident AVC and also revealed a significantly lower rate of AVC progression, compared to their White counterparts. However, the study did not find a difference in incident or progressive AVC risk between the White, Hispanic and Black ethnic groups on univariate analysis. However, in a fully adjusted model, there were no significant differences between any of the ethnic groups (Chinese, White, Hispanic and Black). In another study, Olsen et al. (2005) did not find any difference in ASc prevalence, between African-Americans with and without the disease. In conclusion, ethnicity may

play a role in AS. However, it is probably due to the interaction between environmental, geographical, nutritional and genetic factors that possibly contributes to the differences between various ethnic groups.

Chronic renal failure and dialysis: Although not a traditional atherosclerotic risk factor, patients with chronic renal failure (CRF) have been shown to have a higher incidence of coronary artery disease (CAD) compared to those without CRF. In fact, this is particularly the case in patients with end-stage renal failure on dialysis, whereby they have a higher prevalence of atherosclerosis (Straumann et al. 2002, Kasper et al. 2006) and coronary calcification (Braun et al. 1996; Raggi et al. 2002; Raggi, Bommer & Chertow 2004; Dellegrottaglie et al. 2006; Ix et al. 2007a; Piers et al. 2009). The likely mechanism for this accelerated calcification process is abnormal calcium-phosphorus product metabolism in patients with chronic or end-stage renal failure on dialysis (Straumann et al. 1992; O'Neil 2007), which likely leads to calciphylaxis, that is extra-osseous or 'metastatic' calcification of blood vessels an other soft tissue. Not surprising, cardiovascular disease is the major cause of death in dialysis dependent patients (Kasper et al. 2006). Many studies have revealed higher serum creatinine or CRF to be significantly associated with increased risk in AS incidence (Stewart et al. 1997; Palta et al. 2000; Faggiano et al. 2006; Piers et al. 2009) and progression (Perkovic et al. 2003; Kume et al. 2006). Controversially, a recently published study by Owens et al. (2010) found a higher serum creatinine to be associated with a lower risk of incident AVC. With regards to outcome, patients with CRF are at significantly increased risk of death (Schueler et al. 2010). It is important to note that valvular calcification itself is an

independent predictor of all cause mortality and cardiovascular mortality in dialysis patients (Wang et al. 2003; Varma et al. 2005).

Pathological bone disease, abnormal calcium homeostasis and bisphosphonates: Since the process of calcific AS involves progressive calcification of the trileaflet aortic valve, it should not come as a surprise that abnormal calcium metabolism or homeostasis contributes towards the development and progression of AS. Paget's bone disease and hyperparathyroidism (primary, secondary or tertiary forms) result in abnormal bone turnover, and alters calcium metabolism and homeostasis, leading to elevated serum calcium levels and other by-products. Thus, these pathological diseases have been implicated in the development and progression of AVC or AS (Strickberger, Schulman & Hutchins 1987, Zipes et al. 2005; Kasper et al. 2006). Fascinatingly, there was a recent report of rheumatoid arthritis contributing to rapid progression of AS. Histological examination of the valve leaflets revealed the presence of rheumatoid nodules associated with fibrosis and calcification (Peyrou et al. 2009). Low bone mineral density, especially in osteopenic or osteoporotic individuals, has been found to be independently associated with progression of AS (Aksoy et al. 2005). However, another interesting aspect linking pathological bone diseases to AS involves the pharmacological agents used to treat those diseases. In particular, bisphosphonates may actually confer some protection towards AS development and progression. Bisphosphonates are major pharmacological agents utilised in the treatment of osteporosis, and primarily act by preventing osteoclast-mediated bone resorption (Kasper et al 2006), thus improving bone density. Studies have found bisphosphonate

use to be linked with decreased prevalence of AS (Elmariah et al. 2010a) and its progression (Skolnick et al. 2009), especially in elderly patients with AS. Thus, there is a definite connection between pathological bone disease and AS, and with more research, there may be a role for bisphosphonates in the treatment of AS in the future.

Genetics and AS: With the progress of genetic research, our understanding of the contribution of genetics in the development and progression of AS continues to expand. The genetics of congenital bicuspid aortic valves have been extensively studied (Clementi et al. 1996; Huntington, Hunter & Chan 1997; Cripe et al. 2004). However, genetic studies pertaining to the development of calcific AS have been more difficult to ascertain conclusively. Nonetheless, some progress is being made in this area. An earlier epidemiological French study revealed a clustering of individuals with AS, suggesting a genetic contribution (Le Gal et al. 2005). Probst et al (2006) later showed a similar clustering in the western part of France, thus supporting a genetic hypothesis. In one of the earlier studies involving 100 patients with severe AS, it was discovered that the genetic variant frequency (B allele) located on the vitamin D receptor gene was significantly higher in AS patients compared to controls (Ortlepp et al. 2001). Several other more prevalent genotypes, such as APOE 2/4 and 3/4 (Novaro et al. 2003) and various genetic polymorphisms involving the estrogen receptor-α gene, transforming growth factor-β1 gene, apo B XBaI gene and Interleukin-10 (IL-10) gene, have been found to be more prevalent and significantly associated in patients with ASc or AS (Avakian et al. 2001; Nordstrom et al. 2003; Ortlepp et al. 2004). In addition, mutations involving the NOTCH1 gene have also been associated with AS (Garg et al. 2005;

McKellar et al. 2007; Nigam & Srivastava 2009). It has been suggested that specific mutations in the NOTCH1 gene result in decreased repression of the core-binding factor α1 (Cbfa1) transcription factor (also known as Runx2), thus resulting in increased valvular osteoblast differentiation, contributing to calcification and bone formation of the aortic valve leaflets (O'Brien 2006). Genetic research into AS development is still in its infancy, and with advances in genetic research and techniques, there is hope for a deeper understanding of its contribution in the future.

In conclusion, many factors contribute towards the development and progression of AS, and that it is a much more complex condition than once thought. However, most of the research or correlates of AS development relates purely to clinical associations and not molecular mechanisms; requiring elucidation of the latter.

1.5 Pathogenesis of aortic valve stenosis

1.5.1 The role of mechanical stress and endothelial dysfunction

The aortic valve is subject to significant flow forces when performing its usual function. In particular, the aortic side of the trileaflet valve experiences turbulent circulatory flow causing higher mechanical stress especially in the flexion regions near its attachment to the aortic root, compared to the ventricular surface which undergoes laminar unidirectional flow and less mechanical stress (Freeman & Otto 2005; Butcher et al. 2006). This difference in flow between the two sides is further confirmed by reduced expression of Kruppel-like factor-2 (KLF-2) on the aortic side compared to the ventricular side (Matsumoto et al. 2009), whereby KLF-2 expression is down-regulated by turbulent shear stress (Wang et al. 2006; Atkins & Jain 2007). Over time, the chronic turbulent circulatory forces on the aortic side of the valve likely results in endothelial injury and degeneration of the valve leaflets. Thus, many studies have in fact revealed pathological lesions occurring preferentially on the aortic surface of the valve leaflets (Hurle, Colvee & Fernandez-Teran 1985; Otto et al. 1994; Mirzaie et al. 2002).

When considering the endothelium, it is very important to first consider, arguably, its most valuable function. A major discovery made by Furchgott in 1980 was that endothelial cells produced endothelium-derived relaxing factor (EDRF) following stimulation by acetylcholine (Furchgott & Zawadzki 1980). EDRF was later proven to be nitric oxide (NO) (Ignarro et al. 1987; Palmer et al. 1987). Subsequently, the enzyme endothelial nitric oxide synthase (eNOS) was isolated form bovine aortic endothelial

cells (Pollock et al. 1991), and was found to synthesize NO from L-arginine. NO in-turn activates soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) (Furchgott & Zawadzki 1980; Murad 1996), which causes further signalling of pathways downstream, eventually resulting in vasodilatation of blood vessels. NO is not just a modulator of vascular tone but also inhibits platelet aggregation, smooth muscle proliferation, monocyte and platelet adhesion to the endothelium, LDL oxidation and endothelin production; just some of the many functions of NO. Thus, when endothelial dysfunction ensues, often manifested by decreased NO production, bioavailability or responsiveness, it is commonly a significant contributing factor responsible for diseases such as atherosclerosis, coronary artery disease, peripheral vascular disease, vascular stenosis, hypertension, hypercholesterolemia, transplant rejections, septic shock, erectile dysfunction and many more (Zipes et al. 2005; Kasper et al. 2006; Yetik-Anacak & Catravas 2006).

Even though there are significantly different phenotypic expressions, gene expression, spatial alignment to shear stress, and mechano-transduction properties, between aortic valve endothelial cells (AVECs) compared to vascular endothelial cells (VECs) (El-Hamamsy, Chester & Yacoub 2010), the endothelial cells (ECs) from these two regions also share many similar properties. Similar to the VECs, eNOS expression is up-regulated in AVECs in response to shear stress, to produce NO. The NO released acts directly on the VICs resulting in valve cusp relaxation (El-Hamamsy et al. 2009b), which is important in maintaining valve integrity and function, especially under conditions of high mechanical stress.

Thus, the prolonged exposure of the aortic valve to mechanical stress causing endothelial injury, coupled with the presence of some risk factors (for example male gender, dyslipidaemia, diabetes mellitus, hypertension and smoking) associated with AS, likely result in chronic inflammation and oxidant stress, thus contributing to valvular endothelial dysfunction (Mohler 2004). It is now known that valvular endothelial dysfunction is associated with leaflet degeneration, which also exhibits an up-regulation of proinflammatory adhesion molecules (Ghaisas et al. 2000; Muller et al. 2000). In addition to endothelial dysfunction, the endothelial injury or breakage in the endothelium exposes the underlying VICs to infiltration by circulating inflammatory cells (Mazzone et al. 2004), LDL particles (Mohty et al. 2008), and other cytokines and chemokines, resulting in further oxidative stress, thus contributing to further changes in the subendothelial matrix and cellular components, eventuating in the development of early lesions predominantly on the aortic side of the trileaflet valve (Hurle, Colvee & Fernandez-Teran 1985; Otto et al. 1994; Mirzaie et al. 2002). A recent study by Matsumato et al. (2009) has revealed that there was reduced regenerative capacity of aortic valve endothelial cells (AVECs) due to senescence, and reduced number and migratory capacity of endothelial progenitor cells (EPCs) in patients with severe AS, compared to controls without AS; thus suggesting a pathological link for the destruction of the aortic valve endothelium leading to AS development. In addition, AS is not just solely associated with valvular endothelial dysfunction, but has also been shown to be significantly associated with systemic or vascular endothelial dysfunction (Poggianti et al. 2003). Endothelial dysfunction manifested by uncoupling of NOS has also been found to result in increased oxidative stress in AS (Miller et al. 2008; Towler 2008).

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS. It has now been established to be a marker, mediator and regulator of endothelial dysfunction (Vallance et al. 1992). Elevated ADMA levels have been found to be significantly associated with CAD, renal failure, diabetes mellitus, stroke and post-MI cardiogenic shock; just to name a few (Sibal et al. 2010; Ueda, Yamagishi & Okuda 2010). Furthermore, Ngo et al. (2007) in a study involving 84 patients (42 with AS, and 42 aged-matched controls) showed that the presence of AS was directly correlated with elevated ADMA levels, following analysis by backward stepwise multiple linear regression. More recently, there have been controversial results published on the subject. Cagirci et al. (2011) evaluated 109 patients with mild, moderate and severe AS. The study found that mean ADMA levels were significantly higher in the severe AS group, compared to either the mild or moderate AS groups. In addition, the serum ADMA level was found to be positively correlated with the mean and maximum aortic gradient, and negatively correlated with aortic valve area. Conversely, Ferrari et al. (2010) did not find any significant difference in the plasma ADMA levels in patients with AS compared to healthy non-AS patients. The problem underlying many studies is that several factors shown to be associated with elevated ADMA levels, such as renal failure or diabetes, are also associated with AS risk. Thus, in the absence of large population studies with multivariate analysis, these confounding factors often make attempts to independently associate ADMA with AS risk difficult.

In conclusion, there is a close association between valvular mechanical stress, associated risk factors, endothelial injury and dysfunction, and a potential decrease in NO

production, bioavailability or responsiveness, which may constitute the early processes involved in the pathogenesis of AS.

1.5.2 The role of inflammation in aortic valve stenosis

It is highly probable that aortic valve endothelial dysfunction likely represents at least in part, an early precursor leading to the development of inflammation in AS. Decreased production of endothelial mediators, such as NO and prostacyclin, have been implicated in vessel inflammation (Tedgui & Mallat 2001). It is also known that NO has many protective mechanisms (Wink et al. 1996; Grisham et al. 1999), likely mediated through anti-oxidant pathways (Wink et al. 1998); all of which contribute to decreased inflammation. Thus, the loss of these protective mechanisms via endothelial dysfunction (Rajamannan et al. 2005b; Miller et al. 2008; Towler 2008) are very likely involved in mediating the early processes of aortic valve inflammation.

Another very important aspect of inflammation involves the cellular components that mediate its development and progression, which primarily involves neutrophils, macrophages and lymphocytes (Kasper et al. 2006). Normal aortic valves tend to have minimal or no inflammatory cells present within them, which is in contrast to stenotic aortic valves, which have significantly increased numbers of macrophages and T-lymphocytes present throughout the heavily calcified valvular tissue (Olsson et al. 1994; Otto et al. 1994; Wallby et al. 2002; Akat, Borggrefe & Kaden 2009; Alexopoulos et al. 2010). Neutrophils are not found in calcified aortic valves as they are most often involved in mediating acute inflammatory processes, whereas AS generally represents a

chronic pathological inflammatory state. Monocytes have a half-life of about 12 to 24 hours in the circulation, following which they bind to selective proinflammatory adhesion molecules (for example, vascular cell adhesion molecule-1 [VCAM-1], intracellular adhesion molecule-1 and E-selectin) expressed on the aortic valve endothelium, and subsequently migrate into the subendothelial matrix. Within the valve tissue, these cells differentiate into macrophages (‘big eaters’) (Shahi et al. 1997; Ghaisas et al. 2000; Muller et al. 2000; Kaden 2007). Macrophages in turn are able to secrete a whole host of immune components (for example lysozyme, acid hydrolases, and neutral proteases), cytokines (such as tumour necrosis factor-α, interleukin(IL)-1, -8, -12, and -18), chemokines, reactive oxygen and nitrogen metabolites, colony-stimulating factors, and factors involved in fibroblast stimulation and vessel proliferation (Kasper et al. 2006). In addition to lymphocytes and macrophages; mast cells, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) have also been discovered in stenotic aortic valves, thus revealing the important role of various cellular components, cytokines, chemokines and growth factors in contributing to the development of AS (Kaden et al. 2003; Helske et al. 2004; Kaden et al. 2005a; Isoda et al. 2010).

More recently, proinflammatory cathepsin S (a potent elastase) released from macrophages has been found to accelerate valvular calcification (Aikawa et al. 2009). In addition to cathepsin S, increased expression and activity of elastolytic cathepsins K and V, and their inhibitor cystatin C have been detected in stenotic aortic valves. These cathepsins detected within the diseased valves likely contribute to accelerated destruction of the valvular ECM, thus contributing to AS progression (Helske et al.

2006a). Activated T lymphocytes, valvular endothelial cells and the VICs not only have receptors for TGF-β1, but are also able to produce increasing amounts of this growth factor, especially under inflammatory conditions (Blobe, Schiemann & Lodish 2000; Freeman & Otto 2005). This fascinating cytokine, TGF-β1, has many functions, some of which include inhibition of cell proliferation, increase in cancer cell invasiveness, stimulation of angiogenesis, and immunosuppressive, immuno-stimulatory and tumour suppressive effects. TGF-β1 is a major player in fibrotic disease and atherosclerosis, involved in ventricular remodelling post MI, myocardial regeneration, and is associated with a whole range of human diseases, for example chronic rejection in lung transplantation, gastric cancer, colorectal cancer, chronic viral hepatitis, prostate cancer, breast cancer, hypertension, osteoporosis, idiopathic interstitial pneumonitis, chronic graft-versus-host disease and veno-occulsive disease in transplant recipients (Blobe, Schiemann & Lodish 2000; Azhar et al. 2003; Ikeuchi et al. 2004; Li et al. 2005; Okada 2005). A novel study by Paranya et al. (2001) revealed fascinating results regarding the role of TGF-β1 on the valvular endothelium. The in vitro study showed that under treatment with TGF-β1 or growth in reduced serum, the aortic valve ECs (AVECs) were able to trans-differentiate into a mesenchymal phenotype (expressing α-smooth muscle actin [α-SMA]), while retaining expression for CD31. Furthermore, the study confirmed that only valvular ECs were able to trans-differentiate under these conditions, whereas ECs from venular and other microvascular sites were unable to undergo this transformation. Recently, studies have shown that during inflammation, TGF-β1 mediates the differentiation of VICs into active myofibroblasts, and also contributes to pathological valvular ECM remodelling (Walker et al. 2004). In addition, this

proinflammatory cytokine contributes to the development of valvular calcification (Mohler 2004) and angiogenesis (Pepper 1997).

Studies have indeed confirmed calcified aortic valves having higher levels of TGF-β1 (Jian et al. 2003), and neovascularisation (or angiogenesis) (Soini, Salo & Satta 2003; Chalajour et al. 2004; Mazzone et al. 2004), compared to non-calcified valves. A recent study by Syvaranta et al. (2010) revealed that mast cells and myofibroblasts secreted vascular endothelial growth factor (VEGF), thus contributing to significantly higher neovascularisation of the stenotic aortic valves, compared to the control valves. Interestingly, in vitro cell culture models of porcine (Walker et al. 2004; Kennedy et al. 2009) and sheep (Jian et al. 2003) AVICs treated with TGF-β1 showed formation of calcifying nodules, thus confirming the important role of TGF-β1 and 'activated' myofibroblasts in mediating the early steps involved in initiating aortic valve calcification. These findings were also confirmed in a study by Clark-Greuel et al. (2007) which involved sheep AVICs in culture being treated with TGF-β1. Following treatment with TGF-β1, the study found significantly increased levels of alkaline phosphatase, matrix metalloproteinase-9 (MMP-9) and increased levels of the active form of MMP-2. Thus, it is clearly established that TGF-β1 is in involved in mediating inflammation, likely leading to aortic valve fibrosis and calcification, and represents a pivotal cytokine involved in the pathogenesis of AS (Xu, Liu & Gotlieb 2010).

Matrix metalloproteinases (MMPs) are compounds that are often involved in many inflammatory diseases. These molecules result in ECM remodelling under physiological

and pathological conditions, are able to degrade all components of the ECM (Matrisian 1990; Birkedal-Hansen 1995; Curran & Murray 1999; Curran & Murray 2000), and have been implicated in atherosclerosis (Galis et al. 1994; Nikkari et al. 1995; Galis & Khatri 2002). MMPs have been found to originate from activated valvular myofibroblasts, macrophages, lymphocytes and endothelial cells (Akat, Borggrefe & Kaden 2009). In addition, tissue inhibitors of metalloproteinases (TIMPs), as the name implies, are able to inhibit MMP function, and also exhibit growth promoting properties (Stricklin et al. 1983; Hayakawa et al. 1992; Hayakawa et al. 1994). Studies have revealed the presence of MMPs and TIMPs in calcified stenotic aortic valves, thus suggesting their likely contribution towards AS development (Soini et al. 2001; Kaden et al. 2003; Kaden et al. 2005a). Kaden et al. (2005a) was able to show that activated leukocytes in stenotic human aortic valves secreted TNF-α, which likely stimulates the proliferation of valvular myofibroblasts, which in-turn expresses MMPs resulting in active ECM remodelling in AS. Furthermore, ECM remodelling, which contributes to the variability in the valvular ECM stiffness results in differentiation of VICs into distinct osteogenic or myofibrogenic phenotypes, both leading to calcification of AVICs grown in culture (Yip et al. 2009).

The renin-angiotensin-aldosterone (RAA) system has also been implicated in the development of AS. Firstly, angiotensin-converting enzyme (ACE) has been discovered in sclerotic aortic valves (O'Brien et al. 2002; Gkizas et al. 2010). ACE is a dipeptidase which converts angiotensin I (Ang I) to Ang II (a pro-fibrotic and proinflammatory peptide). It has been suggested that the local production of Ang II contributes to aortic

valve fibrosis (Helske et al. 2004). Furthermore, Ang II may also be produced by macrophages via ACE, and by mast cells via cathepsin G and chymase (O'Brien et al. 2002; Helske et al. 2004; Helske et al. 2006b). Recently, Gkizas et al. (2010) published an 8 week study involving 40 rabbits, whereby some were fed a normal diet, another group was fed a high cholesterol diet (1% cholesterol) alone, and the last group was fed a high cholesterol diet with the addition of an aldosterone inhibitor, eplerenone. The study found that aldosterone receptors were expressed similarly in the control and hypercholesterolemic aortic valves. However, aldosterone blockade resulted in a significant decrease in aortic valve atherosclerotic lesions and calcium deposition in rabbits fed a high cholesterol diet, compared to the group fed the high cholesterol diet alone (without treatment). ACE has another major function that is the break-down of a peptide called bradykinin (Winstanley & Walley 2002). Bradykinin on the other hand is able to mediate antifibrotic effects on fibroblasts. In addition to ACE, neutral endopeptidase (NEP) found in stenotic aortic valves is also able to degrade bradykinin. Interestingly, Helske et al. (2007) revealed that the expression of the pro-fibrotic NEP and bradykinin type-1 receptor was significantly increased in stenotic aortic valves. In particular, the pro-inflammatory cytokine, TNF-α, was implicated in mediating the up-regulation of NEP. Even more intriguing was the finding that NEP inhibition resulted in decreased expression of TGF-β1. Thus, all these recent studies suggest that there may be a role for poly-receptor/molecular targeted therapy, whereby blockade or inhibition of ACE, aldosterone and NEP may synergistically suppress the development and/or progression of AS.

Selected studies have also implicated chronic infection by *Chlamydia pneumoniae* (*C. pneumoniae*) resulting in chronic inflammation of the aortic valve, thus contributing to the development and progression of AS (Juvonen et al. 1997; Nystrom-Rosander et al. 1997). However, other studies failed to show a significant association between *C. pneumoniae* and calcific AS (Andreasen, Farholt & Jensen 1998; Rose 2002; Agmon et al. 2004). Thus, the association between *C. pneumoniae* contributing to the pathogenesis of AS remains uncertain.

Inflammation generally results in significant oxidative stress. In turn, once established, oxidative stress in itself often maintains and/or propagates inflammation in these tissues. Thus, a vicious cycle of oxidative stress and inflammation often contributes to disease development and progression. Liberman et al. (2008) showed that rabbits given a cholesterol-enriched diet and high dose vitamin D_2 for 3 months exhibited significantly increased levels of superoxide and hydrogen peroxide (reactive oxygen species [ROS]) in calcified rabbit aortic valves, especially close to areas of calcification, thus suggesting a role in oxidative stress contributing to AS development. In addition, the study also revealed that cells expressing osteoblastic/osteoclastic markers showed increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits Nox2, p22phox and protein disulfide isomerase, particularly around areas of calcification and highest ROS production. Another interesting finding was that rabbits given the cholesterol and vitamin D_2 diet showed significantly increased aortic valve calcification, which was unexpectedly further enhanced by the superoxide scavenger, Tempol (4-hydroxy-TEMPO). Surprisingly, Tempol-treated rabbits in this study also

showed increased hydrogen peroxide signalling, which was decreased by lipoic acid (LA). This was in contrast to the findings by Kennedy et al. (2009), which revealed decreased calcific nodule formation in porcine AVIC cultures treated with TGF-β1 and Tempol, compared to culture treated with TGF-β1 alone. The study also found that the anti-oxidant, Tempol decreased the production of superoxide. A study by Miller et al. (2008) has also concurred with previous findings that superoxide and hydrogen peroxide levels were significantly increased in stenotic human aortic valves (around calcified areas) compared to normal aortic valves, and that uncoupling of nitric oxide synthase (NOS) was related in part to the increase in superoxide production. The study also found significantly reduced total superoxide dismutase (SOD) activity, and reduced expression of catalase and all 3 SOD isoforms, within calcified areas of the stenotic valves. ROS generation by G-protein-coupled receptor agonists (Ang II, ET-1, phenylephrine) has also been shown to induce the activation of the redox-sensitive transcription factor, nuclear factor-κB (NF-κB), resulting in the development of cardiac hypertrophy. Conversely, anti-oxidants such as N-acetyl cysteine and vitamin E suppressed NF-κB activation in this system (Hirotani et al. 2002). In particular, NF-κB has been implicated in playing an important role in mediating aortic valve inflammation, leading to calcification of the valve leaflets (Elmariah & Mohler 2010b).

In a rabbit model treated with vitamin D_2, Ngo et al. (2008) revealed that stenotic aortic valves exhibited increased levels of the pro-oxidant, thioredoxin-interacting protein (TXNIP) in association with endothelial dysfunction, compared to non-stenotic aortic valves. TXNIP was first discovered in HL-60 human promyelocytic cell lines following

treatment with 1,24-hydroxyvitamin D3, and thus is also known by its alternate name, vitamin D3 up-regulated protein 1 (VDUP1). TXNIP has been shown to bind and inhibit the anti-oxidant, thioredoxin (TRX), thus playing an integral role in modulating cellular redox stress (Chen & DeLuca 1994; Nishiyama et al. 1999; Junn et al. 2000). The mechanism of inhibition occurs whereby TXNIP binds to both the catalytic cysteines of TRX, thus inhibiting its ability to bind to apoptosis signalling kinase-1 (ASK-1) (Saitoh et al. 1998). ASK-1 is a mitogen-activated protein (MAP) kinase kinase kinase, which leads to inflammation and stress-induced cell death (apoptosis) (Ichijo et al. 1997). Thus, unbound ASK-1 under the influence of TNF-α is phosphorylated, resulting in activation of ASK-1, which further activates downstream p38 and Jun-terminal kinase (JNK), leading to increased expression of vascular cell adhesion molecule-1 (VCAM-1). The expression of these cell adhesion molecules encourage adhesion of leukocytes, eventuating in inflammation and development of atherosclerosis (Yamawaki et al. 2005). Thus, Yamawaki et al. (2005) was able to show that under conditions of fluid shear stress, there was down-regulation of TXNIP, rendering free TRX to bind and inhibit ASK-1 and its down-stream proinflammatory events, thus maintaining physiological homeostasis. As such, alterations in this physiological balance likely mediates the development of other pathological conditions, including AS, whereby the turbulent circulatory blood flow on the aortic side of the valve leaflets invokes this molecular proinflammatory pathway, possibly leading to aortic valve endothelial disruption and dysfunction. In addition, TXNIP has also been found to be up-regulated in human tumour cells following stimulation by the proinflammatory cytokine, TGF-β1, which resulted in retardation of tumour cell growth (Han et al. 2003). TXNIP clearly has a

diverse range of functions, and plays a role in mediating cell-cycle regulation, development and function of natural killer (NK) cells, smooth muscle cell proliferation, tumour suppression, cell specific regulation of apoptosis, lipid metabolism and fatty acid utilisation. In addition, TXNIP is also involved in important regulatory processes pertaining to cardiac physiology, cardiac hypertrophy and myocardial cellular viability, especially under conditions of oxidative stress. However, it is vital to realise that TXNIP induces most of its effects by closely interacting with TRX, resulting in the inhibition of this major ubiquitous anti-oxidant, thus promoting intracellular oxidative stress and inflammation (Kim et al. 2007; Watanabe et al. 2010). The TXNIP-TRX system has also been implicated in regulating important endothelial cell mechanisms, such as modulating angiogenic processes, and mediating EC survival, migration and proliferation (Dunn et al. 2010). Interestingly, TXNIP has been found to suppress TNF-α-induced NF-κB activation in hepatogenesis, and conversely, the absence of TXNIP results in TNF-α-induced NF-κB activation leading to increased cell proliferation, and subsequently contributing to the development of hepatocellular carcinoma (Kwon et al. 2010). However, TRX appears to exhibit dual and opposing roles in NF-κB regulation, whereby over expression of TRX within the cytoplasm suppresses the degradation of IκB, thus maintaining NF-κB in an inactivated form bound to IκB. Conversely, TRX within the nucleus reduces the cysteine residues of NF-κB, resulting in the enhancement of NF-κB transcriptional activities (Hirota et al. 1999). Thus, it is likely that the TXNIP-TRX system is involved in the regulation of NF-κB within valvular cells, possibly contributing to inflammation and calcification of the aortic valve leaflets. The TRX system, which consists of TRX, TRX reductase and NADPH, is a ubiquitous thiol-

reducing system that a plays a vital role in regulating cellular redox stress. TRX in turn has been shown to be involved in vascular smooth muscle cell proliferation, protecting endothelial cells from ROS-induced cytotoxicity, exhibits anti-oxidant activity in atherosclerosis, promoting post-ischaemic ventricular salvage and MI size reduction, decreasing reperfusion-induced arrhythmias, and has been implicated in many pathological conditions, especially when TRX is down-regulated or its activity decreased. Furthermore, TRX plasma levels have also been found to be significantly elevated in many cardiac conditions, which includes acute coronary syndrome (including MI), dilated cardiomyopathy, angina and chronic heart failure (World, Yamawaki & Berk 2006). However, the collective contributions of the TXNIP-TRX system in the pathogenesis of aortic valve calcification or AS still remain a mystery. Thus, this thesis will attempt to shed some light on this important question by evaluating this system in response to treatment with the pro-inflammatory cytokine, TGF-β1, in conjunction with the potent vasodilator, nitric oxide (NO). Figure 1.2 illustrates some of the cellular functions of the TXNIP-TRX system.

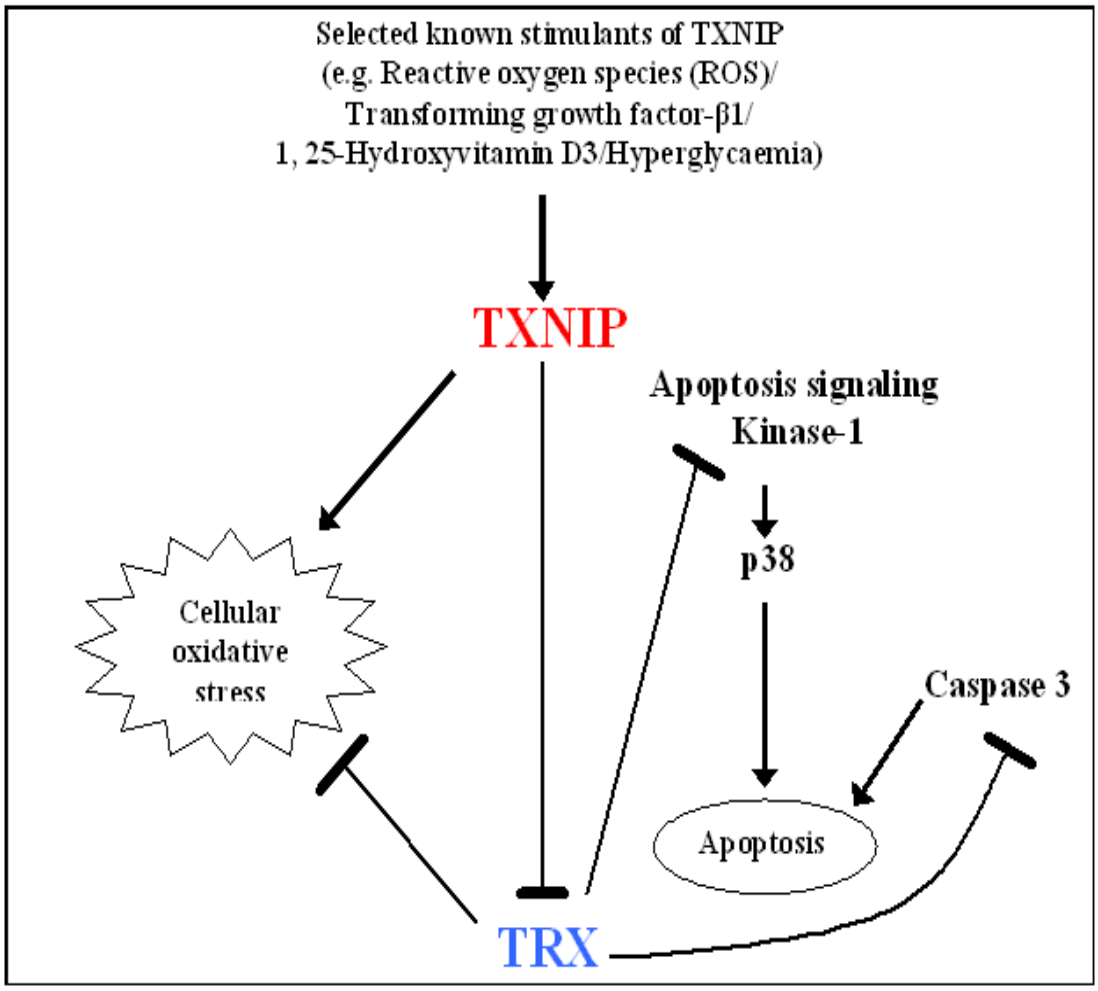

Figure 1.2 Schematic diagram illustrating selected cellular functions/interactions of the TXNIP-TRX system

Another interesting development in AS is the role of the pro-inflammatory hormone, resistin, which is produced by adipocytes, monocytes and macrophages (Jung et al. 2006). Resistin has been linked to insulin resistance, atherosclerosis and coronary calcium content (Steppan & Lazar 2004; Reilly et al. 2005). Mohty et al. (2010) in a recent study involving 114 patients operated for severe AS found that plasma resistin levels were significantly higher in elderly patients (≥ 70 years old, $p<0.0004$), compared

to middle-aged patients (< 70 years old). In addition, the study also found that higher plasma resistin levels in elderly patients were associated with increased AVC and inflammation. Further confirmation that inflammation was involved in the pathogenesis of AS came with the discovery that high sensitivity C-reactive protein (hs CRP) was significantly elevated, and found to be an independent factor associated with severe non-rheumatic AS (in patients free of coronary, carotid or peripheral vascular disease) (Galante et al. 2001), compared to control subjects without AS. Other studies have since confirmed these findings (Sanchez et al. 2006; Jeevanantham et al. 2007; Imai et al. 2008).

In conclusion, it is evident that inflammation, probably precipitated by imbalances between oxidant and anti-oxidant mechanisms, stimulation of proinflammatory cytokines and other factors, and/or induction of inflammatory mechanisms, all generally promote oxidative stress in cells and tissues, thus contributing towards the pathogenesis of AS.

1.5.3 The possible role of atherogenesis in aortic valve stenosis

Lipid accumulation is one of the major hallmarks of vascular atherosclerosis; and as early as four decades ago, researchers had proposed that the lesions present within sclerotic aortic valves were somewhat 'atherosclerotic' in nature (Walton, Williamson & Johnson 1970). Since then, many researchers have also concurred that several features of valvular lesions found in AS are similar to that found in atherosclerosis (Otto et al. 1994; Olsson, Thyberg & Nilsson 1999; O'Brien 2006; Rajamannan 2006; Kaden 2007; Rajamannan 2009; Elmariah & Mohler 2010b). Interestingly, Kuusisto et al. (2005) undertook a necropsy study examining aortic valves from 48 consecutive unselected individuals. None of the study subjects had any evidence of AS, but the study nonetheless revealed that atherosclerotic-like lesions were common in individuals of all age groups, even in young individuals without AS aged between 20 to 40 years; thus concluding that this common phenomenon that often begins in early adulthood may be associated with AS development at a later stage. After all, many of the 'traditional' cardiovascular risk factors of atherosclerosis are also involved in the development and progression of AS, adding further support to this hypothesis. Guerraty et al. (2010) in a study involving adult male swine fed either a hypercholesterolemic or normal diet for a period of 2 weeks and 6 months, showed distinct areas of extracellular subendothelial lipid insudation and scattered early calcific nodules restricted to the aortic side of the valve leaflets in swine fed a hypercholesterolemic diet for 2 weeks, with lesions being more extensive at 6 months. Additionally, many genes were differentially expressed between the aortic and ventricular ECs (1,325 versus 87) of the aortic valve, thus revealing distinct side-specific EC phenotypes. Furthermore, hypercholesterolemic

models induced a protective endothelial phenotype on the aortic side of the valve compared to non-hypercholesterolemic models, whereby there was down-regulation of TNF-α, NF-κB, nuclear receptor co-repressor 2 (a peroxisome proliferator-activated receptor-γ [PPAR-γ] repressor), bone morphogenic protein-4 (BMP-4), annexin A2 and TGF-β1 genes, and up-regulation of glutathione peroxidases 1 and 4, PPAR-γ activator nuclear receptor co-activator 1, and inhibitors of NF-κB (IκA and IκB). Thus, the study by Guerraty et al. (2010) clearly illustrates the protective mechanisms invoked by ECs on the aortic side of the valve leaflets under conditions of turbulent flow and hypercholesterolemia. However, these models were only studied for a period of 2 weeks and 6 months, whereas the prolonged environmental (for example turbulent flow and hypertension) and chemical insults (for example smoking and hypercholesterolemia) experienced by the aortic valve tissue over many years likely results in the eventual loss of these protective mechanisms, possibly contributing to inflammation, fibrosis and eventual calcification of the aortic valve leaflets. With regards to advanced disease, Otto et al. (1994) revealed the presence of subendothelial extracellular lipid accumulation within stenotic aortic valves, which was also confirmed in an earlier study by Sarphie (1986) involving hypercholesterolemic rabbits. Subsequently, the presence of lipoproteins within stenotic human aortic valves was confirmed in later studies (O'Brien et al. 1996; Olsson, Thyberg & Nilsson 1999).

A study involving rabbits fed vitamin D plus a high cholesterol diet resulted in the development of AS (Drolet, Arsenault & Couet 2003), which was also confirmed in a more recent study by Libermann et al. (2008) revealing significant development and

progression of aortic valve calcification in rabbits given a cholesterol-enriched diet and vitamin D_2 for a period of 3 months. Hypercholesterolemia was shown to induce severe AS in genetic knockout mice lacking the gene for the LDL receptor (Weiss et al. 2006). Further support that hypercholesterolemia might contribute to AS development was the discovery that familial hypercholesterolemia contributed to 'atherosclerotic-like' lesions being present on aortic valves, leading to the development and progression of AS (Goldstein & Brown 1973; Sprecher et al. 1984; Kawaguchi et al. 1999).

Many individuals with calcific AS or CAD tend to have normal LDL cholesterol plasma levels (Lamarche et al. 1995), but have disproportionately higher levels of circulating small dense LDL particles (Lamarche, Lemieux & Despres 1999). Tribble et al. (1992) and Bjornheden et al. (1996) suggested that these small dense particles are more able to penetrate tissues and have an increased susceptibility to oxidation, thus making it more atherogenic than the other LDL components. Oxidized LDLs (oxLDLs) in turn have pro-inflammatory and growth promoting attributes, are able to initiate and propagate atherosclerosis, and also have the ability to induce mineralisation and osteoblastic phenotype in vascular cells (Parhami et al. 1997; Hansson 2005; Tedgui & Mallat 2006). Mohty et al. (2008) in a recent study examining 102 explanted severely stenotic aortic valves revealed that increased amounts of circulating small dense LDL particles were associated with an increased AS progression rate and higher accumulation of oxLDLs within those valves. In addition, the study revealed that valves containing the highest amount of oxLDLs had increased expression of TNF-α, and also had significantly higher densities of macrophages (CD68+), leucocytes (CD45+) and T-lymphocytes (CD3+);

thus providing further evidence regarding the proinflammatory nature of these oxidized particles.

Not only does hypercholesterolemia contribute to 'atherosclerotic-like' lesions developing in aortic valves, but also contributes to calcification of the valve leaflets via the Wnt/β-catenin pathway. The Wnt protein acts on the Frizzled (FZD) proteins and the LDL receptor-related protein 5 and 6 (Lrp 5 & 6) co-receptors, causing inhibition of β-catenin degradation. β-catenin in turn is a major player in mediating the effects of Wnt (Clevers 2006). Thus, hypercholesterolemia causes an increase in Lrp5, Wnt and β-catenin in calcified aortic valves, which reveals an important pathway that mediates calcification in AS. In particular, the up-regulation and activation of Lrp5 on VICs is not just related to valve calcification, but likely leads to aortic valve bone formation as well (Rajamannan et al. 2005a; Shao et al. 2005; Caira et al. 2006).

Many animal (Rajamannan et al. 2002; Rajamannan et al. 2005a; Rajamannan et al. 2005b) studies have found that statins reduce the development and/or progression of AVC or AS. Furthermore, a number of non-randomised human observational studies (Aronow et al. 2001; Novaro et al. 2001; Bellamy et al. 2002; Rosenhek et al. 2004b; Osman et al. 2006a; Osman et al. 2006b) supported these conclusions. However, recently published prospective randomised human trials failed to show any significant benefit (Cowell et al. 2005; Rossebo et al. 2008; Chan et al. 2010). Nonetheless, the evidence overall points to some importance in lipids possibly contributing to the development of AS, especially in the early phase of the disease.

1.5.4 The pivotal role of calcification or mineralisation, and 'bone-like' formation in aortic valve stenosis

Calcification and 'bone-like' formation of the trileaflet aortic valve are major hallmarks in calcific valvular AS, which are particularly apparent in all cases of severe AS. In the early stages, turbulent blood flow on the aortic side of the valve cusps and associated hypercholesterolemia, lead to progressive endothelial disruption and dysfunction as manifested by decreased eNOS expression; thus contributing to early mineralisation of the aortic valve (Rajamannan et al. 2005b; Charest et al. 2006). The inflammatory cells, especially the activated T-lymphocytes and myofibroblasts are able to secrete significant amounts of TGF-β1 and TNF-α, and along with IL-1β, result in increased production of bone morphogenic proteins (especially BMP-2 and -4) and MMPs, which in turn cause ECM remodelling, fibrosis, and eventual calcification and bone formation of the aortic valve leaflets (Mohler et al. 1999; Mohler et al. 2001; Jian et al. 2003; Kaden et al. 2003; Kaden et al. 2004a; Kaden et al. 2005a).

In separate cell culture models involving human and canine AVICs, Mohler et al. (1999) discovered that a selected population of the AVICs underwent spontaneous phenotypic differentiation into osteoblast-like cells (stained positively for bone matrix proteins) and formed distinct calcific nodules, which contained hydroxyapatite crystals and dead myofibroblasts. The nodules were also found to exhibit an outer ring of living osteoblast-like cells. In addition, the study also showed that the rate of nodule formation was significantly increased following the addition of TGF-β1 and 25-hydroxycholesterol, compared to untreated controls. Following this, several recent in vitro studies (Jian et al.

2003; Clark-Greuel et al. 2007; Kennedy et al. 2009) have conclusively revealed that AVICs treated with TGF-β1 resulted in significant migration and aggregation of these cells, causing formation of apoptotic-alkaline phosphatase (ALP) enriched nodules, eventuating in progressive calcification of these nodules. Chen et al. (2009) in a study involving cultured porcine AVICs revealed high subpopulation frequencies of mesenchymal progenitors and osteoprogenitors. The mesenchymal progenitors were able to differentiate to osteogenic and chondrogenic lineages, while the osteoprogenitors were able to individually expel bone matrix; thus exposing the propensity of these progenitor cells in contributing to aortic valve calcification. In addition, Yip et al. (2009) showed that porcine AVIC differentiation into osteoblast-like cells or myofibroblasts are distinct processes related to valvular ECM stiffness, both resulting in formation of calcified nodules.

As mentioned earlier, a series of cytokines are up-regulated in AS, leading to increased production of BMP-2, which is found in significant amounts within stenotic aortic valves (Mohler et al. 2001; Jian et al. 2003; Kaden et al. 2003; Kaden et al. 2004a). BMP-2 is then able to activate the transcription factor Msx2, which in turn activates Wnt signalling, resulting in initiation of the osteogenic pathway. BMP-2 is also capable of initiating the chondro-osteogenic pathway via a major transcription factor of bone formation and osteoblast differentiation, known as Cbfa1 (or Runx2) (Banerjee et al. 2001; Krane 2005; Shao et al. 2005). Signalling via the Wnt/Lrp5/β-catenin pathway is essential for formation and proliferation of preosteoblast cells (He et al. 2004). As mentioned previously, this pathway is also invoked in hypercholesterolemia, which

contributes to progressive calcification and bone formation of the trileaflet aortic valve (Rajamannan et al. 2005a; Shao et al. 2005; Caira et al. 2006).

As discussed in the earlier sections, the redox-sensitive transcription factor, NF-κB, plays an important role in mediating valvular inflammation, leading to aortic valve calcification. ROS (Hirotani et al. 2002), IL-1 and TNF-α (Baud & Karin 2001; Kwon et al. 2010), all of which have been detected in significant amounts within diseased aortic valves (Kaden et al. 2003; Kaden et al. 2005a; Miller et al. 2008), mediate the up-regulation of NF-κB in calcified and stenotic aortic valves. β_2-adrenergic receptors (β_2-ARs) have been shown to play an important role in regulating bone resorption and deposition (Suda et al. 1995). Stimulation of these receptors on osteoblasts result in reduced bone formation and increased osteoclastogenesis. In addition, activation of the β_2-ARs stimulates the production of receptor activator of nuclear factor-κB ligand (RANKL), which then binds to RANK on pre-osteoclasts to induce formation of mature osteoclasts leading to bone resorption (Moore et al. 1993; Jimi et al. 1999; Teitelbaum & Ross 2003; Elefteriou et al. 2005). Immunohistochemical analysis of calcified aortic valves demonstrated a high expression of β_1-ARs, β_2-ARs, β_3-ARs and RANK. In addition, human AVICs in culture treated with β_2-agonists showed a significant 5-fold decrease in ALP activity, and a reduction in protein expression of ALP, Cbfa1 and periostin (Osman et al. 2007). Thus, it has been suggested that therapeutic β-AR agonists may prove beneficial in preventing aortic valve calcification. Recently, it has been discovered that toll-like receptors 2 and 4, which are important in mediating inflammation and innate immune system responses, are expressed in significant amounts

in human AVICs. Toll-like receptor (TLR) agonists have also been shown to up-regulate BMP-2 (Meng et al. 2008) in AVICs. Yang et al. (2009) revealed that human aortic valves expressed more TLR2 and TLR4, and that stimulation of these receptors resulted in AVICs expressing higher levels of BMP-2 and Cbfa1/Runx2, along with increased ALP activity and calcified nodule formation. Hence, increased TLR expression and stimulation was linked to osteogenic phenotypic changes in AVICs and NF-κB activation, and thus likely contributing to aortic valve calcification (Meng et al. 2008; Yang et al. 2009).

Receptor activator of NF-κB ligand (RANKL) binds to its receptor RANK on precursor osteoclasts to initiate its differentiation into mature osteoclasts (Teitelbaum & Ross 2003). Osteoprotegerin (OPG) on the other hand is a soluble decoy receptor that binds RANKL, thus preventing it from binding to RANK. Importantly, OPG has been found to inhibit bone resorption, and so its deficiency results in a significantly higher rate of bone loss. OPG and RANKL have both been found to be produced by osteoblasts (Krane 2005). OPG deficiency has been associated with severe vascular calcification, and to an increased expression of RANK and its ligand in the calcified regions. Thus, OPG may in fact be protective and inhibit calcification processes, whereas RANKL may contribute to its development (Bucay et al. 1998; Min et al. 2000). Kaden et al. 2004b revealed that RANKL was significantly expressed in stenotic human aortic valves in contrast to normal control valves, whereas OPG expression was increased in normal aortic valves but significantly lower in stenotic valves. In addition, human AVICs treated with RANKL exhibited significantly increased levels of matrix calcification, ALP activity

and expression, nodule formation, and increased DNA binding of Cbfa1, compared to untreated controls. In addition to promoting aortic valve calcification, RANKL has also been implicated in promoting human AVIC proliferation, and causing an increase in MMP-1 and MMP-2 activity and expression in these cells, thus contributing to ECM remodelling; an important step in AS development (Kaden et al. 2005b). RANKL has also been shown to significantly increase MMP expression in other cell lines, such as in engineered human breast tumour cells (Rucci et al. 2010).

A serum protein, alpha 2-Heremans-Schmid glycoprotein or also known as fetuin-A, is able to significantly inhibit ectopic tissue calcification (Schafer et al. 2003). Studies have revealed that patients with concurrent AS and no diabetes mellitus (DM) have significantly lower serum fetuin-A levels, compared to those without AS and DM (Ix et al. 2007b). Kaden et al. (2007) also showed similar results whereby fetuin-A levels were significantly lower in patients with AS, compared to non-AS controls. These findings were again confirmed in a recent study by Ferrari et al. (2010) showing significantly lower serum levels of fetuin-A in patients with AS, compared to healthy controls. A prospective study involving 238 peritoneal dialysis patients showed that low serum fetuin-A levels were associated with valvular calcification, malnutrition, inflammation and atherosclerosis (Wang et al. 2005). Besides its contribution to AS, Stenvinkel et al. (2005) subsequently revealed that low serum fetuin-A levels in patients with end-stage renal disease (ESRD) were also significantly associated with malnutrition, inflammation and atherosclerosis. Alarmingly, low serum fetuin-A levels in this cohort were significantly associated with cardiovascular and all-cause mortality. Thus, this important

glycoprotein that is synthesized by the liver clearly plays a role in modulating systemic and valvular calcification; thus likely contributing to AS pathogenesis when in short supply.

Recently, tissue factor (TF), anti-calcific osteopontin (OSP) and the thrombin-cleaved pro-inflammatory OSP N-half have been found to be significantly expressed and associated with areas of calcification on the aortic surfaces of severely stenotic aortic valves. Thus, it has been suggested that TF-initiated coagulation results in thrombin generation, which subsequently cleaves OSP and generates the pro-inflammatory OSP N-half that possibly contributes to aortic valve calcification (Breyne et al. 2010). More recent work has revealed a significant increase in the expression of osterix (Osx), SRY-related transcription factor Sox9 and nuclear factor of activated T cells c1 (NFATc1) in VICs of stenotic human aortic valves, compared to normal non-diseased valves (Alexopoulos et al. 2010). Osx is an osteoblast-specific transcription factor acting downstream of Cbfa1, which is able to induce bone formation, a process that is further enhanced by associating with NFATc1 (Stern 2006; Caetano-Lopes, Canhao & Fonseca 2007). Sox9 acts upstream of Cbfa1 and is involved in chondrocyte differentiation and bone formation, and has been implicated in aortic valve calcification (Alexopoulos et al. 2010; Chakraborty, Combs & Yutzey 2010; Peacock et al. 2010). Thus, the newly discovered link between these transcription factors and AS adds further evidence that these important molecular mediators likely contribute towards processes involved in aortic valve calcification and bone formation.

Over time, the aortic valve exhibiting atherosclerotic-like lesions, expresses osteopontin, osteocalcin, alkaline phosphatase, bone sialoprotein, Cbfa1, Sox9 and Osterix (Osx) and a whole range of other proteins and factors, which together likely promote ongoing calcification and bone formation of the tricuspid aortic valve (O'Brien et al. 1995; Mohler et al. 1997; Rajamannan et al. 2001; Rajamannan et al. 2002; Rajamannan et al. 2003b; Kaden et al. 2004a; Rajamannan et al. 2005a; Alexopoulos et al. 2010; Ferrari et al. 2010). Thus, mature lamellar bone, extensive ossification, endochondral bone formation, microfractures and active bone remodelling are often present in these severely stenotic aortic valves (Mohler et al. 2001).

1.6 Histo-pathological changes associated with aortic valve sclerosis / aortic valve stenosis

In the early phase of the disease, there is usually associated mild calcification and some thickening of the valvular leaflets. In addition, due to the persistent turbulent blood flow on the aortic side of the leaflets, there is visible disruption of the valvular endothelium and formation of atherosclerotic-like lesions on that side extending into the underlying fibrosa (Otto et al. 1994). In addition, there is significant accumulation of LDLs, lipoprotein (a) and oxLDLs localised within these lesions (O'Brien et al. 1996; Olsson et al. 1999; Mohty et al. 2008). In particular, the AS valves with the most amount of oxLDLs have been found to contain the highest number of leukocytes, macrophages and T-lymphocytes (Mohty et al. 2008). The presence of inflammatory cells within stenotic valves has also been confirmed in many other studies (Olsson et al. 1994; Wallby et al. 2002; Alexopoulos et al. 2010; Gkizas et al. 2010).

In addition, the ECM remodelling, fibrosis and calcification visible in stenotic aortic valves are in part primarily due to the actions of specific cytokines and proteins, namely TGF-β1, IL-1β, TNF-α, MMP-1, MMP-2 and MMP-9, which have been detected in significant amounts within these diseased valves (Soini et al. 2001; Jian et al. 2003; Kaden et al. 2003; Kaden et al. 2005a; Clark-Greuel et al. 2007). In advanced disease, the progressive inflammation and ongoing ECM remodelling result in significant disarray of collagen fibres throughout the valve leaflets (Kaden et al. 2005a). Charest et al. (2006) studied 30 surgically excised aortic valves with degenerative AS and

compared it with 20 normal aortic valves, and found significant development of blood vessels (neovascularization) and increased expression of SPARC (secreted protein, acidic and rich in cysteine/osteonectin) only in diseased valves. In addition, the blood vessels were located near calcified nodules, under the leaflet border and in rich cellular areas of CD-45+ cells forming cell islands. Soini, Salo and Satta (2003) and Alexopoulos et al. (2010) have also revealed significant angiogenesis present within stenotic aortic valves.

In advanced or severe AS, the diseased valves often exhibit dystrophic calcification (consisting of hydroxyapatite and calcium phosphate), presence of mature lamellar bone with active bone remodelling, microfractures and haematopoietic elements, and also contains myofibroblasts and osteoblast-like cells expressing significant amounts of BMP-2 and 4. In addition, stenotic valves with co-existent bone formation have been found to occasionally contain active or quiescent osteoblasts, and sometimes even osteoclasts (Mohler et al. 2001; Rajamannan et al. 2003b). Thus, the combination of fibrosis, progressive calcification and bone formation of the diseased aortic valve contributes to ongoing narrowing of the aortic valve area to a critical point, resulting in progressively severe obstruction to LV outflow, which confers to an extremely dire set of consequences; mostly resulting in early death when left untreated.

1.7 Pathophysiology of aortic valve stenosis

During the early stage of the disease, ASc poses no significant obstruction to LV outflow. However, with the passage of time, there is progressive fibrosis, calcification and bone formation of the aortic valve, resulting in a significant decrease in the aortic valve area (AVA); whereby AS becomes well established. When the AVA is less than 1.5 cm^2, the obstruction to LV outflow becomes more pronounced resulting in ongoing LV pressure overload, along with the development of a more significant pressure gradient across the aortic valve. Myocardial contractility increases to enable pumping of blood through the ever decreasing aortic orifice in order to maintain a sufficient enough cardiac output and mean arterial blood pressure. Thus, the myocardium increases its contractile force by increasing its muscle mass, that is by undergoing compensatory concentric hypertrophy. Concentric hypertrophy in itself has deleterious effects on the myocardium. It results in the development of LV diastolic dysfunction that impairs coronary perfusion and also decreases coronary reserve, thus collectively contributing to ongoing myocardial ischaemia. In addition to this, coronary perfusion is also affected by improper closure of the stenotic aortic valve, which causes inadequate vortices within the Valsalva sinuses, resulting in suboptimal conditions for proper opening and perfusion of the coronary vessels.

Recently, Lin and Chu (2010) published a report regarding a 79 year man with severe AS, who suffered an acute ST-elevation MI despite having angiographically normal coronary arteries. In addition to the circumstances discussed above that have an effect on

coronary perfusion, reserve and flow, the authors also suggested that increased thrombin generation, platelet activation and hyper-aggregability in patients with AS (Chirkov et al. 2002; Chirkov et al. 2006), likely predisposed to the development of acute coronary thrombosis, thus collectively precipitating an acute MI in this elderly individual.

Furthermore, the development of LV hypertrophy can sometimes by maladaptive, resulting in decreased LVEF and cardiac output caused by excessive ventricular afterload. The 'so-called' compensatory myocardial hypertrophy actually causes inefficient or decreased myocardial contractility and EF, especially when there is inadequate myocardial hypertrophy resulting in increased wall stress coupled with a high afterload. Thus, the heart continues to progressively hypertrophy to compensate for the ever increasing LV afterload until a point is reached, often occurring when the AVA in less than 1.0 cm^2 and the mean valve gradient exceeds 40 mm Hg, whereby the left ventricle begins to progressively dilate due to ongoing myocardial ischaemia and its inability to cope with the increasing pressure. As AS becomes progressively more severe and if left untreated, severe systolic heart failure ensues along with classical symptoms of dyspnoea, angina, syncope and lethargy. These symptoms can occur at any stage of AS, but are particularly more frequent and pronounced in severe AS, conferring an extremely poor prognosis to the affected individual (Hachicha et al. 2007; Bonow et al. 2008; Grimard & Larson 2008; Baumgartner & Otto 2009; Carabello & Paulus 2009; Kurtz & Otto 2010; Maganti et al. 2010).

1.8 Clinical status in aortic valve stenosis

Patients with isolated mild or moderate AS are often asymptomatic, and some patients with severe AS remain asymptomatic for many months or years. In individuals with truly asymptomatic AS, the overall prognosis tends to be fairly comparable to that of the general population, whereby there is a less than 1% per year risk of sudden death (Turina et al. 1987; Horstkotte & Loogen 1988; Kelly et al. 1988; Pellikka et al. 1990; Rosenhek et al. 2000). When patients do become symptomatic, decreased exercise tolerance is often the first symptom manifested in AS. Many then develop symptoms typical of heart failure including angina, dyspnoea, syncope and fatigue. In addition, other associated symptoms that commonly occur are dizziness, arrhythmias and generalised weakness. Angina often occurs due to increased myocardial oxygen demand of the hypertrophied ventricle, reduced coronary perfusion pressure and reserve, and the inability of the heart to maintain sufficient cardiac output to meet the myocardial oxygen demands during periods of exertion or exercise, even in the absence of significant coronary artery disease. Syncope on the other hand often occurs during periods of exercise, whereby the narrowing of the aortic orifice limits the heart from increasing its stroke volume to reach an appropriate cardiac output, and thus limiting its ability to maintain an adequate mean arterial blood pressure to allow for adequate cerebral perfusion. It has also been suggested that an abnormal LV baroreceptor response as a result of significantly increased intraventricular pressure during exercise, results in inappropriately excessive peripheral vasodilatation, thus causing a sudden drop in cerebral perfusion secondary to an acute drop in mean arterial blood pressure, thus

eventuating in syncope. Arrhythmias, in particular atrial fibrillation (AF), can often co-exist with AS. It is usually precipitated by the development of cardiac failure that occurs secondary to long-standing moderate to severe AS. Thus, co-existent AF in patients with AS is often poorly tolerated due to the loss of atrial contraction coupled with a rapid ventricular rate, both contributing to decreased ventricular filling during diastole, thus further exacerbating the failing heart and contributing to ongoing myocardial ischaemia. Other arrhythmias, such as ventricular tachycardia or fibrillation can also occur in patients with AS, but are less common.

Another symptom that is sometimes overlooked in AS patients is that of bleeding, often involving angiodysplastic lesions within the gastrointestinal tract. This condition that is due to acquired von Willebrand deficiency, which is also known as Heyde syndrome, is proposed to occur secondary to the breakdown of large von Williebrand multimers that passage through the severely stenotic aortic valve under conditions of increased turbulent flow forces. As a result of the breakdown of these complex molecules, which are an integral part of the clotting cascade, the affected individual has a much greater propensity to bleed (Marcus et al. 1982; Richards et al. 1984; Vincentelli et al. 2003; Carabello & Paulus 2009; Kurtz & Otto 2010; Maganti et al. 2010). In a major landmark study by Ross and Braunwald (1968), it was revealed that individuals with symptomatic AS were conferred a particularly poor prognosis, whereby AS patients with syncope and angina survived on average for 3 years, those with dyspnoea for 2 years, and patients with associated heart failure survived 1.5 to 2 years on average.

1.9 Possible preventative / medical therapies in aortic valve stenosis: advances to date

Over recent decades, many researchers have evaluated the possible role of various medical therapies in arresting the progression and/or preventing the development of AS. In current practice, medical therapy is aimed primarily at treating AS patients with other associated cardiovascular risk factors, such as hypertension and dyslipidaemia. Thus, it is not utilised in AS patients without any of these risk factors. Some of these therapies will be further evaluated in this section.

HMG-CoA reductase inhibitors ('statins'): As previously discussed in the earlier sections of this introductory chapter, statins, have been shown to be an effective therapy in preventing the onset and/or progression of AS. However, many of these studies showing the benefits of statin therapy have mainly involved several animal studies, non-randomised human observational studies and retrospective cohort studies (Aronow et al. 2001; Novaro et al. 2001; Bellamy et al. 2002; Rajamannan et al. 2002; Rosenhek et al. 2004b; Freeman & Otto 2005; Rajamannan et al. 2005a; Rajamannan et al. 2005b; Osman et al. 2006a; Osman et al. 2006b). Recently published major randomised prospective human placebo-controlled trials have conclusively failed to reveal any significant benefit of statin therapy in preventing or treating AS (Cowell et al. 2005; Rossebo et al. 2008; Chan et al. 2010). It is important to note that many of these trials involved patients with advanced disease, and thus, it is still possible that treatment with

statins during the early stages of disease may still prove beneficial (Xu, Liu & Gotlieb 2010; Miller, Weiss & Heistad 2011).

Angiotensin-converting enzyme inhibitors (ACEIs) *or* ***Angiotensin-2 receptor blockers (A2RBs)***: Helske et al. (2004) suggested that local production of angiotensin II contributed to valvular fibrosis. Several retrospective studies have indeed revealed that ACEIs reduced the rate of aortic valve calcification, but surprisingly failed to reveal any reduction in haemodynamic progression of the disease (Grainger, Witchell & Metcalfe 1995; O'Brien et al. 2005). In addition, a retrospective study by Rosenhek et al. (2004b) revealed that ACEIs failed to slow the progression of AS. Despite this, patients with AS appear to exhibit improved haemodynamics when treated with ACEIs. Currently, the possible role of ACEIs or A2RBs in the treatment of AS, are respectively being evaluated in separate randomised trials (Salas et al. 2011).

Other treatments such as *bisphosphonates* (Skolnick et al. 2009) and *aldosterone inhibitors* (Gkizas et al. 2010) have revealed some promising results, but more studies are required to prove their benefit in the treatment of AS.

In conclusion, despite decades of advances in medical research and technologies, AVR surgery still remains the definitive gold standard treatment in patients with symptomatic or severe AS, thus significantly improving the survival rates and quality of life of those patients (Maganti et al 2010; Belkin et al. 2011). However, a considerable number of individuals with symptomatic or severe AS are denied surgery due to their advanced age

or associated co-morbidities, consequently consigning them to an early death (Iung et al. 2005; Bach et al. 2009; Chitsaz et al. 2011). The advent of trans-catheter aortic-valve replacement (TAVR) is showing promise, especially in those considered unsuitable for surgery. Treatment of AS patients with TAVR has been proven to significantly improve survival rates, compared to those who are managed medically. However, it is still early days for this technology, which is considerably more expensive than conventional AVR surgery, and also has significantly higher associated peri-procedural risks including mortality, especially in less experienced hands (Svensson 2008; Kurtz & Otto 2010; Liff, Babaliaros & Block 2011; Smith et al. 2011). Thus, the possibility of developing medical therapies directed towards slowing or ceasing the progression, or even reversing and preventing the development of AS, would be somewhat of a miracle, especially in the elderly with multiple co-morbidities who have been denied surgery.

1.10 Objectives of the current study

As previously explored throughout this introduction, AS is not just due to simple degenerative calcification, but rather an interplay involving a series of complex patho-physiological mechanisms. However, the role of oxidative stress in the pathogenesis of AS has not been fully explored. Thus, the aim of this study was to further delineate the role of redox/oxidative stress in the development of aortic valve (AV) cell calcification/nodular formation, which contributes to the development of AS. Specifically, this study examined the role of the ubiquitous anti-oxidant (thioredoxin, TRX) and the pro-oxidant (Thioredoxin-interacting protein, TXNIP), and their relative contributions in the development of AV cell calcification/nodular formation. In addition, the effects of oxidative stress on AVIC survival were also studied. More explicitly, the study's experimental components included:

1. A porcine AV tissue culture model consisting of aortic valve interstitial cells (AVICs) was established, which was utilised as a representative model of AV calcification. Subsequently, stimulation of calcification/nodular formation was induced by the pro-calcific cytokine, TGF-β1, as described in previous studies involving porcine AVICs (Walker et al. 2004; Kennedy et al. 2009). Furthermore, potential modulation by TGF-β1-induced calcification/nodular formation on the endogenous anti-oxidant, TRX, and the pro-oxidant, TXNIP, were quantified in this system. The TXNIP-TRX system was also evaluated in response to potential modulation by the TGF-β1 antagonist, SB431542, which is a highly specific and

potent inhibitor of the TGF-β-superfamily type I activin receptor-like kinase (ALK) receptors (ALK4, 5 and 7).

2. DETA-NONOate (NO donor) was added in selected experiments, and its effects on AV calcification/nodular formation relative to changes in intracellular TRX activity and TXNIP levels, were further examined.

3. Cell survival was assessed in porcine AVIC culture models treated with TGF-β1 +/- DETA-NONOate, and the corresponding contributions of the TXNIP-TRX system to changes in cell survival in these experiments were evaluated.

CHAPTER 2

MATERIALS

&

METHODS

2.1 Materials

2.1.1 Materials for primary cell culture preparation

Medium components

- Penicillin/streptomycin [Invitrogen Corporation, UK]. Stock solutions stored at -20°C.
- Fetal calf serum (FCS) [Invitrogen Corporation, UK]. Stock solutions stored at -20°C.
- Low glucose (5.56 mM) Dulbecco's modified Eagle's media (DMEM) [Invitrogen Corporation, UK]. Stock sachets stored at 4°C.
- Sodium bicarbonate [Merck Pty Ltd, Australia]. Powder stored at room temperature.

Medium solution preparation

1 sachet of DMEM was added into a bottle containing 1L of milli Q water, and mixed thoroughly. Penicillin/streptomycin, FCS and 3.7g of sodium bicarbonate were added to the DMEM solution. Subsequently, the solution was sterilised within a sterile laminar flow cabinet [sterile hood] (Gelman Sciences, Australia) utilising a 0.2μm filter (Pall Corporation, USA). Following sterilisation, the media was stored in a fridge at 4°C.

Digest solution (collagenase) components

- Collagenase (0.4mg/ml) [Invitrogen Corporation, UK]. Stored at 4°C.
- Lima Bean trypsin inhibitor (0.5mg/ml) [Invitrogen Corporation, UK]. Stored at 4°C.
- Fatty acid free bovine serum albumin (BSA) (1mg/ml) [Invitrogen Corporation, UK]. Stored at 4°C.
- [Calcium and magnesium] free Hanks' Balanced Salts [Sigma-Aldrich Inc, USA]. Powder stored at 4°C.

Digest solution (collagenase) preparation

To make 20mls of digest solution, 8.0mg of collagenase (0.4mg/ml), 10mg of Lima Bean trypsin inhibitor (0.5mg/ml) and 20mg of fatty acid free BSA (1mg/ml) were dissolved in the [calcium and magnesium] free Hanks' Balanced Salts solution, and subsequently sterilised within the sterile hood utilising the 0.2μm filter. The digest solution was prepared approximately 30 minutes prior to use.

2.1.2 Materials for cell culture passage

Digest solution (trypsin + EDTA) components

- Ethylenediamine tetra-acetic acid (EDTA) [Invitrogen Corporation, UK]. Stored at room temperature.
- Trypsin [Invitrogen Corporation, UK]. Stored at -20°C.

Digest solution (trypsin + EDTA) preparation

To make 25mls of digest solution for cell passaging, 15mg of trypsin and 20mg of EDTA were dissolved in the [calcium and magnesium] free Hanks' Balanced Salts solution adjusted to a pH of 7.4. The solution was subsequently sterilised utilising the 0.2μm filter in the sterile hood. The sterilised solutions were then stored at -20°C for use later in cell passaging.

2.1.3 Materials for aortic valve cell calcification experiments

- Porcine transforming growth factor-β1 **(TGF-β1)** [R&D Systems Inc, USA].

  Solution preparation: The stock solution was made in 4 mM hydrochloric acid (HCL) containing 0.1% BSA, leading to a final TGF-β1 stock solution concentration of 1μg/ml. The solution was subsequently sterilised utilising the 0.2μm filter in the sterile hood, and stored at -80°C in smaller aliquots.

- (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate **(DETA-NONOate)** [Cayman Chemical Co, UK].

  Solution preparation: The stock solution was prepared in 0.01M sodium hydroxide (NaOH) at pH 10, leading to a final DETA-NONOate stock solution concentration of 20mM. The solution was subsequently sterilised utilising the 0.2μm filter in the sterile hood, and stored at -80°C in smaller aliquots.

- 4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate, 4-[4-(1,3-Benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide hydrate, 4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide hydrate **(SB431542)** [Sigma-Aldrich Inc, USA].

  Solution preparation: The stock solution was prepared in dimethyl sulfoxide (DMSO) [Sigma-Aldrich Inc, USA], leading to a final SB431542 stock solution concentration of 10mM. The solution was subsequently sterilised utilising the 0.2μm filter in the sterile hood, and stored at 4°C.

2.1.4. Materials for cell lysis buffer solution

Cell lysis buffer solution components

- Potassium chloride (KCL), NaCL and HEPES (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid) were purchased from Merck Pty Ltd, Australia. All compounds stored at room temperature.
- EDTA [Invitrogen Corporation, UK]. Stored at room temperature.
- Nonidet P40 [Sigma-Aldrich Inc, USA]. Stored at room temperature.
- Phenylmethanesulfonyl fluoride (PMSF), leupeptin and aprotinin were all purchased from Sigma-Aldrich Inc, USA.

Cell lysis buffer solution preparation

The lysis buffer was made on the day of cell collection following termination of cellular calcification experiments. The components above were dissolved in distilled water and stored at 4°C. The final concentration of each of the components were HEPES (20mM at pH 7.9), KCL (100mM), NaCl (300mM), EDTA (10mM), Nonidet P40 (0.1%), PMSF (0.5mM), leupeptin (0.5µg/ml) and aprotinin (0.5µg/ml).

2.1.5 Materials for protein assay

- Bio-Rad protein assay reagent [Bio-Rad Laboratories Inc, AUS].
- Cell lysis buffer solution (see above).
- BSA [Invitrogen Corporation, UK]. Stock solutions (10mg/ml) stored at 4°C.

2.1.6 Materials for thioredoxin (TRX) activity assay

- **Dithiothreitol activation buffer**

  The activation buffer contained 1mg/ml BSA [Invitrogen Corporation, UK], 50mmol/L HEPES [Merck Pty Ltd, Australia] at pH 7.6, 2mmol/L dithiothreitol [Sigma-Aldrich Inc, USA] and 1mmol/L EDTA [Invitrogen Corporation, UK].

- **Reaction mixture**

  The reaction buffer contained 40µl of 0.2mmol/l EDTA, 200µl of 1M HEPES (at pH 7.6), 500µl of insulin solution (10mg/ml) [Sigma-Aldrich Inc, USA], and 40µl of NADPH (40mg/ml) [Sigma-Aldrich Inc, USA]. The solution was prepared and kept on ice approximately 5 minutes prior to utilisation in the TRX activity assay.

- **Thioredoxin reductase** (vials stored at -20°C) and **Thioredoxin** (100 µM TRX stock solutions stored at -80°C). [Both purchased from Sigma-Aldrich Inc, USA].

- **Stopping solution**

  The stopping solution contained 0.4mg/ml 5,5′-Dithiobis(2-nitrobenzoic acid) and 0.25ml of 6M Guanidine HCL in 0.2M Trizma HCL (at pH 8.0). The solution was prepared (kept on ice in the dark) approximately 2 minutes prior to utilisation in stopping the TRX activity assay. All the chemicals in this solution were purchased from Sigma-Aldrich Inc, USA.

2.1.7 Materials for chamber slide fixation and thioredoxin-interacting protein (TXNIP) immunofluorescence staining

- Paraformaldehyde [Sigma-Aldrich Inc, USA]. Powder stored at 4°C.
- Phosphate-buffered solution **(PBS)**. Solution stored at 4°C.
- Fatty acid free bovine serum albumin **(BSA)** (1mg/ml) [Invitrogen Corporation, UK]. Stored at 4°C.
- Triton X-100 **(Triton)** [Sigma-Aldrich Inc, USA]. Solution stored at room temperature.
- Normal goat serum **(NGS)** [Vector Laboratories Inc, USA]. Stored at 4°C.
- ***Primary antibody***: VDUP1 (H-115) **(rabbit anti-TXNIP antibody)** - polyclonal antibody [Santa Cruz Biotechnology Inc, USA]. Stored at 4°C.
- ***Secondary antibody***: biotinylated goat anti-rabbit antibody **(biotinylated antibody)** [Vector Laboratories Ltd, USA]. Stored at 4°C.
- Fluorescein-Avidin D **(FITC-Avidin D)** - binds to secondary antibody [Vector Laboratories Ltd, USA]. Stored at 4°C in the dark.
- ***Negative controls***: Primary antibody replaced by PBS or normal goat serum.
- 4',6-diamidino-2-phenylindole, dihydrochloride **(DAPI nuclear stain- Prolong GOLD)** [Invitrogen Corporation, USA]. Stored at 4°C in the dark.

2.2 Methods

2.2.1 Acquisition of the porcine aortic valve

Hearts were obtained from healthy pigs (housed at The Queen Elizabeth Hospital animal house) killed under anaesthesia in accordance with the principles and procedures approved by the Institute of Medical and Veterinary Science (IMVS)/Central Northern Adelaide Health Service Animal Ethics Committee, and The University of Adelaide Animal Ethics Committee. The pigs were killed following anaesthetic induction with ketamine/xylazine, while on 2% halothane/oxygen maintenance. The whole heart was immediately extracted and placed in a bag containing sterile normal saline, which was placed on ice.

2.2.2 Primary cell culture

The extracted porcine heart was immediately placed inside the sterile laminar flow cabinet [sterile hood] (Gelman Sciences, Australia). All instruments utilised for aortic valve (AV) extraction were sterilised by autoclaving and subsequently with 70% ethanol, followed by Bunsen burner flame sterilisation. The 3 porcine AV leaflets were removed quickly under sterile conditions taking care to avoid any of the attachments to the aortic wall, with the extracted leaflets then washed in the [calcium and magnesium] free Hanks' Balanced Salts solution to remove any remaining blood. The leaflets were then inserted into a sterile test-tube (Greiner Bio-one, Germany) containing 20mls of digest solution (collagenase) (see section 2.1.1), and placed in a moderate speed shaking water bath at 37°C for 50 minutes. This process was performed to ensure the removal of the

aortic valve endothelium. Following agitation, the leaflets were removed and rinsed further in the [calcium and magnesium] free Hanks' Balanced Salts solution. Each leaflet was then gently scraped on both sides with a scalpel blade to remove any remaining endothelial cells (ECs). Following this, the leaflets were washed in low glucose DMEM for about 1 minute. They were subsequently removed and transferred into a new dish containing sterile low glucose DMEM solution, and were cut into little pieces (approximately 0.5mm x 0.5mm). The explants were then transferred into sterile tissue culture flasks (Greiner Bio-one, Germany), each containing 10mls of low glucose DMEM with 10% FCS and 1% penicillin/streptomycin (see section 2.1.1). Finally, the flasks were placed in a tissue culture incubator (Forma Science, USA) at 37°C with 5% CO_2 in air, and 95% relative humidity. This initial culture stage is designated cell passage 0 (P0).

2.2.3 Passaging of cells

The flasks were assessed daily under an inverting microscope (Olympus, Japan), and when the aortic valve interstitial cells (AVICs) within the tissue culture flasks formed a monolayer at 95% confluence, the cells were then collected and subsequently passaged. Firstly, the DMEM solution was discarded and 4mls of digest solution (trypsin + EDTA) was added to each flask using a sterile 10ml pipette (Sarstedt, France). The flasks were then placed back in the incubator at 37°C for 5 minutes. Following this, the flasks were re-checked under the microscope to ensure lifting of AVICs. Once complete lifting was confirmed, a further 6mls of low glucose DMEM was added to each flask and mixed with the existing cocktail of cells and digest solution. The entire solution mixture was

extracted and placed in a sterile test-tube, and subsequently underwent centrifugation at 110 g for 5 minutes at 4°C. Following centrifugation, the supernatant was discarded, and the remaining pellet was re-suspended in 20mls of 10% FCS low glucose DMEM using a sterile pipette. The re-suspended solution was mixed gently and thoroughly to disperse cells. Following complete mixing of the re-suspended solution, 30µls was extracted from the solution and mixed thoroughly with 30µls of Trypan blue reagent in a micro test-tube using a vortex mixer (Ratek, Australia). From the 60µl solution, approximately 20-30µls was extracted utilising an Eppendoff pipette (Eppendoff, Germany) and placed on a cell counting chamber (Neubauer, Germany). Once the cells were counted and the total cells contained within the 20ml re-suspended solution calculated, equivalent volumes of solution containing approximately 1 million cells were extracted and re-constituted in 10mls of 10% FCS low glucose DMEM, and inserted into new sterile tissue culture flasks, which were then placed back in the tissue culture incubator. At this juncture, the cell culture stage is designated passage 1 (P1). The steps described above were repeated for subsequent passaging of cells.

2.2.4 Calcifying nodule formation and cell survival experiments

AVICs cultured between passages 2 and 4 were utilised in experiments. AVICs were plated into 6 and 12 well culture plates containing 10% FCS low glucose DMEM at densities of $0.5x10^6$ cells/well and $0.15x10^6$ cells/well respectively. Once the monolayer of cells within each well reached approximately 90% confluence, the solution was changed to 0.67% FCS low glucose DMEM. At this point, selected chemicals were added to each well and the plates were maintained in the incubator (37°C with 5% CO_2

in air, and 95% relative humidity) to commence the calcifying experiments. Firstly, nodule formation was evaluated in the presence and absence of TGF-β1 (final concentration of 5ng/ml) as described by Mohler et al. (1999). An earlier study performed in our laboratory (Kennedy et al. 2009) showed that porcine AVICs stimulated by TGF-β1 (5ng/ml) produced calcifying nodules, whereby the nodules were stained with the Alizarin red stain, which demonstrated significant deposition of calcium within these nodules (figure 2.1).

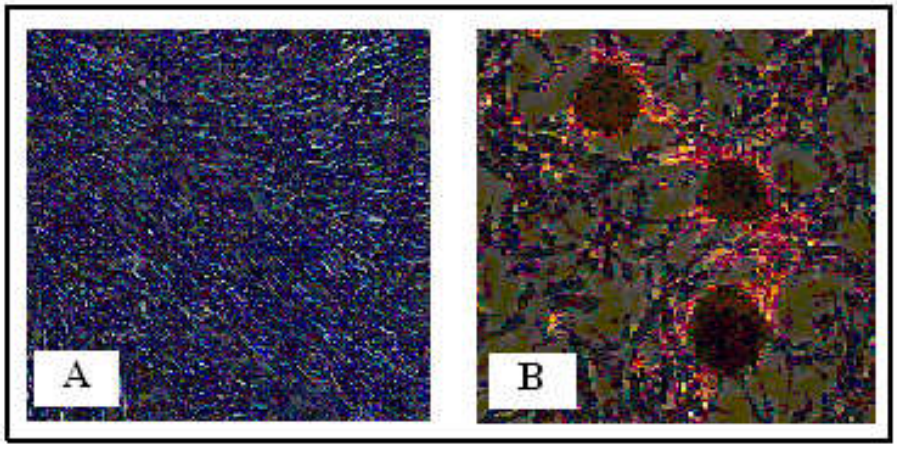

Figure 2.1 (A) Photomicrograph showing porcine aortic valve interstitial cell (AVIC) culture (blue filter). (B) Photomicrograph revealing AVIC calcific nodules (treated with TGF-β1 5ng/ml) stained for calcification with Alizarin red stain

Furthermore, other chemicals under investigation or their respective vehicles were added to selected wells containing TGF-β1 or its vehicle (4mM HCL/0.1% BSA). The specific chemicals under investigation included the NO donor, ***DETA-NONOate*** (final concentration of 20μM), and the TGF-β1 inhibitor, ***SB431542*** (final concentration of

10μM). During experimentation, the medium and study chemicals were replenished twice weekly, except for DETA-NONOate which was replaced daily due to its relatively short half-life. For each experiment, treatments were carried out in triplicate in at least 3 cultures from different pigs. To remove the influence of treatment bias, an optical microscope was utilised to perform nodule counts by observers blinded to the treatments. The nodule count peaked between days 5 and 8, following which the absolute counts within each specific treatment well for all experiments conducted in 6 well plates were collated, and the results expressed as peak nodule count/well. With the exception of the treatment with SB431542, the protocols adopted in these experiments were similar to that described by Kennedy et al. (2009). It is vital to note that all experiments were conducted under strict sterility within the sterile hood. Details of the specific chemicals/treatments involved in the calcifying experiments can be viewed in more detail in section 2.1.3.

Cell survival experiments: Once the peak nodule counts in the 12 well plates were reached between days 5 and 8, the calcifying nodule formation experiments were terminated. The medium solution was then gently removed from all the wells and 300μls of digest solution (trypsin + EDTA) (see section 2.1.2) was added to each well, and the plate was placed back into the incubator for 5 minutes at 37°C. Each well was subsequently assessed under the optical microscope to ensure that all the adherent cells had lifted off the plate. Once complete lifting of cells was confirmed, a further 700μls of low glucose DMEM solution was added to each well, resulting in each well having a total volume of 1ml. Finally, cell counting was performed for each well following the

methods described in section 2.2.3, and the absolute number of cells per well area was calculated. The results were expressed as cell survival/area ($x10^4/cm^2$).

2.2.5 Cell lysis and protein collection

Following peak nodule formation that primarily occurred between days 5 and 8, the experiments were terminated and the medium was removed from all wells. The 6 well plates were placed on ice (non-sterile) and wells washed for approximately 2-3 minutes with cold PBS. Subsequently, the PBS solution was completely removed and 200µl of cell lysis buffer solution (see section 2.1.4) was added to each well. The internal base of each well was scraped thoroughly to remove all adherent cells/proteins, and the lysis buffer containing the lysed cells/cellular proteins were then extracted, and placed in mini-eppendoffs (stored at -80°C). The protein content within the lysis buffer was later quantified using a prescribed method as described below.

2.2.6 Protein assay

Collected cellular proteins were quantified utilising the modified Bradford protein assay (as described by Bradford (1976) with some minor modifications). Firstly, stock BSA standards were prepared in order to construct a protein assay standard curve, which included BSA concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2 and 1.4mg/ml. All the different BSA standard concentrations were made-up in lysis buffer, with each standard having a total volume of 10µl. Then, 1ml of the Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc, AUS) was added into 11 empty 2ml mini-test-tubes, and 10µls of each BSA standard solution was added (in increasing order of

concentration) to the corresponding min-test-tubes. The combined mixture was then mixed using the vortex mixer, and allowed to rest for 5 minutes. The mixture colour changed from blue to brown when protein was added to the reagent solution. Following this, the mixture was then transferred using an Eppendoff pipette into a plastic cuvette [Bio-Rad Laboratories Inc, AUS]. The cuvette was subsequently inserted into a spectrophotometer, and the absorbance was set at 595nm to produce a specific absorbance reading corresponding to a particular protein concentration. Figure 2.2 below illustrates an example of a modified Bradford protein assay standard curve generated during this study.

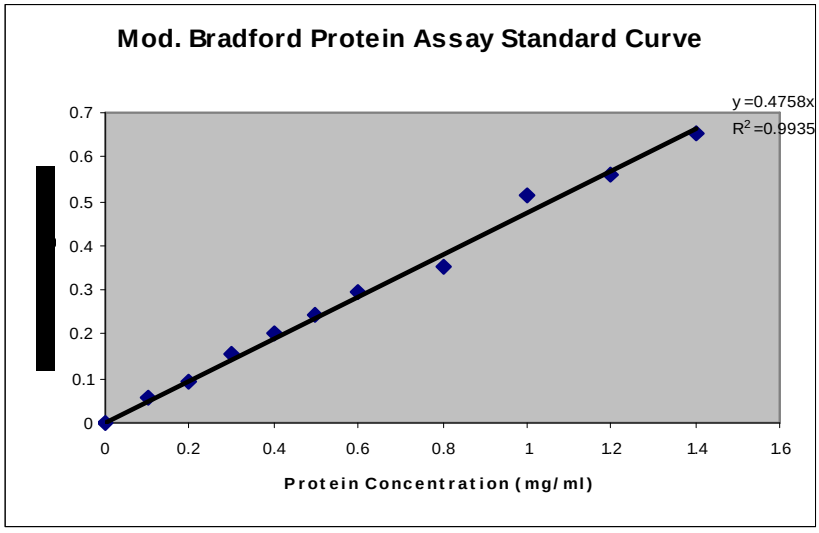

Figure 2.2 Example of a modified Bradford protein assay standard curve

The protein samples collected in lysis buffer (stored at -80°C) following termination of the AVIC calcifying nodule experiments were initially thawed and re-mixed. Then, 5µls from each sample was extracted and inserted into empty 2 ml mini-test-tubes. Each sample then underwent a 1:2 dilution with the addition of 5µls of the lysis buffer

solution, which brought the total protein mixture volume to 10µls. The 10µl sample was then added to the Bio-Rad protein assay reagent as described previously, and the mixture analysed by spectophotometry at an absorbance of 595nm to obtain an absorbance reading corresponding to the particular protein sample. The value obtained was read against the protein assay standard curve to obtain the protein concentration value, which was then multiplied by 2 (due to the earlier 1:2 dilution) to obtain the actual amount of protein contained within that particular sample.

2.2.7 Thioredoxin activity assay

TRX activity was measured using the insulin disulphide reduction method, as described by Holmgren and Bjornstedt (1995), and Wang, De Keulenaer and Lee (2002), with some modifications applied to the assay methodology. A detailed description of the materials utilised in the assay can be elicited from section 2.1.6. Firstly, a TRX activity assay standard curve was established. Stock solutions of 100µM TRX were diluted in lysis buffer to produce specific concentrations in a total volume of 34µls, and were then added into selected wells of a 96-well micro-plate pre-incubated at 37°C in a water bath. Subsequently, 1µl of the dithiothreitol activation buffer was mixed into each well, resulting in a total volume of 35µls per well, and the micro-plate was then incubated at 37°C for 15 minutes. Note that final TRX concentration standards of 0, 100, 200, 400 and 500nM were made-up in a total volume of 35µls respectively. Then, 20µls of the reaction mixture was added to each well, and the reaction was commenced with the addition of 14.45µls (382.5 mUnits) of thioredoxin reductase, along with 14.45µls of distilled water added to control samples. To undergo the reaction, the samples were

incubated at 37°C for a further 20 minutes. The reaction was then ceased by the addition of 250µls of the stopping solution into each well. The 96 well micro-plate was then immediately removed from the water bath and protected from light using an aluminium sheet. The micro-plate was then inserted into a Bio-Rad micro-plate reader, and the absorbance at 415nm was measured. Subsequently, the specific TRX activity units corresponding to each TRX standard was calculated (figure 2.3), and the standard curve was produced utilising the absorbance values obtained (see figure 2.4).

Conversion calculation from TRX concentration to activity

TRX stock at 90% protein purity (20.87 activity Units/mg)

Example: conversion of 200nM TRX to TRX activity (mUnits)

weight = 0.0000002M x 14,000 ***(TRX molecular weight)*** x 0.000035L x 90/100 ***(protein purity)***

= 88.2ng

Thus, activity in 88ng of TRX = 0.0000882mg x 20.87 Units

= ***1.840 mUnits***

Figure 2.3 Example showing calculations for converting TRX concentration (nM) to TRX activity (mUnits)

TRX Conc. (nM)	TRX Activity (mUnits)	Absorbance (Abs) @ 415 nm			
		Abs 1	Abs 2	Average	(-) blank
0	**0**	0.845	0.852	0.8485	**0**
100	**0.9204**	1.033	0.99	1.011	**0.1625**
200	**1.8408**	1.156	1.158	1.157	**0.3085**
400	**3.6816**	1.574	1.589	1.5815	**0.733**
500	**4.602**	1.723	1.744	1.7335	**0.885**

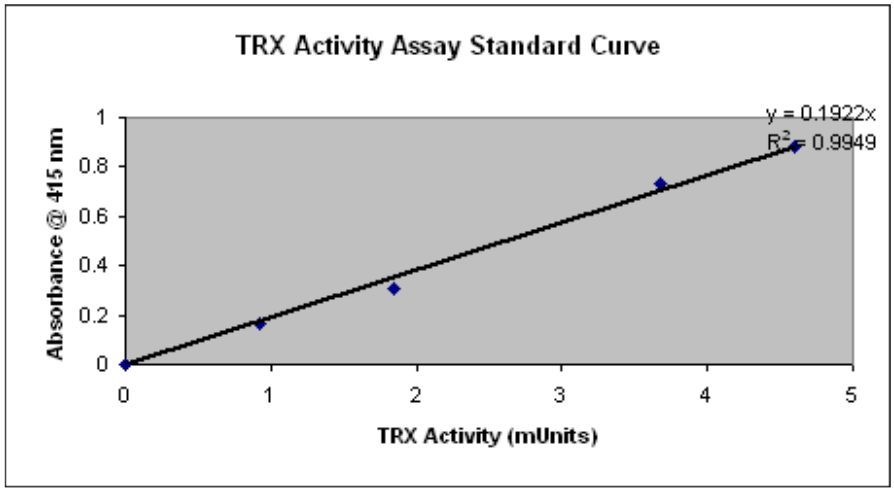

Figure 2.4 Example of a TRX activity assay standard curve produced during the study. (Note: graph was produced by values tabulated in 'bold')

Thus, 40µg/ml of the lysed experimental cellular proteins from each sample was used in the TRX activity assay. Each sample was thawed, and calculated sample volumes containing 40µg/ml of protein were added to respective wells of the 96 well micro-plate. Lysis buffer solution was added to ensure each well contained a total volume of 34µls. Then, the insulin disulphide reaction assay was performed as described in the previous paragraph. Following termination of the assay, the absorbance value obtained at 415nm for each sample was read against the TRX activity assay standard curve to produce the corresponding TRX activity (expressed as mUnits/mg protein) for each particular sample.

2.2.8 Aortic valve interstitial cell (AVIC) calcifying nodule formation experiments on chamber slides, and slide fixation

AVICs were plated on 8 well chamber slides (Sybron, USA) at a density of $3x10^4$ cells/chamber, and grown in 10% FCS low glucose DMEM. Once the monolayer of cells within each chamber reached approximately 90% confluence, the solution was changed to 0.67% FCS low glucose DMEM. At this stage, selected chemicals were added to each chamber and the slides were maintained in the incubator (at 37°C with 5% CO_2 in air, and 95% relative humidity) to commence the calcifying experiments. The calcifying experiments in chamber slides were performed in the same manner as undertaken in the 6 or 12 well culture plates, as described in section 2.2.4. Again, all the same chemicals/treatments were utilised in the experiments, at the same concentrations mentioned in section 2.2.4. Detailed information regarding the chemicals/treatments used can be viewed in section 2.1.3.

Once the experiments were terminated, the medium was removed from all the chambers. To further remove any remaining medium solution, each chamber was gently rinsed for approximately 2 minutes with cold PBS (this step was repeated thrice). The chamber slide was then fixed in 4% paraformaldehyde/PBS (see section 2.1.7) at room temperature for 30 minutes. The fixed chamber slide was then gently washed thrice in cold PBS to ensure complete removal of excess paraformaldehyde, with each wash lasting for approximately 2 minutes. The chamber slide was then submerged in a box containing cold PBS, and stored at 4°C in the fridge overnight for TXNIP immunofluorescence (IF) staining to be performed the following day.

2.2.9 TXNIP immunofluorescence (IF) staining

The cold PBS was gently removed from each chamber slide, and 200µls of 0.2% Trition/PBS was added to each chamber, and left to incubate at -20°C for 10 minutes, in order to allow for permeabilisation of the fixed AVICs. All information regarding the chemicals and antibodies utilised for TXNIP IF staining are fully detailed in section 2.1.7. Then, the Triton solution was removed by gently washing each chamber slide thrice with cold PBS, for approximately 2 minutes each time. Initially, the blocking step was performed by the addition of 300µls of normal goat serum diluted 1:10 in PBS/1%BSA into each chamber, and left to incubate at room temperature for 1 hour. This first step was important in reducing/eliminating non-specific binding of the primary antibody. In addition, positive (full staining protocol adopted) and negative (incorporating various combinations of the staining protocol) controls were established to exclude any non-specific IF staining. Then, 300µls of the primary antibody, anti-TXNIP antibody (diluted 1:200 in PBS/1%BSA), was added into each chamber, and incubated for 2 hours at room temperature. The washing procedure as previously described was repeated to remove any unbound antibodies. Following this, 300µls of the secondary antibody, biotinylated anti-rabbit antibody (diluted 1:300 in PBS/1%BSA), was added to selected chambers, and allowed to incubate at room temperature for 1 hour. Each chamber was again washed with cold PBS according to the methods described earlier. Finally, 300µls of the Fluorescein-Avidin D (FITC-Avidin D) solution (diluted 1:200 in PBS/1%BSA), was added into each chamber, and the slide was incubated for 1 hour at room temperature in the dark. Subsequently, each chamber was washed thrice with cold PBS to ensure removal of any excess FITC-Avidin D solution. Once the

staining procedure was completed, fine-tipped forceps were used to carefully remove the chamber slide walls from the slide base, leaving behind a typical appearing microscope slide. Lastly, two drops of DAPI nuclear stain (see section 2.1.7) mounting medium was placed on the slide surface containing cells, and a cover-slip was gently placed over the microscope slide. The stained slides were assessed using a fluorescence microscope (Nikon Eclipse TE300, Japan) and all chambers in every slide were digitally photographed (minimum of 2 areas photographed/chamber) using a Panasonic CCD camera (Panasonic, Japan). All fluorescent photomicrographs were taken on the same exposure settings. Staining of cells with the DAPI nuclear stain allowed for AVIC localisation under the fluorescence microscope. The TXNIP IF images were subsequently quantified using Image-Pro Plus (Version 6.0, Media Cybernetics Inc), and the data expressed as TXNIP IF intensity/cell (arbitrary units).

2.3 Data analysis

All data were analysed using the Graphpad Prism version 5 software, and the results were expressed as mean ± standard error of the mean (SEM). Multiple comparisons linked directly to the hypotheses being tested, were analysed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. In particular, multiple comparison methodology was utilised in the analyses of:

i. Untreated controls versus TGF-β1 treatment only experiments,
ii. Selected treatments versus TGF-β1 treatment only experiments, and
iii. Selected treatments versus untreated control experiments.

A critical $P<0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

&

DISCUSSION

3.1 Results

3.1.1 Calcific nodule formation experiments in porcine AVIC cultures

3.1.1.1 TGF-β1-induced calcific nodule formation

As previously described (Kennedy et al. 2009), cultures of porcine AVICs were found to form calcific nodules when treated with TGF-β1. However, there was minimal or a complete absence of nodules in cultures not treated with TGF-β1. Calcific nodule formation was significantly increased in porcine AVICs treated with TGF-β1 (5ng/ml) alone, compared to controls (37.19±2.67 vs. 0.33±0.12 (nodule count/well), $P<0.001$, n=4, figure 3.1), with peak nodule formation reached between days 5 and 8. All experiments were performed in triplicate, in 4 cultures of AVICs obtained from different pigs. Furthermore, the TGF-β1 inhibitor, SB431542 (10μM), significantly inhibited TGF-β1-induced nodule formation, compared to cultures treated with TGF-β1 alone (0.42±0.25 vs. 37.19±2.67 (nodule count/well), $P<0.001$, n=4, figure 3.1).

3.1.1.2 DETA-NONOate (20μM) inhibition of TGF-β1-induced nodule formation in AVIC cultures

DETA-NONOate (20μM), which is a direct NO donor, significantly suppressed TGF-β1-induced nodule formation, compared to cultures treated with TGF-β1 alone (9.40±1.28 vs. 37.19±2.67 (nodule count/well), $P<0.001$, n=4, figure 3.1). NO did not completely suppress the effects of TGF-β1, which was a similar

finding previously documented in the same preparation by Kennedy et al. (2009). In AVIC cultures (n=4) treated with either DETA-NONOate (20μM) or SB431542 (10μM) alone, nodule formation did not exceed that of untreated controls (n=4).

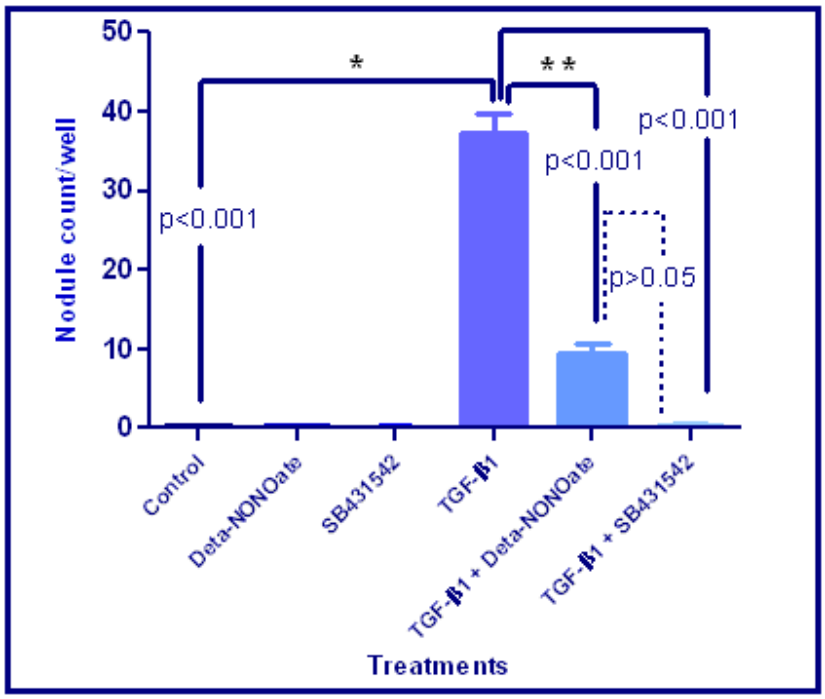

Figure 3.1 As hypotheses tested in the experiments primarily concerned *(a) effects of TGF-β1 on calcific nodule formation in AVIC cultures, and **(b) suppression of these effects by the NO donor, DETA-NONOate; 2 primary comparisons were made with Bonferroni's test. All experiments were performed in triplicate, in 4 different cultures (n=4). *TGF-β1 (5ng/ml) significantly induced nodule formation compared to untreated controls (37.19±2.67 vs. 0.33±0.12 (nodule count/well), P<0.001, n=4). **DETA-NONOate (20μM) significantly suppressed TGF-β1-induced nodule formation, compared to treatment with TGF-β1 alone (9.40±1.28 vs. 37.19±2.67 (nodule count/well), P<0.001, n=4). Note: additional comparisons with Bonferroni's tests are shown above.

3.1.2 Thioredoxin (TRX) activity in AVIC cultures in response to various treatments (TGF-B1 / DETA-NONOate / SB431542)

3.1.2.1 TGF-β1 (5ng/ml) effects on TRX activity in AVIC cultures

The AVIC cultures treated with TGF-β1 (5ng/ml) were found to have significantly lower TRX activity levels, compared to untreated controls (39.94±0.66 vs. 58.96±2.22 (mU/mg protein), $P<0.001$, n=4, figure 3.2). These changes were completely suppressed in the presence of the TGF-β1 inhibitor, SB431542 (10μM) (59.94±1.25 vs. 39.94±0.66 (mU/mg protein), $P<0.001$, n=3, figure 3.2).

3.1.2.2 Effects of DETA-NONOate (20μM) on TRX activity levels in AVIC cultures treated with and without TGF-β1 (5ng/ml)

DETA-NONOate (20μM) increased TRX activity levels in TGF-β1 (5ng/ml) treated AVIC cultures, compared to cultures treated with TGF-β1 alone (59.21±2.49 vs. 39.94±0.66 (mU/mg protein), $P<0.001$, n=4, figure 3.2). These activity levels were statistically similar to those of untreated control cultures (59.21±2.49 vs. 58.96±2.22 (mU/mg protein), $P>0.05$, n=4, figure 3.2). Furthermore, cultures treated with DETA-NONOate (20μM) alone exhibited AVIC TRX activity levels similar to that of untreated controls (59.60±2.40 vs. 58.96±2.22 (mU/mg protein), $P>0.05$, n=4, figure 3.2).

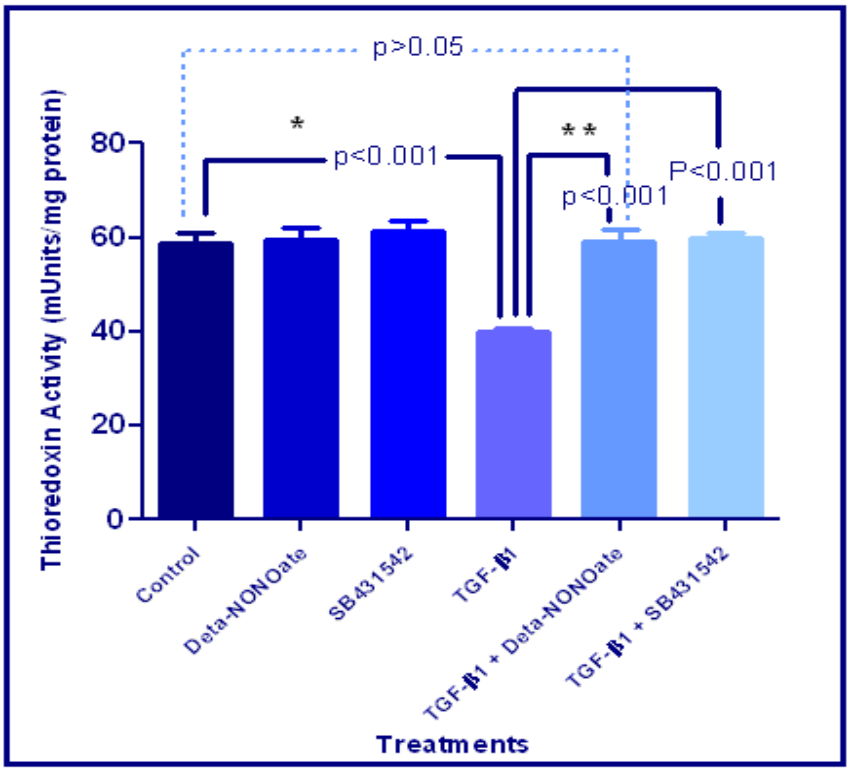

Figure 3.2 As hypotheses tested in the experiments primarily concerned the *(a) effects of TGF-β1 on TRX activity in AVIC cultures, and **(b) potential modulation of TRX activity by the NO donor, DETA-NONOate, in cultures treated with TGF-β1; 2 primary comparisons were made with Bonferroni's test. *TGF-β1 (5ng/ml) suppressed TRX activity compared to untreated controls (39.94±0.66 vs. 58.96±2.22 (mU/mg protein), P<0.001, n=4). **DETA-NONOate (20μM) completely restored TRX activity levels in TGF-β1 treated cultures, compared to treatment with TGF-β1 alone (59.21±2.49 vs. 39.94±0.66 (mU/mg protein), P<0.001, n=4). Note: additional comparisons utilising Bonferroni's test are shown in the graph above.

3.1.3 AVIC TXNIP immunofluorescence (IF) in response to various treatments (TGF-B1 / DETA-NONOate / SB431542)

3.1.3.1 Effects of TGF-β1 (5ng/ml) on porcine AVIC TXNIP immunofluorescence

Porcine AVIC cultures treated with TGF-β1 (5ng/ml) exhibited significantly higher levels of TXNIP IF intensity/cell, compared to controls (17059±204 vs. 7984±423 (arbitrary units), $P<0.001$, n=3, figure 3.3). The TGF-β1 inhibitor, SB431542 (10μM), decreased AVIC TXNIP IF intensity/cell in TGF-β1 treated cultures, compared to those treated with TGF-β1 alone (7670±798 vs. 17059±204 (arbitrary units), $P<0.001$, n=3, figure 3.3).

3.1.3.2 Effects of DETA-NONOate (20μM) on AVIC TXNIP IF in cultures treated with and without TGF-β1 (5ng/ml)

DETA-NONOate (20μM) markedly decreased AVIC TXNIP IF intensity/cell in TGF-β1 (5ng/ml) treated cultures, compared to treatment with TGF-β1 alone (7918±310 vs. 17059±204 (arbitrary units), $P<0.001$, n=3, figure 3.3). In contrast to the incomplete suppression of nodule formation (figure 3.1), the TXNIP IF intensity/cell decreased to levels comparable to that of untreated controls (figure 3.3). Cultures treated with DETA-NONOate alone exhibited levels of TXNIP IF intensity/cell similar to those of untreated controls.

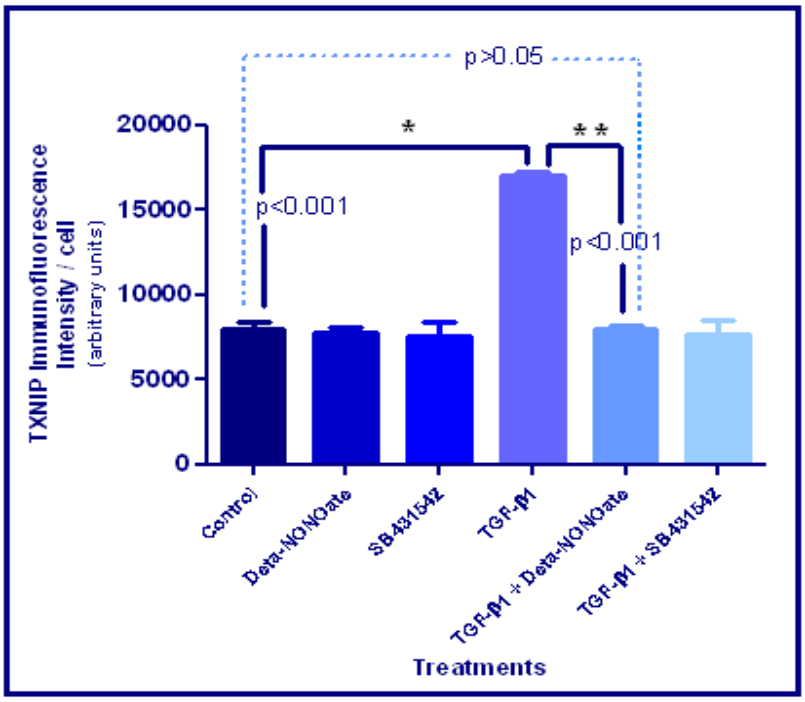

Figure 3.3 As hypotheses tested in the experiments primarily concerned the *(a) effects of TGF-β1 on intracellular TXNIP levels in AVIC cultures, and **(b) potential modulation of intracellular TXNIP levels by the NO donor, DETA-NONOate, in cultures treated with TGF-β1; 2 primary comparisons were made with Bonferroni's test. *TGF-β1 (5ng/ml) significantly induced TXNIP IF intensity/cell compared to untreated controls (17059±204 vs. 7984±423 (arbitrary units), P<0.001, n=3). **DETA-NONOate (20μM) completely inhibited TXNIP IF intensity/cell in TGF-β1 treated cultures, compared to treatment with TGF-β1 alone (7918±310 vs. 17059±204 (arbitrary units), P<0.001, n=3). Note that TXNIP IF intensity/cell in TGF-β1 cultures treated with DETA-NONOate were not statistically different from untreated controls (P>0.05, n=3).

3.1.3.3 TXNIP immunofluorescence images of porcine AVIC monocultures and calcific nodules

Selected AVIC TXNIP IF images produced during the present study following various AVIC culture treatments are shown below. The images were captured using a Panasonic CCD camera (Panasonic, Japan).

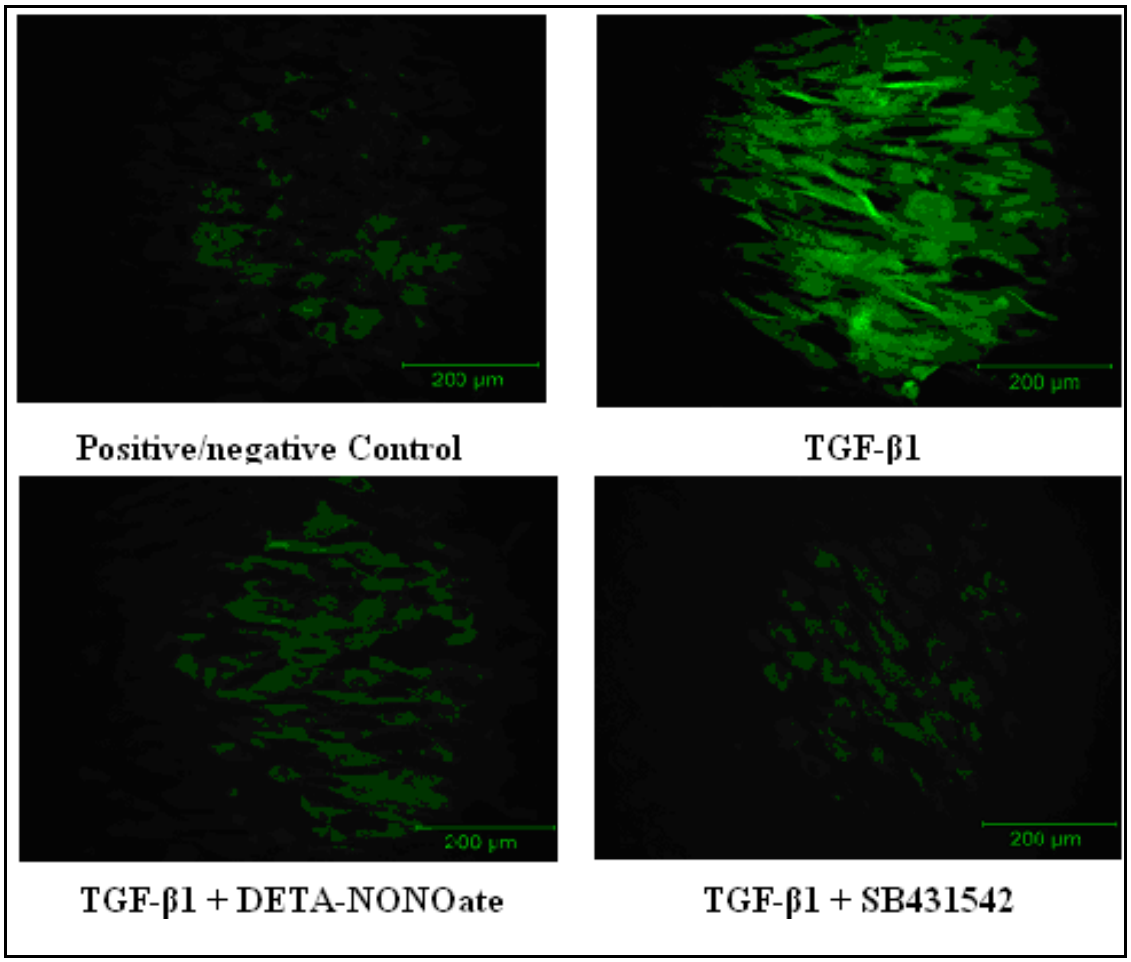

Figure 3.4 Selected images acquired during the study showing AVIC TXNIP immunofluorescence in response to various treatments. The specific treatment(s) are listed below the images. The TXNIP IF intensity was most apparent in the AVIC image treated with TGF-β1 alone.

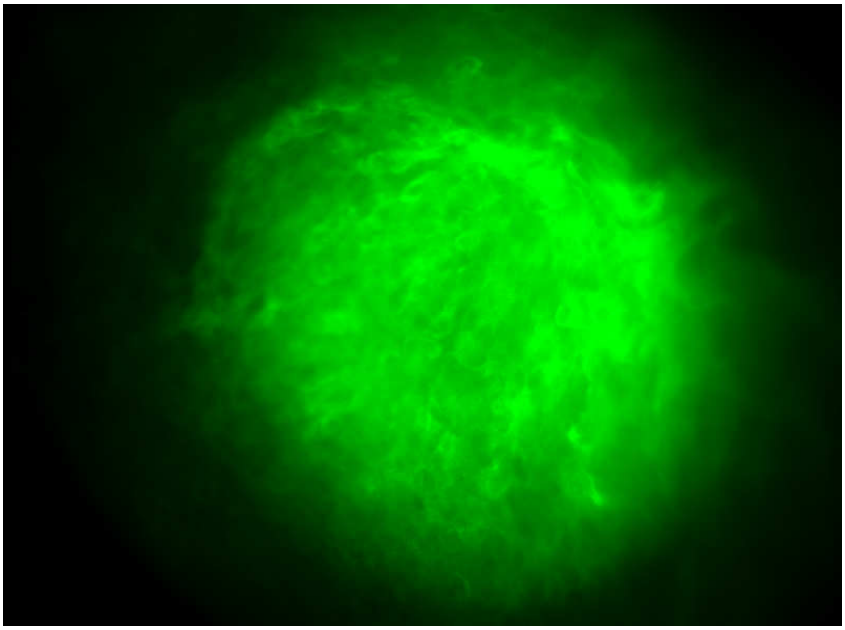

Figure 3.5 The above image shows a TGF-β1-induced calcific nodule following TXNIP immunofluorescence staining.

Nodules formed following various other treatments (DETA-NONOate and SB431542) also exhibited a similar appearance (images following other treatments are not shown) as that of Figure 3.5. It was not possible to analyse the TXNIP IF of the respective nodules as the IF measurements (arbitrary units) exceeded the threshold of the image analysis software. It should be noted however, that all the nodules formed following various treatments stained strongly for TXNIP, indicating that TXNIP was most highly concentrated within calcific nodules (figure 3.5), compared to individual porcine AVICs (figure 3.4). Considering the few number of nodules in DETA-NONOate and SB431542 treated cultures respectively, the differential TXNIP IF may have revealed a much greater difference between those treatments compared to cultures treated with TGF-β1 alone.

3.1.4 Cell survival experiments in porcine AVIC cultures

3.1.4.1 Effects of TGF-β1 (5ng/ml) on porcine AVIC survival

Porcine AVIC cultures treated with TGF-β1 (5ng/ml) exhibited significantly decreased cell survival/area, compared to untreated controls (11.98±0.74 vs. 20.10±0.56 ($x10^4/cm^2$), $P<0.001$, n=4, figure 3.6); consistent with the pro-apoptotic properties of TGF-β1 in various other cell lines (Jian et al. 2003; Sales et al. 2006). These changes were completely reversed by SB431542 (10μM).

3.1.4.2 Effects of DETA-NONOate (20μM) on AVIC survival in cultures treated with and without TGF-β1 (5ng/ml)

DETA-NONOate (20μM) significantly increased porcine AVIC survival/area in TGF-β1 (5ng/ml) treated cultures, compared to cultures treated with TGF-β1 alone (16.93±0.95 vs. 11.98±0.74 ($x10^4/cm^2$), $P<0.01$, n=4, figure 3.6). Interestingly, despite the AVIC survival/area appearing subjectively lower in TGF-β1 cultures treated with DETA-NONOate compared to that of untreated controls, the difference between these two groups were found not to be statistically significant (16.93±0.95 vs. 20.10±0.56 ($x10^4/cm^2$), $P>0.05$, n=4, figure 3.6).

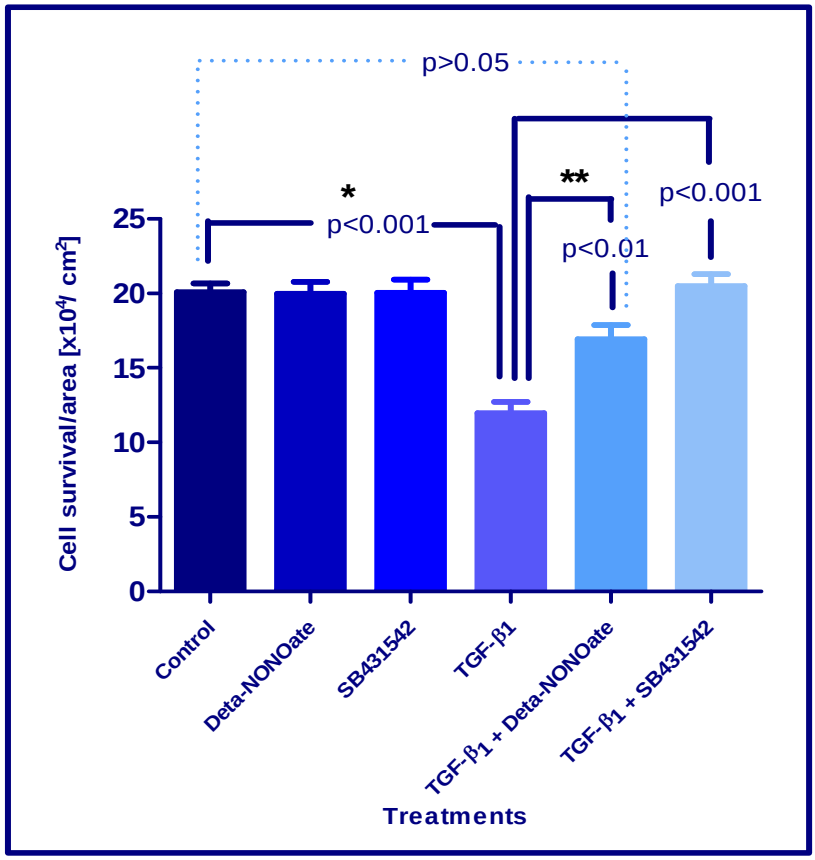

Figure 3.6 As hypotheses tested in the experiments primarily concerned the *(a) effects of TGF-β1 on cell survival in AVIC cultures, and **(b) potential changes in cell survival as modulated by the NO donor, DETA-NONOate, in cultures treated with TGF-β1; 2 primary comparisons were made with Bonferroni's test. All experiments were performed in triplicate, in 4 different cultures (n=4). *TGF-β1 (5ng/ml) significantly decreased cell survival/area, compared to untreated controls (11.98±0.74 vs. 20.10±0.56 ($x10^4/cm^2$), P<0.001, n=4). **DETA-NONOate (20μM) significantly increased cell survival/area in TGF-β1 treated cultures, compared to treatment with TGF-β1 alone (16.93±0.95 vs. 11.98±0.74 ($x10^4/cm^2$), P<0.01, n=4). Note: the graph above shows some additional comparisons.

3.2 Discussion

The key findings of the experiments outlined in this thesis are as follows:-

1) TGF-β1, via a conventional receptor (that is one inhibited by SB431542), induces the following effects in porcine AVIC monocultures:-
    a) Calcific nodule formation; effects similar to those shown previously by Walker et al. (2004) and Kennedy et al. (2009),
    b) Impaired intracellular TRX activity,
    c) Increased intracellular TXNIP IF, and
    d) Decreased overall cell survival.

2) The NO donor, DETA-NONOate, in concentrations previously shown to inhibit TGF-β1-induced nodule formation (Kennedy et al. 2009), :-
    a) Exhibits partial inhibition of calcific nodule formation; effects similar to those demonstrated previously by Kennedy et al. (2009),
    b) Normalises intracellular TRX activity,
    c) Restores intracellular TXNIP IF to baseline control levels, and
    d) Improves overall cell survival.

The mechanisms and implications of these findings merit further discussion.

3.2.1 The effects of TGF-β1 on porcine aortic valve interstitial cells

In this present study, it was confirmed that in porcine AVICs grown in low glucose medium, subsequent treatment with the exogenous pro-inflammatory/pro-calcific cytokine, TGF-β1 (5ng/ml), resulted in significant induction of calcific nodule formation (figure 3.1). These results are consistent with those in several earlier studies performed utilising human and/or animal AVIC lines (Mohler et al. 1999; Jian et al. 2003; Walker et al. 2004; Clark-Greuel et al. 2007; Kennedy et al. 2009).

As discussed extensively in the introductory section of this thesis, TGF-β1 is involved in a whole myriad of functions. More specifically, during periods of oxidative stress resulting in inflammation, TGF-β1 mediates the differentiation of porcine AVICs into active myofibroblasts, and contributes to pathological valvular ECM remodelling (Walker et al. 2004). As mentioned earlier, the current study revealed that the addition of exogenous TGF-β1 to porcine AVIC monocultures resulted in significant increases in calcific nodule formation (figure 3.1); an analogous finding to those in previous studies involving sheep (Jian et al. 2003; Clark-Greuel et al. 2007) and porcine (Walker et al. 2004; Kennedy et al. 2009) AVIC culture models treated with exogenous TGF-β1. Furthermore, the researchers in these earlier studies were able to prove that the TGF-β1-induced nodules formed were indeed calcifying (contained calcium deposits) by showing that the nodules stained heavily for calcium with Alizarin red stain (Clark-Greuel et al. 2007; Kennedy et al. 2009). Also, it was revealed that TGF-β1-induced nodules showed evidence of biomineralisation by exhibiting significantly higher levels

of alkaline phosphatase activity, compared to cultures without TGF-β1 (Jian et al. 2003). With regards to the clinical relevance of those findings, Jian et al. (2003) demonstrated that the ECM of calcified stenotic human aortic valves contained significantly higher levels of TGF-β1, compared to non-calcified normal valves.

Additionally, calcific nodule formation in the present study was further confirmed to be primarily mediated by TGF-β1 as its inhibition by the TGF-β1 antagonist, SB431542, which is a highly specific and potent inhibitor of the TGF-β-superfamily type I activin receptor-like kinase (ALK) receptors (ALK4, 5 and 7), led to complete inhibition of TGF-β1-induced nodule formation (figure 3.1).

In conclusion, the role of TGF-β1 in mediating inflammation is reasonably well established, contributing to AV fibrosis and calcification, and represents a pivotal cytokine that plays a significant role in the pathogenesis of AS (Xu, Liu & Gotlieb 2010; Miller, Weiss & Heistad 2011). Therefore, it can be argued that TGF-β1-induced calcification in vitro represents an appropriate model of clinical processes.

3.2.2 Oxidative stress and AV cell calcification: The possible interplay between TGF-β1 and the TXNIP-TRX system

TGF-β1 has been found to induce the generation of reactive oxygen species (ROS) and contribute to intracellular oxidative stress in a variety of cell types (Yoon et al. 2005; Ismail et al. 2009; Tong et al. 2010; Cui et al. 2011).

There are many intrinsic intracellular anti-oxidant mechanims, such as the glutathione system and superoxide dismutases (SODs) that help regulate and reduce the effects of cellular oxidative stress, which to a large extent is induced by intracellular ROS. As discussed previously, the TXNIP-TRX system also plays an important role in maintaining and regulating intracellular redox balance. The present study showed that treatment of porcine AVICs with exogenous TGF-β1 not only resulted in the formation of calcific nodules, but led to the development and progression of intracellular oxidative stress, as exhibited by the significant increase in the intracellular pro-oxidant, TXNIP (figure 3.3), and corresponding decrease in the activity of the ubiquitous anti-oxidant, TRX (figure 3.2). It has been well documented that intracellular TXNIP, especially when generated in excess, binds to the anti-oxidant, TRX, thus contributing to intracellular oxidative stress, inflammation, and often to apoptosis. When bound to TXNIP, TRX is unable to utilise its catalytic cysteine residues to scavenge intracellular ROS, or to reduce a variety of oxidised intracellular proteins (Harrison 2005; Chung et al. 2006, Kim at al. 2007; Watanabe et al. 2010).

The finding that TGF-β1 contributes to intracellular oxidative stress was further confirmed in previous work performed in our laboratory by Kennedy et al. (2009) utilising similar cell culture protocols (porcine AVICs) treated with TGF-β1 (5ng/ml), which showed that TGF-β1 contributed to the generation of significant amounts of intracellular superoxide (ROS); thus causing these cells to undergo oxidant stress. There is ample evidence that ROS release is increased in AS:- Miller et al. (2008) showed that superoxide and hydrogen peroxide levels were significantly elevated in stenotic human aortic valves, compared to non-stenotic valves. In addition, another study confirmed significant increases in superoxide and hydrogen peroxide levels, along with increased expression of the oxidative enzyme subunits of NADPH oxidase in stenotic human aortic valves, especially around calcifying nodular foci (Liberman et al. 2008). The same study found that rabbits treated with a high cholesterol diet and vitamin D_2 exhibited features of AV sclerosis (ASc), and were shown to have significantly higher levels of superoxide and hydrogen peroxide levels preferentially around calcifying nodular foci, compared to the aortic valves of rabbits on a normal diet. However, no studies to date have demonstrated that ROS generation is TGF-β1-induced in vivo. Thus, in support of the results of the current study, many previous studies provide conclusive evidence that TGF-β1 contributes to oxidative stress, and that intracellular oxidative stress is likely to be a major factor in the pathogenesis leading to the development and progression of AV calcification, culminating in the development of AS.

However, the findings of the present study showing TGF-β1-induced nodule formation in porcine AVIC monocultures exhibiting significantly higher levels of TXNIP

(measured by immunofluorescence [IF]) (figure 3.3), and correspondingly lower TRX activity levels (figure 3.2), provides for a more interesting and complicated scenario. This is mainly because the TXNIP-TRX system, as mentioned earlier, is involved in a number of intracellular processes that vary depending on the cell types and intracellular environment; thus making pathway predictions more complex. To elaborate further on this point, some examples illustrating this complexity will be illustrated. For example, Junn et al. (2000) showed that mouse TXNIP messenger ribonucleic acid (mRNA) expression was strongly induced by the addition of exogenous hydrogen peroxide (200μM) in murine T cell hybridoma cells, and coupled with a significant increase in TRX gene expression, contributed to apoptosis. Thus, the researchers concluded that oxidative stress induced by exogenous ROS (hydrogen peroxide) culminated in the increased expression of both TXNIP and TRX mRNA. Conversely, Wang et al. (2002) showed the TXNIP-TRX system behaving differently in response to hydrogen peroxide. Wang et al. (2002) showed that exogenous hydrogen peroxide (100μM) significantly reduced both the expression and translation (protein levels) of TXNIP in neonatal rat ventricular myocytes, compared to untreated controls. However, the levels of TRX mRNA and protein levels remained unchanged following treatment with hydrogen peroxide. Interestingly, TRX activity levels (measured via the insulin reduction method) significantly increased despite TRX protein levels remaining unchanged following treatment with hydrogen peroxide. Thus, the study suggested that oxidative stress resulted in the suppression of TXNIP, and correspondingly increased TRX activity levels, in spite of TRX mRNA and protein levels in the treatment and control arms remaining unchanged.

Furthermore, many studies have revealed that over-expression of TXNIP in various animal and human cell lines result in enhanced intracellular levels of ROS (Junn et al. 2000; Schulze et al. 2002; Wang et al. 2002; Cheng et al. 2004; Schulze et al. 2004; Yoshioka et al. 2004; Zhou et al. 2011). In conclusion, extensive research has been performed revealing the complexity of the TXNIP-TRX system in different cell types, and in response to various stimuli and environmental conditions. Overwhelmingly, the evidence supports the role of TXNIP as a major inducer of ROS, resulting in TRX activity suppression, thus contributing to intracellular oxidative stress. Thus, these mechanisms provide some plausible explanations as to TGF-β1-induced nodule formation contributing to an increase in intracellular redox stress, as exhibited by the increase in TXNIP IF (figure 3.3), and corresponding decrease in TRX activity (figure 3.2), in porcine AVIC monocultures.

TGF-β1 itself has been shown to significantly induce TXNIP expression. Junn et al. (2000) conclusively showed that primary cultures of mouse lung fibroblast cells treated with TGF-β1 (1ng/ml) exhibited significantly increased levels of TXNIP mRNA expression. The same researchers also indicated that TGF-β1 up-regulated TXNIP expression in TGF-β1-induced erythroid cells. Han et al. (2003) went on to show that human stomach cancer cells treated with various concentrations of TGF-β1 (0, 0.5, 1.0 and 2.0ng/ml) resulted in significant increases in TXNIP mRNA expression, while correspondingly inhibited tumour cell proliferation. In addition, a study performed in mouse mesangial cell lines revealed that TGF-β1 induced TXNIP expression, increased ROS generation, and induced mesangial cell apoptosis (Cheng, Jiang & Singh 2007).

Hamada and Fukagawa (2007) also showed that streptozotocin-induced diabetic mice exhibited significantly increased expression of TGF-β1 and TXNIP mRNA, along with increased renal oxidative stress, compared to normal non-diabetic mice. Thus, these previous studies provide evidence whereby TGF-β1 directly, or via down-stream pathways, leads to the up-regulation of intracellular TXNIP; results supported by the present study (figure 3.3). However, the exact mechanisms involved in TGF-β1 contributing to the up-regulation of TXNIP require further elucidation.

However, making the link between TXNIP and calcific nodule formation makes the evaluation of mechanistic changes somewhat more complicated. At this stage, a likely conclusion would be to infer that the formation of calcific nodules in porcine AVICs is primarily the result of induction by TGF-β1, along with the contribution of intracellular ROS generation and oxidative stress. TXNIP on the other hand may just be an "innocent bystander" that has been up-regulated by TGF-β1 and possibly by ROS generation, with minimal or no impact in contributing to calcific nodule formation. However, it is also plausible that excess intracellular TXNIP may be involved in some downstream pathway that directly results in the formation of calcific nodules. Figure 3.7 illustrates some of the proposed mechanisms that potentially contribute to AV cell calcification and nodule formation.

Thus, one can postulate that if a direct TXNIP-induced calcific nodule formation mechanism exists, blocking the actions of TXNIP directly or via TXNIP gene silencing, may eventuate in inhibition of TGF-β1-induced nodule formation. However, in there lies

the limitations of the present study, and more research is required to further delineate the downstream actions of TXNIP in porcine AVIC culture models, possibly by the utilisation of TXNIP small interfering RNA (siRNA) in TGF-β1 treated cultures.

In conclusion, the results obtained in the present study support the previous evidence that oxidant stress does in fact play a significant role in the development of porcine AV cell calcification, and therefore that it likely contributes to the pathogenesis of AS.

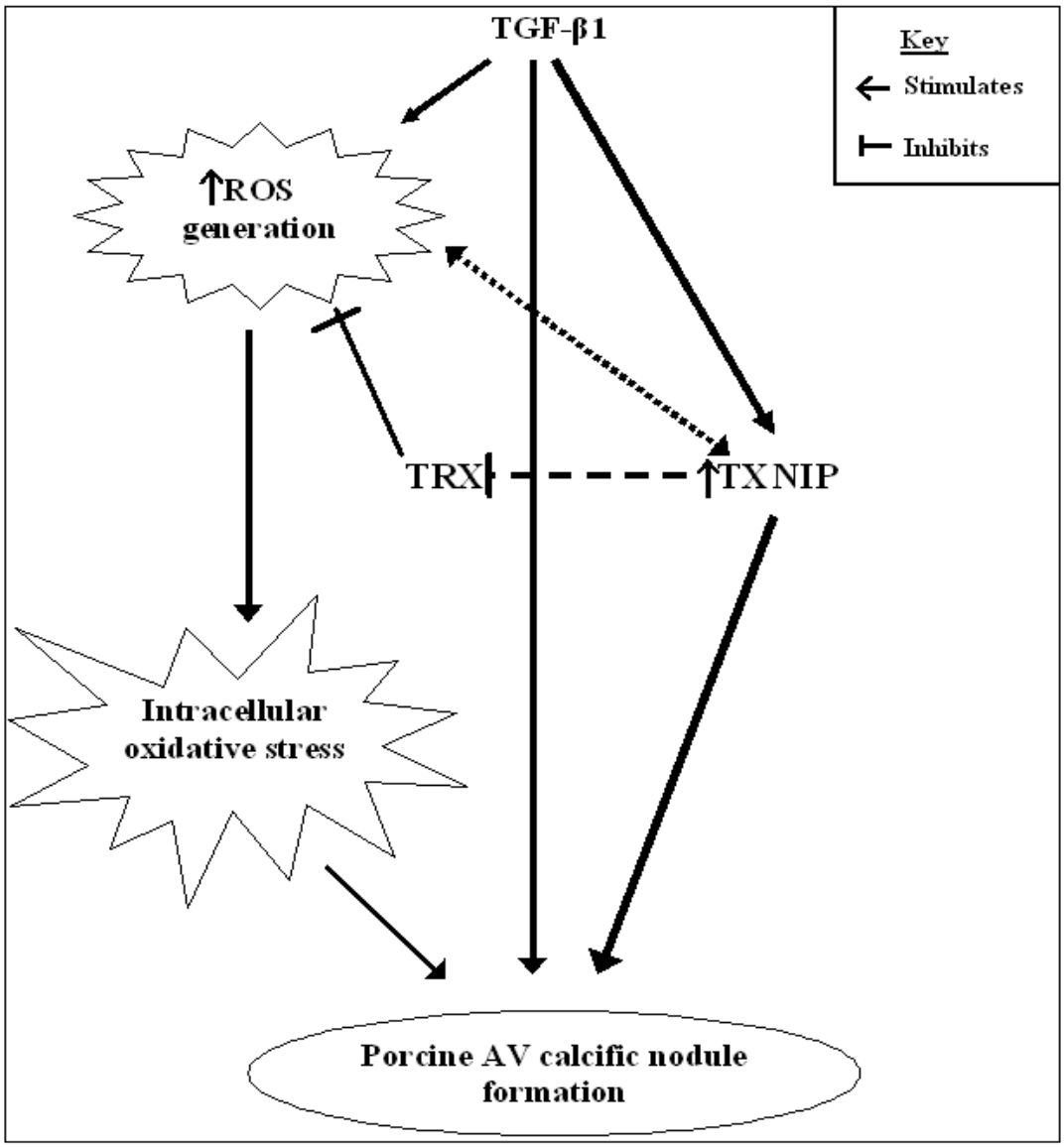

Figure 3.7 Schematic representation illustrating possible mechanistic pathways involving TGF-β1 and the TXNIP-TRX system in contributing to the development of porcine AVIC calcific nodule formation

3.2.3 The attenuating effects of nitric oxide on TGF-β1-induced nodule formation, and its possible interactions with the TXNIP-TRX system

As mentioned in the introductory section, the aortic valve (AV) is subject to significant turbulent flow forces when performing its usual function, especially on the aortic side of the trileaflet valve, resulting in this surface experiencing considerable mechanical stress (Freeman & Otto 2005; Butcher et al. 2006; Xu, Liu & Gotlieb 2010). Over time, the AV may become susceptible to these continued turbulent circulatory forces, probably developing endothelial injury on its aortic side, leading to ongoing inflammation, fibrosis and calcification of the valve. This has been supported by previous evidence demonstrating pathological lesions occurring preferentially on the aortic surfaces of valve leaflets (Hurle, Colvee & Fernandez-Teran 1985; Otto et al. 1994; Mirzaie et al. 2002).

As extensively explored in the earlier sections of the thesis, the endothelial monolayer produces nitric oxide (NO), which is critical to normal valve homeostasis. Importantly, the present study confirmed that the NO donor, DETA-NONOate (20μM), significantly attenuated TGF-β1-induced nodule formation in porcine AVIC monocultures (section 3.1.1.2, figure 3.1), as previously shown in a study by Kennedy et al. (2009). Furthermore, DETA-NONOate appeared to ameliorate TGF-β1's suppression of TRX activity, resulting in the restoration of TRX activity back to control/basal levels (figure 3.2), along with restoring intracellular TXNIP to control/basal levels, as measured by IF (figure 3.3). Previous work by Kennedy et al. (2009) revealed that DETA-NONOate

(20μM) was also able to reduce intracellular ROS generation (superoxide) in porcine AVIC monocultures treated with TGF-β1 (5ng/ml). The study showed inhibition of nodule formation by an agent that directly increased intracellular cGMP (8-bromo cGMP), thus suggesting that NO's inhibition of TGF-β1-induced nodule formation was probably mediated via activation of sGC. A novel study by Kanno et al. (2008) revealed that NO prevented the differentiation of murine vascular smooth muscle cells (VSMCs) into osteoblastic cells by inhibiting TGF-β1 signalling via a cGMP-dependent pathway. In this study, ALP activity was increased in calcifying VSMCs treated with TGF-β1. However, with the addition of the exogenous NO donor, not only was ALP activity inhibited, but expression of other osteoblastic markers including type I collagen, osteocalcin and matrix Gla protein 2 (MDP2) were also significantly inhibited. In addition, the study also showed that NO inhibited mineralisation/calcification of VSMCs (measured by Alizarin red staining). Importantly, the study revealed an important mechanistic pathway whereby NO resulted in the inhibition of TGF-β1-induced phosphorylation of Smad2/3, along with inhibiting the expression of a vital TGF-β1 receptor, ALK5 (a type I receptor). This finding is particularly vital, as ALK5 activation induces the phosphorylation of Smad2/3, which is important for inducing vascular calcification. Furthermore, in the present study, treatment of porcine AVICs with SB431542 (TGF-β1 inhibitor), which also blocks the ALK5 receptor, resulted in the complete inhibition of TGF-β1-induced nodule formation. Thus, collectively, the findings from Kanno et al. (2008), Kennedy et al. (2009) and the present study provide additional support in NO possibly contributing to direct inhibition/interference of TGF-β1 signalling, along with reducing ROS generation and overall intracellular oxidative

stress in porcine AVICs treated with TGF-β1, as exhibited by the reduction in intracellular superoxide generation and TXNIP (pro-oxidant), with the corresponding restoration of intracellular TRX anti-oxidant activity.

Another potential mechanism whereby NO mediates its inhibitory effects on calcific nodule formation and intracellular oxidative stress is via S-nitrosylation of TRX; that is independent of the sGC/cGMP pathway. Human and animal studies have shown that S-nitrosylation of TRX markedly enhanced its anti-oxidant and anti-apoptotic properties, contributing to reduced oxidative stress and increased cellular protection (Haendeler et al. 2004; Tao et al. 2004). S-nitrosylation involves the reversible covalent binding of NO to a cysteine thiol (SH-) group resulting in the formation of S-nitrosothiol (SNO), whereby in the case of TRX, this process brings about the enhancement of its anti-oxidant activity. Interestingly, a study found that statin treatment resulted in S-nitrosylation of TRX in human umbilical vein endothelial cells, thus significantly augmenting TRX activity, and culminating in intracellular ROS reduction (Haendeler et al. 2004). Furthermore, a recent study by Forrester et al. (2009) showed that NO directly suppressed TXNIP gene expression, rendering TRX free to perform denitrosylation of proteins, thus protecting cells from nitrosative stress. However, in the present study, it is less likely that this mechanism was a major contributor to the enhancement of TRX activity in cultures treated with both TGF-β1 and DETA-NONOate, as the positive controls that were treated with DETA-NONOate alone did not exhibit enhanced TRX activity above that of untreated controls (figure 3.2). With regards to NO's contribution to the suppression of intracellular TXNIP as measured by IF (figure 3.3), several

plausible mechanisms can be proposed: for example the overall decrease in intracellular oxidative stress by NO's suppression of TGF-β1 may have led to a decrease in TXNIP expression, or NO may have directly suppressed TXNIP transcription (Schulze et al. 2006; Forrester et al. 2009).

It is important to consider a major and partially analogous study by Schulze et al. (2006) that showed a novel approach to the regulation of the TXNIP-TRX system by NO. This study revealed that rat pulmonary artery smooth muscle cells (RPaSMCs) incubated with the NO donor, S-nitroso-glutathione (GSNO), resulted in the suppression of TXNIP gene expression, while enhancing TRX reductase gene expression. Furthermore, cultures treated with this NO donor demonstrated decreased TXNIP protein levels, with corresponding increases in TRX reductase protein levels. TRX protein levels were not increased by the NO donor, however, it did result in a significant increase in TRX activity levels in RPaSMCs, compared to unstimulated cells. Interestingly, the study also showed that pre-treatment of RPaSMCs with a sGC inhibitor did not lead to inhibition of GSNO-induced changes in TXNIP and TRX reductase gene expression. Thus, the study authors concluded that the findings provided evidence of a sGC/cGMP-independent effect of NO in suppressing TXNIP expression, while inducing TRX reductase expression through redox-regulatory mechanisms. In addition, the researchers showed that NO was able to suppress TXNIP transcription via NO-responsive cis-regulatory elements within the TXNIP gene promoter region, again independent of the sGC/cGMP pathway.

Furthermore, even though DETA-NONOate was able to completely reverse the effects of TGF-β1 on TXNIP IF and TRX activity levels back to baseline/control levels respectively (figure 3.3 and 3.2), it did not completely block TGF-β1-induced nodule formation. However, SB431542 did result in complete blockade of TGF-β1-induced nodule formation. Thus, this suggests that the actions of the TXNIP-TRX system, at minimum, may be partially independent of the effects of TGF-β1.

In conclusion, the results of the present study showed NO attenuating TGF-β1-induced nodule formation, with a resultant increase in intracellular TRX activity, and a corresponding decrease in TXNIP levels, leading to reduced intracellular oxidative stress and porcine AV cell calcification. Potential mechanisms that may be involved in this process are illustrated in figure 3.8. Whether these effects were sGC/cGMP-dependent or not were not explored in the current experiments. Nonetheless, the protective role of NO with respect to AV function has been clearly established. However, in the event that AV endothelial injury and dysfunction ensue, a potential decrease in NO production, bioavailability or responsiveness (for example via impaired sGC activity), coupled with a series of environmental and intrinsic risk factors, would likely contribute to the early processes involved in the pathogenesis of AS.

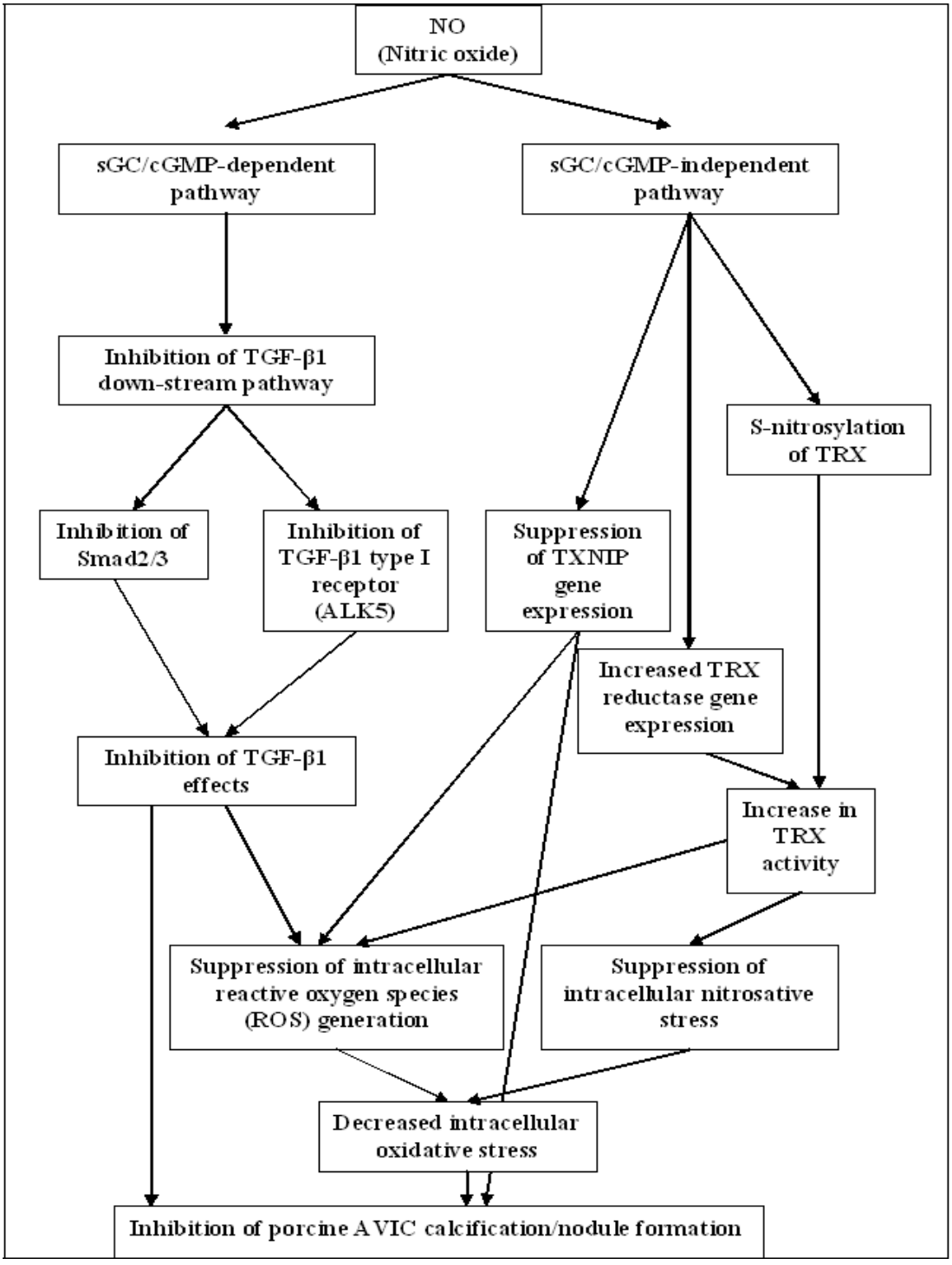

Figure 3.8 Potential mechanisms involving the effects of NO on TGF-β1 and the TXNIP-TRX system, contributing to inhibition of porcine AVIC calcification/nodule formation

3.2.4 The effects of TGF-β1 and NO, and their possible interactions with the TXNIP-TRX system in affecting porcine AVIC survival

In the present study, it was found that TGF-β1-induced nodule formation was associated with decreased overall porcine AVIC survival (section 3.1.4.1, figure 3.6). Furthermore, the attenuating effects of the exogenous NO donor, DETA-NONOate (20μM), on TGF-β1-induced nodule formation was accompanied by an increase in porcine AVIC survival (section 3.1.4.2, figure 3.6), compared to cultures treated with TGF-β1 alone. The present study did not measure cellular apoptosis, and that some of the cells measured in the study may have also been in the process of undergoing apoptosis. However, it is likely that a significantly large number of susceptible cells underwent early apoptosis and cellular lysis prior to termination of experiments, following which the remaining cells in response to various treatments were subsequently quantified. Thus, it can be postulated that the difference in magnitude of results obtained via direct measurement of apoptosis may have been greater in response to individual treatments, but the significant trend in the results derived from the present study would have more than likely been preserved.

Nonetheless, several studies have indeed shown the effects of TGF-β1 contributing to cellular apoptosis. Sales et al. (2006) showed that ovine endothelial progenitor cells stimulated by TGF-β1 exhibited enhanced apoptosis, compared to unstimulated controls. Furthermore, cell culture studies performed in sheep AVICs (Jian et al. 2003) revealed that TGF-β1-induced calcific nodule formation was accompanied by significant

apoptosis. However, subsequent inhibition of apoptosis did not contribute to a reduction in TGF-β1-induced nodule formation in both sheep (Jian et al. 2003) and porcine AVICs (Kennedy et al. 2009). The results of these studies suggest that cellular apoptosis is likely to be an associated finding, rather than being a contributing factor in initiating AV cell calcification and nodule formation.

Adding to the complexity of the above argument is the association and/or interaction between TGF-β1, NO and the TXNIP-TRX system, in relation to porcine AVIC survival. Firstly, not only was TGF-β1-induced nodule formation associated with a decrease in overall porcine AVIC survival, but was also found to be significantly associated with a corresponding increase in intracellular TXNIP IF levels (figure 3.3) and decreased TRX activity levels (figure 3.2). TGF-β1 is not the only "player" contributing to the inhibition of cellular proliferation and induction of apoptosis, but the TXNIP-TRX system also plays a crucial role in these processes. The mechanism of TXNIP inhibition of TRX has been well documented. Firstly, excess intracellular TXNIP binds to both the catalytic cysteines of TRX, consequently inhibiting its ability to bind to apoptosis signalling kinase-1 (ASK-1). ASK-1 is a mitogen-activated protein (MAP) kinase kinase kinase, which leads to inflammation and apoptosis. Thus, unbound ASK-1 under the influence of TNF-α is phosphorylated resulting in activation of ASK-1, which further activates downstream p38 and Jun-terminal kinase (JNK), resulting in increased expression of vascular cell adhesion molecule-1 (VCAM-1), and contributing to cellular apoptosis; eventuating in reduced overall cell survival (Ichijo et al. 1997; Saitoh et al. 1998; Nishiyama et al. 1999; Harrision 2005; Yamawaki et al. 2005; World, Yamawaki &

Berk 2006; Kim et al. 2007). Thus, with regards to the present study, it is feasible to postulate that excess intracellular TXNIP produced in TGF-β1 treated cultures, resulted in its binding to TRX, thereby inhibiting TRX activity, and subsequently leading to a cascade of events contributing to increased cellular apoptosis, and finally culminating in reduced overall cell survival in porcine AVIC monocultures. These pro-apoptotic properties of TXNIP have also been demonstrated in many other cell types, such as cardiomyocytes, cerebellar granule neurons, lymphoma T cells and pancreatic beta cells (World, Yamawaki & Berk 2006; Kim et al. 2007).

The NO donor, DETA-NONOate, was shown in the present study to attenuate the effects of TGF-β1-induced nodule formation, contributing to decreased intracellular TXNIP, increased TRX activity, and correspondingly increased porcine AVIC survival. Thus, since calcific nodule formation is accompanied by increased cellular apoptosis (Jian et al. 2003), then inhibition of nodule formation would result in decreased apoptosis and improved cell survival. Another mechanism for NO to improve cell survival in various cell types is to prevent apoptosis via the S-nitrosylation of caspase-3 by TRX (Mitchell & Marletta 2005; Mitchell et al. 2007). The activation of cysteine proteases, known as caspases, is required for the initiation and execution of apoptosis. Furthermore, caspase-3 in particular plays a vital role in the final apoptotic pathway, and is affectionately termed the ‘executioner’ (Kumar et al. 2010). Mitchell and Marletta (2005) showed that a single cysteine in TRX was capable of a reversible trans-nitrosylation reaction with cysteine 163 of caspase-3, thus inhibiting its pro-apoptotic effects. In addition, a study by Mitchell et al. (2007) went on to show the crucial role of TRX in catalysing the S-

nitrosylation of procaspase-3 (caspase-3 precursor). Thus, these studies reveal a crucial process whereby NO with the aid of TRX, results in the inhibition of caspase-3, and subsequent inhibition of apoptosis. Furthermore, an earlier study (Andoh, Chiueh & Chock 2003) showed that NO inhibited apoptosis in human neuroblastoma cells via activation of cGC, which then activated the cGMP-dependent protein kinase (PKG). The activated PKG subsequently resulted in the protection of cells from lipid peroxidation and apoptosis, inhibition of caspase-3 and -9 activation, and contributed to increased TRX and TRX peroxidase levels. Thus, these earlier studies suggest possible mechanisms by which NO in the present study contributed to the improvement in porcine AVIC survival in cultures treated with TGF-β1.

In conclusion, it is not unreasonable to suggest that TGF-β1 itself has direct pro-apoptotic effects, and is also able to induce intracellular ROS and TXNIP, leading to reduced TRX activity, and thus indirect potentiation of pro-apoptotic effects. These potential mechanisms may contribute to the initiation of apoptosis, and possibly to decreased porcine AVIC survival. Importantly, NO significantly attenuates these effects, whether mediated via sGC/cGMP-dependent pathways (Andoh, Chiueh & Chock 2003) and/or by S-nitrosylation mechanisms (Mitchell & Marletta 2005; Mitchell et al. 2007), resulting in the inhibition of apoptosis, and thus improving overall porcine AVIC survival.

3.3 Study limitations

Despite the present study showing some conclusive and novel results pertaining to the effects of TGF-β1 and NO on porcine AVICs, and the possible associations and interactions with the TXNIP-TRX system, there exist several limitations. Firstly, even though intracellular TXNIP levels were quantified by immunofluorescence (IF) following various treatments, the findings of the current study would have been further strengthened by directly measuring TXNIP gene expression (TXNIP mRNA) and intracellular protein levels. The same can be said for TRX, whereby the present study did not measure TRX gene expression or protein levels, which would have further enriched the current research findings. Furthermore, the current study did not distinguish between the predominantly cytosolic (TRX 1) and mitochondrial (TRX 2) isoforms of TRX, which may have been subject to differential changes. Thus, the present study exhibited a lack of direct evaluation of underlying mechanisms to explain the results obtained.

Secondly, the AVIC culture model adopted in the present study has been widely utilised in previous studies involving various animal species. However, besides AVICs, there are many other different cell types that constitute the AV, such as smooth muscle and endothelial cells. As a result, the present study did not assess the effects of various treatments on other cell types, making extrapolation of the current findings to encompass the entire AV more difficult. Furthermore, despite extreme care being instituted to isolate and perform experimentation on AVICs, the possibility of contamination by

infiltrating cells, and subsequent confounding of results cannot be fully excluded. In addition, any associated AVIC morphological changes in response to the various treatments were not directly assessed in the present study.

Thirdly, the current cell culture model utilised in the present study was evaluated in vitro under highly controlled and sterile conditions, with the addition of various exogenous treatments being performed at specified moments in time. However, the normal AV in vivo is continually subjected to a whole range of mechanical forces, intrinsic risk factors, and often to modulation by multiple cytokines, chemokines, and other compounds. This realisation significantly limits the present study results being extrapolated to the intact AV.

Furthermore, an important inference from the study results is that increased intracellular TXNIP may have been directly involved in inducing calcific nodule formation. However, this hypothesis was not directly tested by direct TXNIP inhibition (example via TXNIP siRNA) in the present study.

Lastly, despite the extensive adoption of animal AV cell culture models mimicking AV calcification in experiments, few researchers have experimented with normal human AVIC culture models. This unfortunately is also a limitation of the present study model; again restricting generalisation to encompass the entire human aortic valve. In fact, many researchers have utilised AVICs from diseased or stenosed aortic valves. However, the cells obtained from these pathological valves pose a number of limitations, including

being subjected to chronic pathological insults in vivo resulting in permanent cellular morphological changes. Consequently, these cell culture models are often resistant to in vitro exogenous testing by various compounds, rendering them less comparable to ‘normal’ human AVICs. Furthermore, the so-called normal aortic valves obtained during autopsies are also not entirely ‘normal’, as these valves would have been exposed to various levels of hypoxia, and likely experienced hypoxia-induced oxidative stress, rendering them relatively unsuitable in controlled experimentation, especially with regards to the intricate study and analysis of cellular oxidative stress.

CHAPTER 4

CONCLUSIONS

&

FUTURE STUDIES

4.1 Study conclusions

The primary conclusions derived from the present study are:

1) TGF-β1, via a conventional receptor (that is one inhibited by SB431542), induces the following effects in porcine AVIC monocultures:-
    a) Calcific nodule formation; effects similar to those shown previously by Walker et al. (2004) and Kennedy et al. (2009),
    b) Impaired intracellular TRX activity,
    c) Increased intracellular TXNIP IF, and
    d) Decreased overall cell survival.

2) The NO donor, DETA-NONOate, in concentrations previously shown to inhibit TGF-β1-induced nodule formation (Kennedy et al. 2009), :-
    a) Exhibits partial inhibition of calcific nodule formation; effects similar to those demonstrated previously by Kennedy et al. (2009),
    b) Normalises intracellular TRX activity,
    c) Restores intracellular TXNIP IF to baseline control levels, and
    d) Improves overall cell survival.

4.2 Future studies and potential therapeutic options

4.2.1 TGF-β1 and aortic valve stenosis: potential therapeutic options

The role of TGF-β1 in contributing to the pathogenesis of AS has been clearly established in many in vitro studies (Jian et al. 2003; Kennedy et al. 2009; Yetkin & Waltenberger 2009; Elmariah & Mohler 2010b; Xu, Liu & Gotlieb 2010; Miller, Weiss & Heistad 2011). However, no study to date has directly evaluated the effects of TGF-β1 on a normal functioning AV in vivo. Thus, an experimental animal model can be established, for example involving rabbits, followed by systemic injection of TGF-β1. The rate of development and progression of ASc/AS in these models can then be assessed with echocardiography, followed by immuno-histological examination of the valve explants following termination of experiments. In addition, this model would be far more representative in exhibiting the effects of TGF-β1 on functioning aortic valves, taking into account the multiple effects of the complex in vivo environment on the TGF-β1-AV interaction. However, one major drawback in this animal model that requires consideration is the unwanted or unexpected systemic effects induced by exogenous TGF-β1 that may confound the results.

Interestingly, there are several drugs/compounds that have been developed that result in direct inhibition of TGF-β1 or its down-stream pathways. For example, several anti-TGF-β1 antibodies have been investigated in Phase I trials related to the treatment of pulmonary fibrosis and animal diabetic nephropathy. Furthermore, other compounds have been developed that inhibit the TGF-β1 receptor (ALK) or Smad 2/3 pathway

(Redondo, Santos-Gallego & Tejerina 2007). Thus, in the future, the possible development of potential therapies directed against AV-specific TGF-β1 or its down-stream pathways, may prove beneficial in slowing, arresting, or even preventing the development of AS. It is also possible that the formation of TGF-β1 might represent a potential therapeutic target. For example the intracellular (predominantly platelet) protein, thrombospondin-1 (TSP-1) may increase expression of TGF-β1 (Crawford et al. 1998; Ahamed et al. 2009) and also inhibit NO signalling (Isenberg et al. 2009); thus inhibition of TSP-1 signalling may retard valve calcification via these 2 mechanisms.

4.2.2 NO: future research and potential therapeutic options in aortic valve sclerosis / aortic valve stenosis

A first step would be to establish an animal model, for example involving rabbits, whereby development of ASc or AS is induced by feeding the rabbits vitamin D_2, following similar experimental protocols as described by Ngo et al. (2008). To date, it has been demonstrated that the ACE inhibitor, ramipril, retards AS development in this model, but ramipril also reduced TXNIP accumulation and preserved the NO effect (Ngo et al. 2011). One possible approach therefore, would be to evaluate the role of NO in AS development in this model via a combination of the following:-

(1) evaluate whether a NOS inhibitor potentiates vitamin D_2 effects and/or nullifies ramipril protection, or

(2) evaluate whether co-administration of a NO donor mimics the ramipril effect.

Direct or indirect increases in NO availability within the valve holds promise in slowing the progression of ASc in affected individuals developing into clinically significant AS.

4.2.3 A crucial link in aortic valve stenosis development: TXNIP–TGF-β1 nexus?

In the present study, it was proposed that up-regulation of TXNIP in porcine AVICs may have been directly involved in the development of AV cell calcification/calcific nodule formation, and possibly directly contributing to AS pathogenesis. A potential study involving porcine AVIC monocultures treated with TGF-β1, with the co-administration of TXNIP siRNA, would confirm or disprove this hypothesis. In the event that TGF-β1-induced nodule formation is found to be inhibited by TXNIP siRNA, then this will provide conclusive evidence that increased levels of intracellular TXNIP directly contributes to the development of calcific nodule formation, and potentially to the development of AS. However, in order for this model to be successful, the knock-down of TXNIP would have to persist to a substantial extent throughout the entire duration of treatment so as to achieve conclusive results. Furthermore, if this hypothesis is confirmed in cell culture models, then animal models can be adopted to hopefully further consolidate this finding. If this approach were to be successful, translation to the clinical arena would be less than specific at this stage: known methods for suppressing TXNIP expression are:-

(1) prevention of hyperglycaemia, and/or

(2) increased NO availability.

Thus, this finding would present a novel therapeutic challenge.

4.3 Final remarks

The present study contributes small, but significant insights into the role of oxidant stress in a cellular model of aortic valve cell calcification. Extrapolating further, one could safely infer from the present results and previous studies that chronic intracellular oxidant stress, along with a number of other physiological, metabolic and pathological mechanisms, contributes to the pathogenesis of AS development and progression. Ultimately, the conclusion to be drawn is that development of AS is theoretically preventable by attenuating critical biochemical pathways. There is clearly a great translational imperative in this area.

CHAPTER 5

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