April 2014

Effects of an Angiotensin II Type 1 Receptor Blocker on Cardiovascular Calcification

Zachary B. Armstrong
The University of Western Ontario

Supervisor
Dr. Kem A. Rogers
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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EFFECTS OF AN ANGIOTENSIN II TYPE 1 RECEPTOR BLOCKER ON CARDIOVASCULAR CALCIFICATION

(Thesis format: Integrated Article)

by

Zachary Brian Armstrong

Graduate Program in Anatomy & Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

**Aims:** Three types of cardiovascular calcification are commonly found in humans: arterial calcification, intimal calcification, and calcific aortic valve disease. Very little is known about the mechanisms driving cardiovascular calcification despite serious clinical implications and a clear association with morbidity and mortality. Indeed, it is even unclear whether the same factors are involved in arterial, intimal, and valvular calcification. The objective of this study was to elucidate the effects of an angiotensin II type 1 receptor blocker (ARB) on the progression of cardiovascular calcification in male New Zealand White rabbits. Where appropriate, statins were examined in conjunction and in combination with ARBs.

**Methods and Results:** *In vivo* and *ex vivo* techniques were used to assess overall disease burden and the extent of calcification including magnetic resonance imaging, micro-computed tomography, histology, and immunohistochemistry. ARB administration significantly inhibited progression of arterial calcification (2.80 ± 1.17 versus 0.01 ± 0.01 % calcified tissue in Cholesterol and ARB-treated, respectively; P < 0.05), but not intimal or valvular calcification. ARB treatment significantly reduced atherosclerotic lesion area when delivered alone (95.50 ± 1.94 versus 61.61 ± 10.17 % lesion area in Cholesterol and ARB-treated, respectively; P < 0.05), but not when combined with statin therapy (92.39 ± 3.25 % in ARB+Statin; P < 0.05 when compared to ARB monotherapy). Finally, ARB-treated animals had significantly increased valvular calcium.

**Conclusions:** This study provides evidence that ARBs robustly inhibit arterial calcification and is the first to suggest ARBs as a novel treatment option for those at risk for cardiovascular calcification. It also suggests that ARBs may not be beneficial for those at risk for intimal or valvular calcification. These disparate results suggest that the three types of cardiovascular calcification are distinct from one another and provides impetus to further examine the underlying molecular mechanisms at play in these debilitating disease processes.
Keywords

aortic valve sclerosis, angiotensin receptor blocker, arterial calcification, atherosclerosis, calcific aortic valve disease, pre-clinical model, renin-angiotensin system, statin, valvular interstitial cell, vascular smooth muscle cell
Co-Authorship Statement

Chapter 2:

Entitled “Angiotensin II type 1 receptor blocker inhibits arterial calcification in a pre-clinical model” was written by Zachary B. Armstrong with inputs from Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers, experimental procedures and data analyses were performed by Zachary B. Armstrong. Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers provided intellectual input.

Chapter 3:

Entitled “Potential negative interaction between statin therapy and angiotensin receptor blockade in atherosclerotic lesion regression” was written by Zachary B. Armstrong with inputs from Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers, experimental procedures and data analyses were performed by Zachary B. Armstrong and Colin P. Carruthers. Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers provided intellectual input.

Chapter 4:

Entitled “Effects of an angiotensin II type 1 receptor blocker on aortic valve sclerosis in a pre-clinical model” was written by Zachary B. Armstrong with inputs from Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers, experimental procedures and data analyses were performed by Zachary B. Armstrong and Colin P. Carruthers. Dr. Derek R. Boughner, Dr. Maria Drangova, and Dr. Kem A. Rogers provided intellectual input.
Acknowledgments

“If I have seen further it is by standing on the shoulders of giants.”
— Isaac Newton, 1676

My time at The University of Western Ontario, both undergraduate and graduate, has been a wholly enriching experience. For nearly a decade I have lived and breathed Western, interacting with thousands of students, staff, and faculty. Not once have I been disappointed by the energy, enthusiasm, and drive of the Western community; it truly is an amazing place.

My time in the Rogers Lab has been equally rewarding. This thesis would not have been possible without the assistance and encouragement of hardworking technicians, fellow students, and good friends. In particular, thank you to Kim Thomaes and Colin Carruthers for making the lab especially interesting.

To my supervisors, Kem and Derek:

Your advice and support has been invaluable to my development as a scientist and as a person. I have always appreciated your counsel and will continue to do so in the months and years to come.

Finally, to my parents, Jim and Kim:

You have supported me every step of the way, even when you could no longer see where I was going. There is no way for me to express my gratitude except to say that, without question, I would not be where I am today if not for you. Thank you.
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List of Abbreviations

ACE, angiotensin converting enzyme
ACEI, angiotensin converting enzyme inhibitor
AngI, angiotensin I
AngII, angiotensin II
ARB, angiotensin II type 1 receptor blocker
AT1R, angiotensin II type 1 receptor
AVS, aortic valve sclerosis
BMP, bone morphogenetic protein
Cbfα1/Runx2, core-binding factor alpha1/runt-related transcription factor 2
CKD, chronic kidney disease
DM, diabetes mellitus
DRI, direct renin inhibitor
HMG-CoA, 3-hydroxy-3-methylglutaryl Co-enzyme A
HU, hounsfield units
IEL, internal elastic lamina
LDL, low-density lipoprotein
MI, myocardial infarction
MRI, magnetic resonance imaging
Msx2, muscle segment homeobox 2
NADPH, nicotinamide adenine dinucleotide phosphate
NF-κB, nuclear factor kappa B
OCN, osteocalcin
PPARγ, peroxisomal proliferator-activated receptor-gamma
RANKL, receptor activator of NF-κB
RAS, renin-angiotensin system
ROS, reactive oxygen species
SMA, smooth muscle actin
SMC, smooth muscle cell
VIC, valvular interstitial cell
VSMC, vascular smooth muscle cell
Chapter 1

1 General Introduction

Cardiovascular calcification can be broadly grouped into three categories: arterial calcification, intimal calcification, and calcific aortic valve disease (Figure 1.1). Each class of cardiovascular calcification is associated with unique pathologies. While arterial calcification is mainly associated with diabetes mellitus (DM) and chronic kidney disease (CKD),\(^1\) intimal calcification and calcific aortic valve disease is found more commonly in the elderly and associated with typical risk factors of atherosclerosis.\(^2-5\)

Clinical consequences of cardiovascular calcification are numerous and diverse. In dialysis patients, arterial calcification is responsible for calcific uremic arteriolopathy, a condition causing necrosis of the skin which has a very high mortality rate.\(^6\) Moreover, arterial calcification is correlated with future cardiovascular events in patients with DM and is a strong predictor of mortality in patients with CKD.\(^7,8\) Intimal calcification is associated with an increased risk of myocardial infarction (MI)\(^9,10\) and may promote plaque instability.\(^11,12\) Patients who have aortic valve disease without concomitant coronary artery disease have a 50% increased risk of MI and cardiovascular death compared to patients who have a normal aortic valve,\(^13-16\) and calcification of valvular tissue is recognized as the primary mode of valve failure in both native and bio-prosthetic valves.\(^17\)

Despite the myriad of clinical implications and the clear association with morbidity and mortality, very little is known about the underlying molecular mechanisms leading to cardiovascular calcification. Indeed, it is even unclear whether the same mechanisms are at play in arterial, intimal, and valvular calcification. Most importantly, there is no preventive therapy available to physicians or patients.

1.1 Renin-Angiotensin System

Dysregulation of the Renin-Angiotensin System (RAS) is well known to promote hypertension and cardiovascular disease.\(^18-20\) The RAS is normally responsible for
Figure 1.1: The sinus of valsalva and three types of cardiovascular calcification.

Arterial calcification presents as large masses localized along the internal elastic lamina and within the tunica media. Intimal calcification typically begins as micro-calcifications within the cholesterol-rich atherosclerotic plaque, which develops in the cellular tunica intima. Calcific aortic valve disease also involves micro-calcifications, typically near the base of the collagenous fibrosa layer of the aortic valve.
maintaining fluid volume and preventing ischemia during fluid loss. The main vasoactive agent, angiotensin II (AngII), induces vasoconstriction and sympathetic activation, raises aldosterone levels, and promotes salt and water retention via the AngII type 1 receptor (AT1R). The canonical RAS cascade is rather simple (Figure 1.2). Angiotensinogen, the precursor peptide that is produced in the liver, is cleaved by renin, an enzyme produced by juxtaglomerular cells in the kidney in response to low blood pressure or low sodium levels. Cleavage of angiotensinogen by renin produces angiotensin I (AngI), which appears to have no biological activity. AngI is further cleaved by angiotensin converting enzyme (ACE), usually in the endothelium, to produce AngII. However, ACE is not the only AngII-producing enzyme in the cardiovascular system. Several groups have shown that mast cell-derived chymase and cathepsin G can also produce AngII in blood vessels, the heart, and heart valves. Angiotensin(1–12), which contains two extra amino acids on the C-terminus of AngI, is the substrate for chymase production of AngII. Regardless of its source, AngII exerts its cellular effects via the AT1R. Certain cell types, including much of the cardiovascular system, also express the AngII type 2 receptor which, when bound by AngII, generally opposes the effects of the AT1R. There are a number of clinically available pharmaceuticals that modulate the RAS (Figure 1.2). The direct renin inhibitor aliskiren first became available in 2007 and inhibits the rate limiting step of the RAS cascade, the conversion of angiotensinogen to AngI, thereby reducing the synthesis of all downstream components. ACE inhibitors (ACEIs), as their name implies, directly inhibit ACE and prevent the conversion of AngI into AngII. They also prevent ACE-mediated degradation of bradykinin which elicits positive cardiovascular effects. Unfortunately, chronic administration of ACEIs sometimes leads to reactivation of AngII, which is linked to poorer outcomes. Finally, AngII type 1 receptor blockers (ARBs) inhibit the binding of AngII to the AT1R and thus they are able to inhibit the function of AngII regardless its source, which is particularly important given the capability of mast cell-derived chymase to produce AngII. Furthermore, the affinity of ARBs for the AT1R provide an opportunity for AngII to bind the AngII type 2 receptor and elicit positive cardiovascular effects. Finally, ARBs are
Angiotensinogen, produced in the liver, is cleaved by renin, an enzyme produced by juxtaglomerular cells in the kidney, to form angiotensin I (AngI). Angiotensin converting enzyme (ACE), typically found in the endothelium, cleaves AngI to form angiotensin II (AngII). AngII acts through the AngII type 1 receptor (AT1R) to elicit vasoconstriction, sympathetic activation, salt retention, and water retention. Pharmaceutical inhibitors of the RAS (shown in red) include direct renin inhibitors (DRIs), ACE inhibitors (ACEIs), and angiotensin receptor blockers (ARBs).

Figure 1.2: The canonical pathway of the renin-angiotensin system (RAS).
generally more tolerable than other antihypertensives, with significantly less cough and angioedema.\textsuperscript{18,36,37}

\section*{1.2 Arterial Calcification}

Arterial calcification, also known as medial artery calcification or Mönckeberg sclerosis,\textsuperscript{38} involves calcification of the internal elastic lamina and elastic fibers within the medial layer of the artery resulting in hardening and increased pulse pressure (Figure 1.1). Commonly associated with advanced age, hypertension, CKD, DM, and osteoporosis, arterial calcification is closely related to cardiovascular morbidity and mortality.\textsuperscript{8,39,40}

\subsection*{1.2.1 Initiation and Progression of Arterial Calcification}

Originally considered a passive, degenerative and, most importantly, irreversible process, arterial calcification is now considered to be a highly regulated process resembling natural bone formation (Figure 1.3).\textsuperscript{41,42} The initiating event appears to be the deposition of hydroxyapatite-like material on degraded or damaged elastin fibers. Vascular smooth muscle cells (VSMCs) cultured in a pre-calcified elastin matrix down-regulated their typical biological markers (\(\alpha\)-smooth muscle actin and myosin heavy chain) and up-regulated markers of osteogenic differentiation including core-binding factor alpha1/runt-related transcription factor 2 (Cbf\(\alpha\)1/Runx2), alkaline phosphatase, and osteocalcin (OCN).\textsuperscript{43} When the calcified conditions were removed, VSMCs reverted to their original phenotype, which suggests some potential for regression. In response to elevated levels of extracellular phosphate, VSMCs release matrix vesicles that contain calcium and phosphate ions, especially if local (matrix gla-protein) or circulating (feutin-A) inhibitors are lost.\textsuperscript{44} It is likely, therefore, that VSMCs transdifferentiate to an osteoblast-like phenotype after the local microenvironment is altered.

\subsection*{1.2.2 Osteoblast Transdifferentiation of VSMCs}

An elegant fate-mapping study by Speer \textit{et al.}\textsuperscript{45} has shown VSMCs are capable of osteoblast transdifferentiation in calcifying arteries. This transdifferentiation was associated with downregulation of smooth muscle cell (SMC) markers and upregulation of the osteoblast transcription factor Cbf\(\alpha\)1/Runx2.\textsuperscript{46} Additional osteoblast transcription
Figure 1.3: The cellular interactions underlying cardiovascular calcification. Arterial calcification (top right) involves calcification of the internal elastic lamina and tunica media, a cellular environment consisting of vascular smooth muscle cells (VSMCs). Intimal calcification (top centre) occurs within the cholesterol-rich atherosclerotic plaque, and environment rich with VSMCs, macrophages, lymphocytes, and mast cells. Calcific aortic valve disease (bottom left) exists primarily in the collagenous fibrosa of the aortic valve, involving native valvular interstitial cells (VICs) as well as macrophages, lymphocytes, and mast cells. Solid arrows represent known interactions; dashed arrows represent unclear or unknown processes.
factors, including muscle segment homeobox 2 (Msx2) and osterix, have also been implicated in VSMC transdifferentiation and in the progression of arterial calcification. A number of factors have been shown to induce VSMC differentiation and promote an osteoblast-like phenotype including fibroblast growth factor-2, tumor necrosis factor-alpha, oxidized-low density lipoprotein (LDL), and bone morphogenetic protein (BMP) 2. Although tremendous progress has been made in this area, the molecular mechanisms underlying this process remain to be fully defined.

1.2.3 RAS and Osteoblast Transdifferentiation

There is growing evidence that vasoactive agents are important modulators of vascular calcification. Naturally existing peptides such as endothelin-1 and urotensin II can promote arterial calcification, while others – adrenomedullin and C-type natriuretic peptide – act to inhibit its progression. Until recently, the role of the RAS and its vasoactive agent, AngII, had not been thoroughly investigated. AngII plays a number of roles in vascular pathology, and was thought to exert its effects by inducing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increasing cellular reactive oxygen species (ROS). In turn, ROS stimulate the expression of BMP2 and the osteoblast transcription factor Cbfα1/Runx2, thereby inducing osteoblast transdifferentiation. The first evidence that AngII affected calcification in VSMCs was from Jaffe and Mendelsohn, who suggested it (along with aldosterone) acted through the mineralocorticoid receptor to promote fibrosis and calcification. More recently, an in vitro study by Jia et al. showed that AngII promoted vascular calcification via Cbfα1/Runx2 and nuclear factor κB (NF-κB). The receptor activator of NF-κB (RANKL) and BMP2 axis has been long implicated in arterial calcification, and a subsequent study suggested that AngII induced vascular calcification in vitro and in vivo via RANKL activation. In turn, RANKL promoted ACE and AT1R, members of the RAS pathway, creating a feedback loop.

1.2.4 Clinical Implications

There is evidence to suggest that arterial calcification, at least in the peripheral arteries, may affect 30–50% of asymptomatic patients in the United States. In patients with
CKD, arterial calcification tends to be more advanced and is associated with increased morbidity and mortality. Indeed, the leading cause of death in CKD patients is cardiovascular disease. In patients with non-insulin-dependent DM, arterial calcification is a strong independent predictor of total, cardiovascular, and coronary heart disease-related mortality. It is also associated with increased risk for MI, stroke, and amputation. Tibial artery calcium score also predicts the short-term risk of amputation in patients with peripheral artery disease. There is also some evidence to suggest that calcification of small blood vessels can lead to necrosis and ulceration of the skin. Clearly, arterial calcification is prevalent in Western society and in dire need of preventive therapy.

1.2.5 Pharmaceutical Management

Blockade of the RAS has been shown to reduce morbidity and mortality in patients with hypertension, atherosclerosis, heart failure, stroke, DM, and CKD, often independent of changes in blood pressure. To date, there have been no clinical studies examining the role of RAS blockade on arterial calcification. However, pre-clinical studies do provide some encouraging evidence. Recent studies have shown that ACEIs – specifically, perindopril and captopril – can prevent the progression of arterial calcification in rat models of CKD and hypertension. An older study in a 5/6 nephrectomized rat model found that enalapril could not suppress arterial calcification but did decrease mortality. The ARB irbesartan has also been shown to prevent arterial calcification in a rat model of hypertension as long as therapy is initiated alongside insult, which in this case was warfarin and vitamin K. Irbesartan is also capable of blocking AngII-induced expression of BMP2 in human endothelial cells.

The effects of other pharmaceuticals have also been examined in relation to arterial calcification, with some success. The calcium channel blocker amlodipine, another antihypertensive, was able to induce regression of arterial calcification in a pre-clinical model. The endothelin receptor antagonist darusentan may also be able to induce regression. Interestingly, there is some evidence to suggest that osteoporosis therapies, including bisphosphonates, may provide benefit to patients with arterial
calcification. Ultimately, however, no pharmaceuticals are clinically indicated for the specific prevention of arterial calcification.

1.3 Intimal Calcification

Intimal calcification occurs within the cholesterol-rich lesions characteristic of atherosclerosis which can result in MI, stroke, or limb ischemia (Figure 1.1). Commonly associated with old age, male sex, hypertension, smoking, and hypercholesterolemia, intimal calcification is a reliable marker of plaque burden and may contribute to plaque instability.

1.3.1 Initiation and Progression of Atherosclerosis

Atherosclerosis is a chronic, progressive disease of the vascular system. In areas predisposed to atherosclerosis, variations in hemodynamic forces can result in adaptive intimal thickening defined as an increase in SMCs and extracellular matrix lacking any inflammatory infiltrate. The initial atherosclerotic plaque (Type I or II), appearing in those as young as age 2, is described as a fatty streak or a visible accumulation of lipid-laden macrophages (foam cells) and is capable of regressing naturally. The advanced atherosclerotic plaque (Type IV, V, or VI), or fibroatheroma, is characterized by a lipid-rich necrotic core covered by a SMC-rich fibrous cap. The rupture of these advanced plaques can lead to either downstream arterial occlusion or localized thrombus formation and subsequent ischemic death of the tissue supplied.

1.3.2 The Vulnerable Plaque

The difference between a stable and a vulnerable plaque is primarily related to the thickness and composition of the fibrous cap. The concept of a vulnerable plaque, or a plaque that is prone to rupture, was first introduced by Muller and Tofler. The vulnerable plaque was described as having a lipid-rich necrotic core and a generally thin fibrous cap, until Burke et al. refined the classification to those plaques with a fibrous cap less than 65 μm thick. They also noted that the fibrous cap of vulnerable plaques often had macrophage infiltration and a loss of SMCs, characteristics which would certainly promote fibrous cap degradation and plaque rupture.
1.3.3 Initiation and Progression of Calcification in Atherosclerosis

Calcification of the atherosclerotic plaque begins as micro-calcifications, typically less than 15 µm in diameter. Micro-calcifications are present in all types of lesions, including fatty streaks, and their abundance increases as atherosclerosis advances. They also precede the appearance of the bone-promoting proteins BMP2 and OCN which suggests that, at least initially, the calcification process in atherosclerotic plaques is distinct from typical bone formation. Micro-calcifications may be initiated by matrix vesicles of apoptotic SMCs or macrophages (Figure 1.3). Once initiated, there is evidence to suggest a highly regulated process which involves several bone-related proteins that promote (BMP2, BMP4, osteopontin, and osteonectin) and inhibit (matrix gla-protein and bone sialoprotein) atherosclerotic calcification. Furthermore, the mineral composition of calcific atherosclerotic plaques is chemically similar to that observed in bone.

1.3.4 Calcification and Vulnerability

There is controversy as to whether plaque calcification stabilizes advanced lesions or promotes rupture. Generally, the presence of calcification is correlated with the incidence of cardiovascular disease, and is associated with increased atherosclerosis progression. While some have reported an association between calcification and stability, others suggest calcification is a marker of vulnerability and may even promote rupture. These disparate reports suggest that the localization of calcification, rather than the volume, may be an important indicator of plaque vulnerability. Indeed, an intravascular ultrasound study suggested that calcification near the base of the lesion increased stability, and a mathematical modeling study suggested that calcification in the fibrous cap may as well. However, several studies have suggested that micro-calcifications within the fibrous cap directly promote plaque rupture. Taken together, these reports suggest that the relationship between calcification and plaque rupture is biphasic. Abedin et al. argue that the principal site of stress is the interface between hard, calcified areas and soft, cellular areas within the plaque; therefore, stress would initially increase as calcification increases until such time that individual calcified
areas enlarge and coalesce, thereby reducing interface area and overall stress. This argument was validated by Motoyama et al., who observed spotty calcification more frequently in ruptured plaques from acute coronary syndrome patients than stable ones from angina pectoris patients. Conversely, they observed large calcification more frequently in stable plaques. Clearly, a deeper understanding of the processes underlying atherosclerotic calcification and its relationship to vulnerability is required.

1.3.5 Clinical Implications

For almost a century, cardiovascular disease has accounted for more deaths than any other major cause in the United States, and a majority of these deaths are a result of atherosclerosis. Coronary arterial calcification is present in 52.9% of men and 32.0% of women over the age of 45, and its severity is predictive of overall cardiovascular risk. Obviously, the acute clinical implications of atherosclerosis depend on the location of individual vulnerable plaques. Rupture of a plaque in the coronary arteries or the carotid can lead to MI or stroke, respectively. Furthermore, the implications of intimal calcification rely on its relationship to plaque vulnerability. This is an area that requires considerably more study, including a reliable mechanism for blockade of calcification.

1.3.6 Pharmaceutical Management

Current clinical guidelines recommend an LDL-cholesterol goal of less than 100 mg/dL in patients at risk for cardiovascular disease, and statins play a role in achieving that goal. Statins, or 3-hydroxy-3-methylglutaryl Co-enzyme A (HMG-CoA) reductase inhibitors, inhibit the rate limiting enzyme in endogenous cholesterol production and thus are frontline drugs for the management of hypercholesterolemia. It is well known that statins reduce the incidence of cardiovascular events, including MI and stroke, and studies have shown that statins alter the progression of subclinical atherosclerosis. Statins are effective at reducing atherosclerotic lesion volume, reducing the size of the lipid core, and reducing progression of carotid intima-media thickness. Pre-clinical models have provided more information about the specific effects of statins, including pleiotropic effects (those not related to cholesterol-lowering). Statins have been shown to reduce expression of monocyte chemotactic protein-1, intercellular adhesion molecule-1,
and vascular cell adhesion molecule-1,\textsuperscript{136,137} down-regulate cyclooxygenase 2,\textsuperscript{138} improve nitric oxide bioavailability,\textsuperscript{139} suppress oxidation of LDL\textsuperscript{140}, and reduce the overall inflammatory burden within atherosclerotic plaques.\textsuperscript{141} The effects of statins on intimal calcification have not been thoroughly examined, although Kizu \textit{et al.}\textsuperscript{142} showed that cerivastatin could inhibit calcification of VSMCs \textit{in vitro}. In contrast, a recent clinical study found progression of coronary calcium was significantly higher in frequent statin users versus those who used statins infrequently.\textsuperscript{143}

Inhibition of the RAS may also have benefits in atherosclerosis. Three of the four prospective clinical trials examining the effect of ARBs on cardiovascular outcomes showed little\textsuperscript{144} or no\textsuperscript{145,146} benefit; however, the OLIVUS (Impact of OLmesartan on progression of coronary atherosclerosis: evaluation by IntraVascular UltraSound) trial found a lower rate of coronary atherosclerosis progression and decreased incidence of major adverse cardio- and cerebrovascular events in patients treated with olmesartan.\textsuperscript{147,148} More recently, Zhao \textit{et al.}\textsuperscript{149} showed that telmisartan reduced plaque size, macrophage infiltration, lipid deposition, and apoptosis in atherosclerotic plaques in a pre-clinical model. Three clinical trials examining ACEIs showed improved cardiovascular outcomes;\textsuperscript{70,150,151} however, two more recent trials showed no benefit.\textsuperscript{152,153} Recently, meta-analysis suggested that ARBs and ACEIs reduce the incidence of cardiovascular death, non-fatal MI, and non-fatal stroke, even in normotensive atherosclerosis patients.\textsuperscript{154} Other clinical studies have shown that ARBs reduce plaque volume\textsuperscript{155} and decrease inflammation.\textsuperscript{156} A pre-clinical study in monkeys also observed a regressive effect of ARBs, albeit on fatty streaks.\textsuperscript{157} Interestingly, aliskiren, a renin inhibitor, was associated with increased progression of atherosclerosis.\textsuperscript{158}

There have also been a number of studies examining various combinations of statin therapy and RAS blockade. Several pre-clinical studies examining statins in combination with ARBs have found an additive reduction of atherosclerosis burden in mice\textsuperscript{159-161} and rabbits.\textsuperscript{162,163} Another pre-clinical study examined the effects of simvastatin and a peroxisomal proliferator-activated receptor-gamma (PPAR\textgreek{g}) agonist and showed that the addition of PPAR\textgreek{g} agonist provided an additive benefit to atherosclerotic plaque
regression;\textsuperscript{164} which is of particular interest since some ARBs have been shown to have PPAR\(\gamma\) agonist activity.\textsuperscript{165-167}

It remains unclear whether aggressive medical management of intimal calcification will provide a reduction in cardiovascular events. However, improving our understanding of the mechanisms underlying calcification could provide an opportunity to prevent it which, in turn, could help reveal its relationship to plaque vulnerability.

### 1.4 Calcific Aortic Valve Disease

Calcific aortic valve disease, encompassing early aortic valve sclerosis (AVS) and clinical aortic stenosis, results in calcification of the aortic valve cusps and hardened, non-pliable valve tissue, decreased orifice area, and increased aortic jet velocity (Figure 1.1).\textsuperscript{168}

Commonly associated with old age, male sex, hypertension, smoking, increased plasma LDL, increased plasma lipoprotein (a), and DM,\textsuperscript{13,20,169-178} calcific aortic valve disease is associated with an increased risk of MI and cardiovascular death.\textsuperscript{13-16}

#### 1.4.1 The Aortic Valve

The normal human aortic valve has a three-layered structure measuring 1 mm in thickness\textsuperscript{169,179} with the fibrosal layer on the aortic side, the ventricularis on the ventricular side, and the spongiosa centrally (Figure 1.1). The fibrosa primarily contains highly organized collagen bundles between which lie valvular interstitial cells (VICs). The ventricularis is less organized and generally less cellular; it contains elastin sheets and some collagen. In contrast, the spongiosa is rich in cells and proteoglycans, but contains little collagen.

#### 1.4.2 Calcific Aortic Valve Disease

A chronic progressive disease, AVS is characterized by five factors: endothelial dysfunction, lipid deposition, chronic inflammation, activation of a local RAS and eventually, tissue calcification. The development of AVS results in distinctive anatomical changes first described by Otto \textit{et al.}\textsuperscript{169} as sub-endothelial thickening and fibrosis, disruption of the basement membrane, and accumulation of both intra- and extra-cellular
lipids. In the same year, Olsson et al. independently reported large numbers of activated T-lymphocytes located in subendothelial areas in close proximity to calcium deposits.

The initiating event in AVS appears to be endothelial cell activation in areas of mechanical stress, thereby predisposing the tissue to infiltration by plasma lipoproteins, a course similar to the development of atherosclerosis (Figure 1.3). In the aortic valve, lipid deposition tends to be localized to the fibrosa where it co-localizes with components of atherogenic apolipoproteins. Over time, lipid particles become oxidized and are taken up by infiltrating macrophages to form foam cells. Oxidized LDL is highly cytotoxic for many cells including endothelial cells and VICs; in addition, native LDL has been shown to co-localize with ACE both in human plasma and aortic valve lesions, creating an environment for the local production of AngII. More recently, Helske et al. described the participation of two additional AngII-forming enzymes: mast cell-derived chymase and cathepsin G.

1.4.3 Angiotensin II in Aortic Valve Disease

The pro-inflammatory and pro-fibrotic effects of AngII are well understood and are often cited as its main contribution to AVS. It is likely, however, that AngII is affecting all aspects of aortic valve disease, including lipid retention, inflammation, endothelial integrity, and calcification. In cardiac fibroblasts and VSMCs, AngII has been shown to induce the production of biglycan, a proteoglycan with enhanced LDL binding properties. Production of biglycan in aortic valves would promote retention of LDL and associated ACE, creating a positive feedback loop for AngII production. Blockade of AngII improves endothelial integrity in a rabbit model of AVS, and also up-regulated endothelial nitric oxide synthase, an enzyme that is generally considered to be cardioprotective. Most importantly, AngII has been shown to transactivate NADPH oxidase in VSMCs, leading to production of ROS. In turn, ROS have been shown to promote vascular calcification, and specifically localize around calcifying foci in human aortic valves.
1.4.4 Clinical Implications

The prevalence of AVS is estimated to be 25–30% in those over age 65 and up to 40% in those over age 75, typically progressing to severe aortic stenosis in 6% of patients over a period of 7 years. Even early AVS without concomitant coronary artery disease is predictive of a 50% increase in cardiovascular death. Considering that the primary cause of valve failure is extensive calcium deposits, it is no surprise that calcified volume correlates with the severity of AVS and is associated with the incidence of coronary events and all-cause mortality.

1.4.5 Pharmaceutical Management

Once AVS is initiated, increased physical activity or a change in diet is not sufficient to alter the disease process. However, an excellent environment exists for preventive, pharmaceutical management. The average time interval from subjective diagnosis of AVS to the development of severe stenosis is eight years, and it is increasingly clear that intervention should start as early as possible.

The overlap in clinical factors associated with AVS and atherosclerosis had suggested a shared disease process, thus the demonstrable benefits of statins on atherosclerosis provided support for their use in AVS. Several pre-clinical studies also promoted the effectiveness of statins for inhibiting hypercholesterolemia-induced cellular proliferation and calcification. In all, twelve clinical studies have evaluated the role of statins on AVS progression but the results have been conflicting. Six small retrospective studies, with a mean follow up of 6–44 months, showed a lower rate of AVS progression in patients treated with statins. Conversely, a larger retrospective study with a mean follow up of 66 months did not observe slower progression. Five prospective trials observed little or no effect of statins on the progression of AVS. The SEAS (intensive lipid lowering with Simvastatin and Ezetimibe in Aortic Stenosis) trial was the largest to date and the only one to measure clinical outcomes. Patients treated with simvastatin and ezetimibe showed a decrease in ischemic cardiac events but there was no effect on outcomes related to the aortic valve. Hamilton et al.
confirmed the ineffectiveness of statins in a pre-clinical model, observing a reduction of inflammation but retention of lipids and the continuation of calcium deposition.

Compared to statins, blockade of the RAS has received relatively little clinical examination as a preventive therapy for AVS. A retrospective study found that ACEIs could not slow the increase in aortic jet velocity associated with AVS; however, a separate retrospective study, specifically examining aortic valve calcium, showed a significant reduction in progression. The ACEI ramipril was effective in reducing progression of AVS, and the ARB olmesartan has been shown to reduce atherosclerotic changes and endothelial disruption in short-term animal models; however, neither study examined effects on calcification. More recently, a clinical study of excised aortic valves observed lower remodeling scores and decreased weight in those valves from patients who had been treated with ARBs. A retrospective study compared the effects of ARBs and ACEIs on AVS and found that only ARBs were effective at reducing progression. A small clinical study of the aldosterone receptor blocker epleronone showed no effect on AVS, but a pre-clinical study examining the early stages of the disease suggested some effects, including a small, qualitative reduction in micro-calcification.

There has been limited examination of other potential therapies for AVS. In an observational clinical trial, a cohort of patients receiving osteoporosis therapy – bisphosphonates, calcitonin, or estrogen receptor modulators – had a lower rate of AVS progression. A pre-clinical trial also had success with bisphosphonates, suggesting they are able to robustly inhibit valve calcification. An experimental recombinant apolipoprotein, Apo-A1 Milano, was successful in reversing AVS in a pre-clinical model, including a qualitative reduction of micro-calcification.

To date, clinical guidelines provide no recommendation for the management of AVS, suggesting additional trials with patients earlier in the disease continuum and with longer follow-up periods are required. In addition, the multitude of mechanisms involved in the progression of AVS suggests that drug therapy may have to be multifactorial.
1.5 Study Rationale and Hypothesis

In summary, little is known about the mechanisms driving cardiovascular calcification despite serious clinical implications and a clear association with morbidity and mortality. Indeed, it is even unclear whether the same mechanisms are at play in arterial, intimal, and valvular calcification. There is some evidence to suggest that the RAS and its vasoactive agent, AngII, are involved in promoting cardiovascular calcification; however, ARBs have not been robustly examined as potential therapies. As such, we set out to test the hypothesis that the ARB olmesartan medoxomil inhibits progression of established cardiovascular calcification in vivo.

1.6 Objectives and Future Directions

1.6.1 Investigate Effects of an ARB on Arterial Calcification in a Pre-Clinical Model

In Chapter 2, a version of which has been published in Cardiovascular Research, we use New Zealand White rabbits to investigate the effects of the ARB olmesartan medoxomil on established arterial calcification. This pre-clinical model has been used successfully by others to investigate potential pharmaceutical therapies. Using micro-computed tomography (micro-CT), histology, and immunohistochemistry, we are the first to show robust inhibition of arterial calcification by an ARB. Calcified areas in our animals displayed a down-regulation of α-smooth muscle actin, a smooth muscle cell marker; up-regulation of BMP2 and the ATIR; and expression of the osteoblast-specific protein OCN.

1.6.2 Investigate Effects of an ARB on Intimal Calcification in a Pre-Clinical Model

In Chapter 3, a version of which has been submitted for publication in the Canadian Journal of Cardiology, we use New Zealand White rabbits to investigate the effects of the ARB olmesartan medoxomil, alone or in combination with atorvastatin, on established atherosclerosis and intimal calcification. The long-term, low-level cholesterol rabbit model we employed for this study was developed previously by our lab and has been
shown to produce significant atherosclerosis over a period of six months with 58% of the aortic surface covered by atheroma and up to 75% of those lesions considered to be advanced.\textsuperscript{230,231} Previous examination of these lesions revealed their similarity to human atherosclerosis; VSMCs and collagen extracellular matrix formed a fibrous cap over a core of lipids, cholesterol crystals, and necrotic debris.\textsuperscript{231} Using classical lesion area analysis, micro-CT, histology, and immunohistochemistry, we show a significant reduction of atherosclerotic burden in animals treated with ARB monotherapy, but not in combination therapy (ARB+Statin). In addition, both ARBs and statins may have slowed progression of intimal calcification.

1.6.3 Investigate Effects of an ARB on Calcific Aortic Valve Disease in a Pre-Clinical Model

In Chapter 4, a version of which has been accepted for publication in the \textit{Canadian Journal of Cardiology},\textsuperscript{232} we use the same animals as in Chapter 3 but examine the effects of the ARB olmesartan medoxomil, alone or in combination with atorvastatin, on established calcific aortic valve disease. Previously, Cimini \textit{et al.}\textsuperscript{233} examined the development of calcific aortic valve disease in our long-term, low-level cholesterol rabbit model.\textsuperscript{230,231} Rabbits have been shown to develop aortic valve thickening, inflammation, and lipid deposition over a period of ten months. While calcification was not observed in the initial report, it was present in aortic valves after 30 months of cholesterol feeding.\textsuperscript{217} We did not observe any demonstrable treatment effects using \textit{in vivo} magnetic resonance imaging. However, \textit{ex vivo} histological and immunohistochemical analyses revealed structural changes to the aortic valve. In particular, ARB-treated animals had significantly increased levels of valvular calcification.

1.6.4 Conclusions and Future Directions

Finally, in Chapter 5, I summarize the results and conclusions of my work. Namely, the disparate effects of the ARB in treating arterial, intimal, and valvular calcification suggest distinct cellular and molecular mechanisms are at play in each disease process. More work needs to be done to fully understand these differences but the robust inhibitory effect of the ARB on arterial calcification cannot be ignored. Therefore, I suggest a
prospective, randomized trial in patients with chronic kidney disease, a group prone to rapid development of arterial calcification. Such a trial would be valuable, given there is currently no preventive therapy for arterial calcification. In all, the studies herein have advanced our understanding of cardiovascular calcification and, together with future work, may lead to novel therapies.
1.7 References


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Chapter 2

2 Angiotensin II Type 1 Receptor Blocker Inhibits Arterial Calcification in a Pre-Clinical Model

Arterial calcification, also known as Mönckeberg sclerosis, involves mineralization of the internal elastic lamina (IEL) and elastic fibers within the medial layer resulting in hardened arteries and increased pulse pressure. Commonly associated with advanced age, chronic kidney disease, diabetes mellitus, and atherosclerosis, arterial calcification is closely related to cardiovascular morbidity and mortality.\(^1\)\(^-\)\(^3\) In the past, it was considered a passive, degenerative and, most importantly, irreversible process.\(^4\) More recently, it has become clear that this process is highly regulated, involving a number of pro- and anti-calcification mediators, and resembles natural bone formation.\(^5\) Indeed, a recent study by Speer et al.\(^6\) has shown that vascular smooth muscle cells (SMCs) are capable of osteoblast transdifferentiation in calcifying arteries. This transdifferentiation was associated with downregulation of SMC markers and concomitant upregulation of the osteoblast transcription factor Runx2/Cbfa1. Although tremendous progress has been made in this area, the molecular mechanisms underlying this process remain to be fully defined.

There is growing evidence that vasoactive agents are important modulators of vascular calcification. Naturally existing peptides such as endothelin-1 and urotensin II can promote arterial calcification, while others – adrenomedullin and C-type natriuretic peptide – act to inhibit its progression.\(^7\)\(^-\)\(^9\) However, the role of the renin-angiotensin system (RAS) and its vasoactive agent, angiotensin II (AngII), has not been investigated. AngII plays a number of roles in vascular pathology, and is thought to exert its effects by inducing NADPH oxidase and increasing cellular reactive oxygen species (ROS).\(^10\) In turn, ROS stimulate the expression of bone morphogenetic protein (BMP) 2 and the osteoblast transcription factor Runx2/Cbfa1, thereby inducing osteoblast transdifferentiation.\(^11\) Blockade of RAS has been shown to reduce morbidity and

\(^{*}\) A version of this chapter has been published: Armstrong ZB, Boughner DR, Drangova M, Rogers KA. Angiotensin II type 1 receptor blocker inhibits arterial calcification in a pre-clinical model. Cardiovasc Res 2011;90:165-70.
mortality in hypertension, atherosclerosis, heart failure, stroke, diabetes, and chronic kidney disease,\textsuperscript{12-17} often independent of changes in blood pressure.\textsuperscript{12,14-15,17}

Here, we test the hypothesis that an angiotensin II type 1 receptor blocker (ARB) can inhibit arterial calcification \textit{in vivo}. We also further characterize the mechanism by which vascular calcification occurs using immunohistochemical methods.

\section*{2.1 Methods}

\subsection*{2.1.1 Pre-Clinical Model}

Male New Zealand White rabbits (1.6 to 2.0 kg, Charles River Laboratories, St-Constant, QC) were fed either regular chow (Control; n = 9) or an atherogenic diet (Cholesterol; n = 9) consisting of 0.5\% cholesterol and 10,000 IU/day Vitamin D\textsubscript{2} for 12 weeks to rapidly induce atherosclerosis and arterial calcification. The treatment group (ARB; n = 6) received the atherogenic diet for 12 weeks with the ARB olmesartan medoxomil (1 mg/kg/day) in the final 4 weeks. Olmesartan medoxomil, suspended in 60\% molasses, was administered daily via oral gavage. Vitamin D\textsubscript{2} and its analogs have been used extensively to induce vascular calcification in animal models,\textsuperscript{18} and there is controversy as to whether or not it contributes to vascular calcification in humans.\textsuperscript{19} After 8 weeks, a subset of the Control (n = 3) and Cholesterol fed (n = 3) groups were euthanized to assess lesion progression at the time of pharmacological intervention. At twelve weeks, animals were euthanized via intravenous ketamine injection and perfused with Hank’s balanced salt solution and heparin (1 U/mL). Immediately upon dissection the thoracic aorta was fixed in 10\% neutral buffered formalin. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal protocols were approved by the University of Western Ontario Animal Use Subcommittee (reference number 2007-023).

\subsection*{2.1.2 Plasma Chemistry}

Blood samples were obtained for the measurement of total cholesterol and inorganic phosphate. EDTA was used in the isolation of plasma, preventing analysis of calcium.
Total cholesterol was measured at weeks 0, 4, 8, 10, and 12 (endpoint) using a WAKO Cholesterol E Kit, according to the manufacturer’s instructions. Inorganic phosphate was examined at endpoint using standard autoanalyzer methods at the London Health Sciences Centre Core Laboratory (London, ON, Canada).

2.1.3 Micro-Computed Tomography

Thoracic aortae were placed in a humidified chamber to prevent dehydration and scanned with an eXplore Locus micro-computed tomography (micro-CT) scanner (General Electric Medical Systems, London, Ontario). Scans were acquired at 80 kVp, 130 mAs and reconstructed with isotropic voxel spacing of 90 μm. Images were analyzed for the presence of calcium using MicroView analysis software (V2.2, GE Medical Systems, London, Ontario). Specifically, the total volume of all voxels containing calcified tissue (identified by setting a threshold level) was calculated and expressed as percent of total vessel volume.

2.1.4 Histological and Immunohistochemical Analysis

Thoracic aortae were embedded in Tissue-Tek OCT compound and frozen in liquid nitrogen-cooled isopentane. Frozen sections (10 μm) were taken as cross-sections through the aorta distal to the first intercostal ostia. Serial sections were stained with Alizarin Red S and von Kossa for calcium deposition. Immunohistochemical studies were performed using the following primary antibodies: mouse anti-α-smooth muscle actin (clone 1A4), mouse anti-angiotensin II type 1 receptor (clone 1E10-1A9), mouse anti-BMP2 (clone 1A11), mouse anti-osteocalcin (clone OC4-30), mouse anti-osteopontin (clone MPIIIB10), and mouse anti-rabbit activated macrophage (clone RAM11). Sections were subjected to single label immunohistochemistry using an Alkaline Phosphatase Substrate Kit (Vector Laboratories) and secondary antibody horse anti-mouse IgG (H + L) Alkaline Phosphatase-conjugate according to the manufacturer’s instructions. Negative controls which omitted the primary antibody were routinely employed.
2.1.5 Statistical Analysis

Data are expressed as mean ± SEM. All analyses were performed using GraphPad Prism (V4.0, GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using a Kruskal-Wallis test with Dunn’s post-hoc test, or one-way repeated measures ANOVA with Tukey’s post-hoc test, as appropriate. P < 0.05 was considered statistically significant.

2.2 Results

2.2.1 Animals and Plasma Chemistry

A subset of Control (n = 3) and Cholesterol (n = 3) animals were euthanized after 8 weeks to assess the extent of disease before administration of the ARB (Supplementary Figure 2.1). A single ARB-treated rabbit died due to unknown causes after a week of treatment (9 weeks total) and was not included in the analysis. To assess systemic effects of ARB treatment we examined plasma levels of total cholesterol and inorganic phosphate. While total cholesterol levels were significantly increased in Cholesterol animals as compared to Control (1394.00 ± 352.16 versus 15.49 ± 6.31 mg/dL in Cholesterol and Control, respectively; n = 6/group; P < 0.001) there was no significant effect of ARB treatment (1531.18 ± 334.51 mg/dL; n = 5; P = ns versus Control; P = ns versus Cholesterol; Supplementary Figure 2.2A). Levels of inorganic phosphate were the same in all groups (3.43 ± 0.17, 4.38 ± 0.40, and 3.83 ± 0.87 mg/dL in Control, Cholesterol, and ARB, respectively; n = 5, 6, and 4; P = ns; Supplementary Figure 2.2B), and within the normal physiological range for New Zealand White rabbits.

2.2.2 Arterial Calcification is Abolished After Treatment with the ARB

To evaluate the effects of ARB treatment on arterial calcification we administered olmesartan medoxomil for the final four weeks of the twelve-week protocol. Calcification was significantly increased in Cholesterol animals (non-detectable versus 2.80 ± 1.17 % calcified tissue in Control and Cholesterol, respectively; n = 6/group; P < 0.01) and, in contrast, was completely inhibited in all but one ARB-treated animal (0.01 ± 0.01 % calcified tissue in ARB; n = 5; P < 0.05 versus Cholesterol; Figure 2.1A). Calcification,
**Figure 2.1: Treatment with the angiotensin II type 1 receptor blocker (ARB) inhibited arterial calcification.** (A) Representative maximum intensity projections, derived from micro-computed tomography (CT) scans, and corresponding quantitation of Control (n = 6), Cholesterol (n = 6) and ARB (n = 5) animals reveals a significant increase in arterial calcification after 12 weeks on the atherogenic diet, except in ARB-treated animals where no significant calcification was detected. Scale bar = 4 mm. Kruskal-Wallis test with Dunns post-hoc test: *p < 0.01 versus Control. †p < 0.05 versus Cholesterol. (B) Histological examination of calcium (Alizarin Red S, top) and calcium salts (Von Kossa, bottom) reveals that they are localized primarily to the internal elastic lamina (IEL) and medial layer, typical of arterial calcification. Scale bar = 500 µm. ND = none detected. Arrows indicate calcification.
when present, was primarily localized to the IEL and media (Figure 2.1B). Some animals (3 of 6 Cholesterol, 2 of 5 ARB) exhibited micro-calcifications within atherosclerotic plaques (Figure 2.2) either in addition to, or independent of, IEL/media calcification. It should be noted that the calcification found in the ARB-treated animals was minor (< 10 μm in size) and was localized to the plaque. These calcifications were too small to be detected by micro-CT because their signal was masked by the surrounding tissue, which occupied the majority of the 90 μm³ voxel.

2.2.3 Calcified Regions Express the Bone-Related Proteins BMP2 and Osteocalcin and Dramatically Increase Expression of the AT1R

To gain insight into the mechanisms underlying this calcification process, we characterized the calcified regions using immunohistochemical methods. Adjacent sections acted as negative controls (omission of primary antibody) and showed no positive staining (data not shown). Calcified areas, and the tissue immediately surrounding them, showed expression of the osteogenic growth factor BMP2 (Figure 2.3). Calcified areas also exhibited increased expression of the bone protein and osteoblast-specific marker osteocalcin (Figure 2.3). In addition, calcified areas display dramatic upregulation of the angiotensin II type 1 receptor (AT1R; Figure 2.3), which has been shown to increase in expression as osteoblasts mature. Calcified areas also showed limited expression of the calcium binding protein osteopontin (data not shown). Corresponding low power images can be found in Supplementary Figure 2.3. Taken together, this strongly suggests the presence of osteoblasts within areas of calcification.

2.2.4 Calcified Areas of the Media are not Associated with SMCs or Macrophages

To eliminate other possible cell types within calcified areas, we examined the expression of α-smooth muscle actin, a marker of smooth muscle cells, and RAM11 as a marker of macrophages. Calcified areas were associated with a downregulation of α-SMA (Figure 2.4). In addition, some areas not associated with calcification also displayed downregulation of α-SMA (Supplementary Figure 2.4), possibly indicating initial
Figure 2.2: Arterial calcification versus micro-calcification of atherosclerotic plaques. Animals fed the atherogenic diet primarily displayed calcification of the IEL and medial layer (arrows). However, histological examination revealed that a number of animals (3/6 Cholesterol, 2/5 ARB) exhibited micro-calcifications within atherosclerotic plaques (arrowheads). It is important to note that these are generally considered distinct processes, and that the minor calcification displayed in the ARB animals was localized to the plaque. Scale bar = 500 µm and 100 µm (inset). Abbreviations as in Figure 2.1.
Figure 2.3: Characterization of calcified regions indicates an osteoblast-like phenotype. Immunohistochemical characterization of calcified regions (arrows) and adjacent sections reveals colocalized expression of the osteogenic growth factor bone morphogenetic protein 2 (BMP2), the bone protein and osteoblast-specific marker osteocalcin (OCN), and dramatic upregulation of the angiotensin II type 1 receptor (AT1R). This data suggests an osteoblast-like phenotype within calcified areas. Scale bar = 500 µm.
Figure 2.4: Calcified regions of the media are not associated with smooth muscle cells or macrophages. Examination of α-smooth muscle actin (α-SMA), a marker of smooth muscle cells, reveals dramatic downregulation in calcified regions (arrows). Furthermore, macrophages are localized specifically to atherosclerotic plaques, and are not associated with areas of calcification. Scale bar = 500 µm.
progression toward an osteogenic phenotype. Interestingly, some of these areas also showed upregulation of BMP2 (data not shown). Macrophages were localized specifically to atherosclerotic lesions and were not observed within the medial layer (Figure 2.4). Corresponding low power images can be found in Supplementary Figure 2.4. Taken together, this suggests a phenotypic switch from vascular to osteoblast-like cells.

2.3 Discussion

The present study is the first to show the dramatic inhibition of arterial calcification by an ARB. Using micro-CT and histology, we have shown that ARB administration, given after induction of disease, can robustly inhibit the calcification observed in animals not so treated. While others have used doses of 25,000-50,000 IU/day Vitamin D₂, we achieved significant levels of vascular calcification in the same time period using 10,000 IU/day. The calcification we observed was localized to the IEL and medial layer, typical of Mönckeberg sclerosis as well as vascular calcification associated with chronic kidney disease and diabetes mellitus. In chronic kidney disease, up to 55% of deaths are cardiovascular related, and cardiovascular mortality is 10-100 times greater than that for age-matched populations. In type 2 diabetes, arterial calcification is a strong predictor of mortality and lower extremity amputation. Inhibiting progression of arterial calcification remains the goal of several groups, but it is generally thought to be irreversible. In contrast, at least one group has shown reversibility of vascular calcification in rats with normal renal function. Here, we provide the first evidence that an ARB is capable of halting progression of arterial calcification.

While the underlying pathologies (diabetes, chronic kidney disease, advanced age) are distinct, the process ultimately leading to vascular calcification is related. Although vascular SMC transdifferentiation to osteoblasts is under intense investigation, the molecular mechanisms are not fully understood. Characterization of the calcific lesions we observed suggests that they result from such a transition. Expression of BMP2, an osteogenic growth factor, in the areas in and around calcification suggests that it is involved in directing this transition, as others have suggested. Calcified areas display
expression of the bone-associated proteins osteopontin and OCN. Osteopontin can be expressed by a range of cell types, including bone cells and macrophages, and has been shown to contribute to arterial calcification.\(^{39}\) Osteocalcin, an osteoblast-specific marker, is often used as a biomarker for bone formation, and is also implicated in calcium ion homeostasis.\(^{22}\) Calcified regions also displayed marked upregulation of the AT1R. The AT1R is commonly expressed in the vasculature, and is responsible for mediating signaling of the RAS. Indeed, we observed diffuse staining for AT1R in aortas from all of our animals. However, recent work by Bandow \textit{et al.}\(^{40}\) has shown that osteoblasts, as they mature, greatly increase expression of the AT1R. We observed regional upregulation of the AT1R in areas associated with calcification, again suggesting an osteoblast-like phenotype. Furthermore, calcified areas showed dramatic downregulation of α-SMA, a smooth muscle marker, providing evidence for a phenotypic switch from vascular to osteoblast-like cell. Here, we provide the first evidence that the RAS is involved in vascular osteogenesis, and that an ARB is capable of modulating the process.

Despite the dramatic effect on calcification, ARB therapy had no effect on systemic disease indicators (hypercholesterolemia and hyperphosphatemia), suggesting its effects are specific to the vascular system, rather than a result of secondary phenomena. As expected, ARB therapy did not normalize total cholesterol.\(^{41-42}\) There was also no effect on plasma levels of inorganic phosphate, either by the atherogenic diet or by ARB therapy. It is important to note that we were unable to reliably quantify changes in the extent of atherosclerosis, as extensive calcium deposition in Cholesterol animals prevented both ultrasound examination and \textit{en face} lipid staining.

Here, we show that angiotensin receptor blockade robustly inhibits the progression of arterial calcification. This form of calcification, commonly associated with advanced age, chronic kidney disease, and diabetes mellitus, is a result of osteoblast transdifferentiation of vascular cells,\(^{6}\) a process replicated here in a pre-clinical model. This study is the first to suggest ARB therapy as a novel treatment option for patients at risk for cardiovascular calcification.
2.4 References


Chapter 3

3 Potential Negative Interaction between Statin Therapy and Angiotensin Receptor Blockade in Atherosclerotic Lesion Regression*

While the mortality rate associated with cardiovascular disease is in decline, it remains the most prevalent cause of death in the United States.\(^1\) Atherosclerosis, the underlying cause of most clinical cardiovascular events, is a chronic disease process involving the formation of atherosclerotic plaques within the arterial intima. These plaques, which contain lipid deposits, inflammatory cells, as well as areas of fibrosis and calcification, are often clinically silent.\(^2\) Vulnerable plaques are those that are considered high-risk for disruption or thrombosis leading to an acute coronary event.\(^3\) Pathologically, they are described as having a large necrotic core, a thin fibrous cap, and inflammatory activity.\(^4,5\)

While calcification correlates with increased atherosclerotic plaque burden,\(^6,7\) the relationship between calcification and plaque vulnerability is less clear. An intravascular ultrasound study suggests that an increase in calcification near the base of the lesion is associated with lower risk of plaque rupture.\(^8\) In contrast, Abedin et al.\(^9\) argue that as plaques begin to calcify there is an initial increase in plaque vulnerability because stress concentrates at the interface between the hard calcium deposits and other, softer components of the plaque. When these calcium deposits begin to coalesce the risk of rupture decreases as the interface area decreases.

The renin-angiotensin system directly influences the progression of atherosclerosis and vascular calcification.\(^10,11\) Angiotensin II type 1 receptor blockers (ARBs) have been shown to cause stabilization,\(^12-14\) or even regression,\(^15,16\) of atherosclerotic plaques in pre-clinical and clinical studies. When ARBs are combined with statin therapy, a synergistic reduction of atherosclerosis burden has been reported.\(^17-19\) However, it should be noted

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* A version of this chapter has been submitted for publication: Armstrong ZB, Boughner DR, Carruthers CP, Drangova M, Rogers KA. Potential negative interaction between statin therapy and angiotensin receptor blockade in atherosclerotic lesion regression. Can J Cardiol 2014.
that these studies introduced treatment before the disease was established, and that the
treatment periods were relatively short (4-10 weeks).

The current study uses a model of dietary hypercholesterolemia to further examine the
effects of the ARB olmesartan medoxomil, alone or in combination with atorvastatin
calcium, on the progression of established atherosclerosis. Interestingly, our results
suggest that ARBs promote regression of advanced atherosclerosis when administered
alone, but not in conjunction with statin therapy.

3.1 Methods

3.1.1 Pre-Clinical Model

The animals used in this study have been described previously.20 Briefly, male New
Zealand White rabbits were fed either regular chow (Control, n = 8) or an atherogenic
diet of 0.25% cholesterol to induce atherosclerosis. After 12 months, a subset of Control
(n = 3) and cholesterol-fed (n = 6) rabbits were euthanized for pathological assessment of
disease progression. The remaining diseased rabbits continued on an atherogenic diet of
0.125% cholesterol and received either no treatment (Cholesterol, n = 6), olmesartan
medoxomil (ARB, n = 7), atorvastatin calcium (Statin, n = 7), or a combination of both
drugs (ARB+Statin, n = 7) for an additional 6 months. Olmesartan (1 mg/kg/day) and
atorvastatin (2.5 mg/kg/day) were administered orally. All animals were individually
housed and cared for in accordance with the Canadian Council on Animal Care and all
protocols were approved by the University of Western Ontario Animal Use
Subcommittee.

3.1.2 Micro-Computed Tomography

Following euthanasia, intact aortae were placed in a humidified chamber to prevent
dehydration and scanned with a Locus Ultra micro-computed tomography (micro-CT)
scanner (General Electric Medical Systems, London, ON). Scans were acquired at
80 kVp, 55 mAs and reconstructed with isotropic voxel spacing of 150 μm. Images were
analyzed for the presence of calcium using MicroView analysis software (V2.2, GE
Medical Systems, London, ON). Specifically, the total volume of all voxels containing
calcified tissue (identified by setting a threshold level) was calculated and expressed as percent of total vessel volume, as described previously.\textsuperscript{21}

3.1.3 Quantification of Lesion Area

Lesion area was quantified in the thoracic aorta as previously described\textsuperscript{22}. Aortae were stained with Oil Red O in propylene glycol for 30 minutes, opened along their ventral surface and pinned flat for \textit{en face} analysis. Lesion area was analyzed by a blinded observer using Adobe Photoshop CS4 Extended (V11, San Jose, CA) and expressed as a percent of total vessel area.

3.1.4 Histological and Immunohistochemical Analysis

Aortic samples (distal to the first intercostal ostia) were cryopreserved using increasing concentrations of sucrose (up to 30\%) then immediately embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) compound and frozen in liquid nitrogen-cooled isopentane. Frozen Sections (10 µm) were taken as cross sections through the aorta. Serial sections were stained with Oil Red O for extracellular lipid deposition and Alizarin Red S for calcium deposits (from Sigma-Aldrich, Oakville, ON, Canada). Immunohistochemical studies were performed using mouse anti-\(\alpha\)-smooth muscle actin (\(\alpha\)-SMA; clone 1A4) and mouse anti-rabbit activated macrophage (clone RAM11). Sections were subjected to single-label immunohistochemistry using an Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) and secondary antibody horse anti-mouse IgG (H+L; Vector Laboratories) Alkaline Phosphatase-conjugate according to the manufacturer’s instructions. Negative controls which omitted the primary antibody were routinely employed. Positive staining areas were calculated by setting a threshold value in ImageJ (National Institutes of Health, Washington, DC) and expressed as a percent of total lesion area.

3.1.5 Statistical Analysis

Data is expressed as mean ± SEM and statistically analyzed by one-sample t-test, unpaired t-test with Welch’s correction, one-way ANOVA with Tukey’s post-hoc test, or Pearson correlation, as indicated. All analyses were performed using GraphPad Prism
(V5, GraphPad Software, Inc., La Jolla, CA). Values of p < 0.05 were considered statistically significant.

3.2 Results

3.2.1 Twelve Months Cholesterol Feeding Induces Advanced Atherosclerotic Lesions

Atherosclerotic lesion area was significantly increased in Cholesterol animals when compared to Controls following 12 months of cholesterol feeding (78.15 ± 14.43 % versus non-detectable for Cholesterol and Control, respectively; n = 6 and 3; p < 0.01; Figure 3.1A). Total volume of calcium in the thoracic aorta, as examined by micro-CT, was also significantly increased in Cholesterol animals (1.81 ± 0.50 versus 0.33 ± 0.08 in Cholesterol and Control, respectively; n = 6 and 3; p < 0.05; Figure 3.1B). Pathological examination of the thoracic aorta (Figure 3.1C) revealed smooth muscle cell and macrophage infiltration, predominantly near the surface of the lesion, the area known as the fibrous cap. Extensive lipid deposition was observed throughout the lesion. Calcium deposition was also observed, forming as punctate nodules, typically near the base of the lesion. Taken together, these hallmarks clearly indicate that animals exhibit advance atherosclerotic lesions after 12 months. This formed the basis upon which progression, stabilization, or regression during the 6 month treatment period could be assessed.

3.2.2 ARBs Cause Significant Regression of Advanced Atherosclerotic Lesions when Delivered Alone, but not when Combined with Statin Therapy

Following the six month treatment period, atherosclerotic lesion area was measured using en face Oil Red O staining. When compared to Control animals, all treatment groups had significantly increased lesion area (non-detectable in Control, 95.50 ± 1.94 % in Cholesterol, 61.61 ± 10.17 % in ARB, 82.50 ± 6.78 % in Statin, and 92.39 ± 3.25 % in ARB+Statin; n = 5, 5, 7, 7, and 6; P < 0.001 for each group when compared to Control, significance not shown; Figure 3.2). When compared to untreated Cholesterol animals, ARB-treated animals had significantly reduced lesion area (P < 0.05). Interestingly, lesion area was also significantly reduced in ARB-treated animals when compared to
Figure 3.1: Significant atherosclerosis progression is achieved after 12 months. A: Atherosclerotic lesion area was significantly increased in Cholesterol animals versus Control. Each data point represents an individual animal with the mean represented by a horizontal line. **P < 0.01; by one sample t-test. B: Total volume of aortic calcium, as measured by micro-computed tomography, was significantly increased in Cholesterol animals versus Control. Each data point represents an individual animal with the mean represented by a horizontal line. *P < 0.05; by unpaired t-test with Welch’s correction. C: Histological and immunohistochemical analyses reveal several markers of atherosclerosis. Lesions in Cholesterol animals were advanced, with smooth muscle cells (as indicated by α-smooth muscle actin) forming a fibrous cap, macrophage (indicated by RAM11) and lipid (indicated by Oil Red O) infiltration, and extensive calcification (indicated by Alizarin Red S) near the lesion base. Aortae from Control animals showed no indication of disease. Inset: high-power image of the area indicated by the box.
Figure 3.2: ARB treatment causes regression of established atherosclerosis.

Atherosclerotic lesion area was significantly increased in Cholesterol, ARB, Statin and ARB+Statin animals when compared to Control (P < 0.001, not shown). Interestingly, lesion area in ARB animals was significantly reduced as compared to both Cholesterol and ARB+Statin animals. Each data point represents an individual animal, with the mean represented by a horizontal line. *P < 0.05; by one-way ANOVA with Tukey’s post-hoc test.
ARB+Statin (P < 0.05), suggesting addition of atorvastatin may interfere with the beneficial effects of olmesartan. It should also be noted that lesion area in ARB animals after treatment (Figure 3.2) appears to be lower than before treatment (Figure 3.1A), consistent with Olmesartan inducing regression of advanced atherosclerotic lesions.

3.2.3 ARBs and Statin Therapy Slows Progression of Atherosclerotic Plaque Calcification

Given the robust inhibitory effects of olmesartan already observed in aortic medial calcification, the aortae in this study were also examined for total calcium volume using micro-CT. Cholesterol rabbits had a significantly increased calcium volume when compared to Control (4.74 ± 1.77 % versus 0.87 ± 0.06 % in Cholesterol and Control, respectively; n = 6 and 5; P < 0.05; Figure 3.3). This increase was not observed in any of the treatment groups (1.69 ± 0.25 % in ARB, 1.62 ± 0.41 % in Statin, and 2.47 ± 0.40 % in ARB+Statin; n = 7, 6, and 6, respectively).

3.2.4 ARBs Do No Affect the Cellular Composition of Atherosclerotic Plaques

Atherosclerotic lesions were advanced, showing all the hallmarks of classical atherosclerosis (Figure 3.4). Migration of smooth muscle cells (as indicated by α-SMA) was robust and typically concentrated on the surface of the lesion, forming a fibrous cap. Extracellular lipids (indicated by Oil Red O) were distributed throughout the entire lesion, but were occasionally found concentrated within the core. Macrophage infiltration (as indicated by RAM11) was also observed throughout the lesion, but was the most intense on the surface. Calcium was observed as small punctate deposits distributed throughout the lesion. While lesion area analysis was performed, no significant differences between groups were observed (Table 3.1). Interestingly, we observed a significant inverse correlation between macrophage ratio and calcium ratio, such that as the ratio of macrophages increased, the calcium ratio decreased (P = 0.025; r = -0.43; Figure 3.5).
Figure 3.3: Pharmaceutical intervention slows progression of atherosclerotic calcium. Total volume of aortic calcium was significantly increased in Cholesterol animals, but not in ARB, Statin, or ARB+Statin animals when compared to Control. Each data point represents an individual animal, with the mean represented by a horizontal line. *P < 0.05; by one-way ANOVA with Tukey’s post-hoc test.
Figure 3.4: Atherosclerotic lesion composition is unaffected by pharmaceutical intervention. Histological and immunohistochemical analyses reveal hallmark features of advanced atherosclerotic lesions including fibrous cap formation and smooth muscle cell (as indicated by $\alpha$-smooth muscle actin) migration to the surface of the lesion; macrophage accumulation (indicated by RAM11) both at the surface of the lesion and within the core; lipid infiltration (indicated by Oil Red O) throughout the lesion, and extensive calcification (indicated by Alizarin Red S) particularly deep within the core of the lesion. Morphometric analysis of lesion composition did not reveal any significant differences between groups (see Table 1). **Inset:** high-power image of the area indicated by the box.
Table 3.1: Lesion composition analysis of thoracic aorta in hypercholesterolemic rabbits.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total Lesion Area (mm²)</th>
<th>SMC Area (mm²)</th>
<th>SMC Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6</td>
<td>8.58±1.61</td>
<td>3.42±0.59</td>
<td>42.32±4.23</td>
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<tr>
<td>Statin</td>
<td>7</td>
<td>6.23±1.34</td>
<td>3.99±1.07</td>
<td>60.90±3.88</td>
</tr>
<tr>
<td>ARB</td>
<td>7</td>
<td>4.30±1.04</td>
<td>2.11±0.48</td>
<td>59.95±12.82</td>
</tr>
<tr>
<td>ARB+Statin</td>
<td>7</td>
<td>6.31±0.70</td>
<td>3.14±0.58</td>
<td>48.68±4.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total Lesion Area (mm²)</th>
<th>MΦ Area (mm²)</th>
<th>MΦ Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6</td>
<td>8.30±1.50</td>
<td>1.28±0.51</td>
<td>13.34±3.86</td>
</tr>
<tr>
<td>Statin</td>
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<td>2.01±0.91</td>
<td>25.63±6.00</td>
</tr>
<tr>
<td>ARB</td>
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<td>0.79±0.33</td>
<td>16.38±4.46</td>
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<tr>
<td>ARB+Statin</td>
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<td>6.21±0.61</td>
<td>1.25±0.25</td>
<td>19.81±3.90</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total Lesion Area (mm²)</th>
<th>Lipid Area (mm²)</th>
<th>Lipid Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6</td>
<td>8.29±1.51</td>
<td>6.04±1.02</td>
<td>76.03±4.34</td>
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<tr>
<td>Statin</td>
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<td>6.26±1.25</td>
<td>5.01±1.31</td>
<td>75.95±6.78</td>
</tr>
<tr>
<td>ARB</td>
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<td>74.34±3.30</td>
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<td>ARB+Statin</td>
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<td>6.51±0.89</td>
<td>5.07±0.70</td>
<td>75.21±4.34</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total Lesion Area (mm²)</th>
<th>Calcium Area (mm²)</th>
<th>Calcium Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholesterol</td>
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<td>8.16±1.63</td>
<td>1.60±0.32</td>
<td>20.99±2.83</td>
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<tr>
<td>Statin</td>
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<td>5.97±1.32</td>
<td>0.92±0.24</td>
<td>15.33±2.55</td>
</tr>
<tr>
<td>ARB</td>
<td>7</td>
<td>4.53±1.08</td>
<td>1.10±0.31</td>
<td>20.64±3.17</td>
</tr>
<tr>
<td>ARB+Statin</td>
<td>7</td>
<td>6.14±0.76</td>
<td>1.13±0.30</td>
<td>17.28±3.53</td>
</tr>
</tbody>
</table>

Total lesion area was determined as the area between the luminal surface and the internal elastic lamina. SMC and Macrophage (MΦ) area were determined as positive areas using anti-α-smooth muscle actin (1A4) and anti-macrophage (RAM11) antibodies, respectively. Lipid and Calcium area were determined as positive areas using Oil Red O and Alizarin Red S staining, respectively. Data are expressed as mean±SEM. ND = non-detectable.
Figure 3.5: Negative correlation between calcium and macrophage ratio in advanced atherosclerotic lesions. The calcium (as indicated by Alizarin Red S) and macrophage (as indicated by RAM11) ratio was determined by dividing the total positive staining area, defined using a threshold value, by the total lesion area, defined as the area between the luminal surface and the internal elastic lamina. Each data point represents an individual animal; P = 0.025; r = -0.43.
3.3 Discussion

The current study examines the effects of an ARB, alone or in combination with statin therapy, on advanced atherosclerotic lesions. ARB treatment induced atherosclerosis regression when delivered alone, but not when combined with Statin therapy, a novel finding that is in contrast with existing pre-clinical studies.\textsuperscript{18,19,23-26} We also observed a significant negative correlation between macrophage content and calcification of atherosclerotic plaques.

Atherosclerotic lesion area was significantly reduced in ARB-treated animals when compared to untreated Cholesterol animals (Figure 3.2), a result reported consistently by others.\textsuperscript{27-31} ARBs induce plaque stabilization,\textsuperscript{32} decrease expression of adhesion molecules\textsuperscript{33} and monocyte adhesion,\textsuperscript{33,34} reduce production of inflammatory cytokines,\textsuperscript{35} and inhibit smooth muscle cell migration.\textsuperscript{36} These pleiotropic effects of ARB therapy may explain its effectiveness in the treatment of atherosclerosis.

However, we also observed significantly reduced lesion area in ARB-treated animals compared to those given ARB+Statin therapy (Figure 3.2). Others pre-clinical studies have reported an additive,\textsuperscript{18,19,23} or even synergistic\textsuperscript{24-26} effect of ARBs and statin therapy in the prevention of atherosclerosis. These studies have all examined the effects of therapy in models which employ genetic mutation to induce hypercholesterolemia. Such models are akin to familial hypercholesterolemia and are not directly translatable to dietary hypercholesterolemia experienced by the majority of Western society. Furthermore, only two of these studies induce atherosclerosis before initiating treatment;\textsuperscript{19,23} the others have limited ability to analyze treatment effects. Only our model specifically replicates the chronic, progressive nature of atherosclerosis and properly examines treatment after disease is established.\textsuperscript{20,22}

While the mechanisms driving smooth muscle cell-mediated calcification in atherosclerotic plaques have been thoroughly examined,\textsuperscript{37,38} the relationship between macrophages and calcification is less clear. In this study we have shown a negative correlation between macrophages and calcium in atherosclerotic plaques, similar to that
observed in coronary arteries.\textsuperscript{39} Conversely, some have suggested that macrophages may directly promote calcification in atherosclerotic plaques by secreting matrix vesicles that act as a nidus for hydroxyapatite.\textsuperscript{40} In addition, Naik \textit{et al.}\textsuperscript{41} have shown that up to 20\% of osteochondrogenic cells in atherosclerotic plaques are bone marrow-derived. Clearly, further studies are required to fully understand the mechanistic interactions between statins and ARBs in the treatment of atherosclerosis.
3.4 References


7. Rumberger JA, Simons DB, Fitzpatrick LA, Sheedy PF, Schwartz RS. Coronary artery calcium area by electron-beam computed tomography and coronary


Chapter 4

4  Effects of an Angiotensin II Type 1 Receptor Blocker on Aortic Valve Sclerosis in a Pre-Clinical Model

Aortic valve stenosis is a chronic progressive disease involving three factors: lipid deposition, chronic inflammation, and tissue calcification. \(^1\)–\(^5\) It is the most prevalent valvular heart disease and the third most common cardiovascular disease behind hypertension and coronary artery disease. \(^6\) It has an incidence of 3–5% in those over the age of 65 while its precursor, aortic valve sclerosis (AVS), is found in over 25% of individuals in the same age group. \(^7\)–\(^9\) Patients with AVS have a 50% increased risk of myocardial infarction and cardiovascular death compared with patients who have a normal aortic valve. \(^10\)–\(^12\) Currently, there are no clinically-approved pharmaceuticals for the prevention of AVS; however, when symptoms develop it is managed by prompt valve replacement, an invasive surgical procedure with high morbidity and mortality. \(^13\)

AVS shares many risk factors with atherosclerosis including advanced age, male gender, hypertension, smoking, increased plasma low-density lipoprotein, increased plasma lipoprotein(a), and diabetes mellitus. \(^1\)–\(^3,14\) This overlap prompted several pre-clinical studies, the results of which suggested statin therapy as an effective intervention. \(^15,16\) However, subsequent clinical trials investigating the effect of statin therapy on AVS proved unsuccessful. \(^17\)–\(^20\) Indeed, only one pre-clinical study has been consistent with clinical trials showing lack of effect; Hamilton et al. \(^21\) suggested Atorvastatin was an effective anti-inflammatory agent, but had no demonstrable effect on lipid deposition or valve calcification.

Blockade of the renin-angiotensin system (RAS) has also been suggested as a potential therapy for AVS. Two conflicting retrospective studies—one showing no benefit of angiotensin converting enzyme inhibitors (ACEIs) compared to statins and another showing a 71% reduction in the progression of valvular calcification—could explain the

\(^*\) A version of this chapter has been accepted for publication: Armstrong ZB, Boughner DR, Carruthers CP, Drangova M, Rogers KA. Effects of an angiotensin II type 1 receptor blocker on aortic valve sclerosis in a pre-clinical model. Can J Cardiol 2014.
lack of prospective clinical trials investigating RAS blockade.\textsuperscript{22,23} More recent pre-
clinical studies have suggested that olmesartan, an angiotensin receptor blocker (ARB), can prevent atherosclerotic changes and endothelial disruption,\textsuperscript{24} while ramipril, an ACEI, can prevent hemodynamic changes in the valve.\textsuperscript{25} Indeed, recent results have prompted two prospective clinical trials investigating the role of ARBs on AVS (ClinicalTrials.gov Identifiers NCT00699452 & NCT01589380).

Given our recent observation that ARBs can robustly inhibit arterial calcification and the established anti-inflammatory effects of statin therapy,\textsuperscript{21,26-28} our objective in the current study was to investigate potential synergistic effects of these two pharmaceuticals in the treatment of AVS.

4.1 Methods

4.1.1 Pre-Clinical Model

Male New Zealand White rabbits (1.6 to 2.0 kg, Charles River Laboratories, St-Constant, QC, Canada) were fed either regular chow (Control, \( n = 8 \)) or an atherogenic diet consisting of 0.25\% cholesterol to induce aortic valve sclerosis.\textsuperscript{29,30} After 12 months, a subset of Control (\( n = 3 \)) and cholesterol-fed (\( n = 6 \)) rabbits were euthanized for pathologic assessment of disease progression. The remaining diseased rabbits continued on an atherogenic diet and were block randomly assigned to four treatment groups receiving either no treatment (Cholesterol, \( n = 6 \)), olmesartan medoxomil (ARB, \( n = 7 \)), atorvastatin calcium (Statin, \( n = 7 \)), or a combination of both drugs (ARB+Statin, \( n = 7 \)) for an additional 6 months (Supplementary Figure 4.1). During the treatment period, dietary cholesterol levels were decreased to 0.125\% to reduce the chance of non-cardiovascular health issues and prolong the life of the animals. Olmesartan medoxomil (1 mg/kg/day) and atorvastatin calcium (2.5 mg/kg/day) were administered via oral gavage, suspended in a solution of 50\% molasses in water. Dosages were based on previous studies using olmesartan medoxomil\textsuperscript{24} and atorvastatin calcium\textsuperscript{21} which showed positive effects in New Zealand White rabbits. At endpoint, animals were euthanized via intravenous ketamine injection and perfused with Hanks’ balanced salt solution and
heparin (1 U/mL). All animal protocols were approved by the University of Western Ontario Animal Use Subcommittee (reference number 2007-023).

4.1.2 Physiological Data

Body weight and blood samples were collected at months 0, 3, 6, 9, 12, 15, and 18 for all animals. Blood samples were used for the measurement of total cholesterol using a WAKO Cholesterol E Kit (Wako Chemicals, Richmond, VA), according to the manufacturer’s instructions.

4.1.3 Magnetic Resonance Imaging

After 12 months of the atherogenic diet, and every three months thereafter, anesthetized rabbits were imaged using a standardized in vivo pulse sequence on a 3.0 Tesla magnetic resonance imaging (MRI) scanner (GE Healthcare, Piscataway, NJ), as previously described. Rabbits were imaged in the supine position with a customized two-channel phased array radio frequency coil positioned over the chest cavity. All imaging was executed using CINE fast spoiled gradient echo (fSPGR) sequences gated to the cardiac cycle (peripheral trigger, arrhythmia rejection window = 30, minimum trigger delay, 30 cardiac phases, 2 segments/view). A finger plethysmograph attached to the rabbit’s ear provided the gating signal. All image analysis was performed off line using ImageJ (National Institutes of Health, Washington, DC). A blinded observer performed aortic valve area planimetry measurements at the moment of maximal valve opening using a double oblique axial image at each time point. In addition, a blinded observer measured thickness in the middle third of the cusp using the best oblique sagittal image of each cusp at each time point. We have found interreader variability for measurements such as these to be moderate with a kappa coefficient of 0.58.

4.1.4 Histological and Immunohistochemical Analysis

Aortic valve cusps (non-coronary) were cryopreserved using increasing concentrations of sucrose (up to 30%) then immediately embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane. Frozen Sections (10 µm) were taken vertically through the cusp and sinus such that sections are taken in
the radial direction. Serial sections were stained with Oil Red O for lipid deposition and Alizarin Red S for calcium deposits (both from Sigma-Aldrich, Oakville, ON, Canada). Immunohistochemical studies were performed using a primary antibody for mouse anti-rabbit activated macrophage (clone RAM11, Sigma-Aldrich, Oakville, ON, Canada). Sections were subjected to single-label immunohistochemistry using an Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) and secondary antibody horse anti-mouse IgG (H + L; Vector Laboratories) Alkaline Phosphatase-conjugate according to the manufacturer’s instructions. Negative controls which omitted the primary antibody were routinely used. Positive staining areas were calculated by setting a threshold value in ImageJ and expressed as a percent of total lesion area.

### 4.1.5 Statistical Analysis

Data is expressed as mean ± SEM and statistically analyzed by unpaired t-test, Kruskal-Wallis with Dunns post-hoc test, or two-way repeated measures ANOVA with Bonferroni post-hoc test, as indicated. All analyses were performed using GraphPad Prism (V5, GraphPad Software, Inc, La Jolla, CA). Values of p < 0.05 were considered statistically significant.

### 4.2 Results

#### 4.2.1 Physiological Data

Body weight increased generally with time but did not differ significantly between treatment groups (Supplementary Table 4.1). Total plasma cholesterol increased significantly in Cholesterol animals, but not Statin-treated animals, when compared to Controls (Supplementary Table 4.1).

#### 4.2.2 Dietary Hypercholesterolemia Induced Significant AVS at 12 Months

After 12 months of cholesterol feeding, cusp thickness in Cholesterol rabbits was significantly increased compared to Control rabbits (0.58 ± 0.03 versus 0.39 ± 0.03 mm for Cholesterol and Control at 12 months, respectively; n = 18 and 9; P < 0.0001; Figure 4.1A), as assessed by in vivo MRI. While valve orifice area was not significantly affected
Figure 4.1: Significant AVS progression is achieved after 12 months. A: Cusp thickness, measured using *in vivo* MRI, is significantly increased in Cholesterol animals versus Control. B: Valve orifice area, a clinical measure of AVS, showed a trend toward disease, but this did not reach statistical significance (P = 0.128). Histological and immunohistochemical analyses reveal several markers of AVS. C: Aortic valves from Cholesterol animals were significantly thickened with marked lipid insudation (Oil Red O). D: Valves from Cholesterol animals display considerable macrophage (clone RAM11) infiltration. E: Valves from Cholesterol animals also show minor signs of calcification (Alizarin Red S). Valves from Control animal showed no indication of disease. A-B: Each data point represents an individual animal (in A: an individual cusp), with the median represented by a horizontal line. Statistical significance is indicated by different superscripts and determined by unpaired t-test. C-E: Representative images shown; scale bar = 500 μm Inset: High-power image of the area indicated by the box; scale bar = 100 μm. AVS, aortic valve sclerosis; MRI, magnetic resonance imaging.
at this time point, there was a trend toward orifice narrowing (0.38 ± 0.03 versus 0.51 ± 0.09 cm$^2$ for Cholesterol and Control, respectively; n = 6 and 3; P = 0.128; Figure 4.1B) consistent with disease progression. Histological assessment of non-coronary cusps from Cholesterol animals showed thickening of the fibrosa and other hallmarks of human AVS including lipid infiltration (Figure 4.1C), inflammation (Figure 4.1D), and calcium deposition (Figure 4.1E). Lipid infiltration, while not present in cusps from Control animals, was extensive in Cholesterol animals and distributed throughout the entire cusp (Figure 4.1C), in contrast with the human disease. Non-coronary cusps from Control animals contained a few macrophages, typically scattered along the ventricular surface of the valve. In contrast, cusps from Cholesterol animals contained extensive macrophage infiltration throughout a thickened fibrosa (Figure 4.1D). Calcification was not found in cusps from Control animals, and only very minor calcified deposits were found in cusps from Cholesterol animals at 12 months (Figure 4.1E). Together, these findings confirm that AVS was well established before pharmaceutical intervention, and provided the basis on which further progression, stabilization, or regression could be assessed.

### 4.2.3 In Vivo Monitoring of AVS Progression did not Reveal Significant Treatment Effects

During 6 months of pharmaceutical intervention, AVS progression was monitored using MRI. While cusp thickness remained significantly greater in Cholesterol rabbits when compared to Control (0.465 ± 0.030 versus 0.388 ± 0.023 mm for Cholesterol and Control at 18 months, respectively; n = 30 and 15; P < 0.05; Figure 4.2A) there was no significant effect of any treatment group. Similar trends were observed in the data for valve orifice area, although they didn’t reach statistical significance; valve orifice area in Cholesterol animals was decreased as compared to Control animals, but no significant effects of treatment were observed (Figure 4.2B).
Figure 4.2: *In vivo* monitoring of AVS does not reveal treatment effect of ARBs, alone or in combination with statin therapy. **A:** Aortic valve cusp thickness was measured at 12, 15, and 18 months using *in vivo* MRI. While cusp thickness in Cholesterol animals was significantly increased from Control, there was no significant effect of ARB, Statin, or ARB+Statin therapy. **B:** Valve orifice area was significantly decreased in Cholesterol animals as compared to Control, but there was no significant effect of ARB, Statin, or ARB+Statin therapy. Data are presented as mean ± SEM. P > 0.05 (not significant) by 2-way repeated measures ANOVA. ARB, angiotensin receptor blocker; AVS, aortic valve sclerosis; MRI, magnetic resonance imaging.
4.2.4 Histological Analysis of Non-Coronary Cusps Revealed Significant Morphological Changes in Response to Pharmaceutical Intervention

Although treatments were unable to produce macroscopic valvular changes, we did observe several changes within the valve microenvironment. As expected, cusps from Control animals showed no signs of lipid infiltration, inflammation, or calcification. While lipid infiltration into the fibrosa was present in cusps from all animals fed the atherogenic diet, morphometric analysis indicated that only cusps from Statin animals had a significantly increased level of lipid when compared to Control (Figure 4.3). Macrophage infiltration was robust with significantly increased levels of macrophage staining in cusps from Cholesterol, ARB, and ARB+Statin animals when compared to Control (Figure 4.4). Interestingly, macrophages in cusps from Cholesterol and ARB+Statin animals typically concentrated on the surface and in the core of the lesion, while macrophages in cusps from ARB animals concentrated only in the core and macrophages in cusps from Statin animals concentrated only on the surface (Figure 4.4). Small, punctate nodules of calcification were observed in some animals, typically at the base of the fibrosa (Figure 4.5). Significantly increased levels of calcium were observed in cusps from ARB and ARB+Statin animals as compared to control. In contrast, cusps from Cholesterol and Statin animals did not have significantly elevated levels of calcium deposition.

4.3 Discussion

The current study examined the effects of an ARB, alone or in combination with statin therapy, on the progression of established aortic valve sclerosis. While clinically-relevant MRI was unable to detect modulation of disease in vivo, some structural changes were observed in the valve cusps ex vivo. When compared to Controls, animals treated with statin therapy alone had significantly increased levels of lipid insudation while animals from other groups (Cholesterol, ARB, and ARB+Statin) had significantly increased macrophage infiltration. More importantly, animals treated with ARBs, alone or in combination with statin therapy, had significantly increased levels of calcification.
Figure 4.3: Lipid insudation is significantly increased in Statin treated animals, but not Cholesterol, ARB, or ARB+Statin treated animals. Histological analyses reveal extensive thickening of the aortic valve with lipid insudation (indicated by Oil Red O) in all groups except Control. Morphometric analysis revealed a significant increase of lipid deposition in Statin treated animals as compared to Control. This increase was not observed in Cholesterol, ARB, or ARB+Statin treated animals. Representative images shown; scale bar = 500 μm. Inset: High-power image of the area indicated by the box; scale bar = 100 μm. Each data point represents an individual animal, with the median represented by a horizontal line. Statistical significance is indicated by different superscripts and determined by Kruskal-Wallis test with Dunns post-hoc test. ARB, angiotensin receptor blocker.
Figure 4.4: Macrophage infiltration is significantly increased in Cholesterol, ARB, and ARB+Statin treated animals, but not Statin treated animals.

Immunohistochemical analyses reveal macrophage infiltration (indicated by RAM11) in all groups except Control. Morphometric analysis revealed a significant increase of macrophage staining in all animals except those treated with Statins. Representative images shown; scale bar = 500 μm. Inset: High-power image of the area indicated by the box; scale bar = 100 μm. Each data point represents an individual animal, with the median represented by a horizontal line. Statistical significance is indicated by different superscripts and determined by Kruskal-Wallis test with Dunns post-hoc test. ARB, angiotensin receptor blocker.
Figure 4.5: Calcification is significantly increased in ARB and ARB+Statin treated animals, but not Cholesterol or Statin treated animals. Histological analyses reveal varying degrees of calcification (indicated by Oil Red O) in all groups except Control. Morphometric analysis revealed a significant increase of calcification in animals who received ARBs alone or in combination with Statins. Cholesterol and Statin treated animals did not have significantly increased amounts of calcification. Representative images shown; scale bar = 500 μm. Inset: High-power image of the area indicated by the box; scale bar = 100 μm. Each data point represents an individual animal, with the median represented by a horizontal line. Statistical significance is indicated by different superscripts and determined by Kruskal-Wallis test with Dunns post-hoc test. ARB, angiotensin receptor blocker.
Despite several successful pre-clinical studies, clinical trials have consistently shown no beneficial effect of statin therapy in the management of AVS. To date, our long-term dietary cholesterol model is the only pre-clinical model which suggests statin therapy has limited potential in the treatment of AVS and is the only pre-clinical model consistent with prospective human trials. As a result, after the failure of statin therapy in the treatment of AVS, we set out to examine the effects of ARBs, a strategy that has been suggested by us and others. Initial results using ACEIs were conflicting; however, two clinical trials studying the effects of ARBs on the progression of AVS are currently recruiting, suggesting continued interest in using ARBs to treat AVS (ClinicalTrials.gov Identifiers NCT00699452 & NCT01589380).

To our knowledge, the current study is the first to use a pre-clinical model to examine the effects of combined ARB and statin therapy in the treatment of AVS. Despite using clinically-relevant measures (Figure 4.2) and ex vivo histological analysis (Figures 4.3-4.5), we did not observe any clinically significant treatment effect of ARBs, alone or in combination with statin therapy. Our findings contrast with those of previous work which suggested RAS blockade may prevent atherosclerotic changes within the aortic valve. It is important to note, however, that the models used in those studies were short-term and therefore could not replicate the slowly progressive nature of clinical AVS. Furthermore, neither study was able to show valvular calcification, a hallmark of advanced AVS. The valves in our study displayed varying degrees of calcification even before the introduction of therapy (Figure 4.1E), suggesting we initiated therapy in the late-stage of the disease process and thereby replicated the clinical practice. Given prevailing opinion that pharmaceutical intervention should be administered early, it remains possible that our intervention was initiated too late to affect the course of the disease.

While the current study was unable to demonstrate significant structural changes, significant changes in the valvular microenvironment were observed. Consistent with previous work done by our lab, we observed an inverse relationship between macrophage infiltration and lipids. In Statin animals we observed significantly increased
lipid deposition when compared to Control animals. Conversely, the Statin group was the only one without significantly increased levels of macrophages. This suggests, as we’ve argued previously, that the primary role of macrophages in AVS is lipid extraction.\textsuperscript{21} Additionally, the localization of macrophages within cusps appeared to change depending on the treatment. Macrophages in the cusps of ARB-treated animals were found predominantly in the core of the valvular lesion, while macrophages in the cusps of Statin-treated animals concentrated on the fibrosa surface. Given recent reports suggesting macrophages may increase vascular calcification in a paracrine manner,\textsuperscript{35} it may be important to understand and manage the distribution of macrophages within the valvular microenvironment if we are to modulate valve calcification.

The current study suggests that angiotensin II type 1 receptor blockers, alone or in combination with statin therapy, may not be a suitable treatment for AVS. While we remain cautiously optimistic about ongoing clinical trials, it has become clear that further research into the unique mechanisms underlying aortic valve disease is required to generate suitable pharmaceutical management.
4.4 References


Chapter 5

5 General Discussion

5.1 Summary of Results and Conclusions

We have utilized two pre-clinical models to examine the effects of an angiotensin II (AngII) type 1 receptor blocker (ARB), olmesartan medoxomil, on the three main classes of cardiovascular calcification: arterial calcification, intimal calcification, and calcific aortic valve disease. Where appropriate or suggested by evidence, statins were examined in conjunction and in combination with ARBs. In vivo and ex vivo techniques were used to assess overall disease burden and the extent of calcification including magnetic resonance imaging, micro-computed tomography, histology, and immunohistochemistry. Interestingly, the ARB olmesartan medoxomil was able to robustly inhibit arterial calcification, but showed little effect in halting intimal or valvular calcification (Figure 5.1). Even when combined with statins, the ARB was unable to inhibit intimal or valvular calcification and, at least in the aortic valve, may have promoted its progression. Taken together, our results suggest that distinct molecular mechanisms may give rise to arterial, intimal, and valvular calcification.

5.1.1 Arterial Calcification

We have provided the first evidence that suggests ARBs as a novel therapy for arterial calcification. Using ex vivo micro-computed tomography and histology, we have shown that an ARB can robustly inhibit arterial calcification well after the disease process is underway. We also provided clues to the underlying molecular mechanisms involving osteogenic differentiation of vascular smooth muscle cells (VSMCs) including early down-regulation of the typical VSMC marker α-smooth muscle actin and upregulation of the osteogenic differentiation marker bone morphogenetic protein-2.

5.1.2 Intimal Calcification

We also examined the effects of an ARB and a statin, alone or in combination, on the progression of atherosclerosis and intimal calcification. ARBs may have induced
Figure 5.1: An angiotensin II type 1 receptor blocker effectively inhibits arterial calcification, but not intimal calcification or calcific aortic valve disease. The angiotensin II type 1 receptor blocker (ARB) olmesartan robustly inhibited arterial calcification within the medial layer of the artery, but may have only slowed progression of intimal calcification. In the aortic valve, the ARB may have promoted the progression of calcification.
significant regression of atherosclerotic lesion area when delivered as monotherapy but not when combined with statins. In addition, the progression of intimal calcification may have been inhibited by both ARBs and statins, although the lack of longitudinal in vivo monitoring means this remains speculative. The effects of ARBs on atherosclerotic lesion composition were unclear.

5.1.3 Calcific Aortic Valve Disease

Finally, we examined the effects of an ARB, alone or in combination with a statin, on the progression of established aortic valve sclerosis (AVS). While in vivo magnetic resonance imaging was unable to detect any demonstrable treatment effects, ex vivo histological examination revealed significant structural changes within valve cusps. Interestingly, animals treated with ARBs, whether alone or combined with statins, had significantly increased levels of calcification in their aortic valve cusps when compared to those from control animals (Figure 4.5).

5.1.4 Three Distinct Mechanisms

While arterial calcification was generally thought to be irreversible, inhibiting its progression remains the goal of several groups.1-6 We were the first to describe the robust inhibition of arterial calcification by an ARB. However, the ARB only inhibited calcification of the tunica media. Indeed, a few micro-calcifications were observed within atherosclerotic plaques of both ARB-treated and untreated animals (Figure 2.2), providing evidence suggesting that ARBs may specifically inhibit arterial calcification of the media, and that the mechanisms giving rise to arterial calcification and intimal calcification are distinct. The failure of ARBs to inhibit progression of intimal calcification (Table 3.1) also supports this theory.

Recently, Speer et al.1 performed an elegant fate mapping study to understand the cell types that give rise to calcification of the media. They showed that transdifferentiation of resident VSMCs to an osteochondrogenic state was the crucial mediator of arterial calcification. More recently, Naik et al.7 have performed a similar fate mapping study examining calcification within atherosclerotic plaques. They found 75–88% of
osteochondrogenic cells observed in atherosclerotic plaques were derived from VSMCs, but they also noted up to 20% were bone-marrow derived. This could provide a clue as to the underlying mechanism whereby ARBs inhibit arterial calcification. If ARBs act directly on VSMCs to exert their anti-calcification effects, it is possible that the bone-marrow derived cells are unaffected and able to continue the calcification process. However, macrophages in the atherosclerotic plaque have been shown to express the AngII type 1 receptor (AT1R) by us (Figure 2.3) and others, and AngII acting through the AT1R in macrophages has been shown to promote atherosclerosis. Therefore, it stands to reason that ARBs would also be effective at blocking the pro-calcific effects of AngII in bone-marrow derived cells. However, it remains possible that the mechanism driving osteochondrogenesis in bone-marrow derived cells is AngII-independent. It is also possible that bone-marrow derived cells secrete additional pro-calcific mediators that reactivate the osteochondrogenic transition in VSMCs in an AngII-independent manner. Clearly, more work needs to be done to understand the mechanisms at play in both arterial and intimal calcification.

The same is true for calcific aortic valve disease. Despite the effectiveness of ARBs at reducing advanced atherosclerotic lesions (Figure 3.2), they have little or no effect on AVS (Figure 4.2). Statins face a similar dilemma when treating atherosclerosis and AVS: effective in the former, but not the latter. Although much has been made of the strong resemblance of AVS to atherosclerosis, it must be remembered that these processes are separate and involve structurally distinct tissues. Arteries involved in atherosclerosis are three layered structures with atherosclerotic lesions developing in the thin cellular intima and the final clinical result being a lipid filled plaque and plaque instability. The aortic valve also has a three-layered structure, but the layer primarily involved in the disease process, the fibrosa, is a highly-organized, collagenous structure and the final outcome is a thickened, calcified valve that fails to perform its required function. Furthermore, the resident cells are distinct: VSMCs play a role in atherosclerosis while valvular interstitial cells (VICs) are present in the valve. VICs, like VSMCs, possess osteochondrogenic potential and contribute to calcification. Osteogenic differentiation of VICs is regulated by many of the same inflammatory factors as VSMCs including C-
type natriuretic peptide, reactive oxygen species, and transforming growth factor-β1. However, VICs are also regulated by additional factors such as matrix stiffness and mechanical stress. Clearly, a multitude of factors are involved in the progression of calcific aortic valve disease and it remains unclear whether the mechanisms underlying calcification of VICs is distinct from that observed in VSMCs. Indeed, the National Heart and Lung and Blood Institute Aortic Stenosis Working Group in 2011 recommended that further research be done to understand the basic biology of calcific aortic valve disease.

### 5.2 Clinical Implications

The most important clinical implication of this work is the suggestion that ARBs may robustly inhibit arterial calcification. To date, there are no methods for pharmaceutical intervention of arterial calcification, a disease that may affect 30–50% of asymptomatic patients and is associated with increased morbidity and mortality, increased risk of amputation, and may lead to necrosis or ulceration of the skin. Despite an incomplete understanding of the underlying mechanisms leading to inhibition, the fact that ARBs are already approved for the treatment of hypertension means that they could rapidly move through clinical trials to assess arterial calcification. The evidence put forward by us and others should provide the impetus to explore the role of ARBs in a clinical setting.

A potential negative interaction between ARBs and statins is another important clinical implication of this work, since hypertension and hypercholesterolemia frequently coexist in patients. According to the United States National Health and Nutrition Examination Surveys from 1988-2010, more than 60% of hypertensive patients were also hypercholesterolemic. Over that same period, dual intervention for hypertension and low density lipoprotein (LDL)-cholesterol rose from 5.0 to 30.7%. It is unclear what proportion of prescribed antihypertensives were ARBs in this survey; however, another study has shown the proportion of patients taking ARBs to be 17–22.6%. Clearly, a significant number of patients are currently prescribed ARBs and statins which may be preventing some of the anti-atherosclerotic effects each drug confers when delivered as monotherapy.
The renin-angiotensin system (RAS) has been the primary focus of several groups searching for pharmaceutical interventions for the clinical management of AVS.\textsuperscript{42-45} Indeed, at the time of writing, two clinical trials were recruiting patients to examine the effects of ARBs. ROCK-AS (the potential of candesartan to retard the progression of aortic stenosis), by Kupari \textit{et al.}, is examining the role of candesartan on inflammation, calcification, lipid deposition, and fibrosis of the aortic valve (ClinicalTrials.gov Identifier: NCT00699452). ALFA (a randomized trial of Angiotensin receptor bLocker, Fimasartan, in Aortic stenosis), by Kim \textit{et al.}, is examining the effects of fimasartan on change in exercise capacity and other symptoms of AVS (ClinicalTrials.gov Identifier: NCT01589380). ALFA is also examining classical hemodynamic measures of AVS including aortic jet velocity and mean pressure gradient across the valve. While we remain cautiously optimistic about these ongoing clinical trials, our pre-clinical model suggests that ARBs may not be suitable for the prevention of AVS. They also provide impetus for a deeper examination of the molecular mechanisms underlying valvular calcification within the complex atherosclerotic milieu that characterizes AVS.

5.3 Limitations

The conclusions drawn in this work are based on pre-clinical models, which are useful tools for understanding mechanisms of disease processes that are difficult or impossible to study in humans. However, they are still based on animals, and may not be directly translatable to clinical practice. It should also be noted, with regard to Chapter 2, that the role of Vitamin D\textsubscript{2} in the progression of arterial calcification in humans remains controversial.\textsuperscript{46,47}

Often, pre-clinical treatment studies initiate the potential therapy alongside the disease-initiating state.\textsuperscript{48,49} If this experimental design were theoretically applied to a clinical setting, it would involve treating children from birth. Others use an atherogenic diet that far exceeds any physiological norm (1 or 2% cholesterol), creating disease states that are morphologically and histologically dissimilar to the human condition and akin to familial hypercholesterolemia or lipid storage disease (>1000mg/dL plasma cholesterol).\textsuperscript{48,50-52} Neither experimental paradigm is ideal since cardiovascular disease is developed slowly,
over the course of a lifetime and treatment is not initiated until the disease process is well established. Our work was done with painstaking commitment to replicate the clinical setting. Animals were administered a low-level cholesterol diet over a long time period and treatment was initiated well after the disease was underway. As an example, rabbits in our studies had approximately 75% of their aortic surface covered by atherosclerotic lesion and a 50% increase in the thickness of their aortic valve before treatment was initiated. In contrast, treatment studies by other groups have described atherosclerotic lesion areas in the range of 12% to 18% at endpoint,\textsuperscript{53,54} and studies that have identified regression of AVS have observed a reduction in valve thickness of only 30%.\textsuperscript{55,56} The experimental design is absolutely crucial when performing pre-clinical studies, and ours has been shown to remain consistent with clinical trials.\textsuperscript{16,57}

The effect of statins in our model - or, indeed, in any animal model - also deserves attention. Statins, or 3-hydroxy-3-methylglutaryl Co-enzyme A (HMG-CoA) reductase inhibitors, lower cholesterol levels by inhibiting HMG-CoA reductase, the rate limiting enzyme in endogenous cholesterol production. Many animal models, including those in rabbits and rats, are based on exogenous cholesterol; they induce high cholesterol through an enriched diet. It is understandable, therefore, to question the effectiveness of a drug that inhibits endogenous cholesterol production when experimental models have very little endogenous cholesterol and induce disease via exogenous cholesterol. However, statins are well known to have anti-inflammatory pleiotropic effects that are unrelated to cholesterol lowering.\textsuperscript{58-61} Since our model is primarily exogenous cholesterol, we are able to study the pleiotropic effects of statins in isolation from their cholesterol lowering effects.

One of the chief methodological limitations in this work is the lack of blood pressure data. Obviously, when examining the effects of blood pressure lowering medication it would be prudent to assess the effects on blood pressure. However, measuring blood pressure in rabbits is notoriously difficult, in contrast to the simplicity of mice and man. Previous studies using an identical dose of olmesartan medoxomil in rabbits measured blood pressure invasively for 30 minutes using the SURFLO Flash catheter at the study
While it is not noted in their manuscript, rabbits surely would have been anesthetized, however slightly, which undermines the reliability of pressure measurements. That being said, they observed no change in blood pressure after 4 weeks of treatment. We suspect that the effects of ARBs we observed are independent of its effect on blood pressure, but can’t be certain without quantitative data.

Another methodological limitation is the lack of hemodynamic data in relation to the aortic valve. Clinical severity of aortic valve disease is assessed using an array of hemodynamic and natural history data including aortic jet velocity, mean pressure gradient, and valve orifice area. In rabbits, standard Doppler echocardiography is used to assess the progression of aortic valve disease. Ultrasonic backscatter has been used with success in humans, rats, and rabbits to measure the echogenicity of the aortic valve as an indicator of disease severity. These methods have also been used to assess the effects of an Apo-A1 mimetic peptide, the angiotensin converting enzyme inhibitor (ACEI) rampril, as treatments of aortic valve disease. While these methods would have been a valuable addition to our work, we did not have the required facilities or technical expertise to perform these measurements. However, we did use MRI to monitor valve thickness and valve orifice area in our model. This showed limited success in detecting the presence of aortic valve disease, but was not sufficiently sensitive to detect changes as a result of treatment.

5.4 Conclusions

This work provides evidence that ARBs robustly inhibit arterial calcification, a disease commonly associated with advanced age, chronic kidney disease (CKD), and diabetes mellitus, and is the first to suggest ARBs as a novel treatment option for those at risk for cardiovascular calcification. It also suggests that ARBs may not be beneficial for those at risk for intimal or valvular calcification which are more commonly associated with an atherosclerotic milieu. These disparate results suggest that the classes of cardiovascular calcification are distinct from one another and provides impetus to further examine the underlying molecular mechanisms at play in these debilitating disease processes.
5.5 Future Directions

Recognizing the fact that there is currently no pharmaceutical therapy available to patients and the robust inhibitory effect of ARBs we observed in our pre-clinical model, arterial calcification should be the primary focus of future research. To date, there have been no prospective, clinical trials specifically examining the role of RAS blockade on arterial calcification. Such a trial would be desirable, particularly if it focused on patients who are prone to rapid development of arterial calcification. To test the applicability of my results, we believe a prospective, randomized trial examining the effects of an ARB on arterial calcification in patients with Stage 3 CKD (glomerular filtration rate of 30-59 mL/min) would be valuable. Exclusion criteria should include subjects on dialysis, subjects with a recent or scheduled kidney transplant, subjects who are pregnant or planning to become pregnant in the next 18 months, and subjects who are currently prescribed ARBs, ACEIs, or direct renin inhibitors. Patients would be randomly assigned to receive either placebo or olmesartan medoxomil (20 mg/day). Primary outcome measures should be the change from baseline in arterial calcification after 18 months, determined using spiral computed tomography. Secondary outcomes could include calcification of the superficial femoral arteries and/or arterial stiffness as measured by pulse wave velocity. A clinical trial by Toussaint et al. described the extent of progression of arterial calcification in CKD patients over a period of 18 months and found the difference from baseline for the entire cohort was +119.6 Hounsfield Units (HU). Using that number, as well as the typical standard deviation they observed (± 250 HU), a sample size of 138 patients (69 each receiving placebo and olmesartan) would be required for 80% power.

Many questions remain about the underlying molecular mechanisms leading to cardiovascular calcification. Experimentally, arterial calcification is the most straightforward of the three diseases to study, at least from a molecular standpoint, and thus should represent the immediate focus of future in vitro studies. At its most basic level, arterial calcification is the calcification of VSMCs in an environment made up primarily of VSMCs, with some paracrine factors from adventitial myofibroblasts and
endothelial cells in the intima. In contrast, atherosclerosis and AVS are each characterized by a complex atherosclerotic milieu. In addition to resident VSMCs or VICs, a typical lesion may also contain macrophage foam cells, lymphocytes, and/or mast cells; even endothelial cells have been shown to influence the process in a paracrine manner.\textsuperscript{72} This complexity makes studying atherosclerosis and AVS \textit{in vitro} extremely difficult. Therefore, a deeper understanding of the mechanisms driving VSMC transdifferentiation in arterial calcification could help focus attention on the most important aspects of that atherosclerotic milieu with regard to intimal or valvular calcification.

Ultimately, much more work needs to be done to understand the basic biology of cardiovascular calcification and its clinical implications. Future research should focus on areas with the potential for greatest impact, namely, clinical studies examining the effects of ARBs on arterial calcification and further investigating the biology underlying all three disease processes.
5.6 References


Appendices

Supplementary Results

Arterial calcification is detected after eight weeks on the atherogenic diet
To assess the state of disease progression before pharmaceutical intervention, a subset of Control and Cholesterol animals were sacrificed after eight weeks on the atherogenic diet. Focal areas of calcification were detected by micro-CT in one Cholesterol animal but did not reach a statistically significant volume (non-detectable versus 0.58 ± 0.58 % calcified tissue in Control and Cholesterol, respectively; n = 3/group; P = ns; Supplementary Figure 2.1A). Subsequent histological examination revealed calcification in a second Cholesterol animal; these regions - reflecting calcification at the initial stages - were too small (~30 µm) to have been identified with the micro-CT protocol we employed. In all cases, calcification was localized to the IEL and extended outward to the media (Supplementary Figure 2.1B), typical of arterial calcification and Mönckeberg sclerosis.
Supplementary Figure 2.1: Arterial calcification, localized to the IEL and medial layer, is present after 8 weeks on the atherogenic diet. (A) Maximum intensity projections, derived from micro-computed tomography (CT) scans, and corresponding quantitation (n = 3/group) show calcification is widespread in one Cholesterol animal, but nonexistent in Control animals. Scale bar = 4 mm. One sample t-test: P = ns. (B) Histological examination of calcium (Alizarin Red S, top) and calcium salts (Von Kossa, bottom) reveals they are localized primarily to the internal elastic lamina (IEL) and medial layer, typical of arterial calcification. Scale bar = 500 µm. ND = none detected. Arrows indicate calcification.
Supplementary Figure 2.2: Treatment with the angiotensin II type 1 receptor blocker has no effect on systemic disease parameters. (A) Total plasma cholesterol was significantly increased in Cholesterol animals as compared to Control, but was unaffected by ARB treatment. One-way repeated measures ANOVA with Tukey’s post-hoc test: $P < 0.001$. (B) Levels of inorganic phosphate were affected neither by the atherogenic diet nor ARB therapy. Kruskal-Wallis test with Dunns post-hoc test: $P = \text{ns.}$
Supplementary Figure 2.3: Characterization of calcified regions indicates an osteoblast-like phenotype. Immunohistochemical characterization of calcified regions (arrows) and adjacent sections reveals colocalized expression of the osteogenic growth factor bone morphogenetic protein 2 (BMP2), the bone protein and osteoblast-specific marker osteocalcin (OCN), and dramatic upregulation of the angiotensin II type 1 receptor (AT1R). This data suggests an osteoblast-like phenotype within the calcified areas. Scale bar = 500 µm.
Supplementary Figure 2.4: Calcified regions of the media are not associated with smooth muscle cells or macrophages. Examination of α-smooth muscle actin (α-SMA), a marker of smooth muscle cells, reveals dramatic downregulation in calcified regions (arrows). Interestingly, α-SMA is also downregulated in some non-calcified areas. Furthermore, macrophages are localized specifically to atherosclerotic plaques, and are not associated with areas of calcification. Scale bar = 500 µm.
Supplementary Figure 4.1: Experimental design. A pre-clinical model was used to investigate the effects of an angiotensin II type 1 receptor blocker (ARB), alone or in combination with a statin, on intimal calcification and atherosclerosis. Male New Zealand White rabbits were fed either regular chow (Control) or an atherogenic diet to induce atherosclerosis (Cholesterol). After 12 months, a subset of Control and Cholesterol rabbits were euthanized for pathological assessment of disease progression. The remaining rabbits continued on an atherogenic diet and received either no treatment (Cholesterol), olmesartan medoxomil (ARB), atorvastatin calcium (Statin), or a combination of both drugs (ARB+Statin) for an additional 6 months.
**Supplementary Table 4.1:** Physiological data

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<td>3.72 ± 0.08</td>
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<td>Month 18</td>
<td>3.65 ± 0.14</td>
<td>3.57 ± 0.06</td>
<td>3.66 ± 0.08</td>
<td>3.83 ± 0.09</td>
<td>3.73 ± 0.05</td>
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<td>Baseline</td>
<td>115.2 ± 4.0</td>
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<td>Month 3</td>
<td>76.1 ± 11.5</td>
<td>602.7 ± 45.9****</td>
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<td>Month 6</td>
<td>30.4 ± 8.4</td>
<td>731.3 ± 65.1****</td>
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<td>Month 9</td>
<td>48.1 ± 6.0</td>
<td>719.2 ± 64.1****</td>
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<td>Month 12</td>
<td>23.1 ± 3.6</td>
<td>614.9 ± 58.4***</td>
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<td>Month 15</td>
<td>26.2 ± 3.7</td>
<td>502.7 ± 94.1*</td>
<td>499.7 ± 118.8*</td>
<td>410.1 ± 59.5</td>
<td>514.3 ± 78.5*</td>
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<td>Month 18</td>
<td>20.0 ± 2.9</td>
<td>400.2 ± 107.2</td>
<td>498.2 ± 122.2*</td>
<td>328.6 ± 46.3</td>
<td>517.0 ± 89.6**</td>
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Data are mean±SEM
*Indicates significantly different from Control (P<0.05).
**Indicates significantly different from Control (P<0.01).
***Indicates significantly different from Control (P<0.001).
****Indicates significantly different from Control (P<0.0001).

All analyses used a Mann-Whitney test or Kruskal-Wallis test with Dunn’s post-hoc test, as appropriate.
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Animal Protocol Approval

2007-023-01::5:

**AUP Number:** 2007-023-01  
**AUP Title:** Imaging of Atherosclerosis, Aortic Valve Sclerosis and Alzheimer's Disease Using Rabbit Models

**Approval Date:** 05/24/2011

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-023-01 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.  
   Health certificates will be required.

**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee
Curriculum Vitae

Name: Zachary B. Armstrong

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2004-2008 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2008-2014 Ph.D.

Honours and Awards:
Renaissance Scholarship of Distinction
2004

Dean’s Honour List
2006-2008

Canadian Institutes of Health Research Strategic Training Fellowship in Vascular Research
2008-2010

Frederick Banting and Charles Best Canada Graduate Scholarship
Master’s Award
2009-2010

Gabriel G. Altmann Research Award
2010

Queen Elizabeth II Graduate Scholarship in Science & Technology
2010-2011

Schulich Graduate Scholarship
2008-2013

Frederick Banting & Charles Best Canada Graduate Scholarship
Doctoral Award
2011-2014

Related Work Experience
Laboratory Coordinator, Mammalian Histology
The University of Western Ontario
2010-2013
Teaching Assistant, Medical Sciences Laboratory
The University of Western Ontario
2008-2010

Peer-Reviewed Publications:
3. Armstrong ZB, Boughner DR, Carruthers CP, Drangova M, and Rogers KA. Potential negative interaction between statin therapy and angiotensin receptor blockage in atherosclerotic lesion reduction. In Review.

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