THE IMPACT OF WEIGHT FLUCTUATION ON ATRIAL SUBSTRATE AND THE PREVENTION OF ATRIAL REMODELLING WITH THE USE OF ANTI-FIBROTICS

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Abstract

Atrial fibrillation (AF) is the commonest sustained arrhythmia in humans and is responsible for a significant socioeconomic burden. Affected individuals can suffer significant symptoms and are at risk of potentially life-threatening complications. Obesity is increasingly recognised as risk factor for the development of this arrhythmia.

Weight fluctuation is common during attempted weight loss and has detrimental cardiovascular effects in human cohort studies, including patients with AF. However, the pathophysiological mechanisms by which this occurs are unclear. The first aim of this thesis is to characterise the electrophysiological effects of weight fluctuation using an obese ovine model.

Previous studies have demonstrated that obesity promotes the development of atrial fibrosis as well as the upregulation of profibrotic factors in atrial tissue. The second major aim of this thesis is to investigate the effect of blockade of these profibrotic receptors on obesity-related atrial remodelling.

Chapter 2 describes the use a fluctuating weight model in order to study the electrophysiological changes over time. Weight fluctuation was associated with progressive changes in atrial electrophysiology. This group demonstrated reduction in conduction velocity when compared to a lean control group, particularly following a second cycle of weight gain followed by weight loss. These changes were less severe when compared to an obese group. Additionally, the changes in conduction were more heterogeneous than in animals with persistent obesity. This resulted in an increased propensity to AF when compared with lean controls.

Chapter 3 investigates the role of endothelin receptor blockade in the prevention of atrial substrate in obesity. Obesity was again induced in ovine subjects and two groups were compared. One was treated with the endothelin receptor antagonist (ERA) bosentan whilst the other acted as a control group. Animals treated with bosentan had attenuation of obesity-related conduction slowing. This was seen on both endocardial and epicardial surfaces. Importantly, there was no effect on either haemodynamics or refractory periods. AF inducibility was also reduced by ERA treatment. Examination of atrial demonstrated reduced fibrosis and downregulation of pro-fibrotic factors with ERA treatment. Importantly, this effect was independent of the TGF-β pathway.

Chapter 4 examines the effect of the TGF-β receptor antagonist tranilast on the obese ovine atrium. A similar model of induced obesity was used to compare tranilast treatment with a control group. Animals receiving tranilast demonstrated attenuation of conduction slowing. Endo and epicardial mapping showed this slowing was heterogeneous across atrial sites, perhaps suggesting a predominantly local mechanism in the development of these electrophysiological changes.

Declaration

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Presentations at Scientific Sessions

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Chapter 1 - Introduction

1.1 Atrial fibrillation and obesity – epidemiology

Atrial fibrillation (AF) is the commonest arrhythmia in humans. The lifetime risk of developing AF at aged 55 is 23.8% in males and 22.2% in females.[1] It is characterised by irregular, chaotic atrial activity and subsequent irregular ventricular contraction. It can cause significant symptoms such as breathlessness, palpitation and is associated with potentially life-threatening complications, in particular, stroke[2], dementia[3, 4] and heart failure.[5] Individuals with AF have 1.5 to 1.9 times greater risk of risk of death.[6] The incidence of AF is increasing and this rise is predicted to continue.[7] This is associated with a dramatic increase the in use of health resources, surpassing both heart failure and coronary artery disease.[8] One contributory factor is the aging population, as the prevalence of AF is higher with increasing age. Less than 1% of 55 to 59 year olds have AF, whereas it affects over 15% of those aged 85 and above.[1] In addition, a number of other conditions are considered risk factors for AF. It has long been recognised that hypertension, diabetes, coronary artery disease, cardiac failure and valvular heart disease contribute to an individual's risk of developing AF.[9] Additionally, the presence of these risk factors increases the risk of thromboembolic events.[10] However, it has also been noted that even after correcting for these known factors, the increase in the prevalence of AF exceeds expectations.[7] This has given rise to the concept of 'novel' risk factors for AF. Obesity is a common, but frequently underestimated example of one of these factors.

The incidence of obesity across the world is reaching epidemic levels, with 60% of Australian adults being overweight or obese.[11] It has doubled between 1980 and

2000, and is projected to increase to over 70% by 2025.[12] Obesity is associated with several of the known risk factors for AF, particularly hypertension, diabetes and sleep apnoea. However, a number of large studies have shown that it also independently increases the risk of AF,[13, 14] with 17.9% of cases attributable to obesity, second only to hypertension.[15] Wanahita et al. performed a meta-analysis of over 78000 patients across 5 population-based cohort studies and concluded that obesity increased the risk of AF by 49%.[16] A recent Danish study recently confirmed these findings with a very large cohort of 271,000 young women. Over a median follow-up period of 4.6 years, the risk of AF was doubled in those with a BMI of 30 to 35 and tripled in those with a BMI of over 35.[17] Each unit increase in BMI increases the risk of AF by 3 to 5%.[18] Obesity is also associated with more severe forms of the disease; progression from paroxysmal to persistent AF is more likely in obese patients.[19]

Interestingly, there are emerging reports of an obesity paradox for patients with established AF. That is, that overweight and obese patients appear to have a lower all cause and cardiovascular mortality.[20] Consistent in these reports however is differential patient populations, frequently with obese patients presenting at a younger age and with fewer comorbidities.[21] Whether this observation is proven in more robust analysis is yet to be seen.

1.2 Atrial Fibrillation - pathophysiology

The pathophysiology of AF is a complex interplay between the fundamental electrophysiological principles of triggered activity in the form of initiating focal ectopic beats, and reentrant activity allowing maintenance of the arrhythmia. Atrial 'remodelling' is a persistent change in atrial structure or function that can both

increase ectopic burden and induce a substrate in atrial tissue, promoting reentry.[22] Remodelling can be anatomical, electrophysiological or molecular. There are numerous contributory factors and underlying conditions, many demonstrating significant overlap in abnormalities within these categories.

Several animal models have been developed to allow study of the properties of the fibrillating or fibrosed atrium. Atrial tachycardia, induced by rapid atrial pacing has demonstrated predominantly electrical remodelling, whereas heart failure models have also induced structural remodelling. Both models are associated with increased inducibility of AF. More recently, an obese animal model has allowed more focussed investigation of the effect on obesity on the structural and electrical properties of the atria.[23, 24] This model demonstrated both electrical and structural abnormalities and increased propensity to AF. Importantly the animals in this model did not develop obstructive sleep apnoea, a significant confounding condition in human studies.

1.2.1 Electrophysiological mechanisms of AF

Ectopic foci act as triggers or initiators of fibrillatory episodes, whereas reentrant, wavelet or rotor mechanisms contribute to the maintenance of these episodes. The degree of maintenance depends on the underlying electrophysiological substrate.

Initiation of AF

Seminal observations by Haissaguerre et al. demonstrated that AF episodes were frequently initiated by ectopic beats originating in the pulmonary veins and were abolished by the application of RF energy.[25] Subsequent studies determined multiple veins were often involved.[26] Based on this, electrical isolation of the pulmonary veins has become a cornerstone of ablation procedures for AF. A small percentage of

patients have a second initiating arrhythmia that may be amenable to ablation.[27,28] Atrial ectopy in paroxysmal AF is more frequent and more premature prior to episodes[29] and excessive atrial ectopy has recently been associated with stroke, even in the absence of AF.[30] The electrophysiology of the pulmonary veins is different to the adjacent atrial tissue and varies between proximal and distal portions of the veins[31], with more marked differences in patients with AF.[32] The relationship of the veins to the atrial tissue is complex, with muscular extensions extending into each structure[33] and the presence of pacemaker-type cells within venous tissue.[34] Conduction between atrium and vein is relatively slow, particularly after extrastimuli, and histological examination of the junction often shows abrupt changes in fibre orientation.[35] Clinically, this is demonstrated by distinct electrical activation by venous tissue.[32]

Sources of ectopy also exist outside the pulmonary veins.[36] These are less common, but may play a greater role in patients with persistent AF, given the reduced effectiveness of pulmonary vein isolation. It is unclear, however, if ablation outside the pulmonary veins has an impact on outcomes.[37]

Maintenance of AF

The mechanisms involved in the maintenance of fibrillation in cardiac tissue have been examined for over a century. Demonstration of circus motion seen in animal models developed into the concept of reentry.[38] It was postulated that atrial flutter and fibrillation both involved a single reentrant circuit, with AF having a smaller and only partially recovered 'excitable gap'. Early computer models of AF suggested that in fact multiple wavelets existed, progressing randomly across the atria.[39] This was later confirmed in animal models [40] and it was theorised that maintenance of AF,

through ‘daughter wavelets’, relied upon a critical mass of tissue, shortened refractory periods and slowed, heterogeneous conduction though the tissue.

The existence of transient rotatory electrical activity or ‘rotors’ was theorised by independent groups as a potential arrhythmia mechanism.[41, 42] A transient, single point acts as a pivot to a spiral wave which can be static or meandering. This propagates across cardiac tissue, interacting with anatomical structures and areas of functional conduction block, resulting in wave fragmentation. Technological advancements in high-density optical mapping allowed visualisation of rotors, initially in fibrillating ventricular tissue,[43] then in AF.[44] Using multipolar basket catheters and advanced phase mapping techniques, rotors have been seen in human subjects in both right and left atria.[45] Subsequent studies have suggested that ablation of these sites may be an effective strategy for invasive management of AF.[46]

1.2.2 Atrial remodelling

Atrial remodelling is a multifactorial process promoting both the occurrence and maintenance of AF. Macroscopic structural abnormalities, in particular atrial dilatation, are present in many clinical conditions associated with the development of AF and are thought to provide a larger surface area for reentrant circuits. Electrophysiological changes were first demonstrated with a rapid atrial-pacing model, with shortening of atrial refractory periods. Subsequent models of induced heart failure and obesity have demonstrated abnormalities in atrial conduction in conjunction with molecular changes such as interstitial fibrosis, ion channel abnormalities, upregulation of profibrotic factors and disruption of cell-to-cell communication. Recent invasive human studies have confirmed many of these abnormalities in patients with risk factors for AF.

Anatomical remodelling

Several large population based studies have shown an association between atrial diameter and incidence of AF.[47] In the Framingham cohort, a 5mm increase in LA diameter was associated with a 40% increase in incident AF.[48] LA dilatation can be caused by, and has adverse prognostic implications in, several conditions associated with AF, in particular hypertension[49], systolic[50] and diastolic[51] heart failure, as well as being prognostic in the general population.[52]

Obesity had the strongest association with left atrial dilatation in a cohort study of 1000 randomly selected German residents, with an odds ratio of 2.4. This was independent from the presence of hypertension, which conferred an odds ratio of 2.2 ($p<0.001$).[53] In patients with established hypertension, obesity doubles the likelihood of having LA dilatation.[54] The Framingham group examined factors influencing incident AF in 5000 patients and found increased BMI was associated with an increased risk of developing AF. After adjustment for clinical confounders using multivariate analysis, increased LA diameter was the only remaining association.[14] Obesity and LA size have also been shown to be independently predictive of progression from paroxysmal to persistent AF.[19]

Electrical remodelling

Seminal work by Wijffel at al involved rapid atrial pacing to induce and maintain AF in goats, the so-called 'atrial tachycardia' model.[55] After only 24 hours of rapid atrial pacing, the duration and rate of AF episodes increased significantly and at 1-3 weeks, AF became sustained. This study coined the phrase 'AF begets AF' and also offered early insight into the electrical remodelling process. The main electrophysiological

abnormality was a reduction in atrial refractory periods, occurring as early as 6 hours, with further reduction at 24 hours. Notably, no change in conduction velocity was noted at any time point. A later study rapidly paced the atria of dogs for 6 weeks. In addition to ERP reduction, there was also a reduction in conduction velocity, which was slower to develop than the changes in ERP.[56] A subsequent study using a similar model demonstrated similar changes in refractory periods within 30 minutes of commencement of high rate atrial pacing. Treatment with atropine and propranolol excluded autonomic tone as a contributory factor and in a subset of animals, calcium channel blockade prevented electrical remodelling, suggesting the involvement of specific ion channels in this pathophysiological state.[57]

Structural remodelling

To avoid the potentially confounding effect of atrial pacing and associated electrical remodelling, Li et al. developed a model of congestive heart failure to investigate the indirect effect on atrial electrophysiology.[58] Rapid ventricular pacing for 5 weeks in a canine model induced LV impairment. The atrial electrophysiology of this CHF group was compared with a rapid atrial pacing (RAP) group and a control group. The RAP group demonstrated reduction in ERP and increased ERP heterogeneity, without change in conduction velocity. In contrast, the CHF group showed no change in tissue refractoriness, but did demonstrate marked localised reduction in conduction velocity. Importantly, these electrical differences were associated with a dramatic increase in the degree of fibrosis, with no significant differences between control and RAP groups. The conduction slowing and ultrastructural changes demonstrated by this study were termed ‘structural remodelling’. Similar findings are associated with animal models of hypertension[59, 60], drug-induced cardiomyopathy[61, 62], coronary

artery disease[62] and, importantly, obesity.[23] The obese model developed by Abed et al. induced obesity over an eight-month period. Progressive atrial structural and electrical abnormalities were demonstrated with increasing weight. Conduction slowing and heterogeneity, without changes in refractory periods, led to increased AF inducibility. On analysis of atrial tissue, obesity was associated with increased atrial fibrosis and upregulation of a number of profibrotic factors.[23]

Human endocardial mapping studies have consistently found abnormalities in the atria of patients with cardiovascular disease. A cohort of individuals with heart failure but no history of AF had detailed right atrial electrophysiological studies. When compared with controls, they had heterogeneous reduction in conduction velocities, higher refractory periods, lower bipolar voltage and more signal fractionation.[63] These findings were strongly suggestive of underlying structural remodelling and similar abnormalities were found in the atria of patients with hypertension,[64] sleep apnoea[65], mitral valve disease[66], atrial septal defects[67] and even in patients with lone AF.[68]

Atrial ectopy

Structural remodelling may increase atrial ectopic burden, which could contribute to the initiation of AF episodes. Ectopic beats can be caused by enhanced automaticity and both early (EAD) and delayed (DAD) afterdepolarisations. Increased automaticity has not been clearly demonstrated to occur in conditions predisposing to atrial substrate, but upregulation of the 'funny current', an important contributor to automatic depolarisation, has been demonstrated in AF substrate models.[69, 70] Late depolarisations are associated with calcium channel abnormalities, and have been found in both atrial pacing and CHF models of AF, with enhancement of calcium

loading.[71, 72] Although early afterdepolarisations have been shown to play a potential role in the pathogenesis of AF, the relationship is not clear.

Molecular remodelling

A multitude of molecular abnormalities are associated with atrial remodelling. Fibrosis, a common finding in diseased organs, underpins many of the electrophysiological abnormalities associated with AF. Numerous pro-fibrotic and inflammatory molecules are involved to varying degrees. There is considerable overlap between these pathways, with multiple complex interactions and feedback loops (figure 1) but the exact mechanisms remain incompletely understood.

Fibrosis

Tissue fibrosis is characterised by fibroblast proliferation, accumulation of extracellular matrix proteins and collagen deposition. It is a feature of most cardiac conditions, particularly post myocardial infarction and cardiomyopathy. Several mechanistic studies have demonstrated that fibrotic tissue also acts as a substrate for AF, with strong associations between atrial fibrosis and conduction abnormalities such as conduction heterogeneity or reduction in conduction velocity, along with increased susceptibility to AF. Induced atrial fibrosis was first demonstrated in the canine heart failure model developed by Li et al. as discussed in the previous section.[58] Subsequent animal studies have demonstrated similar fibrotic processes with associated electrophysiological abnormalities in response to aging,[73] hypertension[59, 60], drug-induced cardiomyopathy[61], coronary artery disease[62] and obesity.[23]

Patients with AF have atrial fibrosis on analysis of surgically excised tissue when compared with patients without AF.[74] This was also found on tissue biopsy samples of patients with lone AF.[75] Recent advances in MRI technology have allowed for the direct visualisation of fibrotic areas in atrial tissue and have shown a strong correlation between the degree of fibrosis and success following AF ablation.[76]

Profibrotic molecules

Endothelin

Endothelin-1 (ET-1) was discovered in 1988 by Yanagisawa et al.[77] The 21-amino acid peptide was shown to be one of the most potent vasoconstrictors in existence. Three other active isoforms (ET-2, 3 and 4) have subsequently been discovered and are less relevant to the cardiovascular system. All isoforms are converted from 'big-ET' molecules by ET converting enzymes. ET-1 is predominantly produced by endothelial cells, with a study of endothelial cell-specific ET-1 knock-out mice demonstrating a 65-80% reduction in plasma and tissue ET-1[78] but is also produced by vascular smooth muscle and cardiomyocytes. Two distinct endothelin receptors were found soon after the existence of the protein. These are both transmembrane G protein-coupled receptors, ET-A and ET-B.[79] Activation of these receptors may have synergistic or opposing effects, depending on the site of the receptors and the physiological state. The predominant effect of endothelin is vasoconstriction, which occurs across multiple organs, with significant involvement in both systemic and pulmonary hypertension.[77, 80, 81] Additional effects include vascular smooth muscle proliferation[82], cardiomyocyte hypertrophy and ET-B mediated vasodilatation through nitric oxide and prostacyclin release.[83]

ET-1 is released from infarcted areas of cardiac tissue[84] and elevation of serum ET-1 correlates with a worse prognosis following MI.[85] Plasma ET-1 also correlates with the severity of stable coronary artery disease even in the absence of left ventricular dysfunction.[86] An ET-1 over-expression mouse model induced dilated cardiomyopathy.[87] Plasma ET-1 is also elevated in patients with heart failure and correlates with severity.[88] Zolk et al. examined ventricular tissue from patients undergoing heart transplantation for end stage dilated cardiomyopathy and found elevation of tissue ET-1 and ET-A, but a reduction in ET-B.[89] Interestingly, blockade of human ET-A receptors was negatively inotropic in normal hearts, but had no effect in a cohort with dilated cardiomyopathy.[90]

Human atrial tissue demonstrates positive inotropy in response to endothelin incubation.[91] One study demonstrated arrhythmic contractions of human atrial tissue in response to ET-1, which were unchanged by blockade of the ET-A receptor.[92] Several studies of its chronotropic effect have reported conflicting results.[93, 94] Analysis of atrial tissue at the time of cardiothoracic surgery have also had discrepant results. One study showed endothelin receptor downregulation in those who were known to have AF, however this was using right atrial tissue.[95] In another study, increased endothelin-1 expression in left atrial tissue was associated with increased AF burden, hypertension and atrial dilatation, which persisted after multivariate analysis.[96] Elevated serum endothelin-1 levels are predictive of failure after AF ablation.[97]

Evidence that endothelin was directly involved in fibrosis was first discovered in patients with systemic sclerosis. ET-1 plasma levels were elevated; more diffuse disease was associated with higher concentrations. Additionally, fibrotic skin and lung

tissue demonstrated elevated ET expression. Subsequent studies determined ET mediates fibrosis in cardiac, lung, renal and hepatic tissue.[98] Guarda et al. first demonstrated endothelin-1 to be directly responsible for cardiac fibrosis. Cardiac fibroblasts maintained in ET-1 had a dose dependant increase in collagen deposition compared with controls.[99] Endothelin exposure is associated with fibrosis, decreased gap junction expression and conduction slowing in ventricular myocytes.[100] Animal models of ischaemic and diabetic cardiomyopathy and obesity-related atrial fibrosis are associated with upregulation of endothelin-A and -B receptors.[101, 102, 23]

Several cytokines and profibrotic factors associated with AF have been shown to promote transcription of endothelin. TGF-β is a powerful inducer of ET upregulation and release in endothelial, vascular smooth muscle and cardiac cells, acting via the ALK-5 cytoplasmic signalling pathway and the Smad transcription factor pathway.[98, 103] Notably, blockade of endothelin receptors prevents the pro-fibrotic actions of TGF-β in the kidneys of hypertensive and diabetic rats.[98] Angiotensin II also induces endothelin production and acts via cytoplasmic protein kinase C,[104, 105] and blockade of angiotensin converting enzyme directly inhibits endothelin production.[106] Angiotensin increases tissue endothelin and induces vascular hypertrophy, an effect blocked by blockade of ET-A receptors. Leptin, a hormone well recognised to be secreted by adipocytes, induces ET-1 in human endothelial cells.[107] Along with TGF-β and angiotensin, leptin also acts via the AP-1 (Fos/Jun) transcription factor.

Transforming growth factor beta

TGF-β plays a central role in fibrosis. It exists in 3 isoforms and is produced by a wide variety of cells (epithelial, endothelial, connective tissue and haemopoietic). Overexpression in animal models results in fibrosis of the kidney, liver and lung and

high levels of the molecule are found in fibrotic organs. It is a potent stimulator of extracellular matrix production and deposition.[108] In cardiac tissue, it induces myofibroblast differentiation and is stimulated by myocardial infarction, cardiac failure and hypertrophy.[109] The Smad pathway plays a central role in the signalling of TGF-β.[110] Overexpression in mice is associated with cardiac hypertrophy, enhanced contractility in response to beta adrenergic agonists, and interstitial fibrosis.[111] This model also demonstrated increased susceptibility to AF, reduced right atrial conduction velocity and increased left atrial conduction heterogeneity.[112] A study by Nakajima et al. demonstrated a model with enhanced local TGF-β activity was associated with atrial but not ventricular fibrosis. This was associated with increased susceptibility to AF.[113] Abed et al. demonstrated obesity-related TGF-β overexpression was associated with atrial conduction abnormalities and increased AF burden.[23] A number of clinical conditions are associated with increased expression of TGF-β in ventricular tissue, including dilated[114], hypertrophic[115] and valvular[116] cardiomyopathy. Patients with AF have increased TGF-β expression in excised atrial tissue, and increased chronicity is associated with higher TGF-β protein and mRNA levels.[117] Increased serum TGF-β is predictive of recurrence following AF ablation, but only in patients with non-paroxysmal AF.[118] It is also associated with atrial fibrosis of excised appendage tissue and persistence of AF one year following the surgical maze procedure.[119]

Angiotensin II is well recognised as a mediator of TGF-β activity in myocardial infarction.[120-122] Angiotensin II stimulation also induces increased expression of TGF-β in neonatal cardiac myocytes and fibroblasts.[123] Animals deficient in in TGF-β do not develop cardiac hypertrophy in the presence of angiotensin II.[124]

Treatment with ET-1 upregulated cardiac TGF-β expression in diabetic rats and ET-1 gene silencing inhibited TGF-β signalling activation.[125] PDGF-A, C and D all induced TGF-β production in cardiomyocytes in a chronic rejection rat model.[126]

Angiotensin II

The renin-angiotensin-aldosterone system (RAAS) is well recognised as having an important role in several cardiac conditions, including hypertension, cardiac failure and atherosclerosis. Inhibition of angiotensin converting enzyme is a cornerstone of clinical heart failure management. RAAS activation is also involved in the pathophysiology of AF. Angiotensin II has the most pronounced pathophysiological effect and activates several profibrotic pathways via the AngII receptor type 1 (AT1) and though the activation of fibroblasts to become myofibroblasts.[22] Mice with overexpression of angiotensin converting enzyme and hence higher AngII levels develop atrial dilatation, fibrosis and AF.[127] In a model of AF-induced atrial fibrosis, AngII mediated the degree of fibrosis via the Smad signalling pathway.[128] Atrial substrate development in a congestive cardiac failure model was associated with upregulation of AngII receptors in atrial tissue, which preceded elevation of plasma levels, suggesting atrial tissue may produce AngII in response to pro-fibrotic stimuli.[129] Similarly, an obese AF animal model demonstrated similar upregulation with increasing atrial fibrosis.[23]

The action of angiotensin II is closely tied to TGF-β. Activation of the AT1 receptor induces production of TGF-β, and AngII-related fibrosis is reliant on the presence of TGF-β.[130] This autocrine loop acts through ALK5, Smad2, 3 and 4 and JNK signalling pathways.[131] Angiotensin has an influence on ET, CTGF and PDGF but the reverse has not been demonstrated.

Connective tissue growth factor

CTGF is a potent profibrotic protein, originally identified on the surface and extracellular matrix of fibroblasts.[132] Initially found to be involved in fibrotic processes in skin, neoplasia and renal disease, it was subsequently determined to be upregulated in atherosclerotic lesions and in ischaemic and infarcted myocardium.[133] Human arterial tissue had undetectable levels of CTGF mRNA and protein, whereas atherosclerotic arteries showed high expression in vascular smooth muscle cells in association with fibrosis.[134] Left atrial tissue of patients with AF shows significant upregulation of CTGF when compared to those in sinus rhythm. Using a mouse model, this was shown to be regulated by AngII though the Rac-1 signalling pathway.[135] TGF-β, AngII and ET-1 have all been shown to induce CTGF activity. CTGF alone is only weakly pro-fibrotic; however in combination with TGF-β, it has significant profibrotic effects and may be required for TGF-β to maximally induce fibrosis.[133] Ruperez at al. studied CTGF expression in rat aortic tissue. Normal animals demonstrated undetectable CTGF. Following AngII infusion, CTGF production was induced after 3 days without evidence of ECM overproduction. At 7 days, CTGF overexpression continued, along with the accumulation of several proteins associated with the ECM. This early induction of CTGF induction is suggestive a central role in AngII-related fibrosis.[134] Elevated CTGF mRNA was found in the ventricular tissue of rats with experimentally induced myocardial infarction. This mRNA induction was prevented by angiotensin receptor blockade.[136] ET-1 upregulates CTGF mRNA expression, promoter activity, and protein production in rat vascular smooth muscle cells.[137] and ET-1 gene silencing inhibits CTGF cardiac expression in diabetic rats.[125] ET-1 infusion induces

CTGF expression in mouse atrial tissue and CTGF silencing reduces expression of some ECM components, suggestive of a downstream action.[138]

Platelet derived growth factor

PDGF is a growth factor involved in cell proliferation, smooth muscle cell growth and angiogenesis and exists in multiple forms, AA, BB, AB, CC and DD, and has 2 receptors, α and β.[133] It is a required element for fibroblast division and has long been demonstrated to play a central role in wound healing.[139] It is predominantly synthesised, stored and released by activated platelets, but is also produced by a wide variety of cells, including those of the endothelium. A transgenic mouse model with overexpression of PDGF developed cardiac fibrosis, hypertrophy and impairment.[140] There is also heightened expression of PDGF and its receptor in atrial tissue, when compared to ventricular, and this difference is exaggerated in heart failure.[141] Exposure of myocytes to myofibroblasts induces shortening of action potentials and reduction in calcium currents and similar effects are seen with direct exposure to PDGF-AB. Pre-treatment with a PDGF-neutralising antibody blocked these effects in both scenarios, strongly suggesting electromechanical remodelling of myocytes can be caused by myofibroblast exposure, whose action may be due to PDGF-AB release.[142] Another study examined pressure overloaded mice and demonstrated upregulation of PDGF-A in cardiac mast cells, with associated atrial fibrosis. Blockade of PDGF receptors attenuated the fibrotic response and reduced AF inducibility.[143]

PDGF-BB was potentiated by ET-1 in aortic smooth muscle cells.[144] This was further demonstrated by Mayyas et al, using microarray analysis of human left atrial appendage tissue to demonstrate that endothelin-1 regulates PDGF expression.[96]

TGF-β-induced growth responses of smooth muscle cells are associated with enhanced production of PDGF-AA but are only partially inhibited by PDGF-AA neutralisation.[145] Angiotensin II stimulates PDGF-B expression in vascular smooth muscle through mechanisms involving Ras-ERK and JNK signalling pathways.[146]

Inflammatory molecules

Inflammation is increasingly recognised as one of the pathological processes in the pathogenesis of AF. The pro-fibrotic factors discussed above are also pro-inflammatory, but other molecules are also involved. Inflammatory cardiac conditions such as pericarditis and myocarditis and inflammatory states such as during the post-operative period, have an association with AF, with the incidence of arrhythmia coinciding with peak C-reactive protein levels.[147] Atrial septal biopsies taken from patients with 'lone' AF revealed two-thirds actually had features of occult myocarditis.[75] Inflammatory cells have been demonstrated in animals with atrial fibrosis.[148]

C-reactive protein

Elevation of C reactive protein in human subjects is associated with both the current and future presence of AF,[149] persistence of AF[150] and is predictive of both unsuccessful cardioversion[151] and failure of catheter ablation.[152] However, there is little data suggestive that CRP is more than just a marker of inflammation rather than an active participant.

Interleukins

IL-6 is thought to be member of the interleukin family most associated with AF and has an active role in its pathogenesis. It is a pleiotropic cytokine produced by T-cells,

macrophages and endothelial cells. Patients with chronic AF have higher IL-6 serum levels that controls in sinus rhythm,[153] as do those with longer duration of AF and larger atrial diameter.[154] Raised IL-6 levels also predict a lower success rate following AF ablation.[155] Elevation of IL-6 was also predictive of the development of AF in over 3000 patients with chronic kidney disease.[156] It is not clear, however if these associations are due to the pro-inflammatory effects of the underlying risk factors associated with AF rather than to the disease itself.[157] Several other interleukins are potentially involved in the pathogenesis of AF. Elevated IL-2, IL-8 and IL-10 (but not IL-6) following cardiac surgery were predictive of post-operative AF.[158, 159]

Oxidative stress

Oxidative stress, in the form of overproduction of reactive oxygen species (ROS), is a likely to play a significant role in both cardiac fibrosis, thrombosis and inflammatory pathways.[160] Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) is a potential enzymic source of ROS and can be induced by rapid atrial pacing and atrial stretch.[161] Angiotensin II also increases NADPH oxidase activity, but importantly, the hypertrophic and fibrotic effects of AngII were suppressed in NADPH-knockout animals.[162] ROS have been shown to damage cardiac myofibrils[163] and cause calcium overload. Analysis of human atrial appendages removed during mitral valve surgery demonstrated increased NADPH activity in patients with a history of AF than in controls.[164]

Gap junction proteins

Gap junction proteins, also known as connexins, connect the cytoplasm of adjoining cells using two hemichannels, each comprised of six connexin proteins.[165] They allow

the passage of both small molecules, typically less than 1KDa in size, and play a critical role in regulation of electrical conduction. Several connexins have been found in cardiac tissue. Cx43 is the most widespread and abundant of these proteins, followed by Cx40, which is generally localised to conduction tissue. Gap junctions permit the transmission of action potentials from one cell to another and, in atrial tissue, are typically found at the end of cardiomyocytes, alongside desmosomes and actin filaments. The arrangement of these connections is unique to atrial tissue and may partially explain the anisotropy commonly found in the atrium. Disruption of connexin function is associated with reduced intercellular conduction and subsequently with AF.[165] Prolonged AF induced by rapid atrial pacing is associated with reduction in connexin expression, with complete reversal following four months of sinus rhythm.[166] AF in surgical patients is also associated reduction of Cx43, as well as with lateralisation of both Cx40 and Cx43 within cells, with tissue from patients in SR confined to the intercalated discs.[74]

Several relevant molecules have been demonstrated to have a direct effect on cardiac connexin expression. Notably, ventricular myocytes exposed to endothelin-1 were found to have both conduction slowing and reduced Cx43 expression on immunofluorescence.[100] In contrast, angiotensin II exposure increased ventricular Cx43 expression.[167] Cx43 has also recently been demonstrated to contribute to TGF-β induced differentiation of cardiac fibroblasts to myofibroblasts.[168] Therapeutic interventions targeting connexins show promising results. Bikou et al. used gene therapy to restore AF-induced Cx43 under-expression and demonstrated reduced progression to persistent AF.[169]

Ion channels

Calcium channels play a major role in the pathophysiology of AF. Early studies of the rapid atrial pacing model demonstrated calcium overload in response to the rapid atrial rates; atrial remodelling was prevented by the calcium channel blocker verapamil and worsened by hypercalcaemia.[57] Patch clamp studies have shown this is associated with reduction in density of L-type calcium currents. The expected reduction in action potential duration was prevented by calcium channel blockade, strongly implicating calcium in the electrical remodelling process.[170] This reduction in L-type calcium currents was later seen in the atrial cells of patients with AF.[171] Transcriptional downregulation of the $Ca_v1.2$ α-subunit underlies this current abnormality,[172] acting via the calmodulin–calcineurin–NFAT system.[173]

Potassium channels, particularly inward rectifiers are responsible for the negative resting membrane potential of the cardiomyocyte. In AF, this becomes more negative, mainly due to increased expression of the channel subunit Kir2.1 causing enlargement of the potassium channel. This results in action potential shortening and may be involved in AF induction, particularly in the setting of vagally mediated or ischaemia-related AF. Voltage gated potassium channels have also been implicated but study findings have been inconsistent.[22] The rapid atrial pacing model is also associated with downregulation of the INa sodium channel. This may play a role in the conduction slowing associated with atrial remodelling but has not been well characterised.[174, 175]

Reverse remodelling

There is some evidence that reversal of atrial substrate can occur if the stimulus for substrate formation is no longer present. Echocardiographic improvement of atrial function and size was seen in a cohort of patients undergoing permanent pacemaker implantation when asynchronous ventricular only pacing was reprogrammed to synchronous dual chamber pacing.[176] Another study examined the right atrium of patients before and after atrial septal defect closure. Post closure right atrial volumes showed significant reduction. There was partial resolution of the refractory period abnormalities but persistence of conduction delay in the form of widely split double potentials at the crista terminalis.[177] John et al. studied patients with mitral stenosis, before and after mitral commissurotomy. Immediately following the procedure, there was a reduction in atrial volume, improvement in conduction velocity and atrial voltage, without changes in refractoriness. A subset of patients were restudied after at least 6 months and demonstrated further reductions in atrial volumes and improvements in conduction and bipolar voltage.[178] An animal study using a ventricular pacing heart failure model found that 5 weeks after termination of pacing and recovery of ventricular function, atrial function improved and propensity to AF was reduced, however atrial conduction remained abnormal and there was no reduction in the degree of fibrosis on histological examination.[179] It is possible that the degree of reversibility is dependant on the severity and duration of the stimulus. Additionally, the length of time required for recovery is unknown.

1.3 Obesity – pathophysiology in relation to AF

It is well recognised that obesity causes conditions associated with the development of AF, in particular, hypertension, diabetes and obstructive sleep apnoea. Several of these conditions often co-exist and elucidating the contribution of each individual condition can be challenging. As well as these indirect associations, the role of fat itself is becoming increasingly clear. Adipose tissue has been demonstrated to play and active role in endocrine and inflammatory processes. In particular, epicardial and pericardial fat may have directly deleterious effects on atrial structure and function.

1.3.1 Associated conditions

Hypertension

Elevated blood pressure is one of the leading causes of AF. In the Framingham cohort, it increased the risk of AF by 40 to 50%.[9] The multitude of mechanisms by which obesity causes hypertension remain incompletely understood. Contributing factors include sympathetic nervous system activation, impairment of natriuresis, upregulation of the renin-angiotensin-aldosterone axis, neurohormonal imbalance and endothelial dysfunction.[180]

Animal models of hypertension have demonstrated atrial dilatation, conduction slowing, predisposition to AF and increased atrial fibrosis in animals treated with unilateral renal artery clipping. Notably, these abnormalities developed in the short-term, and continued to develop over a 4-month period.[59, 181] Similar findings have been demonstrated in alternative hypertensive animal models [60] and endocardially in human subjects.[64]

Diabetes mellitus

The risk of developing AF in diabetes is similar to that of hypertension.[9] Almost 90% of individuals with type 2 diabetes are obese. Insulin resistance is thought to be the underlying pathophysiological mechanism of hyperglycaemia and the subsequent sequelae, but there is also evidence that the major insulin-producing cells, pancreatic B cells, are also dysfunctional. The mechanisms by which obesity causes diabetes are not fully understood, but an inflammatory mediated process in a genetically susceptible individual is the likely cause.[182] There is limited data on the effect of diabetes on cardiac electrophysiology, but one study used a rat model of induced diabetes resulting in conduction slowing and increased ventricular fibrillation inducibility.[183] This was not associated with tissue fibrosis or changes in sodium or potassium currents. No published studies have examined the effect on atrial tissue.

Obstructive sleep apnoea

Obesity is a prominent cause of sleep apnoea, with up to 45% of obese subjects being affected by the condition. It is a clear cause of hypertension and has been heavily implicated in the development of other cardiac diseases, its presence conferring a two to four fold risk of incident AF.[184 185] Fat deposition around the upper airway results in a reduced luminal diameter with subsequent increased collapsibility and increased thoracic fat deposition reduces residual capacity.[186] This causes intermittent hypoxia, with saturations as low as 60% in severe cases, which causes accelerated atherosclerosis[187] and stimulates production of cytokines, in addition to those already produced by adipocytes, with the degree of elevation correlating with disease severity.[188] In addition to elevating system blood pressure, sleep apnoea also

causes pulmonary hypertension, which is reversible through CPAP therapy. As in patients with primary pulmonary hypertension, increased endothelin-1 levels have been found in patients with OSA and this has been postulated as a direct cause of systemic hypertension.[189] Animal studies have shown application of an endothelin receptor antagonist negated the effect of simulated sleep apnoea on cerebral vasculature and oxidative stress.[190] In one study, hypercapnia but not hypoxia, was associated with adverse atrial electrophysiology and increased AF inducibility was seen following normalization of CO_2.[191] In another, recurrent apnoeic episodes in normal weight rats increased inducibility of AF and caused atrial fibrosis and Cx43 downregulation.[192] Multiple studies have suggested severity of sleep apnoea correlates strongly with poor outcomes in patients with AF; treatment of sleep apnoea with CPAP is beneficial, for example in patients following AF ablation, but this requires confirmation in a randomised controlled trial.[193]

1.3.2 Anatomical

Obesity was found to be the strongest predictor of left atrial (LA) size in a large cohort of hypertensive patients[54] as well as in a randomly selected cohort of over 1000 subjects from the general population.[53] Detailed echocardiography examining LA strain rates have also shown a reduction in LA function in obese children.[194] Ventricular effects are also seen with obesity. Although overt systolic dysfunction is uncommon in the absence of concomitant cardiac disease, abnormalities of diastolic function and LV strain have been increasingly recognised.[195, 196]

Pericardial fat

There is increasing evidence that fat surrounding the heart has a direct effect on cardiac tissue. The Framingham data set initially provided a link between pericardial fat and AF. Computed tomography-measured pericardial fat volume, but not intrathoracic or abdominal fat, was associated with the prevalence of AF in a cohort of 3217 patients, 54 of which had AF. This association persisted following adjustment for BMI.[197] Batal and colleagues found that increasing thickness of fat between the left atrium and oesophagus was associated with increasing severity of AF. The median thickness of this fat depot was 0.34cm, 0.39cm and 0.56cm in patients with no AF, paroxysmal and persistent AF respectively. This difference persisted even after multivariate adjustment for age, BMI and LA area.[198] Magnetic resonance imaging is also useful in measuring pericardial fat to predict severity of AF, as demonstrated by Wong et al., who studied 130 patients, 110 of whom had known AF. Both periatrial and periventricular fat were associated with AF severity and were also predictive of outcome following AF ablation. The predictive nature of epicardial fat again persisted following adjustment for other risk factors, importantly including BMI.[199] Venteclef et al. elegantly demonstrated the direct secretory action of epicardial adipose tissue by comparing the effect of human epicardial and subcutaneous on the development of atrial fibrosis in a rat heart. Atrial fibrosis was increased two-fold in tissue incubated with epicardial fat when compared to subcutaneous. Additional analyses determined epicardial fat secreted more MMP1, 8 and 9, Activin A (a member of the TGF-β superfamily) and VEGF. TGF-β1, however, was unchanged between fat types.[200] This increased secretion of cytokines may be due to relative hypoxia of the tissue in the obese state.[201]

1.3.3 Electrophysiological

A study by Abed et al. examined the atrial characteristics in an obese ovine model. With increasing weight gain, atrial conduction slowed and became more heterogeneous, with no effect on refractory periods. AF was more easily induced and has a longer duration in obese animals when compared with control.[23] These findings were extended in a more chronic ovine model of obesity. In this study, Mahajan et al. demonstrated areas of low voltage endocardially, adjacent to regions rich in pericardial fat. In addition, the authors observed the novel feature of pericardial fat infiltration of the adjacent atrial myocardium, potentially a unique component of the substrate for AF in obesity.[24]

In patients undergoing AF ablation, obesity was associated with shortening of ERP in the atrium and pulmonary veins. Although LA conduction velocities were similar in obese and non-obese groups, those with obesity had slower longitudinal PV conduction.[202]

1.3.4 Molecular

Obesity is associated with an inflammatory response, albeit one that is 10 to 100-fold lower than those observed in people with acute inflammatory or immune diseases, as evidenced by elevation in a wide variety of inflammatory markers. Adipose tissue was previously thought to be a bystander in obese individuals, but has more recently been shown to be actively involved in the cardiovascular sequelae of obesity through the secretion of numerous cytokines and hormone-like factors, including leptin, TNF-α, IL-6 and resistin.[203] These pro-inflammatory molecules have a direct effect on endothelial function and are associated with systemic inflammation and oxidative

stress, leading to arterial stiffness[204], atherosclerosis[205] and hypertension.[206] Increased levels of these substances are directly associated with an increase in cardiovascular events.[207, 208]

Obesity also has an effect on factors directly implicated in AF. The study by Abed et al. mentioned in the previous section also revealed upregulation of TGF-β, ET, PDGF and CTGF in atrial tissue correlated with weight and the associated structural and electrophysiological abnormalities.[23]

Analysis of fat in obese rats demonstrated in increased expression of TGF-β[209] and TGF-β expression in human adipose tissue correlates strongly with BMI.[210] It is also released directly from human adipose tissue and this is enhanced by insulin exposure.[211] TGF-β is also involved in regulation of insulin transcription.[212]

Analysis of plasma samples from obese and non-obese subjects showed elevation of ET-1 in obesity, with or without associated hypertension. Following a period of caloric restriction, weight loss was associated with a reduction in ET-1, even in patients without hypertension at baseline.[213] Individuals with obesity have enhanced vasoconstriction when exposed to endothelin.[214] Diabetes-associated cardiac fibrosis was suppressed in endothelin knock-out mice.[125]

Serum AngII is associated with BMI and markers of insulin resistance, with significant reduction with weight loss.[215] Obese rats had an enhanced hypertensive response to angiotensin II, with aldosterone acting as an intermediary hormone.[216] Treatment of well, obese individuals with an aldosterone antagonist improved echocardiographic markers of subtle LV dysfunction and reduced serological markers of fibrosis.[217]

CTGF was upregulated in adipose tissue of obese mice[218] and a recent study by Pellegrinelli et al. found upregulation of CTGF in adipocytes cultured with

decellularised material of adipose tissue and, with correlation of CTGF and the degree of peri-adipocyte fibrosis.[219] Obese rats have higher PDGF plasma levels and mRNA levels in adipose tissue, with macrophages being the dominant source of PDGF expression.[220]

1.3.5 Weight loss

Several animal studies have demonstrated beneficial cardiac changes in response to weight loss, including reduction in oxidative stress and inflammation,[221] and improvements in diastolic dysfunction.[222] Weight loss has been demonstrated to impact the outcome of patients diagnosed with AF. Abed et al. randomised 150 patients with AF to receive a weight management program or standard care. Over 15 months, those receiving weight reduction advice were significantly less symptomatic, and had a lower AF burden, with both fewer episodes and lower cumulative duration.[223] Pathak et al. assessed the effect of aggressive risk factor management in 61 patients undergoing AF ablation, compared with 88 control subjects over a follow up period of over 40 months.[224] The intervention group lost a mean of 13.2 ± 5.4kg, and this was associated with significantly improved outcomes following AF ablation. At the end of the follow up period 32.9% and 87% of patients in the intervention arm were arrhythmia free following single and multiple procedures respectively. In contrast, only 9.7% and 18% of those undergoing standard care were free from AF. A larger cohort with 5-year follow up data confirmed that the improvements in both AF symptomatology and objective measures of AF burden were sustained in the long term.

1.3.6 Weight fluctuation

Fluctuating weight has long been thought to be detrimental to cardiac well-being, even when compared to more obese patients with stable weight. This has been demonstrated in several retrospective population studies. Several studies have showed very significant increases in cardiac mortality in patients with a fluctuating weight, when compared with both lean and obese, stable weight patients,[225, 226] whereas others have not found a correlation.[227] Another study demonstrated significantly lower HDL levels in patients who reported the greatest weight cycling.[228] The LEGACY study analysed 355 patients with AF and obesity. Patients with weight fluctuation had improved outcomes than those with stable or increasing weight, but significantly worse outcomes than those with linear weight loss. Subgroup analysis of the 179 patients with fluctuating weight showed wide fluctuation was associated with adverse cardiac remodelling and increased risk of AF recurrence when compared with those with minimal fluctuation.[229] The pathophysiology behind these findings has not been determined and mechanistic links have been elusive, prominently due to the inability to perform randomised controlled human trials and the heterogeneity of the groups being studied.

1.4 Upstream therapy for AF prevention

Management of AF historically used antiarrhythmic therapy to alter atrial electrophysiology. In the event of failure of these agents, a more interventional approach, for example RF ablation of AF, is subsequently considered. With the discovery of atrial remodelling acting as a substrate for AF, recent focus has shifted to reversal of this substrate to reduce AF burden, treating the arrhythmia ‘upstream’.

1.4.1 Renin-angiotensin-aldosterone system blockade

RAAS blockade is the most widely investigated upstream therapy, with a number of positive human trials. Madrid et al. studied 154 patients undergoing electrical cardioversion of persistent AF. All patients were treated with the antiarrhythmic amiodarone and 79 were also given the Angiotensin II receptor blocker (ARB) irbesartan. After two months, 85% of patients given irbesartan maintained sinus rhythm, compared with 63% in patients who did not. After a median follow up period of 254 days, this remained significantly higher (79% vs 56%, p=0.007).[230] Similar results were found in a trial using an angiotensin converting enzyme inhibitor (ACEi).[231] Treatment with ACEi also reduces the incidence of new AF in several cardiac conditions. Wachtell et al. examined over 9000 patients with hypertension and left ventricular hypertrophy as part of the randomised controlled LIFE trial, comparing antihypertensive treatment with the ARB losartan and the beta-blocker atenolol. New onset AF occurred in 6.8 per 1,000 years of follow up in patients treated with losartan vs 10.1 per 1,000 in those taking atenolol (p<0.001). In patients already diagnosed with AF, losartan was also protective when examining the composite end point of cardiovascular events, stroke, and hospitalisation for heart failure.[232] ACEi also prevents new onset AF following myocardial infarction[233] and in patients with heart failure.[234, 235] In a recent randomised controlled trial of 120 patients undergoing AF ablation, treatment with an ARB improved outcomes in a dose dependant manner.[236] However, several large trials comparing RAAS blockade with alternative anti-hypertensives have not revealed any significant benefit with ACEi or ARBs, either on incident AF or other cardiovascular conditions.[237] The recent ANTIPAF study was the first trial to prospectively examine AF control in patients with known paroxysmal AF

without structural heart disease. After 1 year of follow-up, ARB treatment failed to demonstrate any benefit on AF burden when compared with placebo.[238]

An alternative target of the RAAS system, aldosterone receptor blockade, is used commonly for patients with LV dysfunction, after a mortality benefit was noted in the RALES trial.[239] Treatment with eplerenone improved maintenance of sinus rhythm following cardioversion of long-standing persistent AF.[240] Animal studies suggest reduction of atrial remodelling and AF inducibility in a hypertensive rat model treated with epleronone[241] and canine model treated with spironolactone.[242]

1.4.2 Endothelin-receptor blockade

Following the discovery of increased levels of ET-1 in the serum[243] and upregulation in the lung tissue of patients with pulmonary hypertension[244], blockade of these receptors has been extensively studied, in particular bosentan, an orally active non-peptide antagonist of both endothelin receptor subtypes, with a predominance for ET-A. Early animal studies showed that endothelin-receptor antagonism decreased inflammation, reduced deterioration of pulmonary vessel permeability and prevented the development of fibrosis in subjects with pulmonary inflammation.[245, 246] It was shown to reduce pulmonary arterial pressure and right ventricular hypertrophy with no change in systemic vascular resistance, prior to human trials.[247] Endothelin receptor blockade is now a widely accepted treatment for both mild and severe forms of the disease, following human trials demonstrating clear clinical benefit, namely improved exercise tolerance, haemodynamics and prognosis.[248-250] Macitentan, a newer dual receptor antagonist, has recently completed a phase 3 trial, where it demonstrated a significant effect on mortality, without the hepatotoxicity and fluid retention seen in previous trials of endothelin receptor antagonists.[251]

Studies have also investigated the effect on endothelin-receptor antagonism in left ventricular dysfunction following the discovery that bosentan treatment significantly reduced left ventricular collagen density, improved haemodynamics and offered a survival benefit in a heart failure model in rats[252], and reduced ventricular fibrosis due to hypertension and diabetes.[98, 253] Initial human studies showed an improvement in haemodynamics, however longer term trials did not support their use, predominantly due to the side effect of fluid retention. Recently, a human trial showed ERA treatment slowed the progression of coronary atherosclerosis.[254] Seccia et al. demonstrated that in a hypertensive rat model, blockade of both ET-A and ET-B receptors resulted in reduced cardiac fibrosis, but pure ET-A receptor antagonism did not,[255] evidence that dual receptor blockade may be required for prevention of cardiac remodelling.

1.4.3 Transforming growth factor receptor blockade

TGF-β blockade has been studied in several pre-clinical cardiac models. Studies investigating its role in myocardial infarction have demonstrated potentially conflicting results. Early TGF-β blockade following infarction exacerbated cardiac failure, prevented myocardial remodelling and increased mortality.[256, 257] This was associated with enhanced neutrophil infiltration, increased expression of profibrotic factors, in particular, TNF-α, IL-6, IL-1h, MCP-1 and MMP. In contrast, inhibition late after infarction had a beneficial effect on cardiac remodelling, with reduced collagen deposition.[258] Pressure overloaded rats developed cardiac hypertrophy, fibrosis and diastolic dysfunction, all of which were reduced by blockade of TGF-β.[259] Investigation of atrial effects was possible through the use of a rapid atrial pacing model in dogs. Half of the group were treated with the TGF-β antagonist tranilast. Animals who

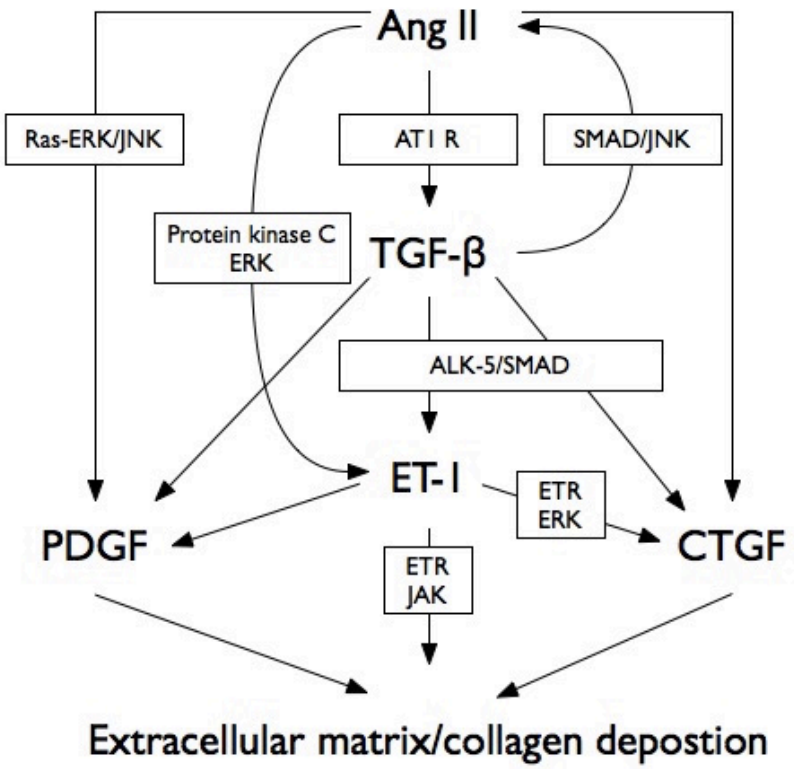
received treatment developed significantly less ERP reduction, conduction slowing, atrial fibrosis, calcium channel reduction and inducibility of AF.[260]

Human studies of tranilast have unfortunately been less successful. Despite two initial studies reporting a reduction in coronary artery restenosis,[261, 262] a large multicentre trial showed the treatment had no effect on any coronary artery disease parameter.[263] Although it is used in Asia as a treatment for asthma and to promote wound healing, minimal data exists to support this.

1.5 Thesis aims

This thesis aims to examine the effect of weight fluctuation on atrial electrophysiology, in comparison with obesity and normal weight. It then investigates the effect of anti-fibrotic treatment on the atrial substrate associated with obesity, particularly in regards to the electrophysiological properties of the atrium. We hope to also grant some insight into the molecular actions of these interventions. Chapter 3 investigates the effect of endothelin receptor blockade and chapter 4 examines the effect of blockade of transforming growth factor.

Figure 1. Profibrotic pathways in atrial fibrosis



Chapter 2 – Weight cycling is associated with progressive atrial remodelling

2.1 Introduction

Obesity has a clear relationship with the development of atrial fibrillation and is associated with increased atrial fibrosis and a pro-inflammatory state.[14,23] Several prospective studies have identified that significant fluctuations in weight, or 'weight cycling' confers an increased risk of developing coronary heart disease and increases all-cause and cardiovascular mortality.[226, 264] Even when weight fluctuation occurs in early life, the effects can be seen over the following 25 years.[265] Fluctuation in weight also causes activation of the sympathetic nervous system with resultant increased heart rate and blood pressure variability, as well as fluctuation in renal and pancreatic function.[266] Weight cycling has a high prevalence in the general population, occurring in 18% of men and 27% of women.[267] This common clinical scenario has recently been found to be associated with increased arrhythmia burden in patients with known AF, in a 'dose-dependant' fashion.[229] The pathophysiology behind these findings is not clear; data is inevitably retrospective and studies often consist of heterogeneous populations and lack standard definitions for weight cycling.

Using an obese ovine model, we sought to examine the effect on atrial electrophysiology in animals with induced weight fluctuation in comparison with both obese and lean controls.

2.2 Methods

Twenty-four ovine (Merino cross) subjects were studied according to National Health and Medical Research Council of Australia guidelines on animal research. Animal research committees of the University of Adelaide and South Australian Health and Medical Research Institute granted approval for the study.

2.2.1 Study Protocol

Animals were randomised into 3 groups: obese, weight fluctuation and lean control. All animals underwent sequential electrophysiological studies as shown in figure 1. The weight fluctuation group had 2 'cycles' of weight gain followed by weight loss. Investigations were performed when animals reached their baseline weight after the first cycle (cycle 1) at 68 weeks. Repeat investigations and terminal studies were performed once the animals again reached their baseline weight (cycle 2) after a further 74 weeks. Eight animals were commenced on high fat diet at study commencement and this was continued for the duration of the study. Seven lean animals were used as controls and had their weight maintained at baseline. These two groups were studied at the same time-points as the weight fluctuation animals to allow comparison. Percutaneous endocardial electrophysiological and electroanatomical evaluation was performed on all animals. Additionally at terminal study, high-density mapping of the epicardial surface was performed using a 90 pole plaque, to allow more detailed electrophysiological study.

2.2.2 Weight control

Obesity was induced using a diet of high fat pellets consisting of wheat, barley and canola seed. Excess voluntary intake was of grass alfalfa silage and hay. As previously

described, the pellets were gradually introduced at 8% excess basal energy requirements and rationed to 70% of the total dry matter intake.

Weight loss was induced by gradual removal of the high fat pellets and the introduction of high quality cereal hay at 75% of usual intake.

Weights were recorded weekly and reported weights were those taken prior to electrophysiological procedures in a shorn and fasted state. Haematological and biochemical indices were monitored using blood collected prior to each invasive procedure.

Weight fluctuation

Animals in the weight fluctuation group were commenced on the high fat diet and this was continued until their weights were 20% higher than baseline. At this stage, the weight loss diet was introduced to reduce the weight back to their baseline weights. Once this was achieved, this constituted one cycle and electrophysiological testing was performed.

2.2.3 Animal anaesthesia

General anaesthesia was used for all procedures. Diazepam 0.4mg/kg was given prior to induction with ketamine 5mg/kg. Isoflurane 2.5% with 4 litres/minute of oxygen was used for maintenance. Non-invasive blood pressure, heart rate, pulse oximetry and end-tidal CO2 were continuously monitored.

2.2.4 Endocardial electrophysiological study

Percutaneous internal jugular and femoral venous access was obtained. Via the internal jugular route, a 10-pole coronary sinus (CS) catheter (2–5–2 mm inter-electrode spacing) was positioned with the proximal bipole at the CS ostium. To allow

left atrial mapping, transseptal puncture was performed using an SL0 sheath and BRK needle. Surface ECG and bipolar endocardial electrograms were monitored continuously and stored on a digital amplifier/recorder system (LabSystem Pro, Bard Electrophysiology, Lowell, MA). Electrogram filters were applied from 30 to 500 Hz.

Electroanatomical mapping

Electroanatomical maps were created in sinus rhythm using the Carto XP system (Biosense Webster, Diamond Bar, CA) as previously described. Right and left atrial maps were recorded using a 3.5-mm tip catheter ablation catheter (Navistar, Biosense Webster, Diamond Bar, CA) with a minimum of 80 points in each chamber. Endocardial contact was confirmed with a combination of electrogram stability, fluoroscopy and the Carto point stability criteria (≤6mm stability in space, ≤5ms in local activation time). Location, voltage and activation timing were recorded at each point. For analysis the atria was divided as follows: Septal right atrium (RA); High lateral RA; Low lateral RA; Posterior left atrium (LA); Lateral LA; and Inferior LA. The following parameters were determined:

1. Regional Conduction velocity
    - The direction of conduction was determined from examination of isochronal maps. 3-5 pairs of points were taken in the direction of conduction, with velocity being calculated using the distance and timing as reported by the mapping system.
2. Signal fractionation
    - This was defined as a signal greater than 50ms duration with four or more deflections.
3. Bipolar voltage

- Low voltage was defined as an area with three contiguous points with a bipolar voltage of <0.5mV.
- Scar was an area with three contiguous points <0.05mV.

4. Atrial volume

Effective refractory periods

Effective refractory period (ERP) was measured at three times the tissue capture threshold at a pulse width of 2ms, using a Micropace EPS 320 stimulator (Micropace, Canterbury, Australia). This was performed from the following 8 sites: Septal RA; High lateral RA; Low lateral RA; Posterior LA; Lateral LA; Inferior LA; Proximal coronary sinus (CS); and Distal CS. Eight S1 stimuli were delivered with S1 cycle lengths of 400 and 200ms, with incremental S2 impulses (10ms increments), commencing at a coupling interval of 100ms. The ERP was the longest S1-S2 interval without atrial capture. The mean of two attempts was recorded. An additional attempt was made if the difference between attempts was greater than 10ms.

AF induction

Inducibility of AF was determined using a burst pacing protocol from the left atrial appendage, performed at the terminal study. Twenty impulses were delivered at the lowest cycle length with 1:1 atrial capture. An episode was defined as irregular atrial activity lasting greater than or equal to 2 seconds. This protocol was repeated five times and the number of episodes and total duration were recorded. Sustained AF was defined as an episode lasting >10 minutes. In the event of sustained AF, no further testing was performed.

Haemodynamic recordings

Non-invasive systolic blood pressure and invasive mean right atrial and systolic right ventricular pressures via the SR0 sheath were recorded at the start of each procedure. Mean left atrial pressure was recorded immediately following transseptal puncture.

2.2.5 Terminal epicardial electrophysiological study

Left lateral thoracotomy was performed to access the left atrium. A custom made 90-pole plaque was placed on the left atrial appendage and connected to the computer-based digital amplifier/recorder system (LabSystem Pro, Bard Electrophysiology, Lowell, MA). Pacing was performed from each of the four corners of the plaque at 400ms. Epicardial electrograms were recorded and the following determined:

1. Effective refractory periods were measured using the same protocol as the endocardial study.
2. Conduction velocity was determined using activation maps analysed by custom software, as previously described.[59] The peak of the largest amplitude deflection on each bipolar electrogram was automatically annotated and manually verified. Local conduction velocity was calculated for each point from triangulated local vectors, allowing subsequent calculation of mean velocity for each map.
3. Conduction heterogeneity was calculated using established phase mapping techniques integrated into the software, as previously described.[59] Absolute conduction phase delay was calculated by subtracting the 5th from the 95th

percentile of the phase difference distribution (P_{5-95}). Conduction heterogeneity index was derived from dividing P_{5-95} by the median (P_{50}).

2.2.6 Statistical Analysis

Normally distributed data was expressed as mean ± standard deviation and compared using unpaired t-test. Analysis of variance was used to detect differences between groups over time. Due to the dependence in observations from the same animal, conduction velocity and ERP across regions were analysed using a mixed effects model ANOVA. Sheep ID was used as a random effect and combinations of group, timepoint and region were used as fixed effects. A maximum of two fixed effects were entered into the statistical model at one time, with testing of both main effects and their interaction. If a significant interaction was present, post-hoc testing was performed. AF duration was non-parametric; therefore log-transformation was performed prior to t-test comparison, with values expressed as median and interquartile range. P-values of less than 0.05 were considered statistically significant. Analyses were performed using SPSS version 23 (SPSS Inc. Chicago, IL).

2.3 Results

2.3.1 Weight, structural and haemodynamic parameters

The changes in weight, body fat, haemodynamics and cardiac structure are shown in table 1a and 1b, following the first (cycle 1) and second (cycle 2) cycles of weight change. The fluctuating weight group returned to baseline weight prior to each study. During cycle 1, the mean maximum weight of this group was 89±4kg, and during cycle 2, the mean weight peaked at 99±6kg.

After cycle 1, the obese group was a significantly higher weight than both lean and fluctuating weight groups. Right and left atrial pressures were significantly higher in this group, and there was a trend towards larger atrial volumes. The fluctuating weight group had numerically higher left atrial pressure and volume than the lean group, but this did not reach statistical significance.

After cycle 2, the obese group again had significantly higher weights than fluctuating and lean animals. When compared with lean controls, obese animals had significantly elevated atrial and right ventricular pressures, larger left atrial volume and a strong trend towards increased systolic blood pressure. At this timepoint, the fluctuating weight group had developed significantly increased left atrial pressure and volume when compared to the lean control group. When compared with the obese group, the fluctuating weight group had a similar left atrial volume, whereas the LA pressure was significantly lower.

2.3.2 Electrophysiological parameters

Conduction velocity - endocardial

Conduction slowing was seen in the obese group and was relatively preserved over time in lean animals, with a significant difference at mid point (1.01±0.06 vs 1.26±0.05m/s, $p<0.001$) and end point study (0.96±0.06 vs 1.25±0.05m/s, $p<0.001$). Following the first cycle, the weight fluctuation group demonstrated no significant difference in overall conduction when compared with lean controls (1.21±0.06 vs 1.26±0.05m/s, p=NS) as shown in figure 2. On examination of individual regions, however, there was significant reduction in conduction velocity at the inferior left

atrial region of the fluctuating group when compared with lean animals (0.99±0.14 vs 1.25±0.13, p=0.001).

At the end of cycle 2, the weight fluctuation group developed significant overall conduction slowing (1.21±0.06 vs 1.07±0.06m/s, p<0.001), whereas the lean control group was unchanged (figure 2). However, the conduction slowing seen in the fluctuating weight group was not as severe as the obese group (1.07±0.06m/s vs 0.96±0.07m/s, p<0.001). Again, regional heterogeneity was present and was more widespread than after cycle 1. Conduction was significantly slower in the fluctuating weight group at the inferior LA (1.02±0.13 vs 1.21±0.13, p=0.001), posterior LA (0.96±0.13 vs 1.29±0.12, p<0.001) and lower lateral RA (0.95±0.15 vs 1.18±0.14, p<0.001) in comparison with lean controls. All of these areas of reduced conduction showed similar velocities to the obese group (p=NS for all 3 regions).

Conduction velocity – epicardial

At endpoint study, mean conduction velocity on the epicardial surface was significant lower in the obese group (0.92±0.05m/s) and in the weight fluctuation group (0.98±0.04m/s) when compared with the control group (1.06±0.05m/s, p<0.01). There were no significant differences between epicardial plaque sites.

Conduction heterogeneity was similar in the obese (1.67±0.33) and control groups (1.72±0.33), but significantly higher in the fluctuating weight group (2.26±0.30 p<0.01).

Effective refractory periods

Endocardial ERPs were not significantly different in any of the 3 groups following the first cycle (figure 4). However, following the cycle 2, there was a significant reduction

in ERP at a drivetrain of 400ms in the group with continuous weight gain, when compared with controls (153±18 vs 178±20ms, p=0.045), with a trend with a 200ms drivetrain (p=0.09). There were no significant regional differences between any groups at either drivetrain cycle length (region*group interaction=NS).

The weight fluctuation group showed no change in ERP over time and was comparable to the lean control group after cycle 1 and cycle 2.

Epicardial ERPs demonstrated similar ERPs between obese and lean groups as shown in figure 5. The weight fluctuation group had significantly higher ERPs than both obese and lean groups with a drivetrain of 200ms, with a trend towards higher ERPs at 400ms. There were no significant regional differences between any groups at either drivetrain (region*group interaction=NS).

AF inducibility

AF duration at the end of the study is shown in figure 6. The obese group had a significantly longer overall AF duration than the lean group (59s [IQR 66.7] vs 4s [IQR 7.0], p<0.001) and significantly more episodes (100% [IQR 30] vs 20% [IQR 40], p<0.001). The weight fluctuation group also had significantly longer AF duration than the lean controls (29s [IQR 77.5] vs 4s [IQR 7], p=0.04). There was also a trend to more episodes in the weight fluctuation group (60% [IQR 70] vs 20% [IQR 40], p=0.08). The obese group had a longer duration of AF than the weight fluctuation group, but this did not reach statistical significance. There was a trend towards the obese group having more AF episodes induced than the weight fluctuation group (100% [IQR 30] vs 60% [IQR 70], p=0.06).

2.4 Discussion

This study presents new information on the impact of weight fluctuation on the atrial substrate and propensity to AF. It provides important insights into the haemodynamic, structural and electrophysiological remodelling of the atria with fluctuations in weight. We have previously demonstrated progressive elevation of cardiac pressures, chamber dilatation, conduction slowing and increased AF inducibility with increasing obesity. This study confirms these findings and additionally demonstrates that weight cycling, when compared to lean controls, is associated with:

1) Reduction in endocardial atrial conduction velocity following the second cycle of weight gain followed by weight loss, despite return to normal weight. This was not seen following the first cycle of weight change.
2) Reduction in epicardial conduction velocity at the end of the study.
3) Increased left atrial pressure and volume. These values were numerically higher following the first cycle, but did not reach statistical significance until after the second cycle.
4) Increased AF duration.
5) No change in endocardial atrial refractory periods and higher epicardial refractory periods

Heterogeneity of conduction slowing

When compared with obese animals, the reduction in conduction velocity was significantly less in animals subjected to weight fluctuation. Notably, the reduction in conduction velocity seen in the weight fluctuation group was heterogeneous across

different atrial sites. This is in contrast to the homogeneous reduction seen with sustained weight gain. This may be due to the studies being performed at an earlier stage of the disease; however, it may be related to the distribution of epicardial fat. It is well recognised that fat contains an array of cytokines, with increased levels in epicardial fat when compared to fat from the abdomen.[268] Recent studies have found fat also directly secretes pro-inflammatory and pro-fibrotic molecules, which act on local structures. In particular, epicardial fat has been shown to directly induce atrial fibrosis.[200] With the fluctuations in weight seen in our study, fat turnover and hence secretory action may be increased, inducing more pronounced local effects. Following the first cycle, although conduction was unaffected overall, a pronounced reduction in conduction was seen in the inferior region of the left atrium. This is in close proximity to the atrio-ventricular groove and its large associated fat depot. Following the second cycle, the posterior left atrium and lower lateral right atrium were similarly affected. The posterior LA also has an epicardial fat depot and previous studies have even demonstrated that, on histological examination, this fat infiltrates the atrial tissue, which may be one of the mechanisms by which abnormalities in conduction and signal voltage occur.[24] The lower lateral RA region is in close proximity to the right atrio-ventricular groove and its associated fat depot. The association between volume of epicardial fat in each area and its secretory action requires further investigation.

Atrial dilatation and pressure elevation

Animals with weight fluctuation developed progressive left atrial dilatation and increased left atrial pressure when compared with lean animals, however this was not as severe as seen in the obese animals. It is unclear whether these abnormalities

persist with prolonged weight normalisation. Previous studies have demonstrated partial reversibility of these structural changes with weight loss, but to date, full reversibility has not been shown.[229]

Effective refractory period reduction

Another finding that has not previously been demonstrated in this model is the significant reduction in endocardial atrial ERP in the sustained obesity group. Previous obese ovine studies found no change in ERP in the intermediate or longer term.[23, 24] Mahajan et al studied animals that became obese over a period of 72 weeks; our study was more than double in duration. This may explain the difference in these findings, and would suggest that changes in conduction occur at an earlier stage, whereas refractory period changes occur later in the disease process. Reduction in ERP has been demonstrated in human subjects by Munger et al. Obese individuals had a reduced ERP across several right and left atrial sites, particularly in the pulmonary veins.[202] However, this study was performed in patients undergoing ablation for highly symptomatic, drug refractory AF, which may contribute to these changes in atrial electrophysiology and may limit extrapolation to the general obese population.

Atrial fibrillation inducibility

The duration of atrial fibrillation during the induction protocol was significantly longer in the weight fluctuation group when compared with lean controls. This is in keeping with investigation by Pathak and colleagues, who examined the outcome of 179 patients with AF and weight fluctuation as part of the LEGACY study.[229] Fluctuation in weight had a negative impact on outcome over a follow up period of 5 years. Patients

with less than 2% fluctuation had a dramatically improved prognosis when compared with those with greater than 5%, with AF free survival of 85% and 44% respectively.

Clinical implications

From a clinical perspective, this study may help in the counselling and management of patients embarking on a weight loss programme. Although some data suggests poorer outcomes following weight cycling, our study shows that, while weight cycling is not optimal with regards to atrial structure and function, persistent obesity is associated with even more pronounced abnormalities.

Limitations

The pathophysiology demonstrated in this ovine study, as with all animal models, may differ with human subjects. The distribution of epicardial fat may vary and different fibrotic pathways may predominate. The degree of weight fluctuation was higher in the second cycle, which may have accentuated the differences between time-points. Although the weight fluctuation group returned to normal weight after both cycles, the abnormal atrial electrophysiology seen after the second cycle may have normalised after a more prolonged period of weight normalisation. Further studies are warranted to determine the permanence of these abnormalities.

2.5 Conclusion

Weight fluctuation is associated with the development of abnormal atrial electrophysiology and propensity to AF, despite return to normal weight. However, these changes are not as severe as with progressive weight gain. These findings may assist in the management of patients undergoing weight loss.

Table 1a and b. Structural and haemodynamic characteristics of obese, fluctuating and lean groups at cycle 1 (top) and cycle 2 (bottom).

Cycle 1	Obese	Fluctuating	Lean	p value (obese vs fluctuating)	p value (fluctuating vs lean)	p value (obese vs lean)
Weight (kg)	90±3	74±6	72±5	<0.001	NS	<0.001
Systolic BP (mmHg)	81±10	77±9	83±7	NS	NS	NS
RV systolic pressure (mmHg)	16±2	14±4	13±3	NS	NS	NS
RA pressure (mmHg)	6.5±2.6	2.9±1.1	2.4±1.2	0.002	NS	0.001
LA pressure (mmHg)	7.5±2.1	5.5±1.2	4.0±1.5	0.1	NS	0.005
LA volume (ml)	55±8	51±11	43±5	NS	NS	0.1

Cycle 2	Obese	Fluctuating	Lean	p value (obese vs fluctuating)	p value (fluctuating vs lean)	p value (obese vs lean)
Weight (kg)	108±7*	77±4	76±4	<0.001	NS	<0.001
Systolic BP (mmHg)	93±13	81±9	79±5	0.08	NS	0.06
RV systolic pressure (mmHg)	18±4	13±3	11±2	0.02	NS	0.002
RA pressure (mmHg)	7.3±2.5	4.6±2.3	2.9±0.8	0.06	NS	0.003
LA pressure (mmHg)	8.5±1.7	5.8±1.6	3.7±0.9	0.008	0.07	<0.001
LA volume (ml)	52±7	47±5	39±3	NS	0.01	<0.001

* $p<0.05$ between cycle 1 and cycle 2.

Figure 1. Study design

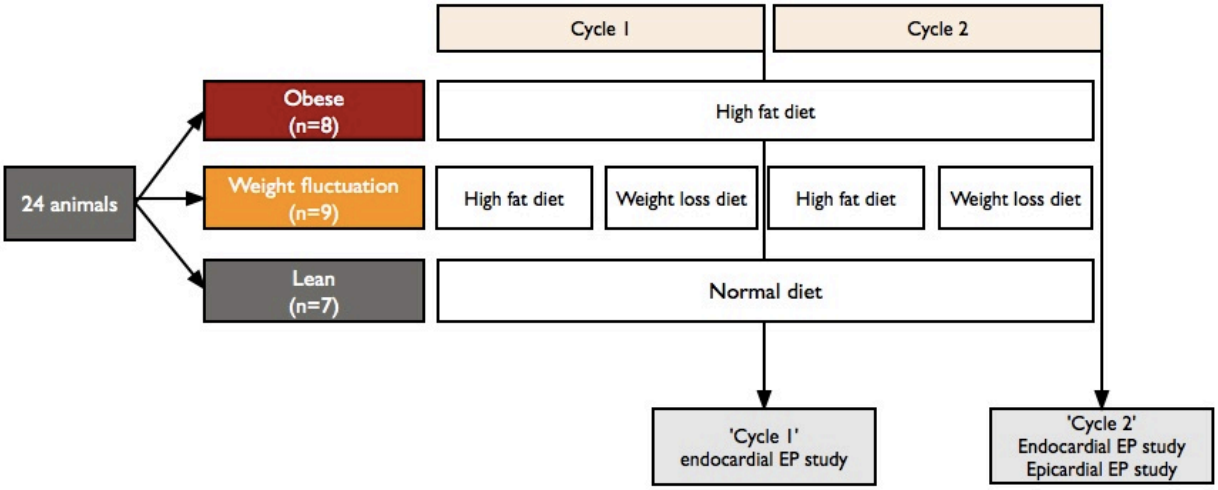


Figure 2. Conduction velocity (CV) following cycle 1 and cycle 2

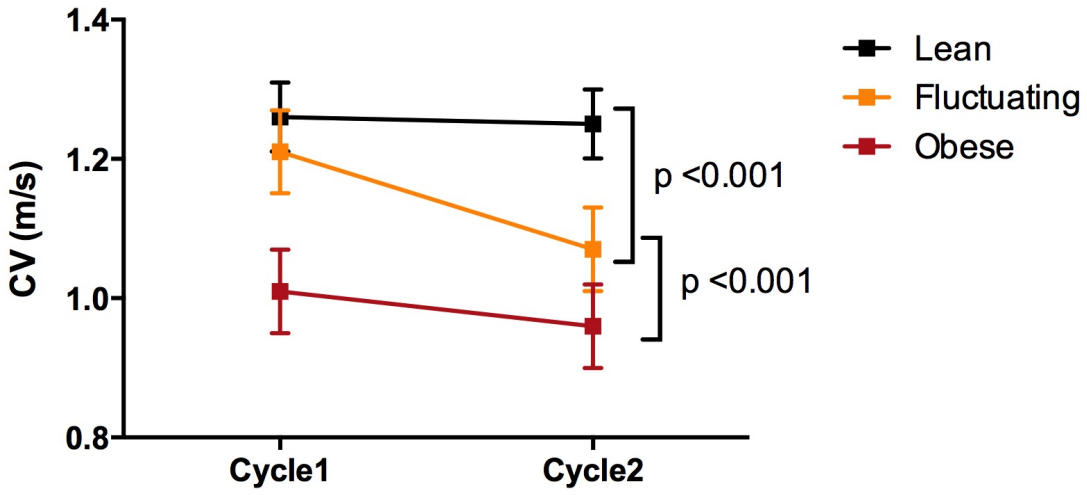


Figure 3. Conduction velocity by region after cycle 1 (left) and cycle 2 (right)

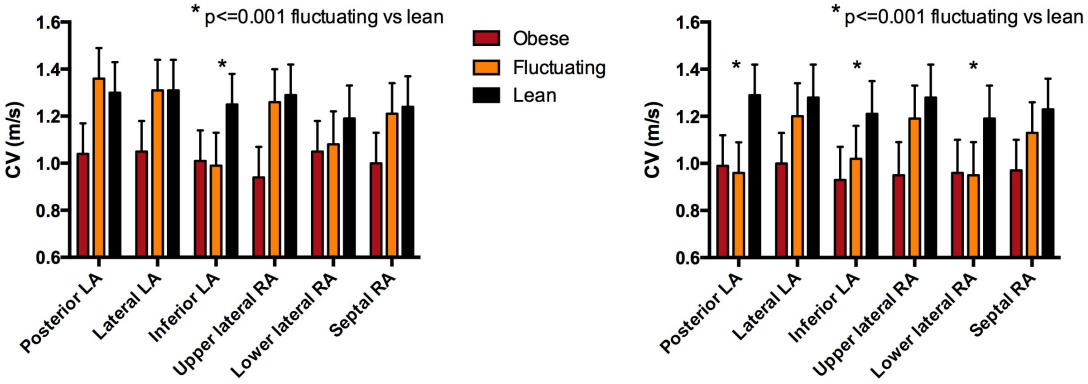


Figure 4. Endocardial effective refractory periods after cycle 1 and cycle 2

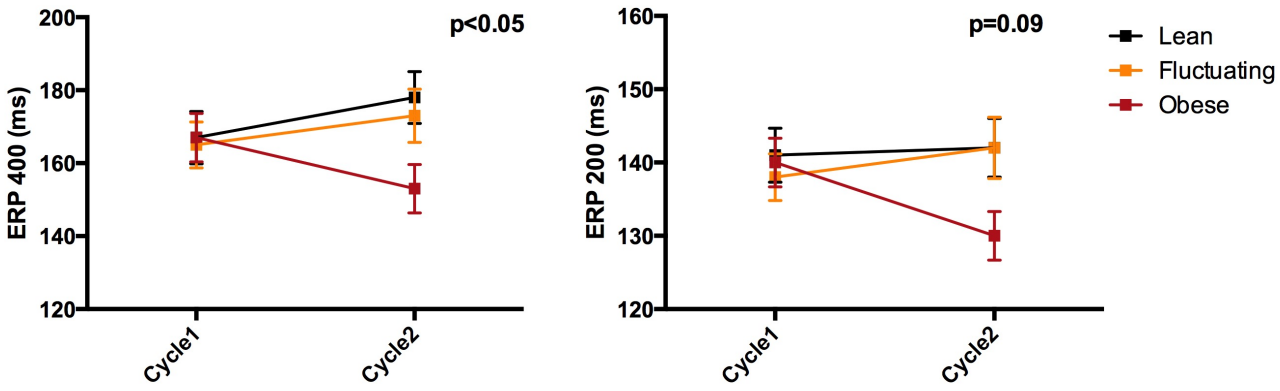


Figure 5. Epicardial effective refractory periods at endpoint study.

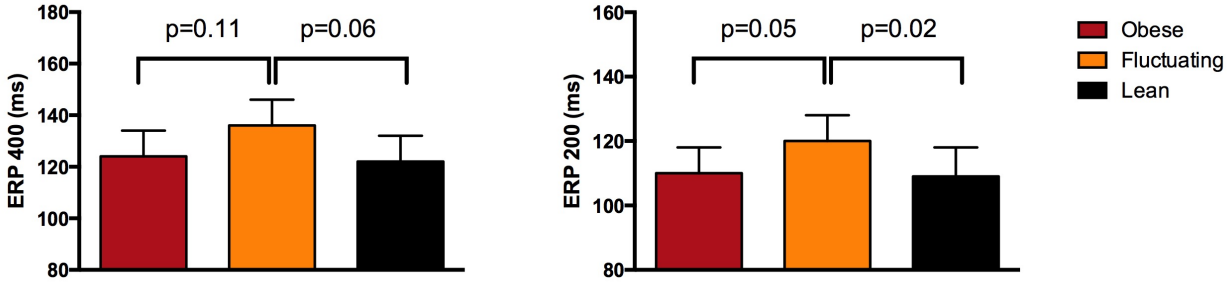
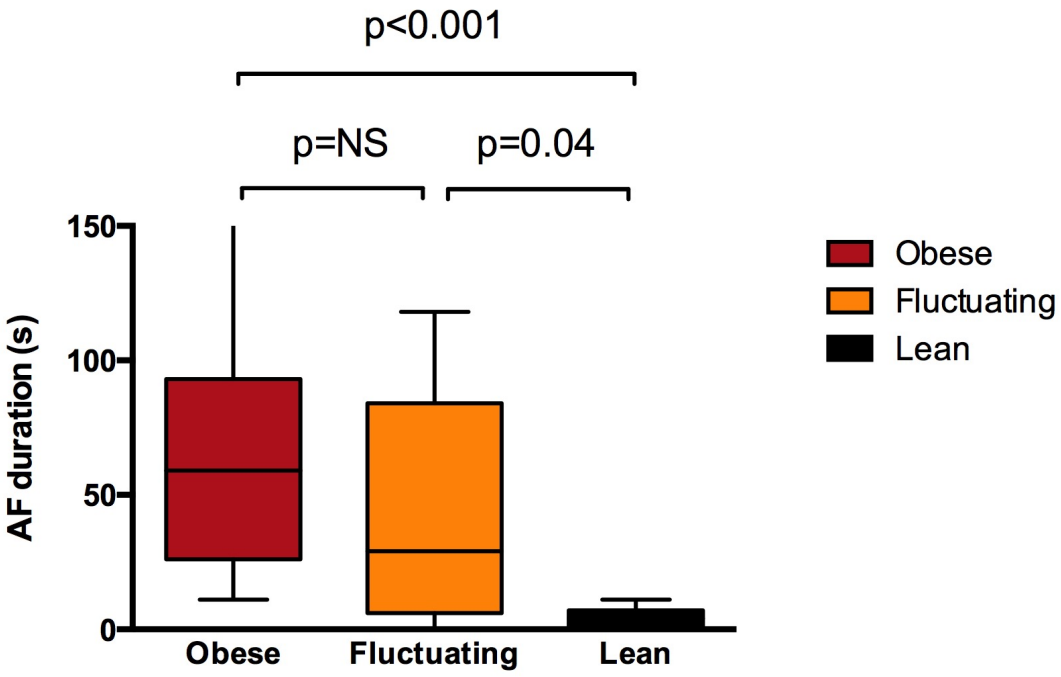


Figure 6. Total AF duration at end point study.



Chapter 3 - The role of endothelin-receptor blockade in the prevention of the substrate for atrial fibrillation – an interventional study in an obese ovine model

3.1 Introduction

Atrial fibrillation (AF) is the most common heart rhythm disorder in adults with its prevalence reaching epidemic proportions.[269] Patients are often highly symptomatic and notable complications include stroke, cardiomyopathy and dementia. This has resulted in a significant increase in hospitalization and cost to the community.[8]

Several factors have been implicated for the burgeoning frequency of AF. In addition to the classical risk factors such as aging and hypertension, obesity has more recently emerged as an independent association with both the development and progression of the disease. Obesity is also at epidemic levels and confers a 49% increased risk of developing AF, the risk increasing in parallel with increasing BMI.[16]

Previous studies have provided direct evidence for the role of obesity in establishing the substrate predisposing to AF.[23, 24] In an ovine model, progressive weight gain was associated with conduction slowing and more inducible AF. Importantly this occurred without the usual confounding factors seen in humans, namely obstructive sleep apnoea and glucose intolerance. In addition, increasing weight was associated with upregulation of profibrotic proteins in atrial tissue. In particular, endothelin-A and B receptors were upregulated as weight increased.

Endothelin causes cardiac fibrosis directly and upregulation is associated with a multitude of cardiac conditions.[99] Endothelin exposure causes conduction slowing in

ventricular tissue[100] and upregulation of endothelin receptors occurs in atrial tissue in a number of heart failure animal models.[101, 102]

Bosentan is an orally active non-peptide endothelin receptor antagonist (ERA) of both receptor subtypes, with an affinity for ET-A. It is used clinically in the management of patients with primary pulmonary hypertension.[250] Later trials examined its use in congestive cardiac failure but failed to show clinical benefit. [270] With the progressive upregulation of ET receptors associated with weight gain and its established role in the fibrotic cascade, a central feature of the substrate for AF, we hypothesised that intervention to block this pathway would prevent the development of the substrate for AF. Therefore, the present study investigates the effect of ERA on the electrophysiological and structural atrial abnormalities associated with obesity in an ovine model.

3.2 Methods

Twenty ovine (Merino Cross) subjects were studied in accordance with the National Health and Medical Research Council of Australia guidelines on animal research. Approval for the study was granted by animal research committees of the University of Adelaide and South Australian Health and Medical Research Institute, Adelaide, Australia.

3.2.1 Study Protocol

The study design is shown in Figure 1. The obese model and intervention are described below. Animals underwent sequential percutaneous endocardial electrophysiological and electroanatomical evaluation at baseline, 30 and 60 weeks. In addition, at each time point, cardiac MRI and DEXA scans were performed. To allow

more detailed electrophysiological characterization, at terminal study, a high-density plaque was utilized to perform epicardial mapping. Following this, tissue was harvested to assess the degree of atrial fibrosis, inflammation and pro-fibrotic protein expression. All procedures are described below.

3.2.2 Obese ovine model

Animals had obesity induced using an ad libitum diet of high fat pellets, predominantly consisting of wheat, barley and canola seed. Excess voluntary intake was of grass alfalfa silage and hay. As previously described,[24] the pellets were gradually introduced at 8% excess basal energy requirements and rationed to 70% of the total dry matter intake. Plasma samples were collected at intervals to ensure stability of haematological and biochemical indices. This was continued for the duration of the study, with gradual increase in weights in all animals over 60 weeks. Weights were recorded weekly and reported weights were those taken in a shorn and fasted state immediately prior to electrophysiological procedures.

Animals were randomly allocated to being either in the control group or to undergo treatment with bosentan. In both groups, feeding was titrated identically to maintain a constant and gradual weight gain.

3.2.3 Treatment (Endothelin Receptor Antagonist) group

Ten animals were randomized to receive bosentan treatment during the 60-week period of weight gain. Pharmacokinetic studies were previously performed to ensure twice daily dosing was appropriate and that significant hypotension did not occur. Therefore, treatment was given orally at the standard human dose of 125mg twice daily. Bosentan powder (Synthesis med chem Pty Ltd, Melbourne, Australia) was

suspended with tragacanth mucilage (125mg/40ml). Animal age, housing conditions and feeding regimen were identical in treatment and control groups.

3.2.4 Animal anaesthesia

All procedures were performed under general anaesthetic. A pre-med of diazepam 0.4mg/kg was given prior to induction with ketamine 5mg/kg. Isoflurane 2.5% with 4 litres/minute of oxygen was used for maintenance. Non-invasive blood pressure, heart rate, pulse oximetry and end-tidal CO2 were continuously monitored.

3.2.5 Sequential endocardial electrophysiological study

Internal jugular and femoral venous access were obtained using a conventional percutaneous approach. A 10-pole coronary sinus (CS) catheter (2–5–2 mm inter-electrode spacing) was positioned with the proximal bipole at the CS ostium. Transseptal puncture was performed using an SL0 sheath and BRK needle using conventional techniques, as previously described.[24]

Surface ECG and bipolar endocardial electrograms were monitored continuously and stored on a computer-based digital amplifier/recorder system (LabSystem Pro, Bard Electrophysiology, Lowell, MA) with storage for offline analysis. Intracardiac electrograms were filtered from 30 to 500 Hz and measured with computer-assisted calipers at a sweep speed of 200mm/s.

Electroanatomical mapping

Electroanatomical maps were created in sinus rhythm using the Carto XP system (Biosense Webster, Diamond Bar, CA) as previously described.[63] Both right and left atria were mapped using a 3.5-mm tip catheter ablation catheter (Navistar, Biosense Webster, Diamond Bar, CA) with a minimum of 80 equally distributed points

collected in each chamber. Endocardial contact was confirmed with a combination of electrogram stability, fluoroscopy and the CARTO point stability criteria (≤6mm stability in space, ≤5ms in local activation time). Location, voltage and activation timing were recorded at each point. For analysis the atria was divided as follows: Septal right atrium (RA); High lateral RA; Low lateral RA; Posterior left atrium (LA); Lateral LA; and Inferior LA. The following were determined:

1. Conduction velocity in each region, determined by the mean velocity between 3-5 pairs of points in the direction of conduction demonstrated by isochronal maps;
2. Signal fractionation, defined as a signal greater than 50ms duration with at least four deflections;
3. Atrial voltage. Low voltage was defined as an area with three contiguous points with a bipolar voltage of <0.5mV. Scar was an area with three contiguous points <0.05mV.

Effective refractory periods

Effective refractory period (ERP) was measured at three times the tissue capture threshold at a pulse width of 2ms. This was performed from the following 8 sites: Septal RA; High lateral RA; Low lateral RA; Posterior LA; Lateral LA; Inferior LA; Proximal coronary sinus (CS); and Distal CS. Eight S1 stimuli were delivered with S1 cycle lengths of 400 and 200ms, with incremental S2 (10ms increments) commencing at a coupling interval of 100ms, using a Micropace EPS 320 stimulator (Micropace, Canterbury, Australia). The ERP was the longest S1-S2 interval without atrial capture. The mean of two attempts was recorded. An additional attempt was made if the difference between attempts was greater than 10ms.

AF induction

Inducibility of AF was determined using a burst pacing protocol from the left atrial appendage, performed at the terminal study. Twenty impulses were delivered at the lowest cycle length with 1:1 atrial capture. An AF episode was defined as irregular atrial activity lasting ≥2 seconds. This protocol was repeated five times and the number of episodes and total duration were recorded. Sustained AF was defined as an episode lasting >10 minutes. In the event of sustained AF, no further testing was performed.

Haemodynamic recordings

Non-invasive systolic blood pressure and invasive mean right atrial and systolic right ventricular pressures were recorded at the start of each procedure. Mean left atrial pressure was recorded immediately following transseptal puncture.

3.2.6 Terminal epicardial electrophysiological study

Left lateral thoracotomy was performed to access the left atrium. A custom made 90-pole plaque was placed on the left atrial appendage. Pacing was performed from each of the four corners of the plaque at 400ms. Epicardial electrograms were recorded and the following determined:

1. Effective refractory periods were measured using the same protocol as the endocardial study.
2. Conduction velocity was determined using activation maps analysed by custom software, as previously described.[59] The peak of the largest amplitude deflection on each bipolar electrogram was automatically annotated and manually verified. Local conduction velocity was calculated

for each point from triangulated local vectors, allowing subsequent calculation of mean velocity for each map.

3. Conduction heterogeneity was calculated using established phase mapping techniques integrated into the software, as previously described.[59] Absolute conduction phase delay was calculated by subtracting the 5th from the 95th percentile of the phase difference distribution (P_{5-95}). Conduction heterogeneity index was derived from dividing P_{5-95} by the median (P_{50}).

3.2.7 Cardiac MRI

Chamber volumes were measured using MRI (Siemens Sonata 1.5 Tesla, MR Imaging Systems, Siemens Medical Solutions, Erlangen, Germany). 6-mm slices were taken through the left atrium and ventricle. Images were taken using electrocardiogram-gating and periodic breath holding. Analyses were performed offline using CVI42 (Circle Cardiovascular Imaging Inc., Calgary, Canada).

3.2.8 Dual-Energy X-ray Absorptiometry (DEXA)

Scans were acquired using a GE-Lunar Prodigy Vision DXA bone densitometer using Encore 13.60.033 software (GE-Lunar, Madison, WI, USA). Scans were performed using Total Body scan protocol in standard or thick mode to allow for variation in tissue depth.

3.2.9 Tissue analysis

At study completion, the heart was removed and the left atrial appendage was isolated and fixed in 10% buffered formalin, paraffin embedded, and 5µm sections cut and stained with Masson's trichrome. For the immunohistochemical study,

polyclonal antibodies to TGF-β (Sigma-Aldrich, St Louis, MO, Cat#SAB4502954), Angiotensin II (Abbiotec, San Diego, CA, Cat#251229), CTGF (Abbiotec, San Diego, CA, Cat#251261), IL-6 (Abbiotec, San Diego, CA, Cat#250717) and PDGF (Abcam, Cambridge, UK, Cat#ab61219) were used at dilutions of 1:500, 1:250, 1:400, 1:500 and 1:6400, respectively, in a standard streptavidin-biotinylated immunoperoxidase technique. All sections underwent antigen retrieval using citrate buffer pH6.0 prior to overnight antibody incubation. This was followed by a biotinylated anti-rabbit secondary (Vector Laboratories, Burlingame, CA, Cat#BA-1000), then washing in phosphate buffered saline. Slides were then incubated with a streptavidin-conjugated peroxidase tertiary (Pierce, Pasadena, CA, Cat#21127). Sections were visualised using diaminobenzidine tetrahydrochloride, washed, counterstained with haematoxylin, dehydrated, cleared, and mounted on glass slides. A minus primary control, as well as a control showing the normal pattern of expression of the antigen in question, was run with each batch of slides.

Images of the tissue were digitally captured from 5 random fields per section at 40x magnification using NanoZoomer Digital Pathology System software (Hamamatsu Phonetics, Japan). Quantitative assessments of collagen content and immuno-staining were made with the appropriate colour range selection using Image-Pro Premium v9.1 (Media Cybernetics Inc., Rockville, MD). Results were calculated in percentage relative to the whole tissue area.

3.2.10 Statistical Analysis

Normally distributed data was expressed as mean ± standard deviation and compared with unpaired t-test. Differences between groups over time were tested using analysis of variance. Conduction velocity and ERP across regions were analysed using

a mixed effects model ANOVA with sheep ID used as a random effect to account for the dependence in observations from the same animal. Fixed effects included combinations of group, time-point and region with a maximum of two fixed effects entered into the statistical model at one time. Main effects and their interaction were tested. If a significant interaction was present, post-hoc testing was performed. AF duration was non-parametric; therefore, log-transformation was performed prior to t-test comparison, with values expressed as median and interquartile range. P-values of less than 0.05 were considered statistically significant. Analyses were performed using SPSS version 23 (SPSS Inc. Chicago, IL).

3.3 Results

3.3.1 Weight, structural and haemodynamic parameters

Table 1 presents the changes in anthropometry, haemodynamic and cardiac structure in the two groups. Both groups increased in weight and body fat equally over 60 weeks. Left and right atrial pressures significantly increased with increasing weight, accompanied by a non-significant increase in systolic blood pressure and RV systolic pressure. There was a non-significant increase in left atrial volume and left ventricular mass with increasing weight but no change in LV volume or function.

There was no significant difference in haemodynamics, chamber volumes, LV mass or function between treatment and control groups at any time-point.

3.3.2 Electrophysiological parameters

Conduction velocity and conduction heterogeneity

Sequential endocardial electrophysiological studies demonstrated that there was progressive endocardial conduction slowing seen with increasing weight in the

control group (p<0.001). The ERA group demonstrated significant attenuation of this conduction slowing when compared with controls (p=0.001) at both 30 weeks (1.24±0.05 vs 1.00±0.05m/s) and 60 weeks (1.15±0.05 vs 0.94±0.05m/s) as shown in Figure 2. This improvement in conduction velocity was homogeneous across all atrial sites (Site*Group interaction=0.17).

At the end of the study period, high-density epicardial mapping of the LA confirmed the marked differences in the conduction properties of the atria. Conduction velocity at 60 weeks was higher in the ERA group than controls (0.98±0.03 vs 0.92±0.03m/s, p=0.001) as shown in figure 3. Epicardial conduction was also less heterogeneous with ERA treatment (1.28±0.05 vs 1.47±0.05, p=0.02).

Effective refractory periods

There was no significant change in ERP with increasing weight, overall or when analysed by chamber. There was no difference in ERP between groups at mid- or endpoint at either cycle length (Figure 4). Terminal epicardial ERP determination demonstrated that there was no significant difference between the groups.

Signal fractionation

Signal fractionation increased with increasing weight in both groups (p<0.001) as shown in figure 5. ERA treated animals had significantly less signal fractionation than control animals at 60 weeks (15.2±3.6 vs 28.6±8.0%, p=0.0001).

Atrial bipolar voltage

There was no significant reduction in endocardial voltage with increasing weight or between groups (5.81±0.99 vs 5.77±1.07mV at endpoint). No areas of low voltage (<0.5mV) or scar were seen in any animal.

AF inducibility

Figure 6 shows the AF burden at endpoint study. The ERA treated group had significantly fewer AF episodes (46.7±28.2 vs 73.3±22.4%, p=0.041). The total AF duration was also reduced by ERA treatment (17.0 [IQR 37.0] vs 47.0s [IQR 139.5], p=0.04). One control animal had sustained AF (>10mins) during testing and was considered to have 100% inducibility. Two animals (1 ERA treated, 1 control) animals had sustained AF prior to induction and were excluded from inducibility and duration analysis.

3.3.3 Histological parameters

Tissue fibrosis

There was a reduction in interstitial fibrosis in animals treated with ERA when compared with controls. Masson's trichrome staining of the LA (figure 7, upper panel) demonstrated a fibrotic area of 3.81±1.73% vs 6.02±1.62% in the ERA treated and control groups respectively (p=0.02).

Pro-fibrotic proteins

Results of immunohistochemistry of left atrial tissue for pro-fibrotic protein expression are shown in figure 8. ERA treated animals had reduced expression of angiotensin II (0.65±0.13 vs 0.84±0.18%, p=0.05), connective tissue growth factor (0.89±0.30 vs 1.60±0.36%, p=0.003), and platelet derived growth factor (1.01±0.44 vs 1.47±0.22%, p=0.04) compared to obese controls. There was no difference in TGF-β expression between groups (2.17±0.64 vs 2.21±0.94%, p=NS).

Inflammation

There was reduced inflammatory protein expression with ERA treatment (figure 9), with reduction in CD68 (0.35±0.09 vs 0.53±0.18, p=0.03) and IL6 expression (1.89±1.12 vs 3.42±1.28, p=0.02).

Gap junctions

There was greater expression of the gap junction protein connexin 43 with ERA treatment than in controls (2.79±0.67 vs 1.94±0.54%, p=0.01). (figure 7, lower panel).

3.4 Discussion

Obesity is increasingly recognized as an important determinant of the "rising tide" in epidemic of AF. Previous studies have demonstrated the evolution of the substrate recognized to predispose to AF with progressive weight gain; however, the mechanisms for these changes have been elusive. The current study undertakes an interventional study to evaluate the role of endothelin receptor pathways in the development of the substrate for AF in obesity to provide new information on the pathophysiological links between these conditions.

Endothelin receptor blockade prevented the development of atrial substrate in an obese ovine model. Treatment with bosentan during weight gain was associated with:

1. Attenuation of the marked conduction abnormalities that result from weight gain, characterised by prevention of conduction slowing, conduction heterogeneity and signal fractionation;
2. Prevention of the structural changes associated with obesity. In particular, there was marked attenuation of the interstitial fibrosis and preservation of gap-junctional content;

3. As a result of these changes there was a reduced vulnerability to AF.

Importantly, these effects observed with ERA treatment, seem to be mediated through a reduction in inflammatory (IL-6) and profibrotic markers (Connective tissue growth factor, Platelet derived growth factor, and Angiotensin II) but not TGF-β. These beneficial effects were observed despite the evolution of progressive obesity and without a significant impact on haemodynamic parameters.

Substrate for AF

Several studies have evaluated the substrate predisposing to the development of AF. These have consistently identified the presence of diffuse atrial fibrosis with the associated conduction abnormalities as being central to the remodelling associated with conditions predisposing to AF. In particular, this has been demonstrated in the preclinical setting in models of heart failure[58], hypertension[59, 60], sleep apnea[192], and coronary ischaemia[271]. Importantly similar observations have also been confirmed in the clinic with areas of low voltage and electrical silence, conduction slowing, and fractionated electrograms.[63, 67, 272] Interestingly similar changes were observed in patients with "lone AF".[68]

Previous animal studies investigating the effect of obesity have demonstrated progressive atrial conduction slowing, increased heterogeneity, signal fractionation and increased propensity to AF. The findings were associated with diffuse, interstitial atrial fibrosis and upregulation of pro-fibrotic proteins.[23, 24] In the clinical setting, atrial electrophysiology has been shown to differ in obese patients with AF, with shorter ERPs and slower conduction into pulmonary veins than in lean patients with AF.[202]

The present study confirms the findings of these prior studies demonstrating the presence of interstitial atrial fibrosis, heterogeneous and slowed conduction, with up regulation of fibrotic and inflammatory markers associated with obesity. Intervention using an endothelin receptor blocker, a recognised cascade that leads to fibrosis, partially prevented the development of these abnormalities with reduced collagen staining on Masson's trichrome, reduced inflammation and increased connexin 43 expression associated with resolution of the conduction abnormalities associated with obesity. Importantly, it reduced vulnerability to AF. Notably, there was no significant difference in haemodynamic parameters between the groups, suggesting that endothelin receptor blockade in obesity may prevent atrial fibrosis directly, without an effect on either systemic or intracardiac pressures.

Mechanisms for the formation of the atrial substrate for AF

Guarda *et al.* first demonstrated endothelin-1 to be directly responsible for cardiac fibrosis. Cardiac fibroblasts maintained in ET-1 had a dose dependent increase in collagen deposition compared with controls.[99] Endothelin exposure is associated with fibrosis, decreased gap junction expression and conduction slowing in ventricular myocytes.[100] Human atrial tissue demonstrates positive inotropy in response to endothelin incubation[91] and AF is associated with endothelin receptor upregulation[95]. Surgical patients with increased endothelin-1 expression in left atrial tissue more commonly had AF, hypertension and atrial dilatation.[96] Gelzer *et al.* found acute ET-A blockade shortened sinus node recovery time in normal pigs, without an effect on atrial or ventricular refractory periods.[273] No studies have previously investigated the effect of endothelin receptor blockade on atrial conduction velocity.

We have previously demonstrated upregulation of endothelin-A and B in atrial tissue of obese ovine subjects.[23] In addition, there was upregulation of other profibrotic molecules, namely transforming growth factor beta (TGF- β), connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), all of which have been directly implicated in atrial fibrosis. Our study investigated these proteins and grants some insight into the mechanisms by which obesity causes fibrosis.

TGF-β is directly involved cardiac fibrosis. Fibroblasts exposed to TGF-β have a dose and time dependent increase in collagen production.[274] In a canine heart failure model, atrial TGF-β was significantly upregulated in association with atrial fibrosis[275] and blockade of TGF-β receptors resulted in reduced fibrosis and propensity to AF.[260] In our study, endothelin receptor blockade reduced fibrosis without an effect on TGF-β expression. This suggests that TGF-β acts independently upstream to endothelin and is in keeping with studies examining lung fibroblasts [276] and vascular endothelial cells.[103] It is likely that TGF-β continues to have a profibrotic effect despite endothelin receptor blockade and could explain why fibrosis is not completely prevented. TGF-β antagonism may represent an alternative therapeutic option for obesity related atrial fibrosis, potentially in combination with ERA treatment.

Mice with overexpression of AngII have increased atrial fibrosis with more inducible AF.[127] Atrial AngII expression is increased in dogs with atrial fibrosis associated with heart failure.[129] In our study, animals treated with ERA showed downregulation of AngII receptors. The angiotensin and endothelin systems have complex bidirectional interactions; our findings may be explained by a negative feedback loop, or may be related to the downregulation of CTGF, which is a mediator of AngII induced vascular fibrosis.[134]

CTGF has previously been found to be a regulator of the effects of endothelin-1 in cardiac tissue. Recchia *et al.* examined the atrial tissue of mice following ET-1 infusion and found increased CTGF expression with increasing concentration of ET-1. Additionally, ET-A and ET-B blockade prevented upregulation.[138] Our results were in keeping with these findings, with significant downregulation of CTGF with ERA treatment. This is strongly suggestive that CTGF acts downstream to endothelin in the fibrotic cascade and that CTGF blockade in addition to endothelin antagonism may not be an effective therapeutic strategy.

PDGF is associated with atrial fibrosis and AF, as demonstrated by a pressure overloaded mouse model.[143] Administration of endothelin had no significant effect on PDGF in rat aortic tissue,[277] but blockade of endothelin receptors reduced PDGF expression in the coronary arteries of rats undergoing cardiac transplantation.[278] Our study was in keeping with the latter study; we demonstrated reduced expression in the atrial tissue of animals treated with ERA than in controls. This offers new insight into the mechanisms of fibrosis in obesity and is suggestive that PDGF acts downstream to endothelin.

IL-6 exposure is associated with cardiac fibrosis and hypertrophy in a mouse model[279] and serum IL-6 has been shown to correlate with human AF more than several other inflammatory markers, including C-reactive protein.[280] ET-1 induces IL-6 release from human vascular smooth muscle cells.[281] Our study is in keeping with this; ERA treatment was associated with reduction in IL-6 expression, suggestive of an anti-inflammatory action.

Connexin 43 is the most prominent gap junction protein in atrial tissue and reduced expression is associated with conduction slowing and fibrosis.[282] ERA treatment was

associated with a higher expression of Cx43, a histological finding supportive of the reduced conduction slowing with demonstrated on both endocardial and epicardial mapping.

Clinical Implications

Using an interventional design, this study demonstrates that ERA can attenuate the formation of the AF substrate due to weight gain and obesity. It demonstrates the central role of the endothelin pathways in the formation of the substrate for AF. Recent studies have highlighted the importance of weight and risk factor management in the management of AF.[224, 229] However, in the clinic, weight loss is not always possible. Additionally, fluctuation in weight is often observed and has a proven negative impact on AF burden.[229] Treatment with an endothelin receptor antagonist may play an important role in the clinical management of patients with obesity related AF and possibly during periods of weight loss if weight fluctuation occurs. ERA use in these clinical scenarios warrants clinical evaluation, along with its potential role in the management of AF associated with other conditions.

Study limitations

This study investigated the effect of endothelin receptor blockade in an obese ovine model. As with all animal models, this may vary from human pathophysiology and the involvement or effect of specific pro-fibrotic pathways may differ. Treatment was given during the development of obesity, which may not allow extrapolation to the clinical scenario of treatment being required once obesity, and hence atrial substrate, has already developed. However, it may prevent the progression of substrate, in particular in patients with weight fluctuation.

3.5 Conclusion

Endothelin receptor pathways are important determinants of the substrate for AF in obesity. Blockade of endothelin receptors partially prevents the development of atrial substrate due to weight gain. This may represent a unique therapeutic target in AF associated with obesity.

Table 1. Structural and haemodynamic characteristics of ERA treated and control groups.

Variable	ERA treated			Control			p value (time)	p value (group)
	Baseline	30 wks	60 wks	Baseline	30 wks	60 wks		
Weight (kg)	68±7	87±9	95±9	68±5	87±7	92±7	<0.001	NS
Body fat (%)	19±6	37±5	43±4	18±6	37±6	41±5	<0.001	NS
Systolic BP (mmHg)	83±18	85±8	89±10	85±15	86±8	90±11	NS	NS
RV systolic pressure (mmHg)	16±3	18±2	19±3	17±2	18±2	19±3	NS	NS
RA pressure (mmHg)	3.0±2.4	5.3±1.8	5.6±2.6	2.8±1.6	5.4±1.8	4.8±2.6	0.006	NS
LA pressure (mmHg)	4.6±2.3	6.9±1.6	6.7±1.5	4.5±2.1	6.9±1.5	6.8±1.9	0.003	NS
LA volume (ml)	49±10	57±9	58±13	52±11	59±8	57±10	NS	NS
LVESV (ml)	69±19	60±11	72±22	69±20	67±9	67±12	NS	NS
LVEF (%)	49±7	50±11	48±8	47±12	48±4	46±6	NS	NS
LV mass (g)	112±10	135±25	139±35	116±10	133±20	133±12	0.17	NS

Figure 1. Study design.

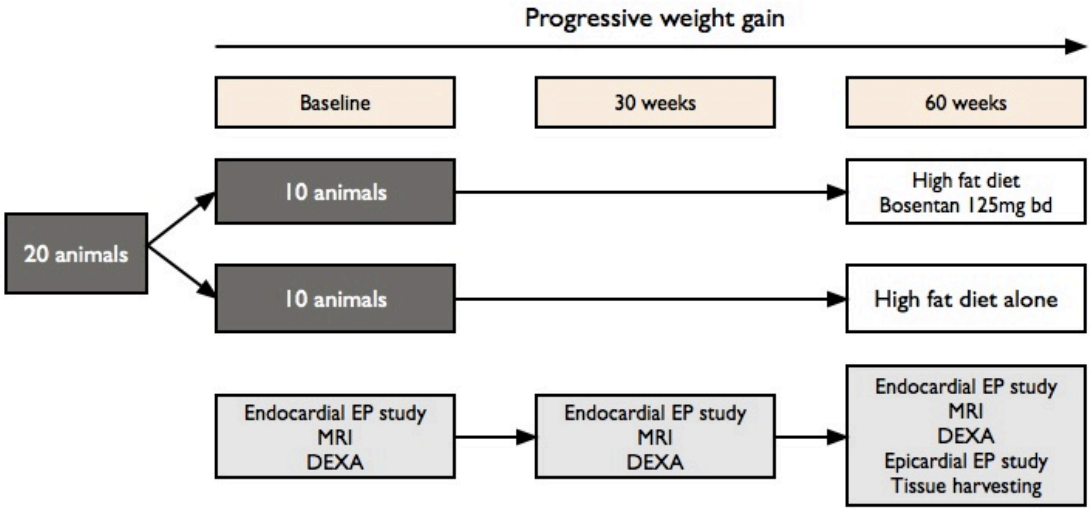


Figure 2. Conduction velocity (CV) at the study baseline, midpoint and endpoint (left) and by region at endpoint study (right).

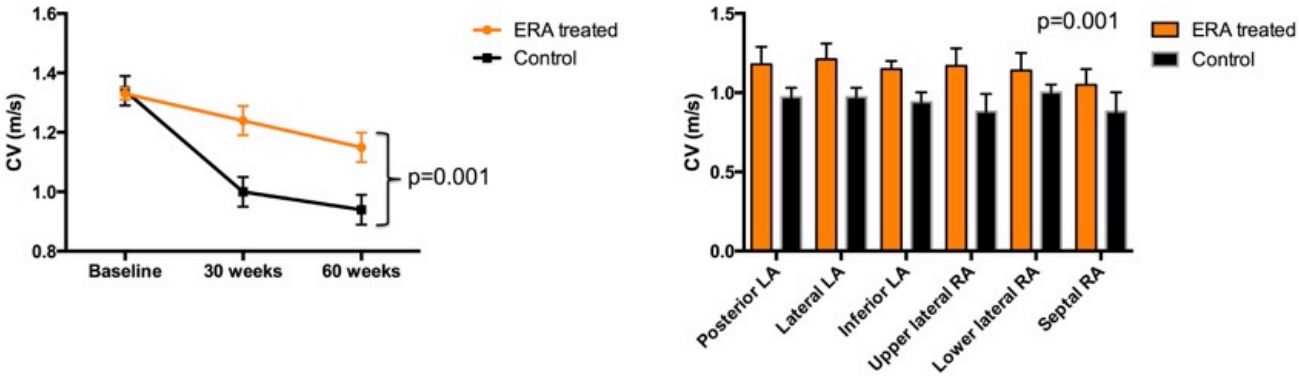


Figure 3. Epicardial conduction velocity (CV) at each corner of the LA plaque (top) and conduction heterogeneity (bottom), with representative examples.

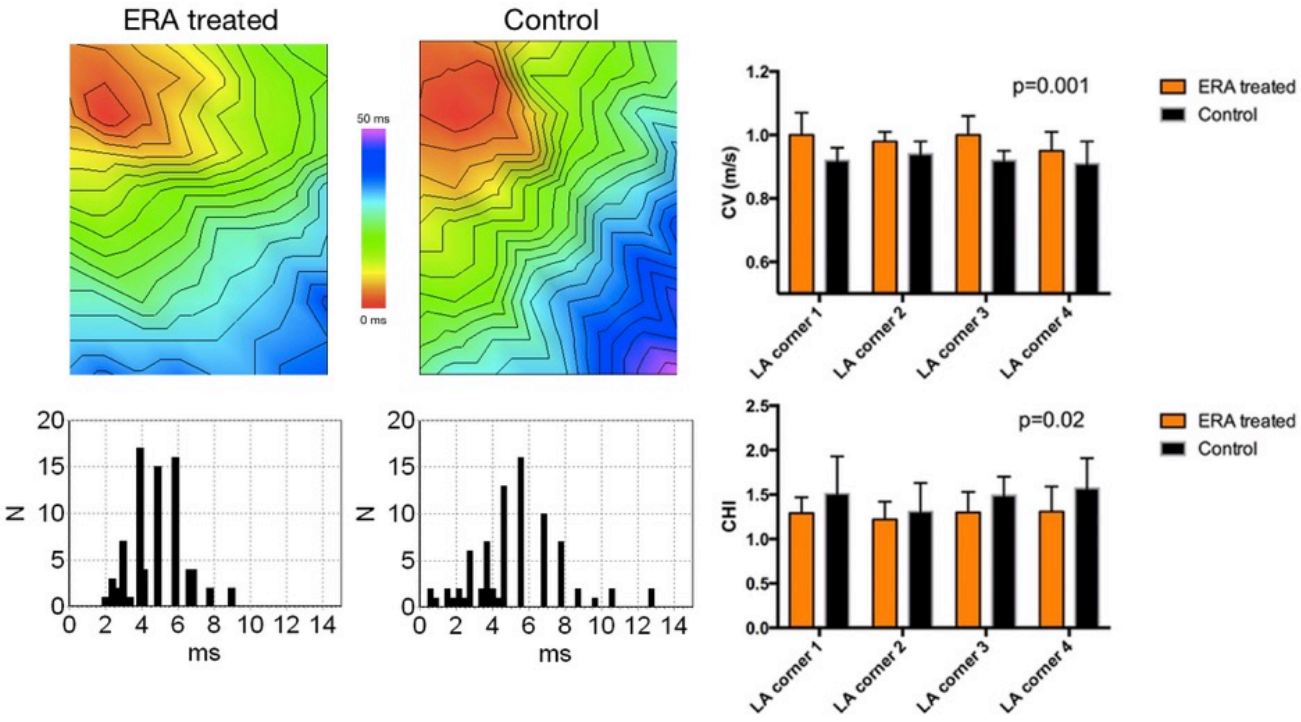


Figure 4. Effective refractory periods (ERP). The upper panel shows endocardial results at study baseline, midpoint and end point, the lower panel shows epicardial results at study endpoint. Left panels are with a drivetrain of 400ms, right at 200ms.

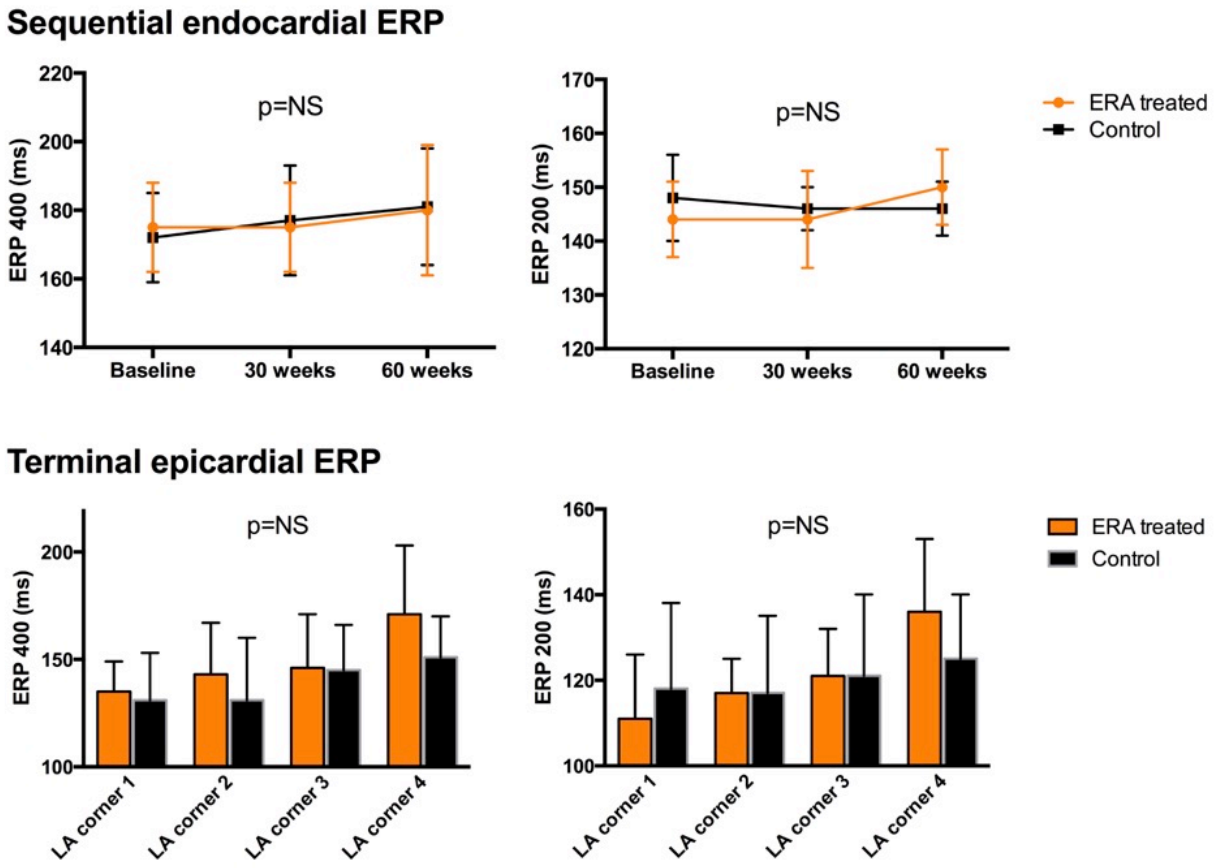


Figure 5. Proportion of fractionated signal at baseline, midpoint and endpoint (top). Representative examples of LA voltage and fractionation maps in both groups at baseline and endpoint (bottom). Pink markers represent points with fractionated signal.

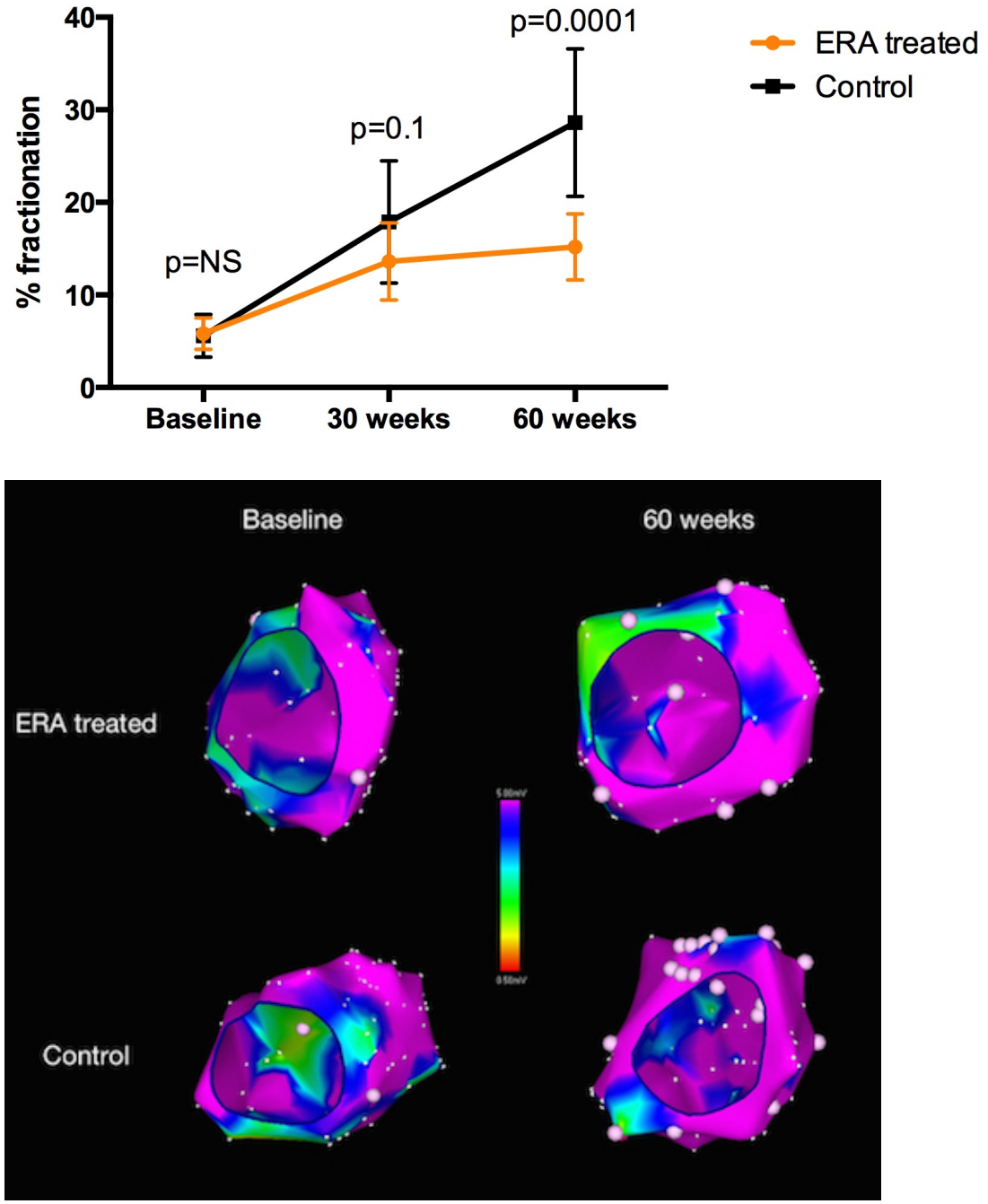


Figure 6. AF inducibility at end point study. The proportion of episodes >2s is shown on the left, the total AF duration (log transformed for statistical analysis) on the right.

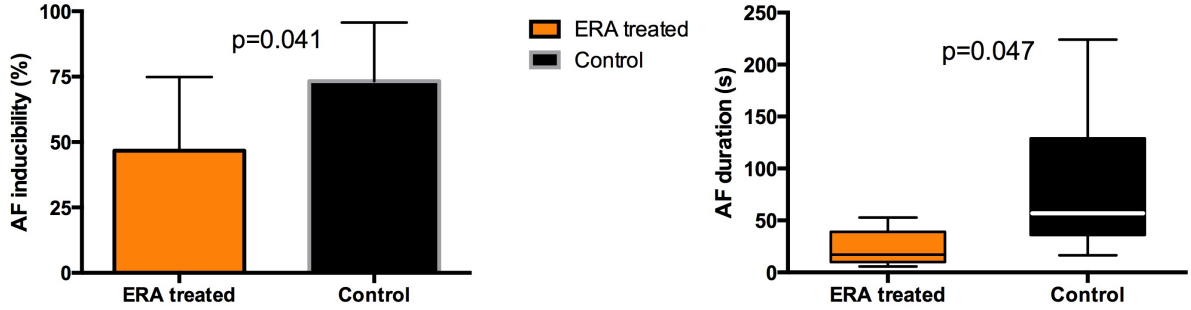


Figure 7. Masson's trichrome staining of LAA tissue at 40x magnification (upper panels). Immunohistochemistry of gap junction protein connexin43 at 40x magnification (lower panels).

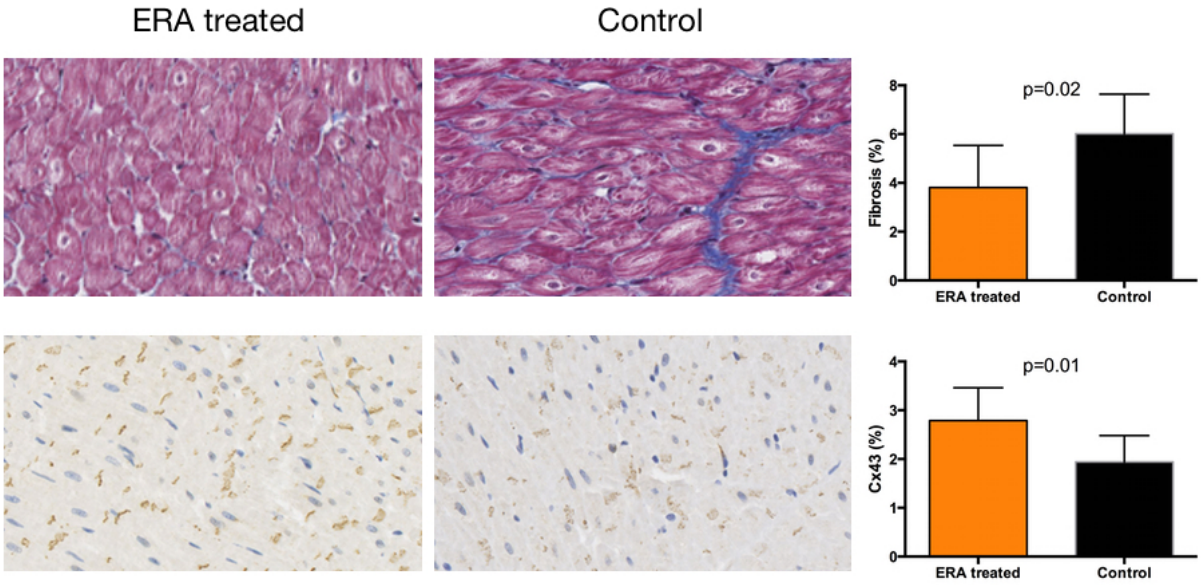


Figure 8. LAA immunohistochemistry of pro-fibrotic molecules (40x magnification). Representative examples are shown on the left and graphs comparing treatment and control groups on the right. TGF-β = Transforming growth factor; AngII = Angiotensin II; CTGF = Connective tissue growth factor; PDGF = Platelet derived growth factor

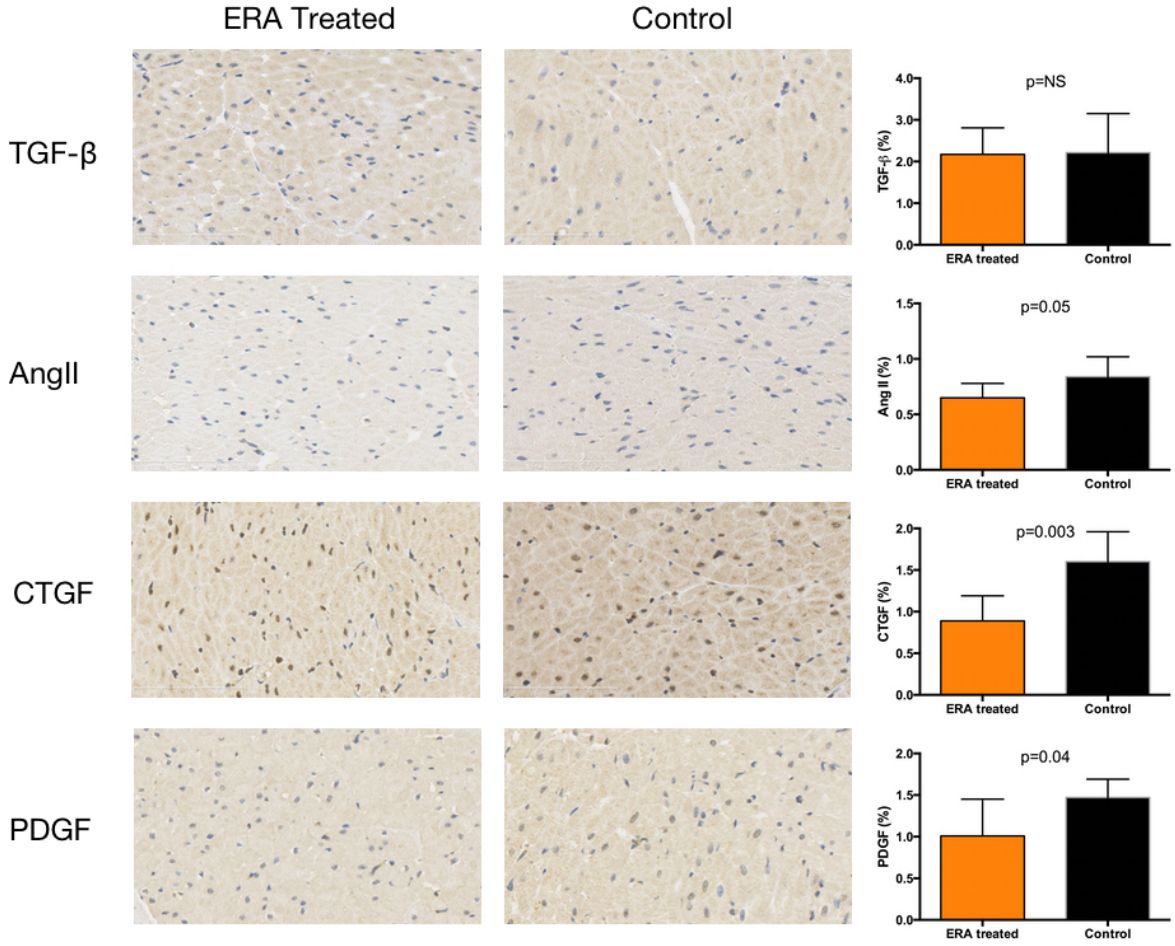
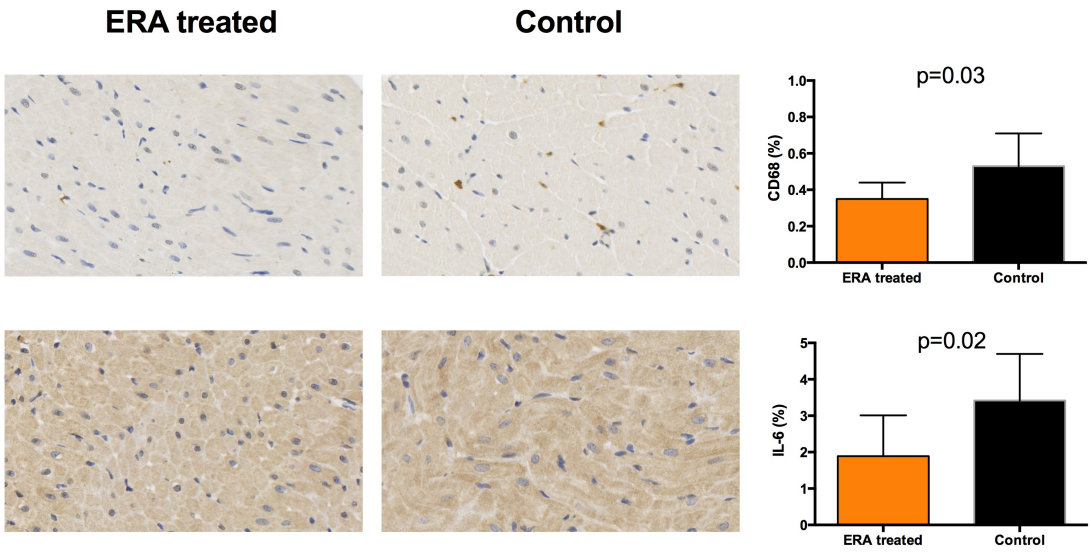


Figure 9. LAA immunohistochemistry of inflammatory molecules (40x magnification). Representative examples are shown on the left and graphs comparing treatment and control groups on the right. IL-6 = Interleukin-6



Chapter 4 – Treatment with tranilast prevents atrial remodelling and AF in an obese ovine model

4.1 Introduction

Atrial fibrillation is the commonest sustained arrhythmia and is responsible for a large socioeconomic burden.[283] The development of AF is increasingly recognised to be due to both electrical and structural remodelling of atrial tissue. Electrical remodelling is generally due to increased heart rates and predominantly leads to reductions in refractory periods. On the other hand, structural remodelling is associated with conduction abnormalities in atrial tissue, promoting electrical reentry and contributing to the maintenance of the arrhythmia. A multitude of conditions are responsible for the development of this remodelling, most commonly advancing age, hypertension, coronary artery disease, heart failure, obstructive sleep apnoea and obesity.

Several pro-fibrotic molecules play a role in the development of atrial fibrosis; transforming growth factor beta (TGF-β) play a central role in the pathophysiology of this process. It induces myofibroblast differentiation in response to several cardiac conditions and overexpression is directly associated with interstitial atrial fibrosis and susceptibility to AF.[112] We have previous demonstrated that TGF-β receptors are upregulated in the atrial tissue of obese animals, along with the development of atrial fibrosis and propensity to AF.[24]

Tranilast (N-(3,4-dimethoxycinnamoyl) anthranilic acid) is an anti-inflammatory/anti-fibrotic compound, predominantly affecting TGF-β. It is used in Japan and South Korea as a treatment for asthma and other allergic conditions, as well as hypertrophic

and keloid scarring. Animal studies have demonstrated benefit in atrial remodelling secondary to heart failure.[260] We sought to examine the effect of tranilast treatment on atrial electrophysiology in an obese animal model.

4.2 Methods

Sixteen ovine (Merino-cross) subjects were studied according to National Health and Medical Research Council of Australia guidelines on animal research. Animal research committees of the University of Adelaide and South Australian Health and Medical Research Institute granted approval for the study.

4.2.1 Study Protocol

Animals underwent sequential investigations at baseline, 32 and 64 weeks, as shown in figure 1. Percutaneous endocardial electrophysiological and electroanatomical evaluation was performed on all animals, along with cardiac MRI to assess cardiac structure and function. Additionally at terminal study, high-density mapping of the epicardial surface was performed using a 90-pole plaque, to allow more detailed electrophysiological study. Following this, cardiac tissue samples were taken to assess atrial fibrosis, inflammation and pro-fibrotic protein expression. All procedures are described below.

4.2.2 Obesity induction

Obesity was using a diet of high fat pellets consisting of wheat, barley and canola seed. Excess voluntary intake was of grass alfalfa silage and hay. As previously described,[24] the pellets were gradually introduced at 8% excess basal energy requirements and rationed to 70% of the total dry matter intake. This was continued for the duration of the study, to allow gradual increase in weights in all animals over

64 weeks. Weights were recorded weekly and reported weights were those taken in a shorn and fasted state immediately prior to electrophysiological procedures. Plasma samples were collected at intervals to ensure stability of haematological and biochemical indices.

At study commencement, animals were randomly allocated to being either in the control group or to undergo treatment with tranilast. Feeding was titrated identically in all animals to maintain a constant and gradual weight gain.

4.2.3 Treatment (tranilast) group

Eight animals were randomised to receive tranilast treatment during the 64-week period of weight gain. Treatment was given orally at a dose of 1g twice daily. Pharmacokinetic studies were previously performed to ensure twice daily dosing was appropriate. Tranilast powder (Synthesis med chem Pty Ltd, Melbourne, Australia) was suspended with tragacanth mucilage (1g/40ml).

4.2.4 Animal anaesthesia

All procedures were performed under general anaesthetic. A pre-med of diazepam 0.4mg/kg was given prior to induction with ketamine 5mg/kg. Isoflurane 2.5% with 4 litres/minute of oxygen was used for maintenance. Non-invasive blood pressure, heart rate, pulse oximetry and end-tidal CO2 were continuously monitored.

4.2.5 Endocardial electrophysiological study

Internal jugular and femoral venous access were obtained using a conventional percutaneous approach. A 10-pole coronary sinus (CS) catheter (2–5–2 mm inter-electrode spacing) was positioned via the internal jugular vein, with the proximal

bipole at the CS ostium. Left atrial mapping was performed after transseptal puncture using an SL0 sheath and BRK needle, as previously described.[24]

Surface ECG and bipolar endocardial electrograms were monitored continuously and stored on a computer-based digital amplifier/recorder system (LabSystem Pro, Bard Electrophysiology, Lowell, MA) with storage for offline analysis. Intracardiac electrograms were filtered from 30 to 500 Hz and measured with computer-assisted calipers at a sweep speed of 200mm/s.

Electroanatomical mapping

Electroanatomical maps were created in sinus rhythm using the Carto XP system (Biosense Webster, Diamond Bar, CA) as previously described.[63] Right and left atrial maps were recorded using a 3.5-mm tip catheter ablation catheter (Navistar, Biosense Webster, Diamond Bar, CA) with a minimum of 80 equally distributed points collected in each chamber. Endocardial contact was confirmed with a combination of electrogram stability, fluoroscopy and the Carto point stability criteria (≤6mm stability in space, ≤5ms in local activation time). Location, voltage and activation timing were recorded at each point. For analysis the atria was divided as follows: Septal right atrium (RA); High lateral RA; Low lateral RA; Posterior left atrium (LA); Lateral LA; and Inferior LA. The following parameters were determined:

1. Regional Conduction velocity
    - The direction of conduction was determined from examination of isochronal maps. 3-5 pairs of points were taken in the direction of conduction, with velocity being calculated using the distance and timing as reported by the mapping system.
2. Signal fractionation

- This was defined as a signal greater than 50ms duration with at least four deflections.

3. Bipolar voltage.
    - Low voltage was defined as an area with three contiguous points with a bipolar voltage of <0.5mV.
    - Scar was an area with three contiguous points <0.05mV.

Effective refractory periods

Effective refractory period (ERP) was measured at three times the tissue capture threshold at a pulse width of 2ms, using a Micropace EPS 320 stimulator (Micropace, Canterbury, Australia). This was performed from the following 8 sites: Septal RA; High lateral RA; Low lateral RA; Posterior LA; Lateral LA; Inferior LA; Proximal coronary sinus (CS); and Distal CS. Eight S1 stimuli were delivered with S1 cycle lengths of 400 and 200ms, with incremental S2 impulses (10ms increments), commencing at a coupling interval of 100ms. The ERP was the longest S1-S2 interval without atrial capture. The mean of two attempts was recorded. An additional attempt was made if the difference between attempts was greater than 10ms.

AF induction

Inducibility of AF was determined using a burst pacing protocol from the left atrial appendage, performed at the terminal study. Twenty impulses were delivered at the lowest cycle length with 1:1 atrial capture. An episode was defined as irregular atrial activity lasting greater than or equal to 3 seconds. This protocol was repeated five times and the total duration was recorded. Sustained AF was defined as an episode lasting >10 minutes. In the event of sustained AF, no further testing was performed.

Haemodynamic recordings

Non-invasive systolic blood pressure and invasive mean right atrial and systolic right ventricular pressures via the SR0 sheath were recorded at the start of each procedure. Mean left atrial pressure was recorded immediately following transseptal puncture.

4.2.6 Terminal epicardial electrophysiological study

Left lateral thoracotomy was performed to access the left atrium. A custom made 90-pole plaque was placed on the left atrial appendage and connected to the computer-based digital amplifier/recorder system (LabSystem Pro, Bard Electrophysiology, Lowell, MA). Pacing was performed from each of the four corners of the plaque at 400ms. Epicardial electrograms were recorded and the following determined:

1. Effective refractory periods were measured using the same protocol as the endocardial study.
2. Conduction velocity was determined using activation maps analysed by custom software, as previously described.[59] The peak of the largest amplitude deflection on each bipolar electrogram was automatically annotated and manually verified. Local conduction velocity was calculated for each point from triangulated local vectors, allowing subsequent calculation of mean velocity for each map.
3. Conduction heterogeneity was calculated using established phase mapping techniques integrated into the software, as previously described.[59] Absolute conduction phase delay was calculated by subtracting the 5th from the 95th

percentile of the phase difference distribution (P_{5-95}). Conduction heterogeneity index was derived from dividing P_{5-95} by the median (P_{50}).

4.2.7 Cardiac MRI

Chamber volumes were measured using MRI (Siemens Sonata 1.5 Tesla, MR Imaging Systems, Siemens Medical Solutions, Erlangen, Germany). 6-mm slices were taken through the left atrium and ventricle. Images were taken using electrocardiogram-gating and periodic breath holding. Analyses were performed offline using CVI42 (Circle Cardiovascular Imaging Inc., Calgary, Canada).

4.2.8 Statistical Analysis

Normally distributed data was expressed as mean ± standard deviation and compared using unpaired t-test. Analysis of variance was used to detect differences between groups over time. Due to the dependence in observations from the same animal, conduction velocity and ERP across regions were analysed using a mixed effects model ANOVA. Sheep ID was used as a random effect and combinations of group, timepoint and region were used as fixed effects. A maximum of two fixed effects were entered into the statistical model at one time, with testing of both main effects and their interaction. If a significant interaction was present, post-hoc testing was performed. AF duration was non-parametric; therefore, log-transformation was performed prior to t-test comparison, with values expressed as median and interquartile range. P-values of less than 0.05 were considered statistically significant. Analyses were performed using SPSS version 23 (SPSS Inc. Chicago, IL).

4.3 Results

4.3.1 Weight, structural and haemodynamic parameters

Table 1 presents the changes in anthropometry, haemodynamic and cardiac structure in the two groups. Both groups increased in weight equally over 64 weeks. Left and right atrial pressures significantly increased with increasing weight, accompanied by a non-significant increase in systolic blood pressure and right ventricular systolic pressure. There was a non-significant increase in left atrial volume and left ventricular mass with increasing weight but no change in LV volume or function.

There was no significant difference in haemodynamics, chamber volumes, LV mass or function between treatment and control groups at any timepoint.

4.3.2 Electrophysiological parameters

Atrial conduction

With increasing weight, progressive slowing of atrial conduction occurred in both groups ($p<0.001$). Animals treated with tranilast had significantly less endocardial conduction slowing than controls ($p<0.001$) at both midpoint and endpoint, as shown in figure 2. On analysis by region at end point study, this change was found to be heterogeneous, with significant differences in conduction across atrial regions. In the left atrium, tranilast treatment was associated with relative preservation of conduction on the posterior (1.18±0.10 vs 0.96±0.06m/s, $p<0.001$) and lateral (1.19±0.06 vs 0.95±0.05m/s, $p<0.001$) walls. In the right atrium, the upper lateral (1.15±0.05 vs 0.89±0.12m/s, $p<0.001$) wall showed significantly higher conduction velocities in tranilast treated animals.

Similarly, epicardial conduction at the 64-week end point study was higher in the tranilast treated group (1.19±0.06 vs 0.95±0.05m/s, p<0.001), as shown in figure 3. This improvement in conduction velocity was homogeneous across all four plaque sites (site*group interaction p=0.21). Notably, there was no difference in conduction heterogeneity between treatment and control groups at any epicardial site.

Effective refractory periods

There was no significant change in ERP with increasing weight. At midpoint study, there was a significant reduction in atrial ERP following 200ms drivetrain in the tranilast treated animals, when compared with controls (136±10 vs 146±10ms). This was not seen with a 400ms drive train and was also not apparent at end point study.

Signal fractionation

Signal fractionation increased with increasing weight in both groups (p<0.001) as shown in figure 5. Tranilast treated animals had significantly less signal fractionation than control animals at 60 weeks (16.7±6.2 vs 29.3±8.8%, p=0.01).

Atrial bipolar voltage

There was no significant reduction in endocardial voltage with increasing weight or between groups. No areas of low voltage (<0.5mV) were seen in any animal.

AF inducibility

The tranilast treated group had significantly less AF during the induction protocol (12s [IQR 36] vs 43s [IQR 110], p=0.05), as shown in figure 6. Two animals (1 tranilast treated, 1 control) had sustained AF (>10mins) during testing and were excluded from inducibility analysis.

4.4 Discussion

This study gives us new information on the mechanism of the abnormalities in atrial electrophysiology seen in obesity. The following effects were seen with tranilast treatment:

1. Attenuation of the marked reduction in endocardial conduction velocity associated with increasing obesity, with associated reduction in signal fractionation. Interestingly, the reduction in conduction velocities with increased weight was seen across all atrial regions, whereas the animals treated with tranilast demonstrated a more heterogeneous pattern, with sparing of certain regions in both atria.
2. Higher epicardial conduction velocity at end point study. This was consistent across all 4 sites on the plaque. There was, however, no difference in local conduction heterogeneity at any site.
3. As a result of this, there was a significant reduction in AF inducibility in the treatment group.

Importantly, these results occurred without effect on haemodynamics or chamber volumes. There was also a minor, but significant, reduction in atrial ERP at midpoint study. However, this was only at one cycle length and similar differences were not seen at endpoint study. Contrary to this, when used in a canine heart failure model, this effect was not seen; rather tranilast appeared to protect against the reduction in ERP associated with the development of heart failure.[260] This may be a treatment effect, however, given the inconsistent changes across time and cycle length, conclusions must be drawn with caution.

Atrial substrate

Changes in atrial electrophysiology have been demonstrated with a wide variety of conditions. These were first noted secondary to prolonged rapid atrial pacing, resulting in reductions in atrial refractory periods and increased susceptibility to AF.[55] Subsequent studies used a heart failure model to induce structural remodelling, with increases in localised conduction slowing and fibrosis of atrial tissue, neither of which were seen in animals subjected to rapid atrial pacing alone.[58] We have previously demonstrated the development of conduction slowing, without ERP change in an obese ovine model. Our study again demonstrated this progressive conduction slowing with increasing weight, without changes in ERP.

TGF-β and cardiac fibrosis

TGF-β has long been recognised to be a central mediator of the fibrotic pathway. Its effect on infarcted, hypertrophic and cardiomyopathic ventricular tissue has been well characterised.[284] More recent work has investigated its relationship with atrial fibrosis. Overexpression of TGF-β in a transgenic mouse model induced heterogeneous conduction in the left atrium, slowed conduction in the right atrium and increased susceptibility to AF.[112] The cardiac profibrotic effects of TGF-β may be enhanced in atrial tissue.[113] Previous studies have demonstrated TGF-β inhibition to have a disease-modifying role. Pressure overloaded rats treated with anti TGF-β antibodies demonstrated dramatically reduced myocardial fibrosis.[259] Tranilast was found, in conjunction with TGF-β mRNA downregulation, to reduce ventricular fibrosis in one study of hypertensive rats,[285] along with reduced mortality.[286] It also prevented cardiac fibrosis in rats with diabetic cardiomyopathy, despite persistent

hypertension and hyperglycaemia.[287] Although initial human studies of its effect on coronary restenosis were encouraging[262], a subsequent multicentre, randomised trial reported no significant effect.[263] Recent investigation has found that it reduces both atrial fibrosis and AF inducibility when used in a canine heart failure model.[260] Our study is in keeping with these findings. Atrial remodelling was prevented by tranilast, as demonstrated by significantly improved endocardial and epicardial atrial conduction velocities in the treatment group. This corresponded with a reduced susceptibility to AF with tranilast treatment.

TGF-β and obesity

We have previously demonstrated the progressive up-regulation of TGF-β receptors in atrial tissue during weight gain, in both the intermediate and long term.[23, 24] This was associated with both increased atrial fibrosis and an increased susceptibility to AF. TGF-β is expressed in adipose tissue and the degree of expression correlates with BMI in human subjects.[209] [210] A recent study used an original atrial organo-culture model to demonstrated that TGF-β was secreted by human epicardial fat in small amounts and this was less than by subcutaneous fat. However, Activin A, a member of the TGF superfamily, was secreted by epicardial fat significantly more than in non-cardiac fat, and its presence induced TGF-β expression.[200]

The distribution of epicardial fat is not uniform. One study showed the SVC, left atrial appendage and left AV groove are adjacent to the largest fat depots.[288] Another found most left atrial epicardial fat was located in the inferior and posterior region, with minimal appendage and lateral fat. This study also demonstrated infiltration of fat into cardiac tissue, in particularly in the posterior wall of the left atrium, which showed marked differences in our study, when compared with the inferior LA.[24] This

may account for the heterogeneous nature of the conduction velocities in tranilast treated animals. Further investigation into the exact mechanism warrants further investigation.

Tranilast effects

Although the effect of Tranilast is predominantly on the TGF-β pathway, it has several other potentially anti-fibrotic and anti-inflammatory effects that may account for some of the observed effects. Macrophages exposed to tranilast demonstrated upregulation of the anti-inflammatory molecule heme oxygenase-1, downregulation of the pro-inflammatory substance cyclooxygenase-2. [289] This resulted in inhibition of prostaglandins, tumour necrosis factor and interleukin-1b and demonstrates the potential confounding effects of tranilast treatment. It also acts antagonistically on angiotensin II, inhibiting the its effects in vascular smooth muscle.[290] In a hypertensive rat model, it inhibited PDGF as well as TGF-β induced collagen synthesis.[291, 292] It may also attenuate the effect of connective tissue growth factor, as shown in renal tissue.[292] It is unclear whether these effects are directly due to tranilast, or whether they are secondary to TGF-β blockade, but further study may be warranted to isolated the exact mechanisms of pure TGF-β blockade.

Study limitations

We used a model of progressive obesity to determine the effect of tranilast treatment. In clinical practice, it is unlikely that treatment will be instated during weight gain rather than once an individual is obese and develops conditions related to this and extrapolation from our study to this likely clinical scenario should be

cautious. However, weight fluctuation is a common in obese individuals and it may be that anti-fibrotic treatments are of benefit in periods of significant weight change.

4.5 Conclusion

Treatment with tranilast attenuates the conduction slowing associated with obesity with minor effects on refractory periods. These conduction changes are heterogeneous across both atria. This reduction in atrial substrate results in reduced inducibility of AF. Tranilast treatment may be of benefit in obese patients with AF.

Table 1. Weight, structural and haemodynamic parameters.

Variable	Tranilast treated			Control			p value (groups)	p value (time)
	Baseline	36 wks	72 wks	Baseline	36 wks	72 wks		
Weight (kg)	64±7	90±10	99±12	63±7	86±7	94±7	NS	<0.001
sBP (mmHg)	85±14	89±10	94±12	83±18	87±9	92±11	NS	NS
RVSP (mmHg)	14±4	18±3	19±4	16±2	17±3	18±3	NS	NS
LA pressure (mmHg)	4.6±1.8	6.8±2.1	6.8±1.5	4.5±2.4	6.9±1.7	6.8±2.1	NS	0.05
RA pressure (mmHg)	2.7±1.9	4.5±1.5	6.6±2.3	2.7±1.6	5.0±1.2	5.0±2.9	NS	0.01
LA volume (ml)	50±7	57±7	61±5	51±11	57±7	57±11	NS	NS
LVEDV (ml)	105±15	131±15	131±19	112±22	129±23	125±17	NS	NS
LV mass (ml)	123±25	133±16	132±12	124±19	134±22	135±12	NS	NS
LVEF (%)	50±5	43±8	52±6	45±14	45±4	46±5	NS	NS

Figure 1. Study design.

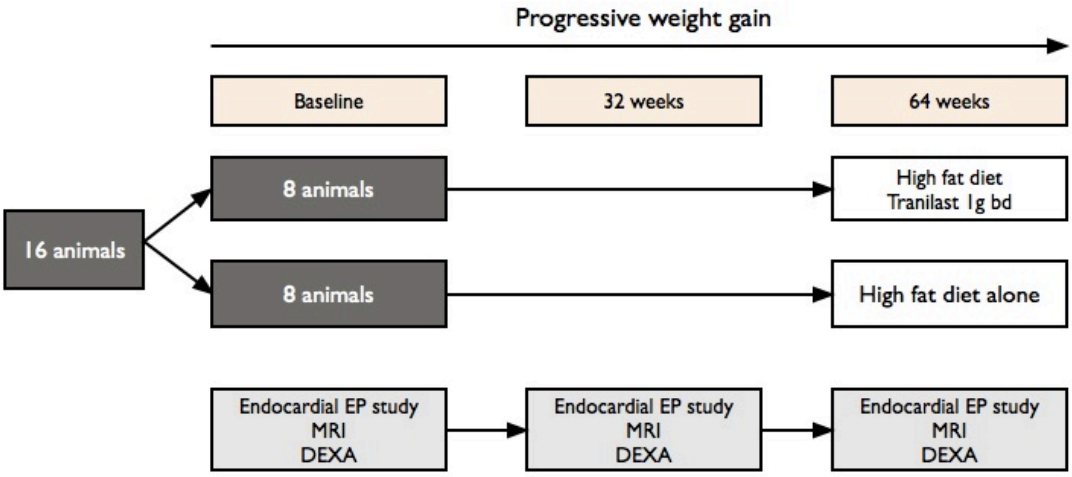
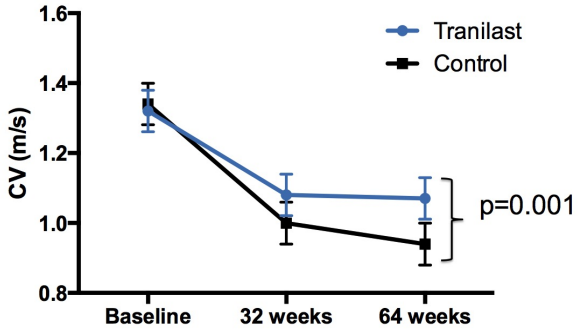


Figure 2. Conduction velocity (CV) at the study baseline, 32 weeks and 64 weeks (left) and by region at endpoint study (right).



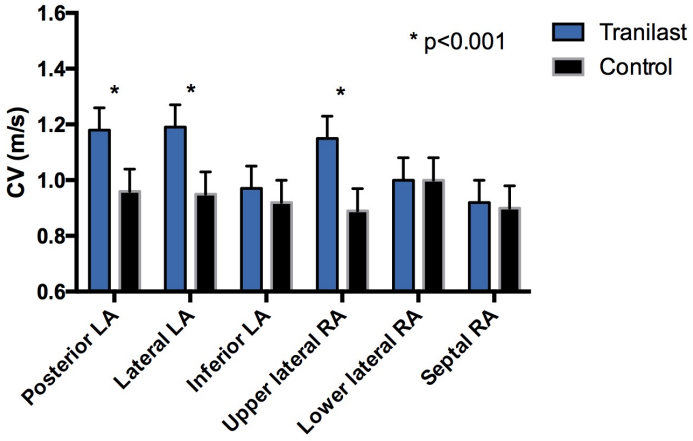


Figure 3. Epicardial conduction velocity (CV) at each site (left) and conduction heterogeneity (right).

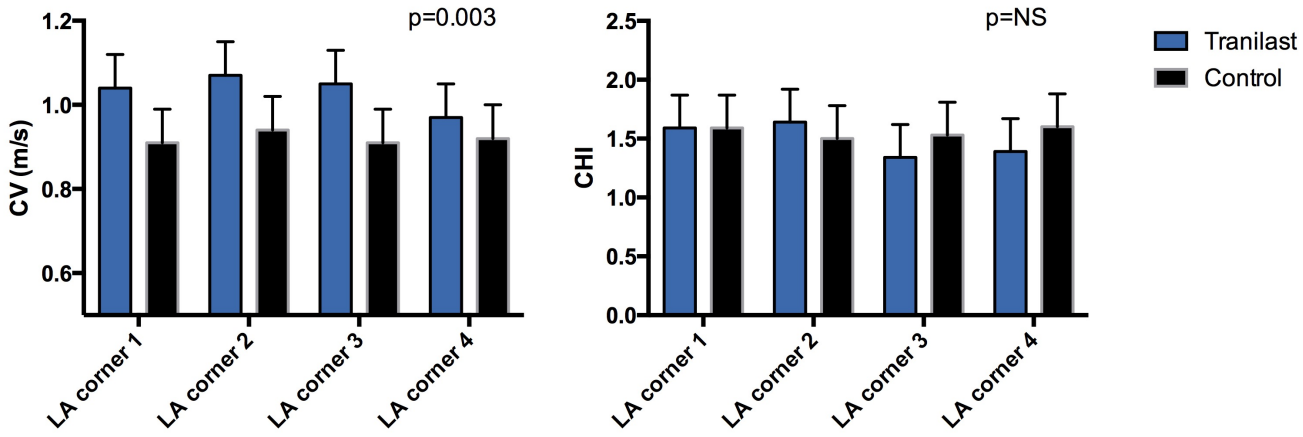


Figure 4. Effective refractory periods (ERP). Endocardial results at each timepoint (upper) and epicardial results at study endpoint (lower). Left panels are at a 400ms cycle length, right at 200ms.

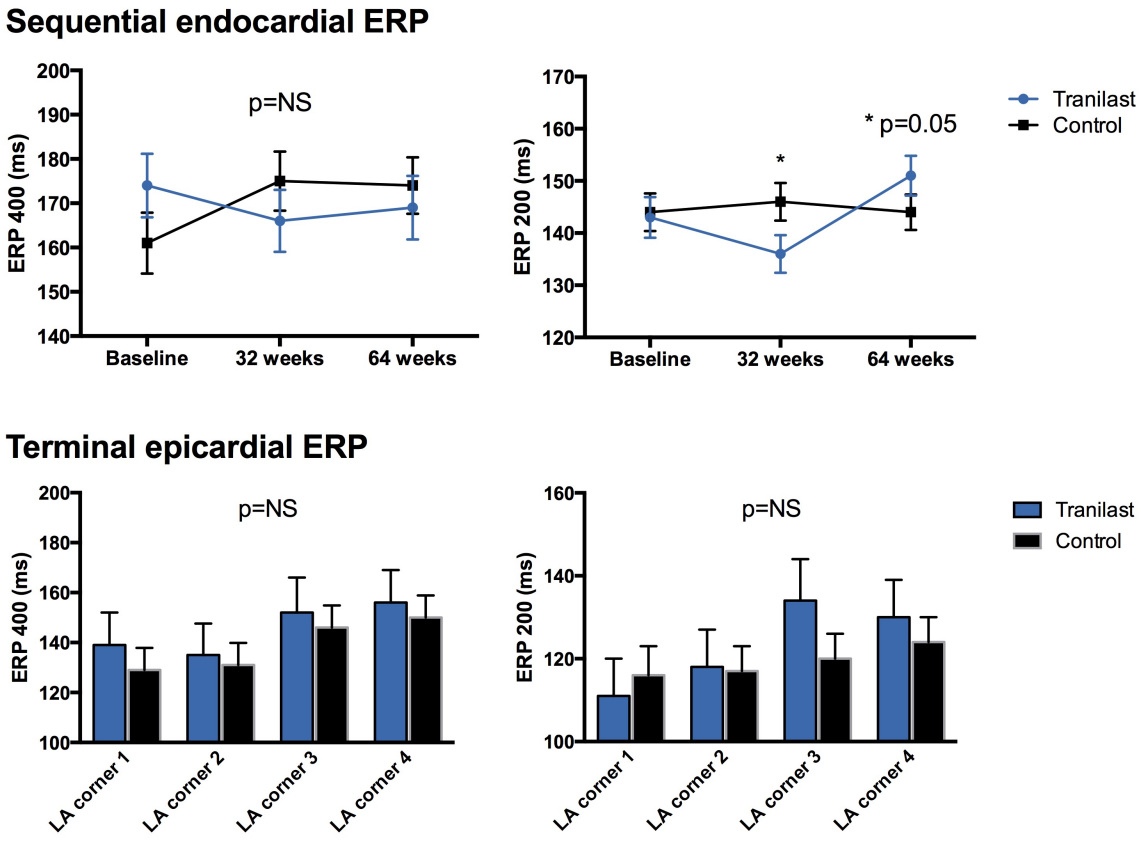


Figure 5. Proportion of fractionated signal at baseline, midpoint and endpoint.

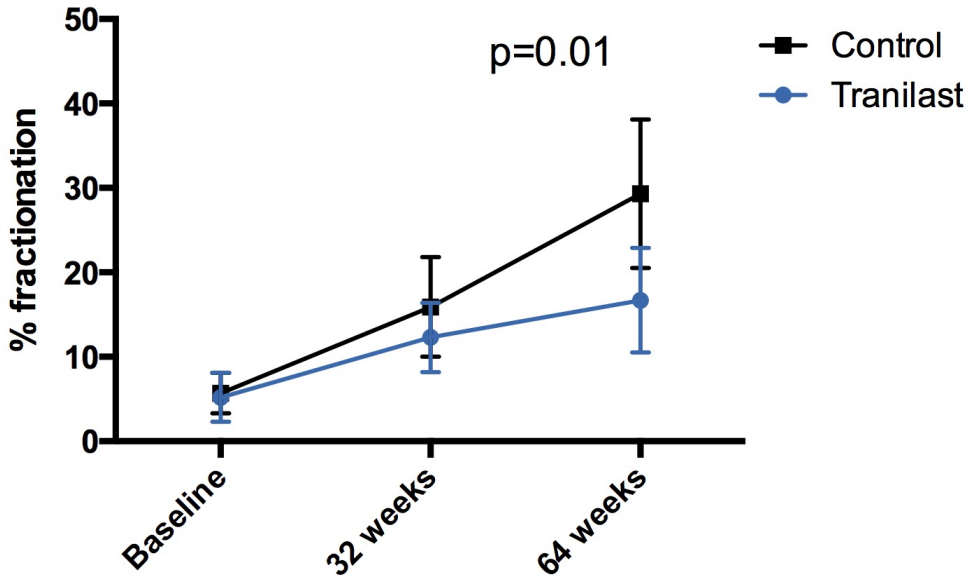
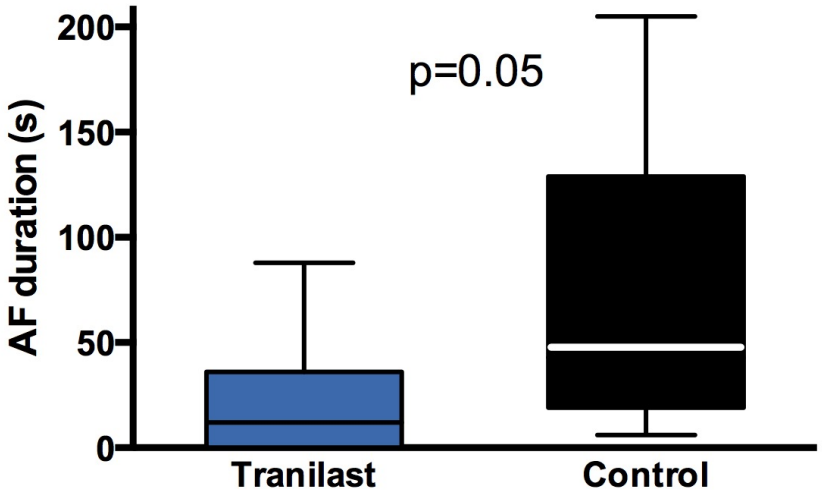


Figure 6. AF inducibility at end point study.



Chapter 5 – Conclusions and future directions

This thesis explores the basis of atrial remodelling in obesity. It examines the effect of weight fluctuation in the process of weight loss and evaluates the role of anti-fibrotics in the prevention of atrial remodelling in an obese ovine model.

The first study compared animals undergoing weight fluctuation with both lean and obese groups. Sustained obesity was associated with a homogeneous reduction in conduction velocities across all atrial sites, as seen in previous studies using this model.[23,24] Animals subjected to weight fluctuation also developed slowing of atrial conduction, but this was less severe and in a more heterogeneous fashion. This was most pronounced at the inferior wall of the left atrium, occurring after the first cycle of weight gain and loss. Following the second cycle, the posterior wall of the LA and lower lateral aspect of the RA were also affected. Epicardial conduction was similarly slowed and was markedly heterogeneous with weight fluctuation but again, the degree of conduction slowing was less than in persistently obese animals.

Additionally, the obese group were found to have reduced endocardial atrial refractoriness, but only after a very prolonged period of obesity. This has not been demonstrated in this obese model previously. Interestingly, previous studies have shown reduction in epicardial ERP alone with sustained obesity, a finding that was not seen in our study. We did, however find that weight fluctuation caused significant elevation of epicardial, but not endocardial, ERP. This highlights the complex and poorly understood discordance between endo- and epicardial electrophysiology. It has long been recognised that there are significant differences between the two surfaces, particularly in areas where the atrial tissue is thickened.[293] However, recent

data has been scarce and one current limitation of epicardial mapping is the limited area available for plaque placement and the difficulty in mapping both surfaces simultaneously. Studies to further characterise these differences could also help to determine the differences in local and systemic factors.

The next part of this thesis examined the effect of pharmacological intervention in the prevention of atrial remodelling, through blockade of both endothelin and TGF-β receptors, molecules that previous studies have shown to be upregulated in obesity.[23,24]

Endothelin receptor antagonism resulted in a marked, homogeneous improvement in conduction velocity across both atria, on both the endocardial and epicardial surface. Importantly, this effect was seen without any effect on haemodynamics or refractory periods and resulted in reduced AF inducibility. Histological analysis of atrial tissue demonstrated a significant reduction in interstitial fibrosis and inflammation with endothelin receptor blockade. Additionally, some insight was granted into the molecular pathways involved; importantly these effects occurred independently of the TGF-β pathway.

Blockade of TGF-β receptors was the focus of the final study. Again, this intervention was effective in reducing the development of obesity-related atrial substrate. In contrast to endothelin receptor blockade, however, this was in a more heterogeneous fashion, with individual areas of the atria demonstrating significant conduction changes, whilst others remained unaffected. There was no effect on haemodynamics with tranilast treatment, however a transient reduction in ERP at midpoint study was noted. A potential explanation for the variation in conduction is the paracrine action of adipose tissue. It is possible that areas in proximity to large

epicardial fat depots are affected differently to those that are not, resulting in more variation in conduction between regions. The direct action of fat on atrial tissue is an important area of study; recent data has used novel techniques to demonstrate that adipocytokines, including members of the TGF-β superfamily, are secreted by epicardial fat, with significant differences to those produced by subcutaneous fat.[200] Further investigation into the distribution and secretory actions of epicardial fat following pharmacological intervention is required to determine the relative local and systemic effect of each pro-fibrotic factor.

The findings of this thesis have important clinical implications. Treatment of obesity related AF is challenging; options are limited and mainly focussed on antiarrhythmic therapy and invasive ablation procedures. The first study could help counsel patients about to commence weight loss. It demonstrates that weight cycling is not an optimal pattern, however it is preferable to persistent obesity. The findings of the following two studies support the use of treatments to target the underlying atrial substrate in obesity and are suggestive that this strategy may reduce the burden of AF in this population.

Future directions

Future mechanistic studies should be directed at more focussed characterisation of the location and volume of fat depots to determine how some disease states and treatments result in a heterogeneous conduction pattern whereas others cause more homogeneous changes. Furthermore, detailed histological analysis and more complex tests of secretory function would grant additional insights into these effects. In addition, the reversibility of an established substrate with treatment using anti-fibrotic agents needs to be confirmed.

From a clinical perspective, both bosentan and tranilast are in routine clinical use for other conditions and have an acceptable side effect profile. Therefore, follow-on human clinical trials examining the effect of these treatments on obese individuals with AF are warranted to determine their efficacy in reducing AF burden. Although the results presented are in a model with progressive weight gain, it could be hypothesised that these interventions are able to reduce adverse atrial remodelling in individuals with stable obesity and this may be especially true in those who have increased adipocyte activity due to significant weight fluctuation. Another important finding of this thesis was that endothelin receptor blockade appears to act independently of the TGF-β pathway. This suggests that there could also be a role for combined treatment, targeting both pathways simultaneously.

In summary, this thesis highlights the detrimental effect of weight cycling on atrial electrophysiology, despite return to normal weight. It also demonstrates the beneficial effects of anti-fibrotic treatment in preventing atrial substrate and reducing AF burden in obesity.

Chapter 6 - References

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