ATRIAL ELECTROPHYSIOLOGICAL & STRUCTURAL CHANGES IN OBESITY & DIABETES MELLITUS

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To my beloved parents, Peter & Stella,

My sister Valerie,

& my partner Wei Wen

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ABSTRACT

Atrial fibrillation (AF) is the most commonly presented arrhythmia in the clinical setting, and its prevalence contributes significantly towards morbidity and mortality rates in the general population. Obesity and diabetes mellitus (DM, type I and type II DM) are recognised, well established independent risk factors of AF which can occur and contribute towards the development of AF both individually and in a concomitant fashion. The pathophysiological processes by which a proarrhythmic atrial substrate is produced in obesity and DM have not been fully elucidated. Further characterisation of the atrial substrate in obesity and DM induced AF is required.

Chapter one addresses the mechanistic components which may contribute towards establishing AF, and discusses the early and current insights underlying the pathogenesis of AF; This chapter describes the current literature available on the electrophysiological and structural components which may lead to the development of a vulnerable atrial substrate; these include the role of the action potential (AP), the relationship between the AP and the effective refractory period (ERP), and the contribution of inflammation and fibrosis towards AF development.

Chapter two investigates the feasibility and result of combined application of simultaneous high density conduction mapping with intracellular membrane potential recording to better understand the genesis and maintenance of arrhythmias in the isolated atria. Described are the ability to observe changes in action potential (AP) morphology at a given recording region, regional differences in AP restitution, lack of correlation between AP duration (APD) and the atrial effective refractory period (ERP), and AP alternans in amplitude, and, duration.

Chapter three assesses electrophysiological and structural changes in a rat model of type I DM (T1DM) using streptozotocin (STZ), which preferentially exerts toxicity to the insulin-producing beta cells of the pancreas to elicit the T1DM phenotype. This chapter demonstrates the impact of untreated T1DM on the atrial myocardium. At the structural level, T1DM animals demonstrated atrial cardiomyocyte hypertrophy with increased fibrosis. At the electrophysiological level, there was an abbreviation of the ERP with increased heterogeneity in conduction, as well as prolongation of the AP.

Chapter four describes the impact of obesity, type II DM (T2DM) and age on the electrical and structural properties of the atria using the Zucker (fa/fa) rat model. This chapter reports cardiomyocyte hypertrophy, increased fibrosis, prolongation of the APD, increased heterogeneity and slowed conduction, with differences in ERP between the left and right atrium of the DM animals. These results highlight the potential difference between the pathogenesis of T2DM from T1DM on the atrial myocardium in the predisposition towards development of AF.

Chapter five summarises the observations made in the T1DM and T2DM studies of chapters three and four respectively; this chapter discusses the similarities and differences shared in the data obtained from the studies, with a brief description of the potential mechanisms involved in DM-induced pathogenesis of AF, Additionally, the potential importance of segregating the diabetic states as having individual and differential influences on the atrial myocardium is highlighted. Future directions and areas of further research conclude this chapter.

THESIS DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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PUBLICATIONS AND COMMUNICATION TO LEARNED SOCIETIES

Chapter two

1. **Manuscript:** Simultaneous conduction mapping and intracellular membrane potential recording in isolated atria. Melissa Neo, David G. Morris, Pawel Kuklik, Dennis H. Lau, Hany Dimitri, Wei-Wen Lim, Prashanthan Sanders, David A. Saint. *Canadian Journal of Physiology and Pharmacology*, 2016, 94(5): 563-569, 10.1139/cjpp-2015-0194 (Accepted manuscript)
2. **Presentation:** Presented at the Cardiac Society of Australia and New Zealand Conference, August 2011, Perth, Australia
3. **Presentation:** Presented at the Faculty of Health Sciences Postgraduate Expo, August 2011, Adelaide, Australia

Chapter three

1. **Manuscript:** A Rodent Model of Streptozotocin-Induced Type 1 Diabetes: A Substrate for Atrial Fibrillation. Melissa Neo, Wei Wen Lim, Dennis H. Lau, Prashanthan Sanders, David A. Saint. (prepared in publication format)
2. **Presentation:** Presented at the Heart Rhythm Society Conference, May 2012, Boston, United States of America
3. **Presentation:** Presented at the the Asia Pacific Heart Rhythm Society Conference, October 2012, Taipei, Taiwan
4. **Presentation:** Presented at the Australian Physiological Society Conference, November 2014, Brisbane, Australia

5. **Presentation:** Presented at the Faculty of Health Sciences Conference, September 2014, Adelaide, Australia
6. **Presentation:** Presented at the Heart Rhythm Society Conference, May 2015, Boston, United States of America
7. **Presentation:** Presented at the Cardiac Society of Australia and New Zealand (CSANZ) Conference, August 2015, Melbourne, Australia

Chapter four

1. **Manuscript:** Obesity, Diabetes and Age: Risk factors for a proarrhythmic substrate for Atrial Fibrillation in a Rat Model of Type II Diabetes Mellitus. Melissa Neo, Wei Wen Lim, Dennis H. Lau, Prashanthan Sanders, David A. Saint. (prepared in publication format)
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6. **Presentation:** Presented at the Heart Rhythm Society Conference, May 2016, San Francisco, United States of America

PRIZES AND AWARDS DURING CANDIDATURE

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ABBREVIATIONS

Abbreviation	Explanation
AF	Atrial fibrillation
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
AP	Action potential
APD_{20}, APD_{50}, APD_{80}, APD_{90}	Action potential duration at 20, 50, 80, 90% of repolarisation respectively
AT1R	Angiotensin receptor type 1
BMI	Body mass index
CAF	Chronic atrial fibrillation
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
CHF	Congestive heart failure
CHI	Conduction heterogeneity index
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CV	Conduction velocity
Cx 40, 43	Connexin 40, 43
DAD	Delayed after depolarisation
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
EPS	Electrophysiology study
ERP	Effective refractory period
fa/fa	Zucker rat fatty gene
GLUT2	Glucose transporter 2
HbA1c	Haemoglobin A1c (Glycated haemoglobin)
hs-CRP	High-sensitivity C-reactive protein
I_{CaL}	L-type Ca^{2+} current
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon-γ
I_{K1}	Inward rectifier K^+ current

I_{Kr}	Rapid delayed rectifier K^+ current
I_{Ks}	Slow delayed rectifier K^+ current
I_{Kur}	Ultra-rapid delayed rectifier K^+ current
IL-1,6,8,10	Interleukin-1,6,8,10
I_{to}	K^+ transient outward current
IVC	Inferior vena cava
Kir	Inwardly rectifying potassium channel(s)
Kv	Voltage gated potassium channel(s)
LA	Left atrium
LDL-C	Low density lipoprotein-C
LV	Left ventricle
MCP-1	Monocyte chemoattractant protein-1
MEA	Multi-electrode array
NCX	Na^+/Ca^{2+} exchanger
NDP	NanoZoomer digital pathology system
NFκB	Nuclear factor kappa-B (light-chain-enhancer of activated B cells)
OSA	Obstructive sleep apnea
P_5, P_{50}, P_{95}	Phase mapping percentiles 5, 50 and 95
PAF	Paroxysmal atrial fibrillation
Pro-BNP	Pro B-type natriuretic peptide
RA	Right atrium
ROS	Reactive oxygen species
RV	Right ventricle
RyR	Ryanodine receptor
SAC	Stretch-activated ion channels
SERCA or SERCA2a	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SHIMP	Simultaneous high density mapping and intracellular membrane potential recording
SR	Sarcoplasmic reticulum
STZ	Streptozotocin

SVC	Superior vena cava
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β1	Transforming growth factor beta 1
TIMP-1	TIMP metallopeptidase inhibitor 1
TNF-α	Tumor necrosis factor-α
Zfr	Zucker fatty rat

CHAPTER ONE

AN OVERVIEW OF ATRIAL FIBRILLATION

1.1 Introduction

1.1.1 General introduction

Atrial fibrillation (AF) is characterised by rapid, irregular and chaotic beating of the atrial chambers of the heart, such that electrical impulses are conducted in a disorganised manner, resulting in desynchrony between atrial and ventricular conduction. This can lead to pooling of blood in the atria and pulmonary veins, increasing the risk of formation of blood clots which can lead to adverse events such as stroke, myocardial infarction and heart failure.

AF has been studied extensively from the early twentieth century, and despite advances in our understanding of the fundamental mechanisms underlying the arrhythmia, questions regarding alterations in atrial electrical and structural properties and the interplay between these properties still remain. An understanding of the key players involved in the process are important in ultimately establishing better risk stratification and therapeutic strategies for patients suffering from AF. These key players include modifications to ion channel gene expression, ion channel function and regulation, as well as the up/downregulation of key pro-fibrotic signalling molecules and their implications in altering the atrial structural myocardium. Heterogeneous alterations to the atrial electrostructural properties may help to explain the complexity of AF and the progression of an associated diseased substrate from paroxysmal AF to

permanent AF. Further advancements in our understanding of the pathophysiology of AF will inevitably enable improvements to be made in the clinical management of AF and in a patient's quality of life(1).

1.1.2 Epidemiological studies

AF is the most common cardiac arrhythmia presented clinically. It has a substantial impact on morbidity and mortality rates in the general population(2). Miyasaka *et al* (2006) investigated the trends in age-adjusted incidence of AF in a community in Olmstead County, Minnesota, over a 21 year period. They reported a relative increase in incidence rate of 12.6% over 21 years, further stating that if this increase was to persist, the number of persons affected by AF would increase to up to 3 fold, affecting approximately 15.9 million individuals by the year 2050(3). AF occurs in 6% of the general population aged 65 years and older and is the most frequent cause of strokes in the elderly. It has been estimated that the treatment and management of AF, in particular, hospital admissions, chronic disease management and related disabilities imposes a substantial annual cost of 1.25 billion to the Australian economy. Additionally, the public health burden of AF has continued to increase at a greater rate than that of other cardiovascular conditions such as heart failure and myocardial infarction. This cardiovascular disease is therefore not only a significant cause for concern for the health care system but also for the health of the general population(4).

AF is a heterogeneous disorder which when presented clinically may vary in origin, clinical profile and natural history(5,6). The genesis of the AF epidemic was initially attributed to an expanding elderly population contributing to the

increase in the incidence and prevalence of AF. In young patients without structural heart disease, AF usually presents itself in either the paroxysmal (self-terminating, lasting less than 7 days) or persistent (non-self-terminating, lasting greater than 7 days) form(7). However, with ageing in the elderly and the presence of concomitant structural heart disease AF usually occurs in the permanent form(8). In addition, previous experimental studies have shown that "AF begets AF", that this cardiovascular disease progresses both in frequency and duration, eventually establishing itself permanently(9,10). The predictors of AF progression have not all been fully identified. The 1998 Framingham study noted that even after adjusting for age, gender and other co-existing cardiovascular conditions, the prevalence of AF continued to increase. Importantly, this arrhythmia continues to remain underdiagnosed as patients are often asymptomatic with approximately 30% of AF patients being unaware of their diagnosis(11). Furthermore, Benjamin *et al* (1998) then showed that AF is independently associated with a 50 – 90% increased risk of premature death(12) and is responsible for approximately 10% of all stroke occurrences(13). This stresses the importance of aggressive intervention and prevention of the reversible risk factors of AF such as obesity, diabetes, obstructive sleep apnoea and hypertension in reducing the socioeconomic impact of the AF epidemic(14).

1.1.3 Treatment of AF

The treatment and management of patients with newly diagnosed AF fall under five domains as recently proposed in the 2016 European Society of Cardiology (ESC) Guidelines(15) (Fig. 1):

1. Acute rate and rhythm control to achieve haemodynamic stability and limit severe symptoms
2. Management of precipitating factors such as through implementation of lifestyle changes and treatment of accompanying cardiovascular conditions to achieve cardiovascular risk reduction
3. Oral anticoagulation therapy in patients at risk for stroke to achieve stroke prevention
4. Assessment of heart rate and need for rate control therapy to achieve improvements in the symptoms, and preservation of the left ventricular function.
5. Assessment of symptoms and determination for need of rhythm control via antiarrhythmic drugs, cardioversion, catheter ablation, surgery for AF to ultimately improve symptoms.

These domains can be briefly summarised into the following three approaches: rate control, rhythm control, and, anticoagulation therapy.

The objective of rate-control is to achieve rate-control of the ventricular response (without necessarily establishing sinus rhythm) by means of the use of beta blockers and channel blockers, which have been shown to elevate the risk of bradycardia and hypotension(16). In the setting of paroxysmal AF, rhythm-control is used to establish long term maintenance of sinus rhythm by means of anti-arrhythmic drugs such as amiodarone, which can have proarrhythmic effects in the ventricle(17,18), and, other adverse effects in the atria even at low doses of therapy(19).

With persistent AF, the main objective is to restore sinus rhythm by means of either electrical cardioversion (catheter and surgical ablation) or pharmacological

cardioversion(20). However, despite advancements in the understanding of AF and its underlying mechanisms, current treatment continues to be suboptimal, with available drugs which are not specific for atrial electrical activity.

Oral anticoagulation therapy lowers the risk of thromboembolic stroke(6,13,21), which is associated with a 2-6% annual incidence in patients with AF. Oral anticoagulation therapies include warfarin, aspirin, clopidogrel, and, more recently, new anticoagulants such as apixaban, rivaroxaban and dabigatran(22). Although prior studies have suggested of the underuse of warfarin in patients with chronic AF(23-26), administration of warfarin has previously been shown to prevent thromboembolic stroke in these patients(13,21,27-29). A 2008 meta-analysis on anti-thrombotic therapy and stroke risk reported that warfarin administration reduced the risk of stroke by 60% with 40% greater efficacy than anti-platelet therapy.

Additionally, recent studies investigating the molecular pathways involved in the risk factors of AF have provided mechanistic and electrophysiological insights into potential alternative/additional therapeutic interventions to reduce the risk of AF(30-32). Detail into the potential risk factors and their management will be addressed in the following section.

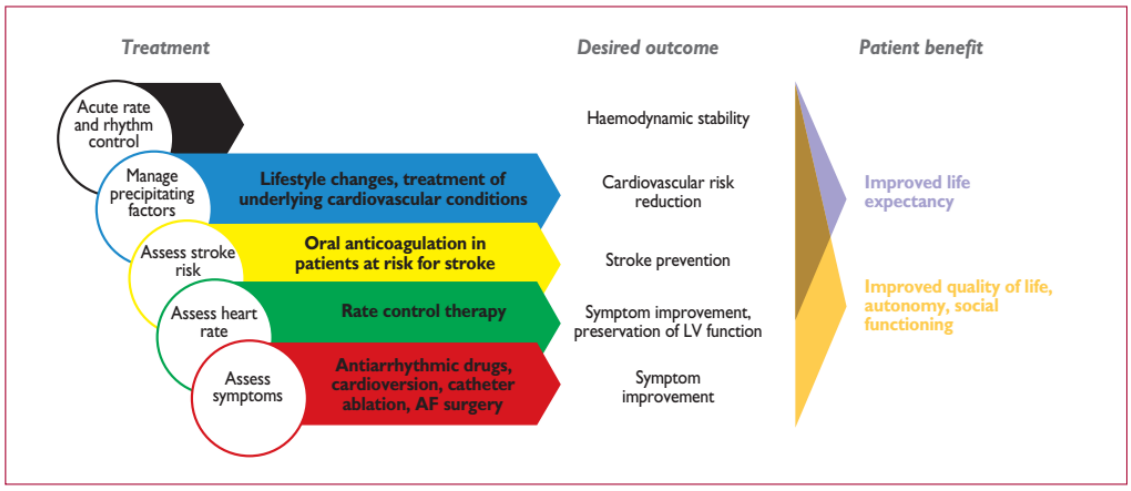

Figure. 1 Acute and chronic management of atrial fibrillation and associated desired cardiovascular outcomes and patient benefits. A brief summary illustrating the treatment strategies involved in the management of atrial fibrillation, beginning from acute rate and rhythm control to achieve haemodynamic stability, to implementation of lifestyle changes and the treatment of other contributing cardiovascular conditions, and, the assessment of stroke risk and symptom severity to ultimately reduce cardiovascular risk, incidence of stroke and achieve improvement in quality of life respectively. LV: left ventricular, AF: atrial fibrillation. **(**Extracted from Kirchoff *et al's* 2016 adaptation from the 4th AFNET/EHRA consensus conference report(33))

1.2 Risk factors of atrial fibrillation

Several risk factors of AF have been described in the literature. These risk factors can be divided into established (Table. 1A) and novel (Table. 1B) risk factors of AF and are briefly discussed below.

Several cardiac disorders have been known to predispose to AF; some of these include pericarditis, mitral valve disease, congenital heart failure and myocardial

infarction(14). Importantly, it has been suggested that AF is an independent risk factor for stroke, predisposing to thrombus formation with risk for thromboembolism and stroke as a consequential sequela. There is a 3 to 5-fold increased risk of incident stroke with AF. Comparative to other attributable stroke risk factors which decline with advancing age, the risk factors associated with AF, stroke risk and death increase dramatically with age from 1.5% to an almost 20-fold increase to 23.5% in those aged 50-59 years and 80-89 years respectively. Benjamin *et al* (1995) reported that left atrial enlargement was significantly related to stroke risk and death, further postulating that left ventricular hypertrophy may be a partial mediator of excess risk by means of left atrial dilation(34). Other clinical risk factors such as advancing age, hypertension, diabetes mellitus, and the presence of an inflammatory state have also been proposed to contribute towards elevated risk for AF development.

1.2.1 Advancing age

The risk of incident AF is related to age, becoming increasingly prevalent among those aged >70 years. Feinberg *et al* in 1995, and more recently Kannel & William (2008) reported that approximately 2.2 million people were diagnosed with AF in the United States(5,35); whilst the presence of AF was estimated to be approximately 2.3% in those aged 40 years, this increased to more than 2 fold (5.9%) in those aged 65 years and older, with approximately 70% of individuals with AF ranging between the ages of 65-85 years(5). Similarly, the increased stroke risk in patients with AF has also been described to be age-dependent. This highlights the potential risks and benefits in the prevention of stroke with antithrombotic therapy in individuals with advancing age. Warfarin is commonly

used in anticoagulant therapy due to its short-term risk of death and severe adverse events, and, its effectiveness in reducing risk for AF-related ischemic stroke. However, it is not without adverse effects, with intracranial haemorrhaging as the most dangerous complication of this anticoagulant; previous research has suggested that elderly patients appear to have a higher risk for haemorrhage(24,36,37).

Anyukhovsky *et al* (2002) proposed that the elevated risk of AF in the elderly may also be attributed to age-related changes in cellular electrophysiology(38). In their study, they investigated action potential characteristics in old canine atria wherein age-related changes in cellular electrophysiological properties may be conducive to a proarrhythmic substrate for AF. Here, they observed disturbances in atrial activation and repolarisation parameters in the older canine atria, reporting significant differences in the contour of the action potential between adult canine atria (aged 1-5 years) and older canine atria (aged >8 years)(38). Application of the L-type calcium current agonist Bay K8644 elevated the plateau and shortened the duration of the action potential further in the older atria compared to the younger adult atria. In contrast, application of nisoldipine, the L-type calcium current blocker, resulted in a depressed plateau in the younger adult atria with no significant differences in the older atria. These results are indicative of a decrease in the depolarising L-type calcium current in the aged atria. Furthermore, Anuyhovsky *et al* (2002) observed a reduction in conduction velocity with a wider time zone during early premature impulse conduction in the aged tissue(38). This was correlated with changes in the content and distribution of the connective tissue, that ultimately modify atrial geometry and performance; Anuyhovsky *et al*

(2002) reported that myocardial fibres were less compacted and the fibrous tissue content was significantly higher in the older atrial tissue(38).

In their more recent 2005 study on chronic AF-induction in aged canine atrial tissue, Anuyhovsky *et al* observed shortening of the action potential duration with a decrease in action potential adaptation as potential mechanisms for the maintenance of sustained AF in both younger and older adult atria(39). Importantly, they noted that whilst increased dispersion of repolarisation may be of importance for AF stabilisation in the younger adult tissue, fibrosis and slowed conduction of the premature impulse may be of importance in the older atrial tissue not only for the initiation of AF during sinus rhythm, but, also for the subsequent stabilisation of AF(39). These results were supported by Kistler *et al* (2004) who investigated the electrophysiological and electroanatomical changes in the human atrium with advancing age(40).

Kistler *et al* (2004), in their clinical study, reported an association between ageing with widespread conduction slowing and anatomically-dependent functional conduction delay and block, increased atrial effective refractory period, reductions in atrial voltage with discrete areas of low voltage, and sinus node dysfunction(40). They postulate that such changes may in part be responsible for the increased susceptibility towards atrial arrhythmias observed with increasing age(40). Taken together, the results from the aforementioned studies indicate that age-related changes in the electrophysiological and structural properties of the atrium may provide a substrate for multiple-wavelet re-entry, such that, even with the increase in refractory period observed by Kistler *et al* (2004), the

conduction changes observed overshadowed this potentially protective mechanism, in favour for the development of AF(40).

1.2.2 Hypertension

Hypertension is a well-established risk factor of AF(12,20,41,42). With nearly a quarter of the world's adult population having been diagnosed, the prevalence of hypertension is of significant cause for concern(43); On its own, hypertension is associated with higher risk for death from other causes as well and individuals with higher blood pressures would have a shorter lifespan, over which AF could develop(44). In women, previous work has shown that even at systolic blood pressures of pre-hypertensive levels <140mmHg, hypertension remained a strong and independent predictor of AF(41). Pre-hypertensive patients have been found to have greater left ventricular diastolic dysfunction and hence greater left atrial stress- such as alterations in left atrial volume to surface ratio- consequentially resulting in larger left atrial dimensions and atrial enlargement(41).

Kistler *et al* in 2006 assessed atrial electrical and structural changes in an ovine model of chronic elevated blood pressure; in this study, pregnant ewes were exposed to a 48 hour period of corticosteroid in their first trimester, and their offspring developed hypertension within the first few months of life which progressed with age(45). They reported in the hypertensive group, atrial myocyte hypertrophy and myolysis, increased collagen fibril deposition, with focal scarring, widespread conduction abnormalities, shortening of the atrial wavelength and an increase in AF duration(45). Similarly, Lau *et al* (2010) in a chronically

instrumented one kidney, one-clip ovine model of hypertension, reported that short-duration hypertension resulted in differences with atrial remodeling characterised by atrial dilatation, increased interstitial fibrosis and inflammation, together with slowed conduction and an increase in the refractory period(46). Following this study, Lau *et al* (2010) investigated the progression of hypertension-induced atrial remodeling(47). They reported elevated effective refractory periods, progressive bi-atrial hypertrophy, left atrial dysfunction with increased inducibility of AF and inflammation following 5 weeks of hypertension(47); Following a 10 week period of hypertension, the investigators observed significant slowing of conduction with increased heterogeneity alongside elevated levels of interstitial fibrosis and greater fractionation during episodes of AF(47). This study demonstrated the importance of hypertension-induced atrial remodeling, reporting that prolonged exposure to hypertension led to progressive remodeling, with atrial fibrosis and inflammation playing an important role in predisposing the atrial myocardium to regional differences in conduction abnormalities and more sustained AF(47,48).

Indeed, these findings were also reflected in the clinical setting, with Medi *et al* (2012), demonstrating an association between pulmonary hypertension and right atrial remodeling; The investigators reported slowing in right atrial conduction with marked heterogeneity in regional conduction together with a lowering in tissue voltage and the presence of regions of electrical silence(49).

1.2.3 Diabetes mellitus

Diabetes mellitus (DM) is a well-established, strong, independent risk factor of AF with an odds ratio of 1.0 - 2.2(42,50-52), and existing in 10-25% of AF

patients(12,53,54). 140 million people worldwide have been diagnosed with DM, and this number has been projected to increase to 300 million by the year 2025(55). Retrospective and epidemiological studies have previously shown that DM is associated with an increased incidence of stroke and death in patients with AF(20,54,56). DM is characterised by impaired glucose tolerance and insulin resistance/deficiency. It is also linked to a cluster of risk factors that fall under the term “metabolic syndrome”, such that metabolic syndrome can be defined by an accumulation of 3 or more of the following atherosclerotic risk factors: obesity (and by extension a proinflammatory state), insulin resistance, lipid abnormalities, elevated triglycerides, hypertension and impaired glucose(57,58). The population attributable risk of AF and metabolic syndrome was reported to be 22%(58). Of the aforementioned risk factors of metabolic syndrome, Chamberlain *et al* (2010) reported that although all components (excluding triglyceride levels) were independently associated with increased AF risk, elevated blood pressure contributed the most towards risk of AF development(58).

DM itself may cause metabolic stress on the atrium through its association with systemic conditions such as the presence of an infection and as a consequence, a proinflammatory state (the role of inflammation is later discussed)(52). Of particular importance, however, is the finding that the risk of AF development increased with a greater number of the aforementioned components of metabolic syndrome, with a 67% increase in risk of incident AF amongst individuals with metabolic syndrome(58,59). Additionally, it has been reported that the beneficial effects of monitoring the components of metabolic syndrome (such as HbA1c and LDL-C levels and blood pressure) to reduce the risk of cardiovascular disease is

effectively lost in patients wherein the goal levels for these components were not met(60). These findings highlight the need for the pathophysiological mechanisms underlying these components in predisposing the diabetic patient to AF to be further investigated.

In experimental models of diabetes, conduction slowing, interstitial fibrosis and greater heterogeneity in the atrial effective refractory period have all been implicated in creating an electrically unstable substrate, in favour of promoting arrhythmogenesis in the diabetic animals(61). In addition, the changes in diabetes-related autonomic function can also alter cardiovascular structure and function, ultimately leading to left ventricular hypertrophy, cardiac dysfunction and autonomic neuropathy. Such diabetes-related changes compromise the ability of the myocardium to remodel, recover and sustain functionality(62).

The contributing mechanisms of diabetes-induced increased susceptibility to AF will be explored in the following chapters. Focus in particular will be placed on the relationship between diabetes, obesity (a condition common to diabetes), and, inflammation (commonly present to both diabetes and obesity), as a potentially important link between diabetes-induced predisposition to AF.

1.3 Novel risk factors of atrial fibrillation

In recent years, substantial efforts have been made to improve our understanding of both the underlying mechanisms of AF and the role of potentially modifiable risk factors of AF. Novel risk factors such as smoking, inflammation, biomarkers such as N-terminal pro B-type natriuretic peptide (NT-proBNP), and early-life

antecedents such as low birth weight and childhood socioeconomic status have been identified(50,63). Of late, research has focussed on obesity and obstructive sleep apnoea as examples of novel risk factors of AF.

1.3.1 Obesity

Obesity is a worldwide public health crisis, with the prevalence of obesity increasing by 31% from 1976 to 1991 in the United States(64). With the continual urbanisation of society, the obesity epidemic may be attributed partially to societal changes that promote inactivity and food consumption such that, an approximate 66% of adults are currently overweight(65). In addition, obesity has been noted to occur in association with most of the risk factors which increases risk of AF; It has previously been shown in an experimental model that progressive obesity resulted in atrial functional, structural and electrophysiological remodeling by means or increased atrial size and volume, atrial interstitial fibrosis and inflammation, slowed atrial conduction with increased conduction heterogeneity and elevated levels of profibrotic factors(66). Obesity alone, has been associated with a subclinical low-grade inflammatory state, which promotes the production of proinflammatory factors that can contribute to the pathogenesis of insulin resistance, impaired glucose tolerance and subsequently lead to the development of DM(67). Studies on obesity have previously demonstrated the activation of the sympathetic nervous system(68), the renin-angiotensin-adlosterone system(69,70), promote oxidative stress(71) and endothelial dysfunction, linking obesity to the well-established aforementioned risk factor of AF- hypertension(72,73). Relling *et* al (2006) in their animal model of high-fat diet induced obesity reported in the absence of overt lipotoxicity, obesity led to

dyslipidemia, impaired cardiomyocyte function, mitochondrial damage with subsequent compromised cardiac function(74). More recently, it was also demonstrated that progressive obesity resulted in atrial functional, structural and electrophysiological remodeling by means of increased atrial size and volume, atrial interstitial fibrosis and inflammation, slowed atrial conduction with increased conduction heterogeneity and elevated levels of profibrotic factors(66). Importantly, evidence of weight-reduction-induced partial reverse-remodeling of the atrial substrate has been reported; Mahajan *et al* (2012) in their experimental model of obesity noted that improvement of connexin 43 expression to control levels with an approximate 30% weight reduction was associated with: a regression of fibrosis in the intercellular tissue, and an improvement in atrial conduction velocity with a reduction in fractionation(75). However, the investigators also reported that epicardial fat infiltration of the atria persisted despite this weight loss, further speculating that the epicardial fat infiltration may be responsible for forming areas of electrical silence in favour a vulnerable substrate for AF at this initial phase of weight loss(75). Despite the persistence in atrial epicardial fat infiltration, the results observed by Mahajan *et al* (2012) demonstrated the significance of weight reduction and reversibility of potential electroanatomical and histological substrates for AF(75).

It is important to note that obesity is often commonly associated with a myriad of accompanying conditions such as the aforementioned AF risk factors of hypertension and DM. Therefore, as management of AF continues to remain challenging, the need for modifying obesity as a risk factor of AF alone, or as an interventional and cautionary step towards preventing the accompanying risk

factors of hypertension and DM, has become an important component in the management of AF in these such individuals. Increased physical activity and/or the implementation of lifestyle and dietary changes may hence have important implications in reducing AF risk and the burden on the public healthcare system.

1.3.2 Obstructive sleep apnea

Obstructive sleep apnea (OSA) has been strongly associated with AF, and is both powerfully and causally related to obesity(76). It has been shown that although the traditional pathophysiological characteristics of obesity have been linked with OSA, OSA too is an independent risk factor of AF, possessing multiple pathophysiological mechanisms that may trigger and promote or maintain atrial arrhythmias. Hypoxemia, large fluctuations in intrathoracic pressure, increased cardiac wall stress, changes in sympathetic and parasympathetic responses and systemic inflammation have all been proposed to have a role. Importantly, Gami *et al* (2004) demonstrated a novel finding that OSA is more strikingly prevalent in patients with AF in comparison to high-risk patients with other cardiovascular diseases(77). Dimitri *et al* (2012) reported significant atrial remodeling in patients with OSA; in this study, atrial remodeling was characterized by atrial enlargement, lowered atrial voltage (indicative of the presence of scarring, fibrosis or a diseased substrate), widespread and site-specific alterations in conduction (notably, a greater number and duration of complex electrograms in the crista, and, slowed left and right atrial conduction velocity respectively) and longer sinus node recovery(78).

In the experimental setting, Iwasaki *et al* (2012) explored the relationship between obesity, OSA and AF; OSA was mimicked by stopping the ventilator supplying

intubated Zucker obese and lean rats, resulting in closure of the airway for 40 seconds(79). The study reported a higher percentage of AF inducibility during OSA in the obese rat compared to their control lean rats. Additionally, acute left atrial dilatation was observed in the obese rat as suggested by the increase in left atrial diameter during OSA(79). The investigators concluded that, in obese individuals wherein OSA may occur concomitantly, forced inspiration-induced increase in left atrial distensibility is related to diastolic dysfunction and may play an important role in the development of a substrate for AF. In an earlier study, Stevenson *et al* (2010) proposed that hypercapnia and not hypoxemia was responsible for the changes in atrial electrophysiology; hypercapnia was associated with prolongation of the refractory period, increased conduction time and delayed recovery of conduction(80). Upon return to eucapnia, vulnerability to AF increased significantly(80).

In the clinical setting, the risk of recurrent AF following cardioversion increases by 2 fold with untreated OSA; Treatment of OSA with continuous positive airway pressure (CPAP) therapy has been shown to lower the risk of recurrent AF(77). In patients with AF, the presence of severe OSA has been associated with AF ablation failure(81). These results are further supported by a 2013 study which demonstrated that CPAP therapy resulted in higher AF-free survival rate and AF-free survival off antiarrhythmic drugs or repeat ablation post pulmonary vein isolation (the cornerstone of AF ablation procedures, which targets the pulmonary veins as regions from which ectopic foci can arise to trigger AF). In addition, the investigators proposed limited value of pulmonary vein isolation in untreated OSA patients(82). As OSA is significantly under diagnosed and patients with OSA are

often left untreated, these results support the need for CPAP therapy in the treatment of AF patients with OSA to ultimately reduce the risk of recurrent AF. Additionally, it is clinically important to screen for the presence of OSA not only in patients with AF but also in those with other co-morbid conditions such as obesity, which itself, is often linked to secondary risk factors such as diabetes which as previously mentioned is also an independent risk factor of AF(76).

There is growing evidence linking OSA with diabetes, in particular, type 2 diabetes. Several studies have reported an inverse relationship between apnea-hypopnea index and insulin sensitivity, independent of age and body mass index (BMI)(83-85). Similarly, Stoohs *et al* (1996) reported that increased BMI accounted for the relationship between sleep disordered breathing and reductions in insulin sensitivity(86). Importantly, in another study, following adjustments for age, sex, race, fasting blood glucose, BMI and weight change, OSA and incident diabetes were found to be independently associated with a hazard ratio of 1.43 (confidence interval, 1.10-1.86). Furthermore, the investigators found that of the patients with more severe OSA, CPAP therapy was associated with attenuation of risk of developing diabetes(87). Similarly, Babu *et al* (2005) reported that HbA1c levels were reduced upon treatment of OSA. These results suggest firstly of the pathophysiological impact of OSA on mediating disturbances in glucose homeostasis, and secondly, of the implications of CPAP therapy in the treatment of OSA in patients with impaired glucose tolerance/fasting glucose levels to reduce risk of diabetes or delay the progression towards development of a diabetic state(88).

1.3.3 Summary

Recent work on weight loss management and reversal of the substrate for AF have been reported; Mahajan *et al* (2012) reported in an experimental model of obesity, an association between weight reduction and partial reverse-remodeling of both the electroanatomic and histological substrate for AF(75). In the clinical setting, Pathak *et al* (2014) reported that aggressive risk factor management was correlated with long-term success of AF ablation(89). Similarly, in 2015, Pathak *et al* reported that long-term management of goal directed weight loss led to significant reduction in AF burden and maintenance of sinus rhythm(90).

Together, all the aforementioned risk factors not only potentiate cardiac dysfunction at the early stages, but also in the longer term with compounding age. Early implementation of strategies which promote weight loss, slow the progression of diabetes and reduce the progression of the cardiovascular risk factors may improve long-term outcomes for 'at risk' AF patients within whom these concomitant conditions may exist.

The tables below show the hazard ratios of some of the established and novel risk factors of AF as compiled by the German Atrial Fibrillation Competence NETwork and the European Heart Rhythm Association(91). The hazard ratios listed were determined individually by each study and ranged between 1.03-3.3 depending on the type and protocol of each study and the duration of follow up. Tables 1A and 1B help illustrate that the treatment of traditional (established) risk factors for cardiovascular disease alone may not be effective in reducing AF risk. The presence of many other contributing risk factors (such as the novel risk

factors in Table. 1B) may have additive/cumulative or interrelated interactive effects that increasing the risk of AF development. Table 1B in particular draws attention to emerging risk factors for AF that may have received less attention, but whose treatment may aid in relieving the increased prevalence of AF in the general population.

Established risk factors for atrial fibrillation	
Risk factor	**Hazard ratio**
Age	
Benjamin *et al*(12)	2.1/2.2
Furberg *et al*(11)	1.03
Psaty *et al*(92)	1.1
Verdecchia *et al*(93)	1.8 (PAF) 2.9 (CAF)
Schnabel *et al*(94)	2.3
Gami *et al*(95)	2.0
Aviles *et al*(96)	1.4
Marcus *et al*(97)	1.1/1.1
Chamberlain *et al*(98)	2.1-5.9
Hypertension	
Benjamin *et al* (12)	1.5/1.4
Furberg *et al*(11)	1.4
Krahn *et al*(42)	1.4
Psaty *et al*(92)	1.1
Schnabel *et al*(94)	1.2 1.8 (Treated hypertension)
Rosengren *et al*(99)	1.7 2.1 (Treated hypertension)
Gammage *et al*(100)	1.4

Aviles *et al*(96)	1.3
Marcus *et al*(97)	1.5
Chamberlain *et al*(98)	1.4/2.2
Diabetes	
Benjamin *et al*(12)	1.4/1.6
Gammage *et al*(100)	2
Marcus *et al*(97)	1.5 2.1
Chamberlain *et al*(98)	1.9

Table. 1A Established risk factors for incident atrial fibrillation

Abbreviations table 1A: (CAF) chronic atrial fibrillation; (PAF) paroxysmal atrial fibrillation (Adapted from Kirchhoff *et al* 2011)

Novel risk factors for atrial fibrillation	
Risk factor	**Hazard ratio**
Obesity/BMI	
Krahn *et al*(42)	1.3
Tsang *et al* (101)	1.5 (BMI >30)
Tedrow *et al*(102)	1.2/1.7 (BMI >25/ > 30)
Frost *et al*(103)	2.3/2.0 (BMI <30 M/F)
Schnabel *et al*(94)	1.2
Rosengren *et al*(99)	1.7 (BMI > 27.5)
Gami *et al*(76)	1.1 (Only <65 years per 1kg/m2)
Wang *et al*(104)	1.5/1.5 (BMI >30 M/F)
Dublin *et al*(105) per unit BMI	1.03
Obstructive sleep apnoea	
Stevenson *et al*(106)	3.0
Gami *et al*(95)	3.3 (Only < 65 years)
Gami *et al*(77)	2.2

Table1B. Novel risk factors for incident atrial fibrillation

Abbreviations: (F) female; (M) male; (BMI) body mass index; (Adapted from Kirchhoff *et al* 2011)

1.4 Mechanisms of AF

1.4.1 History

The knowledge of fibrillatory conduction of the heart muscle dated back to 1850, during which Hoffa and Ludwig produced irregular and weak contractions in the rabbit ventricle using repeated electrical shocks. However, it was in 1874 that the term “fibrillation” was coined by Vulpian who observed and described the irregular, incoherent muscular movements which became fibrillatory contractions of the dog ventricle as “fremissement fibrillaire”(107).

In 1909, James Mackenzie, a Scottish general practitioner in England identified the “A wave” by using an ink-writing polygraph to record and label venous and arterial pulses from patients who presented with irregular pulses(108). He postulated the concept of auricular paralysis to explain the disappearance of the pre-systolic jugular A wave in a female patient who had mitral stenosis with previously regular rhythm and a pre-systolic jugular A wave(109). However, although the autopsy findings of a thin, distended auricle supported this, Mackenzie later questioned his initial findings upon discovering auricular hypertrophy in a patient with the same arrhythmia as well as the restoration of the A wave in patients who experienced irregular rhythms intermittently(109).

It was in 1906 that Arthur Cushny suggested that the fibrillatory contractions he observed in open-chested dogs were similar to the radial artery tracings observed in a woman with delirium auriculae (a condition where the atrial heartbeats follow each other in complete irregularity)(109). Together with the experimental expertise of Thomas Lewis in animal work and cardiac irregularities, and, Willem

Einthoven's invention of the string galvanometer, the irregular waves recorded from a patient with pulsus irregularis perpetuus were found to correspond to the fibrillatory movements Lewis observed in his animal studies. They concluded that the fibrillatory waves on the electrocardiograms corresponded to the fibrillatory movements in the auricle, that this observed irregularity is resultant of auricular fibrillation (atrial fibrillation)(110).

1.4.2 Early insights: rapid atrial ectopic focal activity and single circuit re-entry

In general, there have been two longstanding schools of thought describing the occurrence of AF: a single rapidly firing ectopic focus, and, the presence of multiple re-entrant waves.

Speculation of the underlying mechanism(s) of AF has evolved over time with Garrey proposing in 1914 that AF was due to a "series of ring-like circuits of shifting location and multiple complexity" and that a critical mass of tissue was necessary to establish any form of fibrillation(107). Similarly, Lewis proposed a single circus (Fig. 2) movement during AF with gross changes in the path, which "is used over and over again". Common to both theories is the concept of a single re-entrant circuit generating (Fig. 2) waves with cycle lengths too short to allow for 1:1 conduction and fibrillatory conduction occurs (Fig. 3).

Figure. 2 Leading circle re-entry Schematic diagram illustrating leading circle re-entry. Activity establishes itself in the smallest pathway that can support re-entry forming a single circuit as illustrated by the large black arrow. The small arrows inside the circuit indicate centripetal wavelets which continually emanate to form the central core of the circuit where the tissue is in a refractory state. (Extracted from Comtois *et a*l 2005 Europace(111))

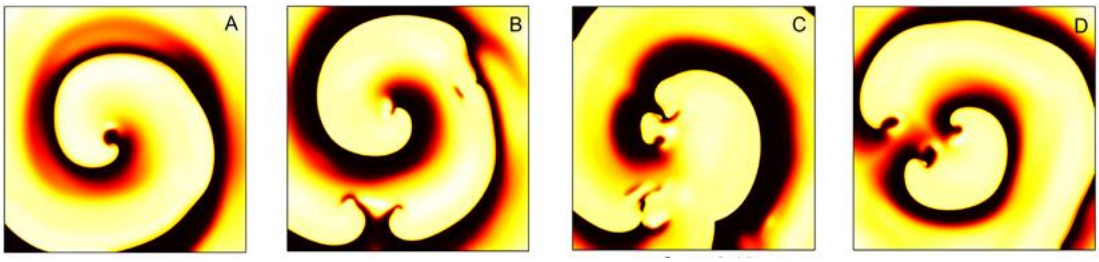

Figure 3. Series of panels showing 2-Dimensional progression from a spiral wave to the initiation of wave breakthrough and the formation of successive multi-wavelets. (A) Single spiral wave (B) Initiation of wave break through (C-D) Formation of multiple wavelets of re-entry. (Extracted from Seenivasan *et al* 2015 Front Physiol(112))

Animal studies investigating the application of aconitine to rabbit atrial tissue(113,114) have shown that a single organised source of re-entrant activity with a regularly firing rapid atrial focus was capable of establishing and sustaining

AF. A focal ectopic occurring outside the sinus node can arise from abnormal impulse formation.

Abnormal focal activity can arise outside the sinus node by means of: (1) an increase in the slope of the inward current for depolarisation, which allows the cell to reach threshold earlier, resulting in the generation of rapidly firing ectopic action potentials; and, (2) afterdepolarisations.

In cardiac cells, intracellular Ca^{2+} is removed from the cell to a large extent by means of an electrogenic process via the transmembrane Na^{+}/Ca^{2+} exchanger (NCX). Three Na^{+} ions are exchanged for a Ca^{2+} ion by using the energy stored in the electrochemical gradient of Na^{+} such that Na^{+} ions flow down its gradient across the plasma membrane in exchange for a Ca^{2+} ion, producing an inward current during removal of Ca^{2+} ions.(115) Afterdepolarisations can occur in conditions that enhance NCX activity; excessive intracellular Ca^{2+} will lead to Ca^{2+} extrusion and an increased inward Na^{+} current which can depolarise the cell to generate an afterdepolarisation. Spontaneous action potentials can then result if the afterdepolarisations reach the threshold potential(116).

1.4.2.1 The multiple wavelet hypothesis

The concept of multiple re-entrant waves remains the long standing theory for AF initiation, and, following Allessie *et al*'s (1976) work in multisite mapping(117), this theory became more widely accepted. Lewis, was the first to propose in 1920 that "we might even assume several circuits, completely or transiently independent of each other, and each controlling for a time material sections of the

muscle”(118,119). In 1959 Moe and Abildskov observed multiple re-entrant wavelets through cholinergic-induced AF in canine hearts. They proposed that AF consisted of randomly distributed re-entrant wavelets which follow pathways that are determined by local atrial refractoriness and excitability; the tissue will more likely still be refractory with longer periods of refractoriness such that the circulating impulse will not be able to reactivate the tissue at a particular site and die out(120).

Allessie and colleagues proposed Moe and Abildskov’s concept as, the “wavelength of re-entry”, whereby the wavelength is the shortest path length capable of sustaining re-entry(121,122). They hypothesised that functional re-entry is able to establish itself naturally in the smallest circuit of the same size as the wavelength. The number of re-excitation waves which can then be accommodated is thus dependent of the size of the atria and the wavelength of the electrical impulse; a smaller wavelength would consequentially require a smaller circuit size, thereby increasing the number of circuits that can be accommodated within the atria, which is ideal for multiple circuit re-entry and the persistence of AF.

1.4.3 Recent insights: rotors and wavelet breakthrough

Recent evidence has suggested focal ectopic activity, single circuit re-entry, and, multiple circuit re-entry may all be involved in the development of AF. The term ‘rotor’ has, in particular, become a household name in the field of cardiac electrophysiology; It refers to an organised source of functional re-entry pertaining particularly to tachycardia and fibrillatory activity. The concept of the

rotor in generating spiral waves was discovered in the late 20th century by Krinsky(123) and Winfree(124), and demonstrated by Davidenko in isolated sheep ventricular muscle in 1990(125). Since then, the major role of rotors as the drivers of cardiac fibrillation in both the experiment and clinical setting has been a focus of investigation(126).

A rotor consists of the curved wavefront and tail of the wave meeting each other at a single point, whereby the tissue at the centre does not remain refractory(127). A spiral wave on the other hand is a two-dimensional representation of the curved vortices generated by the spinning of the rotor (Fig. 3A)(128). The three-dimensional representation of the spiral wave, known as the scroll wave (Fig. 4, left), depicts at the centre of its rotation, a hollow filament (the spiral core) formed by the spiral tip's trajectory as it revolves(Fig. 4, left)(129). This movement of the spiral tip at a singularity point (i.e phase singularity) can be tracked using a technique developed in 1998(130).

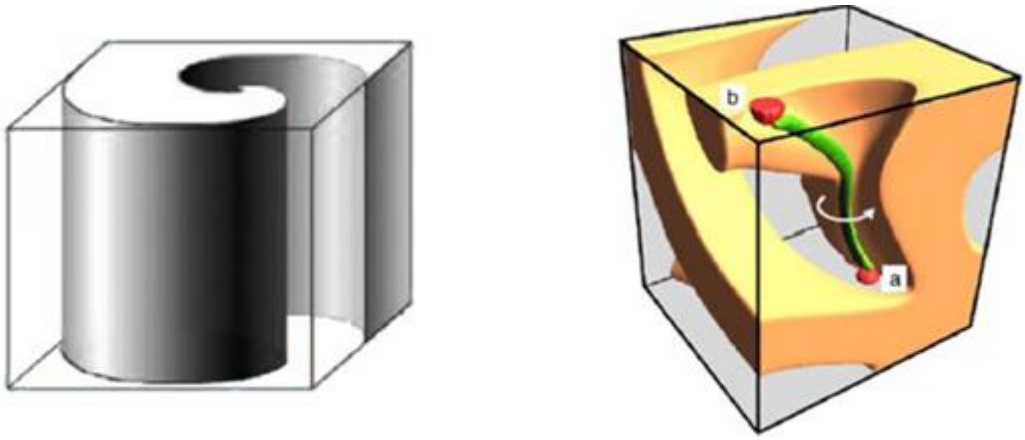

Figure. 4 3-Dimensional representations of rotor mechanisms. Left: Computer model generated representation of a 3-dimensional scroll wave.

Right: Endocardial and epicardial termini points about which the functional re-entry rotor pivots. (Extracted from Krummen *et al* 2015 J Thorac Dis(131))

When the rotor is stationary, it pivots as a phase singularity around a circular path forming the core of the spiral wave as described earlier (Fig. 4, left). If the rotor here spans from the epicardium to the endocardium, then the filament of this spiral would take on a linear cylindrical I-shape (Fig. 4, right). However, when the pivoting point meanders, the trajectory of the rotor can adopt a variety of complex shapes and the phase singularity in this case would move along a trajectory which is dependent on the degree of meandering; the filament here would be a line(126) (Fig. 5).

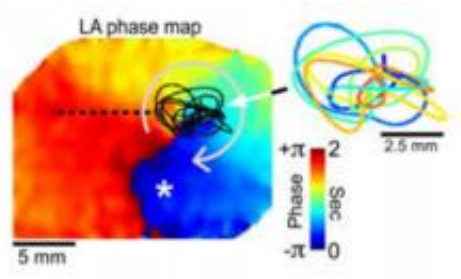

Figure 5. Example of a meandering rotor during an episode of atrial fibrillation in the isolated sheep heart. Optical map of meandering rotor captured during an episode of atrial fibrillation in an isolated sheep heart experiment. Left: Atrial phase snapshot showing re-entrant activity in the left atrial free wall. The inset shows the time-space trajectory of the meandering tip. (Extracted from Pandit & Jalife 2013, Circ Res(126))

Early work by Schuessler *et al*'s 1993 study which challenged the widely accepted concept of two-dimensional atrial activation supported the

aforementioned concept of the three-dimensional nature of rotors; they found a discordance (breakthrough, Fig. 6) in the activation of the epicardium and the endocardium, such that areas with wall thickness >0.5mm presented with greater discordance in rotor activity, highlighting that atrial anatomical dissociation and transmural activation may be crucial in the event of functional block, particularly in sites where the three-dimensional anatomy of the atrium is more complex(132). This was supported by Gray *et al* (1996) who explored the notion that the complex anisotropic structure of the atria is involved in wave propagation(133). They observed incomplete re-entry during AF and its initiation, and that activation did not occur continuously along the re-entrant circuit. This was further supported by the histology which suggested that the thin lines of conduction block observed corresponded with anatomical heterogeneities on the endocardium; more specifically, the sites which showed frequent breakthrough activity were likely related to the major complex and inhomogeneous network of the pectinate muscle bundles, indicating the presence of transmural activation(133).

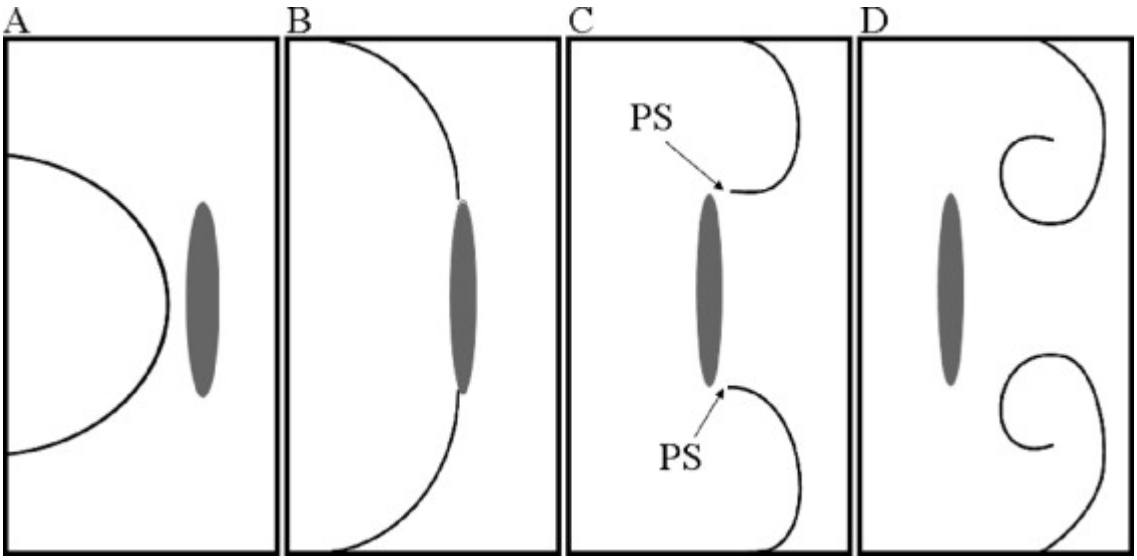

Figure. 6 A step-wise illustration showing the formation of a wavebreak when a wavefront approaches an anatomical obstacle. (A) Movement of a single wavefront towards an anatomical obstacle (shaded grey area) (B) Wavefront meets the obstacle and attempts to circumvent it. **(**C) Wavefront breaks into daughter wavelets which detach from the obstacle forming phase singularities (PS) at each broken end.(D) Each wavelet curls around their respective PS and rotate, producing a figure of 8 pattern **(**Extracted from Vaquero *et al* 2008 Heart Rhythm(127))

In the clinical setting, it has been found that in ventricular tachycardia, a combined endo-epicardial ablation approach was associated with fewer re-admissions and repeat ablations for ventricular tachycardia(134). Importantly, more recent and elegant work by de Groot *et al* (2016) demonstrated the first evidence of asynchronous activation of the endo-epicardial wall during AF in humans(135). This dissociation in the activation of the endo-epicardial wall conduction, when taken together with transmural activation not only provides new sites for re-entry, but may also play a role in explaining why pharmacological intervention with antiarrhythmic drugs may have failed in some AF patients. This highlights the need for better understanding of the quantitative contribution of the aforementioned mechanisms leading to the development and perpetuation of AF(135).

1.4.4 Atrial remodeling in atrial fibrillation

1.4.4.1 Electrophysiological remodeling in AF

AF has previously been suggested to be a self-perpetuating cardiac arrhythmia. Termed “AF begets AF”, the tachyarrhythmia itself can contribute towards changes in the electrophysiological milieu that further perpetuates and sustains AF(10). Morrillo *et al* (1995) demonstrated that chronic rapid atrial pacing would lead to modulation of the atrial substrate such that a reproducible model of sustained AF can be established(136). The investigators demonstrated that the shortest cycle length for AF was found in the posterior left atrium, and postulated that this area may be critical in the perpetuation of AF(136). Following this, Wijffels *et al* (1995) skilfully demonstrated that in chronically instrumented goats, electrical burst stimulation to the atria led to shortening of the atrial effective refractory period (ERP), prolongation of AF duration, and, with longer periods of rapid pacing, progressive increases in spontaneous maintenance of AF which became persistent within 1-2 weeks(10). Additionally, long-term atrial tachycardia has also been shown to prolong conduction of the atrial impulse, which, together with increased heterogeneity in spatial and refractory properties of the atria promotes multiple circuit re-entry.

In the clinical setting, success in the restoration and maintenance of sinus rhythm has been inversely correlated with the duration of the arrhythmia. Indeed, the studies discussed above have demonstrated this. Interestingly, Yu *et al* (1999) demonstrated that the shortening of the atrial refractory period occurring in chronic AF was reversed following cardioversion, with the atrial refractory period reaching a level comparable to that of the control subjects(137). As maintenance of sinus rhythm here is associated with prolongation of the atrial refractory period,

these results suggest that the electrophysiological changes following AF may in fact, be reversible. By deduction, this would suggest that sinus rhythm may beget sinus rhythm.

In contrast, in assessing the efficacy of the implantable cardioverter defibrillator in AF patients, Spurrell *et al* (2004) found that in the majority of AF patients, regular use of the defibrillator did not result in prolonged periods of sinus rhythm between shocks.(138) The investigators reported that following consistent repeated recurrences of AF, there was little variation in the duration of sinus rhythm between the AF episodes. Furthermore, in a small number of patients in which regular use of the device did result in prolongation of sinus rhythm, this occurred between delivered shocks, suggesting that sinus rhythm was not fully restored, and, that sinus rhythm does not beget sinus rhythm. These results suggest that remodeling of atrial electrophysiological substrate may have occurred in these patients(138).

Ausma *et al* (1997) found that atrial tachycardia alone was sufficient to cause atrial ultrastructural changes including amongst others, mitochondrial swelling, myocyte hypertrophy, sarcoplasmic reticulum degeneration(136,139,140), and alterations in the expression of gap junctional proteins connexins 40 / 43(141,142). Studies have also since shown that the proarrhythmic effects of rapid atrial pacing are resultant from tachycardia-induced remodeling of the ion channels.

1.4.4.2 Ion channel remodeling in tachycardia-induced AF remodeling

Modification of ion channel function such as an approximate 70% reduction in the Ca^{2+}-independent transient outward current (I_{to}) and L-type Ca^{2+} channel (I_{CaL}) density have been observed to occur following 6 weeks of tachycardia(1). There is evidence that both I_{CaL} and abnormalities in Ca^{2+} handling are responsible for tachycardia-induced atrial remodeling.

Whilst the application of nifedipine (10 μmol^{L-1}, an inhibitor of I_{CaL}) to a control cell elicited action potential changes akin to those produced by atrial tachycardia, application of BayK8644 to increase I_{Ca}, and inhibition of I_{to} with 4-aminopyridine failed to reproduce the abnormalities produced by atrial tachycardia; these results are indicative of the role of I_{CaL} in altering atrial action potential parameters and the effective refractory period during tachycardia-induced atrial remodeling(143). On the other hand, administration of ryanodine, a sarcoplasmic reticulum Ca^{2+} release inhibitor, to tachycardia-induced remodelled myocytes demonstrated action potentials with morphologies similar to control myocytes, suggesting that Ca^{2+} handling may also be important in atrial electrical remodeling(144).

In the early phase of a rapid paced cultured atrial myocyte model, Ji *et al* (2013) reported reductions of the L-type Ca^{2+} α1c subunit and the K^+ channel Kv 4.3(145). Similarly shortening of the atrial refractory period in paroxysmal and persistent AF have been attributed to alterations in expression levels of K^+ channels, Kv 4.3, Kv 1.5, HERG, mink/KvLQT1, Kir 3.1 / Kir 3.4 and Kir 6.2(146-149). Interestingly, a reduction in mRNA and protein levels were found for several K^+ channels in persistent AF patients. This is in opposition to the reduction in

atrial refractory period found in AF. It has been suggested that this occurs secondarily to reductions in the L-type Ca^{2+} channel, and, that reduced K^+ channel expression levels may have occurred in adaptation to the high pacing rate and to counteract abbreviation of the atrial refractory period(146,150). In addition, studies investigating the role of the Na^+ current ($I_{Na)}$ in atrial myocytes of dogs subjected to rapid pacing the atria, the progressive reduction of I_{Na} density was paralleled with slowing in conduction(151), suggesting that changes in I_{Na} was responsible for the changes in conduction induced by rapid atrial pacing. Indeed, changes of mRNA levels of the Na^+ channel have been found in paroxysmal lone AF patients(146).

Changes to the expression levels or activity of the aforementioned ion channels would have implications for changes at the level of the atrial action potential, which is an important component governing conduction in the atrium.

1.4.4.3 The role of the atrial action potential (AP) in AF

The atria are predominantly composed of fast-channel tissue, and under normal conditions, atrial activation and repolarisation are dependent on 4 phases of the AP. In a control model of the AP, atrial activation occurs at phase 0 of the AP when there's a large inward I_{Na}, which allows the membrane potential of the atrial cells to depolarise at a rapid conduction velocity of approximately 1 m/s in the direction parallel to fibre orientation. This is then followed by AP repolarisation, from its depolarised state back to the resting state by means of a series of time-dependent outward K^+ currents: the short-lasting transient outward current I_{to} (phase 1 of the AP), the ultra-rapid delayed rectifier current I_{Kur} (phases 1 and 2),

the rapid and slow delayed-rectifier currents I_{Kr} and I_{Ks} respectively (phase 3), and, the inward rectifier current I_{K1} (phase 4). The rate of repolarisation is a balance between deactivating I_K, time-independent I_{K1}, as well as the opposing inward current that is generated by the NCX during the late phase of the AP(152).

Variability in AP morphology has often been observed in recordings of human atrial APs. There have been attempts to define the various types of action potentials observed based on their morphological characteristics alongside the underlying ionic current densities. In 1993, Wang *et al* identified three types of APs based on their morphologies using human atrial multicellular preparations to record APs with fine-tipped microelectrodes: type 1 APs were rectangular with a positive plateau, type 2 APs had a spike-and-dome shape with a plateau at ~0 mV and type 3 APs were triangular with little plateau(153). The differences in AP morphologies were attributed to measured increases in the relative densities of I_{to} to I_K from type 1 through to type 3 APs(153). Similarly, Benardeau *et al* in 1996 characterised APs into two main types, type A APs which were spike-and-dome APs with a high plateau, and type B APs which were triangular in shape(154).

In a control model, the AP is predominantly of the type 1 morphology as previously described by Wang *et al* (1993). The aforementioned researchers observed that I_{CaL} density was not responsible for the different shapes of the type 1 through to type 3 APs. In their 1998 study, Courtemanche *et al* used a mathematical model to determine the ionic mechanisms underlying human atrial AP properties(155). They noted that variations in Ito alone could generate many of the differing APs morphology already observed experimentally; here, a large

to intermediate Ito conductance corresponded with APs resembling the triangular type 1, spike-and-dome AP, whilst, a small Ito conductance corresponded with the type 2 AP previously described. This corresponding change in AP morphology and I_{to} amplitude has been observed in young versus adult human atrial myocytes whereby an increase in Ito density was correlated with a shift from the rectangular type 2 AP to the spike-and-dome type 1 AP. The amplitude of I_{to} therefore carries weight as being an important determinant of AP morphology, however, the relative contribution of the remaining currents involved must still be taken into consideration.

The components of the cardiac AP are therefore critical in facilitating the initiation and coordination of the excitation-contraction coupling process which elicits myocardial contraction.(156) This excitation-contraction coupling process is described below: Ca^{2+} enters the cell during cardiac AP depolarisation at phase 1 through L-type Ca^{2+} channels and the NCX; this triggers Ca^{2+}-induced-Ca^{2+}-release from the sarcoplasmic reticulum, enabling Ca^{2+} to bind to troponin C, a myofilament protein forming the regulatory complex attached to thin muscle filaments; Binding of Ca^{2+} to troponin C then exposes a site on the actin molecule to bind to myosin ATPase on the myosin head of the filament; This results in ATP hydrolysis and allows actin and myosin filaments to slide past each other, consequently shortening the sarcomere length; At phase 2, cytosolic Ca^{2+} levels are reduced following removal of Ca^{2+} via the sarco-endplasmic reticulum Ca^{2+} ATPase, and Ca^{2+} no longer binds to troponin-C, resulting in a conformational change that inhibits the actin binding site; A new ATP binds to the myosin head, the sarcomere length is restored, and relaxation occurs, completing the

excitation-contraction coupling process.(157) Therefore, it can be deduced that conditions resulting in variations in the AP would impair this process and potentially lead to cardiac dysfunction.

In the setting of atrial fibrillation, AP duration (APD)-rate accommodation (i.e. shortening of the AP with increasing rate) is impaired, and there is a marked reduction in the refractory period-heart rate relationship in patients who are susceptible. Courtenmanche *et al*'s (1998) model of the AP reasoned that this impaired APD-rate accommodation was likely due to rate-dependent I_{CaL} inactivation together with incomplete deactivation of I_K(158). They deduced from their model of the AP, that strong reduction in I_{CaL} as observed in the setting of atrial disease or via experimental/pharmacological intervention such as blockade of I_{CaL}, can abolish atrial AP rate adaptation. Li and Nattel (1997) also showed that inhibition of I_{CaL} alone was sufficient in abolishing human atrial rate adaptation, highlighting the important role of I_{CaL} in contributing towards propensity for AF(159).

Additionally, atrial APs also show regional variability, adding another layer of complexity to the potential mechanisms underlying arrhythmogenesis. The left atrium has shorter APs than the right atrium due partly to a larger I_{Kr}(160). In the right atrium, the longest APs are in the crista terminalis, with intermediate durations found in the pectinate muscles and the shortest APs occurring close to the atrioventricular ring. These differences in AP morphology are related to discrete differences in the relative size of the ionic currents; Courtenmanche *et al*'s (1998) study showed in particular that variations in plateau currents,

specifically I_{to}, could not only account for the AP heterogeneity reported in human atria, but, may also offer insight into potential mechanisms via which AP heterogeneity can contribute to AF occurrence and sustainability(158).

1.4.4.3.1 *The dynamic relationship between the effective refractory period (ERP) and the atrial AP in AF*

Heterogeneity in the spatial distribution of APs has previously been reported as early as 1989; Spach *et al* (1989) were the first to measure this in adult and canine right atria(161), and it has since been confirmed across both atrial chambers in human and various animal species(162-164). It has been reported that progressive shortening of both the APD and the ERP with increasing distance from the sinoatrial node is an intrinsic protective characteristic of the atrial myocardium. Variations in AP morphology result from regional differences in ionic properties, independent of excitation sequence. Importantly, it is the heterogeneous change in the ionic properties in the setting of a diseased state that acts as a substrate for arrhythmogenesis. Conformational changes in the cardiac Na^+ channel from resting to its active and following depolarisation of the AP, inactivation of the Na^+ channel then return back to its resting state are crucial in determining myocardial excitability. Under normal conditions, the Na^+ channel following full activation and inactivation slowly returns to its resting state, and activation can once again occur; here, the end of the ERP is able to coincide with the end of repolarisation of the AP, therefore APD has been widely used as a measure of the ERP(165). Of late, increased arrhythmogenesis in the ischemic heart has been attributed to enhanced dispersion of repolarisation.(166) Although it is true that the end of repolarisation corresponds with the end of refractoriness

in the well-perfused, well-oxygenated myocardium, this is no longer so in the ischemic heart. In the ischemic myocardium, the recovery of excitability is delayed even following full repolarisation indicating that dispersion of repolarisation is not indicative of heterogeneity in ERP- APD is perhaps not always an index of ERP.

Coronel *et al* (2012) investigated post repolarisation refractoriness in the ischemic heart, and postulated that inhomogeneity in the dispersion of extracellular potassium concentrations between the outer border zone- where anoxia and normal concentrations of extracellular potassium coexist- and the central ischemic zone- where the extracellular potassium levels are higher- forms a substrate for re-entrant arrhythmias(165). In the border/anoxic zone APs with high amplitudes and short durations can be recorded as extracellular levels of potassium are slightly elevated, however in the central ischemic zone, the recorded APs have smaller amplitudes with low upstroke velocity. With this knowledge, they measured APD and ERP in the border and central ischemic zones of a Langendorff-perfused porcine heart subjected to 6 minutes of ischemia via occlusion of the left anterior descending coronary artery. Following ischemia, APD decreased in both central ischemic and border zones, however, whilst refractory period also decreased in the border zone, a marked increase in refractory period was observed in the central ischemic zone, resulting in increasing disparity between APD and ERP.

To add to the complexity of post repolarisation refractoriness inhomogeneity, the duration of the period of relative refractoriness during which a premature stimulus

can elicit a graded response may also contribute to arrhythmogenesis. Conditions such as ischemia and its components of hypoxia and acidosis can invariably alter electrophysiological properties, resulting in a prolongation of the duration of the graded response: the time needed for 95% recovery of the maximum upstroke velocity of the AP. Within the ischemic zone, the premature beat undergoes propagation slowing as a result of post repolarisation refractoriness, decreased local excitability and a reduction in the efficacy of wave front conduction. Slowed conduction in these regions, along with prolonged and heterogeneous refractoriness consequently promotes re-entry.

Allessie *et al* (1977) in exploring the concept of leading circle re-entry demonstrated that the central area of circus movement was activated by centripetal waves, that the fibres which were in and around this core showed double responses of considerable intervals which were unable to propagate beyond the centre(121). This ultimately prevents the double response from shortcutting the circuit, suggesting the presence of post repolarisation refractoriness in contributing to maintenance of re-entry by means of slowed conduction of the graded responses within the circuit core.

The concept of post repolarisation refractoriness highlights the dynamic relationship between APD and ERP; under conditions of altered electrophysiological properties and structural remodeling such as in patients at risk for AF within whom concomitant conditions preside, APD may not correspond with the ERP, and dispersion of repolarisation may underlie the heterogeneity of refractoriness.

1.4.4.3.2 *Repolarisation alternans increases vulnerability to atrial fibrillation*

Alternans in APD is an example of a plethora of repolarisation dispersion possibilities, which can result in fibrillatory activity either directly or accompanied by slowed conduction or ectopic beats. AP alternans can significantly amplify repolarisation gradients, resulting in unidirectional block, which is required for re-entrant waves to establish themselves even in the absence of structural or ion channel heterogeneity(167).

Typically, alternans occur at high heart rates, however, below a critical heart rate, AP repolarisation occurs with the same phase between all regions, otherwise referred to as spatially concordant alternans, where the spatial gradient of repolarisation is not much greater than baseline pacing. At faster heart rates, however, spatially discordant alternans occurs, with the repolarisation of neighbouring regions alternating with the opposite phase(167). Typically, atrial APD alternans and the transition to re-entrant arrhythmogenesis have been reported at fast rates as a result of APD-rate dependence (in accordance with the restitution hypothesis and its effect on the repolarisation gradients)(168,169).

Narayan *et al* (2011) proposed that in remodelled atria of patients with persistent AF, APD alternans could be elicited at slow heart rates, whilst in patients with paroxysmal AF or no AF, APD alternans could be elicited at progressively faster rates(170,171). They observed APD alternans of large amplitude near resting rate in patients with persistent AF, intermediate amplitude and rates in paroxysmal AF, and, small amplitude only at rates >230bpm in control subjects.

Narayan *et al* (2011) additionally observed that oscillations in APD preceded all transitions to AF; during APD alternans at slow rates, an ectopic beat enabled AF initiation(171). At faster rates, transitions into AF occurred via amplified alternans or complex oscillations. The evidence of alternans preceding the transition into AF highlights APD alternans as a key marker of AF susceptibility.

APD alternans as previously mentioned, when occurring at faster rates can typically be explained by the relationship between APD and rate, whereby a maximum slope or steep gradient >1 leads to APD alternans and re-entry. This however is not sufficient to explain Narayan *et al*'s (2011) observation of APD alternans at slower rates where the AP restitution curve is essentially flat in these AF patients(171).

Laurita & Rosenbaum (2008) along with many others have hypothesised that repolarisation alternans can be attributed to beat-to-beat alternans of Ca^{2+} release from the sarcoplasmic reticulum (SR). Evidence has suggested that in isolated cells(172) alternans of the Ca^{2+} transient can occur both in the presence and absence of AP alternans. The underlying mechanisms of this can be partly explained by impaired Ca^{2+} cycling. At faster heart rates, the ability of a cell to release and reuptake Ca^{2+} is diminished; if an AP is initiated before the released calcium and be reclaimed, the amount of Ca^{2+} available in the SR would consequently be reduced. This results in a smaller amount of Ca^{2+} released on the subsequent beat. Given this smaller release of Ca^{2+} and the equivalent time for Ca^{2+} reuptake, a larger amount of calcium will now be present in the SR and available for larger release on the beat to follow, giving rise to our Ca^{2+} alternans.

Ca^{2+} alternans is therefore associated with not only the reuptake of Ca^{2+} (SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase, NCX: Na^{+}-Ca^{2+} exchange) but also the release of Ca^{2+} from the SR through ryanodine (RYR) receptor channels. Dysregulation of intracellular Ca^{2+} via these avenues of Ca^{2+} release and reuptake may result in imbalances in Ca^{2+} load in the SR. Diminished L-type Ca^{2+} currents and altered intracellular Ca^{2+} handling can both lead to Ca^{2+} overload.

SR Ca^{2+} overload can occur at rapid heart rates, during which spatially discordant AP and calcium alternans, and, increased probability of Ca^{2+}-induced- Ca^{2+}-release have been reported to occur. This can lead to activation of the inward NCX current as well as calcium-activated non-selective cation currents, and depolarisation of the cell membrane (delayed after depolarisation, DAD). If the DAD is sufficient to reach the Na+ current excitation threshold, an AP can be fired and initiate re-entry(173).

In diseased and failing hearts, several factors such as altered electrical cell coupling, and electrical and structural remodeling all contribute towards reducing the heart rate threshold required for repolarisation alternans to occur. For example, in heart failure, the 3 factors which promote Ca^{2+} alternans (steeper SR calcium release-load gradient, increase SR calcium leak and reduced rate of SR calcium reuptake) can all occur via phosphorylation of RyRs via β-adrenergic and calcium/calmodulin-dependent protein kinase II (CamKII) signalling and downregulation of SERCA expression(173). In addition to impaired Ca^{2+} handling, structural remodeling in heart failure through fibrosis and reduction of gap junction

conductance have also been shown to reduce the threshold required for spatial discordant alternans to occur(173). Similarly, in the setting of ischemic heart disease, electrical remodeling of the surviving border zone tissue in the infarcted ischemic area of the heart alters Na^+ channel properties such that recovery from inactivation is slowed resulting in heterogeneity of conduction through the tissue, promoting spatial discordant alternans(173).

In AF, atrial tachycardia remodeling promotes AF by abbreviation of atrial refractoriness and reduction of the wavelength of the electrical impulse predominantly through downregulation of the I_{CaL}, and increase in the inward rectifier K^+ currents. This results in a reduction in the APD and consequent shortening of the wavelength of the conducting impulse to ultimately decrease the size of functional re-entry circuit required to establish multi-circuit re-entry and AF. Impaired or abnormal Ca^{2+} handling abnormalities which can occur with atrial tachycardia remodeling include: decrease in the Ca^{2+} transient with slowed decay and concomitant reduction in diastolic intracellular Ca^{2+} can contribute towards atrial dilation and eventually atrial contractile dysfunction in favour of re-entry and the induction of Ca^{2+} alternans and after depolarisations(174).

1.4.5 Structural remodeling in AF

The progression of AF is characterized by self-perpetuating mechanisms which occur with atrial tachycardia remodeling: rate-induced electrophysiological and structural changes in the atrial myocardium that predispose the tissue to re-entry. While the electrophysiological changes in the atrium take place as early as the first few hours of sustained atrial tachycardia(10,175), the structural remodeling

process is slower, occurring in the first days of tachycardia. The mechanisms underlying this structural remodeling process have not been fully elucidated, however, a number of pathways/systems have been proposed; these include: inflammation, oxidative stress, atrial stretching and dilation, and impaired electrical cell-to-cell-coupling.

1.4.5.1 Atrial Fibrosis

Fibrosis is a hallmark of structural remodeling-mediated arrhythmogenesis. Tissue fibrosis results from accumulation of fibrillar collagen deposits which occur mostly as a reparative process to replace degenerating myocardium. Atrial interstitial fibrosis has been shown to increase with age in both humans, and in patients with AF, and, in animal models of ageing, particularly in models of congestive heart failure (CHF)(176). AF is commonly associated with CHF; indeed, it has been shown that atrial interstitial fibrosis increases AF vulnerability in animal models of CHF, indicating that atrial fibrosis creates a substrate that promotes AF. In a dog model of heart failure, atrial fibrosis caused localised regions of conduction slowing along with increased conduction heterogeneity, providing the basis for unidirectional conduction block and macro- re-entry(177).

In the clinical setting, increased levels of fibrosis in patients with AF have been correlated with decreased amounts of connexin 43 expression(178,179) compared to patients in sinus rhythm. However, despite evidence of correlations between AF and atrial fibrosis, the mechanisms and signalling pathways behind atrial fibrosis have yet to be fully discerned. Currently, three interrelated pathways

have been proposed: (1) the renin-angiotensin system, (2) tissue growth factor (TGF)-β1, and (3) the oxidative stress pathways.

Studies have shown that the renin-angiotensin system plays a role in cardiac structural remodeling and in the development of myocardial fibrosis in several diseased states such as CHF(180) and cardiomyopathy(181). Transgenic mouse models of angiotensin-converting enzyme (ACE) overexpression have been shown to result in atrial fibrosis. Indeed, both clinical and animal studies have shown that ACE inhibitors such as enalipril(182,183) and cilzapril(184) were able to reduce the propensity towards AF vulnerability and occurrence(182,185-187) by decreasing the levels of atrial fibrosis. In addition, in animal studies which administered candesartan, an angiotensin II type 1 receptor blocker, they reported both a decrease in atrial fibrosis and AF duration(188); and, prevention of atrial structural remodeling and fibrosis(189).

Furthermore, the use of ACE inhibitors has also been shown to prevent progression from paroxysmal to chronic AF(190), and increase the efficacy of cardioversion of AF(191,192). These data suggest that the renin-angiotensin system is important in atrial fibrosis-mediated AF, and, that the use of ACE inhibitors may be a potential therapeutic intervention to delay progression of atrial fibrosis and AF development.

Increased expression of TGF-β1 has been previously implicated in the development of atrial fibrosis. It is a constitutively active transforming growth factor which has been known to be involved in the tissue wound healing process,

and in angiotensin II-mediated cardiac hypertrophy. Verheule *et al* (2004) investigated increased vulnerability to AF in a transgenic mouse model which overexpressed TGF-β1(193). They reported that although overexpression levels were equal in the atrium and ventricles, selective interstitial fibrosis was observed in the atria whilst ventricular histology remained normal. Importantly, this study also found that the increase in atrial fibrosis corresponded to an increase in conduction heterogeneity and AF vulnerability, suggesting that TGF-β1 may be important in the genesis of atrial fibrosis, and, that atrial fibrosis alone is a sufficient substrate for AF(11). Administration of the drug pirfenidone has been shown to reduce expression of TGF-β1 and tissue fibrosis significantly in a number of animal studies(194-198). Lee *et al* (2006) demonstrated in a canine model of heart failure that administration of pirfenidone resulted in a significant reduction in TGF-β1 expression, atrial fibrosis, conduction abnormalities and AF vulnerability, highlighting TGF-β1 as a potential therapeutic target for atrial fibrosis in patients with AF(199).

Further studies regarding the genesis of atrial fibrosis are still required to better understand its contribution towards AF, and, to improve therapeutic strategies for the prevention of reversal AF-promoting structural remodeling.

1.4.5.1.3 *Inflammation*

There has been evidence of the role of inflammation and oxidative stress in promoting AF. Indeed, in a 1997 study of atrial biopsies from lone AF patients, abnormal atrial histology compatible with a diagnosis of myocarditis occurred in 66% of the lone AF patient cohort(200). Increases in levels of C-reactive protein

(CRP)-a marker of systemic inflammation which promotes monocyte chemoattractant protein (MCP)-mediated chemotaxis, pro-coagulant activity and induces tissue factor secretion- have also been reported in AF patients(201,202), and importantly Aviles *et al* in their large population-based prospective study in 2003 reported that elevated levels of CRP was robustly predictive of increased risk of developing AF and AF recurrence following successful cardioversion(203). Various other inflammatory markers and mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and MCP-1 have also been linked to AF(204).

In contrast to CRP, TNF-α is a pleiotropic pro-inflammatory molecule associated with the pathogenesis of chronic AF. Patients with valvular AF exhibited higher levels of TNF-α, more severe leukocyte infiltration and fibrosis compared to patients either in sinus rhythm or who had other valvular diseases(205). In patients with persistent AF, higher TNF-α levels were also found in comparison to those with paroxysmal AF(206).

Responsible for stimulating the synthesis of acute-phase reaction proteins such as CRP and fibrinogen, as well as the counter regulation of TNF-α, IL-6 has also been implicated as an important inflammatory marker in the generation and perpetuation of AF, with high plasma levels of IL-6 being associated with the presence, duration and increased risk of AF and left atrial enlargement(207,208). Other studies have similarly shown that recurrence of AF following cardiopulmonary bypass grafting, cardioversion and catheter ablation was significantly associated with levels of IL-6. Contrastingly, in Liuba *et al*'s (2008)

study, both IL-6 and CRP levels were not significantly different between their paroxysmal and permanent AF patient cohorts(209).

Lastly, the inflammatory marker IL-8 which promotes neutrophil-mediated organ injury has been proposed to be important in the modulation of platelet-platelet and platelet-leukocyte interactions in several pro-thrombotic and inflammatory states(210). Clinical studies have previously reported elevated levels of IL-8 in patients with permanent AF. However, Li *et al* (2010) reported that levels of IL-6, IL-8 and MCP-1 did not in fact differ between paroxysmal, persistent or permanent AF patients even following adjustments of factors such as age, gender, race, body mass index and heart failure(206).

The relationship between the above inflammatory markers, their different roles in the inflammatory signalling pathway, and, the associated increased predisposition towards/perpetuation of AF has not been completely elucidated(211). Further data and research into whether inflammation is a consequence or cause of AF, or, if in fact it is the presence of AF or other underlying structural disease(s) that results in inflammation is still required.

1.4.5.1.4 *Atrial dilatation*

Left atrial dilatation has previously been implicated in the genesis and persistence of AF by means of atrial stretch. AF can occur secondary to left atrial enlargement, such as in its common association with chronic rheumatic mitral valve disease, whereby valvular obstruction has been proposed to result in elevated atrial pressure, leading to chamber enlargement(212). However, this does not explain

the occurrence of AF in patients with structurally normal hearts, who constitute between 3% and 11% of the AF population(213,214), pre-empting the concept that left atrial enlargement can develop as a consequence of AF, such that a self-perpetuating cycle of progressive left atrial enlargement and arrhythmia can be established. Understanding the underlying mechanism of atrial dilatation-induced stretch would enable further insight into the electrophysiological changes that underlie AF.

In the ventricles, electrophysiological changes in response to mechanical perturbations or changes in hemodynamic loading have been well established. It has been shown that ventricular stretch leads to shortening of the APD and the ERP. Similarly, in the atria, Ravelli *et al* (1994) demonstrated that atrial stretch caused by ventricular contraction modulates the cycle length of atrial flutter in humans(215). In experimental models of atrial dilatation, acute atrial enlargement was associated with proarrhythmic reductions in APD and ERP.

The effects of acute stretch on the atrial myocardium may vary from those of chronic stretch. In animal studies both acute and chronic stretch have been shown to be pro-arryhthmic(216-218). Stretch-activated ion channels (SACs) have been implicated in mediating the electrophysiological changes observed in acute stretch-induced vulnerability to arrhythmia. Non-selective SACs allow for ions such as Ca^{2+} as well as Na^{+} and K^{+} to pass through, whilst selective SACs specifically carry K^{+} or Cl^{-} ions. Blockade of these channels with Gadolinium has been shown to suppress the occurrence of stretch-induced depolarisation in the ventricles(219), and, reduce AF inducibility in the acutely dilated atria(220). Bode

et al (2000) reported a decrease in ERP which was accompanied by increased AF inducibility with progressive atrial stretch. Interestingly, in contrast to the general belief that shortening of the ERP results in shortening of the excitation wavelength in favour of re-entrant activity, Bode *et al* (2000) found that although administration of Gadolinium reduced the inducibility of AF, it did not however alter the atrial ERP response, highlighting that ERP may not fully explain the increased susceptibility to AF during stretch(220). Evidence of atrial stretch-induced spatial dispersion of atrial refractoriness may offer a potential mechanism to explain this observation. It is well known that the structure of the atria is non-homogeneous; this can result in differences in wall stress during changes of intra-atrial pressure, leading to non-uniform distribution of local atrial refractory periods, providing a substrate for the initiation and maintenance of re-entrant activity. Other mechanisms such as stretch-induced proarrhythmic afterdepolarisations have also been proposed to enable AF initiation with administration of caesium chloride to provoke afterdepolarisations reported to have resulted in onset of polymorphic atrial tachycardia which degenerated into AF.

In comparison to acute stretch-mediated changes in atrial electrophysiology, the electrical remodeling in chronic stretch-mediated AF has been linked to the renin-angiotensin system, Studies have shown that atrial tachycardia-induced electrical remodeling via shortening of the ERP and loss of physiologic rate adaption, increases the inducibility and stability of AF(216-218). Accordingly, intracellular Ca^{2+} overload during high-frequency pacing has thought to contribute towards this. However, several, previous studies using verapamil demonstrated that the

L-type Ca^{2+} channel blocker was unable to prevent neither long-term tachycardia-induced shortening of the ERP nor loss of physiologic rate adaptation(10,221,222). Together the results from these studies indicated that although intracellular Ca^{2+} overload may be responsible for short term-induced electrical remodeling, this does not explain the results observed in the long term.

The renin-angiotensin-aldosterone system has previously been reported to be involved in myocardial fibrosis in a number of cardiovascular diseases such as congestive heart failure and in myocardial infarction(223). In clinical studies, patients presenting with primary hyperaldosteronism have an increased risk of developing AF(224). Additionally, locally produced Ang II has been associated with apoptosis and interstitial fibrosis in cardiomyocytes(225). Angiotensin II is a well-characterised pro-fibrotic molecule, which often works in concert with other downstream mediators such as TGF-β1, a growth factor which is central to signalling cascades involved in cardiac fibrosis(226). Mitogen-activated protein kinases are also important mediators of the effects of Angiotensin (Ang) II on the cardiac myocardium(227,228), and over activity of these kinases has been proposed be involved in mediating changes in cardiomyocyte gap-junctional coupling and conduction(229). As conventional antiarrhythmic drug therapies are limited in their effectiveness and may be proarrhythmic(230). ACE inhibitors or Ang II receptor type 1 (AT1R) blockers are an attractive therapeutic alternative.

Kumagai *et al* (2003) first described the use of candesartan (an AT1R blocker) on electrostructural remodeling in rapidly paced canine atria. They discovered that the degree of fibrosis, duration of induced episodes of AF improved with

admission of candesartan(188). Similarly, Kumagi *et al* (2003) investigated the effects of candesartan on long-term atrial electrostructural remodeling. They reported that although candesartan was not able to prevent shortening of the atrial ERP following one week of rapid pacing, the AT1R antagonist was interestingly able to significantly reduce the frequency of inducibility and the duration of AF following five weeks of rapid atrial pacing(188). These results suggest that AT1R blockade may offer similar benefits to ACE inhibition in improving susceptibility to AF and structural remodeling in favour of a proarrhythmic substrate(188,231).

Current literature available of left atrial dilatation-induced AF suggests that perhaps SAC blockade with agents such as gadolinium(219), or ACE inhibitors and AT1R blockers such as enalapril(232) and candesartan respectively, may represent a novel therapeutic anti-arrhythmic target to abolish the proarrhythmic influence of atrial stretch on the initiation of AF. Further studies are still need to better define the role of these different molecular pathways in altering atrial electrostructural physiology to increase risk of AF incidence.

1.4.5.1.5 *The role of gap junctions and connexins in the pathology of AF*

It is very well known that AF is characterised by erratic electrical activity in the atrium which can lead to several adverse events such as impairment of atrial contractility, pooling of blood in the atrium promoting thrombosis and increasing the risk of embolic stroke(63,233). In recent years, there has been great interest in the area of gap junctions and connexins and, in particular their role in electrical cell coupling and smooth conduction of the electrical impulse through the cardiac

myocardium. The importance of gap junction function as a therapeutic target for AF was first highlighted over 15 years ago by Spach and Starmer(234). Since then, our understanding of the diversity of connexins in the heart has greatly increased, as have the number of studies investigating their role in the pathogenesis and treatment of AF.

Gap junctions contain connexons which consist of 6 transmembrane ion-channel proteins each; these transmembrane subunits are otherwise known as connexins. These connexins when formed into a connexon can line up in the gap junctions of adjacent cardiomyocytes and attach, allowing the passive movement of ions and small molecules <1 kDa in size between cells, essentially coupling adjacent cells electrically. The heart itself expresses a variety of connexins, with different regions of the heart exhibiting a particular profile of connexin(s); the atria predominantly express connexins (Cx) 40 and 43. Given the importance of connexins in bridging both cell-to-cell coupling and uniformity of conduction of the AP between cells, modifications in the distribution and balance of connexin expression resulting in heterogeneous conduction and refractoriness would be proarrhythmic(233). It is crucial for gap junction channels to be located at the intercalated disks between adjacent myocytes to enable rapid and smooth propagation of the AP between cardiac myocytes. Indeed, there has been evidence of post-translational modification, in particular connexin phosphorylation having important roles in both localization and function of connexins(235). In AF, changes in both total connexin expression and lateralisation of connexons to the margins of atrial cardiomyocytes have been reported(141,236-242). However, data assessing the relationship between

distribution and expression of Cx 40 and 43 in the atria remains controversial and somewhat contradictory.

Transgenic animal models have been used as a means of understanding the role of connexins in the setting of AF. While some Cx 40 knockout mice studies indicated frequent inducibility of atrial tachyarrhythmias with no inducible atrial arrhythmias in the heterozygous or wild type mice(243), other studies have suggested that Cx 40 knockout mice were resistant to carbachol-induced atrial tachyarrhythmia and fibrillation(244). Leaf *et al* (2008) reported that both either partial (Cx $40^{+/-}$) or complete deletion (Cx $40^{-/-}$) abolished heterogeneity in conductance between the left and right atria, suggesting that Cx 40 may be involved in establishing interatrial conduction velocity heterogeneity(245). Interestingly, G60S mutant Cx 43 mice (Cx $43^{G60S/+}$) exhibited severe atrial tachyarrhythmia compared to the Cx 40 knockout mouse. The investigators reported atrial fractionated patterns consistent with clinical observations in these Cx $43^{G60S/+}$ mice(243).

Cell cultures have previously been used to assess for the relationship between cell alignment and changes in intracellular conductance(246,247). In the interest of understanding the degree of contribution and the proportion of Cx 40 / 43 required for functional atrial gap junctional conduction, experiments using cell cultures such as those by Lin *et al* (2010) have also been conducted(248,249). Using cultured neonatal mouse atrial myocytes and dual whole cell patch clamp, Lin *et al* (2010) quantified the functional contribution of Cx 40 and 43 towards atrial gap junctional conductance. They reported that both Cx 40 and 43 appear

to make equal contributions towards total gap junction conductance(248). In contrast, an earlier study by Beauchamp *et al* (2006) noted that the ratio of Cx 40 / 43 expression was a key determinant of propagation, with increases in Cx 43 and Cx 40 expression conveying faster and slower conduction respectively(250).

In 2011, Igarashi *et al* presented a novel method of gene therapy and sought to improve myocardial conduction by controlling Cx gene expression via epicardial gene painting, a technique that allowed for homogeneous and complete transmural atrial gene transfer of Cx 40 and 43(251). Their study reported that atrial gene transfer of either Cx 40 or 43 was successfully able to preserve atrial conduction and prevent development of AF in a porcine model of tachycardia-induced AF(251). These results highlight modulation of Cx function as a potentially attractive therapeutic target for the treatment of AF, however, further investigation addressing the limitations, differing models and contradicting results need to be conducted.

1.5 Type 1 and type 2 models of diabetes employed in this thesis

The use of animal models has been crucial to developing a range of AF models in clinically relevant pathological substrates. The Zucker rat was first discovered by scientists investigating if the propensity for obesity could be inherited, labelling this gene the ‘fatty gene’ (fa/fa)(252). Lois and Theodore Zucker found that at times, the Zucker strain would produce morbidly obese rats which did not typically present with type 2 diabetes, exhibiting signs of hyperinsulinemia in the absence of hyperglycaemia. From this original colony, further morbidly obese Zucker rats arose, displaying unusually elevated levels of blood glucose with accompanying

insulin resistance. It was through selective inbreeding and outbreeding of these rats that the Zucker fatty rat (Zfr) and a rodent model of type 2 diabetes which has since been well recognised was formed(252).

Zfr inherit the autosomal Mendelian recessive trait whereby a missense mutation of the leptin receptor lies in the ‘fatty gene’ (fa/fa)(253). These rats in comparison to their lean litter mates typically present with obesity, hyperphagia, hyperinsulinemia and hypertriglyceridemia. Their obese phenotype can be characterized by the hypertriglyceridemia resultant from increased hepatic production of very low density lipoproteins. Adipocytes gather in size and number particularly in the subcutaneous area, where the number of fat cells and the fat depot is the largest(253).

In comparison to type 2 diabetes, there is currently no genetically-specific rodent model for type 1 diabetes. However, diabetogenic agents such as alloxan and streptozotocin are the most prominently used and widely recognised in experimental models of type 1 diabetes(254). Both alloxan and streptozotocin induce insulin deficiency via similar yet differing pathways. Both agents share the trait of being beta-cell selective cytotoxic glucose analogues which are taken up via the GLUT2 glucose transporter, causing beta cell death via necrosis. However, the method by which necrosis and beta cell death occur differ between these analogues, with activation of reactive oxygen species (ROS) in the case of alloxan, and DNA alkylation in the case of streptozotocin being the primary modes of toxic action. Although alloxan is more useful as a model compound for understanding the underlying mechanisms of ROS-mediated beta-cell toxicity,

due to its relatively unstable nature (with a short half-life of 1.5min at ph 7.4 at 37 ºC), streptozotocin has tended to be the preferred agent of choice in terms of reproducibility of a diabetic metabolic state in animal experiments(254).

The two aforementioned models of diabetes: (1) type 2 diabetic: fa/fa Zucker rat, (2) type 1 diabetic: streptozotocin-induced Zucker rat, have consequently been chosen here as appropriate models for investigating the potential modifiable substrates of AF in an attempt to better understand and ultimately seek to reduce the predisposition of obese, type 1 and type 2 diabetic individuals to the development of, or the maintenance of AF.

1.6 Further research and future directions

At present, little is known of type 1 diabetes and its pathophysiological impact on the atrial myocardium at both the electrophysiological and the structural level in favour of a substrate for AF. Interestingly, no study has compared the relationship between type 1 and type 2 diabetes: do they share a common underlying pathway of pathogenesis in favour of AF? Are the structural and electrophysiological determinants of re-entry identical or different between these two diabetic conditions? Does one particular diabetic state increase an individual's predisposition more so than another?

Evidence of differences in pathology of type 1 and type 2 diabetes-mediated atrial cardiomyopathy have been individually reported. However current literature offering a comparison between the two diabetic states is lacking. Perhaps the first step towards providing some answers would be to determine the

electrophysiological and structural parameters which govern/underlie the mechanisms of type 1 diabetes-mediated AF. Secondly, to identify common observations made between the impact of the two diabetic states on the electrophysiological and structural properties of the atrial myocardium. Thirdly, to investigate potential pathophysiological pathways that the diabetic states may share or differ in. Research addressing these areas would give an opportunity for some insight into the diabetic myocardium as a proarrhythmic substrate for AF.

This thesis aims to assess these two diabetic states as individual risk factors contributing towards the pathogenesis of AF. The techniques employed in each study were kept constant, and the same measurements were made in each study to allow for the identification of common and differing electrophysiological and structural components to be made between type 1 and type 2 diabetes mellitus. More specifically, the following chapters will assess for differences in animal characteristics, intracellular action potential repolarisation, and atrial conduction.

Statement of Authorship

Title of Paper	Simultaneous Conduction Mapping and Intracellular Membrane Potential Recording in Isolated Atria
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Name of Principal Author (Candidate)	Melissa Neo
Contribution to the Paper	Design of study Performance of experiments in the study and follow up analysis and interpretation of data obtained Drafting of manuscript
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 10/8/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Melissa Neo

From: David Morris <davidmorris12@gmail.com>
Sent: Wednesday, 24 August 2016 2:40 PM
To: Melissa Neo
Subject: Re: Statement of authorship

Dead Melissa,

I approve the manuscript, please take this email as affirmation of my approval for the purpose of the required forms.

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On 24 Aug 2016 2:53 p.m., "David Morris" <davidmorris12@gmail.com> wrote:
Dead Melissa,

I approve the manuscript, please take this email as affirmation of my approval for the purpose of the required forms.

David Morris
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On Tue, Aug 23, 2016 at 6:59 PM, Melissa Neo <melissa.neo@adelaide.edu.au> wrote:
Dear David,

I have attached the Author Statement form for the following published thesis chapter:

Simultaneous conduction mapping and intracellular membrane potential recording in isolated atria

The thesis chapter was accepted in the Canadian Journal of Physiology and Pharmacology.

If you are unable to sign the form (physically/electronically) could you please provide a written emailed approval for the above manuscript?

Kind regards,
Melissa

CHAPTER TWO

SIMULTANEOUS CONDUCTION MAPPING AND INTRACELLULAR MEMBRANE POTENTIAL RECORDING IN ISOLATED ATRIA

2.1 Introduction

To understand the genesis and maintenance of atrial arrhythmias, detailed evaluation of the structural and electrophysiological characteristics of atrial tissue is necessary. Electrophysiological techniques generally available are electro-anatomical mapping, optical mapping, or electrode arrays. Electro-anatomical mapping is not feasible in small animals, because of the small size of the heart relative to the resolution of the recording, while optical mapping, which can provide excellent information on action potential (AP) propagation and duration (255), is limited by the use of electro-mechanical uncouplers such as butanedione monoxime (256) to obviate movement artefacts. These agents may have considerable effects on tissue electrophysiology (256,257). We have previously demonstrated the feasibility of accurate assessment of conduction patterns such as re-entrant pathways, rotors, or wave-break patterns in small hearts by contact mapping using a multi-electrode array (MEA)(258).

A notable limitation of both MEA and optical mapping is the lack of membrane potential information, accurate measurement of rate of AP upstroke, AP amplitude, or true action potential duration (APD). This information is only available from intracellular recordings. We, therefore, investigated the feasibility of membrane potential recording with sharp intracellular electrodes while

simultaneously mapping AP propagation and refractory periods with a high density electrode array, a technique we have called SHIMP (Simultaneous High density mapping and Intracellular Membrane Potential recording). Using this technique, we show that there are regional variations in AP restitution curves in rat atria in vitro, and that these regional differences are frequency dependent. Self-sustaining re-entry rarely occurred in our studies using healthy Young rats, but when it did we directly observed APD alternans at different sites in the re-entrant pathway while simultaneously mapping the AP conduction.

2.2 Methods

All experimental procedures involving animals were approved by the Animal Ethics Committee of the University of Adelaide. All procedures were in accordance to the guidelines outlined in the Australian code for the care and use of animals for scientific purposes (NHMRC 2013). A total of 7 male Sprague Dawley rats aged 12 weeks were studied.

2.2.1 Tissue preparation

The right atrium was carefully excised from anaesthetised rats under full anaesthesia (ketamine (75 mg/kg) and medetomidine (0.5 mg/kg)) and used for immediate electrophysiological study using a custom built MEA system as previously described (258), coupled with simultaneous intracellular recording.

2.2.2 Electrophysiological study

During each electrophysiological study, the atrial tissue was positioned with the epicardial surface in contact with the MEA (Fig. 1). The stimulation sites (sites 1 and 2) were in the right atrial appendage, and the recording regions (regions A and B) were at the right-hand side (Fig. 1) in the right atrial free wall. The tissue was superfused with bicarbonate-buffered solution containing: (mmol/L) NaCl 130, KCl 4.0, $NaHCO_3$ 24.2, NaH_2PO_4 1.2, $MgCl_2$ 0.6, $CaCl_2$ 2.2, glucose 12, and bubbled with carbogen (95% O_2/5% CO_2), maintained at pH 7.35 and 37.5 °C. Electrograms were sampled at 4 kHz and filtered from 10–500 Hz. Pacing stimuli were delivered to either of 2 stimulation sites via bi-pole pairs of array electrodes at a current of 0.1 mA with a pulse width of 0.5 ms.

2.2.3 Intracellular recording

A sharp aluminosilicate glass microelectrode (100 MΩ, filled with 3 $molL^{-1}$ KCl) was inserted from the endocardial side of the tissue and intracellular membrane potential recordings made with a high input impedance amplifier (WPI model 725), digitised at 10 kHz, 16-bit resolution and recorded and analysed with Chart 5 (AD Instruments). The electrode was pulled to be sufficiently “springy” that it could follow tissue movement without compromising the recording. Signal ground was via a silver/silver chloride wire in the bath. Action potentials (APs) were recorded using this electrode alternately from 2 regions (region A, region B) during atrial pacing from 2 stimulation sites (site 1, site 2) at opposite corners of the MEA (Fig. 1). The entire apparatus was mounted on a vibration free table and contained in a grounded Faraday cage.

2.2.4 Parameters measured

2.2.4.1 Electrophysiological study

Three measurements of refractoriness (effective refractory period, ERP) were recorded for each pacing cycle length at the first stimulation site, and repeated at the second stimulation site during a standard S1–S2 pacing protocol, which consisted of eight basic (S1) stimuli followed by a premature (S2) stimulus in decrements of 10 ms. The ERP was defined as the longest S1–S2 interval not resulting in a propagated response. As previously described by (258), conduction velocity (CV) and heterogeneity of conduction (CHI) parameters were determined using semi-automated custom-made plaque analysis software (Nucleus Medical) to obtain an activation map for each pacing cycle length. CV was calculated using triangulated local vectors formed between groups of 3 electrodes, and CHI was determined using an established phase mapping technique whereby the largest phase difference between a group of 4 electrodes is plotted on a phase map allocated with percentile scores such that P_5-P_{95} is representative of the total range of maximal differences and the formula of (P_5-P_{95}/P_{50}) is reflective of conduction inhomogeneity(259).

2.2.4.2 Intracellular recording

Measurements from a total of 80 APDs at 80% of repolarisation (APD_{80}) were recorded from atrial preparations (N = 4) using Peak Parameters (Chart 5, AD Instruments) to obtain restitution curves at each pacing cycle length of 400, 300, 200, and 100 ms. Exclusion criteria comprised of a baseline membrane potential of less than –60 mV, dV/dT of under 10 000 mV/s, and an overshoot of less than 10 mV.

2.2.4.3 Statistics

All data are expressed as mean ± SEM. A univariate analysis of variance (ANOVA) was performed to assess for average CV, CHI, ERP, and APD between stimulation sites. To assess for the relationship between APD and ERP, the Spearman's correlation coefficient was determined. A Student's *t* test was used to determine APD and amplitude alternans during tachyarrhythmia. A *p* value <0.05 was considered significant.

2.3 Results

2.3.1 Mapping and intracellular measures during pacing

ERP was not significantly different when stimulating at site 1 (32.27 ± 1.89 ms) or site 2 (33.33 ± 2.93 ms) ($p = 0.76$). Similarly, CV and CHI were not significantly different between stimulation sites 1 and 2; CV: 0.21 ± 0.01 m/s stimulation site 1 vs. 0.18 ± 0.01 m/s site 2, and, CHI: 3.18 ± 0.22 stimulation site 1 vs. 3.66 ± 0.31 site 2. The mean ± SEM resting membrane potentials were: –71.23 ± 0.27 mV region A and –72.65 ± 0.19 mV region B.

Tissue electrical heterogeneity was readily apparent. This heterogeneity was seen not only at different recording regions, as one might expect, but also at the same recording region when the tissue was stimulated at a different site. For example, APD_{80} at recording region A was significantly different when paced at stimulation site 1 compared with pacing at stimulation site 2 when comparing between stimulation sites for each pacing cycle length ($p < 0.05$, Fig. 2A). There was no interaction between APD_{80} and pacing cycle lengths when APs were initiated from stimulation sites 1 or 2 (both $p > 0.05$). Hence, the AP properties at

a given site depended on the direction of propagation of the AP across that site. This directional dependence of APD_{80}, however, was not observed at recording region B where APD_{80} was not significantly between AP initiated at stimulation sites 1 and 2 when comparing between stimulation sites for each pacing cycle length ($p > 0.05$, Fig. 2B). The restitution curves at the 2 sites was also different, at region A, the expected shortening of APD with decreasing cycle length was seen, but the curve at region B was essentially flat. Similar results were found for APD_{50} measured at recording region A, with a significant difference found between stimulation at sites 1 and 2 ($p < 0.0001$). This difference in AP initiated from the 2 stimulation sites was not observed when recording from recording region B for APD_{50} ($p = 0.24$). However, when assessing for APD_{20}, significant differences were found between stimulation sites 1 and 2 in recording regions A and B (both $p < 0.0001$).

2.3.2 APD and ERP

The shortening of APD_{80} at shorter cycle lengths when stimulated at either site (Fig. 2A) was not reflected in a shortening of ERP, which did not differ significantly at any cycle lengths ($p > 0.05$) The correlation coefficient (Spearman's) showed a slight negative correlation between APD_{80} and ERP: for region A, r was –0.255($p = 0.032$) and for region B, r was –0.245 ($p = 0.029$).

2.3.3 SHIMP of tachyarrhythmia episodes

Figure 3 shows simultaneous intracellular membrane potential recording (left) during 1 observed episode of tachycardia in a single atrial preparation, triggered by attempting to determine ERP at a pacing cycle length of 100 ms (S1), with S1–

S2 interval of 50 ms. Close inspection of the intracellular recordings revealed AP alternans in duration and in amplitude. During this self-sustaining re-entrant tachyarrhythmia, AP measurements were made for 10 alternating APs during each of 3 successive 1 s periods. APD alternans was present in periods A–C. The mean APD_{80} during period A was 32.76 ± 0.40 ms for AP_n vs. 29.78 ± 0.63 ms for AP_{n+1} ($p < 0.0001$). During periods B and C, the mean APD_{80} for AP_n vs. AP_{n+1} were: Period B, APD_{80} = 32.36 ± 0.09 vs. $APD80_{n+1}$ = 30.12 ± 0.13 ms ($p < 0.05$), and Period C, $APD80_n$ = 30.42 ± 0.0008 vs. $APD80_{n+1}$ = 29.48 ± 0.14 ms ($p < 0.01$). Similar results were seen for APD_{50} and APD_{20}. AP amplitude alternans was present in periods A and B but not in period C (periods A and B, both $p < 0.0001$) before resolving (period C, $p = 0.36$). The mean overshoot amplitudes were as follows: AP_n –13.22 ± 0.19 vs. AP_{n+1} –16.06 ± 0.63 mV (Period A), AP_n –14.03 ± 0.07 vs. AP_{n+1} –16.28 ± 0.06 mV (Period B), AP_n –15.36 ± 0.12 vs. AP_{n+1} –15.22 ± 0.07 mV (Period C). This intracellular recording was from region B, as illustrated in the right panel, which also shows the local activation map of 1 of the tachycardia beats with superimposed isochrones (2 ms). Conduction velocity alternans was also often apparent during re-entrant tachyarrhythmias (Fig. 4).

2.4 Discussion

In these experiments, we combined the use of 2 established techniques, high density electrode array mapping (which records extracellular potentials) and intracellular membrane potential recording. To our knowledge, these 2 techniques have not yet been combined in this way. This combination allows one to assess changes in AP morphology along with changes in conduction velocity.

While similar information can be obtained from optical mapping techniques, our approach has the advantage that absolute membrane potential is recorded, the temporal resolution is very high, and there is no need to paralyse the tissue to avoid motion artefacts. In these pilot studies, we aimed to validate the technique. We show that APs can be stably recorded, and that membrane potential (ranging from –60 mV to –80 mV) (260-262) and conduction velocities (263) agree with published values for isolated superfused rat atrial tissue.

Use of SHIMP allowed us to make some intriguing observations:

1. That the AP morphology at a given recording region varies depending on the direction of propagation of the AP across that region (Fig. 2). This is undoubtedly due to the well-known electrical anisotropy of atrial tissue. Presumably, this results in a different capacitative load to which the cell is coupled in different tissue directions, and so the AP morphology is subtly changed (264-267).

2. That AP restitution curves can be different at different recording regions. Regional differences in restitution curves will act to enhance heterogeneities in repolarisation at different cycle lengths, and may act to pre-dispose the atria to arrhythmias. Studies showing these anisotropic differences have been done in simulations and cell monolayers (268), and on hearts from genetically engineered animals (269), although most of these are in ventricle. The atrial tissue used in these studies were from healthy animals, we therefore speculate that the regional differences

observed will be exaggerated in diseases such as diabetes, and will contribute to the arrhythmogenic substrate in these diseases.

3. That APD and ERP are not necessarily correlated. Although it is generally assumed that APD and ERP are closely correlated (e.g. (270)), it is well documented that in some circumstances, such as in ischemia, changes in APD and ERP can be dissociated from one another(271). Here we show a slightly negative, but statistically significant correlation between APD and ERP (with APD shortening at increased pacing rates as ERP lengthens (Fig. 2); this is inconsistent with that reported in humans(272), where APD/ERP ratio was invariant with changes in cycle length. It is possible that this lack of coherence between APD and ERP is species-dependent, or may be due to the recording conditions. This result would need to be verified in vivo, or in arterially perfused tissues.

4. We were able to directly observe AP alternans in both amplitude and duration during the mapping of an episode of re-entrant arrhythmia.

2.4.1 Advantages and disadvantages of the technique

This combination of techniques may allow more detailed examination of the mechanisms of arrhythmogenesis in small animal models, such as in diabetic or hypertensive rat models, or in genetically modified mouse models. It may also be possible to use this approach to screen drugs for either antiarrhythmic efficacy or arrhythmogenic potential in appropriate small animal models more cheaply and quickly than in large animal models. For example, it is well known that discordant

alternans is much more arrhythmogenic than concordant alternans(273,274). Adding a second intracellular recording electrode would enable this to be directly investigated along with the simultaneous mapping of the re-entrant pathway under different stimulus parameters, enabling greater insight into the mechanisms underlying the genesis of atrial fibrillation.

FIGURES & CAPTIONS

Figure 1. Schematic drawing of multi-electrode array (MEA) system with simultaneous intracellular membrane potential recording. Top view of the combined setup has been shown together with a schematic drawing demonstrating the orientation of the right atria on the MEA. The excised right atria were positioned with epicardial surface in contact with the MEA (downwards), the free wall to the right and the tricuspid valve to the left. IVC = location of Inferior Vena Cava, SVC = location of Superior Vena Cava. Locations of stimulation sites (sites 1 and 2, via the electrode in the MEA) and the recording regions (regions A and B) are shown.

Figure 2. Action potential duration at 80% of repolarisation (APD_{80}) measured from region A (A) and region B (B) in n = 4 atrial preparations (10 action potentials per site per atrial preparation per pacing cycle length). (A) APD_{80} was significantly different between pacing sites (one and two) for all cycle lengths ($p < 0.0001$). (B) APD_{80} was not significantly different between stimulation sites (one and two) across all pacing cycle lengths in region B. (Closed and open circles indicate stimulation sites 1 and 2 respectively)

Figure 3. Right atrial tachycardia simultaneous high-density mapping with intracellular membrane potential recording during a standard S1-S2 pacing protocol in a single atrial preparation. Observation of action potential alternans during recording of one episode of tachycardia. Upper panel: Intracellular membrane potential recording during a 6s period of re-entrant arrhythmia lasting 1min 29s. Action potential properties were measured during

period A, B and C as indicated. Upper right panel: Local activation map between tachycardia beats with superimposed 2 ms isochrones. The white circle indicates region from which membrane potential recording of tachycardia episode following S1-S2 pacing (S1: 100ms, S2: 50ms) was measured. Lower panels: Three representative magnified views of action potential recordings in periods A, B and C. Action potential amplitude alternans was apparent early in the period of sustained arrhythmia. As the arrhythmia progressed, this alternans in duration dissipated over time.; AP amplitude alternans was apparent during period A and B, but not C; Action potential duration alternans was observed in all periods A to C; identical spear-headed arrows and round-ended arrows indicate differences in AP duration and amplitude in period A respectively.

Figure 4. Conduction velocity alternans observed in a single atrial preparation. Section of recording (400 ms sweep) from the MEA during re-entrant arrhythmia showing conduction velocity alternans. The interval for the propagated response to return to a given point on the rotor pathway is shown below recording.

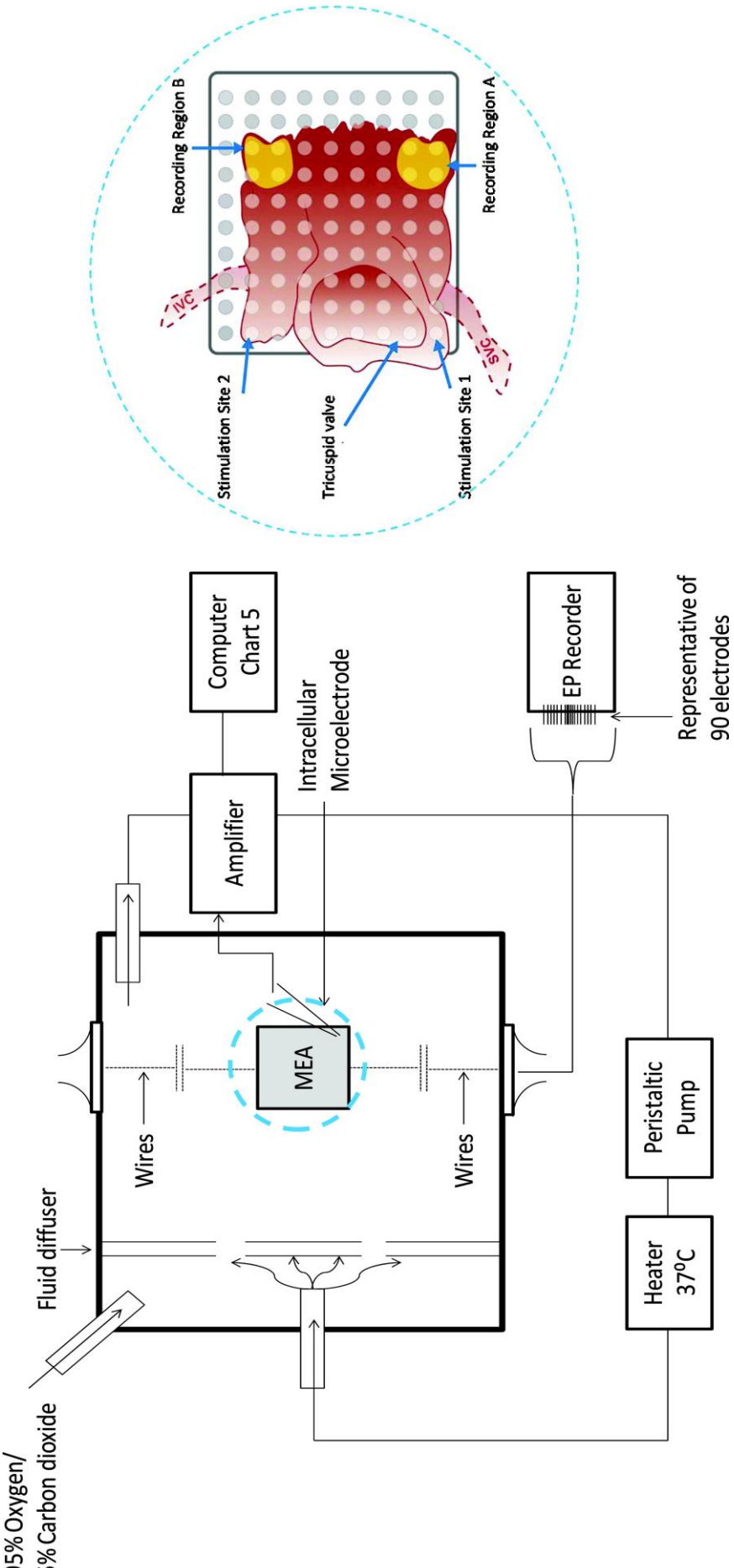
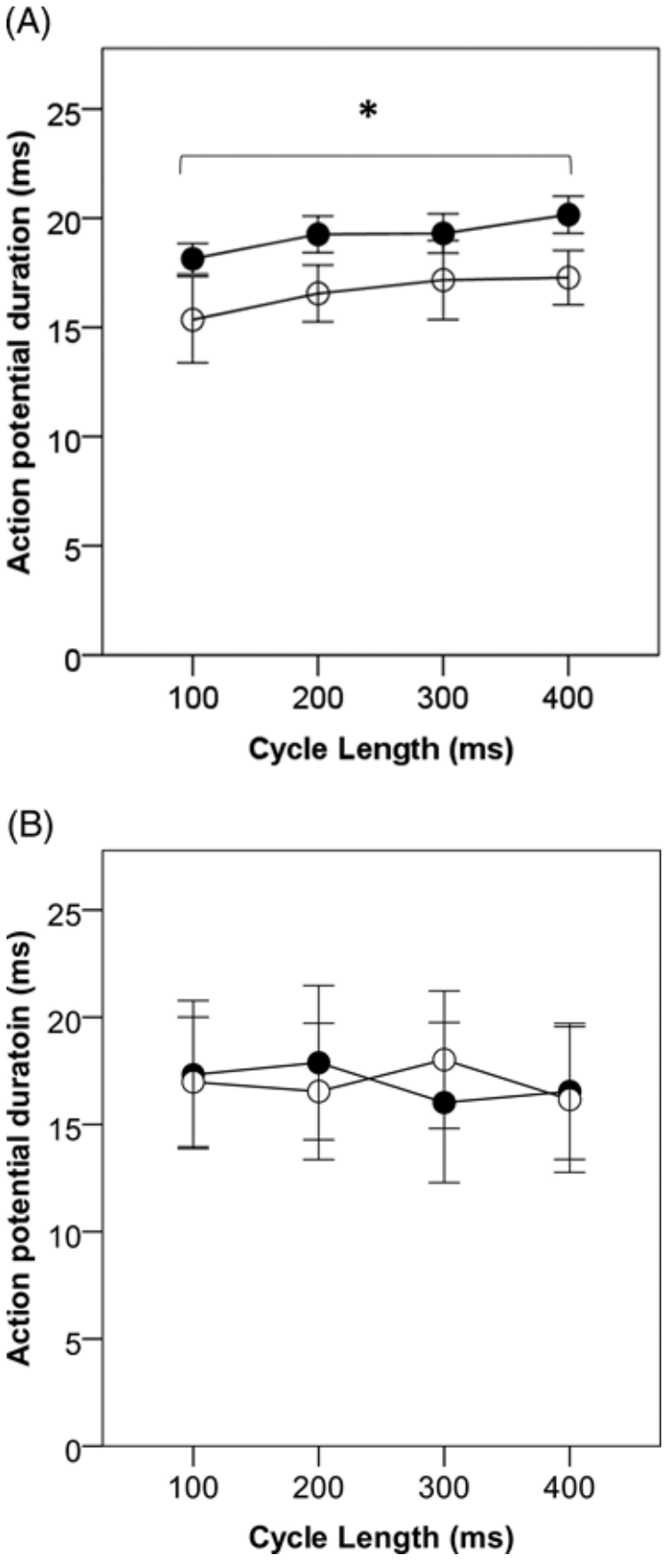

Recording Region B
Recording Region A
IVC
SVC
Stimulation Site 2
Tricuspid valve
Stimulation Site 1
Computer Chart 5
Amplifier
Intracellular Microelectrode
EP Recorder
Representative of 90 electrodes
MEA
Wires
Wires
Fluid diffuser
95% Oxygen/ 5% Carbon dioxide
Peristaltic Pump
Heater 37°C

Fig.1

Fig. 2


(A)
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Action potential duration (ms)
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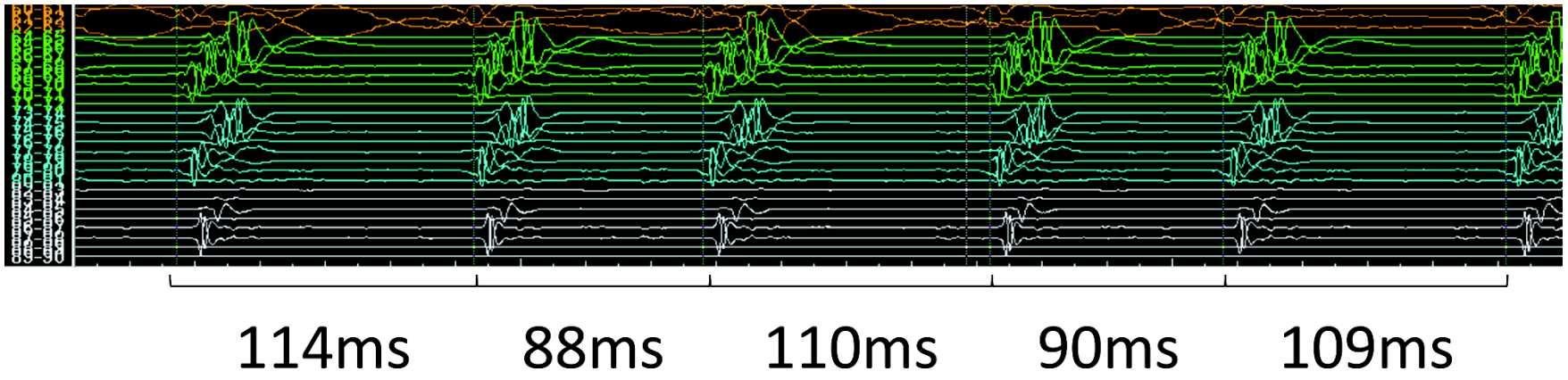
Fig.4



Statement of Authorship

Title of Paper	A Rodent Model of Streptozotocin-Induced Type 1 Diabetes: A Substrate for Atrial Fibrillation
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	n/a

Principal Author

Name of Principal Author (Candidate)	Melissa Neo
Contribution to the Paper	Design of study Performance of experiments in the study and follow up analysis and interpretation of data obtained Drafting of manuscript
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 15/8/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature	Date: 10/08/16

CHAPTER THREE

A RODENT MODEL OF STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES: A SUBSTRATE FOR ATRIAL FIBRILLATION

3.1 Introduction

There is an increased prevalence of cardiovascular complications in patients with type 1 and type 2 diabetes mellitus (T1DM and T2DM respectively)(275,276). In particular, patients with T1DM have been associated with at least a 10-fold increase in risk of attaining cardiovascular disease in comparison to the non-diabetic age-matched population(276,277). DM is an independent risk factor of atrial fibrillation (AF)(12), a commonly presented arrhythmia responsible for considerable morbidity and mortality in the general population. Importantly both DM(278) and AF(279,280) have been identified as independent risk factors for stroke, and consequentially, death. However, epidemiological data delineating the two diabetic states and their individual role in predisposing to AF is lacking. Unlike T2DM, the relationship between T1DM and the pathogenesis of atrial AF in particular, remains at large, with few studies investigating the T1DM atrial substrate.

Previous studies have shown that the pathophysiology of T1DM has been associated with abnormalities in contractile activity, sarcoplasmic reticular function, myocardial metabolism, and disturbances in heart rhythm(281-283). Contractile dysfunctions include reduced amplitude of contraction, prolonged time course of contraction and relaxation, and, in the rat heart, prolongation of the transmembrane action potential (AP)(284-287). Although the underlying

mechanisms of these complications remain incompletely understood, evidence from epidemiological studies has suggested a strong association between the level of glycaemia and the occurrence of such complications(288). While some studies have demonstrated an association between hyperglycaemia and cardiovascular disease(289), others have noted no reduction in the occurrence of cardiovascular disease even with long term intensive therapy(290).

Alloxan and streptozotocin (STZ) are two recognised diabetogenic agents that have been widely used in animal experimental models of T1DM, with the latter glucose mimetic being the predominantly preferred agent used due to its stability and longer half-life(254). Treatment of adult rats with STZ produces a diabetic state characterised by weight loss, polydipsia, polyuria, glycosuria, hyperphagia, hypoinsulinemia with accompanying hyperglycaemia(291). Studies investigating the pathophysiology of STZ-induced T1DM have postulated that defective Ca^{2+} signalling mechanisms -such as reduction in L-type Ca^{2+} current (I_{CaL}), decreased Ca^{2+} uptake and release from the sarcoplasmic reticulum, and, reduced rate of Ca^{2+} efflux via Na^{+}-Ca^{2+} exchanger (NCX)- partly underlie the contractile defects observed in T1DM-mediated cardiovascular disease(292-294). Other studies such as Ward & Crossman *et al*'s 2014 study have also attributed the impairment of excitation-contraction coupling and consequential contractile dysfunction observed in DM to alterations in sarcoplasmic reticulum Ca^{2+} reuptake, a reduction in the t-tubule distribution, increased levels of type I collagen and decreased myofilament sensitivity to Ca^{2+}(295). It is important however, to note that these aforementioned studies have focussed primarily on the ventricular myocardium. Additionally, patients with diabetes have a high incidence of diabetic

cardiomyopathy owing to potential prolongation of the QT-interval(296). Currently, little is known of the effect of STZ-induced T1DM on the atrial myocardium, in particular, the predisposition of type 1 diabetic patients to atrial fibrillation (AF), a cardiac arrhythmia that is responsible for significant morbidity and mortality in the general population, and, is the most commonly presented cardiovascular disease clinically(63).

This study investigated STZ-induced T1DM changes in atrial electro-structural physiology. To better understand the pathogenic mechanisms of the type 1 diabetic atrial substrate in promoting AF, we investigated changes in the atrial action potential, conduction parameters, effective refractory period (ERP), cardiomyocyte size and degree of fibrosis.

3.2 Methods

All experimental procedures involving animals were approved by the Animal Ethics Committee of the University of Adelaide. All procedures were in accordance to the guidelines outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition, 2004 endorsed by the National Health and Medical Research Council of Australia. Animals were housed individually under identical conditions upon administration of saline or streptozotocin, in accordance to the animal welfare officer and the Laboratory of Animal Services, University of Adelaide. Animals were fed standard chow and water *ad libitum*.20 Zucker rats were studied in total (Control: N = 10, T1DM: N = 10).

3.2.1 Induction of T1DM with Streptozotocin (STZ)

At 3 months of age, Zucker rats were randomly selected to receive one of two treatment options via a single intraperitoneal injection of either saline (Control animals) or 30 mg / kg of STZ (T1DM animals) dissolved in a citrate buffer solution (0.1 $mmolL^{-1}$ citric acid, 0.1 $mmolL^{-1}$ sodium citrate). At 6 months of age, animals were sacrificed by exsanguination and their atrial myocardium used for electrophysiological study. The fasting glucose levels of each animal was measured prior to commencement of each study.

3.2.2 Tissue preparation

Rats were anaesthetized with an intraperitoneal injection of Ketamine (75 mg / kg) and Domitor (0.5mg / kg). Heparin (2-U / g body weight) was administered following anaesthesia. A midline thoracotomy incision was then performed following deep anaesthesia to enable the heart to be removed and the right and left atria (RA, LA respectively) excised for separate and immediate electrophysiological study.

3.2.3 Electrophysiological study with high density mapping and simultaneous intracellular action potential recording

Each atrium was placed in the same cranial-caudal and medial-lateral orientation, with the epicardial surface in contact with a custom built 9 x 10 multi-electrode array (MEA) with a total of 90 electrodes (0.1mm diameter, 0.5 mm pitch, 0.3 mm in height) yielding 80 bipolar electrograms. The MEA was housed in a sealed acrylic chamber where the tissue was superfused with bicarbonate-buffered

solution containing the following (in mmolL^{-1}): NaCl 130, KCl 4.0, $NaHCO_3$ 24.2, NaH_2PO_4 1.2, $MgCl_2$ 0.6, $CaCl_2$ 2.2, glucose 12, and bubbled with carbogen (95% O_2 / 5% CO_2), and, maintained at physiological pH 7.35 at 37.5 ºC. A lightweight nylon mesh was placed over the tissue to ensure constant contact with the MEA during the experiments.

Together with the use of this custom built MEA system as previously described(258), simultaneous action potential recordings were obtained by inserting an aluminosilicate microelectrode of 100 MΩ resistance (filled with 3M KCl solution) into the endocardial surface of the atrium at a given recording region. The MEA was connected to a computerised recording system (LabSystem Pro; Bard Electrophysiology, Lowell, MA, USA) using high-density connectors and cables. Electrograms were sampled at 4 kHz and filtered from 10-500 Hz. A pacing stimulus was delivered to two stimulation sites during a standard S1-S2 pacing protocol at a current of 0.1 mA with a pulse width of 0.5 ms at four pacing cycle lengths beginning at 400 ms with 100 ms decrements. Intracellular AP recordings were made with a high input impedance amplifier (WPI model 725), digitised at 10 KHz, with 16-bit resolution, and, were both recorded and analysed with Chart 5 (AD Instruments). The entire apparatus was mounted on a vibration free table and housed in a grounded Faraday cage.

3.2.4 Parameters measured

3.2.4.1 Animal characteristics

Prior to each experiment, fasting blood glucose levels, systolic blood pressure (using the standard tail cuff method) and body weight measurements were made.

Upon completion of each experiment, chamber weights for the LA, RA, left and right ventricles (LV and RV respectively) were measured.

3.2.4.2 Electrophysiological Study

Tissue refractoriness (effective refractory period, ERP) was evaluated using a drive train stimulus of 8 beats (S1) followed by delivery of a premature stimulus (S2) at decrements of 10ms beginning from an initial coupling interval of 200ms. ERP was defined as the longest S1 - S2 interval not resulting in a propagated response. Three measurements of refractoriness were recorded for each pacing cycle length (100, 200, 300 and 400 ms) at each stimulation site.

Conduction velocity (CV) and conduction heterogeneity (conduction heterogeneity index, CHI) were determined using semi-automated custom made plaque analysis software (Nucleus Medical) to obtain a single activation map for each pacing cycle length(258). Manual verification was conducted for each annotation by annotating the local activation time to the maximum deflection of the largest amplitude from baseline in the bipolar electrogams obtained. CV calculations were made from triangulation of local vectors of electrodes as previously validated(297). CHI was evaluated using previously described phase-mapping techniques(259). The phase distribution of conduction was calculated using the largest difference in activation between each of 4 consecutively occurring adjacent electrodes. Absolute conduction phase delay was obtained by determining the difference between the 5^{th} and 95^{th} percentiles of the phase distribution (P_5-P_{95}). This value was divided by the median of the phase

distribution (P_{50}) to obtain the CHI. The total activation time was evaluated as the longest time taken for conduction for a given map.

3.2.4.3 Intracellular Recording

100 action potential duration (APD) measurements at 90, 50 and 20% of repolarisation (APD_{90}, APD_{50}, APD_{20}) were obtained using Peak Parameters (Chart 5, AD Instruments) for each atrial preparation. APs with a baseline membrane potential of less than -60 mV, dV / dT of <10,000 mVs^{-1} and an overshoot of <10 mV were excluded. In the LA, intracellular APs were recorded from *N* = 10, Control and *N* = 9, T1DM atrial preparations. In the RA, intracellular APs were obtained from *N* =8, Control and *N* =10, T1DM atrial preparations.

3.2.4.4 Histology

At the end of each experiment, the atria were fixed in 10% formalin for histological analysis (Control: LA and RA *N* =7 preparations per group, T1DM: LA and RA *N* =9 preparations per group). Atrial preparations were then embedded in wax as per standard routine procedures. To assess for atrial cardiomyocyte size and the degree of fibrosis, transverse sections of atrial preparations were sliced (4µm, Lecia RM2235 Rotary Microtome), mounted onto slides coated with albumin, and stained with haematoxylin and eosin, and Masson's Trichrome respectively. Slides were then scanned at 400 x magnification using the NanoZoomer Digital Pathology (NDP) system (Hamamatsu Photonics, Hamamatsu, Japan). Five fields from each photomicrograph were then selected at random and exported. To assess for cardiomyocyte size, the cross-sectional diameter of 100 cells per atrium were analysed independently in a blinded fashion using NDP view 2

(Hamamatsu Photonics, Hamamatsu, Japan). The degree of fibrosis was assessed with Masson's Trichrome staining, whereby collagen depositions were stained blue. Photomicrographs were processed via colour enhancement (Adobe Photoshop CC ver. 14.1.2, Adobe Systems, CCA, USA)(298) and the pixel content staining of each atrium was measured as a percentage of the total tissue area (Image J ver 1.7.0, NIH, USA)(298) with a batch macro after background subtraction with colour correction.

3.2.4.5 Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples were collected via cardiac puncture prior to excising the heart and the atria from Control (*N* = 6) and T1DM (*N* = 6) animals and placed in 4 mL EDTA tubes. Blood collection was conducted using a 16 G needle and a 5 mL syringe containing heparin. The EDTA tubes were centrifuged at 1500 g at 4 ºC for ten minutes. Following this, the plasma was transferred into 1.5mL Eppendorf tubes and stored in a -20 ºC refrigerator. Plasma samples were analysed for peptide levels of the following Interleukin- (IL)-1β, -6, -10, interferon-γ (IFNg), intercellular adhesion molecule-1 (ICAM-1), L-Selectin, monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and tumour necrosis factor alpha (TNF-α). Plasma samples were analysed in accordance with the instructions provided by the manufacturers, using multiplex sandwich ELISA arrays (Rat Cytokine Array Q2 (QAR-CYT), Ray biotech, GA, USA).

Statistics

All data are expressed as mean ± SEM. Data with a p value < 0.05 was considered significant. Unpaired student's t Test was used to compare Control and T1DM data for animal characteristics, ELISA, cardiomyocyte size and percentage fibrosis. To assess for significant differences in the ERP, conduction velocity (CV), conduction heterogeneity (CHI) and APD between control and T1DM groups across pacing cycle lengths of 100, 200, 300 and 400 ms, a generalised mixed linear model was used.

3.3 Results

3.3.1 Animal characteristics

T1DM animals were not significantly different to Control animals in terms of body weight (p = 0.07) and chamber weights (LA: p = 0.61, RA: p = 0.25, LV: p = 0.78, RV: p = 0.16) (Table. 1). As expected, T1DM animals showed significant signs of hyperglycaemia compared to their age-matched Controls (T1DM: 18.47 ± 1.19 $mmolL^{-1}$ vs Control: 13.69 ± 1.26 $mmolL^{-1}$, p = 0.04) (Table. 1). Interestingly, T1DM animals presented with elevated systolic blood pressures compared to Control animals (T1DM: 131.54 ± 3.93 mmHg vs Control: 129.92 ± 4.93 mmHg, p =0.02) (Table. 1)

3.3.2 Electrical remodeling

3.3.2.1 High density map study

A detailed electrophysiological study (EPS) was conducted on all atrial tissue preparations. There was an abbreviation in the ERP across all pacing cycle lengths in both LA (p < 0.0001, Fig. 1, left) and RA (p < 0.0001, Fig. 1, right) with a concomitant significant increase in conduction heterogeneity (both RA and LA

$p < 0.0001$, Fig. 2) in T1DM animals compared to their age-matched Controls. In the LA, the CHI was as high as up to 2 fold in T1DM animals compared to Controls, and approximately 1.5 fold more in the RA (Fig. 2, Fig. 3), with T1DM animals showing faster average CV in both LA ($p < 0.0001$, Fig. 4, left) and RA ($p < 0.0001$, Fig. 4, right).

3.3.2.2 Intracellular action potential study

APD_{90} was significantly prolonged in T1DM animals compared to Controls in both atria (LA and RA both $p < 0.0001$, Fig. 5). This was consistently observed for APD_{50} and APD_{20} for both LA and RA and displayed in Table. 2, which shows a comparison between Control and T1DM APD at each repolarisation parameter measured irrespective of the pacing cycle length.

3.3.3 Structural remodeling (Histology)

To assess for T1DM-induced changes in atrial cardiomyocytes size, the diameter of individual cardiomyocytes was measured in both LA and RA (Fig. 6). 20 cardiomyocyte diameters were obtained from 5 randomly selected regions per atrial preparation. Evidence of atrial cardiomyocyte hypertrophy was found, with T1DM LA and RA preparations showing significantly larger cardiomyocyte diameters when compared to Control animals (LA: Control 8.80 ± 0.12 μm vs T1DM 11.98 ± 0.14 μm, $p < 0.0001$; RA: Control 8.34 ± 0.12 μm vs T1DM 11.20 ± 0.29 μm, $p < 0.0001$).

Mason's Trichrome staining was used to investigate atrial structural remodeling in the T1DM animal by measuring for differences in levels of fibrosis (Fig. 7). T1DM animals had significantly elevated levels of fibrosis compared to their

Control counterparts in both LA (Control 0.19 ± 0.08 % vs T1DM 0.79 ± 0.20 %, $p = 0.001$) and RA (Control 0.28 ± 0.15 % vs T1DM 0.51 ± 0.19 %, $p < 0.0001$).

3.3.4 Plasma serum levels of inflammatory biomarkers

Plasma serum levels of IL-1β, IL-6, IL-10, IFN-γ, ICAM-1, L-selectin, TIMP-1 and TNF-α were elevated in T1DM animals compared to Control animals (Table. 3). However, this was only significantly increased for levels of ICAM-1 (Control 444.88 ± 419.31 pg / mL vs T1DM 5911.75 ± 393.11 pg / mL, $p = 0.03$). In contrast, levels of MCP-1 were significantly reduced in T1DM animals compared to Control animals (Control 1298.21 ± 113.20 pg / mL vs T1DM 828.97 ± 140.98 pg / mL, $p = 0.03$).

3.4 Discussion

This study presents new information on the nature of the atrial remodelling that results from diabetes induced by a low-dose STZ injection (T1DM). We found the following features:

1. Atrial conduction abnormalities characterised by an increase in conduction velocity, and an increase in conduction heterogeneity;
2. Abbreviation of the atrial refractoriness;
3. Prolonged the intracellular atrial action potential at all repolarisation parameters measured;
4. Structural abnormalities characterised by atrial cardiomyocyte hypertrophy and increased levels of atrial fibrosis;

These changes present a structural and electrical substrate for conduction instability and for a re-entrant arrhythmia to potentially establish itself.

Patients with DM exhibit a high incidence of diabetic cardiomyopathy and metabolic dysregulation resulting from systemic changes as well as alterations in structural, mechanical and electrical properties of the heart(299). In the present study, following a single intraperitoneal injection of STZ, T1DM animals presented with hyperglycaemia at levels similar to those previously reported in other studies(300,301). 12 weeks following administration of either STZ or saline solution for T1DM and Control animals respectively, animals were not significantly different in body weight ($p > 0.05$) and chamber weights ($p > 0.05$). In contrast, Ding *et al* (2006) reported a reduction in body weight following an 8-week period post STZ injection. This was not dissimilar to other studies which have employed murine models of STZ-induced diabetes(302,303).

3.4.1 Systemic changes

In the clinical setting, patients with insulin-dependent T1DM have an increased risk of developing hypertension secondary to diabetes-induced nephropathy. Patients within whom nephropathy does not develop, on the other hand, remain normotensive(304); Although this study did not seek to investigate the functional impact of STZ-induced diabetes on the kidney, elevated levels of systolic blood glucose were observed ($p = 0.02$), suggesting the hypertension reported in this STZ-induced model of T1DM may have occurred secondarily to the diabetes-induced nephropathy. Interestingly, although previously reported clinically and associated with the occurrence of nephropathy of the glomeruli in insulin-dependent diabetic patients, this cannot be said for experimental studies of T1DM. Current available literature on experimentally induced diabetes is lacking in data on T1DM-induced hypertension. This study is the first to report such changes in

blood pressure in the STZ-induced T1DM Zucker rat. Our results were in contrast to clinical and experimental observations of the relationship between hypertension and risk of AF. Several studies have previously reported that hypertension is associated with increased risk of AF and stroke(42,46,47,305-307).

3.4.2 Atrial electrical changes

The most prominent and lethal change in electrical properties in the diabetic heart is prolongation of the Q-T interval(296) or prolonged dispersion of the Q-T interval as a result of lengthening of the cardiac APD(308,309). There is evidence that chronic diabetes induced by STZ injection can not only prolong APD via marked reduction in outward K^+ currents I_{to} (transient outward current), I_{kr} (delayed rectifier current) and I_{ss} (steady state current) but also, increase the incidence of myocyte arrhythmic contraction in rat ventricular myocytes. Interestingly, enothelin-1 receptor antagonist, bosentan has previously been shown to attenuate these changes(301). Prolongation of the APD has been speculated to also contribute towards reduced diastolic filling and stroke volume at high heart rate, and may decrease the endocardial-epicardial APD gradient, distorting the temporal pattern of repolarisation as a result(310). In addition to the downregulation of the outward K^+ currents, depressed levels of mRNA and protein expression of Kv 4.2 and Kv 1.2 which contribute towards I_{to} and I_{ss} respectively have been reported in diabetic rat myocytes in comparison to control rat myocytes(311,312). While some studies have reported prolongation of the AP as early as 4-6 days(285,313) following diabetes induction, others have found contrasting results indicating that such changes in electrophysiology required at

least a 30 week time period of sustained diabetes(314). This current study showed that a short term, 3 month period at approximately half the dose of the latter study (which required 65 mg/ kg of STZ), was sufficient for electrophysiological changes to be observed in the atrial myocardium.

It is important to note that differences in ventricular and atrial preparations in response to STZ-induced changes are crucial. While many studies have investigated diabetes-induced ventricular changes, few have assessed for alterations in the atrial AP and atrial myocardium. Interestingly, it has been observed that although lengthening of the APD occurred earlier in the ventricular myocardium than in the atrial myocardium, prolongation of the APD was most pronounced at earlier phases of repolarisation in atrial preparations(315). Similar to our study's findings, other researchers have reported that elongation of the APD occurred as early as within 1 week of diabetes(316). It has been proposed that lengthening of the AP at the early phase of repolarisation is likely due to suppression of K^+ currents(313,317), whilst elongation at the final stage of repolarisation is probably resultant of Ca^{2+}-overload induced enhanced NCX activity in the diabetic myocardium. However, with such conflicting observations, it is likely that the diabetes-mediated changes may also be largely dependent on differences in experimental conditions such as STZ dosage, duration of diabetes, and, diversity in anatomical atrio-ventricular composition (i.e. differences in ion channel distribution and currents between the atria and ventricles)(315).

This study demonstrated that following a 12 week period of STZ, atrial APD was significantly prolonged in both LA and RA preparations at all repolarisation

parameters measured regardless of pacing cycle length. Together with slowing of AP repolarisation and lengthening of the APD, a reduction in the ERP with concomitant increased heterogeneity in conduction was observed. These findings are novel to this study. Two other studies investigating T1DM-induced alterations in the AP and conduction of the AP have reported similar results.

Pacher *et al* (1999) observed significant time-dependent prolongation of the atrial AP at 25, 50, 75 and 90% of repolarisation(318). Watanabe *et al* (2012) assessed for STZ-induced diabetes in 8 week old male Wistar rats with a dosage of > 2 fold used in this study(61). Using optical mapping, they investigated rate-dependent alterations in conduction velocity and heterogeneity in the RA. They observed prolongation of APD at 80% of repolarisation (APD_{80}) with an accompanying greater coefficient of variation of APD_{80} as well as an increase in conduction slowing and conduction heterogeneity in the diabetic animals compared to controls(61). In contrast to Watanabe *et al*'s (2012) study, we observed an increase in conduction in both LA and RA preparations. Along with a greater heterogeneous conduction in the T1DM atrial preparations, these results suggest of the complexity and non-uniformity of conduction in the diabetic atria(61).

Interestingly, Howarth *et al* (2007) who administered a similar dosage of STZ (60 mg / kg) to that of Watanabe *et al* (2012), showed that APD was significantly prolonged in not only the RA but also in the sinoatrial node and in the RV of diabetic rats(61,319). This however, was not observed in the LA and LV. The authors postulated that it is the regional defects in the expression and/or electrophysiology of the sinoatrial nodal ion channels that underlie heart rhythm disturbances in the diabetic rat(319). In addition, these authors further postulated

that alterations in gap junction connexin protein expression in the sinoatrial node may partly underlie electrophysiological defects in STZ-induced diabetic rat(320). Watanabe *et al* (2012) demonstrated for the first time reduced expression of the connexin (Cx) protein Cx 40 in the STZ-induced diabetic atrium. The authors proposed that together with a reduction in Cx 40, increased interstitial fibrosis were responsible for conduction slowing and heterogeneity observed in the diabetic atrium(61). Although Cx expression was not explored in this study, it can be speculated that alterations in Cx 40 / 43 expression and distribution may underlie the slowing of conduction and increase in conduction heterogeneity observed, as the changes in electrophysiological parameters observed in this study were similar to that of Watanabe *et al*'s (2012) study(61).

3.4.3 Atrial structural changes (Histology)

When assessing for evidence of structural remodeling, T1DM animals were found to have larger left and right atrial cardiomyocyte diameters when compared to the Control animals. This was indicative of atrial cardiomyocyte hypertrophy in the T1DM group. Interestingly, although there were no differences in LA and RA weights in Control and T1DM animals, atrial cardiomyocyte hypertrophy was observed. In addition, Masson's trichrome stained photomicrographs showed more pronounced levels of fibrosis in the T1DM animals as opposed to their age-matched Controls. These results were in support of other studies such as Huynh *et al* (2012) who demonstrated that hyperglycaemic T2DM db / db mice showed evidence of adverse structural remodeling via cardiomyocyte hypertrophy, myocardial fibrosis and increased apoptosis in the ventricles(321). Picatoste *et al* (2013) similarly reported that administration of sitagliptin, an anti-glycaemic drug,

attenuated the hypertrophy, fibrosis and cardiac cell death, and, reduced the expression levels of pro-apoptotic, hypertrophic and fibrotic factors which were previously upregulated in the T2DM Goto-Kakizaki rat(322). Importantly, in the STZ-induced and OVE26 T1DM mouse, Li *et al* (2011) reported increased cardiomyocyte cross-sectional area and increased myocardial collagen deposition with elevated levels of hypertrophic and fibrotic collagen genes(323). These investigators postulated that these results were associated with an increase in calpain activity. They further reported that deficiency of Capn4 or overexpression of calpastatin resulted in a reduction in myocardial hypertrophy and fibrosis, postulating that these effects may have occurred from normalization of nuclear factor-kB (NF-kB) and metalloproteinase activity(323). Indeed, the NF-kB pathway has previously been proposed to underlie diabetes-induced atrial remodeling in favour of a proarrhythmic substrate for AF(324).

3.4.4 Inflammatory biomarkers

Inflammation has been recognised and implicated in the pathophysiology of diabetes-induced risk of cardiovascular disease(325). The inflammatory signalling cascade is an attractive upstream approach for many researchers, and, identification of pro-inflammatory and/or pro-fibrotic markers associated with diabetes-induced predisposition to AF is a growing avenue for therapeutic targets(326).

This study investigated a number of inflammatory markers and cytokines. The adhesion molecule which mediates leukocyte adhesion in the endothelium and is part of the atherosclerotic process(327), ICAM-1, was elevated in the plasma

serum of T1DM animals. Previous studies have associated ICAM-1 with the excess pathogenesis of cardiovascular disease in diabetic patients with adverse renal complications(327) as well as diabetic retinopathy(328). Similarly, in the setting of AF, it has been proposed that AF may regulate expression of ICAM-1 on endothelial cells in the LA. Upregulation of levels of ICAM-1 may also increase leukocyte-endothelia adhesion and increase the risk of thrombus formation, and stroke in AF. Studies have demonstrated that neutralizing antibodies to cytokines and adhesion molecules significantly attenuated the inflammatory response of venous thrombosis(329). Levels of IL-1β, IL-6, IL-10 and TNF-α, TIMP-1 and L-Selectin although elevated in T1DM animals, were not found to be significantly different when compared to Control animals. Literature regarding inflammatory biomarkers and T1DM is lacking. However, many of the inflammatory biomarkers assessed in this study have been implicated in patients with AF. IL-6, IL-8, IL-10, TNF-α and MCP-1 have all been independently associated with AF(206). In the absence of significant vascular disease and normal renal function, alterations in levels of TIMP-1 have been associated with T1DM(330). Interestingly, T1DM animals had lower levels of MCP-1 compared to Controls. Comparatively, heightened levels of MCP-1 have been implicated as a critical marker of diabetic renal inflammation and the progression towards diabetic nephropathy(331). The relationship between heightened levels of MCP-1 in T1DM and AF is not known, however, it has been reported that patients with AF have significantly higher concentrations of MCP-1 than in the unaffected population(332). Although little is known of the role of inflammatory biomarkers on the myocardium in T1DM patients, it may be deduced that patients in whom both T1DM and AF is already prevalent, the morbidity and mortality risk may be further exacerbated. Therefore,

in patients with AF and/or T1DM, administration of neutralizing antibodies to cell adhesion molecules which aid in the inflammatory process may help reduce inflammation-induced increase in risk of AF.

3.4.5 Conclusion

The results from this study demonstrate that STZ-induced diabetes can alter the atrial substrate at the electrophysiological and structural level in favour of a proarrhythmic substrate for AF. We demonstrated for the first time an abbreviation of the effective refractory period, increased conduction heterogeneity, increased rate of conduction, prolongation of the atrial action potential, with elevated levels of interstitial fibrosis and atrial cardiomyocyte hypertrophy.

Literature regarding the impact of T1DM on the atrial myocardium is limited. Variations in experimental protocol, such as in the animal or species of used, dosage of STZ and in the duration of diabetes induction may impart explain the different observations made. Additionally, the administration of insulin would also be an important factor to consider as it may potentially reduce the diabetes-induced pathologically adverse effects on the atrial myocardium. Further investigation into this area of T1DM and its impact on the atrial myocardium is still required to better understand the underlying predisposition of the diabetic patient towards AF.

FIGURES & TABLES

Parameters	Control	T1DM	P Value
Body weight (g)	353.80 ± 4.46	373.40 ± 8.53	0.07
Fasting blood glucose ($mmolL^{-1}$)	13.69 ± 1.26	18.47 ± 1.19	0.04*
Systolic blood pressure (mmHg)	129.92 ± 4.93	131.54 ± 3.93	0.02*
LA (mg)	18.54 ± 1.33	20.12 ± 2.70	0.61
RA (mg)	36.93 ± 3.56	45.61 ± 6.24	0.25
LV (mg)	425.69 ± 27.19	412.12 ± 40.20	0.78
RV (mg)	202.69 ± 10.59	233.70 ± 17.58	0.16

Table 1. Animal Characteristics. Type 1 diabetic animals (T1DM) were not significantly different in body weight when compared to their age-matched Control animals (p = 0.07). T1DM animals were significantly more hyperglycaemic (p = 0.04) and had higher systolic blood pressures (p = 0.02) compared to Control animals. Atrial and ventricular weights were not significantly different between groups (p > 0.05). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle. * indicates p < 0.05

Left Atrial APD (ms)			
	Control	T1DM	*p* Value
APD90	25.46 ± 0.98	33.61 ± 0.90	$p < 0.0001$
APD50	11.66 ± 0.37	14.83 ± 0.25	$p < 0.0001$
APD20	5.46 ± 0.21	6.86 ± 0.16	$p < 0.0001$

Right Atrial APD (ms)			
	Control	T1DM	*p* Value
APD90	25.26 ± 1.08	42.91 ± 1.42	$p < 0.0001$
APD50	8.37 ± 0.46	14.37 ± 0.75	$p < 0.0001$
APD20	2.88 ± 0.17	5.21 ±0.30	$p < 0.0001$

Table. 2 Left and right atrial action potential durations at 90, 50 and 20% ($APD_{90,50,20}$) of repolarisation in type 1 diabetic (T1DM) and Control animals at a pacing cycle length of 400ms. Top: Left atrial $APD_{90, 50, 20}$ were significantly prolonged in T1DM animals compared to Control animals ($p < 0.0001$). Bottom: Right atrial $APD_{90, 50, 20}$ were longer in T1DM animals compared to Controls ($p < 0.0001$).

Biomarker (pg/mL)	Control	T1DM	P Value
IL-1β	14.41 ± 1.34	32.46 ± 10.61	0.15
1IL-6	6.87 ± 4.19	10.95 ± 7.20	0.64
IL-10	1620.95 ± 337.59	2538.61 ± 636.22	0.24
IFNg	0.57 ± 0.14	0.72 ± 0.13	0.48
ICAM-1	4444.88 ± 419.31	5911.75 ± 393.11	0.03*
L-Selectin	583.52 ± 90.51	1066.28 ± 218.41	0.08
TIMP-1	8837.97 ± 1438.32	14648.95 ± 3256.79	0.15
TNF-α	304.59 ± 38.42	415.47 ± 75.23	0.23

Table 3. Plasma serum levels of biomarkers of inflammation. Type 1 diabetic animals (T1DM) had significantly elevated levels of ICAM-1 ($p < 0.05$) compared to Controls. Levels of IL-1β, IL-6, IL-10, IFNg, L-Selectin, TIMP-1 and TNF-α were not significantly different between T1DM and Control animals. * indicates $p < 0.05$

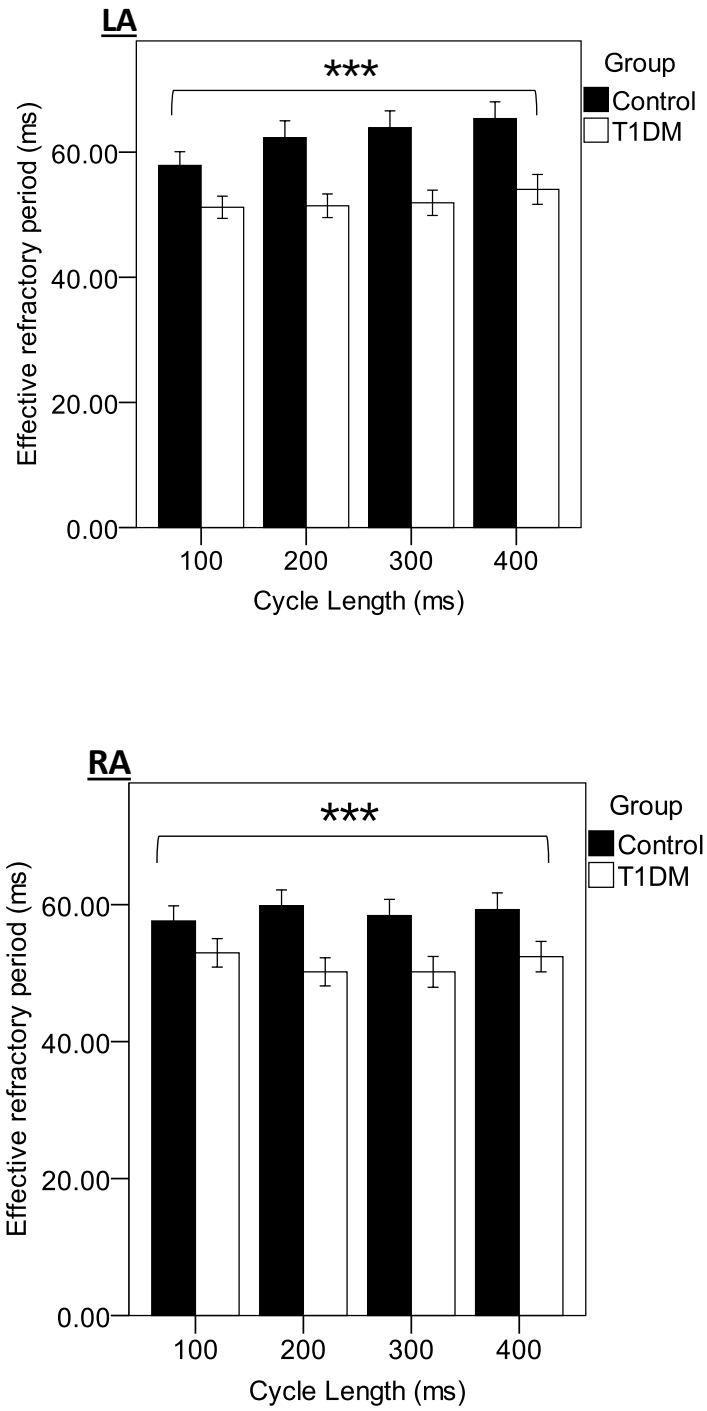

Figure 1. A comparison of type 1 diabetic (T1DM) and Control left (LA) and right (RA) atrial effective refractory periods (ERP) during standard S1-S2 pacing at 100, 200, 300 and 400 ms. LA (top) and RA (bottom) ERP were shortened significantly in the T1DM group compared to the Control group (both LA and RA $p < 0.0001$). *** indicates $p < 0.0001$

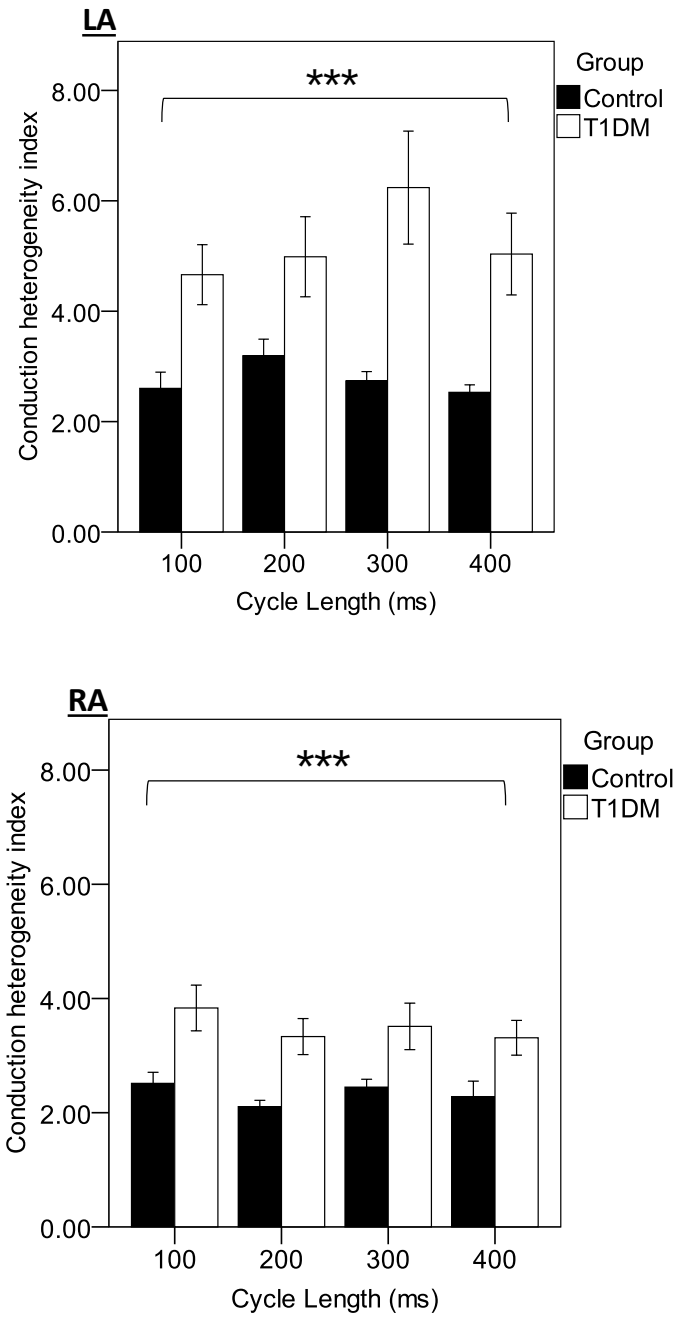

Figure 2. A comparison of conduction heterogeneity in type 1 diabetic (T1DM) and Control left (LA) and right (RA) atria. Conduction of the electrical impulse was significantly less uniform in the LA (top) and RA (bottom) as indicated by the higher conduction heterogeneity index value compared to Controls ($p < 0.0001$). *** indicates $p < 0.0001$

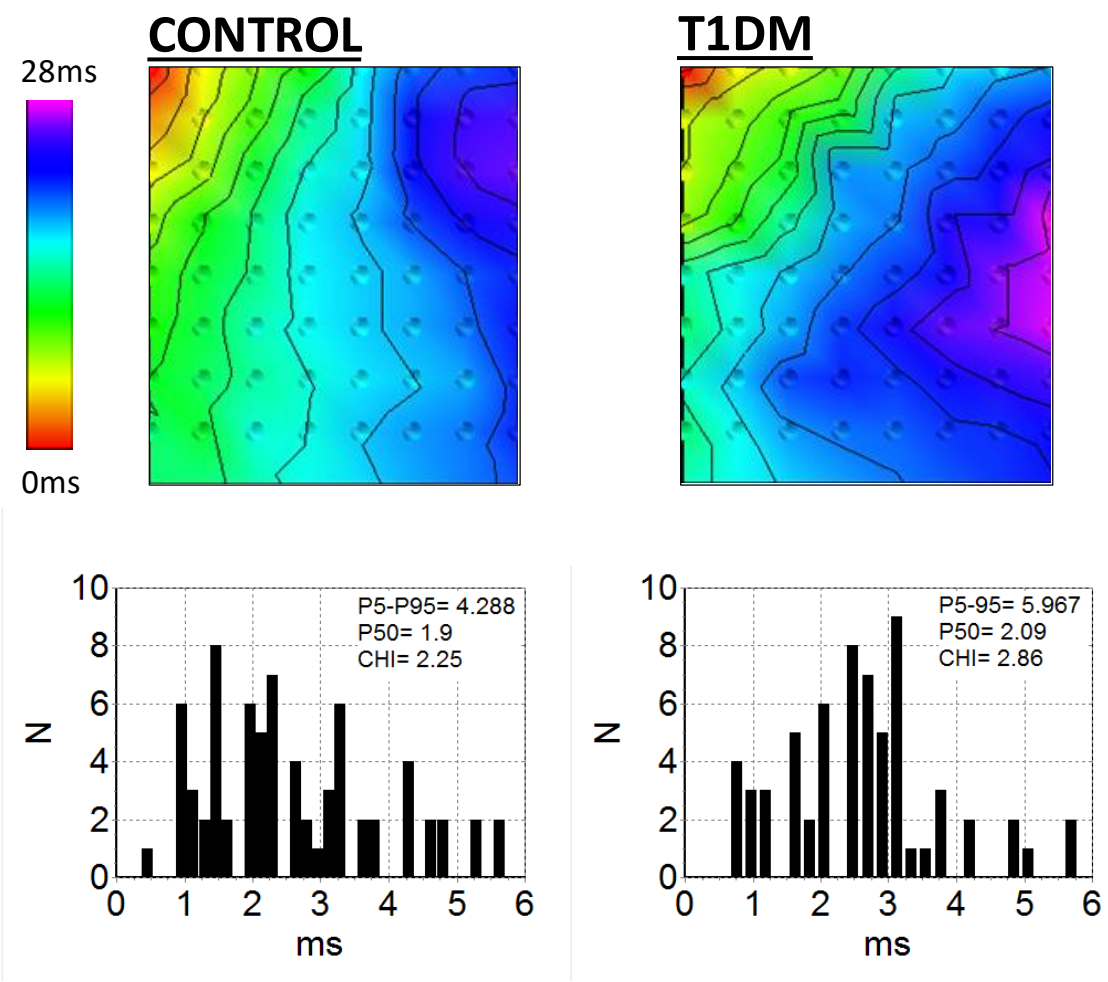

Figure 3. Representative activation maps and phase histograms from the left atrium (LA) of a single Control and type 1 diabetic (T1DM) animal. Example activation maps with superimposed 2 ms isochrones, and, corresponding phase histograms from the LA of a single Control (left) and T1DM (right) animal.

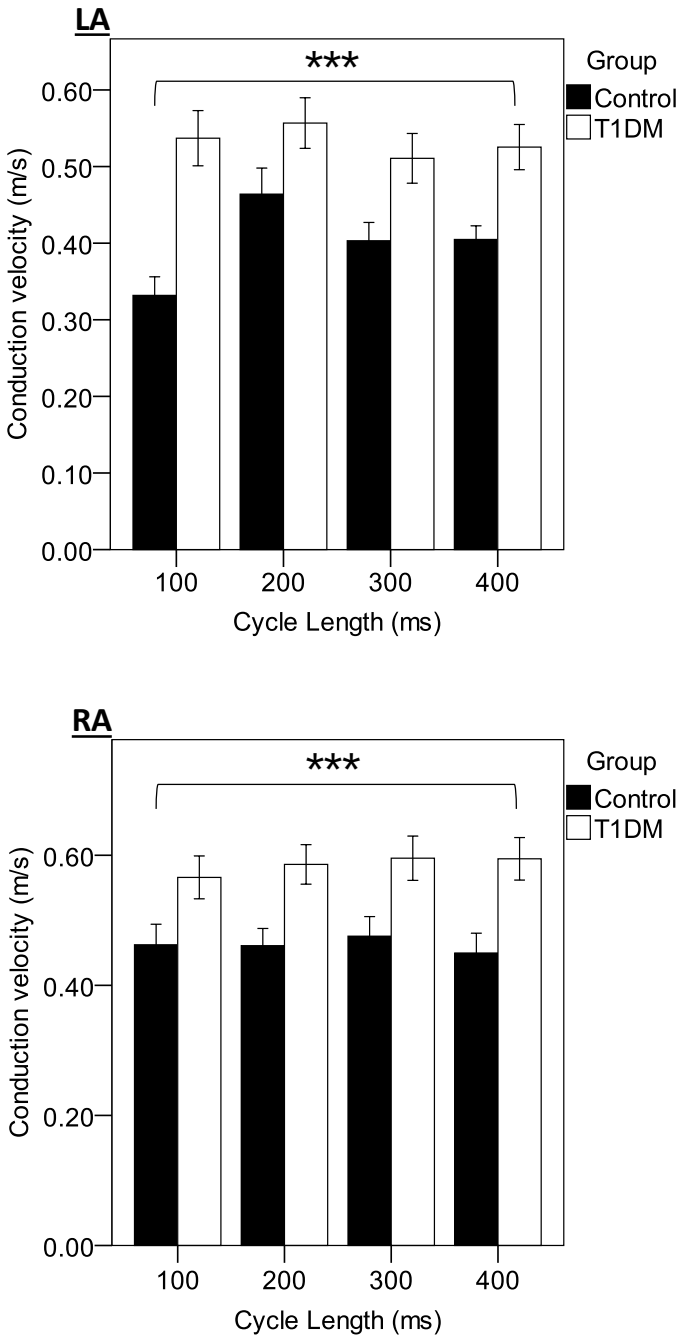

Figure 4. A comparison of conduction velocities in type 1 diabetic (T1DM) and Control left (LA) and right (RA) atria. Average conduction velocities were greater in T1DM animals in both the LA (top) and RA (bottom) compared to controls (both LA and RA, $p < 0.0001$). *** indicates $p < 0.0001$.

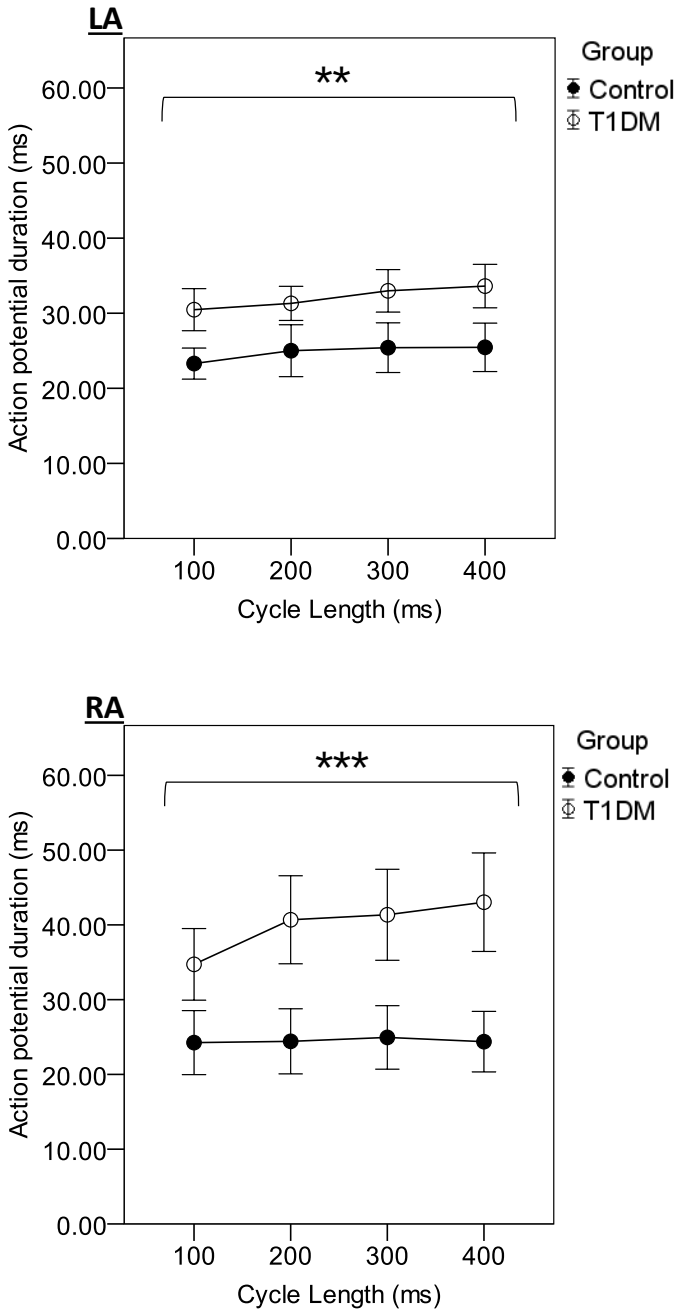

Figure 5. A comparison of action potential duration at 90% of repolarisation (APD_{90}) in type 1 diabetic (T1DM) and Control left (LA) and right (RA) atria. T1DM animals had significantly prolonged APD_{90} compared to Control animals in the LA (top, $p < 0.01$) and RA (bottom, $p < 0.0001$). ** indicates $p < 0.01$, *** indicates $p < 0.0001$.

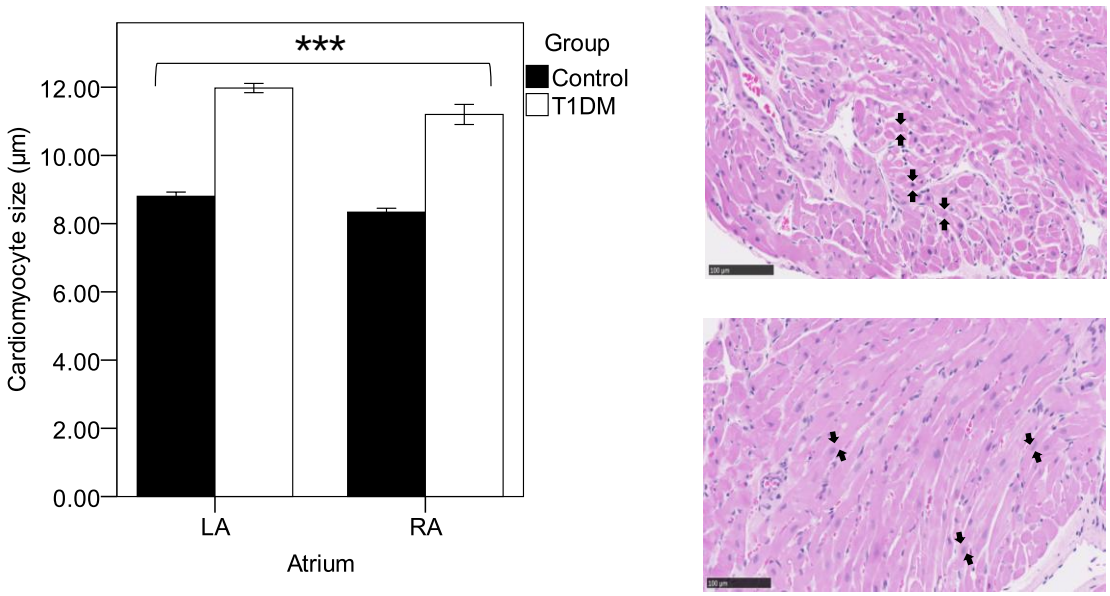

Figure 6. Cardiomyocyte diameters of left (LA) and right (RA) atrial preparations from Control and type 1 diabetic (T1DM) animals. Left: LA and RA cardiomyocytes were significantly enlarged in the T1DM group compared Controls (LA and RA: p < 0. 0001) Right: Example haematoxylin and eosin photomicrographs at 400 x magnification from the LA preparations of a single Control (top) and T1DM (bottom) animal. Scale bars show 100 µm. The space between each pair of black arrows indicates the presence of a cardiomyocytes. *** indicates $p < 0.0001$.

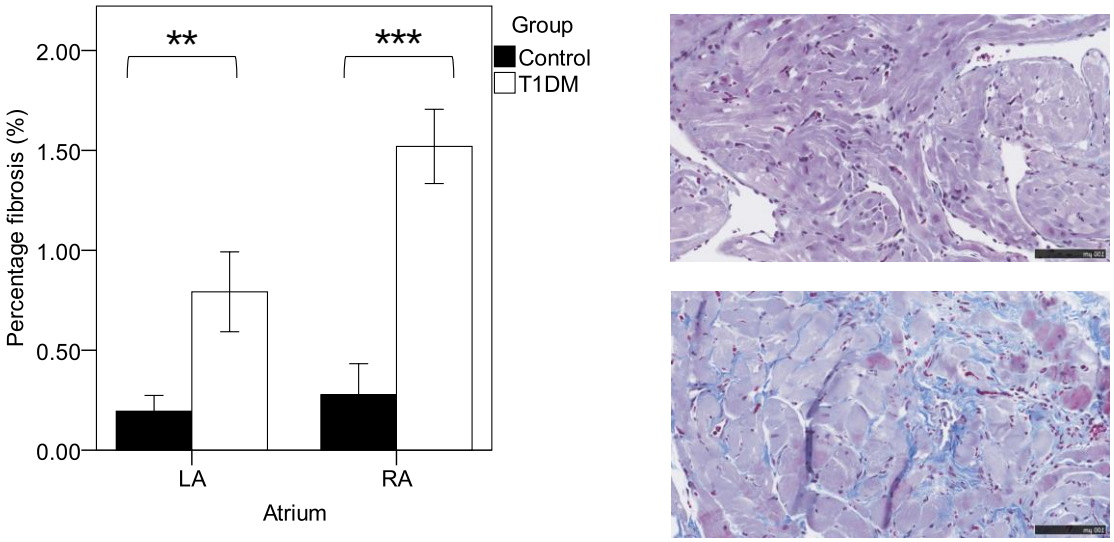

Figure 7. Percentage of fibrosis in left (LA) and right (RA) atrial preparations from Control and type 1 diabetic (T1DM) animals. Left: Percentage of fibrosis in T1DM animals were > 3 fold compared to Control animals (LA: $p < 0.01$ and RA: $p < 0.0001$) Right: Example Mason's trichrome photomicrographs at 400 x magnification from the LA preparations of a single Control (top) and T1DM (bottom) animal. Scale bars show 100 µm. ** indicates $p < 0.01$, *** indicates $p < 0.0001$.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Melissa Neo
Contribution to the Paper	Design of study Performance of experiments in the study and follow up analysis and interpretation of data obtained Drafting of manuscript
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 10/8/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Wei-Wen Lim
Contribution to the Paper	Performance of experiments in the study Analysis and interpretation of research data obtained Drafting of the manuscript
Signature	Date: 11/08/2016

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Contribution to the Paper	Analysis and interpretation of research data obtained Drafting of the manuscript
Signature	Date: 06/09/2016

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Name of Co-Author	Professor David A. Saint
Contribution to the Paper	Design of the study Analysis and interpretation of research data obtained Drafting and reviewing structure and content of important parts in the manuscript
Signature	Date 10/08/16

CHAPTER FOUR

DIABETES AND AGE: RISK FACTORS FOR A PROARRHYTHMIC SUBSTRATE FOR ATRIAL FIBRILLATION IN A RAT MODEL OF TYPE II DIABETES MELLITUS

4.1 Introduction

Atrial fibrillation (AF) is the most commonly presented cardiac arrhythmia clinically. The prevalence of AF is increasing with the ageing population; approximately 0.4% of the general population suffer from AF, of which > 6% occurs in the elderly aged above 80 years old(5,20). AF is associated with increased morbidity and mortality in the general population and is a major risk marker for increased stroke incidence with age and associated cardiac abnormalities(279,280). As a result, identification of potentially modifiable risk factors for AF has become an attractive means towards achieving the goal of reducing AF risk(12,14,333). Advanced age, hypertension and cardiovascular disease have all been previously identified as risk factors of AF. Diabetes mellitus (DM) is associated with most of the above risk factors(334-338), and is recognised as a strong, independent risk factor for AF, with an odds ratio of 1.4 (CI 1.0-2.0)(12). DM is additionally associated with a further increase in the incidence of stroke and death in patients with AF(54). Akin to AF, the prevalence of DM increases with age, affecting approximately 140 million people worldwide, and, an estimated 2 fold increase in diabetic individuals has been projected to occur by the year 2025, affecting up to 300 million people in the world(55).

The association between DM (in particular, non-insulin dependent, type 2 diabetes, T2DM) and obesity is common knowledge, previously being demonstrated in both cross-sectional(334,339) and prospective studies(340-342). Obesity plays a major role in the pathogenesis and aetiology of T2DM, and, also occurs in association with aforementioned risk factors such as hypertension. The World Health Organization estimated that the prevalence of obesity would reach 180 million worldwide in 2010(343). DM and obesity are both independent risk factors for increased morbidity and mortality in cardiovascular disease. However, it is unclear whether obesity itself predisposes to AF.

Taken together, there are a considerable number of electrophysiological and structural factors that can contribute towards obesity and DM-mediated increased risk of AF. These factors often coexist in the ageing individual. However, the relationship shared between AF, obesity and DM with progressing age is yet to be fully understood. Currently, there is no single study which assesses the impact of hyperglycaemia, obesity and advancing age on the electrophysiological and structural changes in the diabetic rat atria.

This study explores the impact of DM, obesity and age on the electrophysiological and anatomical parameters of the isolated rat atrium. Using a well-established rodent model of T2DM, we determine if action potential parameters, tissue conduction and changes in the structural properties of the atrial myocardium occur in the ageing, obese, diabetic rat.

4.2 Methods

All experimental procedures involving animals were approved by the Animal Ethics Committee of the University of Adelaide. All procedures were conducted in accordance to the guidelines outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition, 2004 endorsed by the National Health and Medical Research Council of Australia.

Animals were housed under identical conditions, at, and, in accordance to the animal welfare officer and the Laboratory of Animal Services, University of Adelaide. Animals were fed standard chow and water *ad libitum* until the appropriate age for experiments to be conducted.

The Zucker rat employed in this study is a recognised model of non-insulin dependent T2DM and obesity. Its origins and characteristics have been previously described(253,344,345).

4.2.1 Tissue preparation

At 3 months and 10 months of age, Zucker rats were sacrificed by exsanguination and their atrial myocardium used for electrophysiological study. 40 Zucker rats were studied in total (Control (Zucker lean): N = 10 per age group, T2DM (Zucker obese): N = 10 per age group).

Rats were anaesthetized with an intraperitoneal injection of Ketamine (75mg / kg) and Domitor (0.5mg / kg). Following anaesthesia, Heparin (2 U / g body weight) was administered. Upon deep anaesthesia, a midline thoracotomy incision was performed, the heart removed and the left and right atria (LA and RA respectively) excised for separate and immediate electrophysiological study.

4.2.2 Electrophysiological study

A custom built multi-electrode system as previously described by Lau *et al* (2010) was used for each experiment(258). Atrial orientation was kept consistent for each experiment and placed in the same cranial-caudal and medial-lateral orientation, with the epicardial surface in contact with a custom built 9 x 10 multi-electrode array (MEA) consisting of a total of 90 electrodes (0.1 mm diameter, 0.5 mm pitch, 0.3 mm in height) and yielding 80 bipolar electrograms. The atrial tissue was superfused with bicarbonate-buffered solution containing the following (in $mmolL^{-1}$): NaCl 130, KCl 4.0, $NaHCO_3$ 24.2, NaH_2PO_4 1.2, $MgCl_2$ 0.6, $CaCl_2$ 2.2, glucose 12, and bubbled with carbogen (95 % O_2 / 5 % CO_2), and, maintained at physiological pH 7.35 at 37.5 ºC, in an acrylic chamber. To ensure constant contact with the MEA, a lightweight nylon mesh was placed over the tissue for the duration of each experiment. The entire apparatus was mounted on a vibration-free table and housed in a grounded Faraday cage. The MEA was connected to a computerised recording system (LabSystem Pro; Bard Electrophysiology, Lowell, MA, USA) using high-density connectors and cables. Electrograms were sampled at 4 kHz and filtered from 10-500 Hz.

A pacing stimulus was delivered to two stimulation sites during a standard S1 - S2 pacing protocol at a current of 0.1 mA with a pulse width of 0.5 ms at four pacing cycle lengths beginning at 400 ms with 100 ms decrements. AP recordings were simultaneously obtained during atrial pacing using an aluminosilicate microelectrode of 100 MΩ resistance (filled with 3 M KCl solution) inserted into the endocardial surface of the atrium at a single recording region. Intracellular AP

recordings were made with a high input impedance amplifier (WPI model 725), digitised at 10 KHz, with 16 bit resolution. All AP recordings and analyses were conducted with Chart 5 (AD Instruments).

4.2.3 Parameters measured

4.2.3.1 *Animal characteristics*

Body weight, non-fasting blood glucose levels and systolic blood pressure measurements were determined before commencement of each experiment. The standard tail cuff method for rodents was applied for systolic blood pressure measurements. Upon completion of each experiment, chamber weights for the left and right atria (LA, RA), left and right ventricles (LV and RV respectively) were measured.

4.2.3.2 *Electrophysiological study*

Tissue refractoriness (effective refractory period, ERP) was evaluated using a drive train stimulus of 8 beats (S1) followed by delivery of a premature stimulus (S2) at decrements of 10ms beginning from an initial coupling interval of 200 ms. ERP was defined as the longest S1 - S2 interval not resulting in a propagated response. Three measurements of refractoriness were recorded for each pacing cycle length (100, 200, 300 and 400 ms) at each stimulation site.

Conduction velocity (CV) and conduction heterogeneity (conduction heterogeneity index, CHI) were determined using semi-automated custom made plaque analysis software (Nucleus Medical) to obtain a single activation map for each pacing cycle length(258). Manual verification was conducted for each

annotation by annotating the local activation time to the maximum deflection of the largest amplitude from baseline in the bipolar electrogams obtained. CV calculations were made from triangulation of local vectors of electrodes as previously validated(297). CHI was evaluated using previously described phase-mapping techniques(259). The phase distribution of conduction was calculated using the largest difference in activation between each of 4 consecutively occurring adjacent electrodes. Absolute conduction phase delay was obtained by determining the difference between the 5^{th} and 95^{th} percentiles of the phase distribution (P_5-P_{95}). This value was divided by the median of the phase distribution (P_{50}) to obtain the CHI. The total activation time was evaluated as the longest time taken for conduction for a given map.

60 to 150 action potential duration (APD) measurements at 90, 50 and 20% of repolarisation (APD_{90}, APD_{50}, APD_{20}) were obtained using Peak Parameters (Chart 5, AD Instruments) for each atrial preparation at each pacing cycle length, from a single recording region (Young RA: Control *N* = 5 (75 AP), T2DM *N* = 4 (60 AP), Young LA: Control *N* = 4 (60 AP), T2DM *N* = 5 (75 AP), Old RA: Control *N* = 9 (135 AP), T2DM *N* = 9 (135 AP), Old LA: *N* = 10 (150 AP). T2DM *N* = 10 (150 AP)). Exclusion criteria were applied to the APs selected; APs with a baseline membrane potential of less than -60mV, dV / dT of < 10,000 mV / s and an overshoot of < 10 mV were excluded. In the LA, intracellular APs were recorded from *N* = 10, Control and *N* = 10, T2DM atrial preparations. In the RA, intracellular APs were obtained from *N* = 8, Control and *N* = 10, T2DM atrial preparations.

4.2.3.3 *Histology*

Upon cessation of each experiment, the atria were fixed in 10% formalin and embedded in wax for histological analysis; Haemotoxoylin and eosin and Masson's trichrome stains were conducted in *N* = 6 LA and RA preparations of Young Control and T2DM groups, and, *N* = 10 LA and RA preparations of Old Control and T2DM groups. Transverse sections of atrial preparations were sliced (4µm, Lecia RM2235 Rotary Microtome), mounted onto slides coated with albumin, and stained with haematoxylin and eosin, and, Masson's Trichome to assess for cardiomyocyte size and presence of fibrosis respectively. Slides were scanned at 400x magnification using the NanoZoomer Digital Pathology (NDP) system (Hamamatsu Photonics, Hamamatsu, Japan). Five fields were chosen at random from each photomicrograph and exported. Cardiomyocyte size was determined by measuring the cross-sectional diameter of 100 cells per photomicrograph per atrium independently and in a blinded fashion using NDP view 2 (Hamamatsu Photonics, Hamamatsu, Japan). Masson's Trichrome staining enabled collagen depositions to be stained blue, allowing for the degree of fibrosis to be determined. Photomicrographs were processed via colour enhancement (Adobe Photoshop CC ver. 14.1.2, Adobe Systems, CCA, USA)(298). The pixel content staining of each atrium was measured as a percentage of the total tissue area (Image J ver 1.7.0, NIH, USA)(298) with a batch macro following background subtraction with colour correction.

4.2.3.4 Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from Control and T2DM animals (*N* = 12 per group, *N* = 6 per age group) via cardiac puncture and prior to excision of the atria. Blood

collection was conducted using a 16 G needle and a 5 mL syringe containing heparin and placed in 4 mL EDTA tubes which were centrifuged at 1500 g at 4 ºC for ten minutes. The plasma was then transferred into 1.5mL Eppendorf tubes and stored in a -20 ºC refrigerator. Plasma samples were analysed for levels of the following peptides: Interleukin (IL)-1β, -6, -10, interferon-γ (IFN-γ), intercellular adhesion molecule-1 (ICAM-1), Leptin, L-Selectin and monocyte chemoattractant protein-1 (MCP-1). Plasma samples were analysed using multiplex sandwich ELSIA arrays (Rat Cytokine Array Q2 (QAR-CYT), Ray biotech, GA, USA) conducted in accordance to the instructions provided by the manufacturer.

Statistics

All data are expressed as mean ± SEM. A two-way ANOVA was performed to assess for significant differences in animal characteristics, histological and ELISA data between Control and T2DM animals across both age groups. A generalised mixed linear model was used to assess for significant differences in the effective refractory period, conduction velocity, conduction heterogeneity and action potential duration between groups at pacing cycle lengths of 100, 200, 300 and 400 ms. Data with a p value < 0.05 was considered significant.

4.3 Results

4.3.1 Animal characteristics

T2DM animals had significantly greater body weights (Young Control 271.69 ± 22.02 g, T2DM 446.44 ± 10.32 g; Old Control 428.18 ± 11.43 g, T2DM 451.45 ± 12.67 g, p < 0.0001), significantly higher systolic blood pressure levels (Young

Control 123.49 ± 2.24 mmHg, T2DM 141.69 ± 3.12 mmHg; Old Control 123.72 ± 3.14 mmHg, T2DM 157.33 ± 4.31 mmHg, $p < 0.0001$) and hyperglycaemia (Young Control 19.35 ± 0.74 $mmolL^{-1}$, T2DM 29.56 ± 1.62 $mmolL^{-1}$; Old Control 21.51 ± 1.21 $mmolL^{-1}$, T2DM 29.42 ± 1.38 $mmolL^{-1}$ $p < 0.0001$) when compared to control age-matched animals in both the Young and Old groups. RA and LA weights in T2DM animals were comparable to age-matched Controls (RA, LA, $p > 0.05$, Table. 1).

4.3.2 Atrial electrical remodeling

4.3.2.1 High density map study

The following parameters were measured to assess for the presence of atrial electrical remodeling in T2DM: ERP, CV and CHI. A prolongation of the ERP was observed in the RA preparations of T2DM animals compared to Controls ($p < 0.0001$, Fig. 1, right). This was not reflected in the LA wherein no significant differences were found between groups ($p > 0.05$, Fig. 1, left). The CHI was higher in T2DM animals, suggesting marked heterogeneity in conduction compared to their age-matched Controls in both the LA and RA (LA, RA $p < 0.0001$, Fig. 2, Fig. 3). In addition, a slowing in conduction however was observed in both the LA and RA of T2DM animals (LA and RA both $p < 0.05$, Fig. 4).

4.3.2.2 Intracellular action potential study

APD_{90} was significantly prolonged in T2DM animals compared to Controls in both atria (LA $p < 0.01$, RA $p < 0.0001$, Fig. 5). Similarly, this was also observed in the LA and RA for APD_{50} (LA $p < 0.05$, RA $p < 0.0001$) and APD_{20} (LA $p < 0.05$, RA $p < 0.0001$); Table. 2 details $APD_{20,50,90}$ in the LA and RA.

4.3.3 Atrial structural remodeling (Histology)

A total of 100 cardiomyocytes were assessed for each atrial preparation (Young Control and T2DM *N* = 600 cardiomyocytes per group, Old Control and T2DM *N* = 1000 cardiomyocytes per group). Atrial cardiomyocytes of T2DM animals displayed a profound increase in cardiomyocyte diameter irrespective of age compared to Controls (Fig. 6, LA Young: Control 7.58 ± 0.24 µm vs T2DM 12.58 ± 0.26 µm, LA Old: Control 7.67 ± 0.07 µm vs T2DM 12.13 ± 0.18 µm, $p < 0.0001$; RA Young: Control 7.57 ± 0.06 µm vs T2DM 12.58 ± 0.15 µm, RA Old: Control 7.67 ± 1.30 µm vs 12.13 ± 2.71 µm, $p < 0.0001$).

Mason's Trichrome staining showed evidence of atrial structural remodeling as indicated by significantly elevated levels of fibrosis in T2DM animals regardless of age in both LA and RA preparations (Fig. 7, LA Young: Control 0.79 ± 0.10 % vs T2DM 2.87 ± 0.51 %, LA Old: Control 1.04 ± 0.09 % vs T2DM 1.32 ± 0.14 %, $p < 0.0001$; RA Young: Control 1.00 ± 0.17 % vs T2DM 2.95 ± 0.69 %, RA Old: Control 1.43 ± 0.15 % vs T2DM 1.98 ± 0.23 %, $p < 0.0001$).

4.3.4 Plasma serum levels of leptin and inflammatory biomarkers

The plasma serum levels of leptin and the inflammatory biomarkers measured for Young and Old Control and T2DM animals have been combined and can be found in Table 3. Plasma serum levels of IL-1β, IL-6, IL-10 and IFN-γ were not significantly different between groups ($p > 0.05$, Table. 3) T2DM animals had markedly higher levels of leptin in the plasma in contrast to their age-matched Controls (Control 24.33. ± 12.04 pg / mL vs T2DM 10421.67 ± 4944.40 pg / mL,

$p < 0.05$). Plasma serum levels of the following inflammatory biomarkers were elevated in T2DM animals compared to Controls: ICAM-1 (Control 4102.67 ± 404.93 pg / mL vs T2DM 6260.08 ± 802.00 pg / mL), L-selectin (Control 648.50 ± 69.49 pg / mL vs T2DM 917.83 ± 130.42 pg / mL) and MCP-1 (Control 721.67 ± 138.88 pg / mL vs T2DM 1255.17 ± 293.10 pg / mL), with plasma levels of only L-selectin being significantly different between groups ($p < 0.05$).

4.4 Discussion

This study examined the impact of T2DM in two different age groups on the electrophysiological and structural properties of the atria as a proarrhythmic substrate for AF. The key results from this study can be summarised as follows: At the structural level T2DM is associated with (1) atrial cardiomyocyte hypertrophy and (2) increased degree of interstitial fibrosis. At the electrophysiological level T2DM is associated with (1) increased conduction heterogeneity with (2) prolongation of the APD. It is apparent from the primary observations above that irrespective of age T2DM is associated with marked atrial structural change that manifests predominantly in an increased heterogeneous conduction that is accompanied by an added undertone of prolongation of action potential repolarisation, which may ultimately produce a vulnerable substrate for AF.

There is an increasing body of evidence that associates T2DM with an increase in risk of cardiovascular disease, with coronary heart disease accounting for 50 % of all deaths, and, stroke accounting for a further 15 % in individuals diagnosed with T2DM(346). In addition, the risk of cardiovascular disease has also been

associated with the “ticking clock” hypothesis- that the risk of cardiovascular disease increases before the onset of clinical diabetes (in pre-diabetic individuals)(347). Similarly, the prevalence of AF, of which diabetes is a strong, independent predictor, increases with age, affecting approximately 6% of those aged 65 years(348). In order to investigate the “ticking clock” hypothesis and the impact of diabetes on the atrial myocardium, two representative age groups, Young (3 months Old) and Old (10 months Old) were selected for Control (Zucker lean) and T2DM (Zucker obese) animals.

4.4.1 Animal characteristics

T2DM animals were significantly heavier ($p < 0.0001$) and presented with higher systolic blood pressures indicative of hypertension ($p < 0.0001$), and as expected, elevated levels of plasma glucose regardless of age ($p < 0.0001$) (Table.1). These results were similar to previous studies which have utilised the Zucker diabetic fatty rat (Zfr). Peterson *et al* (1990) reported body weights of the Zfr from 5 to 40 weeks of age; These body weights were within the range observed in our study for animals of similar ages(344,349). Similarly, Kurtz *et al* (1989) and Kasiske *et al* (1985) reported elevated systolic blood pressures in the anaesthetized T2DM rat via the tail cuff technique which was also employed in this study(350,351). The non-fasting glucose levels reported in our study were also consistent with those of Seino et al (2013); these investigators reported non-fasting plasma glucose levels as high as 462.30 mg / dl (approximately 25 $mmolL^{-1}$) by the age of 21 weeks in the T2DM rat(352).

4.4.2 Atrial electrophysiological remodeling

The electrophysiological study performed composed of two components: high-density mapping and intracellular action potential recording. Accordingly, the four electrophysiological parameters measured can be grouped under one of the aforementioned components- high density mapping: atrial ERP, CV, and CHI; intracellular action potential recording: $APD_{20,50,90}$.

Recent experimental studies have proposed that the increased susceptibility to AF in diabetic animals is due to slowing of conduction as a consequence of increased interstitial fibrosis(353) and increased atrial refractoriness(354). As previously discussed, fibrosis is an important factor in atrial structural remodeling. In addition, its role in modifying electrical conduction through the atria is also part and parcel of establishing a substrate for AF through electrical remodeling of the atria.

The main features of atrial electrical remodeling include (1) shortening of the ERP, (2) increased dispersion of refractoriness and (3) conduction delay due to loss of frequency adaptation(355). Kato *et al* (2006) demonstrated that the increase in interatrial conduction time was resultant of diabetes-mediated increase in fibrotic deposition(353). Indeed, Zhang *et al* (2014) demonstrated that atrial fibrosis, increased interatrial conduction time as well as LA diameter were related to AF inducibility(356). Similarly, Liu *et al* (2012) demonstrated interatrial conduction delay with increased atrial ERP dispersion and prolongation of the atrial ERP in diabetic rabbits(357). The added layer of complexity of fibrosis-induced structural remodeling observed by Zhang *et al* (2014) may lead to and explain the amplification in the disparity of atrial refractoriness observed in this study(355), and, by Liu *et al* (2012)(357). In this study, we observed a lengthening of the ERP

in the RA of T2DM animals compared to Controls ($p < 0.0001$, Fig. 1, right) that was not reflected in the LA ($p > 0.05$, Fig. 1, left); the disparity between refractoriness of the LA and RA results in interatrial dispersion of the refractory period, which on its own plays a major role in atrial arrhythmogenecity and development of AF.

As previously mentioned, conduction delay or prolonged conduction time may promote re-entry and increase AF susceptibility. This was in support of our data which showed that average CV was slower in the LA and RA of the T2DM animals compared to Controls ($p < 0.05$, Fig. 4). Similarly, Kato *et al* showed that atrial activation time was prolonged in GK rats compared to control Wistar rats, indicative of conduction slowing in the diabetic rat atria(353). In addition, these investigators also reported that the mean atrial cycle length of the isolated heart of GK rats were significantly longer than the control rats during sinus rhythm.(353) In the present study, conduction heterogeneity through the T2DM atrial tissue was significantly less uniform, with greater heterogeneity of conduction by 2 fold or greater than Control animals in both the LA and RA ($p < 0.0001$, Fig. 2, Fig 3). This suggests that heterogeneity of repolarization is an important altered electrophysiological parameter in diabetes.

Watanabe *et al* (2012) elegantly described the multiple electrophysiological changes that occur in the diabetic atrium, amongst these were atrial conduction delay, heterogeneous conduction, and, prolongation of APD(61). The patch-clamp study conducted by Zhang *et al* (2014) revealed prolongation of APD_{90} and APD_{50} with a reduction in Na^+ current density and a contrasting increase in I_{CaL}(356). The authors proposed that whilst the interatrial conduction delay may

be partially explained by a decrease in Na^+ current density, the increase in I_{CaL} density may have acted as an ion foundation for prolongation of the APD and dispersion of the ERP(356). APD was significantly and consistently prolonged across all repolarisation parameters ($APD_{20,50,90}$, Table. 2) observed in the LA and RA of the T2DM animals compared to Control animals in our study (APD_{90}, Figure. 5). This was consistent with Zhang *et al*'s (2014) findings in the diabetic rat(355).

Several potential mechanisms involving modulations in ion channel expression and current density may contribute towards lengthening of the AP in the diabetic atrium. Augmentation of the Na^+/Ca^{2+} exchanger (NCX) current through increased cytosolic Ca^{2+} and downregulation of sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) can lead to activation of the forward mode of the NCX current and also prolong APD(358,359).

An earlier study by Shimoni *et al* (1994) showed that the two major repolarising currents, the transient outward K^+ current (I_{to}) and steady-state K^+ current (I_{Kss}) were attenuated in diabetic ventricular myocytes(360). Watanabe *et al*'s (2012) recent study demonstrated that the prolongation in the cycle length of atrial tachycardia and that of the APD in the diabetic rat was associated with increased susceptibility to induction of atrial tachycardia(61). It has been shown that reduction in the K^+ current can lead to lengthening of the APD and the production of proarrhythmic early afterdepolarizations(361). Similarly, caesium-induced loss of K^+ channel activity not only prolonged APD but also promoted AF in anaesthetized dogs in another study(362). Shimoni *et al* (1998) reported contrasting results, noting that whilst I_{Kss} was enhanced, no significant change in I_{to} was found. Amplification of the I_{Kss} current is correlated with a shortening of

the APD, and, although this was observed in the fructose-fed T2DM rat atrial AP compared to controls, the reduction in APD was not significant. Such discrepancies in results however, may be the result of different diabetic sates, differences in methodology, varying models of experimental diabetes and chamber-specific compositions of ion channels/ion channel type. The earlier studies from Shimoni *et al* are examples of alternate models of experimental diabetes; The investigators employed a streptozotocin-induced model of type 1 diabetes (T1DM) in their earlier 1994 study(360), whilst their latter study was a fructose-fed T2DM model(363).

It remains to be determined if the underlying mechanism(s) of the two diabetic states and their role in AF should be addressed separately or under the single bracket of 'diabetes mellitus'. The work from Shimoni *et al* (1998) suggests of differential changes in the ionic currents in the T1DM and T2DM rat, and that, perhaps at the level of the K^+ current itself, such a difference between diabetic states exists(363). Importantly, the changes in APD observed indicate that repolarization abnormalities may contribute towards the mechanisms underlying increased susceptibility to AF in the diabetic atrial myocardium. Further studies investigating the mechanisms involved in APD prolongation have to be conducted to further elucidate its role in electrical remodeling in the diabetic atrium.

4.4.3 Atrial structural remodeling

Interestingly, although both LA and RA chamber weights were not significantly different between T2DM animals and Controls (LA, RA $p > 0.05$, Table. 1), there was a marked increase in cardiomyocyte diameter in both age groups (LA, RA *p*

< 0.0001, Fig. 6). This suggests that in addition to hypertrophy, atrial cardiomyocyte apoptosis and necrosis may have also occurred. A higher percentage of atrial fibrosis was also observed in the T2DM LA and RA compared to Control animals irrespective of age (LA, RA $p < 0.0001$, Fig. 7). Interestingly, the extent of fibrosis was reduced in the Old T2DM animals in both the LA and RA compared to the Young animals (LA, RA $p < 0.0001$, Fig. 7). Our study is the first to have reported these results. We postulate that this may have been in part, due to a faster rate of progression of fibrosis in the Young animals, such that despite a potentially lower rate of progression of fibrosis, fibrosis levels increased in the T2DM animals It has previously been reported that extracellular matrix turnover rates were consistently different between young and old animals with up to a 30 fold difference(364); Younger animals have been postulated to have a higher regenerative capability of connective tissues compared to older animals(364). This may have implications for the differences in fibrosis levels observed in our study; such that a reduced regenerative capacity may be also be indicative of a reduced degeneration of the extracellular matrix components resulting in a less severe increase in fibrosis levels in the older animals. Taken together, these are novel findings unique to our study as no previous study has investigated cardiomyocyte diameter and the percentage of fibrosis in the atria of the diabetic rat, with a large number of studies focusing predominantly on ventricular tissue. One such example is a recent study by Huynh *et al* (2012)(321). These investigators used the *db / db* diabetic mouse- a T2DM murine model that has been well recognised for over 40 years(365)- to determine the effects of coenzyme Q10 on ventricular diastolic dysfunction and structural remodeling in the diabetes. Huynh *et al* (2012) showed that untreated db / db diabetic mice

exhibited adverse structural remodeling in the ventricular tissue(321). This included ventricular cardiomyocyte hypertrophy, myocardial fibrosis and increased apoptosis. Treatment with coenzyme Q10 was able to reduce both cardiomyocyte hypertrophy and myocardial fibrosis(321). On the other hand, the study conducted by Fredersdorf *et al* (2004) may offer a better comparison to our findings, having also used the Zfr rat. Fredersdorf *et al* (2004) observed, once again, in the ventricular tissue, that cardiomyocyte diameter and perivascular fibrosis were increased in the Zfr rat at 19 weeks of age compared to the non-diabetic control rat(349).

Visually, upon closer inspection, the Control photomicrographs are composed of closely aligned longitudinal atrial cardiomyocytes which are positioned in an orderly fashion (Fig. 6 & 7). In contrast, the atrial cardiomyocytes in the T2DM photomicrographs do not appear to be resemble the orderly, seemingly uniform and longitudinal structure as seen in the Control group; instead the structure and alignment of these atrial cardiomyocytes are varied and distorted (Fig. 6 & 7). Furthermore, the T2DM photomicrographs suggest there the endomysial space between myocardial fibres is greater compared to Controls, which may have been further exacerbated with age (Fig. 6 & 7). These observations are structural hallmarks of diabetic cardiomyopathy; it has been shown that increased cardiomyocyte width correlates with compensatory ventricular hypertrophy(366), which in the case of concentric ventricular hypertrophy causes a distortion in the alignment of the cardiomyocytes which may lead to contractile dysfunction and heart failure(367). Histological analysis of the non-hypertensive ageing heart alone showed a progressive loss pf cardiomyocytes due to necrosis and apoptosis, with the remaining cardiomyocytes undergoing hypertrophy(368).

Eghbali *et al* (1989) further showed that left ventricular collagen content increase almost two fold in the senescent rats compared to the young controls(369).

Xu *et al* (2004) demonstrated that during AF, collagen type I increased gradually from that observed in patients with non-documented AF through to patients with permanent AF(370). They further showed that more collagen I was found in patients in the permanent and persistent AF groups who experienced more frequent and longer durations in AF. Increased collagen I deposition can contribute towards a substrate for electrical remodeling via heterogeneous fibre thickening and fibre disarray, increasing heterogeneity of conduction through the atria, potentially playing a key role in establishing re-entry in AF(370).

Several inflammatory biomarkers and cytokines as well as plasma serum levels of leptin were examined in this study. Plasma serum levels were markedly higher in the T2DM animals compared to Controls irrespective of age ($p < 0.0001$). This is consistent with other literature which demonstrated, at ages 9 to 12 weeks, leptin receptor levels ranged between 5-20 fold higher in the Zfr rat compared to the Control lean rat(371,372).

The association of an inflammatory state with obesity and insulin resistance has been previously described(373). Studies have shown that obesity is a state of chronic inflammation associated with elevated plasma levels of CRP(374), TNF-α(375,376) and IL-6(376). CRP has been recognised as the most robust and reproducible marker indicative of vascular inflammation(377), and is synthesized in response to IL-6 and IL-1(378). In AF, CRP and IL-6 are the most commonly studied markers of inflammation. High-sensitivity-CRP (hs-CRP) levels have

been correlated with risk of future cardiovascular events such as stroke, sudden cardiac death, AF and myocardial infarction.(379) In patients with AF, levels of hs-CRP are notably higher compared to control patients in sinus rhythm, rising further in persistent AF patients. In addition, such higher levels of hs-CRP have also been associated with longer AF duration and left atrial enlargement(380). Although CRP and TNF-α levels were not measured in this study, IL-6 was one of three interleukins examined. IL-6 is a pleiotropic cytokine, possessing roles in pro-inflammatory and cyto-protective responses(381). IL-6 not only stimulates the synthesis of CRP but it is also responsible for the synthesis of several other acute-phase agents, amongst them are TNF-α, serum amyloid-A and IL-1β(382). In our study, plasma serum levels of IL-1β, IL-6, IL-10 as well as IFN-γ were not found to be significantly different between groups ($p > 0.05$, Table. 3). This is in support of Conway *et al*'s (2004) study which found no association between IL-6 levels and patients with AF(383). In contrast, a greater number of studies have found an increase in IL-6 levels in patients with AF compared to healthy controls(384-386). Similarly, although plasma serum levels of ICAM-1 and MCP-1 were elevated in the T2DM group compared to Controls, this was not found to be significant between groups ($p > 0.05$, Table. 3). It has previously been shown that the insulin resistant state in T2DM may promote inflammation, and that insulin has an anti-inflammatory effect; low dose infusion of insulin can reduce the generation of reactive oxygen species, supress NADPH oxidase expression, nuclear factor κB (NF-κB) binding, and facilitate suppression of ICAM-1 and MCP-1(387). This may provide an explanation for the moderate increase in ICAM-1 and MCP-1 levels observed in the T2DM arm of our study. Lastly, we examined differences in L-selectin expression between groups. L-selectin is a cell adhesion

molecule which has been shown to mediate leukocyte recruitment during chronic inflammation(388). Levels of L-selectin were greater in the T2DM animals compared to Controls ($p < 0.05$), this was in contrast to studies which have reported that in patients with T2DM, a drop in soluble L-selectin has been proposed as a marker for symptomatic coronary artery disease. Similarly, in patients with T2DM and silent myocardial infarction, levels of L-selectin are reduced compared to control patients(389). There is increasing evidence establishing the relationship between inflammation and AF, our results in part suggest that the inflammatory state in T2DM may play an important role as a supporting link between T2DM and the development of AF.

4.4.4 Conclusion

Currently, experimental studies investigating the different diabetic states of T1DM and T2DM are greatly lacking. In addition, whether the same mechanisms demonstrated in the diabetic atrium occur in the diabetic ventricle should also be evaluated. The present study has demonstrated that regardless of age, in the presence of obesity, atrial structural remodeling, characterized predominantly by extensive interstitial fibrosis may be one of the major mechanisms of T2DM-induced AF.

FIGURES & TABLES

	Young		Old		
	Control	T2DM	Control	T2DM	P Value
Body Weight (g)	271.69 ± 22.02	446.44 ± 10.32	428.18 ± 11.43	451.45 ± 12.67	< 0.0001***
Systolic blood pressure (mmHg)	123.49 ± 2.24	141.69 ± 3.12	123.72 ± 3.14	157.33 ± 4.31	< 0.0001***
Non-fasting glucose (mmol/L)	19.35 ± 0.74	29.56 ± 1.62	21.51 ± 1.21	29.42 ± 1.38	< 0.0001***
RA weight (mg)	35.87 ± 4.89	60.70 ± 3.78	43.58 ± 4.41	33.41 ± 2.42	0.53
LA weight (mg)	13.88 ± 3.27	24.92 ± 3.20	32.06 ± 4.19	20.50 ± 1.27	0.62

Table 1. Animal Characteristics. Type 2 diabetic animals (T2DM) were significantly heavier ($p < 0.0001$), had higher systolic blood pressure levels ($p < 0.0001$) and were hyperglycaemic ($p < 0.0001$) compared to their age-matched Control animals. RA and LA weights were not significantly different in T2DM animals compared to their age-matched Controls ($p > 0.05$). Abbreviations: RA (right atrium), LA (left atrium), RV (right ventricle), LV (left ventricle). *** indicates $p < 0.0001$ between Control and T2DM animals regardless of age.

LA	Control	T2DM	P Value
APD_{20}	7.79 ± 0.45	11.30 ± 0.99	< 0.05*
APD_{50}	13.41 ± 0.63	19.12 ± 1.47	< 0.05*
APD_{90}	29.95 ± 1.29	41.83 ± 2.69	< 0.01**
RA	Control	T2DM	P Value
APD_{20}	5.88 ± 0.25	9.01 ± 0.44	< 0.0001***
APD_{50}	11.23 ± 0.33	17.17 ± 0.89	< 0.0001***
APD_{90}	28.11 ± 0.89	41.91 ± 2.55	< 0.0001***

Table 2. Action potential duration (ms) at 20, 50, 90% of repolarisation ($ADP_{20,50,90}$) in the left (LA) and right (RA) atria of Control and type 2 diabetic (T2DM) animals. $APD_{20,50,90}$ was significantly prolonged in the LA (APD_{20} and APD_{50} $p < 0.05$, APD_{90} $p < 0.01$) and RA ($APD_{20,50,90}$ $p < 0.0001$) of T2DM animals compared to Controls.

Biomarker (pg/mL)	Control	T2DM	P Value
IL-1β	34.92 ± 7.90	25.17 ± 2.99	0.10
IL-6	11.45 ± 4.24	13.55 ± 4.00	0.47
IL-10	1673.00 ± 273.39	2437.08 ± 314.71	0.35
IFN-γ	0.50 ± 0.19	0.75 ± 0.18	0.62
ICAM-1	4102.67 ± 404.93	6260.08 ± 802.00	0.08
Leptin	24.33 ± 12.04	10421.67 ± 4944.40	0.04*
L-selectin	648.50 ± 69.49	917.83 ± 130.42	0.02*
MCP-1	721.67 ± 138.88	1255.17 ± 293.10	0.08

Table 3. Plasma serum levels of leptin and biomarkers of inflammation. Type 2 diabetic animals (T2DM) had significantly elevated levels of Leptin ($p < 0.05$) and L-selectin ($p < 0.05$). Plasma serum levels of ICAM-1 ($p = 0.08$) and MCP-1 ($p = 0.08$) were also moderately elevated in T2DM animals compared to Controls. * indicates $p < 0.05$

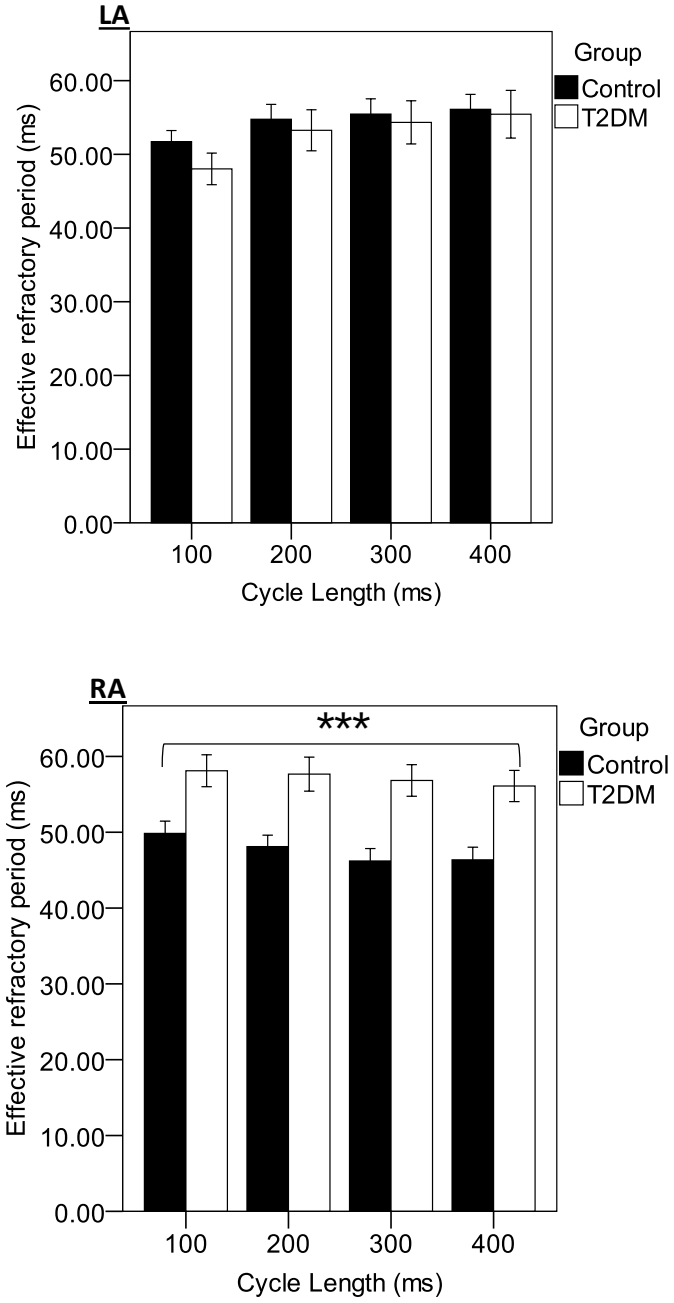

Figure 1. Effective refractory periods (ERP) of left (LA) and right (RA) atria of Control and type 2 diabetic (T2DM) animals during standard S1-S2 pacing at 100, 200, 300 and 400 ms. The graphs illustrate pooled data (Young and Old) for Control, and T2DM animals. Top There were no significant differences in ERP between groups ($p > 0.05$) in the LA. Bottom: The ERP was markedly prolonged in T2DM animals compared to Controls ($p < 0.0001$). *** indicates $p < 0.0001$

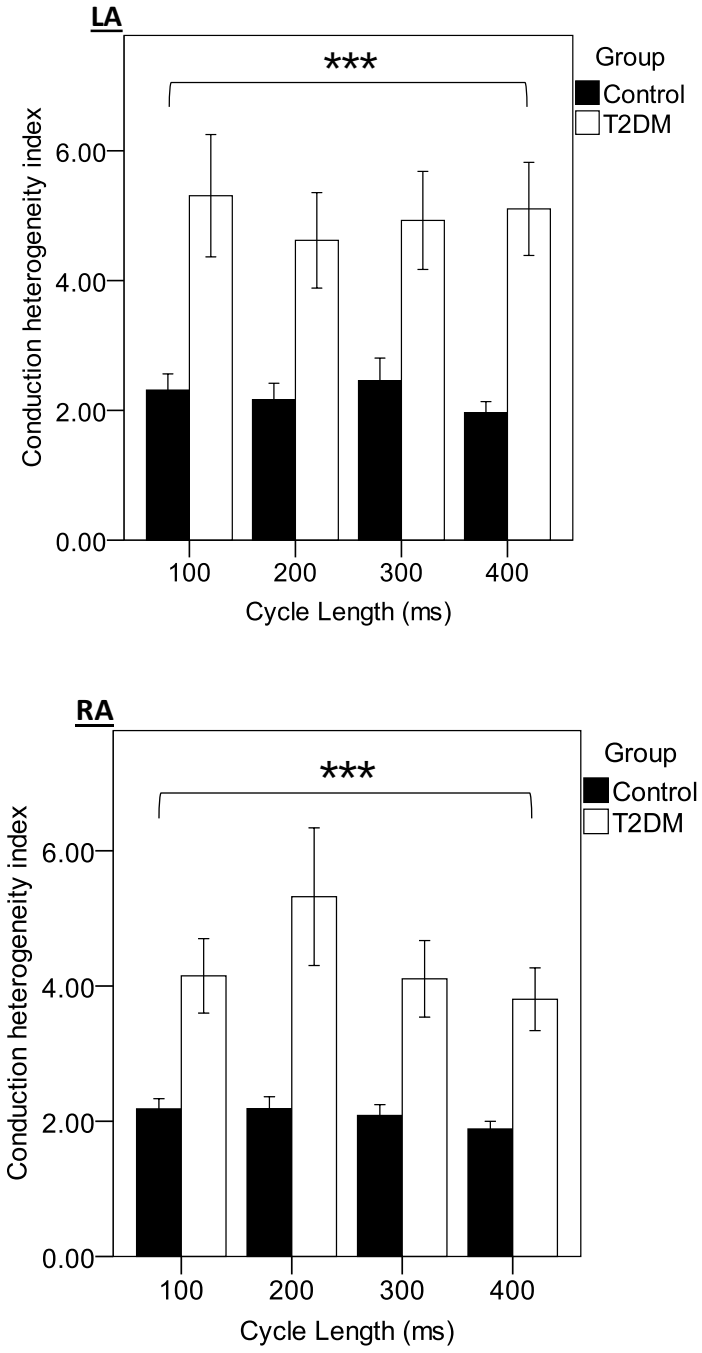

Figure 2. Conduction heterogeneity in the left (LA) and right (RA) atria of Control and type 2 diabetic (T2DM) animals. The graphs illustrate pooled data (Young and Old) for Control, and T2DM animals. Heterogeneous conduction was observed in the LA (top) and RA (bottom) of T2DM animals compared to Controls (LA, RA $p < 0.0001$). ******* indicates $p < 0.001$

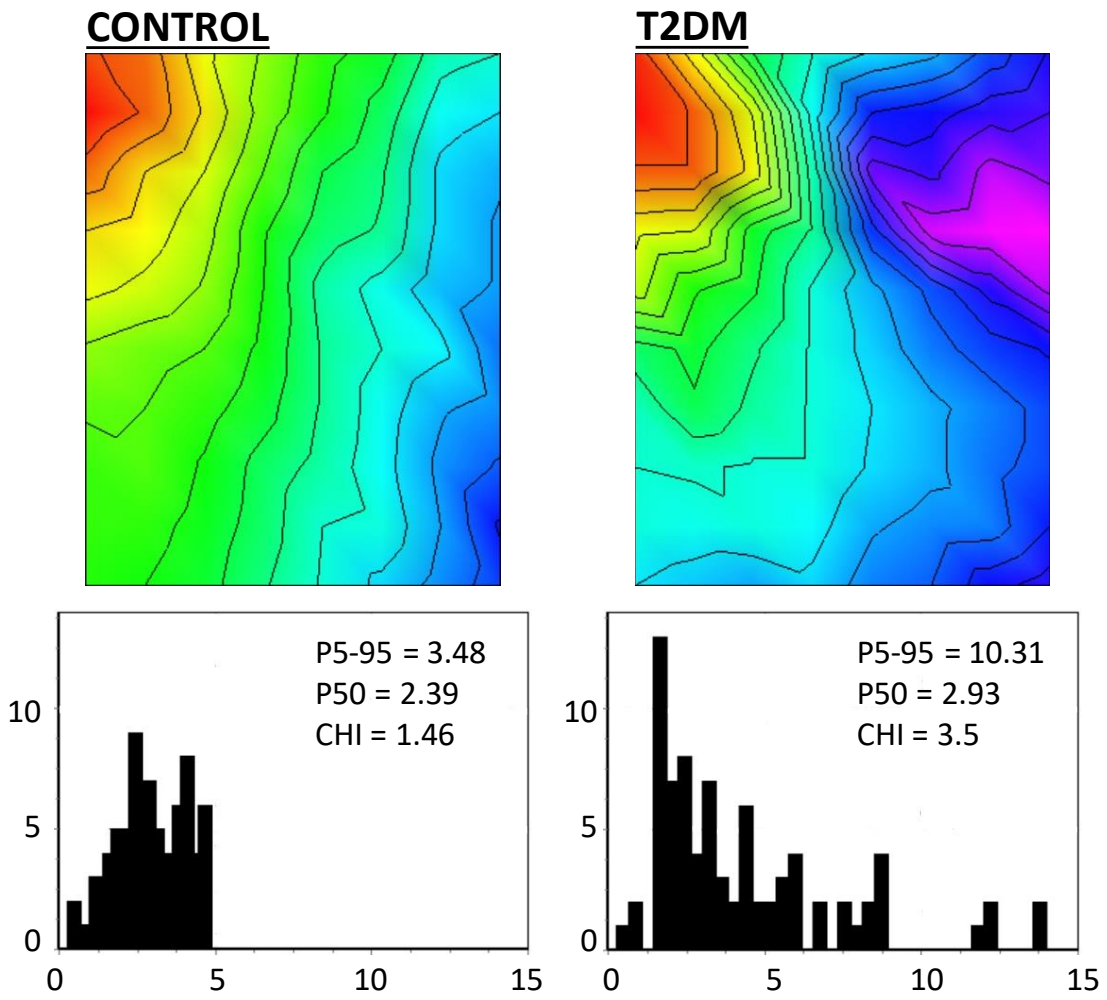

Fig 3. Representative activation maps and phase histograms from the left atrium of a single Control and type 2 diabetic (T2DM) animal. The activation maps and phase histograms contain pooled data (Young and Old) for Control, and T2DM animals. Activation maps with superimposed 2ms isochrones are shown (top) with the corresponding phase histograms (bottom) from the left atrium of a single Control (left) and T2DM (right) animal at a pacing cycle length of 200ms.

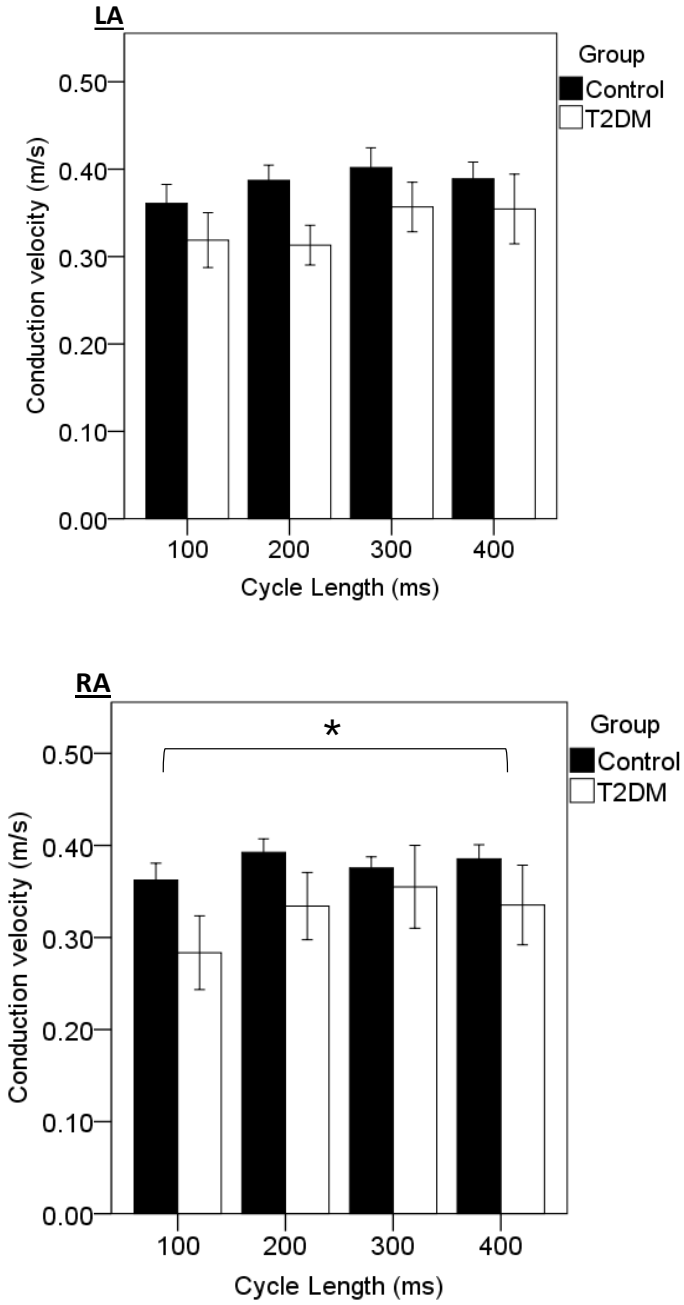

Figure 4. Average conduction velocity in the left (LA) and right (RA) atria of Control and type 2 diabetic (T2DM) animals. The graphs illustrate pooled data (Young and Old) for Control, and T2DM animals. Conduction slowing was observed in both the LA (top) and (RA) of T2DM animals compared to Controls (LA, RA $p < 0.05$). * indicates $p < 0.05$

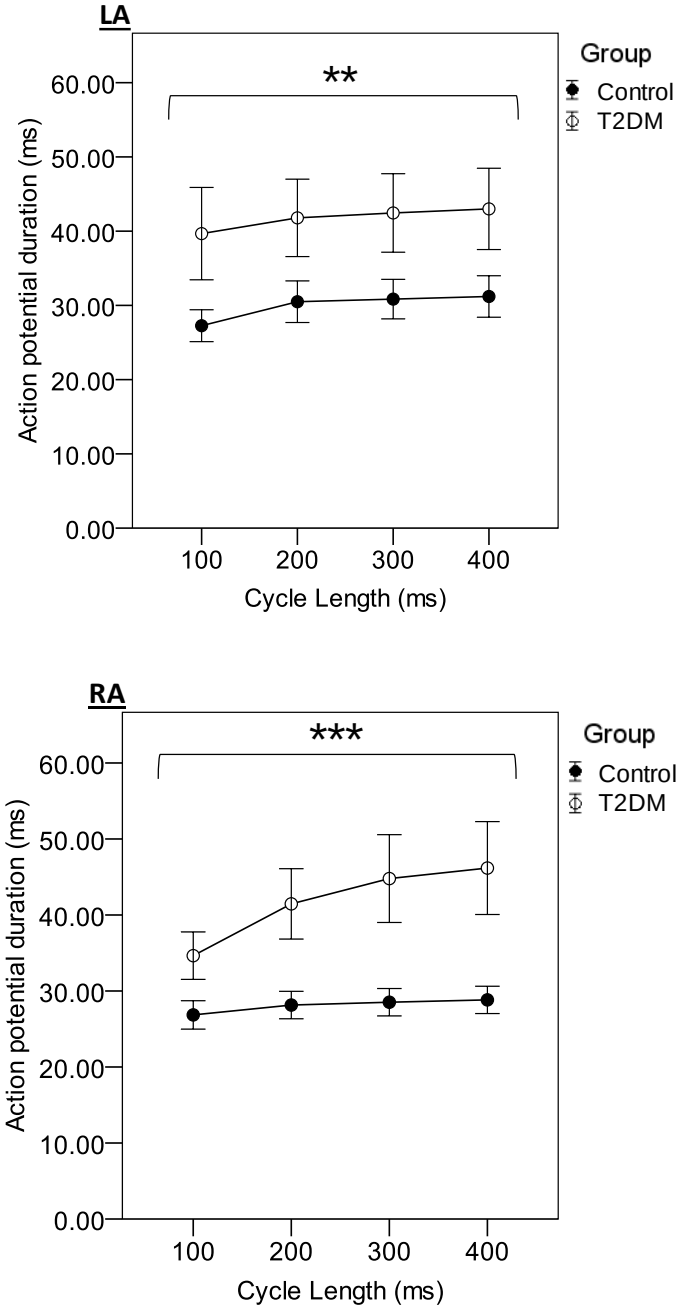

Figure 5. Action potential duration at 90% of repolarisation (APD_{90}) recording from the left (LA) and right (RA) atria of Control and type 2 diabetic (T2DM) animals. The graphs illustrate pooled data (Young and Old) for Control, and T2DM animals. APD_{90} was markedly lengthened in the T2DM animals compared to Controls in both LA (top) and RA (bottom) (LA p <0.01, RA p < 0.0001). ** indicates p < 0.01, ***indicates p < 0.0001

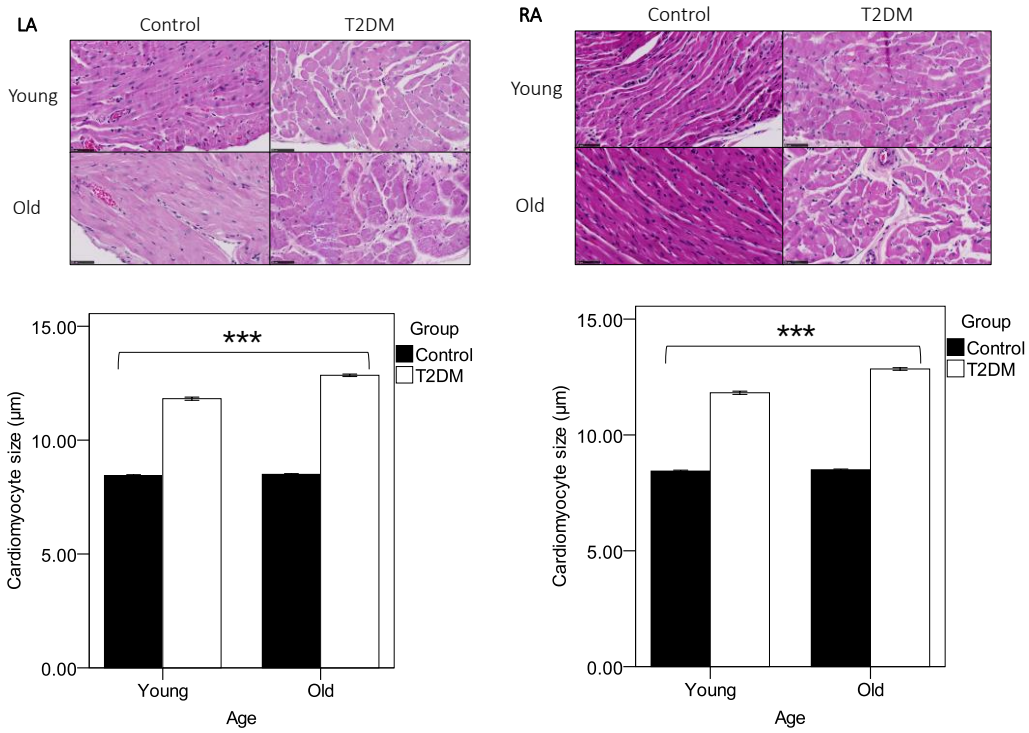

Figure 6. Cardiomyocyte diameters of left (LA) and right (RA) atrial preparations from Control and type II diabetic (T2DM) animals. Top: Representative haemotoxoylin and eosin photomicrographs at 400 x magnification from LA (left) and RA (right) preparations of Control and T2DM animals at 3 months (Young) and 10 months (Old) of age. Scale bars show 50 µm. Bottom: LA (left) and RA (right) cardiomyocytes were significantly enlarged in T2DM animals compared Controls (LA, RA: $p < 0.0001$). *** indicates $p < 0.0001$.

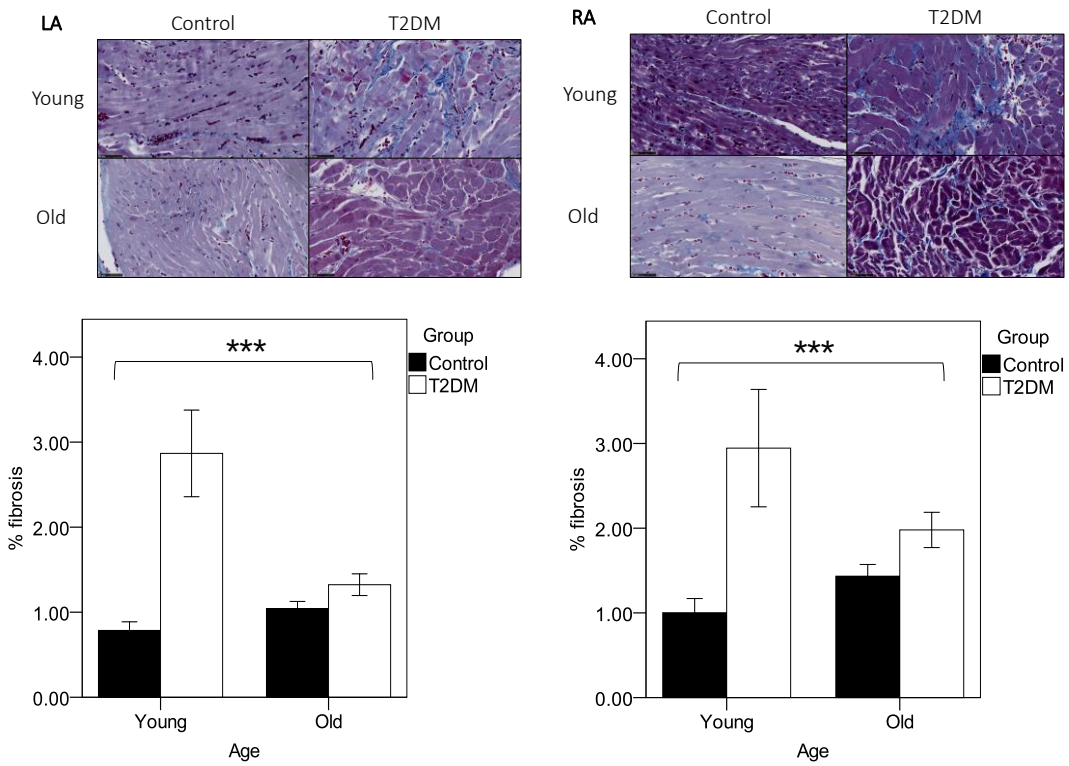

Figure 7. Percentage of fibrosis in left (LA) and right (RA) atrial preparations from Control and type I diabetic (T2DM) animals. Top: Representative Mason's trichrome photomicrographs at 400x magnification from the LA preparations of a Control and T2DM animals at 3 months (Young) and 10 months (Old) of age. Scale bars show 50 µm. Bottom: Percentage of fibrosis was significantly greater in T2DM animals compared to Controls in the LA (left) and RA (right) (LA, RA $p < 0.0001$). *** indicates $p < 0.0001$.

CHAPTER FIVE

FINAL DISCUSSION

5.1 Type 1 and type 2 diabetes mellitus as individual risk factors of atrial fibrillation

Atrial fibrillation (AF) is the most common clinically presented arrhythmia and its prevalence continues to rise(390,391). Current therapeutic treatment options for AF are not without limitations(392-394), and despite the progress and extensive research over almost a century, the underlying mechanisms have yet to be completely revealed, with questions left unanswered. New and further insights into the pathophysiology of AF and the potential modification of contributing risk factors such as obesity(103,395), and diabetes mellitus (DM) are still required(348,396).

Current literature on the underlying mechanisms of obesity- and DM-induced AF have yet to be fully elucidated; it is unclear for example, whether the inflammatory response in AF, obesity and DM share similar pathways in producing a proarrhythmic substrate. At present, the available literature addressing DM-mediated induction of AF neglects to address DM as two characteristically different conditions, often grouping the pathophysiological mechanisms of type 1 (T1DM) and type 2 (T2DM) DM-induced predisposition to AF under a single bracket. T1DM and T2DM, which can otherwise be referred to as insulin-dependent and non-insulin-dependent DM respectively, differ in definition by their response to or availability of insulin; While T1DM accounts for approximately 5-10% of DM individuals in the general population(397), it is a chronic auto-immune

disorder wherein pancreatic beta cell destruction leads to insulin deficiency which occurs comparably earlier in life than the insulin responses observed in T2DM(397); In contrast, T2DM is a chronic metabolic disorder wherein defects in the secretion and action of insulin(398) are often accompanied by obesity(101,103,391,395) and impaired glucose tolerance(59,399-401); both of which have been proposed as contributing risk factors of AF. These differences are reflected in the experimental models of T1DM and T2DM.

There are several ways to induce DM: (1) partial or full surgical removal of the pancreas, (2) non-surgical administration of agents such as streptozotocin (STZ) or alloxan, (3) breeding of genetically pre-disposed animals to obtain monogenic and polygenic animals, and (4) feeding of diet-specific chow.

The following discussion will focus predominantly on: (1) the STZ-induced model of T1DM (chapter three) and (2) the monogenic model of T2DM in the fa/fa Zucker rat (Zfr) (chapter four), and, in addition, some reference to unpublished data obtained from a collaboration using (3) the polygenic NONcNZO10/LtJ (NON) mouse model of T2DM, will also be made, in an attempt to review/identify similarities and/or differences in T1DM and T2DM substrates for AF.

5.2 Mechanisms of diabetes mellitus-induced atrial fibrillation

The mechanism of AF can be explained via two fundamental processes: trigger and substrate, both of which are dependent on the electrophysiological and anatomical substrate of the atria(1,116,152).

5.2.1 Atrial electrical remodeling

Atrial electrophysiological remodeling in AF can occur via rapid atrial rate- atrial tachycardia remodeling, and generally results in shortening of the action potential duration (APD) and a consequential abbreviation of the atrial effective refractory period (ERP)(402).

We assessed changes in both APD and ERP in both the left (LA) and right (RA) atria which has not been previously done in the setting of DM. We observed similar results in both STZ-induced and Zfr models of T1DM and T2DM respectively. In each study, irrespective of the atrial chamber, the pacing cycle length, and, the age of the animal, APD was significantly prolonged at each repolarisation parameter measured ($APD_{20,50,90\%}$). Upon comparison, compared to their age-matched control animals, the prolongation of APD observed in the T2DM animals was significantly greater ($p < 0.0001$, chapter four) as opposed the T1DM animals ($p < 0.05$, chapter three). In support of these results, the collaborative study on the NON mouse reported lengthening of APD_{90} in the LA of the DM animals compared to controls across all pacing cycle lengths.

Potential mechanisms proposed to lengthen APD in DM include an increase in the Na^+/Ca^{2+} exchanger (NCX) current and attenuation of K^+ currents(360). Downregulation of the sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) has been shown to occur in the setting of DM;(359) This results in reduced reuptake of intracellular Ca^{2+} which can prolong APD via NCX, or, at increasingly faster heart rates, due to abnormal Ca^{2+} handling, lead to inactivation of I_{CaL} and shortening of the AP(61). Increased NCX can then act to promote development

of AF by afterdepolarization-related ectopic activity which can trigger re-entry if presented with a vulnerable substrate for AF, or, by resulting in atrial tachycardia-induced electrical remodeling, producing a proarrhythmic substrate for re-entry.

The transient outward K^+ current (I_{to}) and the steady-state K^+ current (I_{KSS}) have also been demonstrated to prolong APD in ventricular myocytes(360). Shimoni *et al* (1998) noted that different models of DM did not produce the same modifications in K^+ currents(363). The investigators employed STZ, and carbohydrate fructose/sucrose-enriched diet to induce T1DM and T2DM respectively(363). Using right ventricular single myocytes, Shimoni *et al* (1998) noted no reduction in I_{to}, prolongation of I_{Kss} and maintenance of a short AP in the T2DM model(363). Comparatively, I_{to} was found to be attenuated and APD prolonged in the T1DM model such that APD was markedly shorter in the T2DM animals than that of the T1DM animals when compared to control APs(363). The investigators proposed that as I_{Kss} is inhibited in the insulin-deficient state (T1DM) and augmented in the insulin-resistant (T2DM) state wherein insulin levels are in excess, the hyperinsulinaemic state found in T2DM is responsible for the control-like AP configuration observed. The investigators further observed that insulin has a tone-dependent effect on cardiac K^+ currents, that the I_{KSS} density changes in response to changes in insulin levels(363).

Although the relative contributions of NCX, L-type Ca^{2+} current ($I_{CaL)}$ and K^+ rectifier currents in DM have not been fully elucidated, the prolongation of the APD observed in our experimental studies of DM indicate that perhaps downregulation or inactivation of the K^+ rectifier currents may have greater precedence in than Ca^{2+} entry in the T1DM setting.

In addition, the observations made by Shimoni *et al* (1998) may offer some explanation as to the conflicting results found in our studies whereby prolongation of the atrial AP was not necessarily reflected in prolongation of the ERP(363).

In chapter four, together with the increase in $APD_{20,50,90}$, we observed no change and a lengthening in the atrial ERP in the LA and RA respectively. Assuming that the APD_{90} is correlated with the ERP, prolongation of the AP would lead to lengthening of the ERP. Some evidence of this was also observed in the NON mouse study, whereby young 10 week old DM mice had significantly longer ERP together with prolonged APD_{90} compared to controls. In contrast, the prolongation of $APD_{20,50,90}$ in the LA and RA in chapter three was accompanied by no change in ERP and shortening of the ERP in the LA and RA respectively. These contrasting results support the notion that the electrophysiological changes in T1DM and T2DM may be mediated differently. As previously discussed, while the K^+ rectifier currents may play a greater role in T1DM, perhaps Ca^{2+} entry may play a greater role in T2DM as suggested by the contrasting shortening of the ERP found in chapter four.

It has been shown that modulation of the I_{Ca} via inactivation of I_{Ca} (short term change)(155), or, by downregulation of the I_{Ca}-α subunit mRNA (namely the Cav1.2 α subunit) (long term change), in AF can decrease I_{Ca} and abbreviate the ERP to promote AF(143,149,150,403,404). This decrease in I_{Ca} was also reported in experimental models of DM; Lu *et al* (2007) reported decreased I_{CaL} in the cardiac myocytes of T1DM Akita mice, with reduced expression of L-type Ca^{2+} channel density and decreased phosphatidylinositol 3-kinase (PI3K) signalling(405). This reduction in I_{CaL} was rescued to control levels upon insulin treatment or intracellular infusion of PI 3,4,5-triphosphate ($PI_{(3,4,5)}P_3$). The

investigators postulated that the decrease in Ca^{2+} entry through the L-type Ca^{2+} channel in T1DM may be mediated by insulin deficiency-induced reduction in PI3K(405). These investigators reported a positive shift in voltage dependence of both activation and inactivation of the L-type Ca^{2+} channel, which was normalised upon administration of $PI_{(3,4,5)}P_3$ for channel inactivation, but not so for channel activation. These results suggest that in the setting of T1DM, of the reduced density of L-type Ca^{2+} channels present, a smaller fraction of these channels are likely to open during conductance of an AP, reducing intracellular Ca^{2+} entry leading to a proarrhythmic shortening of the AP, and, depressed cardiac contractility which is a hallmark of diabetic cardiomyopathy(406).

5.2.2 Atrial structural remodeling

The contribution of the inflammatory cascade towards the development of AF is well recognised(407-409); The inflammatory cascade consists of a myriad of inflammatory cytokines including the aforementioned tumor necrosis factor- α (TNF-α), interleukins -1, -6 and -8 (IL-1, IL-6 and IL-8 respectively), and transforming growth factor-beta (TGF-β), with IL-6 being the primary stimulator of acute-phase proteins, in particular, C-reactive protein (CRP) which is a widely used and recognised indicator of inflammation in the clinical setting(96,407-410). Obesity alone is characterised by low-grade and systemic inflammation(387,411,412) and elevated levels of TNF-α, an important inflammatory cytokine which contributes significantly towards insulin resistance, has been found in the adipose tissue of rodents with DM(413). In the clinical setting, TNF-α has been found to be overproduced in the adipose tissues of obese individuals. Administration of recombinant TNF-α has been shown to

impair insulin action, while the absence of functional TNF-α in obese mice improved insulin sensitivity, suggesting that elevated levels of TNF-α is an important feature linking both obesity and DM(413,414).

Secreted by both macrophages and leucocytes, TNF-α, activates several inflammatory mediators and cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of matrix mmetalloproteinase-1 (TIMP-1), and L-selectin, all of which were measured in our studies using enzyme-linked immunosorbent assays of plasma serum. TNF-α, IL-1, IL-6 levels were not significantly different between DM animals and controls in both chapters three and four. Similarly, TIMP-1 levels were not significantly different between groups in chapter three. ICAM-1 levels were found to be significantly elevated in our T1DM and T2DM animals compared to control animals. In the T2DM study, L-selectin was found to be elevated in the DM animals compared to control animals. Although plasma serum levels of TNF-α were not significantly different between groups, the possible activation of TNF-α cannot be dismissed as expression levels of TNF-α mRNA were not assessed using Western blot analysis. Activation of the TNF-α/NF-κB/TGF-β pathway has been proposed to be critical in the progression diabetic nephropathy(415-417), and more recently in atrial remodeling (Fig. 1)(324).

In DM, myocardial NF-κB activity can be stimulated by high concentrations of circulating glucose and increased glycation of lipoproteins(418) which can enhance the generation of reactive oxygen species (ROS) in DM and potentiate

the inflammatory response(419). Indeed, studies employing STZ-induced rodent models of DM have reported higher levels of NF-κB activity in diabetic spontaneously hypertensive rats and Sprague Dawley rats(420,421). Fu *et al* (2015) explored the used of probucol, a potent antioxidant(324); They reported that probucol may assist in reducing protein levels of NF-κB by minimizing the detrimental effects caused by oxidative stress, production of ROS, mitochondrial dysfunction and cell death, ultimately preventing DM-induced cardiac hypertrophy, reducing the propensity towards development of proarrhythmic substrate for AF(422). This was evidenced by attenuation of left ventricular hypertrophy, LA dilatation, and reductions in levels of malondialdehyde, superoxide dismutase, myeloperoxidase, IL-1 and TNF-α, upon administration of probucol to the diabetic group. The investigators also reported extensive interstitial fibrosis in LA cardiomyocytes. This was in support of the results found in our studies. Irrespective of the form of DM in our studies, levels of fibrosis in the LA and RA of diabetic animals were significantly higher than control animals.

In our STZ-induced T1DM and Zfr T2DM rat studies, fibrosis levels were approximately three fold or greater in the LA and RA of diabetic animals compared to control. Comparatively, the level of fibrosis was significantly greater only in the older NON T2DM mice and not so in the younger NON T2DM mice. Progressive cardiac fibrosis is a hallmark of ageing-associated diastolic dysfunction(423). Indeed, cardiomyopathy is known as an independent age-related pathophysiologic condition of DM(406,424). Experimental animal(425) and human(426,427) studies have reported of an age-related increase in collagen deposition indicative of elevated levels of fibrosis. In comparison, a

higher percentage of fibrosis was present in the young DM animals in our Zfr T2DM study. This increase in fibrosis in the DM group persisted with age, however, the degree of fibrosis was less prominent than that exhibited by the younger DM animals.

In addition, we observed an increase in conduction heterogeneity in the DM animals of each study. In chapter four, elevated levels of fibrosis may impart explain the heterogeneity in conduction as well as the corresponding slowing of conduction in both the LA and RA. In contrast, in chapter three, there was an increase in average conduction velocity in the T1DM animals compared to controls. In our T1DM and T2DM studies, no significant differences in LA and RA chamber weights were found in the DM group compared to controls. In chapter three, our results showed a significant increase in LA and RA cardiomyocyte diameter, indicating that myocyte apoptosis and/or necrosis may have occurred. This DM-induced increase in cardiomyocyte diameter was likewise observed in the LA and RA of our young (3 months) and old (10 months) Zfr T2DM rats in chapter four. This was again observed in the findings from a collaborative study on the NON T2DM mouse; the LA of both young (10 weeks) and old (30 weeks) mice had a significantly larger cardiomyocyte diameters compared to their age-matched controls. Taken together, comparative to the T2DM Zfr model (chapter four) wherein the toxicity of STZ was not present, the increase in average conduction velocity observed in chapter three may have been resultant from an accumulation of myocyte apoptosis/necrosis (from the toxicity of STZ) together with cardiomyocyte hypertrophy, potentially overshadowing the possible conduction delay that can occur with increased fibrosis. Indeed, large cell size in

rabbits with heart failure have been reported to have increased myocardial conduction velocity(428).

Release of angiotensin (AT) II in the cardiac myocardium has been proposed to activate endogenous pathways leading to cell death(429), and, promote cardiac myocyte hypertrophy(430). Fiordaliso *et al* (2012) examined the effects of STZ-induced DM and application of an AT1 receptor blocker on the progression of myocyte apoptosis and necrosis, as well as myocyte number and cell size over a four-week period(431). An aggregate myocyte loss of 30 % with an accompanying 14 % increase in myocyte volume of viable cells were reported in diabetic rats. Additionally, levels of AT II increased several fold, with a concomitant increase in AT II-positive cells and the number of AT II sites per myocyte. Administration of AT1 receptor antagonist resulted in inhibition of AT1 receptor upregulation and angiotensinogen, attenuating synthesis of AT II and consequently preventing myocyte death. Fiordaliso *et al* (2000) therefore demonstrated that DM-induced cardiomyopathy was resultant of myocyte apoptosis and hypertrophy mediated in part by AT II(431). Additionally, other studies have also demonstrated that increased TNF-α expression can lead to increased expression of proto-oncogenes which can accelerate the rate of general protein expression, increase protein content and cardiomyocyte cell size(432).

The results discussed here, suggest that the increase in atrial cardiomyocyte diameter observed in chapters three and four may be resultant of not only the

increase in myocyte volume as postulated by Fiordaliso *et al* (2000)(431), but, may also be mediated by TNF-α and the NF-κB pathway.

5.3 Conclusion

The mechanisms of DM-induced atrial remodeling are multi-layered and complex, occurring at the electrophysiological and structural level, with a myriad of contributing factors. It is likely that not one but several mechanisms both separate and interrelated that lead to atrial remodeling and impairment of atrial electro-mechanical function in DM alone, and, particularly in the presence of concomitant conditions common to DM such as obesity. Animal models have provided a platform into attaining insights into the pathophysiological mechanisms of AF and its potentially modifiable risk factors such that observations and therapeutic interventions made in the clinical setting can be better understood, reassessed/refined, and, for novel approaches to be developed.

The need for further experimental studies and clinical investigation are still required in order for this complex atrial arrhythmia to be treated more effectively to achieve better quality of life for individuals suffering from AF in the general population.

5.4 Limitations

Further experimental studies extending from this research would benefit from addressing the limitations of the studies in this the thesis. These limitations have been briefly discussed below.

In chapter two, the use of a single microelectrode was a notable limitation in identifying regional differences in AP morphology; indeed the use of a second microelectrode would have enabled these regional differences in AP morphology to be determined, particularly, in the setting of T1DM and T2DM, whereby differences in regional AP morphologies may help delineate the pathophysiology of these diabetic states and perhaps, also provide an explanation as to the potential role of glycaemic control and insulin between T1DM and T2DM.

By extension, identification of the SA node would further help classify such regional differences in AP morphology. Determination of the SA node location, the pacemaker site of the RA, would have offered greater insight into signal transduction at the level of the AP in the DM atrium; for example, would changes in the SA nodal AP alter signal conduction through the rest of the atrial tissue and impact the excitation-contraction coupling process? Accordingly, this would also lead to the investigation of expression levels of both connexins and the ion channels which regulate the cardiac AP; down regulation of SA nodal connexin and ion channel expression levels alone would subsequently lead to signal transduction impairment and alterations in the atrial AP morphology in the SA node. Similarly, down regulation of connexin and ion channel expression common to both the SA node and other RA and LA regions would consequentially also have an impact on electrical conduction through the atria in favour of establishing an electrically unstable and proarrhythmic substrate for AF; in addition, in following with the latter point, identification of other anatomical sites such as the pectinate muscles and crista terminalis prior to the commencement of AP recording would also have to be determined. Simultaneous recordings of intracellular APs from each of these regions would perhaps then require a series

of carefully placed microelectrodes; further work in the development of the technique (SHIMP, described in chapter two) employed in these studies will hence be required to allow for the use of multiple recording sites.

Another notable limitation of the studies, would be the ability to cross compare the parameters measured between the two different models of DM. Although the animals used in the T1DM and T2DM study were approximately 12 weeks of age (at the commencement of the study protocols), the administration of STZ over the 3 month period of diabetes induction in the T1DM study resulted in age differences between the animals in the T1DM and T2DM studies. Age-matched comparisons could therefore not be made between these diabetic states; this then prevented the animals from being grouped accordingly to their level of hyperglycaemia such that the impact of varying degrees of hyperglycaemia, and, perhaps also the progressive increase in plasma glucose levels on the electrophysiological and structural parameters measured could not be determined. To appropriately assess if varying levels of hyperglycaemia resulted in progressive electrophysiological and structural remodeling of the atria, additional groups of age-matched 3 month and 10 month old STZ-induced T1DM animals, and, 6 month old T2DM obese animals would be required; this would not only allow for the age group comparisons to be made across the two diabetic states, but also allow for the impact of DM to be assessed progressively, from 3 months of age, through to 6 months and 10 months of age; this may ultimately equip us with further information as to the presence of similarities or differences between the impact of T1DM and T2DM on the electrophysiological and structural properties of the atrial myocardium over time.

There were also a number of factors which should have also been considered during the design and execution of the protocols. Some of these factors/gaps/inconsistencies in the experimental protocols have been listed below; The measurement of fasting plasma blood glucose levels were conducted only in the T1DM study and not so in the T2DM study whereby non-fasting blood glucose levels were obtained. As a result, this disparity between the type of plasma blood glucose measured led to a large range in the plasma glucose data, highlighting the need for the correction of such inconsistencies between the T1DM and T2DM protocols. The measurement of fasting plasma blood glucose levels in both studies would have given additional insight into the role of hyperglycaemia and potentially the role of insulin as well; one would be able to better assess if the degree of hyperglycaemia in one diabetic state and the accompanying electrophysiological and structural changes that may occur are comparable to those observed in the other diabetic state. and perhaps help determine if an individual is at a greater risk of developing AF if diagnosed with one particular diabetic state (i.e would the T2DM insulin resistant state or the T1DM insulin deficient state provide a more proarrhythmic substrate for AF?)

A range of parameters were also not measured in these studies. Notably, these measurements include: (1) resting heart rate (to assess if differences in basal heart rates are associated with increased atrial remodeling between diabetic states), (2) HbA1c and insulin levels (which enable better characterisation of the degree of DM in each study), (3) connexin expression levels of Cx 40 and 43 as well as ion channel expression levels of the AP-determining Ca^{2+} (L-type Ca^{2+}

channels) and K^+ currents (I_{to}, I_{Kss}) (to establish a link between the changes in the AP morphology observed with the components which determined electrical conduction), as well as (4) the frequency of spontaneous and pacing-induced episodes of tachyarrhythmias (which would provide an indication of atrial electrical instability and vulnerability towards developing AF).

It may also be of importance to note that the atrial response to DM may differ between the species of animal employed; the impact of DM on the electrophysiological and structural properties of the atria of a Sprague Dawley rat may in fact differ from that of the Zucker rat; these differences may potentially mask or even exacerbate the vulnerability of the diabetic atrium to AF depending on the species at hand. Although the results from the first study in chapter two, which employed the use of the Sprague Dawley rat were not compared with those of chapters three and four, which used the Zucker rat, further studies involving these two diabetic states may benefit from a comparison between the impact of DM on the atrial myocardium of different animal species to assess if, for example, the diabetic state achieved from a single peritoneal injection of STZ in our T1DM study in the Zucker rat is similarly reflected in the Sprague Dawley rat.

Lastly, the sample size differences in each study was also a notable limitation, with sample sizes ranging from $N = 4$ in chapter two to $N = 10$ in chapters three and four. Undoubtedly, a larger sample size would have been beneficial to each study, in particular, in the assessment of the influence of the degree of hyperglycaemia on the atrial myocardium, as well as the determination of regional differences in AP morphology between the diabetic states.

The improvement and addition of these aforementioned factors above would have been beneficial towards a better understanding of the potential underlying mechanisms of DM-induced AF in these studies.

5.5 Future directions

Recent studies have begun to explore the concept of glycaemic control- and by extension the potential role of insulin- and the processes that mediate remodeling of the DM cardiac myocardium. For many years, the risk of adverse cardiovascular events in DM has been associated with elevated glycated haemoglobin and fasting levels of glucose(288,289,433-435). Of late, evidence from large-scale clinical trials have emerged, demonstrating the potential adverse effects of intensive glycaemic control in increasing risk of mortality in patients(436,437). Saito *et al* (2014) investigated whether fluctuations in glucose increased atrial fibrosis and risk of AF more so than a persistent state of hyperglycaemia in the LA of STZ-induced DM Sprague Dawley rats. DM has been previously associated with mitochondrial dysfunction and recurrent hypoglycaemic episodes have been shown to increase mitochondrial free radical release(438,439). Additionally, intermittent and high exposure to glucose levels have resulted in heightened oxidative stress response and apoptosis in endothelial cells(440,441) and in cardiac myoblast H9c2 mouse cells respectively(442). Saito *et al* (2014) demonstrated in their study that cardiomyocytes alternately exposed to high and low glucose concentrations had significantly higher ROS levels in comparison to cardiomyocytes exposed to normal-to-low concentrations of glucose(443). They reported a greater degree of overexpression of Txnip, NADPH oxidase and TNF-α in the group exposed to the

largest fluctuation in glucose levels. These investigators postulated that it was not hyperglycaemia alone, but rather, fluctuations in glucose levels and perhaps the extent to which these glucose levels oscillate that facilitate increases in ROS generation, stimulation of TGF-β and promotion of fibrosis in DM(443). This study suggests that patients exposed to frequent episodes of hypoglycaemia may be at a higher risk of developing AF, and highlights the need for risk stratification in the management of glycaemic control in patients with DM.

The impact of weight loss on risk of AF is another avenue which has been explored in recent times. Weight loss through lifestyle adjustments and exercise is an attractive and non-invasive option of targeting obesity as a modifiable risk factor to lower the incidence of AF. It was previously shown that in patients with symptomatic paroxysmal and persistent AF, aggressive risk factor management and weight reduction improve echocardiographic parameters, reducing interventricular septal thickness and left atrial size. Long term, sustained weight loss has also been associated with reduced AF burden(90). Pathak *et al* (2014) demonstrated that aggressive risk factor management and weight loss corresponded with long term success of AF ablation(89). Pathak *et al* (2015) further showed that weight loss of >10% resulted in a 6-fold increase in probability of arrhythmia-free survival. Experimental studies of obesity have also shown that calorie-restricted weight reduction is associated with attenuation in left ventricular hypertrophy, improvement in diastolic function(444), reduced inflammation, oxidative stress and fibrosis(445).

These studies illustrate the need for weight loss reduction strategies to be implemented as a means of targeting obesity, minimizing risk of concomitant

conditions such as T2DM to ultimately reducing risk of adverse cardiovascular events.

It is also important to recognise that there is evidence that suggests that there may be differences in the pathogenesis of T1DM and T2DM. Literature delineating the two states of DM and their potential differences or commonalities are few and far between. Research which acknowledges and investigates these DM states as potentially exerting a different impact on the DM cardiac myocardium is very much limited. Such differences have been shown as previously mentioned, by Shimoni *et al* (1998) where insulin was proposed to have a tonic effect on the I_{Kss} specific to the T1DM insulin-dependent group only(363); in contrast, the changes observed in I_{to} were found only in the T2DM non-insulin dependent group. Bugger & Abel (2010) discussed some potential differences and changes in myocardial mitochondrial energetics in T1DM and T2DM hearts, proposing that the underlying mechanisms of ROS-induced dysfunction of the mitochondria may not overlap completely uniformly between the DM states; these investigators postulate that in T2DM, the presence of fatty acid-induced mitochondrial uncoupling demonstrates that mitochondrial production of ROS may play a more central role, with insulin resistance as an important mediator(439). CIRKO mice enable the investigation of impaired myocardial insulin signalling in the absence of accompanying systemic metabolic conditions found in T2DM; these mice exhibit an age-dependent decrease in contractile function which was associated with reduced insulin-stimulated glucose uptake, decrease in oxidation of glucose and fatty acids, impaired mitochondrial respiration, and reduced rate of ATP synthesis. In comparison, the myocardial

mitochondrial dysfunction in T1DM may be resultant of increased cardiac fatty oxidation due to increased levels of fatty acid oxidation proteins, and, impaired mitochondrial respiration rates. These results demonstrate that despite an overlap of mechanisms underlying mitochondrial dysfunction in cardiac cells, the mitochondrial uncoupling in T2DM may be dependent on impaired cardiac insulin signalling(446).

It can be deduced that it is unlikely for a single mechanism to be responsible for the DM-induced increased propensity towards developing a vulnerable substrate for AF. As discussed, it is rather a combination of several mechanisms (potentially associated with accompanying conditions such as obesity and age) acting separately at the electrophysiological and structural level as well as in an interactive/dependent manner to reduce conduction velocity, shorten the action potential, abbreviate the refractory period and increase conduction heterogeneity to ultimately lead to the incidence, maintenance and perpetuation of AF.

Additional studies both in animal models and in the clinical setting are still required to further elucidate these mechanisms, better understand the crucial and fundamental mechanisms underlying the pathogenesis of AF, and, ultimately to provide useful insight into the development and refinement of therapeutic strategies that enable improvement in health outcome, quality of life, and, relieve the burden on our healthcare system.

FIGURE & CAPTION

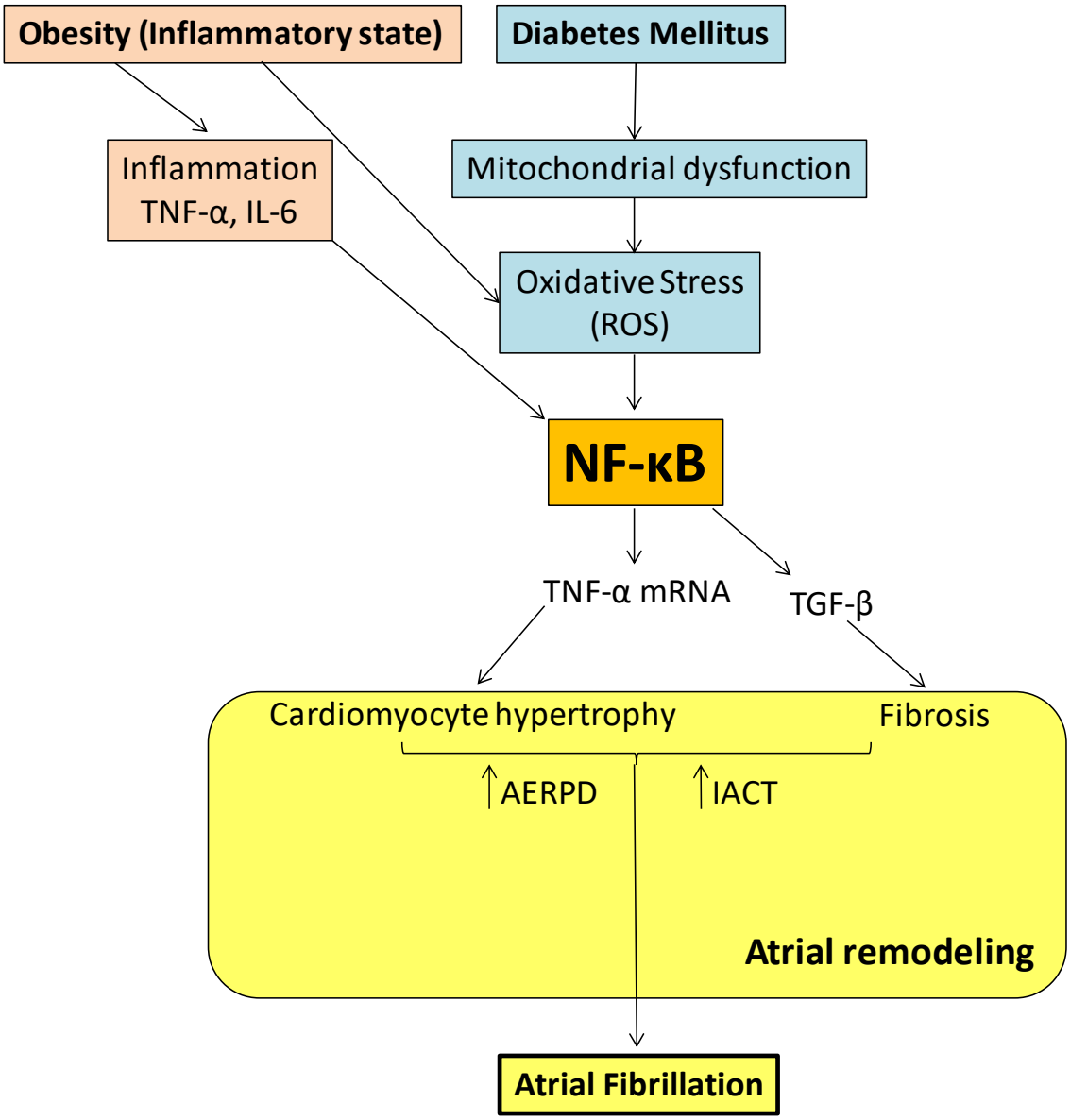

Figure 1. Flow chart showing NF-κB system as a focal pathway mediating diabetes mellitus (DM)-induced remodeling. Activation of the NF-κB system can be induced by a number of factors such as oxidative stress and mitochondrial dysfunction. This increases the expression of TNF-α and TGF-β, promoting atrial myocyte hypertrophy and fibrosis, important proarrhythmic substrates for atrial fibrillation. (Adapted from Fu *et al* 2015)

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