CARDIAC REMODELLING IN HYPERTROPHIC CARDIOMYOPATHY AND DIABESITY

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To my parents, Yong Meng and Florence,

my sister, Shu Ping,

and my partner, Melissa

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ABSTRACT

Atrial fibrillation (AF) is the most common arrhythmia encountered in clinical practice and contributes to significant morbidity and mortality risks in patients. Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiac disorder and whilst ventricular cardiomyopathy has been well characterised, the atrial substrate contributing to AF has not.

Chapter 2 assessed the underlying atrial electrophysiological, structural and biomarkers alterations in transgenic mice with a monogenic mutation in Troponin-I as a model of HCM. Also described are the changes in the atrial substrate with chronicity of HCM (30 vs. 50 weeks of age). Mice with established HCM (30 weeks) demonstrate increased bi-atrial mass associated with atrial myocyte hypertrophy, increased fibrosis and inflammatory cell infiltration. Electrophysiological parameters demonstrate decreased action potential durations, normal refractoriness, normal but heterogeneous conduction in the HCM atria. Conversely, biomarkers of extracellular matrix remodelling and inflammation were not altered in 30-week old HCM mice. With older age, HCM mice demonstrate progressively increased refractoriness and slowed conduction, as well as changes in biomarkers for extracellular matrix remodelling and inflammation.

Chapter 3 characterizes electrocardiography (ECG) changes that are indicative of conduction time within compartments of the heart and HRV as a measure of cardiac autonomic function in HCM mice. HCM mice demonstrated slowed atrial and atrioventricular conduction (as measured by P wave duration

and PR intervals respectively), as well as depressed HRV (as shown by decreased standard deviation of RR intervals (SDRR), coefficient of variation of RR intervals (CVRR), and standard deviation of heart rate (SDHR)). No significant age-related difference was observed in all ECG and HRV parameters.

Although diabetes and obesity (collectively termed as diabesity) are increasingly prevalent in humans and independently associated with AF development; the atrial substrate accounting for AF has not been fully understood. Animal models used in diabetes research are often of monogenic aetiology that demonstrates severe clinical phenotypes uncharacteristic of humans. Studies in polygenic models that better represent human diabesity are warranted.

Chapter 4 describes electrophysiological and structural changes in the atria of young (10-week old) and matured (30-week old) polygenic NONcNZO10/LtJ (RCS10) mice prone to diabesity development with high fat diet and/or increasing age. Young RCS10 mice with diet-induced progressive obesity and hyperglycaemia demonstrated increased refractoriness and action potential durations, slowed and heterogeneous conduction, increased myocyte hypertrophy, fibrosis and inflammatory cell infiltration in the atria. Matured RCS10 mice demonstrated similar electrophysiological and structural substrates with the exception of unchanged atrial refractoriness and action potential durations. Furthermore, biomarkers of extracellular matrix remodelling and inflammation in this model were altered.

Chapter 5 examines ECG and HRV modulations in the RCS10 mice with age and diet-induced diabesity. RCS10 mice demonstrated atrial and atrioventricular conduction delay (as evident by increased PR intervals and progressively prolonged P wave durations respectively). RCS10 mice also demonstrated reduced SDRR and RMSSD indicative of reduced HRV. Age was not a significant contributor of ECG and HRV changes. Increased severity of obesity and diabetes had a minor but significant correlation to worse P wave duration and depressed RMSSD.

THESIS DECLARATION

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PUBLICATIONS AND COMMUNICATION TO LEARNED SOCIETIES

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Chapter 3

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Chapter 4

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Chapter One

Literature Review

1.1. INTRODUCTION

Atrial fibrillation (AF) is the most common cardiac rhythm disturbance treated in clinical practice, accounting for one third of hospitalizations for cardiac arrhythmias (1). In AF, the electrical discharges within the atrium are rapid and irregular, resulting in asynchronous atrial depolarisation without efficient hemodynamic contraction. Increased age is a major determinant of AF with a doubling in prevalence with each decade of age in individuals aged ≥50 years (2). The prevalence of AF is recognized as a major public health epidemic worldwide, and contributes to substantial morbidity and mortality in the population (1). This is primarily mediated through the dislodgement of thrombus from the fibrillating atrial appendage leading to stroke. One in six ischemic strokes occurs in AF patients and this increases to one in three elderly patients over the age of 75 (3). AF-related strokes are also twice as fatal, more likely to recur, and cause severe functional deficits (4).

Due to the high risk of AF patients developing cardio-embolic strokes, anticoagulation therapies such oral vitamin K antagonists or aspirin are often prescribed (5). Current therapies for AF include either rhythm- or rate-control strategies in patients. Under rate-control strategies, the ventricular rate is controlled with the use of beta-blockers or calcium channel blockers in the absence

of any attempt to restore or maintain sinus rhythm. Rhythm control strategies aim to convert to and maintain individuals in sinus rhythm; these include pharmacological approaches with anti-arrhythmic agents, ablative therapies, surgical approaches, and cardioversion (6). While anti-arrhythmic drugs such as amiodarone can be effective in the maintenance of sinus rhythm, they are associated with adverse effects of pro-arrhythmias and increased mortality risk (7). Additionally, atrial contractility may be reduced following surgical approaches causing stasis and increase risk of thromboembolism (8). At present, whilst catheter ablation may improve quality of life in drug-refractory patients, there is insufficient evidence showing that catheter ablation improves survival or the risk of developing stroke and heart failure (9). Furthermore, ablative therapies do not always guard against AF recurrences (10). The current lack of pharmacological specificity and elevated risks for adverse events in the present therapies have made the understanding of AF mechanisms and subsequent translation of basic science concepts into clinical practice an attractive avenue for research in the hope of improving success rates with reduced risks.

1.1.1. Incidence and Prevalence of Atrial Fibrillation

AF is recognized as a burgeoning epidemic of the millennium (11). It has been projected that 12-15 million individuals in the United States will be affected by AF by 2050 (12), and the direct costs to the country has been estimated at $6 billion annually (13). In the United Kingdom, AF accounted for 0.9-2.4% of total national health expenditure in 2000, of which 50% was attributed to hospitalization alone (14). In Australia between 1993-2007, hospitalization due to AF was significantly

increased by 155% as compared with myocardial infarction (50%) and heart failure (-2%) demonstrating the enormous public health care burden of AF (15). The increase in hospitalizations may in part be explained by the aging populations worldwide. A systematic review of the current worldwide epidemiological data on AF confirms higher rates of AF in older age groups where males aged 75-79 years have a 2- and 5-fold increased prevalence of AF compared to males aged 65-69 and 55-59 respectively. Improvements in medical care have also resulted in prolonging exposure to traditional as well as novel risk factors for AF (16,17).

1.2. RISK FACTORS FOR ATRIAL FIBRILLATION

1.2.1. Major Risk Factors for Atrial Fibrillation

1.2.1.1. Age

Age is an independent risk factor for developing AF, with a 100-fold increase in prevalence in octogenarians compared to individuals younger than 55 years (18). With advances in medicine and therapies, the global population is expected to age further putting more people at risk of developing AF. The aging process clearly affects both the electrophysiology and structural substrates in the myocardium (19). The majority of elderly patients develop AF in the setting of structural heart disease, whereas only small percentages have a primarily electrical disorder as the cause of AF (18). Importantly, the number of prescribed drug use increases with age due to co-morbidities, which makes drug interactions more likely and side-effects more risky in the elderly population (20).

In older age, it has been demonstrated that action potential duration and the effective refractory period lengthens, and the atrioventricular conduction slows (21,22). Age-related fibrosis is known to occur in the ventricles resulting in a reduction in ventricular compliance and a greater ventricular diastolic pressure which restricts atrial flow and increases atrial pressure and diameter (20). Additionally, experimental data from several large (23,24) and small (25,26) animal models of AF demonstrates that age-related fibrosis also accumulates in the atria, whereas others do not (27,28). The presence of interstitial fibrosis can have implications on the electrophysiology of the atrial myocardium by producing areas

of localized conduction slowing and heterogeneity, providing a substrate of unidirectional block and macro-reentry circuit formation (29).

1.2.1.2. Congestive Heart Failure

Heart failure (HF) and AF frequently coexist (30) and HF can predispose to the development of AF and vice versa (31). The increased morbidity and mortality with the onset of AF in HF patients may be potentially mediated by a high ventricular rate resulting in a loss of atrial contraction and irregular ventricular filling time, or thromboembolism (32-35). Of note, increasing evidence suggest that genetic factors may be associated with an increased risk of AF in HF patients and AF is more pronounced in HF patients than the general population (36). In HF patients, the atria often undergo hypertrophy and eventually become dilated as a result of chronic pressure and volume overload. This increase in surface area allows for multiple wavelet formation (37) and thus increases AF probability (38). Chronic atrial dilatation can activate stretch-activated channels (11,39,40), which alters the atrial electrophysiology and can cause heterogeneous reduction in effective refractory periods, conduction slowing and spontaneous triggered activity (41,42).

1.2.1.3. Hypertension

Hypertension is the most common risk factor associated with AF, primarily due to its high prevalence in the general population (43). In a 38-year follow-up of the Framingham cohort, it was found that hypertension conferred a 1.5- and 1.4-fold increased risk of AF development in males and females respectively (2). Left

ventricular hypertrophy usually occurs as a maladaptive response to chronic pressure overload due to systolic hypertension and is an important risk factor for AF development. Hypertension is also related to structural changes in the left atrium that are associated with AF development. These include left atrial dilatation, changes in mechanical function, altered electrophysiology, and increased ectopic activity (41,42), all of which contribute to AF.

1.2.1.4. Diabetes Mellitus

Diabetes mellitus (DM) is one of the most insidious epidemics affecting humans and is projected to increase from 135 million in 1995 to 300 million worldwide by 2025 (44). A meta-analysis by Huxley et al. (2011) estimates that patients with DM have 40% greater risk of developing AF (45). The possible mechanisms of how DM relates to AF remain to be elucidated. However, hypotheses include: 1) insulin resistance, 2) inflammation, 3) left ventricular hypertrophy, and 4) atrial mechanical stress.

1.2.1.5. Myocardial Infarction

Myocardial infarction (MI) is one of the most common causes of death in Australia. However, as medical practice improves patient outcomes, it has been suggested that 20% of MI survivors go on to develop AF (46,47). MI confers a 1.4- and 1.2-fold increased risk of developing AF in males and females respectively (2). Post-MI AF is postulated to be due to a lack of ventricular function and decreased left ventricular ejection fraction (48,49), resulting in an increase in atrial pressure and volume and consequently AF. Alternatively, atrial ischemia due to coronary artery

disease affecting the atrial branches independent of increased filling pressures has been associated with new-onset AF post-MI (50).

1.2.2. Novel Risk Factors for AF

1.2.2.1. Obesity

Obesity is associated with ventricular diastolic dysfunction (51), pro-inflammatory state (52), cardiac autonomic dysfunction (53), and increased atrial size (54,55) – all of which are known to promote AF (56). Therefore, it comes as no surprise that recent studies have demonstrated the association of obesity to atrial fibrillation (57-60). A meta-analysis by Wanahita et al. (2008) demonstrates that obesity increases the risk of developing AF by 49% in the general population and the AF risk increases in parallel with increased body mass index (BMI) (61). Obesity also implicates progression of paroxysmal to permanent AF (55). Furthermore, weight reduction and risk factor management reduces atrial fibrillation symptom burden and improves beneficial cardiac morphology (62).

Recent evidence suggests that cardiac or pericardial adipose tissue has direct implications of AF development. Atrial pericardial fat in AF patients has been associated with the presence and severity of AF, as well as long-term AF recurrence following first-time AF ablation (63). Furthermore, these associations persisted after adjustments for body weight and were stronger than other systemic measures of adiposity including body mass index and body surface area. Potential mechanisms underlying the association of pericardial fat to AF development may be via 1) paracrine activity due to secretions of cytokines that

influence inflammation and extracellular matrix remodelling and 2) infiltration of the atrial myocardium resulting in disorganization in conduction propagation and local conduction block contributing to an arrhythmogenic substrate (64).

1.2.2.2. Metabolic Syndrome

Metabolic syndrome (MetS) involves a cluster of factors including obesity, hypertension, insulin resistance, dyslipidaemia. MetS is a growing epidemic in the United States and currently affects 20% of the population (65). In the past few decades, several small cohort studies have suggested that weight at birth may implicate adult onset of metabolic disorders including hypertension (66), diabetes (67,68), and obesity (69). Subsequently, Curhan et al. (1996) confirmed in large cohorts studies that low birth weights correlated to adult hypertension, diabetes and obesity in men (70) and adult hypertension and obesity in women (71).

Recent evidence suggest that MetS is associated with an increased risk of AF (72,73) and also an increased risk of developing recurrent AF following catheter ablation (74). Additionally, Chamberlain et al. (2010) demonstrated that the risk of AF was increased with each of the components of MetS, and cumulative risk of AF increased with the number of risk factors present (75).

1.2.2.3. Smoking

Cigarette smoking is a leading cause of cardiovascular diseases such as hypertension, myocardial infarction and cardiac arrhythmias in humans, and smoking cessation alleviates these cardiovascular risks (76-78). However, the role

of cigarette smoking as a new risk factor for AF is unclear. Large population studies have been conflicting. The Rotterdam study suggests that smoking results in an increased risk of AF (79). In contrast, after age-adjustments, smoking was a weak risk factor for AF occurrence in the Framingham Study (16). Nonetheless, this may be due to a less important role of cigarette smoking to AF development in the presence of other confounding comorbidities. Furthermore, published case reports suggest that nicotine, the main ingredient of cigarette smoke, can lead to serious and sometimes fatal cardiac arrhythmias (80-83), highlighting the pro-arrhythmic effects of nicotine alone. Chronic smoking in humans has been demonstrated to result in atrial fibrosis due to the effects of nicotine (84) and atrial fibrosis has been postulated as a substrate for AF by impairing local atrial conduction and promotes induction of re-entry circuits, causing abnormal electrical activity and consequently fibrillation (85,86).

1.2.2.4. Inflammation

Inflammation has been found to contribute to some types of AF such as post-cardiac surgery and pericarditis (87,88); and implicated in genetic causes of elevated inflammatory cytokines (89). Inflammation has also been implicated in lone AF by the presence of inflammatory infiltrates, myocytes necrosis, and fibrosis producing a substrate to sustain and progress AF (90).

1.2.2.5. Familial AF

Atrial fibrillation is often regarded as a condition resulting from structural and electrical remodelling in the atria due to cardiac and systemic disorders. However,

a proportion of patients that do not present with clinical comorbidities develop unexplained AF, known as 'Lone AF'. It is only in recent years that studies have documented evidence of inherited AF (91-93), of which accounting for 30% of lone AF patients having a family history of AF. However, recent studies have demonstrated atrial structural abnormalities in lone AF patients that may contribute to the development and progression of AF (90,94). These structural abnormalities suggest evidence for a consistent atrial substrate in AF patients, even in the absence of comorbidities.

1.2.2.6. Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiovascular disease affecting 1 in every 500 of the general population (95). AF is the most prevalent cardiac arrhythmia clinically presented in patients with HCM and a significant contributor to heart failure progression and embolic stroke deaths (96-99). A recent meta-analysis by Guttmann et al. (2013) observed that AF was prevalent in 22% of HCM patients, with an annual AF incidence rate of 3 per hundred patients (100). This suggests a 4- to 6-fold increased likelihood of AF in HCM patients compared to the general population (43,101).

1.3. MECHANISMS OF AF

Despite significant research being done in understanding AF development, the underlying mechanism(s) responsible for the pathogenesis of this disease remains uncertain. There are currently four major theories attributed to AF development: a focal source of ectopy originating from pulmonary veins (102), coexistence of multiple re-entry circuits (103) or a stable, localized spiral wave emitting multiple 'daughter' wavelets (104), and more recently transmural conduction leading to electric dissociation between the endocardial muscle bundle network and the epicardial surface (EED) (105).

1.3.1. Rapid Ectopic Activity

In a healthy heart, the sinus node which is intrinsically the fastest pacemaker determines the heart rate. However, spontaneous initiation of AF may be triggered by rapid sources other than the sinus node (102,106-109), with the majority of atrial premature beats originating in the pulmonary veins (102).

Automaticity in areas outside the sinus node may be due to a change in the balance of inward and outward currents, such that the predominance of inward currents leads to progressive diastolic depolarization allowing for the premature reaching of current threshold and the cell fires rapidly as a result (110). Afterdepolarizations may also contribute to abnormal focal activity. This may be due to Ca^{2+} dysregulation when there is a reduction in sarcoplasmic reticulum reuptake and/or extrusion through the electrogenic transmembrane Na^{+}/Ca^{2+} exchanger (110). The inward current generated by the latter process can lead to

cellular depolarization resulting in afterdepolarizations that may generate ectopic beats if and when the threshold potential is reached. Furthermore, studies utilising experimental atrial tachypacing as a method for rapid sources have clearly demonstrated progressive, and sometimes persistent, changes in atrial electrophysiology that translates to progression of atrial arrhythmias (Chapter 1.4.1).

Clinical therapies for suppressing abnormal automaticity or triggered activity includes rate control approaches such as β-adrenergic receptor blockers and calcium and sodium channel blockers (106) or rhythm control strategies such as anti-arrhythmic drugs (111) and radiofrequency ablation (102,107). Interestingly, catheter ablation therapy has been recently proposed as a superior first-line treatment of AF over the use of rate control and anti-arrhythmic medications by preventing sources of atrial premature beats and thereby the primary cause of fibrillatory conduction (112). However, it should also be noted that structural substrates such as atrial fibrosis are associated with conduction abnormalities and perpetuation of re-entrant arrhythmias (113), and as such may not be rectified solely through ablation. Recently, results from the DECAAF study suggest that post-ablation recurrent arrhythmias are associated with increased atrial fibrosis (114). This further complicates AF ablation strategies in structural heart disease which often presents with extensive fibrosis, and may have long-term implications on AF recurrence in this subset of patients.

1.3.2. Single and Multiple Circuit Re-entry

A dissociation of refractoriness between different zones of tissue can result in re-entry formation thereby causing abnormal impulse propagation (110). Re-entry can exist either as a single rotor with regular yet rapid firing, multiple unstable and self-sustaining rotors or even a transition between the two (104,110,115).

The multiple-circuit re-entry theory has been the long standing predominant notion for initiation of AF, supporting the maintenance of continuous electrical activity by coexistence of simultaneous wavelets varying in spatial and temporal characteristics propagating through excitable tissue (110). The excitability of the atria is dependent on the cellular, structural and functional alterations in the atrial tissue. The number and size of wavelets (110), slowed myocardial conduction velocity (116), alterations in action potential duration (APD), effective refractory period (ERP) and consequent changes in action potential wavelength (117), and spatial heterogeneity in impulse propagation (118) can all contribute to the initiation and sustenance of AF.

The concept that AF induces atrial remodelling which in turn further increases the likelihood of arrhythmia maintenance is well established (117). This is primarily manifested by a shortening of the ERP, which reduces the functional wavelength of wave propagation and facilitates functional reentry circuits. Furthermore, increasing evidence suggests that atrial fibrosis and its resultant role in conduction heterogeneity can be an alternative substrate for AF sustenance, presenting with prolonged rather than shortened refractoriness in animal models

of congestive heart failure (29) and hypertension (119). Additionally, contributors of slowed conduction velocity such as the reduction in fast sodium current (I_{Na}), increased axial resistance due to gap junctional decoupling, and fibrosis change resulting in myocyte-myocyte and myocyte-fibroblasts decoupling have been demonstrated in pathophysiological conditions predisposing to arrhythmogenesis through slow conducting re-entry circuits (116).

1.3.3. Endocardial-epicardial Wave Breakthrough

Apart from automaticity and re-entrant arrhythmias, recent evidence proposes electric dissociation between the endocardial and epicardial muscle bundle network (EED) as a new 3-dimensional substrate for the induction of AF in goats, explanted human hearts and persistent AF patients (120-123). Present hypotheses for the cause of breakthrough events include transmural conduction, transmural micro-reentry, or ectopy foci (102,121,124). Eckstein et al. (2013) demonstrated with simultaneous endo-epicardial mapping of the left atrial free walls in goats that the leading mechanism of epicardial breakthrough was transmural conduction (86%) with ectopic focal discharges being a limited source in breakthrough (13%) and 1% being unexplained (105). Their data also suggested that transmural micro-reentry was an unlikely contributor of breakthrough events due to the lack of increased incidence non-peripheral waves preceding a breakthrough event at half the AF cycle length which would be expected for transmural micro-reentry (105).

On the other hand, heterogeneous conduction properties like fibrosis can cause the disruption of transmural connections leading to more breakthrough

events (121). Breakthrough events are rarely due to single rotors but often wide-spread, not clustered and not repetitive in both computer simulations (125) and persistent AF in the goat model(105), lending support to the existence of multiple wavelets resulting in fibrillatory conduction rather than local sources of AF. Furthermore, recent studies by Hansen et al. (2015) suggest that AF is driven by intramural re-entry anchored to interstitial fibrosis and angle differences in transmural fiber orientation in explanted human hearts (123). This study contrasted with earlier studies by Eckstein and colleagues (2013) and may be due to several experimental differences. These include differences in experimental model, optical mapping vs. high density surface electrode mapping, and right as opposed to the left atria. Additionally, the added use of gadolinium-enhanced magnetic resonance imaging of the atrial wall structure in the latter study allows for the 3D mapping of intramural conduction; contrasting from the interpretation of intramural conduction in the prior study based on overlapping of two planes of conduction on the endo-epicardial surface in both time and direction. The latter study demonstrates with histological evidence that intramural re-entry pathways are anchored to areas of fibrosis, opposing fiber orientation, and thickness of the myocardium that would not be seen by endocardial and epicardial surface mapping alone.

1.3.4. Summary

Over the last century, we have seen an evolvement in our understanding and knowledge in regards to mechanisms that initiate and maintain AF. Despite evidence of the various theories, there is no clear mechanism that lies superior

over the others. There is a current gap in the knowledge bridging the underlying atrial substrate to the initiation and/or sustenance of AF.

1.4. ATRIAL REMODELLING IN AF

AF and congestive heart failure (CHF) are two conditions commonly encountered in clinical practice with both leading to an array of processes resulting in changes in atrial function and structure, cumulatively known as atrial remodelling (126). As will be further explained below, the progression of atrial remodelling in these two diseases take on a very different profile despite both often coinciding and have reciprocal relationships.

Atrial remodelling comprises of several changes within the atria including electrophysiological, structural and contractile changes that may be responsible for the genesis and maintenance of AF. Electrophysiological remodelling refers to alterations in atrial action potential profile and duration due to changes in ionic currents, atrial refractoriness, conduction velocity, and heterogeneity of atrial conduction and atrial refractoriness, all of which provides an "electrical" substrate for re-entry mechanisms by creating a dispersion of non-uniformed repolarization in the myocardium resulting in conduction block and re-entry. Alternatively, changes in structural substrates leading to increased tissue anisotropy such as accumulation of tissue fibrosis and gap junctional remodelling within the myocardium may be a slower occurring 'second factor' responsible for longer time course of sustained AF and recurrence of AF.

1.4.1. Atrial Tachycardia-induced Remodelling: "AF begets AF"

It has been known for some time that AF is a progressive arrhythmia with chronicity rates depending on the underlying aetiology (127). However, close to 20%

of patients with paroxysmal AF go on to develop sustained AF even in the absence of progressive underlying heart disease (128), suggesting that AF itself results in electrophysiological and/or structural changes within the atria promoting the perpetuation of AF. This was first demonstrated in dogs and goats whereby chronic rapid pacing or fibrillation resulted in reductions in ERP and stabilization of increasingly sustained AF (129,130). AF-induced electrical remodelling has also been confirmed in humans (131). Furthermore, Lu et al. (2008) demonstrated with ganglionated plexi ablation that the interruption of the intrinsic cardiac autonomic nervous system prevented the progression of paroxysmal AF to persistent AF, suggesting autonomic modulation to be important in atrial electrical remodelling (132).

In early studies, Attuel et al. (1982) demonstrated that increased vulnerability to sustained atrial tachyarrhythmia correlated with a loss of physiologic adaptation of the ERP to increasing heart rates (133). Similarly, virtually all studies of tachycardia-induced atrial electrical remodelling over time courses ranging from days to weeks demonstrated decreased atrial ERP and reduced rate adaptations of ERP (129,134-136). Interestingly, even AF as short as several minutes has been demonstrated to decrease ERP (137). Decreases in ERP result in a reduction in minimal wavelength required for the electrical wave to travel to support re-entry and thereby allow more functional re-entry circuits to co-exist in the atria and decreases the chances of spontaneous termination (138,139). Atrial tachycardia also results in atrial conduction slowing (129,134,136), this in turn reduces wavelength by increasing the chances of the re-entry circuit to be re-initiated following recovery from refractoriness. The spatial

heterogeneity of atrial ERP is also an important factor to determine the self-propagation of AF. Spatial heterogeneity of refractoriness increases over time in dogs subjected to rapid atrial pacing, and has been shown to be a predictor of atrial tachycardia-induced changes in AF duration (136). Additionally, changes in atrial ERP caused by sustained atrial tachycardia are spatially variable among and within atrial regions resulting in increased ERP heterogeneity and contributing to atrial remodelling that promotes AF (140).

Decreases in APD and APD restitution mirror the ERP and ERP rate adaptation caused by rapid pacing in dogs and lends support to the notion of APD changes as the mechanism for ERP alterations caused by tachycardia (141). Shortening of the APD can be either due to an increase in outward repolarising current or a decrease in inward depolarising current. The major contributors of the outward current and plateau phase of the inward current lie primarily in potassium (K^+) ions and calcium (Ca^{2+}) ions respectively. Therefore, it was hypothesized that AF-induced shortening of the APD was due to an upregulation of K^+ channels or a downregulation of Ca^{2+} channels. However, Van Wagoner et al demonstrated that the channels carrying the transient outward current (I_{to}) as well as the sustained potassium current (I_{Ksus}) were reduced instead of elevated in patients with chronic AF(142). Additionally, the expression of $Kv_{1.5}$ (the alpha subunit of the delayed rectifier K^+ channel) was reduced by more than 50%. Taken together, these findings suggested that K^+ channels were not a sole contributor of the observed shortening of APD in chronic AF patients. Yue et al. (1997) studied the alternative hypothesis in a canine model of rapid-pacing induced atrial fibrillation, where they demonstrated the density of the transient outward current

(I_{to}) and the L-type Ca^{2+} current to be markedly reduced (141). On the other hand, no changes in voltage clamp studies of I_{K1}, I_{Kr}, I_{Ks}, $I_{Kur.d}$, I_{CaT}, or I_{ClCa} were observed. The shortened action potential in the remodelled cells also resembled those in normal cells subjected to nifedipine (an L-type Ca^{2+} blocker) which was restored upon administration of an L-type Ca^{2+} channel agonist, thereby suggesting that APD changes were largely due to reduced $I_{Ca,L}$. Yue and colleagues further went on to demonstrate reduced mRNA concentrations of Kv4.3 (putative gene encoding transient outward current channels), the α_{1c} subunit of L-type Ca^{2+} channels and the α subunit of the cardiac Na^{+} channel in the same model, which was paralleled by a decrease in protein expressions of Kv4.3 and Na^{+} channel α subunits; therefore providing an possible explanation for the self-perpetuating nature of AF (143). These findings were mirrored in human subjects whereby the action potential was shortened in atrial myocytes from chronic AF patients due to a reduction in $I_{Ca,L}$ and I_{to} coupled with increases in I_{K1} and I_{KACh} (144).

This leads one to question as to why the atrial myocytes adapt in such a way to tachycardia pacing. One theory is that atrial electrical remodelling occurs as a result of Ca^{2+} overload due to the rapid activation resulting in a continuously open state of the L-type Ca^{2+} channel. Therefore, alterations of ion channels, particularly the decreased $I_{Ca,L}$, occurs to subsequently shorten the action potential duration and refractoriness (143,144).

Rapid atrial activation through AF itself or by rapid atrial pacing causes electrical remodelling via decreased atrial ERP, slowed conduction and increased electrophysiological heterogeneity (129,130). However, it is unlikely that rate-

related remodelling forms the substrate predisposing to AF development. This is observed in the longer time course of development of sustained AF not running in parallel with the refractoriness alterations nor the cumulative effects of repetitive 1-month episodes of AF (130,145). This supports the notion of a 'second factor' needed for the persistence of AF, of which tissue anisotropy due to changes in local gap junctional proteins or tissue fibrosis may be the cause (146).

Electrical coupling in the atria is mediated by gap junctional proteins, of which connexin40 (Cx40) and Cx43 are the two principle connexins co-expressed in combinations in intercalated discs between cardiac myocytes (147,148). Cx43 is the predominant connexin expressed by cardiomyocytes and Cx40 is expressed in lower quantities (148). Gap junctional remodelling can play a part in AF-induced atrial remodelling by contributing to abnormal impulse propagation and arrhythmia; however, findings to date have been contradictory. Elvan et al. (1997) demonstrated an increase in Cx43 protein in the atria dogs with pacing-induced AF which was reversed with ablation (135), left atrial tissue in AF patients demonstrated increases in Cx40 and Cx43 compared to patients in sinus rhythm (149), whereas there was no change in connexin43 and a heterogeneous distribution of Cx40 observed in the goat model of AF (150). Furthermore, the speed of atrial impulse propagation is only affected when connexins are reduced by more than 40% (151). Spatial heterogeneities of connexins may therefore be the more important factor for AF by altering the route of impulse propagation and resulting in a fragmented wavefront. Redistribution of Cx40 to predominate at the lateral borders have been observed in human atrial samples from patients suffering from AF and are suggested to contribute to dispersion and heterogeneity

in tissue anisotropy contributing to perpetuation of re-entrant pathways (152,153). An additional complexity in gap junctional remodelling lies in the co-expression of multiple connexins having differing properties in propagation velocities. Using in vitro expression systems, Cx40 when expressed alone results in high conductance gap junctions (154). However in cultured neonatal atrial myocytes with co-expression of Cx40 and Cx43, progressive reduction in Cx40 expression against a background of normal Cx43 expression using partial or total knockout mice lead to further increases in conduction velocity (155). Notably, whilst specific expression of the junctions and their channels at the molecular level are important in inter-myocyte electrophysiological properties using in vitro systems, determining gap junction expression at the molecular level in pathological states can be technically challenging.

On the cellular level, Morillo et al. (1995) demonstrated for the first time changes in the structure of atrial myocytes due to rapid atrial pacing (129). Since then, several following studies in dog and goat models of AF (135,156-159) have shown that AF-induced structural changes in atrial myocytes include myocyte hypertrophy, glycogen accumulation, myolysis, alterations in connexin expression, changes in the mitochondrial shape, fragmentation of sarcoplasmic reticulum, homogeneous nuclear chromatin distribution, and changes in the amount and localization of structural cellular proteins.

1.4.2. Atrial Structural Remodelling: "Atrial Remodelling of a Different Sort"

As the mechanisms of "AF begets AF" explains the progression of AF but not the underlying predisposing substrate for AF initiation, investigations have since been concentrated on evaluating atrial abnormalities in conditions that predispose to AF development. A study by Li et al. (1999) in a canine model of congestive heart failure (CHF) demonstrated a different atrial remodelling to that commonly seen in tachycardia-induced remodelling (29). Rather than a decrease in refractoriness favouring AF maintenance as is seen in "AF begets AF," their study demonstrated a significant increase in ERP at shorter cycle lengths which was associated with increased conduction heterogeneity and extensive interstitial fibrosis was also observed. These findings are in concert with those seen in dogs with mitral valve fibrosis (160) and cats with cardiomyopathy (161), both of which develop spontaneous AF, as well as in a canine model of atrial enlargement due to mitral regurgitation (162).

Atrial enlargement appears to be a common structural trait in most experimental models of CHF (29), mitral valve disease (160,162), and ventricular cardiomyopathy (161). Left atrial size (163) and volume (164) are also important risk factors for AF development. Boyden and colleagues found increased connective tissue accumulation between hypertrophied myocytes in the dilated atria of dogs and cats with mitral valve disease and cardiomyopathy that develop spontaneous AF, with degeneration and loss of myofilaments (160,161). They also found transmembrane action potential was not altered in the dilated atria lending support to the fact that the enlarged atria may accommodate multiple re-entry

circuit formation simply by increasing surface area. Li et al. (1999) also demonstrated that treatment with class III agent dofetilide strongly suppressed CHF-related AF, consistent with a re-entry mechanism, whereas flunarizine that suppresses abnormal automaticity, did not significantly affect either duration or inducibility of AF in their dog model (29), suggesting that re-entry mechanisms may play a more important role than triggered activity in development of AF at least in the setting of heart failure.

Fibrosis is a fundamental hallmark of atrial structural remodelling, contributing to a sustained substrate for AF progression. Local conduction abnormalities have been observed in relation to fibrotic tissue infiltrates in atrial tissue preparations (165), in left atrial appendage tissue of patients with rheumatic mitral stenosis (166), and animal models of hypertension (28,119), obesity (167), and heart failure (29,168). Therefore, it appears that fibrosis may be a common endpoint in a range of different cardiac diseases. A major electrophysiological change in the mentioned animal models consists of an increase in spatial heterogeneity in atrial conduction velocity which may be explained by the development of regional fibrosis resulting in the electrical uncoupling of adjacent myocytes. Myocardial electrical impulse propagation is governed by low-resistance connexin channels located in the gap junction of adjacent myocytes, which can be disrupted by fibrosis interrupting inter-myocyte coupling. As a result, electrical propagation in the transverse direction is reduced, leading to focal areas of slowed conduction and block, thereby increasing heterogeneity and promoting focal and macro-reentry mechanisms driving AF (113).

Tissue fibrosis commonly occurs as a reparative process to replace degenerative myocardial cells with reactive fibrosis through accumulation of fibrillar collagen deposits, resulting in interstitial expansion (169,170). Interestingly, the atria myocardium is more susceptible to fibrosis as compared to the ventricles, at least in the setting of ventricular tachypacing-induced remodelling (171). Atrial fibrosis involves a complex interplay among neurohormonal and cellular mediators including angiotensin II (AngII), transforming growth factor (TGF)-beta 1, platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF).

The renin-angiotensin-aldosterone system has been implicated in the development of myocardial fibrosis in hypertensive heart disease, myocardial infarction, cardiomyopathy and chronic heart failure (172), as well as *in vitro* experiments where AngII and aldosterone stimulate collagen synthesis (173,174). Additionally, AngII also suppresses the activities of matrix metalloproteinase I, a key enzyme that degrades interstitial collagen, thereby synergistically resulting in progressive interstitial collagen accumulation in the myocardium (173). Several animal studies have since shown that administration of ACE inhibitors and angiotensin 1 receptor blockers attenuated atrial fibrosis with consequent reduction in AF duration and/or inducibility (175-179).

TGF-β1 is a pivotal player in the signalling cascade, as a downstream mediator of AngII effects, and the two work hand in hand in the process of cardiac fibrosis (180). The stimulation of AngII has been demonstrated to upregulate both

mRNA and protein expression of TGF-β1 in cardiomyocytes and cardiac fibroblasts (181,182). Furthermore, ACE inhibitors or angiotensin 1 receptor blockers significantly reduced TGF-β1 levels in hypertrophied (183) and infarcted hearts (184), lending support to the notion that TGF-β1 is mediated, at least partially, through the effects of AngII. Additionally, cardiac overexpression of TGF-β1 causes atrial fibrosis, heterogeneous conduction and increased propensity for AF (185,186). Interestingly, the selective fibrosis occurring in the atria and not the ventricles in this model have significant implications by suggesting that TGF-β1 is a crucial activator of atrial fibrosis and that regional differences exist within cardiac remodelling in the sense that the atria is more particularly prone to fibrosis.

Inflammation is another crucial initiator of atrial fibrosis because pro-inflammatory stimuli such as oxidative stress and cytokines, growth factors, AngII and other hormones, stimulate fibroblast proliferation, migration and differentiation into myofibroblast (187,188). Of note, myofibroblasts are rarely seen in healthy atrial myocardium, but are the predominant cell type in diseased states (187). Myofibroblasts in turn play a pivotal role in the fibrotic process by secreting more growth factors, cytokines, matrix metalloproteinases (MMPs) and extracellular matrix proteins (189,190). Importantly, enhanced MMP expression and activity degrades extracellular matrix proteins, fuels the turnover of connective tissue and in turn results in increased extracellular matrix deposition (191,192) and predisposes to AF occurrences (193,194). Pro-inflammatory cytokines including tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β) are present in post-infarct myocardium and have been shown to upregulate the

angiotensin 1 receptor density through the process of NF-κβ activation in cardiac fibroblasts (195).

1.4.3. Summary

Initial studies in animal models demonstrated the progression of AF whereby artificial maintenance of AF resulted in electrophysiological changes in the atria, contributing to increased AF inducibility and stability, termed “AF begets AF”. However, the mechanisms did not seem to explain for all forms of AF, especially those with underlying atrial substrates, leading to a second theory “atrial remodelling of a different sort”. Indeed, increasing evidence suggests that the complex atrial substrates promote the sustenance of AF in their own ways that are very different from those of atrial tachycardia-induced AF. To be able to treat clinical AF effectively, we need greater understanding in the substrates, electrophysiological or structural, that contributes to AF in the setting of different disease states.

1.5. CARDIAC AUTONOMIC NERVOUS SYSTEM AND ATRIAL FIBRILLATION

1.5.1. Physiology of Heart Rate Variability

The heart is an organ that is richly innervated by the parasympathetic and sympathetic limbs of the ANS, which can modify the rate as well as strength of contraction. Although the heart is self-sufficient in regulating heart rate via intrinsic cardiac pacemaker cells (in most cases the sino-atrial (SA) node is the pacemaker of the heart), these functions are largely regulated by autonomic influences (196). Normal variation in heart rate as seen in healthy humans is due to autonomic neural regulation (either vagal or sympathetic) of the heart and the circulatory system (197), in response to match cardiac output with body needs during various stimuli such as physical and mental stress, postural changes, and exercise. The atrium, in particular the SA node and atrioventricular (AV) node, receives both vagal and sympathetic stimulation, whereas in the ventricles autonomic innervation is predominantly sympathetic in origin (198). Additionally, the heart also has afferent receptors that can subserve various reflexes originating from the heart (199).

Instantaneous fluctuations of heart rate mirror the interplay between ongoing perturbations to the cardiovascular system in response to its regulatory mechanisms. Heart rate variability (HRV), which is the temporal variation between consecutive heart beats, is thought to reflect the ability of the heart to adapt to

changing circumstances, and can be used as a tool to investigate the overall state of the autonomic nervous system (ANS) and cardiac health (200).

The vagal and sympathetic nervous systems both bring about changes to the heart rate via a complex, dynamic, and often antagonistic interaction (201,202). These interactions are altered via the activity of the second messenger system (cyclic AMP and cyclic GMP) through to the innervated cardiac cells.

1.5.2. Vagal Contributions to Heart Rate Variability

Parasympathetic nervous stimulation is supplied by the vagal nerves, innervating the SA and AV node, and the atrial muscle. Parasympathetic innervations on the ventricular muscle remains controversial depending on the animal model, and is thought to be of trivial effect if any in humans (199). Vagal efferents from the medulla extend to postganglionic nerves that innervate the atria via ganglia located in cardiac epicardial fat pads that are anatomically located around great vessels such as the pulmonary veins (203). Neurotransmission through the vagal pathway is mediated via nicotinic receptors in the ganglia and muscarinic receptors on target tissue (204). The most obvious effect of vagal stimulation is heart rate reduction either through inhibiting sympathetic contribution presynaptically and/or direct hyperpolarization of sinus node cells (204). Acetylcholine released as a result of enhanced parasympathetic activity increases the permeability of the SA node to potassium ions (K^+) by slowing the closure of K^+ channels, resulting in a reduction in the rate of spontaneous action potentials via a reduction in resting potential, thereby decreasing the rate of spontaneous

depolarization (196). Therefore, a low sympathetic activity or high parasympathetic activity, primarily from trauma, allergic reactions and inhalation of irritants, decreases the firing rate of pacemaker cells and the HR (205).

1.5.3. Sympathetic Contributions to Heart Rate Variability

The cardiac sympathetic nerves innervate the entire heart, including the SA and AV node, and the atrial and ventricular myocardium (199). The sympathetic nervous system controls the heart action in emergency and exercise situations, speeds up the heart rate through its effects on the pacemaker tissue. Noradrenaline released from the sympathetic nerve endings decreases K^+ permeability by accelerating inactivation of the K^+ channels, thereby causing a drift to threshold under sympathetic influence and permits more frequent action potentials and a correspondingly faster heart rate (196).

Sympathetic stimulation or parasympathetic inhibition, in response to stress, exercise and heart disease, results in acceleration of the heart rate by increasing the firing rate of the pacemaker cells in the sino-atrial node.

1.5.4. Heart Rate Variability and Atrial Fibrillation

The most important clinical application of HRV lies in its overwhelming presence in pathophysiological states and its usefulness in risk stratification of patients at risk of mortality. A depressed HRV has been implicated in patients with epilepsy (206), myocardial infarction (207), hypertension (208), obesity (53), diabetes (209), and congestive heart failure (210,211). Decreased HRV has also been

implicated in identifying patients at risk for sudden cardiac death (212,213) and prospective risk for developing cardiac events such as angina pectoris, coronary insufficiency, myocardial infarction, and congestive heart failure (214).

Recent evidence suggests that the autonomic nervous system plays an important role in AF initiation and sustenance. In 1997, Armour et al. (1997) highlighted the anatomical findings that concentrations of intrinsic cardiac autonomic nerves converge in epicardial fat pads, termed ganglionated plexi, innervating great vessels such as the pulmonary veins (203). Additionally, the pulmonary veins contribute to the majority of atrial ectopy in AF, and ablation of the pulmonary veins appears to be effective in terminating AF (102). Taken together, this suggests that autonomic regulation may be important in AF development. Several studies have since demonstrated favourable results with ablation of ganglionated fat pads (135,215,216), whereas others have shown increased vulnerability (217) and lack of long term suppression of AF induction in canine models (218). Similarly, clinical studies have demonstrated conflicting results (219-221).

Nonetheless while the contributions of the autonomic regulation to AF initiation and maintenance is debatable, changes in HRV, which acts as a non-invasive measure of cardiac autonomic function, have been implicated in AF. Changes in HRV have been reported in the last 5 mins prior to an episode of AF reflecting two types of AF initiation: 1) vagal tone whereby a decrease in low frequency power (LF) and increase in high frequency power (HF) is observed or 2) sympathetic tone with an increase in LF and decrease in HF (222,223). Vagal-

mediated AF tend to occur in patients with structurally normal hearts, whereas sympathetic tone plays a greater role in patients with structural heart disease (224). Furthermore, a decrease in RR interval complexity and altered fractal properties in RR interval dynamics, both independent measures of HRV, has been demonstrated in lone AF patients preceding spontaneous AF initiation (225). Interestingly, Tomita et al. (2003) demonstrated differences in HRV alterations before and after episodes of paroxysmal AF in patients suspected of vagal AF and sympathetic AF did not correlated with duration or termination time of AF (226). Lastly, it has been noted that in AF patients that have undergone cardioversion, presenting with a high LF/HF ratio is associated with a 2-fold risk of having AF recurrence (227). Notably, most of these studies either looked solely into lone AF, without structural heart disease, or did not state any structural heart disease in their subject population.

1.5.5. Summary

The complex interplay between vagal and sympathetic arms of the autonomic nervous system likely contributes AF initiation and maintenance. However, operative neural modulation as a therapy to terminate AF has remained inconclusive thus far. HRV as a surrogate indicator of autonomic regulation is a useful tool in risk stratification in a variety of cardiac diseases, as well as being implicated in AF development. Current evidence suggests a heightened sympathetic tone in patients with vagal AF, and vice versa, contributing to the initiation of AF (222,223) and its termination (226). However, the majority of studies have been concentrated on patients without underlying structural heart

disease. Further studies are warranted in patients who have underlying risk factors for AF in order to elucidate their contributions towards AF initiation and maintenance.

1.6. METABOLIC SYNDROME, OBESITY, DIABETES AND ITS ROLE IN AF DEVELOPMENT

1.6.1. Metabolic Syndrome

MetS consists of a combination of atherosclerotic risk factors that includes hypertension, obesity, insulin resistance, and dyslipidaemia, many of which are also independent risk factors for AF (72,228). Hypertension is one of the most prominent risk factors for AF (43), and confers an increased risk of developing AF by 1.5 and 1.4 times in males and females respectively (2). Although data from individual studies regarding the association of diabetes and AF have been conflicting, a meta-analysis by Huxley et al. (2011) estimates that patients with type 2 diabetes have a 40% increased risk of developing AF (45). Furthermore, it has been suggested that insulin resistance may be the critical factor in type 2 diabetes and hypertension responsible for AF development (229). Obesity is associated with a 49% increased risk of AF in the general population (61).

Taking these factors into consideration, it is very likely that metabolic syndrome is possibly related to AF development. Indeed, obesity and hypertension have been recognized as factors correlating to atrial stretch and dilatation (230,231). Patients with MetS demonstrate moderately increased atrial dimensions associated with increased risk of developing new-onset AF as compared to patients without MetS (232). Additionally, inflammation and oxidative stress has been associated with MetS and its individual components (233-235). Considering that inflammation has been implicated in atrial

remodelling through inflammatory infiltrates and fibrosis (90), it is reasonable to assume that these remodelling processes provide the necessary substrate that facilitates AF in the context of MetS. Furthermore, MetS is also independently associated with increased AF recurrence risk following coronary artery bypass graft surgery (236) and catheter ablation (237,238). Together, these suggest that despite successful arrhythmia cure with intervention, the underlying substrate promoting AF may continue to progress in the MetS population.

1.6.2. Association of Obesity to AF development

Adult obesity has risen to global epidemic proportions in the past few decades. Body mass index (BMI), a measure of human body shape, has been associated with increased risk of death, type 2 diabetes, cardiovascular morbidity and mortality (239,240). Similarly, AF incidence similarly has encountered a parallel rise, and is also a significant contributor to cardiovascular morbidity and mortality (2). A systematic review of the literature has since revealed an excess AF risk of 49% due to obesity in the general population that increases in parallel with the BMI (61). Obesity also exacerbates the progression of AF (55). Furthermore, obesity has been demonstrated as a modifiable risk factor where weight reduction and risk factor management reduces AF burden and improves cardiac structure (62).

Obesity is associated with ventricular diastolic dysfunction (51), pro-inflammatory state (52), cardiac autonomic dysfunction (53), and atrial size (54,55) – all of which are known to promote AF (56). Increased left atrial size appears to be a particularly strong predictor of AF. In the first prospective study associating

obesity and AF, Wang et al. (2004) demonstrated a 4-5% excess AF risk for each unit of BMI increase (231). Furthermore, the association remained significant following adjustments for cardiac co-morbidities including myocardial infarction, hypertension and diabetes, but not with adjustment for left atrial size. This suggests that AF risk in obese subjects is, at least in part, mediated through an enlarged atrial size, a similar finding observed in subjects with MetS (232,241). In the MONICA/KORA study, age, obesity, and hypertension independently predicted for left atrial enlargement; of which obesity being the most powerful predictor and the combination of hypertension and obesity demonstrating the largest atrial size (54).

Although the mechanisms associating obesity, left atria enlargement, and AF are not fully understood, it is possibly multifactorial involving a combination of hemodynamic dysfunction (51), autonomic dysfunction (53), inflammation (52) and activation of the renin-angiotensin-aldosterone system (188), all of which results in pathological atria structural and electrical remodelling and contributing to arrhythmogenesis.

1.6.3. Atrial Remodelling in Obesity

Pericardial adipose tissue depots have recently been implicated in AF development (63,242,243). Furthermore, pericardial fat is associated with AF presence, AF severity, left atrial volumes, and AF recurrence following ablation (63). Furthermore, total and interatrial epicardial fat are independently associated with left atrial structural remodelling in AF patients (244), and epicardial fat thickness

correlates to enlarged atrial dimensions and diastolic dysfunction in morbidly obese patients (245). Additionally, adipocytes secrete pro-inflammatory cytokines enhancing inflammation and oxidative stress in patients with obesity and MetS. Recent evidence suggests that adipocytes are capable of modulating electrophysiological characteristics and contribute to atrial arrhythmogenesis (246). In a study by Lin et al. (2012), this effect has been postulated to be in part due to secretary cytokines that prolongs action potential duration and changes the resting membrane potential which in turn decreases the depolarizing threshold and facilitates arrhythmia induction (246). Notably, incubation of atrial myocytes in this study with supernatant produced a lesser extent of changes, suggesting at least some direct effects of adipocyte on modulation of myocyte electrophysiological properties.

In rodent studies, mice and rat fed diets consisting of 45%-46% fat of total calories develop several cardiac effects including oxidative stress, inflammation, hypertrophy, fibrosis, apoptosis, contractile dysfunction, and cardiomyocyte intracellular Ca^{2+} defects (247-251). On the other hand, other studies report absence of overt cardiac remodelling and contractile dysfunction despite higher fat content diets (60%), indicating that specific lipid components of the diet (i.e. the composition of saturated and unsaturated fatty acids) are important in fatty acid metabolism and cardiac dysfunction was likely due to changes in fatty acid utilization pathways (252-254). Of note, these studies were conducted on ventricular tissue and the effects of obesity directly on the atria are less established.

In an ovine model of obesity, Abed et al. (2013) demonstrated that by using an ad libitum calorie-dense diet, progressive obesity was associated with atrial electro-structural remodelling (167). These changes included an increased atrial size, slowed and heterogeneous conduction as measured by epicardial electrophysiology studies, increased fibrosis, inflammatory cell infiltrate, and lipidosis that were associated with spontaneous and more persistent AF. This model in a following study has also demonstrated atrial structural and endocardial electro-anatomical remodelling (255), which can be partially reversed with weight loss (256).

1.6.4. Association of Diabetes with AF development

Diabetes mellitus (DM) and pre-diabetes continues to be a rising epidemic affecting humans worldwide with diabetes prevalence projected to reach 439 million worldwide by 2030, a 54% increase up from 2010 (257). DM is characterized by hyperglycaemia arising from defects in insulin secretion, insulin action, or both (258). There are 2 major classifications of DM, namely type I DM (T1DM; insulin-dependent) characterised by a lack of insulin production due to cellular-mediated autoimmune destruction of insulin-producing β-cells of the pancreas and type II DM (T2DM; insulin-resistant) characterised by insulin resistance and relative insulin deficiency. T2DM accounts for 90% or more of the total diabetic population (44). Nonetheless, T1DM is also on the rise and its incidence is projected to exceed 30 per 100 000 people worldwide annually. T2DM confers a 40% excess risk of developing AF (45). The possible mechanisms of how DM relates to AF remains to be fully elucidated, however several hypotheses

include: 1) insulin resistance, 2) inflammation, 3) left ventricular hypertrophy, and 4) atrial mechanical stress as possible underlying mechanisms.

A large community-based study in Sweden by Ostgren et al. (2004) found that patients with both DM and hypertension had 3-fold increased risk of developing AF than control patients (229). However, combined DM and hypertension was no longer significant for AF following adjustment for insulin resistance, suggesting insulin resistance to be a common underlying mechanism behind hypertension and DM. Inflammation is another possible mechanism that may influence AF development, as pro-inflammatory cytokines such as C-reactive protein and IL-6 have been shown to be similarly elevated in cohorts of patients with DM (259) as well as AF patients (260). In the Framingham study, Rutter et al. (2003) found that the LV mass and wall thickness was increased in patients with worsening glucose intolerance and insulin resistance (261). Left ventricular hypertrophy in itself is a major risk factor for AF (163) and AF risk was increased by 28% for every 4-mm increase in left ventricular thickness (2).

1.6.5. Atrial Remodelling in Diabetes

Abnormal glucose metabolism has been implicated in changes in the atrial substrate, and contributing to AF development and/or recurrence. Chao et al. (2010) demonstrated through bi-atrial electro-anatomical mapping that patients with DM or impaired fasting glucose demonstrated inter-atrial conduction delay, decreased voltage in both atria, and increased AF recurrence following catheter ablation (262). Inter- and intra-atrial electromechanical delay as measured with

tissue Doppler imaging was also identified in patients with impaired fasting glucose (263) and T2DM (264).

One serious, yet often overlooked, complication of DM is cardiovascular autonomic neuropathy (CAN) (265) which consists of damage to the autonomic nerve fibers innervating the heart and blood vessels, which can result in dysfunctional heart-rate control and impaired vascular dynamics (266). CAN has been associated with abnormalities in left ventricular systolic and diastolic dysfunction in patients with chronic T2DM (267) as well as T1DM (268). Recent studies suggest that CAN, in particular the sympathetic arm, has direct implications on heterogeneous electrophysiological properties in the atria. Olgin et al. (1998) demonstrated in dogs that heterogeneous sympathetic denervation of the atrial epicardium by phenol application in 3 distinct areas of the right atrium resulted in inducible sustained AF and increased dispersion of refractoriness and AF cycle length (269). On the other hand, Jayachandran et al. (2000) demonstrated that rapid pacing in dogs led to increased heterogeneity of sympathetic innervation that is spatially related to heterogeneity of atrial refractoriness as measured with AF cycle length (270). Furthermore, increased heterogeneity of atrial refractoriness was seen with sympathetic nerve stimulation in streptozotocin-induced diabetic rats, and parasympathetic nerve stimulation increased AF incidence (271). These studies suggest that the cardiovascular autonomic system plays a crucial role in regulation of atrial electrophysiology and neural remodelling can negatively affect atrial conduction, resulting in AF development. However, the chronic effects of CAN and atrial structural remodelling were not investigated and should not be disregarded.

Although the balance of evidence indicates that DM is likely an independent risk factor for AF, its arrhythmogenic substrates remain unclear. Recently, Watanabe et al. (2012) demonstrated greater atrial tachycardia vulnerability, increased conduction slowing and heterogeneity, prolonged and spatially dispersed action potential duration, and incidence of APD alternans in the right atrium of streptozotocin-induced diabetic rats (272). They also reported increased interstitial fibrosis and decreased expression of connexin40 in the right atrium of diabetic rats, suggesting atrial structural remodelling may be implicated in AF development in this rat model. In a genetic model of type II diabetes, Kato et al. (2008) reported that the left atrial appendage demonstrated increased atrial tachycardia vulnerability, longer intra-atrial conduction, increased fibrotic deposition but no change in refractoriness in the isolated heart. Furthermore, the atria of streptozotocin-induced diabetic rats demonstrates diffuse interstitial fibrosis with abundant expression of receptor for advance glycation end products (RAGE) and CTGF, which was prevented with the use of an inhibitor of advance glycation end products (AGEs) formation (273). This suggests that the AGEs-RAGE system mediates atrial structural remodelling in diabetic rats, at least in this particular model.

1.7. HYPERTROPHIC CARDIOMYOPATHY AS A CAUSE FOR AF

1.7.1. Hypertrophic Cardiomyopathy and its association with AF development

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiac disease affecting 1 in 500 of the general population (95). It is clinically characterized by a small left ventricular cavity and marked hypertrophy of the myocardium with myofibril disarray in the absence of any cardiac or systemic secondary causes of hypertrophy (274,275). Although initial studies in tertiary care centres associate HCM patients with a poor prognosis (276,277), subsequent studies in unselected populations have since suggested a generally benign course in most patients with an overall mortality rate of 1-2% per annum (96,278-283). Nonetheless, a significant proportion of HCM patients are subjected to several adverse outcomes including progression to heart failure, increased risk of strokes predominantly due to atrial fibrillation, and the most unpredictable risks for sudden cardiac death (95). AF contributes to a variety of adverse clinical profiles with a 3-fold increased risk of HCM-related deaths in HCM patients with AF compared to patients in sinus rhythm (284). AF is also a common disease variable (two-thirds of the HCM patients in a large population study) associated with progressive heart failure (285), proving to be the most important factor in heart failure evolution overall. The increase in HCM-related deaths in AF patients is believed to occur due to: 1) loss of atrial systole leading to deterioration of cardiac function, 2) rapid ventricular rate resulting in less filling and a resultant greater degree of outflow obstruction, and 3) stroke-related death (284).

The fundamental cause of HCM has been elucidated to lie predominantly with mutations in the sarcomeric proteins (275,286). Importantly, mutations within the beta-myosin heavy chain (β-MHC), cardiac myosin binding protein C (MyBP-C), cardiac troponin T (TnT) and the cardiac troponin I (TnI) sarcomeric proteins account for 60-70% of all cases of HCM (286-288). It should also be noted that clinical disease expression is variable between different mutations in sarcomeric proteins. For example, patients with β-MHC mutations tend to develop mild or severe HCM, whereas MyBP-C mutations develop late onset HCM, troponin-T mutations results in mild hypertrophy with risk of sudden death, and troponin-I mutations in patients tend not to develop sudden death in the absence of severe disease (289). The mechanisms by which mutations in the sarcomere cause left ventricular hypertrophy remain unresolved, although it has been suggested that myocardial hypertrophy acts in compensation to the sarcomeric dysfunction in HCM (286,290). This is supported by findings that functional abnormalities often precede development of cardiac hypertrophy and myocyte disarray in both human (291,292) and animal HCM (293). This hypothesis is further supported by the up-regulation of compensatory cardiac hypertrophy markers including insulin-like growth factor-I(294), atrial natriuretic peptide (295), endothelin-I (296), and brain natriuretic peptide (297). Furthermore, the possible reversal of interstitial fibrosis and/or hypertrophy by angiotensin II blockade (298), and statins (299,300) substantiates a “secondary” nature of the histological and morphological phenotypes in HCM. Noticeably, there is a current lack of literature investigating the prospective and retrospective cardiac remodelling process and their correlation to arrhythmogenesis in HCM.

Despite the advances in the treatment and management of HCM, current therapies of affected individuals remain suboptimal. This is due to the heterogeneous clinical course of HCM varying between benign asymptomatic forms to most dramatically, sudden cardiac death (SCD). Despite the substantial literature regarding the pathophysiology and natural history of HCM (95), the mechanistic linkage between the genetic basis of developing HCM and its heterogeneous manifestation remains poorly understood.

1.7.2. Atrial Remodelling in Hypertrophic Cardiomyopathy

Recent meta-analyses by Guttmann and colleagues revealed atrial fibrillation to be present in 22% of HCM patients, with an annual AF incidence of 3 per 100 patients (100). This suggests a 4- to 6-fold increased likelihood of AF in HCM patients compared to the general population (43,101). Guttmann et al. (2013) also identified left atria size and age to be significant predictors for AF development and stroke in the majority of studies (100). Age is a well-established risk factor for AF development (16), Olivotto et al. (2001) however demonstrated in a community-based HCM population, that unlike the general population, more than a third of this population developed AF prior to 50 years of age (284).

One proposed mechanism behind the increased AF prevalence in HCM lies in diastolic dysfunction and/or mitral regurgitation that increases left atria pressure leading to chronic dilatation and increased size, which in turn influences left atria electrical properties (301,302). However, Olivotto et al. (2001) showed that the left atria size, but not left ventricular thickness nor outflow obstruction,

independently predicted AF development in their cohort (284). Losi et al. (2004) suggested that a decreased LA function, a measure of LA dysfunction and dilatation as determined by M-mode echocardiography to be a predictor of AF (303). Additionally, Maron et al. (2014) also recently demonstrated left atrial structural and functional changes in decreased left atria ejection fraction and increased left atria end diastolic volume, without significant changes in left ventricular outflow tract gradient and wall thickness, by cardiac magnetic resonance associating AF occurrence with HCM (304). These factors also reliably identified a small subset of 41 HCM patients at risk for future AF development upon prospective follow-up. These results suggest that atrial dysfunction, irrespective but not excluding ventricular dysfunction, is directly related to AF susceptibility.

An alternative hypothesis yet to be determined is the coexistence of atrial myopathy, which may determine LA dysfunction and the development of AF due to fragmentation of atrial conduction; this hypothesis is indirectly supported by observations that individuals with a specific Arg633His β-MHC mutation, having less cardiac hypertrophy as compared to other β-MHC mutations, have a higher risk of developing AF (305). Furthermore, atrial myopathy may provide the explanation for AF development in the absence of LA dilatation in a minority of patients (284). To date, there is no clear evidence of atrial myopathy in HCM. However, left atrial myopathy has been previously suggested in patients with idiopathic dilated cardiomyopathy who developed left atrial systolic dysfunction that could not be explained by LA dilatation or tension at the end of atrial systole (306).

Increasing emphasis has been placed in recent years on establishing animal models replicating phenotypes of human HCM due to two main benefits, 1) full access to tissues, cells and invasive techniques that would otherwise be restricted in human studies, and 2) carefully controlled animal model variables to separate concomitant factors in the clinical setting. Most transgenic animal models to date have replicated a combination or all of human HCM phenotype consisting of myocardial fibrosis, hypertrophy, and myofibrillar disarray, and left ventricular dysfunction in mice (307-309), rats (310,311), rabbits (300,312), and cats (313). Notably, most if not all of these histological findings were determined in ventricular tissue, and not in the atria. Furthermore, involvement of the atria in cardiac remodelling is less investigated, with only a few studies noting atria enlargement in transgenic models (314-316). Only one study to date has demonstrated cardiomyocyte disarray and sarcomere dysgenesis in the atria of a mouse model of MyBP-C mutation (317), although in this study they did not observe significant heart enlargement nor myocardium wall thickening presumably due to the relatively young age at time of experimentation mirroring the clinical picture in HCM patients with this mutation.

1.8. SUMMARY AND DIRECTIONS

It is now well-established that AF involves a highly complex interplay of triggers, substrate and modulating factors influencing arrhythmia perpetuation. Given the rising prevalence of AF with age, the number of people affected by AF is expected to rise significantly. The lack of highly effective therapies further complicates the AF epidemic. The major reason for this lies in our incomplete understanding of the complex pathophysiology of the arrhythmia, especially in the context of differing cardiovascular morbidities.

In diabetes, obesity, and metabolic syndrome, the majority of animals studies conducted thus far have concentrated on monogenic causes of the pathophysiology phenotype such as the db/db mice, ob/ob mice, Zucker Lean and Fatty rats. Whilst these studies have clearly demonstrated direct abnormalities in cardiac structure and electrophysiology, they often exhibit extreme disease phenotypes not commonly observed in humans. Therefore, these models do not adequately represent human conditions where a polygenetic nature arising from dietary, environmental factors and life-style habits likely contribute to the disease. Recent studies have established new recombinant congenic models of diabetes and obesity that more accurately reflect the human phenotypes (318-320). It will be interesting to examine the effects of diabetes and obesity in such models and its corresponding effects on atrial electrical, structural and autonomic remodelling and their corresponding impact on AF.

In HCM, it is well established that monogenetic mutations in sarcomeric proteins are the main cause of left ventricle hypertrophy. Additionally, compounding mutations in multiple genes can also be the cause of HCM and often results in a more severe phenotype (316,321). However, there is a lack of understanding in atrial response in this disorder and if intrinsic atrial myopathy co-exist that may explain the increase AF incidence in HCM.

Finally, the possibility of reverse remodelling of the atria as a therapeutic option to treat AF remains to be investigated in these models, and would be interesting to determine if pharmacological agents capable of reversing atrial remodelling are translatable to human studies to treat AF.

Statement of Authorship

Title of Paper	Atrial Electrophysiological and Structural Remodelling in a Murine Model of Hypertrophic Cardiomyopathy from Troponin-I Mutations: Implications for Atrial Fibrillation
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Principal Author

Name of Principal Author (Candidate)	Wei-Wen Lim
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article
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 7/10/2015

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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Wei Wen Lim

From: Anand Ganesan
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Dear Wei Wen,
I am in the USA and do not have access to printer or Adobe PDF writer.

I do approve the manuscripts. Please take this email as affirmation of my approval for the purpose of the form.

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ANAND GANESAN MBBS (Hons.) PhD FRACP CEPS CCDS

Michel Mirowski Fellow, Heart Rhythm Society
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2) Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabesity
3) Atrial Electrophysiological and Structural Remodelling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes
4) Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy

Also included is the 'Statement of Authorship and Location of Data' form for the manuscript "Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy" accepted in Clinical and Experimental Pharmacology and Physiology.

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Kind Regards,
Wen

WEI WEN LIM, BSc(Hons)
PhD candidate

Centre for Heart Rhythm Disorders | University of Adelaide

Chapter Two

Electrophysiological and Structural Remodelling of the Atria in Hypertrophic Cardiomyopathy: Implications for Atrial Fibrillation

2.1. INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiac disease affecting 1 in 500 of the general population (95). Although frequently undiagnosed, HCM is also a global disorder across all geographical regions, regardless of race groups and gender differences (322). HCM is associated with significant adverse outcomes such as risks of developing sudden cardiac death (1-2% per annum) often in young patients (96,278,279,281,283,323,324), whereas progressive heart failure and increased risk of strokes develops in the older cohort (95). Atrial fibrillation (AF) is the most common rhythm disorder affecting patients with HCM and confers an added risk of developing heart failure, death and stroke (284). A recent meta-analysis revealed the overall prevalence of AF to be 22.5% in HCM patients (325), suggesting a 4 to 6-fold increased likelihood of AF in HCM patients compared to the general population (43,326). Increased left atrial (LA) size and age are major predictors for AF and stroke development (325). In a large community-based HCM population assembled from Italy and the US, Olivotto et al. (2001) demonstrated that AF prevalence increased with age, but was not rare in younger patients (36% developed AF at ≤50 years of age) (284). Similarly, a recent

large single-centre study by Siontis et al. (2014) demonstrated that AF was more prevalent in older HCM patients and was associated with worse survival compared to AF-free HCM patients (327). Interestingly in this study, AF was associated with larger left atria, larger wall thickness and worse cardiopulmonary exercise tolerance; however, AF was less common in HCM patients with the obstructive phenotype. With the economic burden posed by AF being on the rise (14,328), there is a need for better targeted therapies of AF particularly in the HCM cohort where AF incidence occur at a younger age compared to the general population with greater mortality risks.

Thromboembolism prophylaxis with oral anticoagulation is recommended for all HCM patients with paroxysmal, persistent or permanent AF regardless of the CHA2DS2-VASc score due to the high stroke risk despite the younger age of AF presentation in HCM patients (329,330). Treatment of AF in HCM patients in accordance to European Society of Cardiology and American Heart Association guidelines involves rate control therapies with the use of beta blockers and calcium channel antagonists, or the use of rhythm control strategies such as antiarrhythmic agents and radiofrequency ablation (329,330). However, it should be noted that randomized controlled trials with rhythm control therapies in HCM patients with AF are currently lacking. The limited published data on the use of amiodarone in HCM patients suggests safety and efficacy in maintaining sinus rhythm (331), however no significant benefit was observed for sinus rhythm maintenance over treatment with beta blockers and calcium channel blockers (96,284). Long-term benefits of radiofrequency ablation in HCM patients with drug-resistant AF are also unclear, however early success rates (50-60%) in HCM

patients have been comparable to that of other heart diseases (332-335), with left atria size and older age being the major reasons for failure of AF suppression (332,333).

Underlying atrial myopathy creates the substrate for AF development (336). However, the evaluation of the atrial substrate in HCM that predispose to the development of AF remains uncertain. Better delineation of the progression of the atrial substrate in HCM may allow for the early prevention of atrial myopathy and AF development. The aim of the current study was to characterise the atrial substrates that predispose to AF in HCM. In particular, we utilised an established Gly203Ser cardiac troponin-I transgenic male mouse that has been previously shown to develop characteristic features of human HCM by 21 weeks of age (337), to characterise the atrial structural and electrophysiological abnormalities and blood biomarkers alterations due to HCM. In addition, given the change in the occurrence of AF with age, we also evaluated the impact of age on the HCM atria.

2.2. METHODS

2.2.1. Animal Model

HCM was characterised in the Gly203Ser cardiac troponin-I transgenic mouse model as previously described (337). The mouse model was bred on the C57BL/6 genetic background and has been shown to have a normal life span. Transgenic (HCM) mice with the mutation develop phenotypic hallmarks of HCM by 21 weeks of age. A total of 12 HCM animals were studied in 2 groups at age 30 and 50 weeks of age. As a control group, non-transgenic C57BL/6 (control) mice were used. Again these have been previously demonstrated to be indistinguishable from transgenic wild-type overexpression mice (337). A total of 12 control animals were studied in 2 groups at age 30 and 50 weeks of age.

HCM and control mice were bred by crossing a HCM female with a wild-type male mouse, and were tail genotyped at 2-3 weeks of age for identification. Mice were then allocated to the two groups. Mice were housed at controlled temperature (24° C) and lighting (12-hour light-dark cycles) with free access to standard chow and water ad libitum. All experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2.2. Heart Rate and Blood Pressure

Non-invasive tail-cuff systolic blood pressures and heart rates of mice were measured using the NIBP controller (AD Instruments, Australia) under anaesthesia

with intraperitoneal injection (75mg/kg Ketamine (100mg/ml) and 0.5mg/kg Medetomidine (1mg/ml)) immediately prior to sacrifice. Depth of sedation was checked periodically for paw- and tail-pinch reflexes. Reported values are derived from a mean of 3 consecutive readings.

2.2.3. Electrophysiology Study (Multi-Electrode Array)

At the respective endpoints for sacrifice (30 or 50 weeks of age) and following anaesthesia, a single intraperitoneal injection of heparin (2-IU/g body weight) was administered prior to blood sampling. A midline thoracotomy incision was performed and the heart promptly removed and rinsed with ice-chilled bicarbonate buffer solution. The left atrium was carefully dissected from the heart and the epicardial surface placed onto the multi-electrode array (MEA) housed within a flow chamber irrigated with bicarbonate buffered solution (in mM: 130 NaCl, 4 KCl, 0.6 MgCl, 24 $NaHCO_3$, 1.2 NaH_2PO_4, 12 D-glucose, 1.5 $CaCl_2$) maintained at 37 ^oC and pH 7.4 when aerated with 95% oxygen and 5% carbon dioxide, as previously described (338). The MEA was custom made, consisting of 6 x 6 electrodes of 0.1 mm diameter and 0.5 mm inter-electrode distance yielding a total of 25 bipolar electrograms. Electrograms from the MEA digitally sampled at a rate of 2 KHz and filtered from 10-500 Hz. These were recorded to commercially available computerized recording system (LabSystem Pro, Bard Electrophysiology, USA). This system allows off-line analysis using digital callipers at a sweep speed of 200 mm/s. Stimulation was performed a commercially available cardiac stimulator (Micropace EPS cardiac stimulator, Micropace Pty Ltd, Australia).

For consistency, the placement of the atrial tissue was oriented in the same cranial-caudal and medial-lateral orientation with the epicardial surface in contact with the electrodes. A nylon weighting harp was placed over the tissue to improve contact with underlying electrodes. Stimulation of the atrial tissue was performed from two corners of the plaque (Stimulation site 1: inferior LA appendage (LAA), site 2: LA free wall (LAFW)) at twice the pacing capture threshold with a pulse width of 0.5 ms (see Figure 1).

2.2.4. Atrial Refractoriness

Refractoriness of the tissue was evaluated from the two corners of the plaque as outlined previously using an eight beat (S1) stimuli drive train followed by a premature (S2) stimulus delivered in 10 ms decrements from an initial coupling interval of 100 ms. Effective refractory period (ERP) was defined as the longest S_1-S_2 interval failing to propagate an electrical impulse. This was repeated at 4 cycle-lengths (100, 200, 300, and 400 ms). ERP measurements were conducted thrice during each CL and averaged. Induced arrhythmia was assessed during ERP testing and characterised as self-propagating atrial arrhythmias lasting more than 2 seconds.

2.2.5. Conduction Analysis

Conduction was assessed during constant capture at each CL using the local activation time maps. Activation maps were generated offline using semi-automated custom designed software to determine mean conduction velocity (CV) and conduction heterogeneity index (CHI). The impact of premature extra-stimuli

was also evaluated to determine the effects of functional conduction abnormalities and performed by evaluating the shortest coupled extra-stimulus that resulted in a propagated response. Each annotation was manually verified by annotating the local activation time to the maximum deviation of the largest amplitude from the baseline on bipolar electrograms. CV was calculated from local vectors within each triangle of electrodes (85), while CHI was assessed using established phase-mapping techniques (118). In brief, the phase distribution was obtained by calculating the largest difference in activation between every 4 adjacent electrodes. Absolute conduction phase delay was established through subtracting the 5^{th} from 95^{th} percentile of the phase distribution (P_{5-95}), which was then divided by the median (P_{50}) to derive the CHI. Total activation time (TAT) was defined as the longest conduction time in a given annotated map.

2.2.6. Intracellular Action Potential Recording

To perform simultaneous intracellular action potential recordings during electrophysiology studies, an aluminosilicate glass electrode filled with 3M KCl (100MΩ) was inserted in the atrium endocardium and intracellular membrane potential recordings made with a high input impedance amplifier (WPI model 725) (339). Action potentials were digitised at 10 kHz, 16-bit resolution and recorded and analysed with Chart 5 (AD Instruments, NSW, Australia). Signal ground was via a silver/silver chloride wire in the bath (MEA electrodes are allowed to float relative to this ground). Action potentials were recorded from opposing two regions (Recording region 1, region 2) of the MEA from the two stimulation sites during atrial pacing (see Figure 1).

2.2.7. Structural Analysis

The left atrial tissue was fixed in 10% formalin before being wax embedded following standard routine procedures. Transverse sections (6μm) were cut (Leica RM2235 Rotary Microtome), mounted on to albumin coated slides and stained with Masson's Trichrome to determine the extent of fibrosis (blue staining), and presence of inflammatory infiltrates with H&E straining. Histological slides were scanned at 40x magnification using the NanoZoomer Digital Pathology System (Hamamatsu Photonics, Hamamatsu, Japan). Five fields at 400x magnification were randomly selected per atrium, and images were exported and myocyte cross-sectional diameter (100 cells in total per atrium) were independently analysed by a blinded observer using the ruler function on NDP view 2 (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with Adobe Photoshop CC ver. 14.1.2 (Adobe Systems, CA, USA) in accordance to published protocols (340), before the pixel content of staining for each atrium was measured relative to the total tissue area using ImageJ (ver. 1.7.0; NIH, USA) with a batch macro following background subtraction.

2.2.8. Multiplex Enzyme-linked Immunoassay

Blood samples were collected immediately before heart excision in 4 mL EDTA tubes with 1 mL heparinised syringes with a 21G needle via cardiac puncture in the left ventricle. Tubes were then centrifuged for 10 minutes at 1,500 g at 4 °C refrigerated centrifuge and the plasma was transferred using a Pasteur pipette into 1.5 mL microcentrifuge tubes and stored at -20 °C. To explore the potential

mechanisms by which HCM leads to the substrate for AF, plasma samples were analysed for peptide levels of matrix metalloproteinase (MMP)-2 and -3, pro-MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), transforming growth factor beta-1 (TGFβ1), tumour necrosis factor alpha (TNFα), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) using multiplex sandwich ELISA arrays (Custom Quantibody Array, Raybiotech, GA, USA) according to manufacturer's instructions.

2.2.9. Statistical Analysis

All data are presented as means ± standard error of means (SEM) unless indicated otherwise. Two-way ANOVA was conducted to determine differences of age and HCM mutation contributions on animal characteristics using GraphPad Prism 6 (GraphPad Software Inc., USA). Two-tailed student *t* test was used as appropriate to identify for strain differences when ANOVA was significant. General linear model analysis of variance (GLM ANOVA) was used to determine significant differences of age and HCM mutation contributions on electrophysiological and histological parameters as appropriate using PASW Statistics 18 (IBM Corp., USA). Statistical significance was established at $P < 0.05$.

2.3. RESULTS

2.3.1. Animal Characteristics

Table 1 presents the detailed animal characteristics of both groups. There were no significant differences in body weight, heart rates and blood pressures between HCM and control mice at both age groups, although significantly lower blood pressures were noted in old mice as compared to young mice (85.8 ± 0.6 vs. 94.9 ± 0.4 mmHg; $P < 0.001$).

Young HCM animals demonstrated a greater left ventricular and septal wall thickness compared to controls (both $P < 0.0001$) along with significant bi-atrial hypertrophy evident by the increased of left and right atrial mass ($P < 0.01$ and $P < 0.05$ respectively) (Table 1). Similarly, old HCM animals demonstrated ventricular and bi-atrial hypertrophy compared to controls. Increased age was not a significant factor for any parameter for cardiac hypertrophy in HCM mice.

2.3.2. Atrial Action Potential Duration and Effective Refractory Period

Atrial APD20, 50 and 90 were significantly reduced in young HCM mice compared to controls (APD20: 4.74 ± 0.07 vs. 8.93 ± 0.25 ms, APD50: 12.19 ± 0.32 vs. 19.94 ± 0.90, APD90: 33.09 ± 1.5 vs. 49.31 ± 2.75 respectively; all $P < 0.001$; Figure 2A). Looking at rate adaptations changes in APD20, 50 and 90, the decrease in APD20, 50 and 90 were uniformly reduced in the young HCM mice compared to controls across all pacing cycle lengths tested (Figure 2B-D). Similarly, older HCM animals demonstrated reduced APD20, 50, and 90 as compared to controls (Figure 2E-H).

In contrast, we observed that changes in atrial ERPs were not similar to APD results (Figure 3). Atrial ERP in the young HCM mice was not different from controls across all pacing intervals (51 ± 6 vs. 52 ± 3 ms respectively at pacing interval of 200 ms; P = NS). At 50 weeks of age, HCM mice demonstrated significant prolongation of ERP at across all pacing intervals (78 ± 4 vs. 57 ± 2 ms respectively at pacing interval of 200 ms; $P < 0.001$). Additionally, a significant decrease in ERP was observed at faster pacing rates (400 vs. 100 ms pacing) in both HCM and control mice (HCM: 84 ± 5 vs. 71 ± 4 ms respectively; Control: 64 ± 3 vs. 54 ± 2 ms respectively; both $P < 0.01$).

2.3.3. Atrial Conduction

Figure 4 shows the activation time maps and corresponding phase histograms from representative LA of HCM and control mice during S1 pacing at 200 ms. Young HCM mice demonstrate unchanged conduction velocity (0.323 ± 0.023 vs. 0.341 ± 0.014 m/s respectively at 200 ms pacing; P = NS) but increased conduction heterogeneity (2.29 ± 0.27 vs. 1.88 ± 0.17 respectively at 200 ms pacing; $P = 0.001$) across all pacing cycle lengths as compared to age-matched controls. In the older cohort, HCM animals demonstrated a reduction in conduction velocity (0.251 ± 0.012 vs. 0.353 ± 0.021 m/s respectively at 200 ms pacing; $P = 0.001$) and also greater conduction heterogeneity index (2.21 ± 0.10 vs. 1.76 ± 0.10 respectively at 200 ms pacing; $P = 0.001$) across all pacing cycle lengths compared to controls (Figure 5).

2.3.4. Arrhythmia Induction

There were no incidence of self-propagating atrial arrhythmias lasting more than 2 seconds observed during the standard extra-stimulus pacing protocol in the current study.

2.3.5. Atrial Structural Remodelling

Representative images of transverse sections of the left atrium from (A) young and (B) old mice stained with H&E and Masson's Trichrome are illustrated in Figure 6. HCM mice atria demonstrates increased cardiomyocyte hypertrophy (Figure 6C, left), increased interstitial fibrosis (Figure 6C, middle), and increased inflammatory cell infiltration (Figure 6C, right) as compared to controls. Additionally, no statistical difference was observed between older mice compared to young mice for any of the atrial structural parameters assessed.

2.3.6. Extracellular Matrix Remodelling

To investigate biomarkers of extracellular matrix remodelling that may explain the elevated atrial fibrosis in the HCM atria, we assessed the plasma concentrations of MMP-2, MMP-3, pro MMP-9, TIMP-1, and TGFβ1 in HCM and control mice at both 30 and 50 weeks of age (Figure 7). No significant changes in plasma levels of MMP-2, -3, pro MMP-9, TIMP-1 and TGFβ1 were observed in the young HCM mice as compared to controls. In contrast, significant elevations in circulating MMP-2 and MMP-3 were observed in old HCM mice compared to age-matched controls (MMP-2: 208.0 ± 41.5 vs. 128.2 ± 7.4 ng/mL, MMP-3: 5.5 ± 0.7 vs. 2.0 ± 0.4 ng/mL; $P <$

0.05 and $P < 0.01$ respectively). Pro MMP-9 and TIMP-1 and TGFβ1 levels were not statistically altered in the old HCM mice as compared to controls.

2.3.7. Changes in Cellular Adhesion and Inflammation

To investigate the increased inflammatory cell infiltration in the HCM atria, we assessed plasma concentrations of TNFα, ICAM-1, and VCAM-1 in both HCM and control mice at both ages (Figure 8). Young HCM mice demonstrated unchanged concentrations of TNFα, ICAM-1, and VCAM-1 compared to controls. In contrast, plasma levels of VCAM-1 were elevated in the old HCM mice as compared to controls (13.9 ± 0.8 vs. 11.8 ± 0.3 ng/mL; $P < 0.05$), whereas concentrations of TNFα and ICAM-1 were not different to controls.

2.4. DISCUSSION

Using an established model of hypertrophic cardiomyopathy, this study characterises the structural and electrophysiological remodelling of the atria that potentially predisposes to the development of AF. This study presents the following new information regarding the atrial remodelling in HCM compared to controls:

1. Both atria were enlarged and demonstrate an increase in atrial myocardial mass
2. Marked structural abnormalities with myocyte hypertrophy, cellular infiltrates and interstitial fibrosis
3. Electrophysiological abnormalities within the atria were observed from a young age with abbreviation of the action potential duration, and an increase in conduction heterogeneity. However, abnormalities in refractoriness and conduction velocity were only observed with increasing age.
4. Older HCM mice demonstrated increased circulating levels of MMP-2, MMP-3, and VCAM-1, which were not changed in the younger cohort.

These observations demonstrate that HCM is associated with an abnormal atrial substrate that is further potentiated with increasing age. This milieu of abnormalities may in part result in the increased burden of AF in patients with HCM.

2.4.1. Substrate predisposing to AF

Rapid atrial activation, either through artificial AF maintenance (130) or rapid atrial pacing (129), has been demonstrated to induce electrical alterations such as decreased atrial refractoriness, slowed and heterogeneous conduction thereby promoting AF, namely termed 'AF begets AF'. Whilst this may explain the progression of paroxysmal to permanent AF, it does not explain for the substrate leading to AF development. Li et al. (1999) previously demonstrated that experimental congestive heart failure in dogs modifies atrial substrates by promoting atrial interstitial fibrosis, resulting in slowed and heterogeneous conduction and thereby increasing inducibility and duration of AF (29). These findings have also been demonstrated in humans with congestive heart failure (341). Localized conduction abnormalities have also been observed implicating AF in the setting of atrial ischemia (342,343), obesity (167,344), obstructive sleep apnoea (345), hypertension (119,346), mitral valve fibrosis (347), cardiomyopathy (161,348), and mitral regurgitation (162). Likewise, slower and heterogeneous atrial conduction associated with increased age was observed in the current model of HCM. However, in contrast to the notion of 'AF begets AF' where atrial refractoriness is known to be progressively reduced to drive the stability of AF (130), atrial refractoriness has been variably reported to be either decreased (342), unchanged (167,344,345) or increased (119,346,348) depending on the underlying heart disorder. These contrasting results may perhaps suggest that atrial refractoriness is less likely to represent a defined component of the substrate for AF in all types of heart disorders.

2.4.2. Atrial Remodelling in HCM

Most transgenic mouse models to date have replicated a combination or all of the human HCM phenotype, mainly consisting of ventricular myocardial fibrosis, hypertrophy, and myofibrillar disarray, and left ventricular dysfunction (314,337,349-351). Atrial enlargement has occasionally been observed in some HCM mice models (349,350) and not others (351); however the characterisation of electrophysiological and structural substrates have not been investigated. We observed significant atrial and myocyte hypertrophy in troponin I mutant HCM mice in the current study. Atrial enlargement is a common structural trait in experimental models of CHF (29), mitral valve disease (162,347), and ventricular cardiomyopathy (161). Additionally, LA size and volume are important risk factors for AF development (163,352). However, we did not observe any inducible AF despite achieving significant atrial enlargement with slowed and heterogeneous atrial conduction in this mouse model of HCM. This may be due to a lack of an appropriate trigger to perpetuate AF in the presence of vulnerable atrial substrate. Alternatively, increased refractoriness in the older HCM mice may counteract the slower conduction by normalizing the electrical impulse wavelength that accounts for re-entrant activity (353). Nonetheless, our observations of increased refractoriness in the older HCM mice are in agreement with human studies demonstrating age-associated increased refractoriness (19). Our observations of reduced atrial APD recorded from the atrial endocardium in HCM mice may also reflect spatial and transmural heterogeneity in both APD and ERP in the atrium. Transmural repolarization differences in APD have been previously demonstrated in mouse ventricular tissue (354) and may be replicated in atrial myocardium.

Fibrosis is a fundamental hallmark of atrial structural remodelling contributing to a sustained substrate for AF progression. Local conduction abnormalities have been observed in relation to fibrotic tissue infiltrates in atrial tissue preparations (165), in LA appendage tissue of patients with rheumatic mitral stenosis (166), animal models of hypertension (338,355), obesity (167) and heart failure (29,356). Similarly, interstitial fibrosis and inflammatory infiltration are key histological findings in the current study. A major electrophysiological change in the aforementioned animal models consists of an increase in spatial heterogeneity in atrial conduction velocity which may be explained by the development of regional fibrosis resulting in the electrical uncoupling of adjacent myocytes. Myocardial electrical impulse propagation is governed by low-resistance connexin channels located in the gap junction of adjacent myocytes, which can be disrupted by fibrosis interrupting inter-myocyte coupling. As a result, electrical propagation is reduced, leading to focal areas of slowed conduction and block, contributing to development of AF substrate (113).

Alterations in plasma matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), key proteins involved in the homeostasis of collagen turnover and resultant fibrosis, have been demonstrated in adverse structural and functional manifestations of ventricular remodelling in the setting of hypertension (357), myocardial infarction (358), and HCM (359-361). Increased circulating TIMP-1 and MMP-2 have been observed in HCM patients with systolic dysfunction (359), elevated plasma MMP-2, MMP-9 and NT-proBNP have been

observed in HCM patients (360), whereas MMP-2 correlates with reduced LV systolic function and MMP-9 associated with small LV size and degree of LV hypertrophy in HCM patients (361). Additionally, the effect of aging changes the balance of plasma MMPs and TIMPs with one study demonstrating elevated MMP-2, -7, TIMP-1, -2 and -4, with decreased MMP-9 with increasing subject age (362). In atrial biopsies, downregulation of TIMP-2 and upregulation of MMP-2 correlates with development of sustained AF in patients with cardiomyopathy and heart failure (363). Furthermore, plasma levels of MMP-9, MMP-3, and TIMP-4 independently predicted AF recurrence in post-cardioversion patients (364). In the current study, we observed elevations in plasma MMP-2 and -3 in old HCM mice although pro-MMP-9 and TIMP-1 levels were unchanged. In contrast, plasma levels of TGFβ1 in HCM mice were not statistically altered. This may have been due to high variability in the severity of disease progression in these mice. In our study model of moderate HCM, increased age differentially modulates biomarkers of extracellular matrix remodelling in the pathogenesis of HCM possibly reflecting the severity and/or chronicity of the disorder.

Inflammation has been found to contribute to certain types of AF such as post-cardiac surgery (87) and pericarditis (88). Inflammatory infiltrates have been observed in atrial endothelium in AF patients and animal models (162,365), and inflammatory biomarkers are elevated in AF patients and associated with progression of AF (366,367). Our results demonstrate a progressive increase in inflammatory infiltration in the atrial myocardium in older mice with HCM. Cardiac mast cells have been suggested to contribute to fibrosis leading to AF (368) and is

associated with the pathogenesis of dilated cardiomyopathy, cardiac hypertrophy and heart failure (369-372). Furthermore, TNFα is released from cardiac mast cells, initiating the cytokine cascade leading to myocyte ICAM-1 induction (373). Schultz et al. (2014) have previously demonstrated that circulating levels of ICAM-1 were significantly greater in AF patients compared to subjects with supraventricular tachycardia and non-AF controls (374). ICAM-1 levels in our study may therefore be unchanged as our model did not demonstrate inducible AF. We also found increased inflammatory infiltration without significant changes in circulating TNFα and ICAM-1 levels; however, we did not study their expression in the atrial myocardium. In contrast, the increase in circulating VCAM-1 levels, an endothelial promoter of monocyte and lymphocyte recruitment and a marker of inflammation (375,376), was observed in older mice with HCM.

2.4.3. Clinical Implications for AF

Evidence suggest that atrial myopathy plays a crucial role in providing a substrate that predisposes AF development (336). However, the delineation of the pathophysiological traits in the diseased atria in the setting of HCM has not been adequately characterized although atrial size, fibrosis and inflammation are likely perpetuators for AF development. Catheter ablation therapies have been proposed as possible first-line treatment of AF over the use of rate control strategies or anti-arrhythmic medication for rhythm control by treating the primary causes of fibrillatory conduction (112). In light of recent evidence from the DECAAF study suggesting that left atrial fibrosis is associated with arrhythmia recurrence (114), catheter ablation strategies may not be effective given the fibrotic, inflamed and

hypertrophied atria in HCM. This may also explain the reason behind early success rates of catheter ablation in HCM patients to be around 50-60% (329,330), that is considerably lower than the 80-90% success of catheter ablation in AF patients of unspecified comorbidities (5). Nonetheless, large randomised studies of catheter ablation in HCM patients with AF are currently lacking and are warranted. Alternatively, the use of pharmacological agents of renin-angiotensin-aldosterone blockade (377,378), antioxidants (379), statins (300) and calcium channel antagonists (380) have shown promise in reversal or prevention of ventricular cardiomyopathy in transgenic models of human HCM although their effects on the atria remains to be studied.

2.4.4. Study Limitations

This study aimed to characterise the atrial substrate associated with HCM predisposing to AF development. A detailed examination of the temporal course of atrial remodelling in parallel to the severity and rate of ventricular pathology in HCM that may help delineate the cause of atrial myopathy was not undertaken. More robust analysis of molecular remodelling using mRNA profiling may be possible with these models in future studies. The pulmonary veins that are a known source of ectopic beats that initiate AF (381) were excluded from the left atrial preparation in the current study. Notably, none of the animals evaluated had incidence of AF. In addition, the initiation and maintenance of AF is recognised to be related to the complex interplay between triggers, initiators and the substrate (382). This study has only provided information on the potential substrate for AF. We have studied only one of the transgenic models of HCM, and the atrial

substrates presented here may not necessary replicate all other sarcomeric protein mutations.

2.5. CONCLUSIONS

To conclude, mice with established HCM demonstrate key pathological findings of atrial myopathy with overt structural and electrophysiological abnormalities in the atrial tissue. Additionally, increased age resulted in significant ERP prolongation, slower conduction velocity, and elevations in some biomarkers of extracellular matrix remodelling and inflammation. These changes may potentially explain the age-related predisposition of HCM patients to the development of AF. Modulation of the atrial electrophysiological and structural changes may therefore represent new therapeutic approaches in the early treatment and prevention of AF in HCM.

TABLE 1. ANIMAL CHARACTERISTICS AND PATHOLOGY ASSESSMENT

	30 weeks of age		50 weeks of age		P-value (Strain)*	P-value (Age)‡
	Controls	**HCM**	**Controls**	**HCM**		
Body Weight (g)	31.3 ± 1.2	30.3 ± 1.0	30.3 ± 0.3	31.3 ± 0.5	0.99	0.99
Heart Rate (bpm)	295.5 ± 14.9	254.9 ± 20.4	268.7 ± 7.3	273.83 ± 12.4	0.2	0.8
Blood Pressure (mmHg)	94.5 ± 2.8	95.3 ± 1.5	86.4 ± 1.6‡	85.2 ± 1.7‡	0.7	**0.0005**
LA weight (mg)	3.9 ± 0.4	6.8 ± 0. 9*	3.7 ± 0.1	8.0 ± 2.0*	**0.0072**	0.6
RA weight (mg)	4.1 ± 0.4	5.3 ± 0.6*	3.6 ± 0.3	4.4 ± 0.5*	**0.0116**	0.07
LV wall thickness (mm)	1.2 ± 0.1	1.5 ± 0.1*	1.2 ± 0.1	1.7 ± 0.1*	**<0.0001**	0.3
Septal wall thickness (mm)	1.0 ± 0.1	1.3 ± 0.1*	0.9 ± 0.1	1.4 ± 0.1*	**<0.0001**	0.4

* $P < 0.05$ for strain on two-tailed student *t* test

FIGURE LEGENDS

Figure 1 (Left) Schematic drawing of the multi-electrode array (MEA) system with simultaneous intracellular action potential recording. **(Right)** Orientation of the excised left atrium with epicardium surface down on the MEA, the atrial free wall on the bottom and the atrial appendage on the top (Image reproduced and redrawn from Neo *et al.*, Reference 350).

Figure 2 Action potential durations at 20, 50 and 90% repolarisations in the HCM atria. **(A)** Young HCM mice demonstrated reduced APD20, 50, and 90 compared to control mice ($P < 0.001$ vs. controls). **(B to D)** Atrial APD20, 50, and 90 were reduced in young HCM compared to controls across all pacing cycle lengths tested (all $P < 0.001$ vs. controls). **(E)** Similarly, older HCM mice had decreased APD20, 50 and 90 compared to controls ($P < 0.001$ vs. controls). **(F to H)** Atrial APD20, 50, and 90 were reduced in old HCM compared to controls across all pacing cycle lengths tested (all $P < 0.001$ vs. controls). * $P < 0.001$.

Figure 3 Atrial effective refractory periods in the HCM mice**. (A)** Young HCM mice demonstrate unchanged ERP compared to controls across all pacing cycle lengths. **(B)** Old HCM mice exhibit increased ERP across all pacing cycle lengths in the 50-week old HCM mice were observed ($P < 0.001$ vs. control). Pacing intervals at 400 ms demonstrated significantly higher ERP compared to pacing at 100 ms intervals in both HCM and control mice ($P < 0.01$ vs. 100 ms). ** $P < 0.01$ and *** $P < 0.001$.

Figure 4 Representative LA time maps and corresponding phase histograms of mice at 30-weeks **(A)** and 50-weeks **(B)** at 200 ms pacing. HCM mice demonstrate unchanged total activation time but increased conduction heterogeneity when young progressing to slowed heterogeneous conduction in older HCM mice. Isochrones (black lines) are constructed at 1ms intervals. Red indicates the start of the electrical impulse and violet represents the longest activation time.

Figure 5 HCM mice demonstrated age-related slowing and heterogeneous conduction in the atria. **(A)** Mean conduction velocity was unchanged in 30-week old HCM mice as compared to controls **(left)**, whereas slowed atrial conduction was observed in 50-week old HCM mice across all pacing cycle lengths **(right)** ($P < 0.001$ vs. controls). **(B)** Increased conduction heterogeneity was observed in both 30-week old **(left)** and 50-week old HCM mice **(right)** as compared to controls across all cycle lengths tested (both $P < 0.001$). *** $P < 0.001$.

Figure 6 HCM mice exhibited myocyte hypertrophy, elevated interstitial fibrosis and inflammatory cell infiltrate in the atria. **(A)** Photomicrographs of transverse sections of the LA stained with H&E **(top)** and Masson's Trichrome stain **(bottom)** from young HCM mice demonstrated increased inflammatory cell infiltration **(arrows)** and interstitial fibrosis **(stained blue)** in the atrial myocardium. **(B)** Similarly, older HCM mice demonstrate elevated inflammatory cell infiltration **(arrows)** and interstitial fibrosis **(stained blue)** in the atrial myocardium as compared to controls. Scale = 50 μm. **(C)** Pooled data on LA cardiomyocyte diameter **(left)**, atrial fibrosis **(middle)** and inflammatory cell infiltrate **(right)**

shows that atrial myocyte hypertrophy, interstitial fibrosis and inflammatory cell infiltrates were greater in HCM mice compared to controls. No significant age differences were observed. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 7 Circulating Markers of Extracellular Matrix Remodelling in HCM mice. Plasma levels of MMP-2, MMP-3, pro MMP-9, TIMP-1 and TGFβ1 were unchanged in young HCM mice. In contrast, older HCM mice demonstrated elevated plasma levels of MMP-2 and MMP-3 compared to controls ($P < 0.05$ and $P < 0.01$ vs. controls respectively). Although plasma levels of TGFβ1 were elevated in old HCM mice compared to controls, this was not statistically significant ($P = 0.189$). * $P < 0.05$ and ** $P <0.01$.

Figure 8 Circulating Markers of Inflammation and Cell Adhesion Molecules in HCM mice. Plasma levels of TNFα, ICAM-1 in both young and old HCM mice compared to controls. In contrast, old HCM mice showed elevated VCAM-1 levels in comparison to age-matched controls ($P < 0.05$). * $P < 0.05$.

FIGURE 1

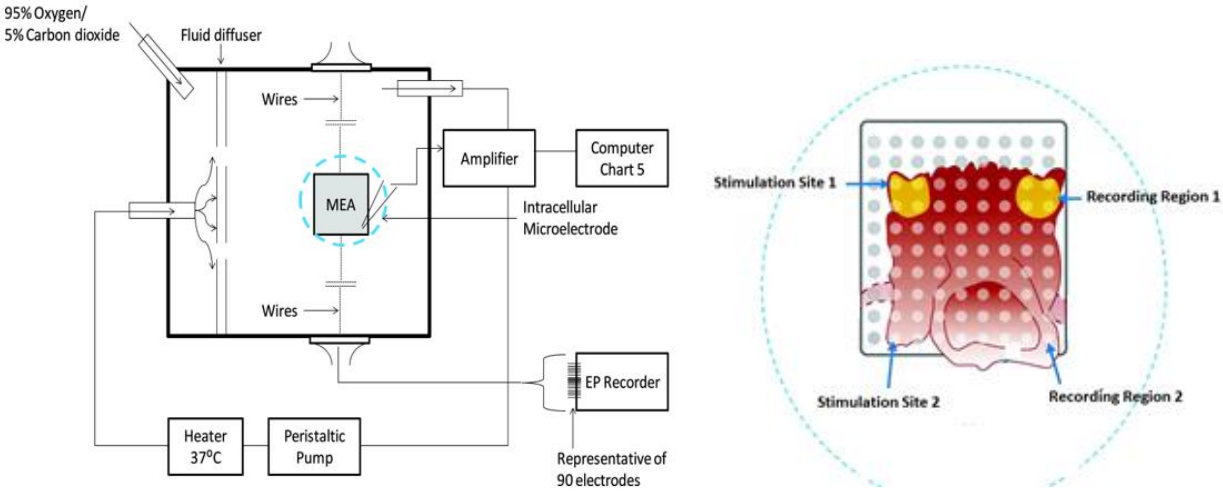

95% Oxygen/
5% Carbon dioxide
Fluid diffuser
Wires
Amplifier
Computer
Chart 5
MEA
Intracellular
Microelectrode
Wires
EP Recorder
Heater
37°C
Peristaltic
Pump
Representative of
90 electrodes
Stimulation Site 1
Recording Region 1
Stimulation Site 2
Recording Region 2

FIGURE 2

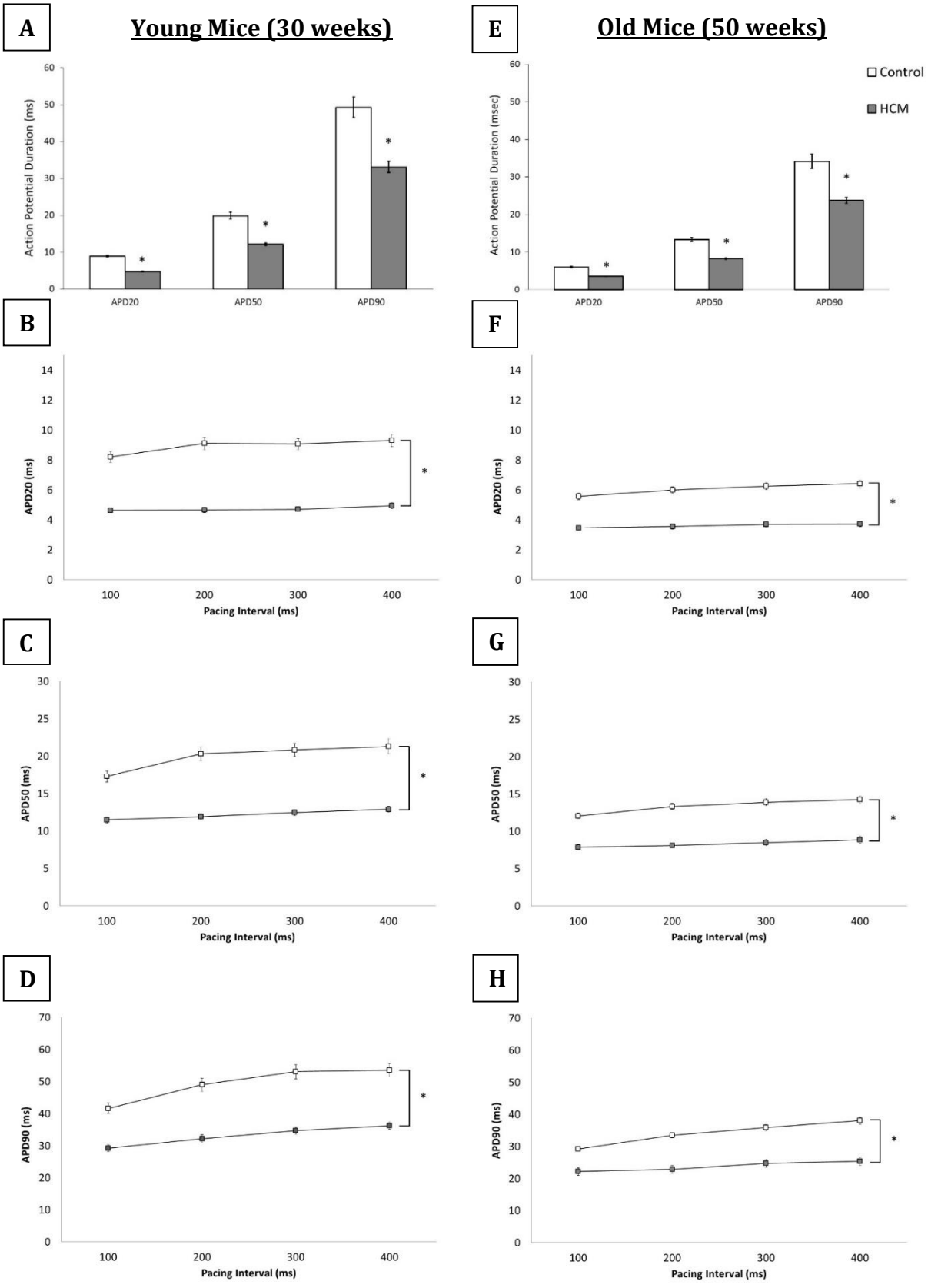

A
Young Mice (30 weeks)
E
Old Mice (50 weeks)
Control
HCM
Action Potential Duration (ms)
Action Potential Duration (msec)
APD20
APD50
APD90
B
F
APD20 (ms)
C
G
APD50 (ms)
D
H
APD90 (ms)
Pacing Interval (ms)
100
200
300
400
*

FIGURE 3

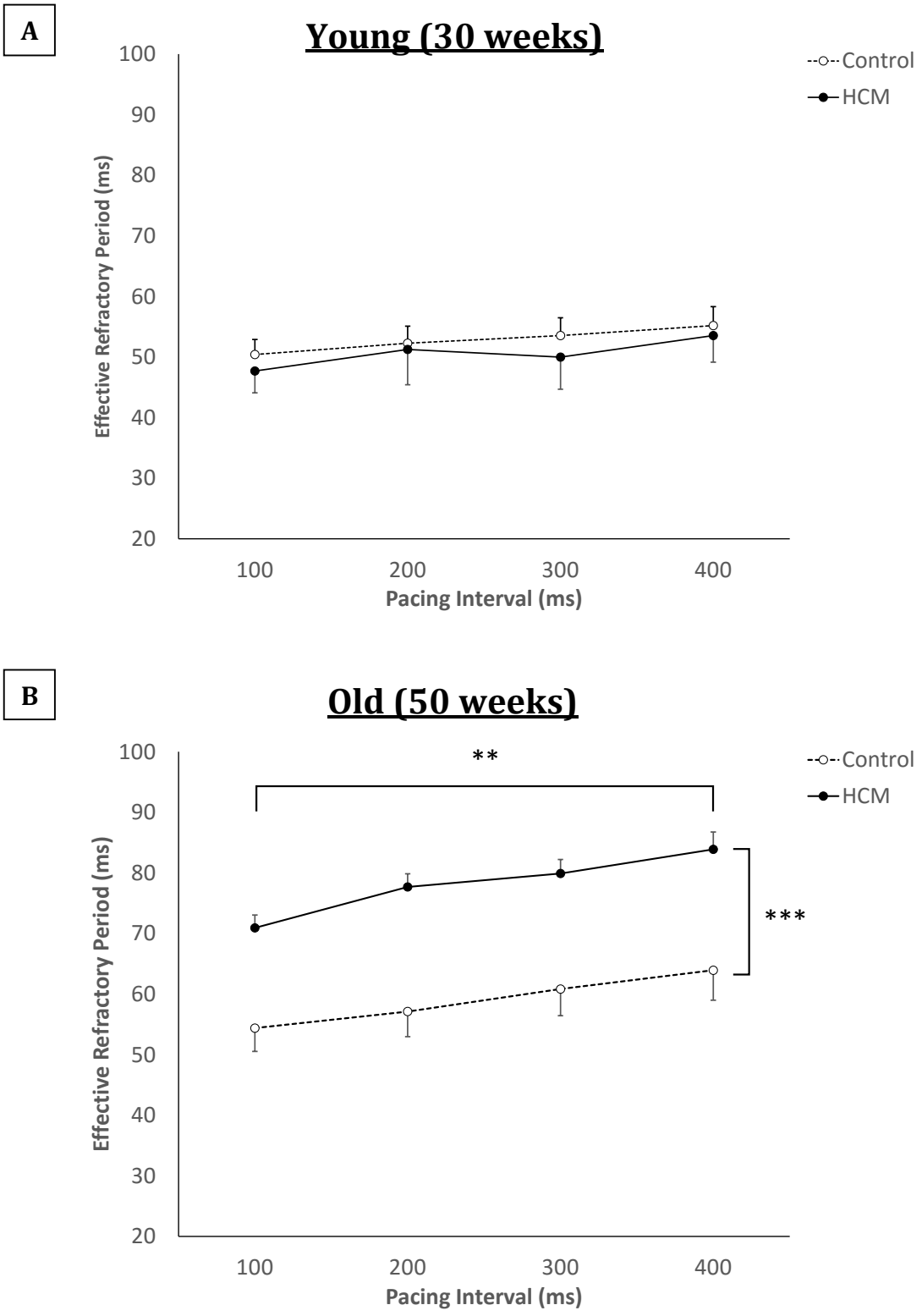

A
Young (30 weeks)
Control
HCM
Effective Refractory Period (ms)
100
90
80
70
60
50
40
30
20
100
200
300
400
Pacing Interval (ms)
B
Old (50 weeks)
**
***
Control
HCM
Effective Refractory Period (ms)
100
90
80
70
60
50
40
30
20
100
200
300
400
Pacing Interval (ms)

FIGURE 4

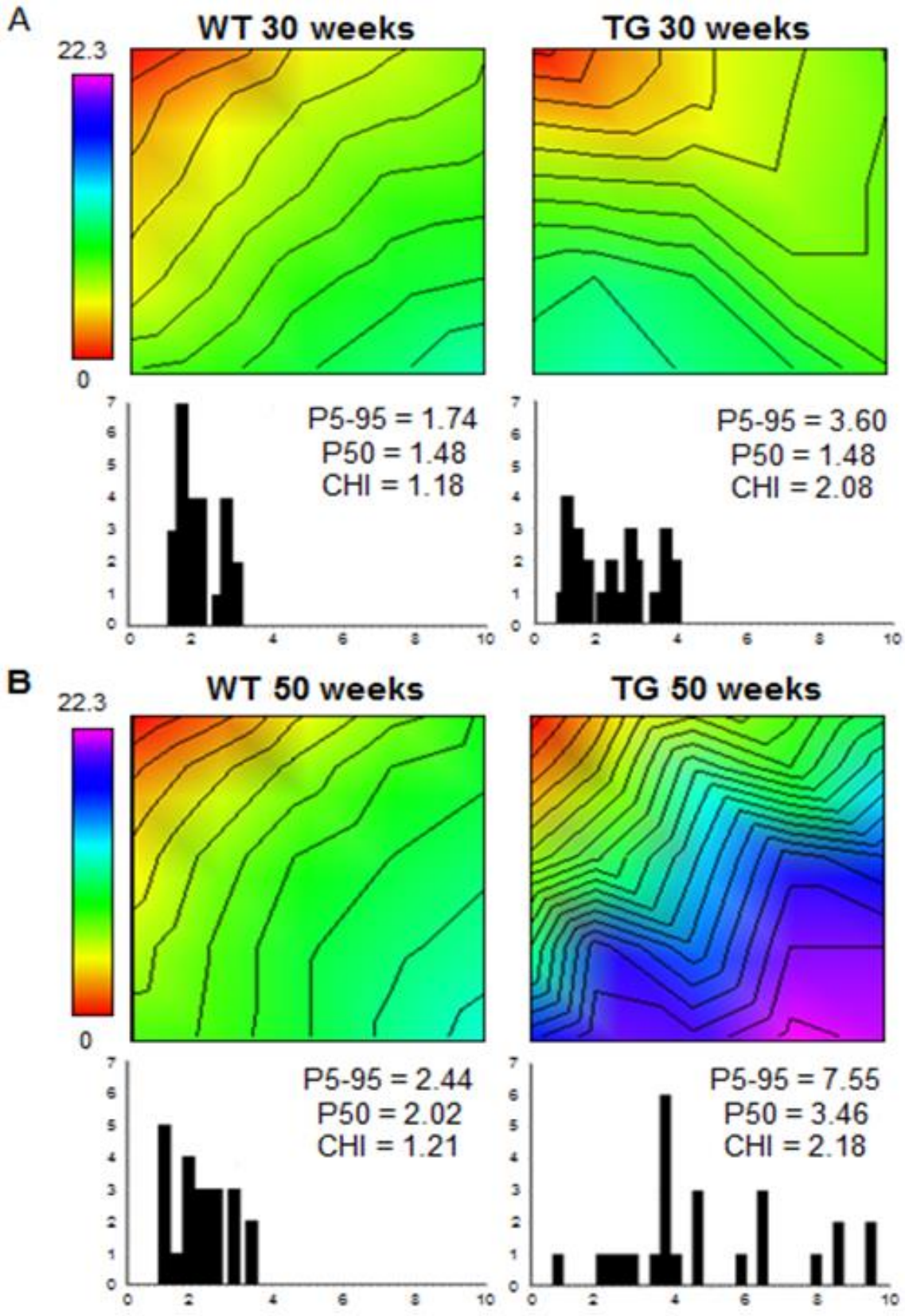

A
WT 30 weeks
TG 30 weeks
22.3
0
P5-95 = 1.74
P50 = 1.48
CHI = 1.18
P5-95 = 3.60
P50 = 1.48
CHI = 2.08
B
WT 50 weeks
TG 50 weeks
22.3
0
P5-95 = 2.44
P50 = 2.02
CHI = 1.21
P5-95 = 7.55
P50 = 3.46
CHI = 2.18

FIGURE 5

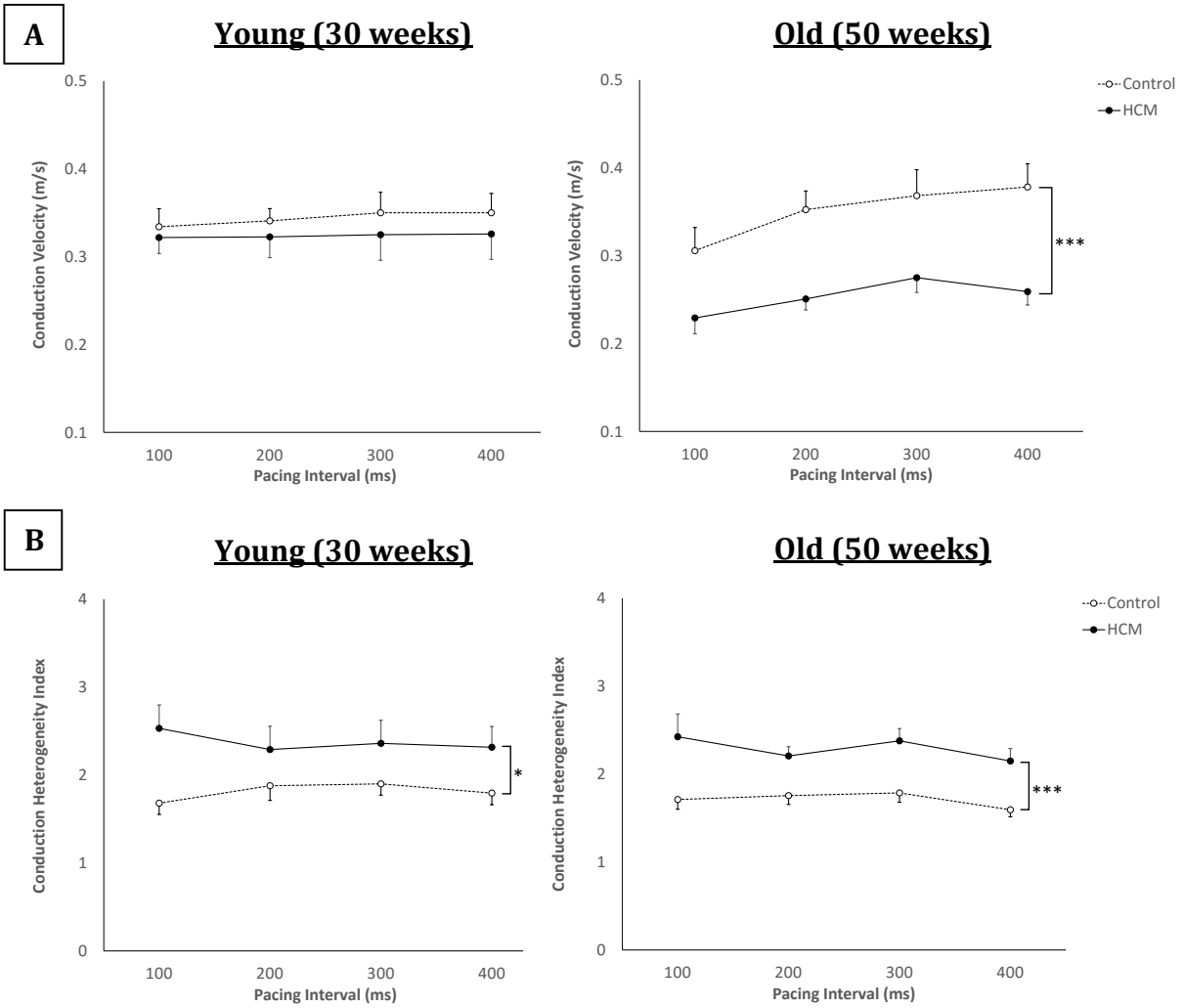

A
Young (30 weeks)
Old (50 weeks)
Control
HCM
Conduction Velocity (m/s)
0.5
0.4
0.3
0.2
0.1
100
200
300
400
Pacing Interval (ms)
***
B
Young (30 weeks)
Old (50 weeks)
Control
HCM
Conduction Heterogeneity Index
4
3
2
1
0
100
200
300
400
Pacing Interval (ms)
*
***

FIGURE 6

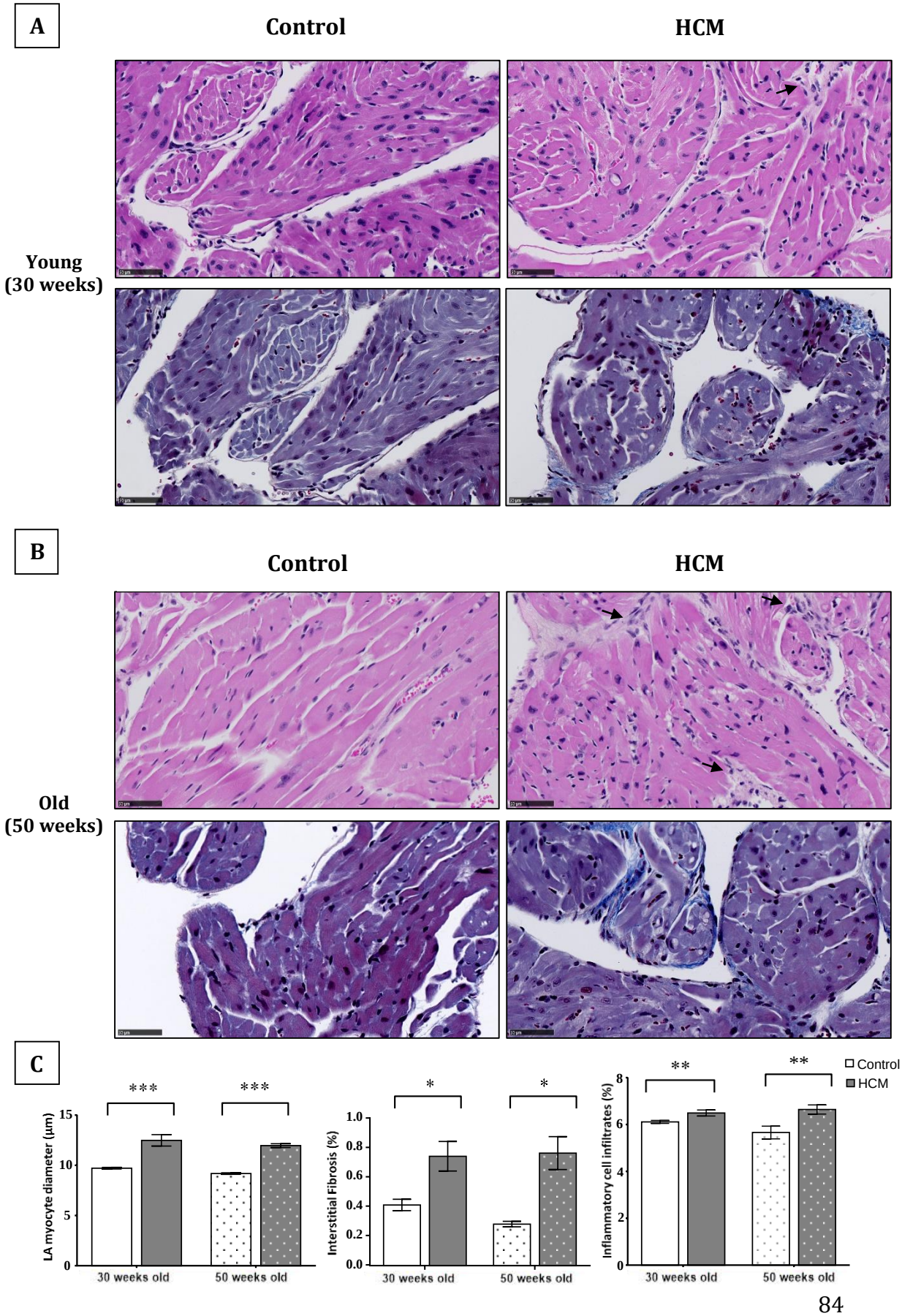

A
Control
HCM
Young
(30 weeks)
B
Control
HCM
Old
(50 weeks)
C
LA myocyte diameter (µm)
Interstitial Fibrosis (%)
Inflammatory cell infiltrates (%)
30 weeks old
50 weeks old
Control
HCM

FIGURE 7

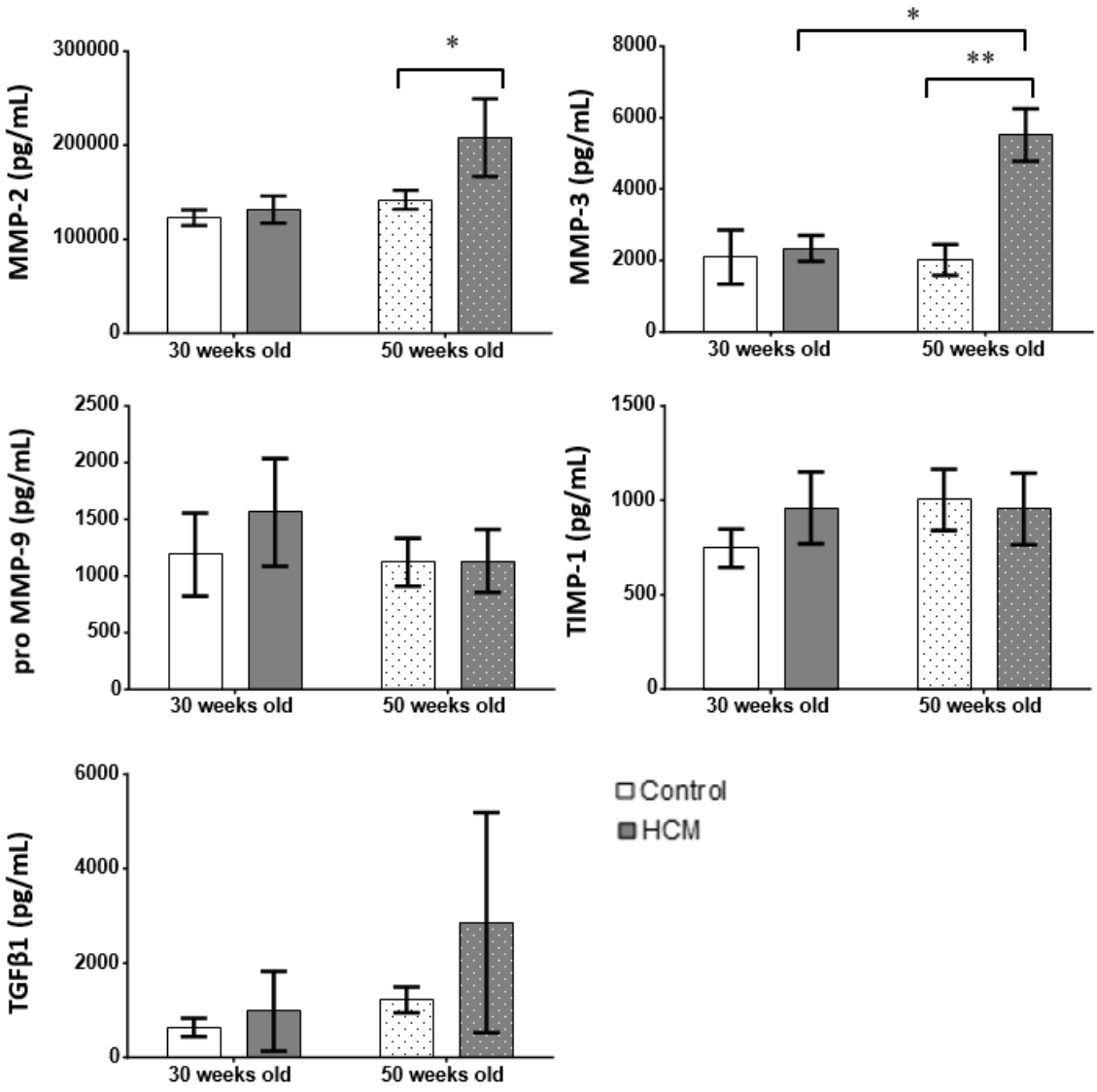
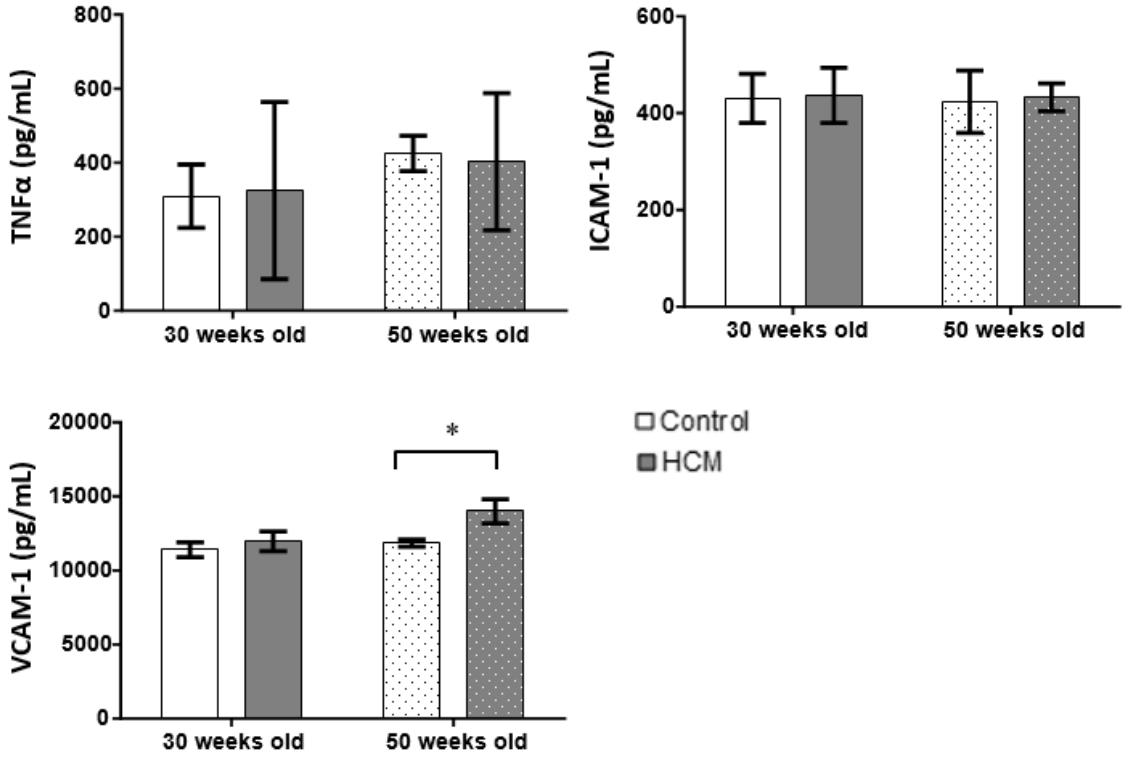

MMP-2 (pg/mL)
300000
200000
100000
0
*
30 weeks old
50 weeks old
MMP-3 (pg/mL)
8000
6000
4000
2000
0
*
**
30 weeks old
50 weeks old
pro MMP-9 (pg/mL)
2500
2000
1500
1000
500
0
30 weeks old
50 weeks old
TIMP-1 (pg/mL)
1500
1000
500
0
30 weeks old
50 weeks old
TGFβ1 (pg/mL)
6000
4000
2000
0
30 weeks old
50 weeks old
Control
HCM

FIGURE 8


TNFα (pg/mL)
800
600
400
200
0
30 weeks old
50 weeks old
ICAM-1 (pg/mL)
600
400
200
0
30 weeks old
50 weeks old
VCAM-1 (pg/mL)
20000
15000
10000
5000
0
*
30 weeks old
50 weeks old
Control
HCM

Statement of Authorship

Title of Paper	Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Clinical and Experimental Pharmacology and Physiology

Principal Author

Name of Principal Author (Candidate)	Wei-Wen Lim
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article
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 6/10/2015

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

Name of Co-Author	Mathias Baumert
Contribution to the Paper	analysis and interpretation of research data drafting significant parts of the article
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Name of Co-Author	Dennis Lau
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Signature	Date 8/10/15

Name of Co-Author	Tatiana Tecotzman
Contribution to the Paper	analysis and interpretation of research data
Signature	Date 02/12/15

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Contribution to the Paper	conception and design of the project drafting significant parts of the article
Signature	Date 26-11-15

Name of Co-Author	Prashanthan Sanders
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article or revising it critically for important intellectual content
Signature	Date 24/11/15

Name of Co-Author	David Saint
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article or revising it critically for important intellectual content
Signature	Date 06/10/15

Please cut and paste additional co-author panels here as required.

Wei Wen Lim

From: Anand Ganesan
Sent: Saturday, 7 November 2015 7:49 AM
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Subject: RE: Author Statements

Dear Wei Wen,
I am in the USA and do not have access to printer or Adobe PDF writer.

I do approve the manuscripts. Please take this email as affirmation of my approval for the purpose of the form.

Best regards,

Anand

ANAND GANESAN MBBS (Hons.) PhD FRACP CEPS CCDS

Michel Mirowski Fellow, Heart Rhythm Society
NHMRC Australian Early Career Health Practitioner Fellow
CRICOS Provider Number 00123M

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1) Electrophysiological and Structural Remodelling of the Atria in Hypertrophic Cardiomyopathy: Implications for Atrial Fibrillation
2) Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabesity
3) Atrial Electrophysiological and Structural Remodelling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes
4) Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy

Also included is the 'Statement of Authorship and Location of Data' form for the manuscript "Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy" accepted in Clinical and Experimental Pharmacology and Physiology.

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Kind Regards,
Wen

WEI WEN LIM, BSc(Hons)
PhD candidate

Centre for Heart Rhythm Disorders | University of Adelaide

Chapter Three

Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy

3.1. INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiac disease affecting 1 in 500 of the general population (95). Although initial studies in tertiary care centres associate HCM patients with a poor prognosis (276,277), subsequent studies in unselected populations have suggested a generally benign course in most patients with an overall mortality rate of 1-2% per annum (96,278,279,281-283,323). Nonetheless, a significant proportion of HCM patients are subjected to several adverse outcomes including progression to heart failure, increased risk of stroke predominantly due to atrial fibrillation, and risks for developing sudden cardiac death (95).

The clinical course of HCM in human patients is very variable ranging from benign carriers to sudden cardiac death particularly in young patients (95). This is fundamentally due to the variety of inter- and intragenic mutations of the individual sarcomeric proteins. Furthermore, the clinical phenotype is different between mutations in different proteins. For example, patients with beta myosin heavy chain (β-MHC) mutations tend to develop mild or severe HCM, whereas

myosin binding protein-C (MyBP-C) mutations develop late onset HCM, troponin-T mutations results in mild hypertrophy with risk of sudden death, and patients with troponin-I (TnI) mutations tend not to develop sudden death in the absence of severe disease (289). Additionally, recent evidence suggests multiple mutations can occur in the same HCM patients, often resulting with greater severity in clinical disease (321). This highlights the importance of identification of mutations within HCM families, and greater risk stratification in this population. Animal models of HCM, such as the Gly203Ser cardiac TnI mice, serve as a tool in isolating disease pathogenesis due to the targeted mutations without any co-morbidity as is often found in human patients.

Heart rate variability (HRV), the naturally occurring temporal variation between consecutive heart beats, is thought to reflect the overall state of the autonomic nervous system and cardiac health (200). Depressed HRV has been previously implicated in patients with congestive heart failure (210,211), identifying patients at risk for sudden cardiac death (212,213). Changes in HRV have also been identified prior to atrial fibrillation (AF) initiation (223), as well as predictive of AF recurrence following ablation (227). As these clinical sequelae are not uncommon in the course of HCM, we propose that similar changes in HRV may be present in HCM.

Previous studies characterizing the heterozygous MyBP-C mutant mice past 125 weeks of age demonstrated later onset of less severe hypertrophy, unchanged electrophysiological interval conduction times and less inducible arrhythmias compared to the alpha myosin heavy chain (αMHC) mutant mouse (314). In

contrast, homozygous MyBP-C mutant mice at 55 weeks of age whilst demonstrating normal HRV as well as electrophysiological interval conduction times compared to heterozygous mutation and wild type controls, developed more inducible ventricular arrhythmias (383). Although ventricular effects of HCM have been well characterised in the Gly203Ser cardiac TnI transgenic male mouse (337). The electrophysiology and cardiac autonomic changes that occur in this model have not been studied. The present study sought to characterise ECG and HRV changes in this model of HCM. As several HCM mutations have been associated with late onset HCM in the elderly (384,385). and the TnI mutant mice being previously demonstrated having normal lifespans at 52 weeks of age (337), we investigated age-related ECG and HRV changes in 30- and 50-week old mice in attempt to identify any potential long-term changes underlying the course of HCM.

3.2. METHODS

3.2.1. Animals

We used the Gly203Ser cardiac TnI transgenic mouse model that has been previously described[1] (337). The mouse model was bred on the C57BL/6 genetic background and has been shown to have a normal life span. TG mice with the mutation develop phenotypic hallmarks of HCM by 21 weeks of age. We used non-transgenic C57BL/6 mice as controls, as they have been previously demonstrated to be indistinguishable from transgenic wild-type overexpression mice (337). TG and Con mice were bred by crossing a TG female with a Con male mouse, and were tail genotyped at 2-3 weeks of age for identification. Mice were then allocated to two age groups (n=7-11 per group), 30 weeks or 50 weeks of age before experiments were performed. Mice were housed at controlled temperature (24°C) and lighting (12h light-dark cycles) with free access to standard chow and water ad libitum. All experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.2. Data Processing and Analyses

All ECG recordings were analysed with the investigator blinded to the animal groupings. ECG and HRV parameters were analysed offline with the respective modules from LabChart Pro (ADInstruments, NSW, Australia). ECG parameters including P-wave duration, PR, QRS, QT, and QTc intervals were determined in 150-200 consecutive beats, with averages generated for every four successive beats. We used the rate-corrected Bazett's formula to calculate for QTc values. HRV

[1] The cohort of animals used in this study was separate from that of Chapter Two.

quantification was conducted in 2 mins continuous ECG tracings by the use of time-domain analysis, and frequency-domain analysis. Time-domain parameters included total beats, mean RR intervals, SDRR, CVRR, average HR, SD HR, and (RMSSD. CVRR was calculated as a ratio by the formulae SDRR/meanRR as per LabChart Pro software. Frequency-domain parameters included TP, LF (0.15-1.5 Hz) expressed in normalized units, HF (1.5-5 Hz) expressed in normalized units, and the LF/HF ratio. These frequency spectra selected were in accordance with the majority of mice HRV studies (386).

3.2.3. Statistical Analysis

All values are reported in mean ± standard deviation unless indicated otherwise. Analysis of variance (ANOVA) was used to determine significant differences, and Tukey's post-hoc test was conducted to identify between group differences. A value of $p<0.05$ was used to determine significance.

3.3. RESULTS

3.3.1. Study Design

We studied four groups of mice: (1) Non-transgenic controls (Con) at 30 weeks of age; (2) Gly203Ser transgenic mice (TG) at 30 weeks of age; (3) Con at 50 weeks of age; and (4) TG at 50 weeks of age. Following anaesthesia, mice underwent 30-minute surface 3-lead electrocardiography recording and ECG and HRV parameters were analysed offline.

3.3.2. ECG Parameters

Overall ECG results are summarised in Table 1 with example ECG tracings from 50-weeks old mice presented in Figure 1B. General linear model ANOVA demonstrated no significant differences in age and strain × age interactions across all ECG parameters tested. QRS, QT, and corrected QT (QTc) intervals do not significantly differ between TG mice compared to controls at both age groups. On the other hand, we observed significantly prolonged P-wave duration in TG mice compared to controls at both ages ($p<0.001$). A significant prolongation of PR interval was also observed in TG mice compared to controls ($p<0.001$).

3.3.3. HRV Parameters

Figure 1 depicts ECG, heart rate (HR) fluctuations, and power spectra in representative 50-week old mice and Figure 2 illustrates Poincaré plots depicting the correlation of consecutive RR intervals in representative 50-week old mice. Summarized HRV results are presented in Table 2. General linear model ANOVA

demonstrated no significant differences in age and strain × age interactions across all HRV parameters tested. There were no significant changes in total beats, beat-to-beat (RR) intervals, average HR, root mean square of successive heart-beat interval differences (RMSSD) between the two mouse strains. In contrast, standard deviation of RR intervals (SDRR) was significantly decreased in TG mice compared to controls ($p<0.01$). Additionally, coefficient of variation of RR intervals (CVRR) and standard deviation of heart rate (SDHR) were both significantly decreased in TG mice compared to controls (both $p<0.01$). Lastly, total power (TP) was significantly decreased in TG compared to controls ($p<0.05$), although low frequency (LF), high frequency (HF) and LF/HF ratio was not significantly different between mouse strains.

3.4. DISCUSSION

This study has shown that Gly203Ser cardiac TnI transgenic mice demonstrate several electrophysiological and heart rate variability alterations:

1. Prolonged atrial and atrioventricular conduction as evident by a longer P-wave and PR interval duration
2. Depressed overall HRV evident by a decrease in time-domain parameters of SDRR, CVRR, and SDHR
3. Age upon established HCM phenotype did not significantly impact electrophysiological and heart rate variability in TG mice

Previous studies by Tsoutsman et al. (2006) have identified that animals from this mouse model develop all characteristic features of human HCM by 21 weeks of age (337). In that study, with the primary focus on the ventricular myocardium, they demonstrated left ventricular hypertrophy, increased PR intervals, myocyte hypertrophy, myofibrillar disarray, interstitial fibrosis, and abnormal myocyte Ca^{2+} cycling. In accordance with their ECG data, we observed that in addition to PR prolongation, HCM mice show prolonged P-wave duration, demonstrating atrial and atrioventricular conduction slowing. Interestingly, no significant change was observed in ventricular depolarisation and repolarisation times (QRS, QT and QTc; Table 1) in this animal model despite evident ventricular hypertrophy (337). In a different model of HCM, Berul et al. (1997), in the setting of an *in vivo* electrophysiological study with concurrent 12-lead ECG recordings, demonstrated evidence of prolonged repolarization times (QT and QTc) in 30-week old αMHC403 mice (387), as well as gender differences with males demonstrating greater

histopathology and hemodynamic derangements (388). On the other hand, mice with homozygous MyBP-C mutations demonstrate normal ECG intervals with normal sinus node, atrial, and ventricular conduction and repolarisation despite prominent histopathology and ventricular dysfunction (383). Variability in the electrophysiological phenotype in different models may implicate genotype-specific risk factors, in addition to other important environmental or somatic contributions that contribute to disease and clinical outcome (389).

Notably, TnI together with troponin C and T forms the troponin complex and acts as a molecular switch mediating muscle contraction through actin-myosin interactions depending on Ca^{2+} concentrations (390). The mechanisms of slow atrial and atrioventricular conduction may therefore be due to Ca^{2+} handling abnormalities with expression of mutant troponin I. This was suggested by observations of reduced protein interactions between the troponin I with troponin T and C in the transgenic mouse, as well as increased decay constant in cardiomyocytes (337). Alternatively, atrial enlargement and intra- as well as inter-atrial conduction slowing due to local disruptions of fibrosis or gap junctional remodelling can also contribute to prolonged P wave durations and PR intervals (391-393).

Further, we have demonstrated depressed HRV in our model of TnI mutations that is not seen in the mouse model of homozygous MyBP-C mutations where HRV was found to be unchanged (383). Our findings of decreased SDRR, CVRR, and SDHR are indicative of depressed vagal activity in the TnI mutant mouse. Similarly, global HRV parameters in HCM patients such as standard deviation of normal beat-

to-beat interval (SDNN), RMSSD and specific vagal influences on HRV such as the proportion of beat-to-beat time difference by more than 50 ms (pNN50) and HF were reduced (394-396), and correlated well to severity of heart failure (397,398). Furthermore, predictors for parasympathetic withdrawal in HCM patients were attributed to degree of left ventricular hypertrophy, enlarged left atrium and small left ventricular end-systolic dimension (395,398). However, genetic mutation contributions have not been identified in these clinical studies. The shift towards compromised parasympathetic regulation may be related to increased left ventricular wall thickness and decreased end-diastole diameter previously observed in this mouse model (337).

It is generally accepted that HF power is a marker of vagal tone (399), whereas LF power reflects both vagal and sympathetic activity (400). Hence, the LF/HF ratio is believed to be an indicator of sympatho-vagal balance (399). However, recent evidence has suggested that that LF and LF/HF ratio do not accurately reflect sympatho-vagal balance (401,402). Furthermore, the results from frequency domain measures of vagal or sympathetic activity in HCM patients are inconclusive. Some studies suggest a reduction in vagal control with a reduction of HF power (394-396,398), whereas Fei et al. (1995) demonstrated normal HF power with reduced LF power and LF/HF ratio in HCM patients, suggesting of reduced sympathetic tone instead (403). In the current study, we did not observe any significant changes in LF, HF, and LF/HF ratio in HCM mice.

3.4.1. Study Limitations

The influence of anaesthetics is known to cause depress sympathetic tone (404). However, unlike other murine strains, C57BL/6 mice have robust vagal tone making them more comparable to that of humans featuring vagal dominance (405,406). Likewise, other experimenters have assessed cardiac autonomic function by measuring HRV in mice under other anaesthetics such as urethane, isoflurane, thiopental, and a combination ketamine, acepromazine and xylazine (407-411). Nevertheless, we observed significant differences in various HRV parameters in the HCM mice despite similar treatment compared to controls. Telemetric ECG is the ideal methodology of reflecting true resting heart rates of the mice (404). However, the majority of studies with implantable telemetric devices employ a designated 2-4 post-surgery recovery days (411-414), when post-surgical resting heart rates can remain elevated up to 10 days (386,415). This suggests that surgery-related stress or other implantation-related factors could have a significant impact on HRV as well. The design of 50-week old TG mice may not be long enough to observe late progression of HCM and hence demonstrating a lack of significant difference compared to 30-week old mice. However, there is no existing data to date in the TnI mutant mice characterizing further deterioration of the cardiac phenotype past 52 weeks of age.

3.4.2. Conclusions

In summary, the results from the present study demonstrate electrophysiological and heart rate alterations in the Gly203Ser cardiac TnI mutant mouse model of HCM. The prolongation in atrial conduction may implicate electro-anatomical

changes within the atria that remain to be investigated. Furthermore, a depressed HRV in these mice may reflect the degree of disease severity; although we could not correlate HRV parameters to mortality rates as the strain of mice demonstrate normal longevity (337). The current model of HCM does not demonstrate spontaneous arrhythmias (337), although sudden deaths and inducible arrhythmias have been reported infrequently in other models (349,383,387). Utilizing various animal models with differing genetic mutations may increase our understanding of cardiac autonomic regulation and improve outcomes in HCM, a disease with variable clinical outcomes. More research is warranted to minimise adverse autonomic traits and improve cardiovascular outcomes in HCM.

TABLE 1. COMPARISON OF ECG PARAMETERS BETWEEN CONTROL AND TG MICE AT 30 AND 50 WEEKS OF AGE

	30 weeks		50 weeks	
	Con (n=11)	TG (n=7)	Con (n=7)	TG (n=8)
P wave duration (ms)	11.00 ± 1.04	13.49 ± 1.97***	10.63 ± 1.77	14.43 ± 1.80***
PR interval (ms)	40.38 ± 4.67	44.89 ± 2.01***	42.07 ± 4.52	47.90 ± 4.16***
QRS interval (ms)	11.37 ± 1.50	11.21 ± 1.26	11.43 ± 1.54	10.77 ± 1.10
QT interval (ms)	21.12 ± 1.44	21.28 ± 1.46	21.35 ± 1.15	21.55 ± 0.90
QTc interval (ms)	47.39 ± 3.86	44.74 ± 4.68	45.45 ± 4.30	45.42 ± 4.51

*** $p<0.001$ vs. age-matched controls

TABLE 2. COMPARISON OF HRV PARAMETERS BETWEEN CONTROL AND TG MICE AT 30 AND 50 WEEKS OF AGE

		30 weeks		50 weeks	
		Con (n=11)	TG (n=7)	Con (n=7)	TG (n=8)
	Total Beats (n)	594.20 ± 36.36	582.30 ± 60.76	575.40 ± 43.79	573.40 ± 59.70
	RR interval (ms)	202.40 ± 12.59	208.10 ± 21.14	209.90 ± 17.72	210.90 ± 21.42
	HR (bpm)	297.50 ± 18.49	291.10 ± 30.48	287.60 ± 23.15	287.10 ± 29.63
Time-domain	**SDRR (ms)**	3.33 ± 0.85	2.23 ± 0.56**	3.28 ± 0.54	2.48 ± 0.80**
	CVRR (%)	1.6 ± 0. 5	1.0 ± 0. 2***	1.6 ± 0. 2	1.1 ± 0.3***
	SD HR (BPM)	4.98 ± 1.54	3.05 ± 0.45***	4.49 ± 0.67	3.30 ± 0.70***
	RMSSD	3.78 ± 1.00	3.18 ± 1.15	3.91 ± 1.04	3.33 ± 1.66
Frequency-domain	**Total power (μs^2)**	15.19 ± 5.57	9.92 ± 5.73*	19.76 ± 8.25	12.44 ± 9.03*
	LF (nu)	15.37 ± 6.68	13.50 ± 6.18	15.07 ± 4.13	14.79 ± 6.44
	HF (nu)	72.95 ± 10.45	79.00 ± 10.33	74.65 ± 7.38	73.79 ± 10.94
	LF/HF ratio	0.23 ± 0.14	0.18 ± 0.11	0.21 ± 0.06	0.21 ± 0.10

* denotes $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. age-matched controls

FIGURE LEGENDS

Figure 1 Example of ECG recording, single ECG tracing, HR fluctuations, and power spectra of 50-week old mice. (A) ECG recordings from representative TG and control mice. (B) TG mice demonstrate longer P wave duration and PR interval compared to control. Black bar represents 10 ms. (C) Comparison of beat-to-beat changes in RR intervals plotted against successive beats. (D) Power spectra of the RR signal from transgenic and control mice. VLF = 0 – 0.15 Hz, LF = 0.15 – 1.5 Hz, and HF = 1.5 – 5 Hz.

Figure 2 Poincaré plots depicting the correlation between consecutive RR intervals in representative 50-week old mice. TG mice demonstrated diminished scattering of the plot compared to controls, highlighting the hallmark of a decreased HRV.

FIGURE 1

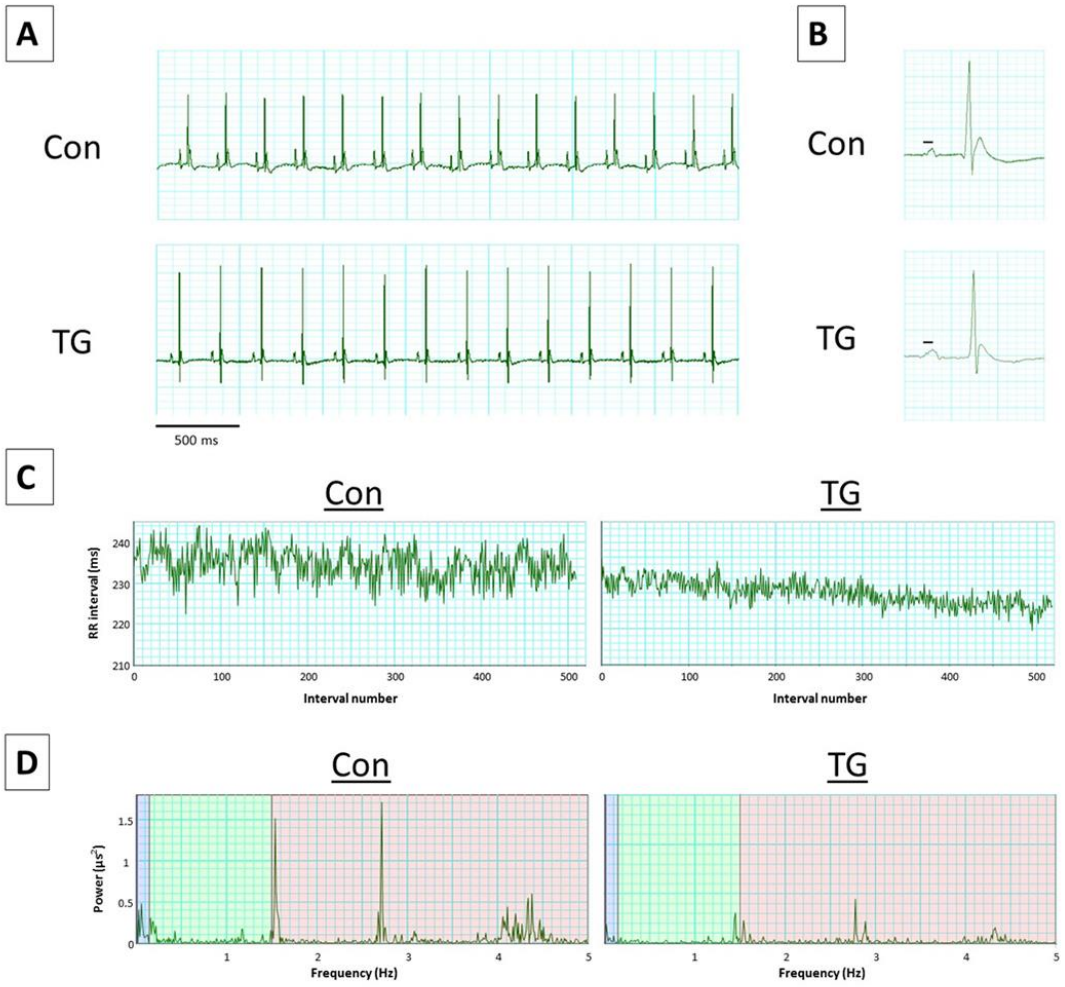
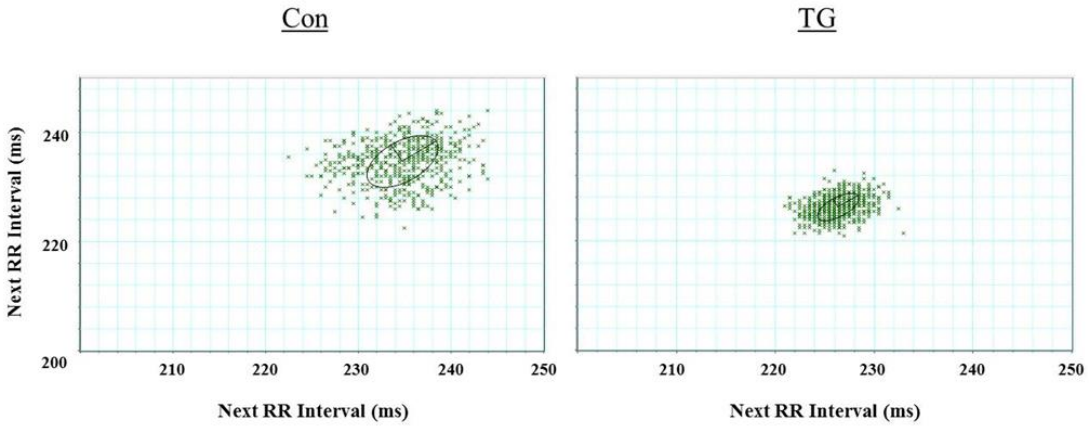

A
Con
TG
500 ms
B
Con
TG
C
Con
TG
RR interval (ms)
240
230
220
210
0
100
200
300
400
500
Interval number
Interval number
D
Con
TG
Power (μs²)
1.5
1
0.5
0
1
2
3
4
5
Frequency (Hz)
Frequency (Hz)

FIGURE 2


Con
TG
Next RR Interval (ms)
240
220
200
210
220
230
240
250
Next RR Interval (ms)
210
220
230
240
250
Next RR Interval (ms)

Statement of Authorship

Title of Paper	Atrial Electrophysiological and Structural Remodeling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Wei-Wen Lim
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article
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 7/10/2015

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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Wei Wen Lim

From: Anand Ganesan
Sent: Saturday, 7 November 2015 7:49 AM
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Subject: RE: Author Statements

Dear Wei Wen,
I am in the USA and do not have access to printer or Adobe PDF writer.

I do approve the manuscripts. Please take this email as affirmation of my approval for the purpose of the form.

Best regards,

Anand

ANAND GANESAN MBBS (Hons.) PhD FRACP CEPS CCDS

Michel Mirowski Fellow, Heart Rhythm Society
NHMRC Australian Early Career Health Practitioner Fellow
CRICOS Provider Number 00123M

From: Wei Wen Lim
Sent: Saturday, 7 November 2015 7:38 AM
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Dear Anand,

I've resent the Author Statements forms for my Thesis Chapters manuscripts requiring your signatures.

The Thesis Chapters include:

1) Electrophysiological and Structural Remodelling of the Atria in Hypertrophic Cardiomyopathy: Implications for Atrial Fibrillation
2) Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabesity
3) Atrial Electrophysiological and Structural Remodelling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes
4) Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy

Also included is the 'Statement of Authorship and Location of Data' form for the manuscript "Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy" accepted in Clinical and Experimental Pharmacology and Physiology.

If you are not able to sign the forms physically or electronically, can you please provide a written approval through email that you approve of the manuscripts?

Kind Regards,
Wen

WEI WEN LIM, BSc(Hons)
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Chapter Four

Atrial Electrophysiological and Structural Remodelling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes

4.1. INTRODUCTION

Both type II diabetes mellitus and obesity have risen to global epidemic proportions over the past few decades (416,417), and are associated with elevated risk of developing atrial fibrillation (AF). Given the growing aging population worldwide, AF has similarly encountered a progressive increase in prevalence with significant implications on patient morbidity and mortality, and health care burden (418). Meta-analyses of cohort and case-control series determine that diabetes and obesity confer approximately 40% and 50% elevated risk of AF respectively (45) (61).

In the Atherosclerosis Risk in Communities (ARIC) study, diabetes but not pre-diabetes attributed an increased 35% risk of developing AF (419). Similarly, insulin-resistance, a common manifestation of pre-diabetes and obesity, did not independently correlate with new-onset AF in the Framingham Heart Study (420). In the Women's Health Study, investigators found that whilst baseline diabetes was a modest but significant risk factor for AF development, increased AF risk was associated with other diabetes-related comorbidities such as obesity over time

(421). Furthermore, poor glycaemic control resulting in glucose fluctuations and/or hypoglycaemic episodes has been linked to AF in diabetic patients (422,423). Obesity contributes to the progression of paroxysmal to permanent AF (424), and weight reduction and risk factor management have demonstrated significant alleviation of the AF burden and improvements to the cardiac structure (62,425,426). Pathophysiological links between diabetes, obesity and AF are multi-factorial and can include insulin resistance, cardiac autonomic, electrophysiological, and structural remodelling. Such factors are known to contribute to oxidative stress and can alter myocardial electrical properties (273,427).

Several mouse models have been developed to reflect human obesity and diabetes (428). The two most commonly used mouse models for diabetes and obesity research are the leptin-deficient (ob/ob) and leptin-resistant (db/db) mice of monogenic causes of disease. However, leptin signalling which has been suggested to be crucial for development of atrial fibrosis and AF is impaired in these models (429,430). Therefore, polygenic mice models presenting a more accurate representation of the complex metabolic abnormalities resembling the progressive development of human disease (431), provides for an attractive tool to investigate atrial remodelling.

One method to mimic the polygenic nature of human metabolic syndrome is by enriching fat content of diets in established rodent models. Prolonged feeding of high fat diets in susceptible rodent models is known to increase 10-20% body weight, whereas effects on blood glucose, insulin levels and sensitivity have been

largely variable depending on the diet type (432). The polygenic NONcNZO10/LtJ (RCS10) mouse is one such model derived from the combination of independent risk-conferring quantitative traits from the New Zealand Obese and the Non-obese Non-diabetic mice (433). These mice develop fasted hyperglycaemia, liver and skeletal muscle insulin resistance at 8 weeks and transitions to moderate obesity, and chronic hyperglycaemia by 13 weeks (433,434). The condition of maturity-onset obesity-induced diabetes has been termed as 'diabesity' (433). RCS10 mice do not develop severe hypercorticism, hyperphagia, severe insulin resistance and morbid obesity unlike monogenic diabesity (434).

The current study sought to characterise the effects of age maturity and differential diets on atrial electrophysiological and structural remodelling in the RCS10 polygenic mouse model of maturity-onset obesity and diabetes. We also characterised the plasma biomarkers involved in extracellular matrix remodelling and inflammation and the impact of sustained diabesity in the current model.

4.2. METHODS

4.2.1. Animal Models

Male and Female NONcNZO10/LtJ (RCS10) and SWR/J (SWR) mice were purchased from Jackson Laboratory (Bar Harbor, USA) and a breeding colony was set up for each strain and maintained in the animal facility in the University of Adelaide. RCS10 mice weaned onto 10% fat diets develop moderate obesity without hyperplasia and transitions from impaired glucose tolerance to stable non-fasting hyperglycaemia by 10 weeks of age (435). RCS10 mice develop obesity and hyperglycaemic state by 13 weeks with increased insulin resistance in skeletal muscle, liver, and heart associated with increased organ-specific lipid content (433). Moderate elevation in plasma insulin is observed, and pancreatic islets transitions from hypertrophy and hyperplasia to atrophy by 24 weeks of age (435). The SWR mouse is a Swiss-derived inbred strain that is resistant to diet-induced obesity and does not exhibit impaired glucose tolerance (Strain #000689, Jackson Laboratory, Bar Harbour, USA).

Mice were housed at controlled temperature (24°C) and lighting (12h light-dark cycles) with free access to normal chow (5% fat), high fat diet (HFD; 10% fat), and HFD with chocolate bar supplement (Choco) and water ad libitum. We attempted to drive obesity in young RCS10 mice by supplementing HFD with chocolate bars (268 calorie breakdown: 50% fat, 42% carbohydrate, 8% protein) that were replenished fortnightly to ensure ad libitum supply. All experiments were approved by the University of Adelaide Animal Ethics Committee and

conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.2.2. Blood and Urine Glucose levels

At the respective endpoints for sacrifice (30 or 50 weeks of age), mice were weighed and anaesthetised with an intraperitoneal injection of ketamine (75mg/kg) and medetomidine (0.5mg/kg). Depth of sedation was checked periodically for paw- and tail-pinch reflexes. Following anaesthesia, heparin (2-IU/g body weight) was administered. Blood glucose levels were measured with the Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany) and urine glucose measured with Keto-Diastix (Bayer, Indiana, USA).

4.2.3. Electrophysiology Study

A midline thoracotomy incision was performed under deep anaesthesia and the heart promptly removed and placed in ice-cold bicarbonate-buffered solution. The left atrium was then carefully dissected from the heart and the epicardial surface placed onto the MEA housed within a flow chamber irrigated with bicarbonate buffered solution (in mM: 130 NaCl, 4 KCl, 0.6 MgCl, 24 $NaHCO_3$, 1.2 NaH_2PO_4, 12 D-glucose, 1.5 $CaCl_2$) maintained at 37°C and pH 7.4 when aerated with 95% oxygen and 5% carbon dioxide (338).

Electrophysiology studies were conducted with a custom-designed multi-electrode array (MEA), consisting of 6 x 6 electrodes of 0.1mm diameter and 0.5mm inter-electrode distance yielding a total of 25 bipolar electrograms (338).

The MEA was attached to a cardiac stimulator (Micropace EPS cardiac stimulator, Micropace Pty Ltd, Australia) and a computerized recording system (LabSystem Pro, Bard Electrophysiology, USA). For consistency, the placement of the atrial tissue was oriented in the same cranial-caudal and medial-lateral orientation with the epicardial surface in contact with the electrodes. A nylon weighting harp was placed over the tissue to improve contact with underlying electrodes. Stimulation of the atrial tissue was provided individually from the two corners (corner 1: inferior left atria appendage (LAA), corner 2: left atrial free wall (LAFW) with 1ms bipolar constant current pulses at 2-times threshold. Electrograms were sampled at 2 kHz and filtered from 10-500Hz. Inducible arrhythmias were identified and characterised as self-propagating atrial arrhythmias lasting more than 2 seconds during electrophysiology study.

4.2.4. Intracellular Action Potential Recording

Action potential recordings were conducted simultaneously with the electrophysiology study (339). Briefly, an aluminosilicate glass electrode filled with 3M KCl (100MΩ) was inserted in the atrial endocardium and intracellular membrane potential recordings made with a high input impedance amplifier (WPI model 725), digitised at 10 KHz, 16-bit resolution and recorded with Chart 5 (AD Instruments). Signal ground was via a silver/silver chloride wire in the bath (MEA electrodes are allowed to float relative to this ground). Action potentials were recorded from opposing two regions (region 1, region 2) of the MEA from the two stimulation sites (corner 1, corner 2) during atrial pacing. Action potential

duration at 90% of repolarization (APD90) was measured offline with the use of Chart 5 (peak parameters module, AD Instruments).

4.2.5. Atrial Refractoriness

Electrograms were recorded by employing eight beat (S1) stimuli drive train followed by a premature (S2) stimulus delivered in 10ms decrements from an initial coupling interval of 100 ms. Atrial effective refractory period (ERP) was defined as the longest S_1-S_2 interval failing to propagate an atrial depolarization. This was repeated at 4 cycle-lengths (100, 200, 300, 400ms CL). ERP measurements were conducted thrice during each CL and values averaged.

4.2.6. Conduction Analysis

Conduction was assessed during S1 pacing and local activation time maps were generated offline using semi-automated custom designed software (Nucleus Medical, Australia) to determine mean conduction velocity (CV) and conduction heterogeneity index (CHI). Functional conduction was also evaluated for the last S2 extra-stimulus pacing that resulted in a propagated response. Each annotation was manually verified by annotating the local activation time to the maximum deviation of the largest amplitude from the baseline on bipolar electrograms. CV was calculated from local vectors within each triangle of electrodes (85), while CHI was assessed using an established phase-mapping technique (118). In brief, the phase distribution was obtained by calculating the largest difference in activation between every 4 adjacent electrodes. Absolute conduction phase delay was established through subtracting the 5^{th} from 95^{th} percentile of the phase

distribution (P_{5-95}), which was then divided by the median (P_{50}) to derive the CHI. Total activation time (TAT) was defined as the longest conduction time in a given annotated map.

4.2.7. Structural Analysis

The LA was fixed in 10% formalin before being wax embedded following standard routine procedures. Transverse sections (6μm) were cut (Leica RM2235 Rotary Microtome), mounted onto albumin coated slides and stained with Masson's Trichrome to determine the extent of fibrosis (blue staining), and presence of mast cell infiltrates with H&E straining. Histological slides were scanned at 40x magnification using the NanoZoomer Digital Pathology System (NDP) (Hamamatsu Photonics, Hamamatsu, Japan). Five fields at 400x magnification were randomly selected per atrium, and images were exported and myocyte cross-sectional diameter (100 cells in total per atrium) were independently analysed by a blinded observer using the ruler function on NDP view 2 (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with Adobe Photoshop CC ver. 14.1.2 (Adobe Systems, CA, USA) in accordance to published protocols (340). The pixel content of staining for each atrium was measured relative to the total tissue area using ImageJ (ver. 1.7.0; NIH, USA) with a batch macro following background subtraction with colour correction.

4.2.8. Slide-based Multiplex ELISA

Blood samples from 10 weeks old mice (n=6 per age per strain) were collected in 4mL EDTA tubes with 1mL heparinised syringes with a 21G needle via cardiac

puncture in the left ventricle prior to heart excision. Tubes were then centrifuged for 10 minutes at 1,500g at 4°C refrigerated centrifuge and the plasma was transferred using a Pasteur pipette into 1.5mL Eppendorf tubes and stored at -20°C. Plasma samples were analysed for peptide levels of leptin, adiponectin, matrix metalloproteinase (MMP)-2 and -3, pro-MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), transforming growth factor beta-1 (TGFβ1), tumour necrosis factor alpha (TNFα), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) using multiplex sandwich ELISA arrays (Custom Quantibody Array, Raybiotech, GA, USA) according to manufacturer's instructions.

4.2.9. Statistical Analysis

All data are presented as means ± standard error of means (SEM) unless indicated otherwise. General linear model analysis of variance (GLM ANOVA) was used to determine significant differences of age and HCM mutation contributions on electrophysiological (ERP, CV, TAT and CHI) and histological parameters as appropriate using PASW Statistics 18 (IBM Corp., USA). Multiple comparisons with Tukey's post hoc test were conducted to identify strain and diet interactions. Statistical significance was established at $P < 0.05$.

4.3. RESULTS

4.3.1. Animal Characteristics

Table 1 highlights the animal characteristics in RCS10 mice and SWR controls. In young 10-week old mice, RCS10 mice fed CHOC diet had significantly heavier body weights than controls (33.3 ± 0.4 g vs. 22.3 ± 0.6 g, $P < 0.001$), whereas NOM and HFD diet did not significantly affect body weight. Additionally, post-hoc analysis demonstrated that CHOC diet significantly accelerated weight gain in young RCS10 mice compared to NOM and HFD diets (both $P < 0.001$). Unfasted blood glucose levels were significantly greater in young RCS10 mice fed CHOC diet ($P < 0.001$), but were unchanged with NOM and HFD diet. Lastly, urine glucose was not detectable in all SWR animals, but were elevated in all young RCS10 mice fed NOM, HFD, and CHOC diets with a trend towards greater glycosuria in RCS10 mice fed increasing fat concentrated diets (diet interaction: P = 0.073).

Older 30-week old RCS10 mice demonstrated significantly increased body weights compared to controls across all three diets (all $P < 0.001$). With sustained diabesity, 30-week old RCS10 mice fed NOM, HFD and CHOC diets developed hyperglycaemia compared to controls (all $P < 0.001$). Additionally, there was no significant diet interaction on blood glucose levels in RCS10 mice. Urine glucose levels were significantly elevated in 30-week old RCS10 mice fed NOM, HFD, and CHOC diets compared to controls (all $P < 0.001$). However, diet interaction on urine glucose levels was not significantly greater with increasing fat concentrated diets.

Additionally, we looked at circulating levels of adiponectin and leptin in the young RCS10 mice that have demonstrated diet-induced accelerated obesity. Adiponectin levels did not differ between RCS10 and SWR animals irrespective of diet feeding. Plasma leptin levels were significantly elevated in RCS10 mice fed CHOC diets compared to controls (2902.7 ± 515.9 vs. 70.8 ± 61.4 pg/mL, $P < 0.001$), whereas leptin levels in RCS10 mice fed NOM or HFD diets did not differ from controls.

4.3.2. Atrial Effective Refractory Period and Action Potential Duration in Diabesity

Figure 1 and 2 demonstrates atrial ERP and APD90 in RCS10 mice and SWR controls respectively. Atrial ERP was increased in 10-week old RCS10 mice fed HFD and CHOC diets compared to controls (49.3 ± 2.9 vs. 39.5 ± 3.4, $P < 0.05$; 75.7 ± 4.7 vs. 54.2 ± 2.2 ms, $P < 0.001$ respectively), but did not significantly differ in the NOM diet group. Figure 1B depicts changes in atrial ERP with increasing pacing rates. GLM ANOVA revealed an overall increased ERPs in RCS10 mice fed CHOC diet compared to controls ($P < 0.001$), with greatest difference observed at higher pacing rates (100 ms: 83.9 ± 8.8 vs. 54.2 ± 15.7 ms, $P < 0.01$). In contrast, sustained diabesity in RCS10 mice resulted in unchanged ERPs compared to controls.

Similarly, APD90 was greater in 10-week old RCS10 mice fed NOM, HFD and CHOC diet compared to controls (31.7 ± 0.3 vs. 29.1 ± 0.4 ms, $P < 0.001$; 37.4 ± 0.3 vs. 25.1 ± 0.2 ms, $P < 0.001$; 44.5 ± 0.8 vs. 26.1 ± 0.3 ms, $P < 0.001$ respectively). Additionally, GLM ANOVA revealed that APD90 was similarly reduced in both

RCS10 and SWR animals fed NOM, HFD and CHOC diets at increased pacing rates (cycle-length interaction: all $P < 0.01$) without significant strain*cycle-length interaction. Increased age at 30 weeks demonstrated unchanged APD90 in RCS10 animals as compared to controls.

4.3.3. Slowed and Heterogeneous Atrial Conduction in Diabesity

Figure 3 illustrates representative activation time maps of 10- and 30-week old RCS10 and SWR animals and their respective phase histograms. Development of diabesity resulted in progressive and significant conduction slowing and heterogeneity with increasingly fat diets in the young RCS10 mice. In the older age group, RCS10 mice with established diabesity demonstrated progressively heterogeneous conduction with increasing fat concentrated diets, whereas conduction velocity was significantly slowed compared to controls in all diets.

In young mice, all RCS10 animals fed NOM, HFD, and CHOC diets demonstrate reduced conduction velocity compared to controls (0.303 ± 0.007 vs. 0.327 ± 0.006 m/s, $P < 0.05$; 0.269 ± 0.009 vs. 0.309 ± 0.008 m/s, $P < 0.01$; 0.244 ± 0.007 vs. 0.314 ± 0.006 m/s, $P < 0.001$ respectively; Figure 4A). Additionally, GLM ANOVA revealed significant diet interactions ($P < 0.05$), with RCS10 animals fed HFD and CHOC diets demonstrating significantly lower CV compared to NOM diet animals on post hoc analyses ($P < 0.01$ and $P < 0.001$ respectively), but were not statistically different between HFD and CHOC diet animals. Additionally, conduction velocity was reduced in RCS10 mice fed NOM, HFD and CHOC diets

across all pacing cycle-lengths, without significant cycle-length interactions (Figure 4B). 10-week old RCS10 mice also demonstrated progressive increase in conduction heterogeneity with increasing fat content diets with significant increase CHI in HFD and CHOC diets compared to controls (1.90 ± 0.06 vs. 1.67 ± 0.03, $P < 0.01$; 2.30 ± 0.07 vs. 1.75 ± 0.05, $P < 0.001$ respectively), but not in NOM diet animals (1.70 ± 0.05 vs. 1.76 ± 0.05; Figure 5A). In addition, the elevated CHI in RCS10 mice fed CHOC diets was significantly greater than RCS10 mice fed NOM and HFD diets (both $P < 0.001$). The increase in CHI in RCS10 mice fed HFD was predominantly evident at 300 and 400 ms pacing (both $P < 0.01$), whereas RCS10 mice fed CHOC demonstrated elevated CHI across all pacing cycle-lengths tested ($P < 0.001$).

Conduction velocity was significantly reduced in all 30-week old RCS10 mice fed NOM, HFD, and CHOC diets compared to controls (0.260 ± 0.005 vs. 0.320 ± 0.005, $P < 0.001$; 0.283 ± 0.005 vs. 0.328 ± 0.008, $P < 0.001$; 0.255 ± 0.005 vs. 0.308 ± 0.007, $P < 0.001$; Figure 4A), without significant diet interaction observed. Additionally, the reduction in CV was significant across all cycle-lengths in all 3 diets (Figure 4B). Older RCS10 mice at 30 weeks of age demonstrated overall increased CHI across NOM, HFD, and CHOC diets compared to controls (2.02 ± 0.05 vs. 1.71 ± 0.08, $P < 0.05$; 2.61 ± 0.07 vs. 1.56 ± 0.10, $P < 0.001$; 2.59 ± 0.06 vs. 1.47 ± 0.04, $P < 0.001$ respectively; Figure 5A). GLM ANOVA revealed significant diet interactions with older RCS10 mice fed HFD and CHOC diets similarly demonstrated increased CHI compared to the NOM diet on post hoc analyses ($P < 0.001$ and $P < 0.05$), without significant difference between HFD and CHOC diets.

The increased conduction heterogeneity in RCS10 mice fed NOM diets was evident increasing pacing rates, whereas CHI was increased across all pacing cycle-lengths in RCS10 mice fed HFD and CHOC diets (both $P < 0.001$).

4.3.4. Arrhythmia Inducibility in Diabesity Mice

We observed self-propagating atrial tachyarrhythmias during electrophysiological study in 1 SWR (30 weeks; HFD), 1 RCS10 (10 weeks; NOM), 1 RCS10 (30 weeks; HFD), and 3 RCS10 (30 weeks; CHOC). Averaged arrhythmia duration in the single SWR mouse was 3.0 ± 0.4 s (total 2 episodes). Averaged arrhythmia duration in RCS10 mice was 3.6 ± 0.5 s (total 17 episodes). Unfortunately, this study was not sufficiently statistically powered to assess the effect of diet and strain on inducibility of arrhythmias.

4.3.5. Atrial Histopathology in Diabesity

Figure 6 and 7 are representative photomicrographs of atrial myocardium from 10- and 30-week old RCS10 and SWR animals stained with H&E or Masson's Trichrome respectively, and Figure 8 illustrates the overall cardiomyocyte hypertrophy, fibrosis accumulation and inflammatory infiltration in the atrial myocardium.

10-week old RCS10 animals fed NOM, HFD, and CHOC diet demonstrated increased cardiomyocyte diameter compared to controls (8.33 ± 0.23 vs. 7.78 ±

0.17 μm, $P < 0.001$; 8.35 ± 0.14 vs. 7.60 ± 0.16 μm, $P < 0.001$; 8.76 ± 0.22 vs. 7.65 ± 0.20 μm, $P < 0.001$ respectively; Figure 8A). Additionally, no significant diet interaction was observed with cardiomyocyte hypertrophy. We observed a progressive increase in diet-induced fibrosis accumulation in 10-week old RCS10 mice compared to controls with increasing fat content diets (strain interaction: $P < 0.001$, diet interaction: $P < 0.05$, and strain*diet interaction: $P < 0.05$). Fibrosis was increased in 10-week old RCS10 animals fed HFD and CHOC diets compared to controls (2.80 ± 0.53 vs. 0.81 ± 0.08 %, $P < 0.05$; 3.31 ± 0.81 vs. 0.70 ± 0.05 %, $P < 0.05$ respectively) but not were unchanged with NOM diet (1.11 ± 0.08 vs. 0.84 ± 0.16 %, $P = 0.171$; Figure 8B). Additionally, post hoc analyses demonstrated significantly greater fibrosis in RCS10 animals fed CHOC diet compared to NOM diet ($P < 0.05$). Lastly, inflammatory cell infiltration was elevated in 10-week old RCS10 mice compared to controls without significant influence of diet (strain interaction: $P < 0.001$, diet interaction: $P = 0.4$). RCS10 mice fed NOM, HFD and CHOC demonstrated greater inflammatory cell infiltrates compared to controls (4.89 ± 0.32 vs. 4.01 ± 0.25, $P = 0.06$; 5.12 ± 0.30 vs. 3.94 ± 0.14, $P < 0.01$; 5.57 ± 0.43 vs. 4.11 ± 0.28, $P < 0.05$ respectively; Figure 8C).

30-week old RCS10 animals fed NOM, HFD, and CHOC diet demonstrated increased cardiomyocyte diameter compared to controls (8.74 ± 0.16 vs. 8.08 ± 0.13 μm, $P < 0.01$; 8.74 ± 0.12 vs. 8.11 ± 0.21 μm, $P < 0.01$; 8.81 ± 0.38 vs. 8.06 ± 0.23 μm, $P < 0.01$ respectively; Figure 8A). Additionally, no significant diet interaction was observed with cardiomyocyte hypertrophy. We observed a progressive increase in diet-induced fibrosis accumulation in 30-week old RCS10

mice compared to controls with increasing fat content diets (strain interaction: $P < 0.01$, diet interaction: $P < 0.05$, and strain*diet interaction: $P < 0.05$). Fibrosis was increased in 30-week old RCS10 animals fed NOM, HFD and CHOC diets compared to controls (2.07 ± 0.42 vs. 0.62 ± 0.05 %, $P < 0.05$; 2.29 ± 0.55 vs. 0.78 ± 0.07 %, $P < 0.05$ respectively; 4.50 ± 0.91 vs. 0.55 ± 0.12 %, $P < 0.01$; Figure 8B). Additionally, post hoc analyses demonstrated significantly greater fibrosis in RCS10 animals fed CHOC diet compared to NOM diet ($P < 0.05$). Lastly, inflammatory cell infiltration was elevated in 30-week old RCS10 mice compared to controls without significant influence of diet (strain interaction: $P < 0.001$, diet interaction: $P = 0.2$). RCS10 mice fed NOM, HFD and CHOC demonstrated greater inflammatory cell infiltrates compared to controls (4.87 ± 0.32 vs. 3.84 ± 0.08, $P < 0.05$; 5.24 ± 0.23 vs. 3.99 ± 0.14, $P < 0.01$; 5.76 ± 0.29 vs. 3.85 ± 0.28, $P < 0.001$ respectively; Figure 8C).

4.3.6. Biomarkers of Extracellular Matrix Remodelling

Figure 9 illustrates the plasma concentrations of MMP-2, MMP-3, pro-MMP-9, TIMP-1 and TGFβ1 in RCS10 and SWR animals. Plasma MMP-2 levels were significantly elevated in RCS10 mice compared to controls (70.1 ± 4.5 vs. 56.4 ± 4.1 ng/mL, $P < 0.05$) with no significant effect with increasing fat content diets ($P = 0.14$). Increase in pro-MMP-9 levels trended towards significance in RCS10 mice compared to controls (300.3 ± 77.2 vs. 134.1 ± 30.7 pg/mL, $P = 0.055$) without significant difference between diets ($P = 0.23$). TIMP-1 was significantly decreased in RCS10 animals compared to controls (138.4 ± 32.2 vs. 361.9 ± 40.3 pg/ml, $P < 0.001$). Additionally, TIMP-1 levels in RCS10 mice fed CHOC diets trended towards further decrease compared to normal diets ($P = 0.062$). TGFβ1 was significantly

increased in RCS10 mice compared to controls (704.2 ± 131.5 vs. 39.1 ± 22.4 pg/mL, $P < 0.001$). Additionally, the increased plasma concentration of TGFβ1 was greatest in RCS10 mice fed CHOC compared to NOM and HFD ($P < 0.001$ and $P < 0.01$ respectively). On the other hand, plasma MMP-3 was unchanged between RCS10 and SWR mice.

4.3.7. Biomarkers of Inflammation/Leukocyte and Monocyte Activation

Figure 10 represents plasma concentrations of TNFα, ICAM-1 and VCAM-1 in RCS10 and SWR controls. Plasma levels of TNFα was significantly elevated in RCS10 mice compared to controls (246.6 ± 17.0 vs. 117.8 ± 23.0 pg/mL, $P < 0.001$), regardless of diets fed ($P = 0.321$). Overall concentration of ICAM-1 was significantly elevated in RCS10 mice compared to controls (223.2 ± 24.6 vs. 148.2 ± 15.4 pg/mL, $P < 0.05$), regardless of diet ($P = 0.145$). VCAM-1 was significantly increased in RCS10 mice compared to controls (12.4 ± 0.3 vs. 10.9 ± 0.2 ng/mL, $P < 0.001$). RCS10 mice fed CHOC diet demonstrated significantly greater VCAM-1 levels compared to NOM diet.

4.4. DISCUSSION

In this study, we have demonstrated in NONcNZO10/LtJ (RCS10) mice, a polygenic model of diabesity that the following occurs:

1. RCS10 mice demonstrate maturity-onset diabetes and obesity (young vs. old RCS10 fed normal diets).
2. This maturity-onset of diabesity can be progressively accelerated with diets of higher fat content, demonstrating a parallel increase of leptin levels but normal adiponectin levels (young mice fed normal to CHOC diet).
3. RCS10 mice demonstrated progressively increased atrial refractoriness and APD in the young but not the older animals with established diabesity.
4. Diet and age-related slowed and heterogeneous conduction in the atria.
5. RCS10 mice demonstrate atrial myocyte hypertrophy and diet-induced increase in fibrosis and inflammatory infiltrates.
6. Diabesity mice demonstrate altered plasma concentrations of proteins involved in extracellular matrix maintenance and inflammatory cell recruitment.

We believe that this is the first study that has characterised electrophysiological and structural remodelling in the atria of the polygenic RCS10 mouse. These findings have significant implications by highlighting diet-induced changes in the atrial myocardium that can predispose to atrial fibrillation in a polygenic cause of diabetes and obesity, a closer mimic to the human diabesity syndrome.

The polygenic RCS10 mouse model was developed to harvest the quantitative trait loci contributing to both diabetes and obesity, exhibiting diabesity at frequencies of 90-100% in males when fed an elevated fat diet (434). Our results demonstrated diet-induced increase in body weights and glucose levels in both the bloodstream and urine with progressively elevated fat content in post-weaning diets in the young animals. Surprisingly, the RCS10 mice in the current study were obese and hyperglycaemic despite the 5% diet after 30 weeks of age. We do not know of any long-term studies that investigated the use of 5% fat diets in these animals. Although it has been noted that >11% fat diets, instead of the original 6% fat, was required to drive diabesity in these animals following re-derivation into a higher barrier colony (434). Our results may suggest that the RCS10 mice develop maturity-onset diabetes and obesity even at the low fat diets and the rate of maturation may be accelerated with greater fat content diets. Alternatively, in-breeding of the RCS10 mice at our animal facility may have contributed to increasing susceptibility of the RCS10 offspring to diabetes and obesity despite the low fat diet.

Increased plasma leptin levels in 10-week old RCS10 mice were observed when fed chocolate supplemented high fat diet, but not in the normal and high fat diet groups. Leptin, an adipocyte-derived hormone, inhibits β cell insulin secretion through both direct and indirect mechanisms (436). Additionally, circulating leptin levels have been suggested to reflect body lipid content in mice (437). As expected, we observed a progressive increase in leptin levels of RCS10 mice weaned onto normal to high fat and chocolate diets that parallels the increase in body mass. Despite the fact that obese patients often demonstrate reduced adiponectin levels

(438,439), we did not observe any differences in adiponectin levels of RCS10 mice fed all 3 diets. Adiponectin is an adipocyte secreted hormone that increases insulin sensitivity by reducing triglyceride levels in the muscles and liver (440). Circulating adiponectin has been previously shown to correlate positively insulin sensitivity and improvement in body mass index (438,441). Notably, adiponectin has been previously demonstrated to be transiently elevated in the RCS10 mouse at 6 weeks before declining over a period of 16 weeks (431). This transient increase in adiponectin levels may reflect the transient insulin resistance also observed in human patients (442), and may explain for the unchanged adiponectin levels in animals with chronic hyperglycaemia in the current study.

4.4.1. Electrophysiology of the Diabesity Atria

Atrial ERP and APD90 were significantly greater in the 10-week old RCS10 mice fed chocolate-supplemented high fat diet in parallel with the significant weight gain and hyperglycaemia. In contrast, sustained diabesity resulted in normalization of the ERP and APD. The reasons for this remain unclear. However, we noted that atrial myocytes were larger in the 30-week old that may be a manifestation of atrial stretch or dilatation which is known to result in shortening of the atrial ERP (443). Furthermore, oxidative stress can contribute to ERP shortening (444), although we did not investigate changes in reactive oxygen species expression in the current study. ERP and APD prolongation has been previously observed in some diabetic studies (272,445), as well as no change in ERP despite AF vulnerability (271,446-448).

With progression of hyperglycaemia and obesity with increasing fat diets, the isolated atria in RCS10 animals demonstrate progressive reductions in conduction velocity and heterogeneous conduction propagation. These findings are similar to that of other established diabetic models that have reported slowed inter- or intra-atrial activation time (445,446), reduced conduction velocity and increased heterogeneity (272). Likewise, other models of obesity such as the high caloric diet fed sheep results in progressive decrease in conduction velocity and heterogeneity, whereas atrial refractoriness was unchanged (167,449). These were primarily associated with structural remodelling including atria enlargement, fibrosis and inflammatory infiltrates.

In total, we observed 1 SWR control and 5 RCS10 diabesity mice demonstrated self-propagating atrial arrhythmias during the electrophysiological study. However, there was insufficient statistical power to identify any diet or age effect on the vulnerability of arrhythmias in the current study. The reasons for the low incidence of inducible arrhythmias remains unclear but may have been due to the small size of the mouse atria to sustain arrhythmias in the setting of the isolated atria protocol.

4.4.2. Histopathology of the Diabesity Atria

In the current study, we observed myocyte hypertrophy in RCS10 mice regardless of diet that may be due to the polygenic cause for diabesity. Myocyte hypertrophy in the ventricles of diabetic and/or obese animals have been commonly reported by others (450-453), although evidence of atrial myocyte hypertrophy has been

scarce. Additionally, increased interstitial fibrosis and inflammatory cell infiltration was associated with progressively increased fat-enriched diet in the current model of diabesity. Likewise, others have observed increased atrial fibrosis in other diabetic models that can contribute to the vulnerable substrate for arrhythmias (272,273,446). Furthermore, inflammatory infiltrates such as macrophages and leukocytes are known to secrete growth factors, such as TGF-β1, that activate fibroblasts to secrete pro-fibrotic and pro-apoptotic factors resulting in extracellular matrix deposition and apoptosis in the diabetic and/or obese myocardium (453,454). Similarly, we have observed increased plasma TGF-β1 levels in the RCS10 mice. It has been postulated that fibrosis can disrupt inter-myocyte coupling, resulting in areas of slowed conduction and block and contributing to a sustained substrate for AF development (113). Accordingly, enhanced atrial fibrosis by overexpressing TGF-β1 in mice has been demonstrated to alter electrophysiological properties and increase AF vulnerability (455).

4.4.3. Biomarkers of Extracellular Matrix Remodelling and Inflammation in Diabesity

We observed elevated plasma concentrations of MMP-2, pro-MMP-9, TGF-β1, unchanged MMP-3, and decreased TIMP-1 in the RCS10 mouse. In contrast, Adi et al. (2012) demonstrated altered gene expressions in a variety of extracellular matrix remodelling proteins including increased MMP-2, increased MMP-3, unchanged MMP-9, increased TIMP-1 in the visceral adipose tissue of 25 week old RCS10 mice compared to age-matched SWR controls when fed a 27% fat diet (456). The disparity between our findings and Adi et al. (2012) may reflect the

differences in sampling (plasma vs. visceral adipose tissue), high fat diets (11% fat vs. 27% fat), type of analyses (protein vs. gene expression) or age (10 weeks vs. 25 weeks). Our findings of elevated plasma concentrations of MMP-2 and/or pro-MMP-9 are in accordance with diabetic patients (457-459) or patients with dyslipidaemia (460), but not others (461). In streptozotocin-induced type I and II diabetes rats, increased activity and expression of MMP-9, but not MMP-2, were observed in the vasculature and plasma (462). On the other hand, alterations in plasma TIMP-1 levels have been controversial in diabetes with some reporting an increased in TIMP-1 levels (457,458), whereas other reporting a decrease (461). Decreased plasma TIMP-1 levels have also been previously reported in obese children with hypoadiponectinaemia (463). Recent evidence have suggested that adiponectin increases TIMP-1 secretion through macrophages (464), whereas adiponectin levels were unchanged in the current study. Alternatively, TIMP-1 levels are known to be decreased in patients with diabetic nephropathy (465) and RCS10 mice are known to develop diabetic nephropathy (466). Noting that TIMP-1 is a potent inhibitor of activated MMPs, a decrease in TIMP-1 levels and a concurrently increase in MMPs may suggest enhanced extracellular matrix turnover. Coupled with elevations in the concentration of pro-fibrotic TGF-β1, this may explain for our observations of increased fibrosis in the atria.

Recent evidence suggests that type II diabetes mellitus is a condition mediated by inflammatory cytokines (467). In obesity-linked insulin resistant animals (ob/ob mouse, db/db mouse, and Zucker fatty rats), Hotamisligil et al. (1993) demonstrated that TNFα expression is increased in adipocytes and insulin

resistance in these animals can be modulated by neutralisation of TNFα (468). In the RCS10 mice, we noted diet-induced increases in TNFα, as well as cell adhesion molecules ICAM-1 and VCAM-1 concentrations. Increased expression of ICAM-1 and VCAM-1 have previously been associated with increased glomerular macrophage recruitment in streptozotocin-induced diabetes (469). The diet-induced elevation of these inflammatory cytokines may explain for our observation of increased inflammatory infiltrates in the atrial myocardium in the RCS10 mice.

4.4.4. Clinical Implications for AF

Evidence suggest that atrial myopathy plays a crucial role in providing a substrate that predisposes AF development (336). In particular, recent interests have been focused on atrial fibrosis based on modern MRI techniques used to visualize atrial fibrosis in humans that have demonstrated prognostic benefits in AF treatment (114). The current model demonstrates primary atrial histological and electrophysiological substrates that are associated with diabesity and fatty diets. Treating the underlying atrial histopathology with anti-inflammatory, anti-oxidant, and anti-fibrotic agents shows promise in improving the atrial substrate in diabesity. In addition, lifestyle modifications such as targeted weight loss goals and risk factor management have been shown to reduce AF burden in humans (425), and may likely elicit its benefits by treating the primary atrial myopathy. Identification of altered biomarkers may also be useful in assessment of AF risk and identify molecular pathways that can be targeted for treatment.

4.4.5. Study Limitations

Plasma biomarker assays were only carried out in the young group in order to identify early changes in biomarkers of the RCS10 mice in response to the different diets, which to the best of our knowledge has not been demonstrated before. We did not investigate if changes in these biomarkers are altered with increased age. Further studies looking at gene and protein expression are warranted to identify how these markers change in response to increasing age, dietary or lifestyle modifications.

4.5. CONCLUSIONS

This study has identified key pathological changes in both structural and electrophysiological remodelling of the atria in response to high-fat feeding of mice with polygenic diabesity. Additionally, biomarkers of extracellular matrix remodelling and inflammation are altered in the diabesity mouse. Further work is warranted to investigate if physical activity, dietary modifications, and/or pharmacological interventions can modulate these atrial electrophysiological and structural remodelling.

TABLE 1. ANIMAL CHARACTERISTICS

Young Mice (10 weeks)

	NOM		HFD		CHOC	
	SWR	RCS10	SWR	RCS10	SWR	RCS10
BW (g)	23.3 ± 0.7	25.0 ± 1.2	23.9 ± 0.7	26.3 ± 0.5	22.3 ± 0.6	33.3 ± 0.4***/ǂǂ
Blood glucose (mmol/L)	11.6 ± 0.9	12.8 ± 1.6	12.6 ± 1.2	15.6 ± 2.1	10.5 ± 1.2	26.4 ± 2.4***/ǂǂ
Urine glucose (mmol/L)	N.D.	11.1 ± 4.0***	N.D.	19.8 ± 4.1***	N.D.	31.0 ± 8.5***
Adiponectin (ng/mL)	14.4 ± 0.5	14.7 ± 0.8	15.9 ± 0.4	15.6 ± 0.6	14.8 ± 1.2	15.7 ±1.3
Leptin (pg/mL)	Below LOD	Below LOD	Below LOD	806.2 ± 497.0	212.5 ± 93.8	2902.7 ± 515.9***/ǂ

Old Mice (30 weeks)

	NOM		HFD		CHOC	
	SWR	RCS10	SWR	RCS10	SWR	RCS10
BW (g)	30.3 ± 0.6	38.0 ± 2.4***	29.0 ± 0.4	37.0 ± 1.4***	29.0 ± 0.9	40.7 ± 2.1***
Blood glucose (mmol/L)	13.0 ± 0.6	25.7 ± 3.4***	10.7 ± 3.0	20.7 ± 2.1***	11.9 ± 1.8	26.4 ± 2.9***
Urine glucose (mmol/L)	N.D.	21.3 ± 8.2***	N.D.	27.8 ± 6.2***	N.D.	41.7 ± 8.8***
Adiponectin (pg/mL)	----	----	----	----	----	----
Leptin (pg/mL)	----	----	----	----	----	----

*** $P < 0.001$ vs. age-matched SWR. ǂ $P < 0.01$ and ǂǂ $P < 0.001$ vs. NOM diet. Multiplex ELISAs were conducted for circulating adiponectin and leptin levels in the young animals but not in the old animals.

FIGURE LEGENDS

Figure 1. Diet-induced early prolongation of atrial effective refractory period (ERP) in RCS10 mice that normalised with sustained diabesity. (A) Mean atrial ERP in 10-week old (left) and 30-week old mice (right) fed NOM, HFD, or CHOC diets. The graph was pooled for pacing cycle lengths. (B) Mean atrial ERP in 10-week old (top) and 30-week old mice (bottom) at increasing pacing rates. * P <0.05, ** P < 0.01, *** P < 0.001 vs. diet-matched SWR, ## P < 0.001 vs. strain-matched NOM and HFD.

Figure 2. Diet-induced early prolonged atrial action potential duration at 90% repolarisation (APD90) in RCS10 mice that normalised with sustained diabesity. (A) Mean APD90 in 10-week old (left) and 30-week old mice (right) fed NOM, HFD, or CHOC diets. The graph was pooled for pacing cycle lengths. (B) Mean APD90 in 10-week old (top) and 30-week old mice (bottom) with pacing rate adaptations at increasing pace rates. * P <0.05, ** P < 0.01, *** P < 0.001 vs. diet-matched SWR, ## P < 0.001 vs. strain-matched NOM and HFD.

Figure 3. Representative activation time maps and corresponding phase histograms. (A) Activation time maps were constructed with corresponding phase histograms in representative 10-week old RCS10 and SWR mice. Young RCS10 mice demonstrated progressive conduction slowing with associated increase in conduction heterogeneity when fed HFD and CHOC diets. (B) Activation time maps were constructed with corresponding phase histograms in 30-week old RCS10 and

SWR mice. Slowed and heterogeneous conduction were observed in all RCS10 mice fed all three diets, with progressive increase in conduction heterogeneity when fed increasing fat content diets. Isochronal bars were constructed at 1 ms intervals and a time bar presented with red indicating the pacing site and violet representing the longest activation time recorded.

Figure 4. Diabesity-induced slowed atrial conduction velocity (CV) with diet and age. (A) Mean atrial CV in 10-week old (left) and 30-week old mice (right) fed NOM, HFD, or CHOC diets. The graph was pooled for pacing cycle lengths. (B) Mean atrial CV in 10-week old (top) and 30-week old mice (bottom) at increasing pacing rates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. diet-matched SWR, ǂ $P < 0.001$ vs. strain-matched NOM.

Figure 5. Diabesity-induced atrial conduction heterogeneity index (CHI) with diet and age. (A) Mean atrial CHI in 10-week old (left) and 30-week old mice (right) fed NOM, HFD, or CHOC diets. The graph was pooled for pacing cycle lengths. (B) Mean atrial CHI in young mice (top) and old mice (bottom) at increasing pacing rates. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. diet-matched SWR, ǂ P < 0.001 vs. strain-matched NOM.

Figure 6. Diabesity-associated cardiomyocyte hypertrophy and inflammatory cell infiltration in atrial myocardium. (A) Representative haematoxylin and eosin stained atrial sections from 10-week old mice fed NOM, HFD, and CHOC diet. (B) Representative haematoxylin and eosin stained sections of atrial myocardium

from 30-week old mice fed NOM, HFD, and CHOC diet. Atrial myocardium of RCS10 mice demonstrated larger cardiomyocytes and increased density of inflammatory cell infiltration (white arrows) with increasing fat content diets. Black bar represents 50 μm in length.

Figure 7. Diabesity-associated interstitial fibrosis in atrial myocardium (A) Representative Masson's Trichrome stained atrial sections from 10-week old mice fed NOM, HFD, and CHOC diet. (B) Representative Masson's Trichrome stained sections of atrial myocardium from 30-week old mice fed NOM, HFD, and CHOC diet. Atrial myocardium of RCS10 mice demonstrated evidence of increased infiltrating collagen deposition (stained blue). Black bar represents 50 μm in length.

Figure 8. Diet-induced atrial cardiomyocyte hypertrophy, fibrosis and inflammatory cell infiltration in RCS10 mice. (A) Cumulative data on atrial myocyte diameter in 10- and 30-week old RCS10 and control mice. (B) Cumulative data on atrial fibrosis was in 10- and 30-week old RCS10 and control mice. (C) Cumulative data on inflammatory cell infiltration in 10- and 30-week old RCS10 and control mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 9. Biomarkers of extracellular matrix remodelling. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. diet-matched controls; ## $P < 0.01$, ### $P < 0.001$ vs. strain-matched NOM and HFD.

Figure 10. Biomarkers of inflammatory mediators. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; # $P < 0.05$ vs. strain-matched NOM.

FIGURE 1.

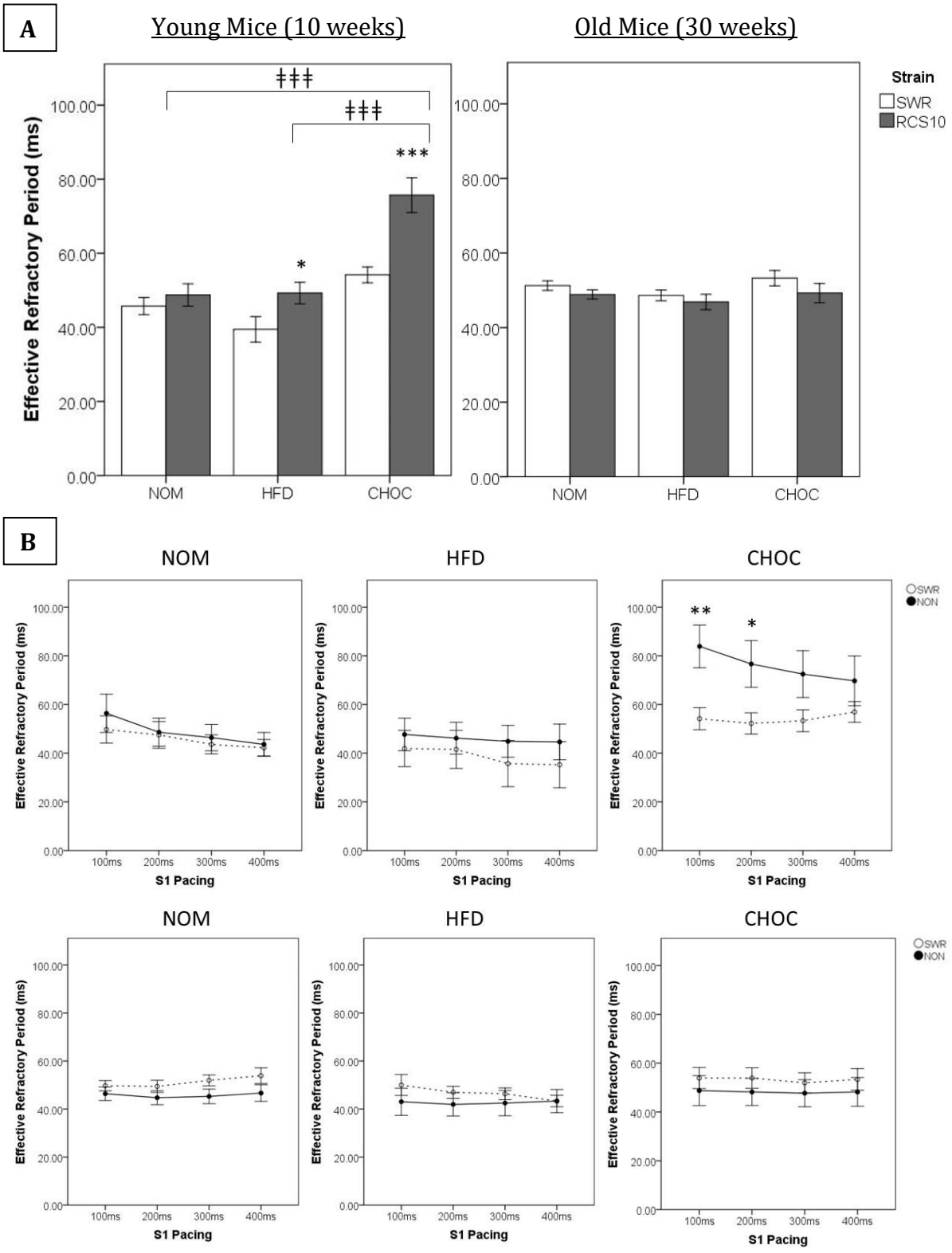

A
Young Mice (10 weeks)
Old Mice (30 weeks)
Strain
SWR
RCS10
Effective Refractory Period (ms)
NOM
HFD
CHOC
B
S1 Pacing
100ms
200ms
300ms
400ms
SWR
NON

FIGURE 2.

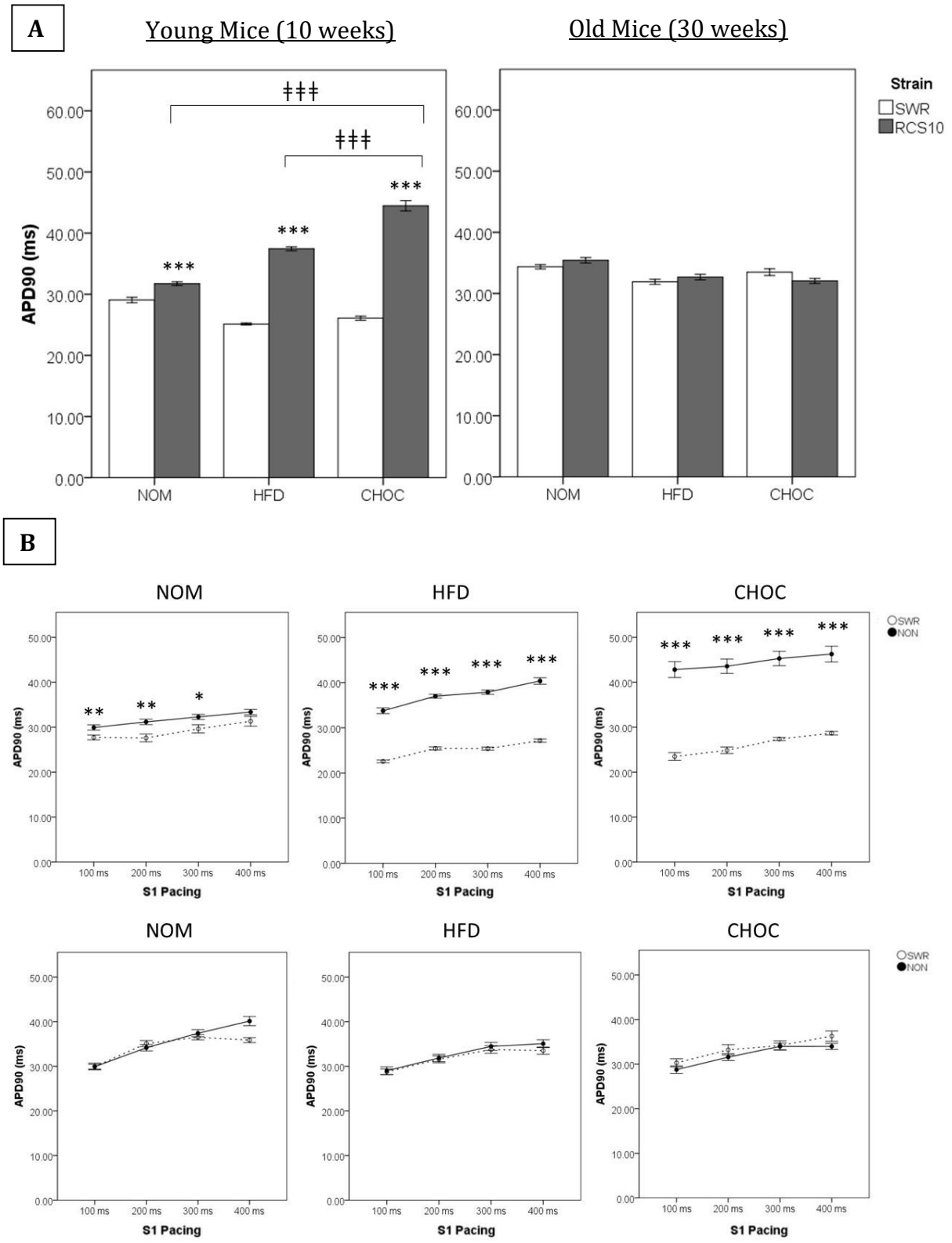

A
Young Mice (10 weeks)
Old Mice (30 weeks)
Strain
SWR
RCS10
APD90 (ms)
NOM
HFD
CHOC
B
S1 Pacing
100 ms
200 ms
300 ms
400 ms
SWR
NON

FIGURE 3.

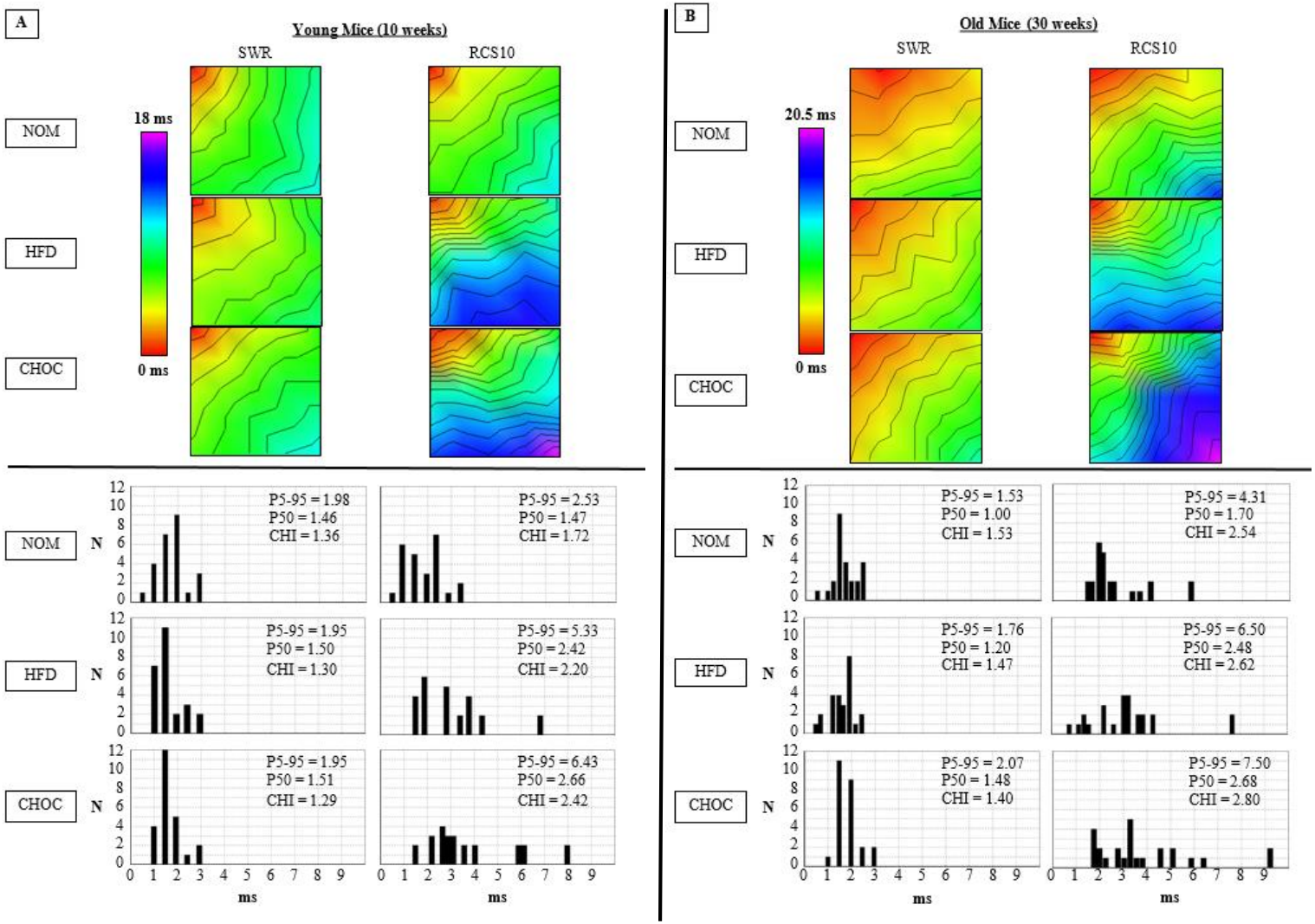

A
Young Mice (10 weeks)
SWR
RCS10
NOM
HFD
CHOC
18 ms
0 ms
P5-95 = 1.98
P50 = 1.46
CHI = 1.36
P5-95 = 2.53
P50 = 1.47
CHI = 1.72
P5-95 = 1.95
P50 = 1.50
CHI = 1.30
P5-95 = 5.33
P50 = 2.42
CHI = 2.20
P5-95 = 1.95
P50 = 1.51
CHI = 1.29
P5-95 = 6.43
P50 = 2.66
CHI = 2.42
N
ms
B
Old Mice (30 weeks)
SWR
RCS10
NOM
HFD
CHOC
20.5 ms
0 ms
P5-95 = 1.53
P50 = 1.00
CHI = 1.53
P5-95 = 4.31
P50 = 1.70
CHI = 2.54
P5-95 = 1.76
P50 = 1.20
CHI = 1.47
P5-95 = 6.50
P50 = 2.48
CHI = 2.62
P5-95 = 2.07
P50 = 1.48
CHI = 1.40
P5-95 = 7.50
P50 = 2.68
CHI = 2.80
N
ms

FIGURE 4.

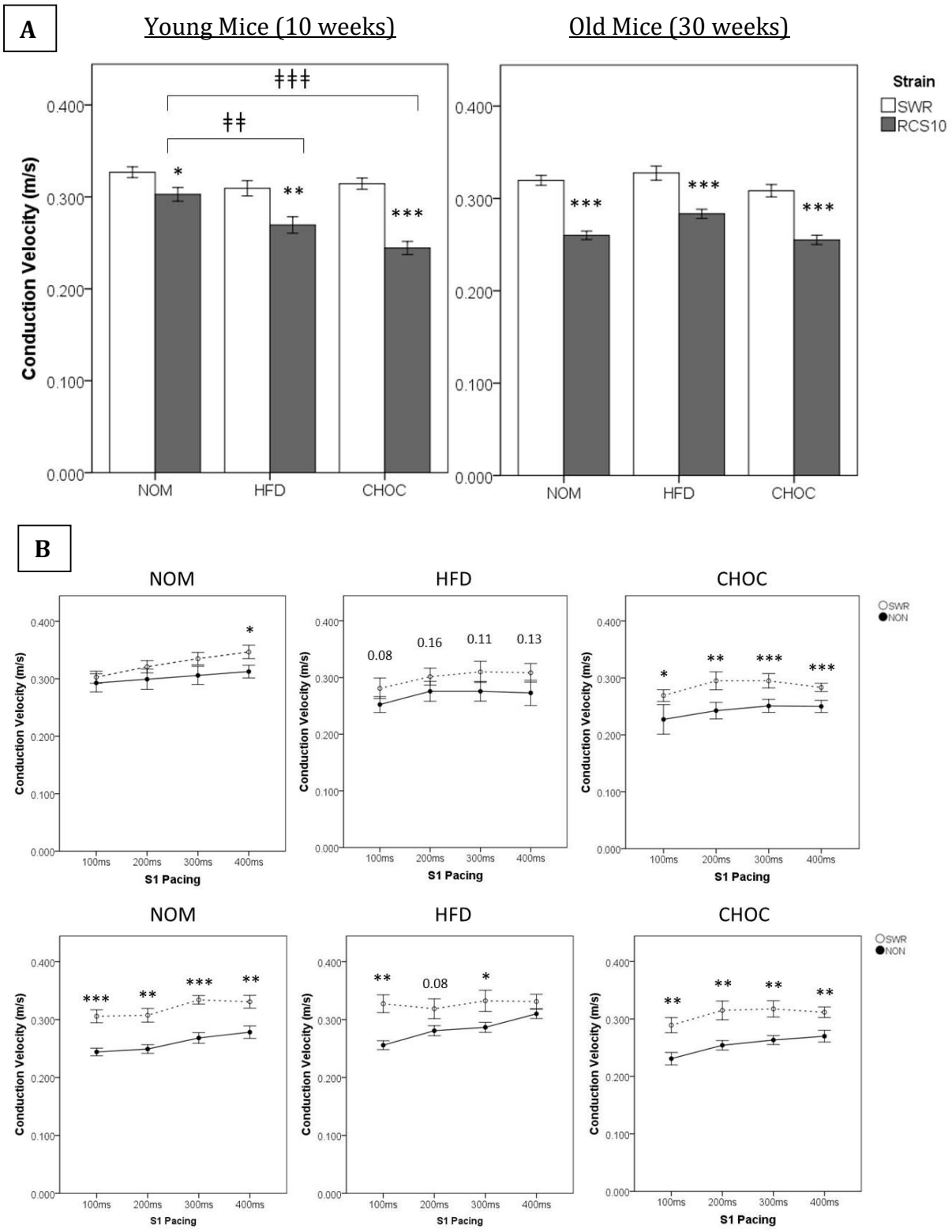

A
Young Mice (10 weeks)
Old Mice (30 weeks)
Strain
SWR
RCS10
Conduction Velocity (m/s)
0.400
0.300
0.200
0.100
0.000
NOM
HFD
CHOC
‡‡‡
‡‡
*
**
***
B
NOM
HFD
CHOC
SWR
NON
S1 Pacing
100ms
200ms
300ms
400ms
0.08
0.16
0.11
0.13

FIGURE 5.

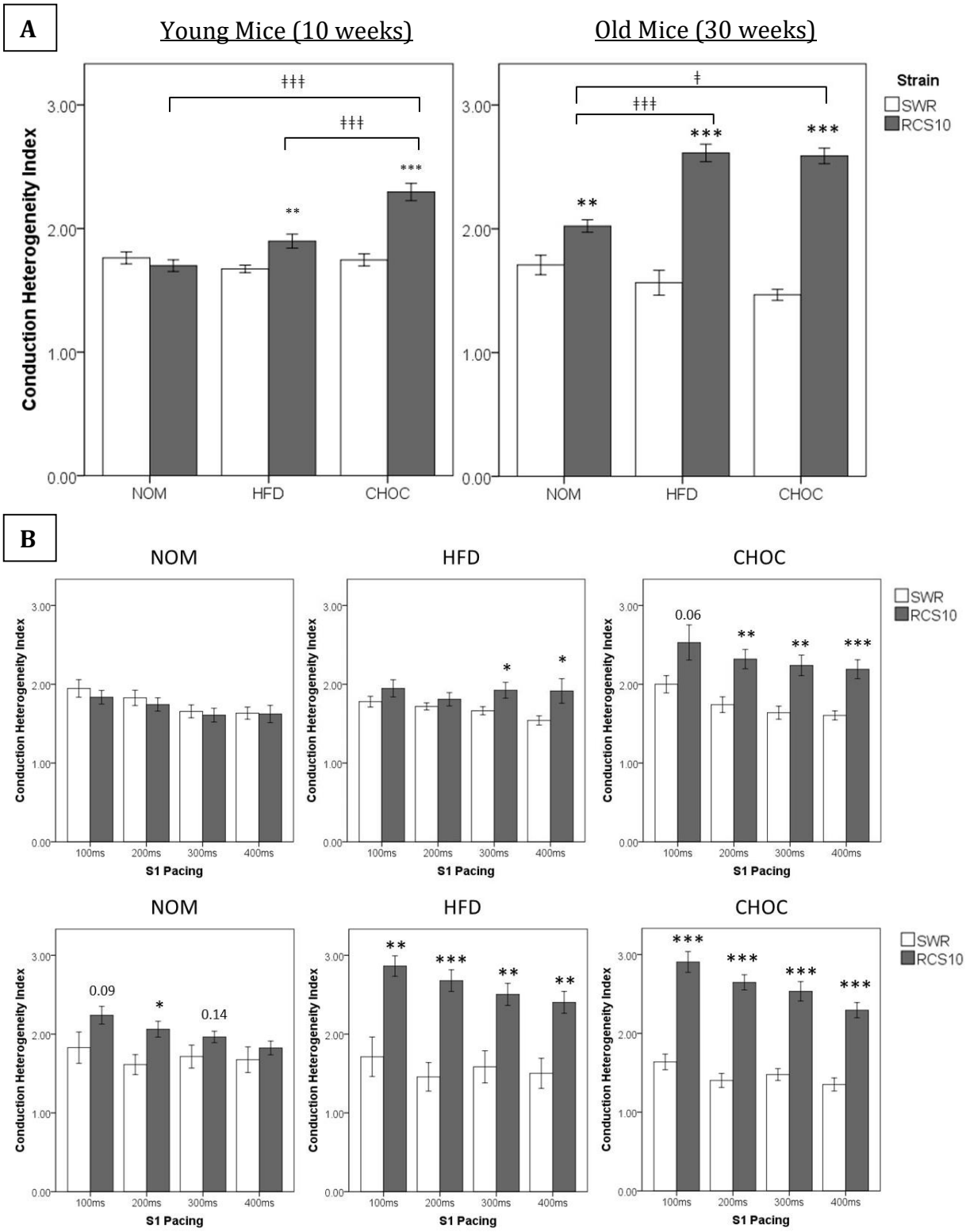

A
Young Mice (10 weeks)
Old Mice (30 weeks)
Conduction Heterogeneity Index
Strain
SWR
RCS10
NOM
HFD
CHOC
B
S1 Pacing
100ms
200ms
300ms
400ms
0.06
0.09
0.14

FIGURE 6.

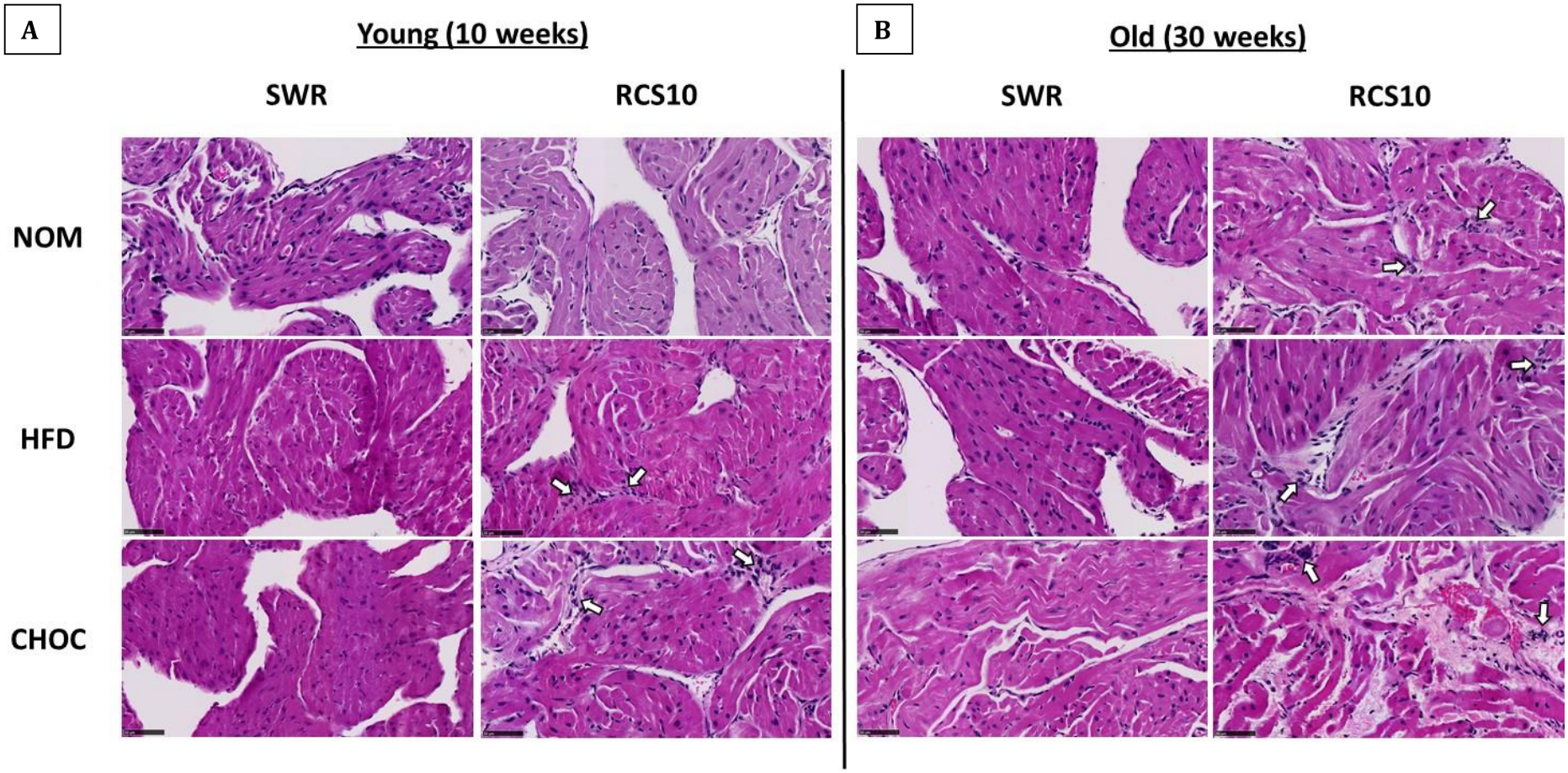

A
Young (10 weeks)
SWR
RCS10
NOM
HFD
CHOC
B
Old (30 weeks)
SWR
RCS10

FIGURE 7.

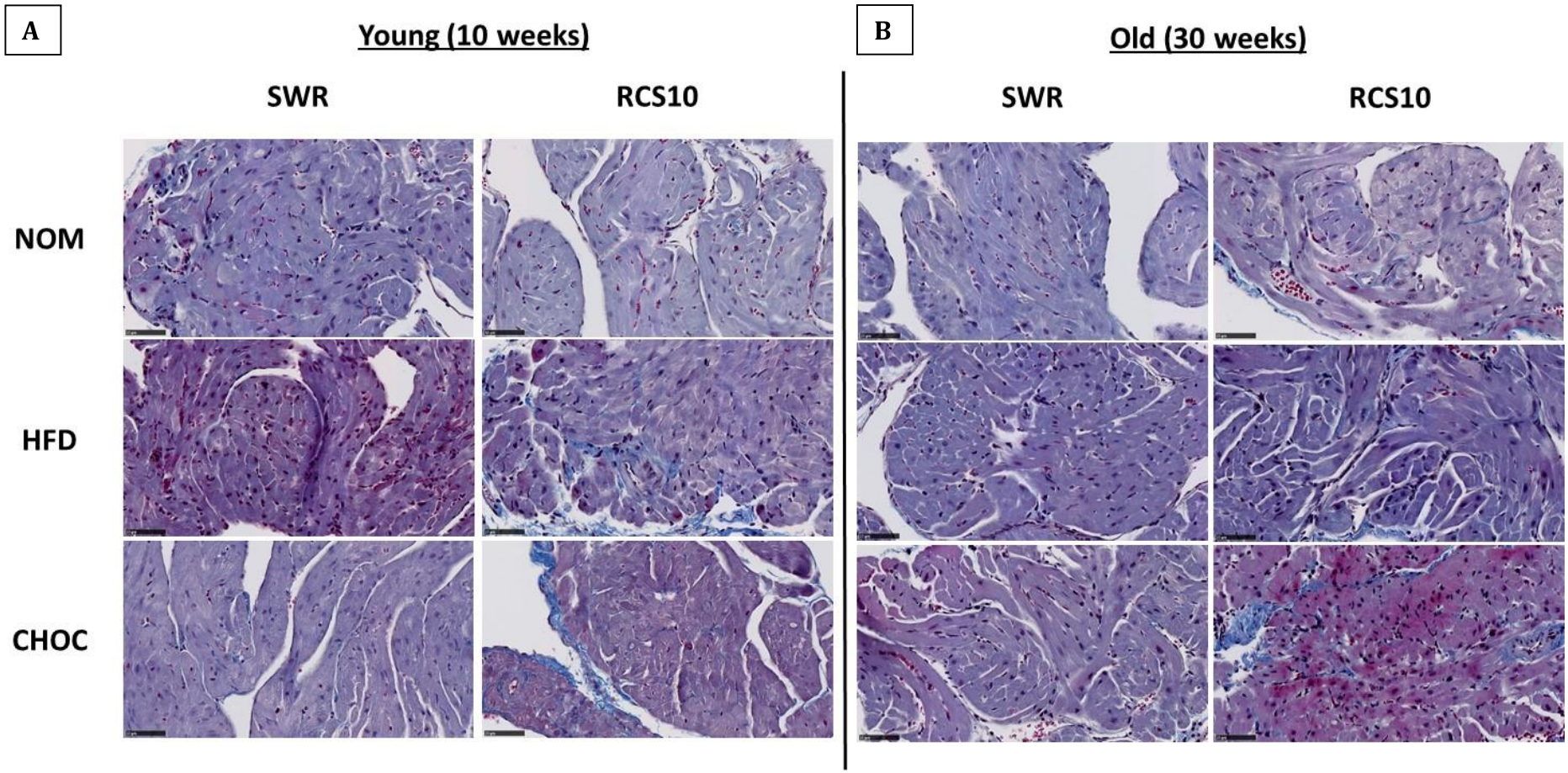

A
Young (10 weeks)
SWR
RCS10
NOM
HFD
CHOC
B
Old (30 weeks)
SWR
RCS10

FIGURE 8.

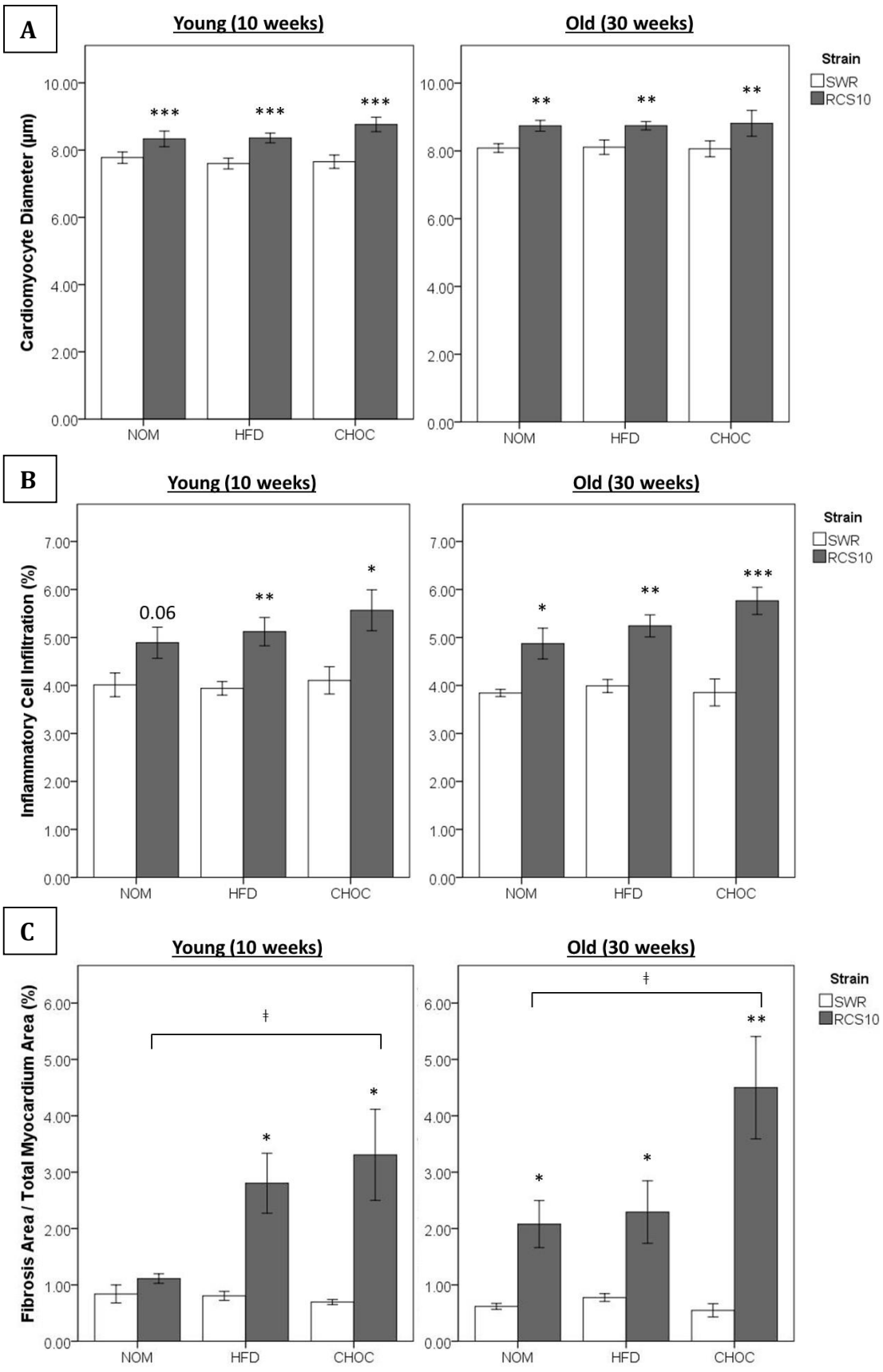

A
Young (10 weeks)
Old (30 weeks)
Strain
SWR
RCS10
Cardiomyocyte Diameter (µm)
10.00
8.00
6.00
4.00
2.00
0.00
***
**
NOM
HFD
CHOC
B
Young (10 weeks)
Old (30 weeks)
Strain
SWR
RCS10
Inflammatory Cell Infiltration (%)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.06
*
**
***
NOM
HFD
CHOC
C
Young (10 weeks)
Old (30 weeks)
Strain
SWR
RCS10
Fibrosis Area / Total Myocardium Area (%)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
‡
*
**
NOM
HFD
CHOC

FIGURE 9.

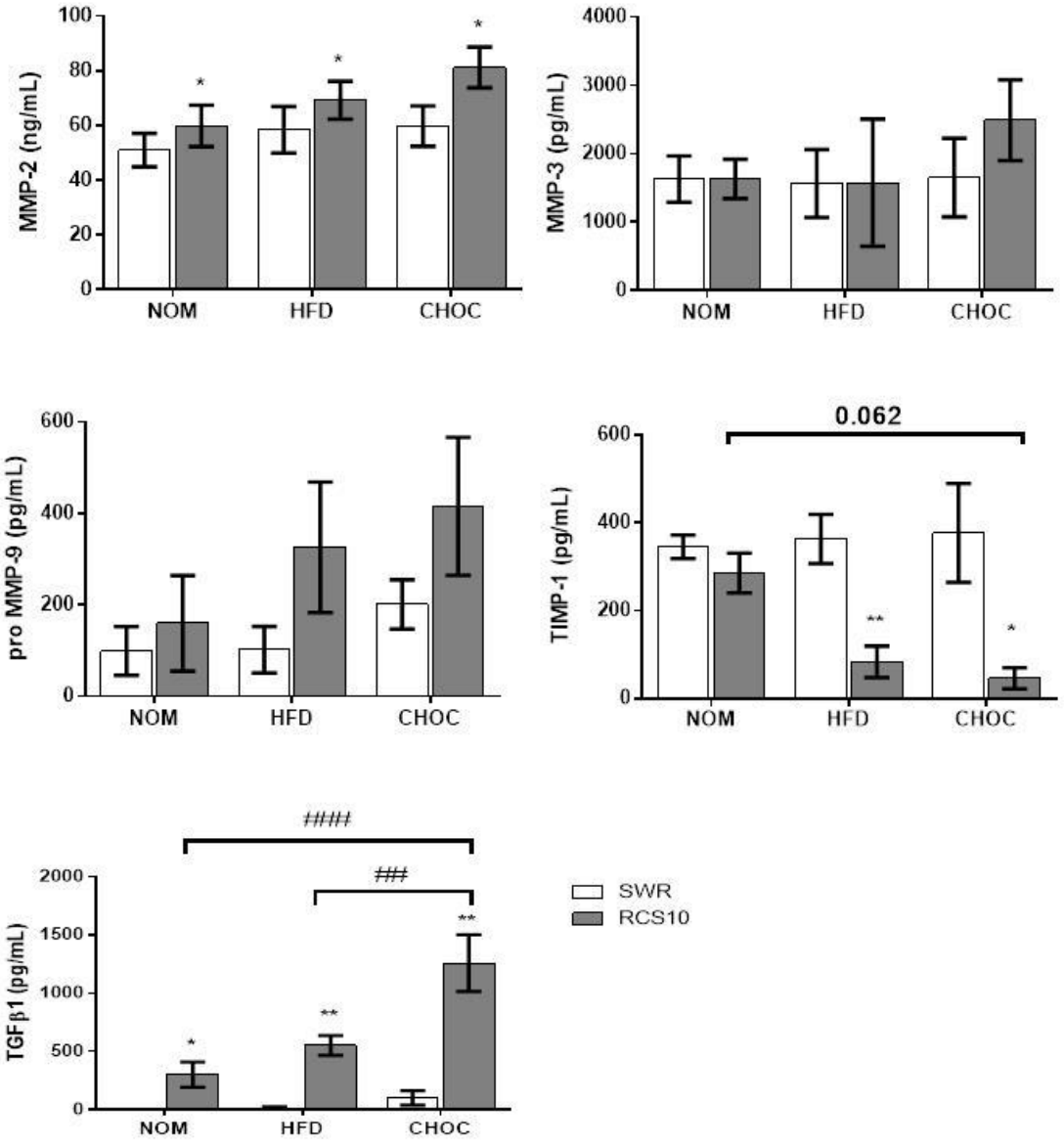
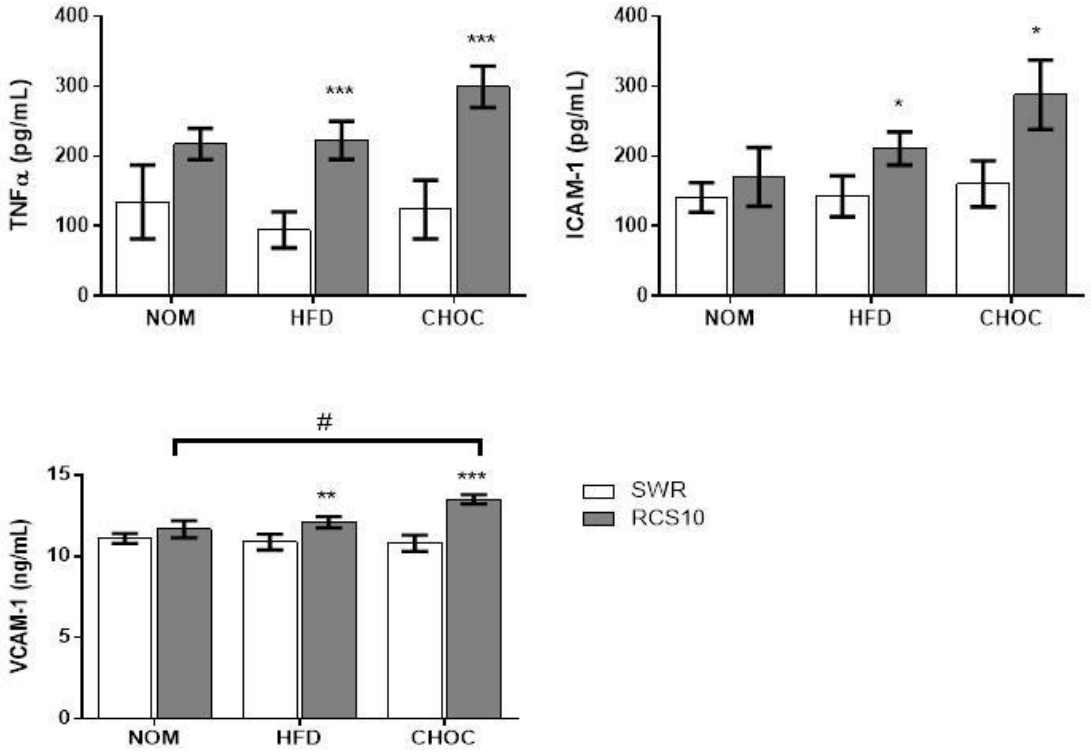

MMP-2 (ng/mL)
100
80
60
40
20
0
NOM
HFD
CHOC
MMP-3 (pg/mL)
4000
3000
2000
1000
0
pro MMP-9 (pg/mL)
600
400
200
TIMP-1 (pg/mL)
0.062
TGFβ1 (pg/mL)
2000
1500
1000
500
####
##
SWR
RCS10

FIGURE 10.


TNFα (pg/mL)
400
300
200
100
0
***
***
NOM
HFD
CHOC
ICAM-1 (pg/mL)
400
300
200
100
0
*
*
NOM
HFD
CHOC
VCAM-1 (ng/mL)
15
10
5
0
#
**
***
NOM
HFD
CHOC
SWR
RCS10

Statement of Authorship

Title of Paper	Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabetes and Obesity
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Wei-Wen Lim
Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article
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 7/10/2015

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	Melissa Neo
Contribution to the Paper	analysis and interpretation of research data drafting significant parts of the article
Signature	Date 7.10.15

Name of Co-Author	Mathias Baumert
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Signature	Date 8/10/15

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Signature	Date 7/12/2015

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Signature	Date 26.11.15

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Signature	Date 26/10/15

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Contribution to the Paper	conception and design of the project analysis and interpretation of research data drafting significant parts of the article or revising it critically for important intellectual content
Signature	Date 07/10/15

Please cut and paste additional co-author panels here as required.

Wei Wen Lim

From: Anand Ganesan
Sent: Saturday, 7 November 2015 7:49 AM
To: Wei Wen Lim
Subject: RE: Author Statements

Dear Wei Wen,
I am in the USA and do not have access to printer or Adobe PDF writer.

I do approve the manuscripts. Please take this email as affirmation of my approval for the purpose of the form.

Best regards,

Anand

ANAND GANESAN MBBS (Hons.) PhD FRACP CEPS CCDS

Michel Mirowski Fellow, Heart Rhythm Society
NHMRC Australian Early Career Health Practitioner Fellow
CRICOS Provider Number 00123M

From: Wei Wen Lim
Sent: Saturday, 7 November 2015 7:38 AM
To: Anand Ganesan
Subject: Author Statements

Dear Anand,

I've resent the Author Statements forms for my Thesis Chapters manuscripts requiring your signatures.

The Thesis Chapters include:

1) Electrophysiological and Structural Remodelling of the Atria in Hypertrophic Cardiomyopathy: Implications for Atrial Fibrillation
2) Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabesity
3) Atrial Electrophysiological and Structural Remodelling in Diabesity: Implications for Arrhythmogenesis in Diet-induced Obesity in a Murine Model of Type II Diabetes
4) Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy

Also included is the 'Statement of Authorship and Location of Data' form for the manuscript "Slowed Atrial and Atrioventricular Conduction and Depressed HRV in a Murine Model of Hypertrophic Cardiomyopathy" accepted in Clinical and Experimental Pharmacology and Physiology.

If you are not able to sign the forms physically or electronically, can you please provide a written approval through email that you approve of the manuscripts?

Kind Regards,
Wen

WEI WEN LIM, BSc(Hons)
PhD candidate

Centre for Heart Rhythm Disorders | University of Adelaide

Chapter Five

Depressed HRV and Slowed Atrial and Atrioventricular Conduction in Diabesity: A Polygenic Mouse Model of Diabetes and Obesity

5.1. INTRODUCTION

Both type II diabetes mellitus (DM) and obesity are rising epidemics affecting millions of humans worldwide (44,470). Additionally, obesity itself promotes development of insulin resistance and can lead to type II DM (471,472). In the Nurses' Health Study whereby 85,000 females underwent a 120 itemised, semi-quantitative food frequency questionnaire and were followed up from 1980-1996, apart from excess body fat being the most important determinant of diabetes, increased dietary intake of trans-fat and increased glycaemic load also predicted risk of DM development (473). In contrast, increased intake of dietary fibre and polyunsaturated fat was found to reduce DM risk. This study underscores dietary contributions in the promotion and prevention of DM.

Heart rate variability (HRV), a measure of the temporal variation of consecutive heart beats, is a non-invasive indicator of cardiac autonomic function (200). Depressed HRV has been previously implicated in various metabolic disorders such as hypertension (208), obesity (53), diabetes (209), and metabolic syndrome (474). In the Atherosclerosis Risk in Communities study, diabetic subjects

demonstrated depressed HRV at baseline and a more rapid temporal decline in HRV over a 9 year follow up compared to non-diabetics (475). Similarly, the Framingham Heart study also reported lower HRV in diabetic subjects compared to those with normal fasting glucose (476). However, these studies did not adequately address the contributions of obesity which is often concomitantly present in diabetic patients. In small case-control studies, obesity has been associated with reduced HRV and weight loss through diet, gastroplasty or bariatric surgery improves these autonomic disturbances (53,477,478).

Previous studies looking into HRV modulation, as a measure of cardiac autonomic neuropathy, in rodent models include the Streptozotocin-induced (STZ) rat and mouse (479-481), leptin receptor-deficient db/db mouse (482,483), Zucker diabetic fatty rat (484), Akita mouse (485) and Goto-Kakizaki rat (486). Most of these studies demonstrated depressed HRV parameters (time- and/or frequency domain analyses of HRV) in the presence of diabetes but not others (483). Additionally, although decreased nerve sympathetic nerve density has been observed in most models (485,487,488), the changes in norepinephrine content have been variable and inconsistent (487,489-491). One possible explanation for this disparity of results is the severity of disease in different animal strains and duration of chronic diabetes (ranging from 1 to 9 months of chronic diabetes). However, several limitations remain in the existing models of diabetes, especially animal models of type II DM. The current models for type II DM are mostly derived from monogenic causes which may not fully represent the clinical presentation in humans. Additionally, the presence of obesity which is common in type II diabetes is itself a modulator of HRV (492), and has not been sufficiently factored into the

HRV modulations from previous studies. It is well established that long-term high-fat diet consumption not only results in increased body weight but also can cause insulin resistance and that itself is a risk factor for developing cardiac autonomic dysfunction (493,494). Additionally, free fatty acids and visceral fat have been demonstrated to correlate well with indices of HRV in high-fat induced obese rats (494). However, most high-fat diet consumption studies investigating neuropathy in rodent models employ diets of variable but extremely high fat contribution (ranging from 36-59% energy from fat) (494-496).

The NONcNZO10/LtJ (RCS10) male mouse is a polygenic model of diet-induced type II DM and obesity, generated from the a cross of the New Zealand Obese and Non-obese Non-diabetic mice (433). RCS10 mice are thought to provide a more accurate representation of human diabetes and obesity by producing moderate obesity through polygenic interactions rather than massive obesity elicited by monogenic mutations in the leptin/leptin receptor axis. RCS10 mice weaned onto a moderately high fat diet (10-11% fat) have been characterised to develop moderate obesity, maturity-onset chronic hyperglycaemia (12-20 weeks), glucose intolerance, visceral obesity, dyslipidaemia, moderate liver steatosis and pancreatic islet atrophy (434,497). Additionally, diet differences in fat content have obvious implications on the genetic contributions of RCS10 mice. Adi et al. (2012) demonstrated that RCS10 mice fed control chow (27% calories in fat, 19% protein, 54% carbohydrates) demonstrated alterations in visceral adipose tissue expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), proteins involved in extracellular matrix regulation and remodelling, when compared to either a diet of high protein and fish oil

and/or SWR/J mice, a strain of mice that is diet-resistant to obesity and diabetes (456). This suggests that both diet regulation and genetic contributions play important roles in driving obesity and progression of type II diabetes in this model. Furthermore, high fat diets in RCS10 mice demonstrate elevations of peroxisome proliferator-activated receptor-γ gene expression (456), known to contribute to adipocyte hypertrophy and insulin resistance (498).

It is currently unclear if the polygenic model of diet-induced diabetes and obesity is associated with cardiac autonomic dysfunction. In this study, we aim to determine the impact of diabetes, diet contribution to obesity, and age on ECG characteristics and HRV parameters in a polygenic mouse model of diabetes and maturity-onset obesity.

5.2. METHODS

5.2.1. Mouse Models

NONcNZO10/LtJ (RCS10) and SWR/J (control) mice were obtained from The Jackson Laboratory (Bar Harbour, ME) and housed in an in-house animal facility (The University of Adelaide, SA) to set up breeding colonies. SWR/J mice are suggested as diet-resistant non-obese controls for physiologic comparisons (Jackson Laboratory). Male mice were randomly allocated in two steps: 1) according to age groups, one at 8-10 weeks of age and the other at 30 weeks of age and 2) according to diets, standard chow (NOM: 5% fat by calories, Meat-free Rat and Mouse Cubes, Specialty Feeds, Glen Forrest, Australia), high fat chow (HFD: 10% fat; SF06-105, Specialty Feeds, Glen Forrest, Australia), and high fat chow with chocolate bar supplement (CHOC: 268 calories breakdown: 50% fat, 42% carbohydrate, 8% protein) that was replenished every fortnight[1]. Mice were housed at controlled temperature (24°C) and lighting (12h light-dark cycles) with free access to respective chow and water ad libitum. All experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.2.1. Mouse Electrocardiography

3-lead ECG studies were performed in deeply anesthetized mice. Powerlab with LabChart v8.0.5 software (ADInstruments, NSW, Australia) was used for electrogram acquisition. In brief, mice were anesthetized with an intraperitoneal

[1] The cohort of animals used in this study was separate from that of Chapter Four.

injection of 75mg/kg ketamine and 0.5mg/kg medetomidine. Sedation state was checked by toe- and tail-pinch testing. Mice were placed on a heating pad maintained at 37°C, and gel-coated electrode pads were attached to both front feet and left back feet. Each mouse then underwent 30 minutes of continuous ECG recording. The ECG channel was filtered between 0.5 to 150 Hz and sampled at 1 kHz.

5.2.2. Data Processing and Analyses

ECG recordings were grouped and blinded to the analysing experimenter. ECG and HRV parameters were analysed offline with the respective modules from LabChart Pro (ADInstruments, NSW, Australia). ECG parameters including P-wave duration, PR, QRS, QT, and corrected QT (QTc) intervals were determined in 150-200 consecutive beats, with averages generated for every four successive beats. We used the rate-corrected Bazett's formula to calculate for QTc values. HRV quantification was conducted in 2 mins continuous ECG tracings by the use of time-domain and frequency-domain analyses. Time-domain parameters included the mean of normal R-R intervals (RR), standard deviation of normal RR intervals (SDRR), average heart rate (HR) and root mean square of successive heartbeat interval differences (RMSSD). Frequency-domain parameters included total power (TP), low frequency (LF; 0.15-1.5 Hz) expressed in normalized units, high frequency (HF; 1.5-5 Hz) expressed in normalized units, and the LF/HF ratio. These frequency spectrums selected were pre-set in LabChart Pro in accordance to mouse characteristics.

5.2.3. Statistical Analysis

All values are reported in mean ± standard error unless indicated otherwise. General Linear Model (GLM) ANOVA was used to determine significant differences between strain, diet and age factors, and Tukey's post-hoc test was conducted to determine diet differences. Student t-test was used to compare inter-group differences when ANOVA was significant. Linear regression was conducted to determine correlation of P-wave duration and RMSSD values to increased body weight or blood glucose levels. A value of $p<0.05$ was used to determine significance.

5.3. RESULTS

5.3.1. Animal Characteristics

Figure 1 illustrates the body weight, unfasted plasma and urine glucose levels of RCS10 and SWR/J mice. Both young and old RCS10 mice fed NOM diets were not significantly overweight (**young:** 26.2 ± 1.2 g vs. 24.0 ± 0.7 g, p=NS; **old:** 33.7 ± 1.7 g vs. 30.4 ± 0.4 g, p=NS) nor diabetic compared to age-matched controls (**young:** 14.5 ± 1.5 mmol/L vs. 11.1 ± 0.9 mmol/L, p=NS; **old:** 13.7 ± 1.2 mmol/L vs. 13.2 ± 0.6 mmol/L, p=NS). Young RCS10 mice fed HFD trended towards increased body weights (17% difference) (27.4 ± 1.7 g vs. 23.4 ± 0.7 g, p=0.06) and significant hyperglycaemia (plasma glucose: 20.7 ± 2.6 mmol/L vs. 12.6 ± 1.2 mmol/L, $p<0.05$) compared to controls. Additionally, increased age resulted in progressed obesity (30% difference body weight). Young RCS10 mice fed CHOC developed severe (41% difference) obesity (32.5 ± 0.5 g vs. 23.4 ± 0.6 g, $p<0.0001$) and hyperglycaemia (plasma glucose: 23.4 ± 2.2 mmol/L vs. 10.5 ± 1.1 mmol/L, $p<0.001$) which persisted with increased age. Older age was associated with increased body weight regardless of strain or diet ($p<0.001$) possibly due to maturation growth, but no significant age effect was seen in unfasted plasma and urine glucose levels. Presence of glycosuria was uniformly greater in RCS10 mice in all diets whereas urine glucose was not detectable in all SWR/J controls (Figure 1C).

5.3.2. ECG parameters

Figure 2 illustrates representative ECG recordings depicting example of prolonged p-wave duration in young and old RCS10 compared to age-matched SWR/J mice fed HFD diets. P-wave duration was prolonged in all RCS10 mice compared to controls in

NOM diets (**Young:** 15.3 ± 0.7 vs. 11.0 ± 0.6 ms, p<0.05; **Old:** 18.3 ± 0.3 vs. 12.1 ± 0.3 ms, p<0.001), HFD (**Young:** 19.7 ± 0.9 vs. 13.0 ±0.5 ms, p<0.0001; **Old:** 18.2 ± 0.7 vs. 11.7 ± 0.5ms, p<0.0001) and CHOC diets (**Young:** 19.2 ± 1.2 vs. 12.2 ± 0.4 ms, p<0.0001; **Old:** 18.5 ± 0.5 vs. 12.5 ± 0.7 ms, p<0.0001) (Figure 3B). PR intervals were elevated in RCS10 mice compared to SWR/J controls (p<0.001) without significant diet effect. Increased age had a small but significant increased PR interval regardless of strain (Age: p<0.05; Strain*Age interaction: p=NS). No significant difference in ECG parameters QRS, QT and QTc intervals were observed in RCS10 mice of all diet and age groups.

5.3.3. HRV parameters

Figure 4 depicts representative ECG, HR fluctuations and power spectrum of 10 weeks old SWR/J and RCS10 mice fed CHOC diet. As observed in this example, RCS10 mice demonstrated reduced HR fluctuations when compared to SWR/J mice (Figure 4B).

In time-domain analyses of HRV, mean RR intervals and average heart rate were not significantly different between RCS10 and SWR/J mice in all diets and age groups (all P=NS, Figure 5A and 5B). Both young and old RCS10 mice fed NOM diets had unchanged SDRR and RMSSD as compared to age-matched SWR/J controls (Figure 5C and 5D). In RCS10 mice fed HFD diet, SDRR was significantly reduced in young mice (3.41 ± 0.35 ms vs. 5.18 ± 0.54 ms, p<0.05) and trended to be reduced in older mice (3.12 ± 0.25 ms vs. 4.52 ± 0.58 ms, p=0.051). When fed CHOC diet, RCS10 mice demonstrated reduced SDRR compared to age-matched SWR/J mice (**young:** 2.46 ± 0.22 ms vs. 5.12 ± 0.73 ms, p<0.001; **old:** 2.66 ± 0.13 ms vs. 4.66 ± 0.38 ms, p<0.01). No significant age and diet effect in SDRR was observed (both p=NS). In RCS10 mice

fed HFD, RMSSD trended towards reduction in young mice and was significantly reduced in old mice (**young:** 2.62 ± 0.38 vs. 3.91 ± 0.55, p=0.08; **old:** 1.82 ± 0.30 vs. 4.13 ± 0.48, p<0.01). When fed CHOC diet, RCS10 showed reduced RMSSD compared to SWR/J controls (**young:** 2.38 ± 0.35 vs. 3.98 ± 0.47, p<0.05; **old:** 2.23 ± 0.23 vs. 3.74 ± 0.24, p<0.01). No significant age effect in RMSSD was observed (p=NS), however RCS10 mice fed HFD and CHOC diets trended towards lower RMSSD in both diets compared to NOM diets (Strain*Age interaction: p=0.079).

In frequency-domain analyses of HRV, RCS10 mice demonstrated increased LF power compared to SWR/J controls, however this did not reach statistical significance (23.9 ± 0.7 vs. 22.2 ± 0.7 n.u., p=0.078; Figure 6A). Additionally, increased age and fat-content in diets trended towards significance for LF power (Age: p=0.089; Diet: p=0.055). HF power was significantly reduced in RCS10 mice compared to controls (76.2 ± 0.8 vs. 78.3 ± 0.7 n.u., p<0.05; Figure 6B), with no significant age and diet effect observed (both p=NS). LF/HF ratio was not significantly altered between RCS10 and SWR/J mice in all diet and age groups.

5.3.4. Association of P-wave and RMSSD to Obesity and Hyperglycaemia

Figure 7 depicts the distribution of increasing body weight and blood glucose levels according to mouse strain and diets. SWR/J mice in all three diets remained relatively lean and normoglycaemic whereas RCS10 mice fed HFD or CHOC diets tend to demonstrate greater body weights and blood glucose levels.

Figure 8 depicts linear regression correlates of P-wave durations and RMSSD to body weight and blood glucose levels. P-wave duration was positively correlated with

increasing body weight and blood glucose levels in the RCS10 mice (R^2 value: 0.113 and 0.108 respectively, both $p<0.05$). Conversely, SWR/J controls demonstrated poor correlation of the P-wave duration to body weights or blood glucose (R^2 value: 0.012 and 0.084 respectively) and was statistically different to RCS10 animals ($p<0.001$). RCS10 mice demonstrated reduced RMSSD compared to SWR/J controls ($p<0.01$), and a negative correlation of RMSSD to increased body weight (R^2 value: 0.156, $p<0.05$). Additionally, RCS10 mice trended towards significantly reduced RMSSD compared to controls when assessed for blood glucose levels ($p=0.074$), and increased blood glucose levels correlated with further reduction in RMSSD ($p<0.01$). Conversely, SWR/J controls demonstrated poor correlation of the RMSSD to body weights or blood glucose (R^2 value: 0.018 and 0.001 respectively).

5.4. DISCUSSION

To determine the effects of diet-induced diabetes and obesity on cardiac autonomic regulation and electrophysiology, we studied a relatively new and understudied polygenic mouse model of Type II diabetes. The RCS10 mouse is a strain of mice that varies in progression of diabetes and/or obesity based on the diet and the maturation age. In this study, we characterised the following ECG and HRV alterations using non-invasive ECG recordings in a polygenic model of diet-induced diabetes and obesity:

1) RCS10 mice fed normal low fat content diets do not develop overt diabetes and obesity and have normal HRV but demonstrate slight to moderate prolongation of the atrial conduction as evidenced by increased p-wave durations.
2) RCS10 mice fed HFD or CHOC diets develop diabetes and obesity demonstrating severe slowing of atrial conduction and reduced heart rate variability as evident by time-domain HRV parameters of reduced SDRR and RMSSD and trended towards significant increase and decrease of LF and HF power respectively.
3) Prolonged p-wave duration and depressed RMSSD had a weak but significant correlation to increased obesity and hyperglycaemia.
4) Older age in RCS10 mice was not a main contributor to more marked ECG abnormalities and reduced HRV.

Cardiac function is controlled by cardiac autonomic innervations through a balance of sympatho-vagal modulation and reflected through HRV. Although autonomic

neuropathy has been originally thought to be a late complication of diabetes, HRV has been demonstrated to detect small changes in the autonomic dysfunction occurring much earlier in the clinical course of diabetes (499). Cardiac autonomic dysfunction has been described in a variety of diabetic animal models. In Type I diabetes, rodent models such as the STZ-induced, NOD and Akita mouse commonly demonstrate a decrease in both heart rate and HRV (479-481,491). In Type II diabetic rodent models including the db/db mice, Goto-Kakizaki rat, Zucker Diabetic Fatty rat, and high-fat diet induced obese rats have commonly demonstrated decreased HRV, however the average HR change in these studies have been inconclusive. Some studies have demonstrated a decrease in HR due to diabetes (483,486), whereas others have shown an increase (482,484,493). It should be noted that assessment of HRV has not been consistent throughout, such as 1) methods of HRV analysis including time-domain, frequency-domain or both, 2) the use of β-adrenergic and/or muscarinic blockade has been variable, and 3) method of ECG recording (telemetric, restraint, or under anaesthesia). One study demonstrated that feeding state of the animal can have a significant impact on HR and HRV, suggesting that glycaemic state itself can alter HR and HRV at the time of recording (500). Soltynsinska et al. demonstrated in the anesthetized db/db mouse model of Type II diabetes that hypoglycaemia due to fasting resulted in a decrease in HR, and increased time-domain HRV parameters of standard deviation of the difference between successive RR intervals (SDARR) and percentage of normal consecutive RR intervals differing by >6 ms (pRR6) whereas frequency-domain analyses of HRV were unchanged. In contrast, in fed animals HR and HRV were unaffected (500). In the current study, we observed unchanged HR, diet-induced decrease in SDRR and RMSSD, and unchanged frequency-domain analyses of HRV

in anesthetized unfasted RCS10 mice. Our results contrasts with the prior study in HR and HRV despite animals being unfasted, and may be explained by the difference in animal strain (RCS10 vs. db/db) and age (10 and 30 weeks vs. 8 weeks). Our results in this polygenic model suggest similar depression of HRV compared to previous monogenic models, this despite being a closer mimic to human obesity/diabetes syndrome (497).

To the best of our knowledge, we believe that this is the first study that has looked at non-invasive ECG recordings in a polygenic model of type II diabetes. In our study, we observed diet-specific prolongation of P wave durations in the diabetic and obese mice compared to non-obese non-diabetic controls is in the absence of any ventricular depolarisation and repolarisation abnormalities. P wave duration is known to be highly correlated with inter-atrial conduction times both in normal and patients with LA enlargement (501). Increased maximum P-wave duration and P-wave dispersion have been previously demonstrated in diabetic as well as obese patients (502-505). Autonomic nervous system activity is known to affect the p-wave duration. β-adrenergic blockage is known to prolong p-wave duration whereas parasympathetic blockade shortens p-wave duration (506). Additionally, patients with prolonged p-wave durations have an increased risk of developing atrial fibrillation (507-509) and it is postulated that prolonged p-wave duration may be due to areas of delayed atrial conduction predisposing to AF. In isolated hearts or atria of diabetic animals, inter-atrial conduction time and intra-atrial conduction has been shown to be prolonged in concert with increased atrial interstitial fibrosis and higher AF vulnerability (272,445,446,510).

5.4.1. Clinical Implications

Evidence of depressed HRV in the polygenic mouse model for diabesity are consistent with studies in monogenic disorders such as leptin deficiency. Additionally, the polygenic cause is a closer representation of human diabesity with less severe organ defects (434). The results from the current study likely suggest electrophysiological abnormalities in ECG and HRV in humans with diabetes and obesity. Evidently depressed HRV has been observed in cohort and case-controlled studies in relation to diabetes and obesity independently (53,475-478). However, their combined and individual contributions to HRV in a population where multiple metabolic disorders are concomitantly expressed remain to be investigated. Linear correlation studies also suggest that depressed RMSSD and prolonged P-wave duration are weak but significantly correlated with the progression of obesity and hyperglycaemia in the polygenic mouse.

5.4.2. Study Limitations

Firstly, we did not monitor food consumption as food was given ad libitum and we acknowledge that the effects of glycaemic status in these animals may impact heart rate and HRV. Future studies could identify the differences in HRV of these animals under fasting and non-fasting conditions. We chose to give chocolate supplement in addition of HFD to boost obesity in the RCS10 mice because extremely high fat diets often lose their solid pellet form and are not as palatable to the RCS10 mice. Chocolate bars were replenished every two weeks for both strains of mice, with visual observation that both RCS10 and SWR/J mice consumed the chocolate. Our ECG recordings were conducted under anaesthesia and it is known that

anaesthesia reduces the power of HRV and conclusions from the frequency domain analysis should be drawn with caution (511). However, all our animal groups underwent the same protocol and were randomly allotted and blinded before ECG and HRV analyses. Therefore, any significant difference is unlikely to be due to anaesthesia alone.

5.5. CONCLUSIONS

In conclusion, diabetes of polygenic causes results in slowed atrial conduction and depressed HRV. Prolonged P-wave duration was observed in ECG recordings from diabetic and obese mice. High fat diets and chocolate supplemented diets significantly prolonged p-wave durations and demonstrated depressed RMSSD in the diabetic and obese mice. Moreover, there was a slight but significant correlation of these markers to both increased hyperglycaemia and body weight. The results highlighted in the current study suggest of dietary-induced electrophysiological abnormalities and cardiac autonomic dysfunction in polygenic diabetes. Further studies are warranted to investigate effects of managing body weight and glycaemic state on these changes.

FIGURE LEGENDS

Figure 1. RCS10 mice develop obesity and diabetes at 10- and 30-weeks of age when fed high fat (HFD) and chocolate supplemented (CHOC) diets. (A) Body weights of 10- and 30-week old RCS10 mice were significantly greater compared to SWR/J controls when fed HFD and CHOC diets post-weaning. 30 week old mice regardless of strain demonstrated elevated body weights compared to 10 week old mice (Age: $p<0.001$; Age*Strain interaction: $p=0.151$). **(B)** Elevated levels of unfasted blood glucose were demonstrated in 10- and 30-week old RCS10 mice when fed HFD and CHOC diets post-weaning. The increase in unfasted blood glucose of RCS10 mice fed HFD and CHOC diets was significantly greater compared to RCS10 mice fed NOM diets (Strain*Diet interaction: $p<0.01$). No significant age effect was observed (p=NS). **(C)** Elevated urine glucose levels were observed in RCS10 mice fed all three diets whereas urine glucose was not detectable in SWR/J mice. The increase in urine glucose was significantly greater in all RCS10 mice compared to SWR/J controls regardless of age and diet (Strain: $p<0.001$; Age: $p=0.112$; Diet: $p=0.292$).

Figure 2. Prolonged P-wave durations in diabetic mice. (A) Representative surface ECG recordings demonstrating prolonged P-wave durations in young and old RCS10 mice fed HFD diet (dashed lines).

Figure 3. ECG recordings demonstrate slowed atrial and AV node conduction delay but normal ventricular conduction in RCS10 mice as compared to SWR/J controls. (A) PR interval, a measure of AV node conduction, was elevated

in RCS10 mice compared to SWR/J controls ($p<0.001$) without significant diet effect (p=NS). 30 weeks old mice demonstrated increased PR intervals compared to younger mice regardless of strain (Age: $p<0.05$; Strain*Age interaction: p=0.586). **(B)** Both 10 and 30 weeks old RCS10 mice demonstrated similarly prolonged P-wave durations compared to SWR/J mice (Strain: $p<0.001$; Age: p=0.317). Additionally, RCS10 mice fed HFD and CHOC diets demonstrated significantly prolonged P-wave durations compared to RCS10 mice fed NOM diets (Strain*diet interaction: $p<0.001$). **(C-E)** QRS intervals, QT intervals and corrected QT intervals were not significantly different between RCS10 mice and controls in all diets and both age groups (all p=NS).

Figure 4. ECG, HR fluctuations and power spectra of 10 weeks old SWR/J and RCS10 mice fed CHOC diet. (A) One-lead ECG recorded from representative RCS10 and SWR/J (control) mice. (B) Comparison of beat-to-beat changes in RR intervals plotted against successive beats. (C) Power spectrum of the RR signal from RCS10 and control mice. VLF = 0-0.15 Hz, LF = 0.15-1.5 Hz, and HF = 1.5-4 Hz. (D) Poincaré plot demonstrating distribution of RR intervals for successive R-R beats from RCS10 and control mice.

Figure 5. Time domain indices of HRV parameters. (A) RCS10 mice demonstrated similar mean RR intervals as compared to SWR/J controls in all diets and age groups. **(B)** RCS10 mice demonstrated similar mean HR as compared to SWR/J controls in all diets and age groups. **(C)** RCS10 mice had significantly reduced SDRR as compared to SWR/J controls when fed HFD and CHOC diets, but was not significantly reduced in the NOM diets. No significant age and diet effect

was observed (both p=NS). **(D)** RMSSD was significantly reduced in RCS10 mice as compared to SWR/J controls when fed the HFD and CHOC diets, but was unchanged in the NOM diets. Reduced RMSSD in RCS10 mice fed HFD and CHOC diets trended towards significance when compared to RCS10 mice fed NOM diets (strain*diet interaction: p=0.079). No significant age effect on RMSSD was observed (p=NS).

Figure 6. Frequency domain indices of HRV parameters. (A) RCS10 mice demonstrated increased LF power compared to SWR/J controls but this did not reach statistical significance (Strain: p=0.078). Additionally, increased LF power in 30 weeks old RCS10 mice fed HFD trended towards significance (Diet: p=0.055, Age: p=0.089). **(B)** RCS10 mice demonstrated reduced HF power compared to SWR/J controls (Strain: p<0.05), although no significant diet and age effect was observed (both p=NS). **(C)** LF/HF ratio was not significantly altered in RCS10 mice compared to SWR/J controls (p=NS).

Figure 7. Distribution of increasing body weight and blood glucose levels in accordance to mouse strain and diets. SWR/J mice given all 3 diets remained relatively lean and normoglycaemic whereas RCS10 mice fed HFD and/or CHOC diets were more obese and hyperglycaemic.

Figure 8. Correlation of P-wave duration (top) and RMSSD (bottom) to body weight (left) and blood glucose levels (right). (A & B) RCS10 mice that developed significantly prolonged p-wave durations had a small but significant positive correlation to both increasing body weight and blood glucose level. **(C)**

RCS10 mice with increasing body weight had a small but significant negative correlation with RMSSD. **(D)** RCS10 mice trended towards significantly reduced RMSSD and increase blood glucose levels correlated with further reduction in RMSSD.

FIGURE 1.

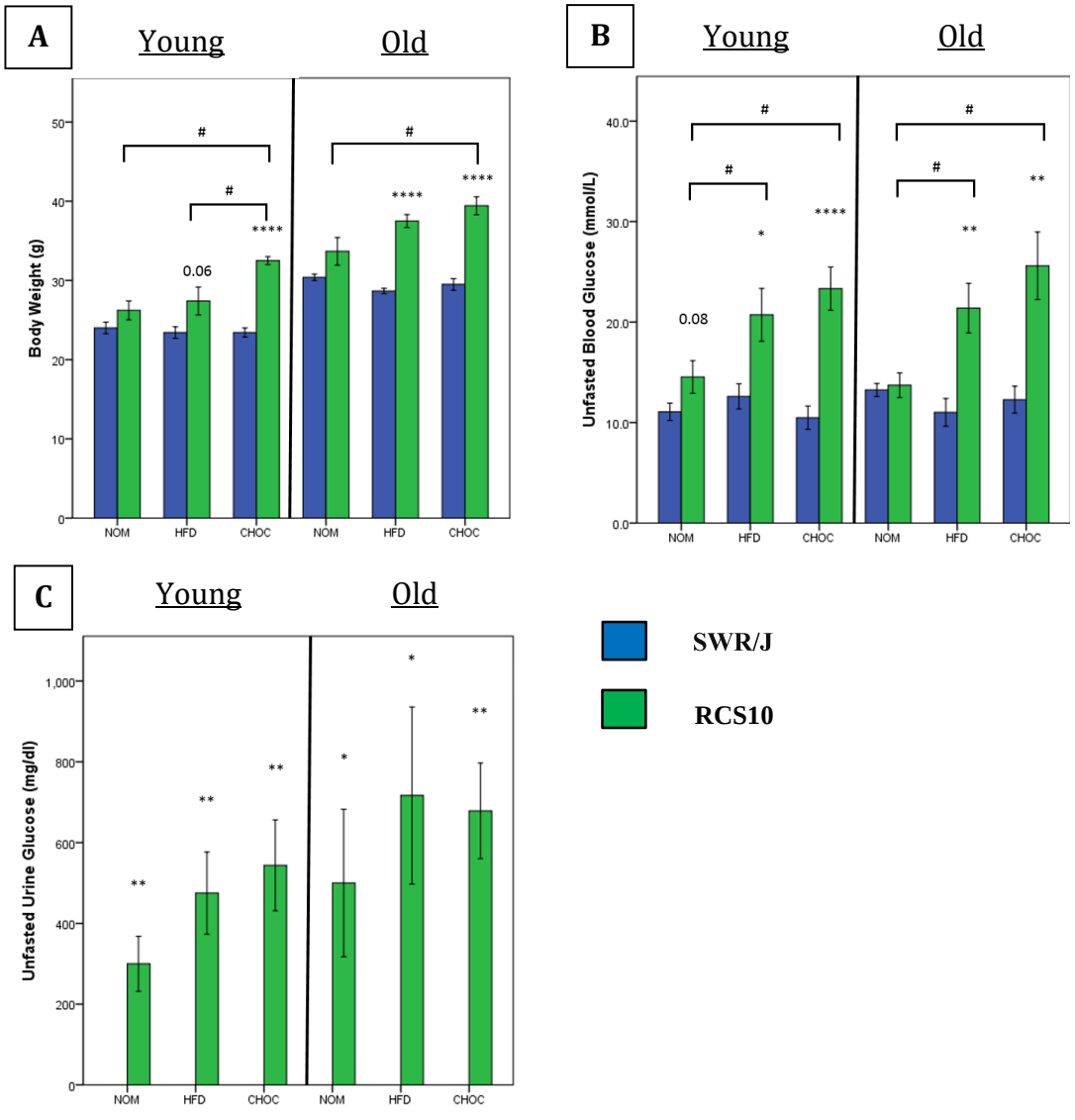

A
Young
Old
Body Weight (g)
0.06
B
Young
Old
Unfasted Blood Glucose (mmol/L)
0.08
C
Young
Old
Unfasted Urine Glucose (mg/dl)
NOM
HFD
CHOC
SWR/J
RCS10

FIGURE 2.

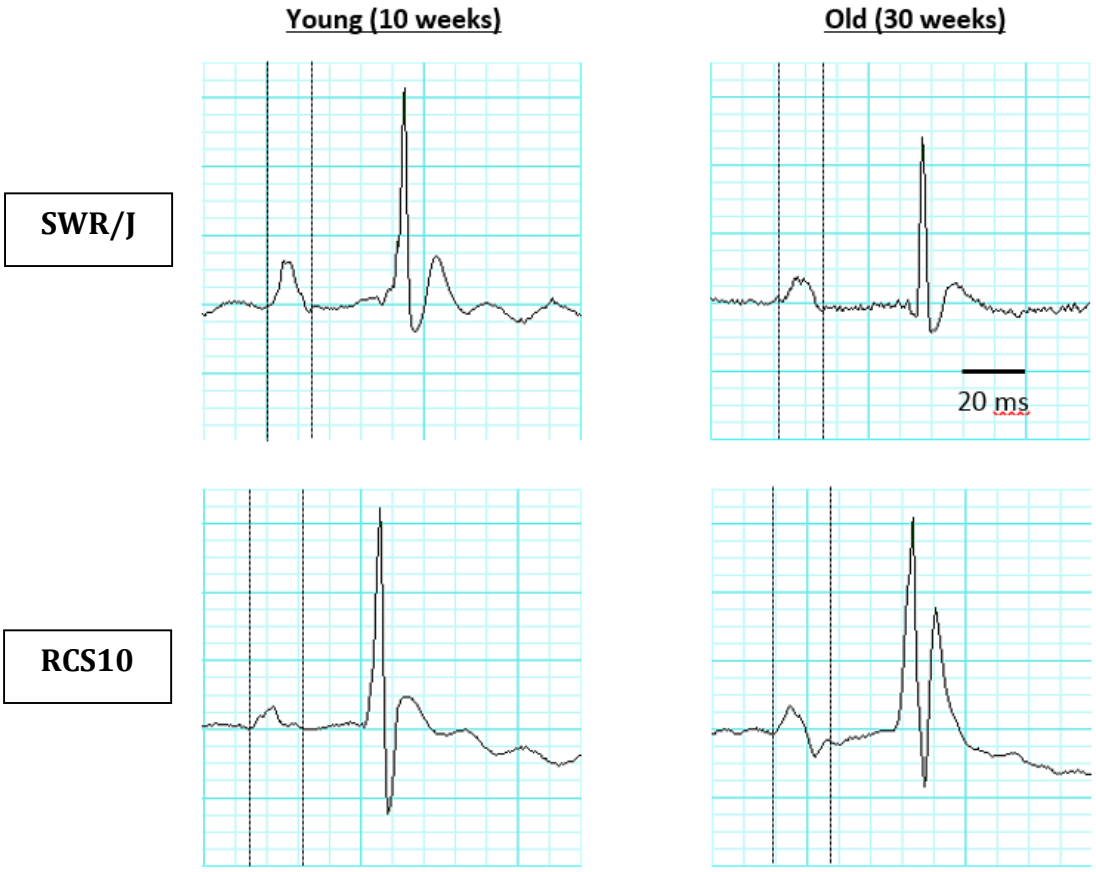


FIGURE 3.

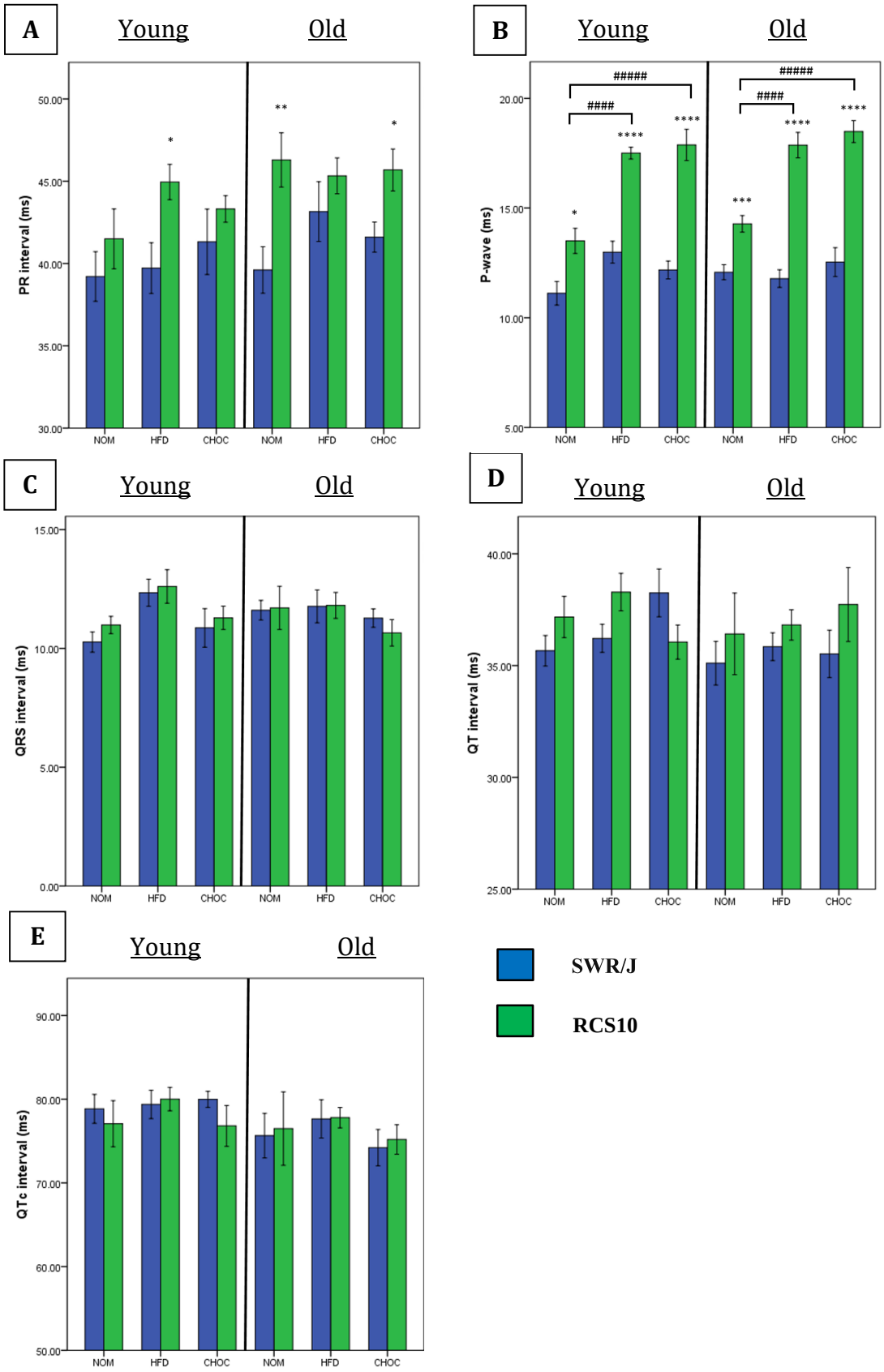

A
Young
Old
PR interval (ms)
50.00
45.00
40.00
35.00
30.00
NOM
HFD
CHOC
B
P-wave (ms)
20.00
15.00
10.00
5.00
#####
####
C
QRS interval (ms)
15.00
10.00
5.00
0.00
D
QT interval (ms)
40.00
35.00
30.00
25.00
E
QTc interval (ms)
90.00
80.00
70.00
60.00
50.00
SWR/J
RCS10

FIGURE 4.

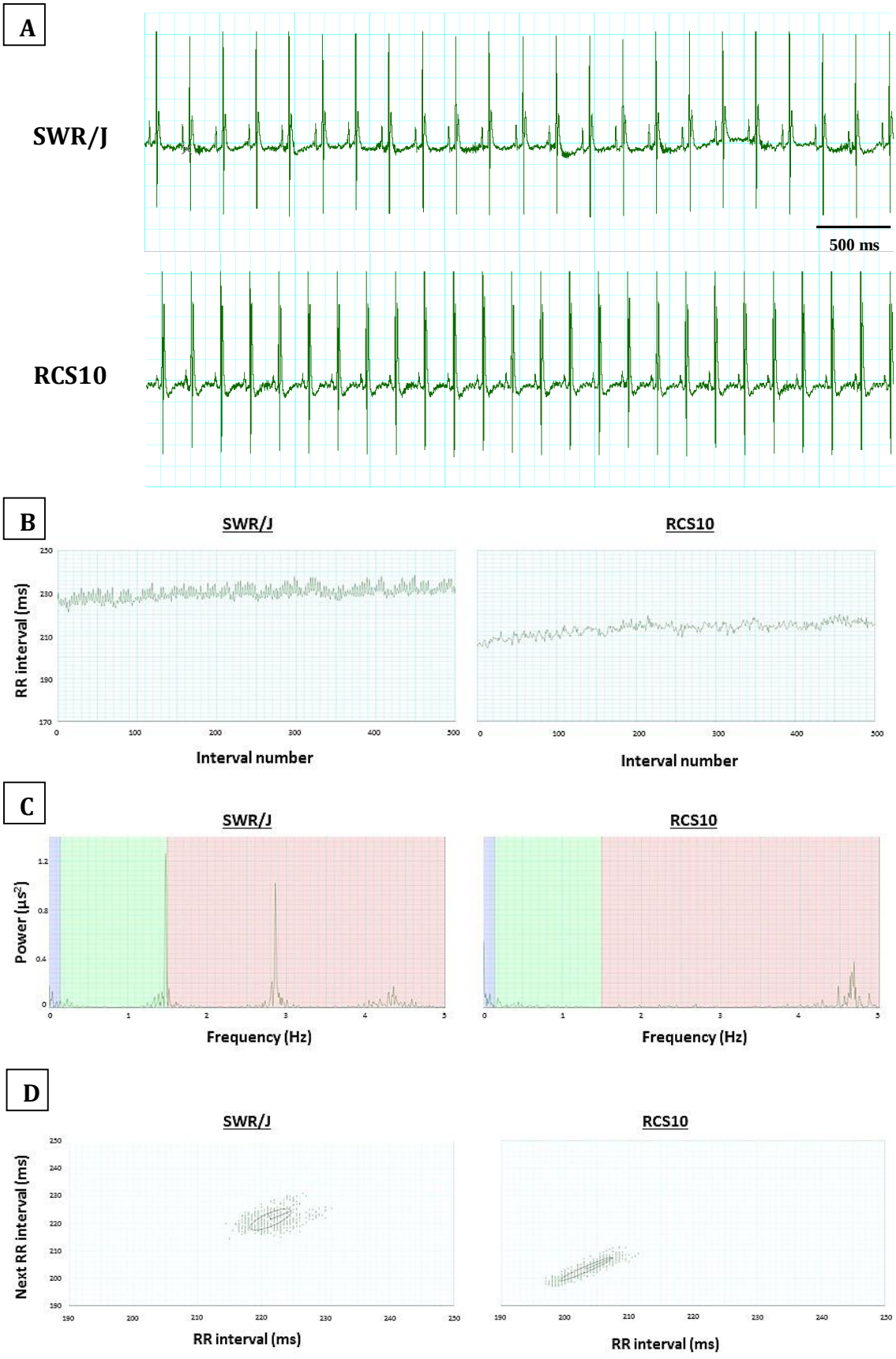

A
SWR/J
500 ms
RCS10
B
SWR/J
RCS10
RR interval (ms)
Interval number
Interval number
C
SWR/J
RCS10
Power (µs²)
Frequency (Hz)
Frequency (Hz)
D
SWR/J
RCS10
Next RR interval (ms)
RR interval (ms)
RR interval (ms)

FIGURE 5.

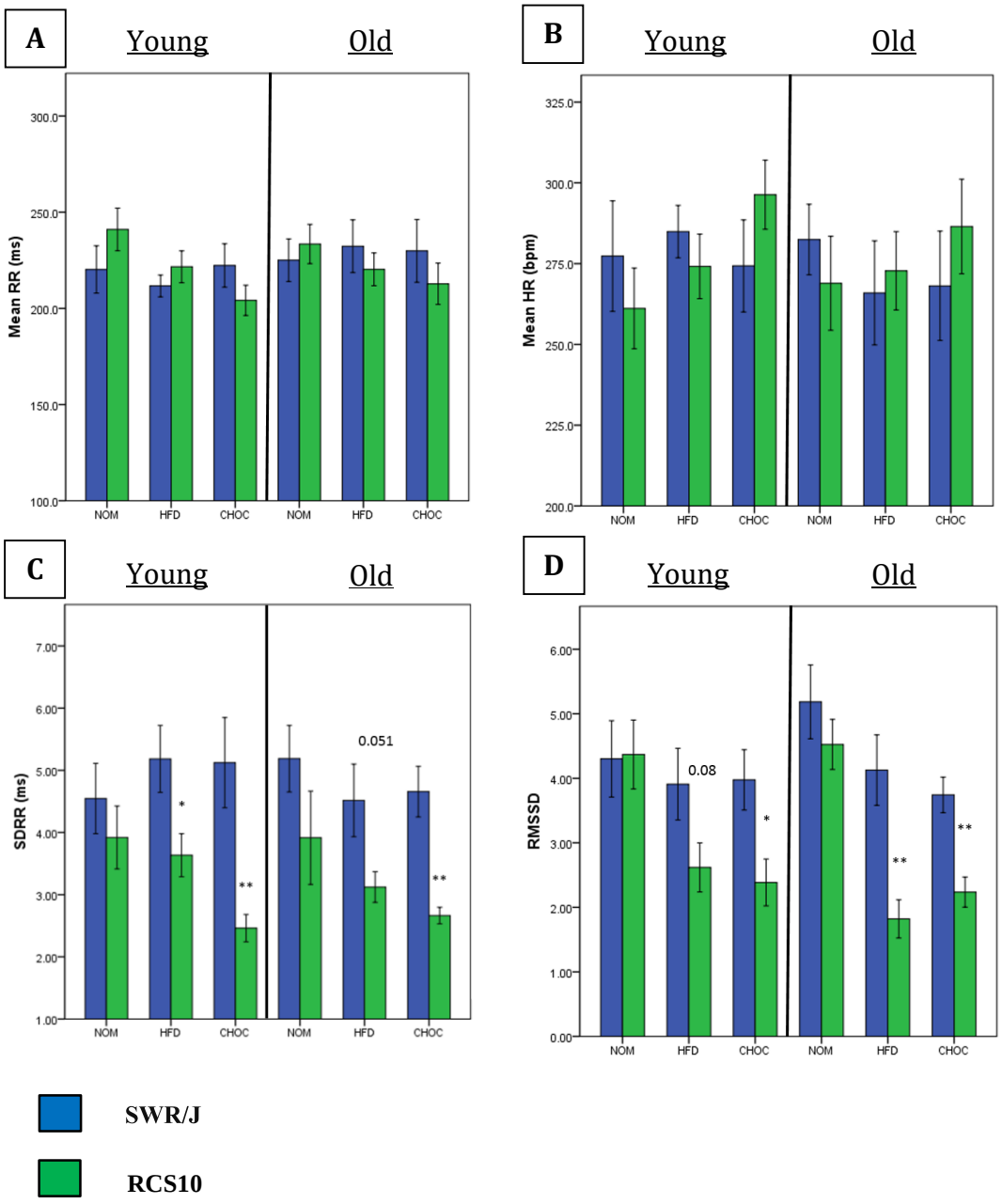

A
Young
Old
Mean RR (ms)
300.0
250.0
200.0
150.0
100.0
NOM
HFD
CHOC
NOM
HFD
CHOC
B
Young
Old
Mean HR (bpm)
325.0
300.0
275.0
250.0
225.0
200.0
NOM
HFD
CHOC
NOM
HFD
CHOC
C
Young
Old
SDRR (ms)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.051
*
**
**
NOM
HFD
CHOC
NOM
HFD
CHOC
D
Young
Old
RMSSD
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.08
*
**
**
NOM
HFD
CHOC
NOM
HFD
CHOC
SWR/J
RCS10

FIGURE 6.

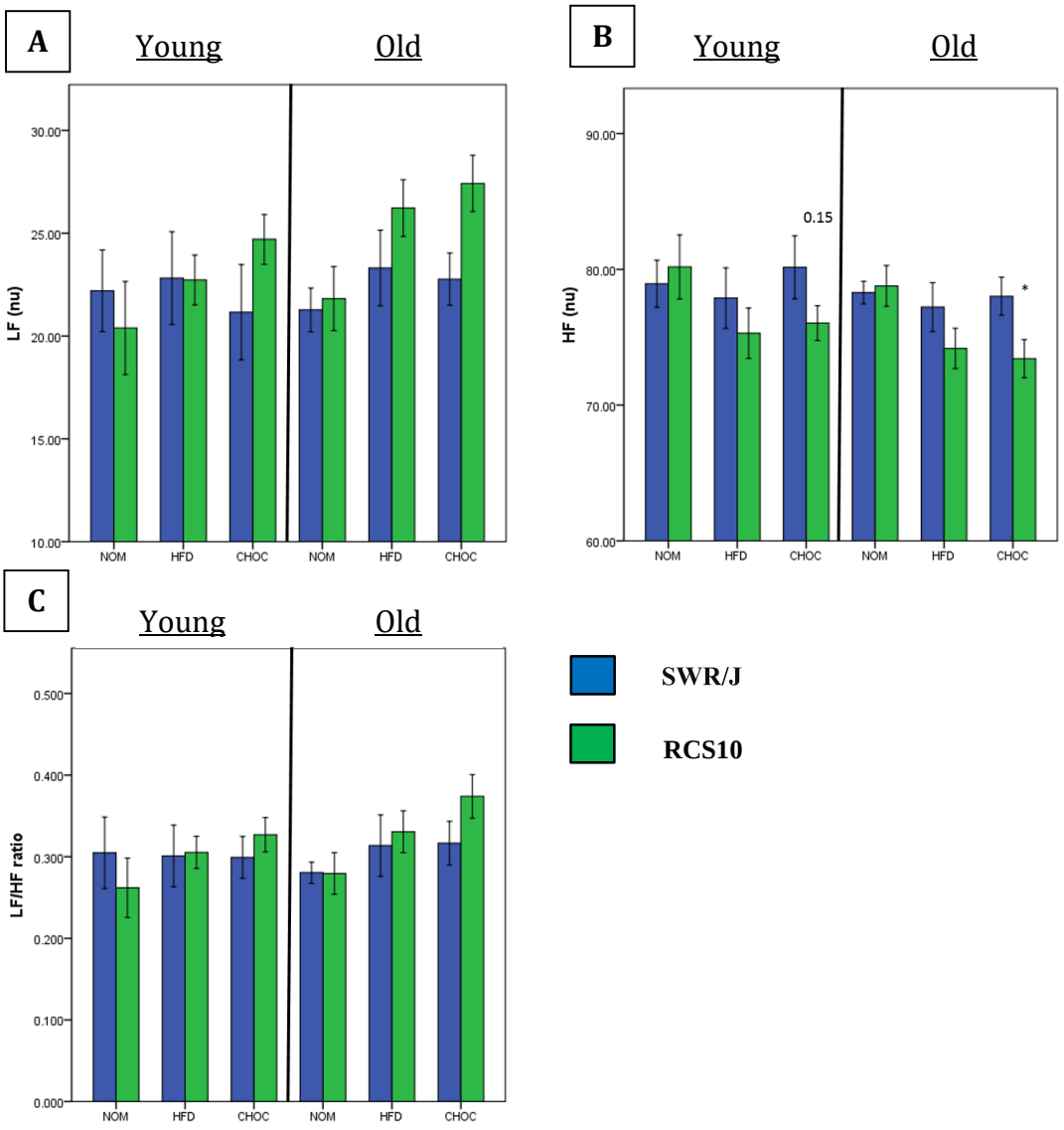

A
Young
Old
LF (nu)
30.00
25.00
20.00
15.00
10.00
NOM
HFD
CHOC
NOM
HFD
CHOC
B
Young
Old
HF (nu)
90.00
80.00
70.00
60.00
0.15
*
NOM
HFD
CHOC
NOM
HFD
CHOC
C
Young
Old
LF/HF ratio
0.500
0.400
0.300
0.200
0.100
0.000
NOM
HFD
CHOC
NOM
HFD
CHOC
SWR/J
RCS10

FIGURE 7.

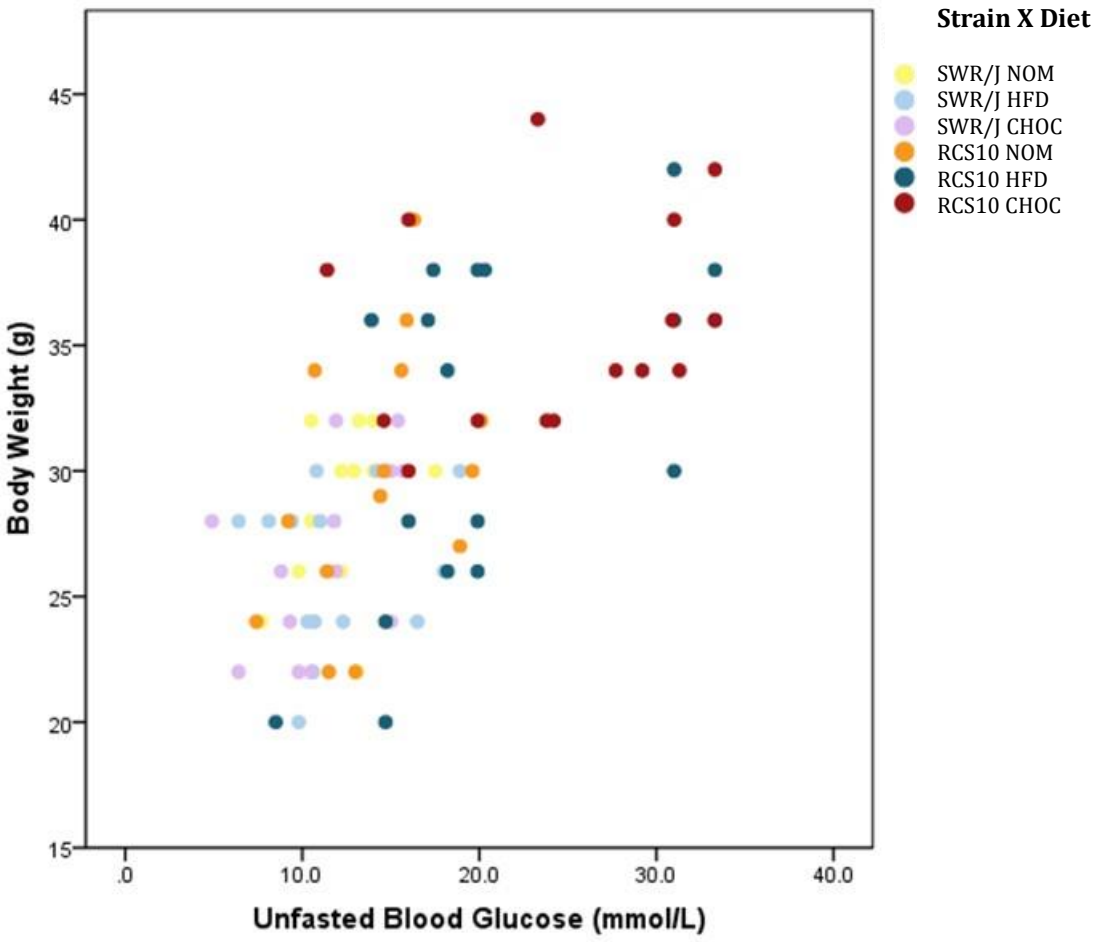
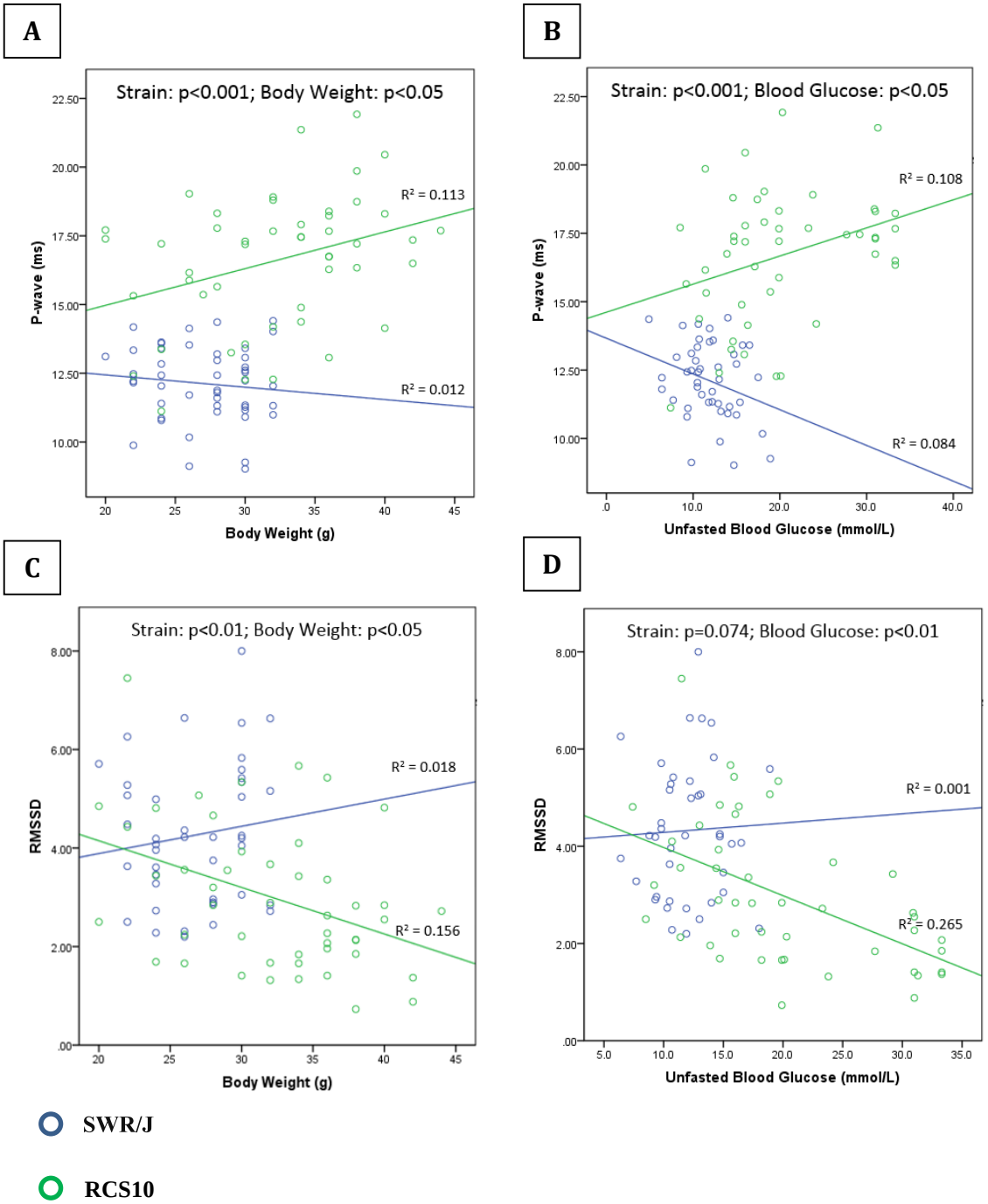

Strain X Diet
SWR/J NOM
SWR/J HFD
SWR/J CHOC
RCS10 NOM
RCS10 HFD
RCS10 CHOC
Body Weight (g)
45
40
35
30
25
20
15
.0
10.0
20.0
30.0
40.0
Unfasted Blood Glucose (mmol/L)

FIGURE 8.


A
Strain: p<0.001; Body Weight: p<0.05
P-wave (ms)
Body Weight (g)
R² = 0.113
R² = 0.012
B
Strain: p<0.001; Blood Glucose: p<0.05
P-wave (ms)
Unfasted Blood Glucose (mmol/L)
R² = 0.108
R² = 0.084
C
Strain: p<0.01; Body Weight: p<0.05
RMSSD
Body Weight (g)
R² = 0.018
R² = 0.156
D
Strain: p=0.074; Blood Glucose: p<0.01
RMSSD
Unfasted Blood Glucose (mmol/L)
R² = 0.001
R² = 0.265
SWR/J
RCS10

Chapter Six

Final Discussions

6.1. ATRIAL REMODELLING IN HYPERTROPHIC CARDIOMYOPATHY: IMPLICATIONS FOR ATRIAL FIBRILLATION

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiac disorder with variable mortality outcomes including sudden cardiac death, thromboembolic strokes and end-stage heart failure (95). Importantly, inter- and intra-genic heterogeneity makes HCM a complex disorder demonstrating phenotypic variability with differences in severity of hypertrophy and fibrosis, age of presentation, and the risk for sudden cardiac death (289). Notably, whilst sudden cardiac death mostly affects young HCM patients, death due to heart failure and strokes mostly occur during midlife and beyond (283). Importantly, atrial fibrillation (AF) itself is an important risk factor for heart failure development and thrombo-embolic strokes (283,284,512).

AF occurs in 22% of the HCM population and HCM patients with AF are known to have worst survival (presumably due to heart failure and stroke) to non-AF HCM patients (325,327). AF development in HCM is often thought to be due to left atrial (LA) enlargement as a result of elevated preload pressure from the dysfunctional ventricles (513), resulting in secondary atrial myopathy. However,

intrinsic atrial myopathy cannot be disregarded as mutations in the sarcomeric proteins that cause left ventricular and septal hypertrophy in HCM are likely to affect the atrial myocardium as well. Noting that LA enlargement, function (contractility as measured by ejection fraction), and inhomogeneous conduction and refractoriness (as measured with p-wave duration and dispersion) have been suggested to predict for AF risk in HCM (513), relatively little is known about the role of atrial remodelling in HCM progression. A proposed pathogenesis of HCM has been summarized in Figure 1.

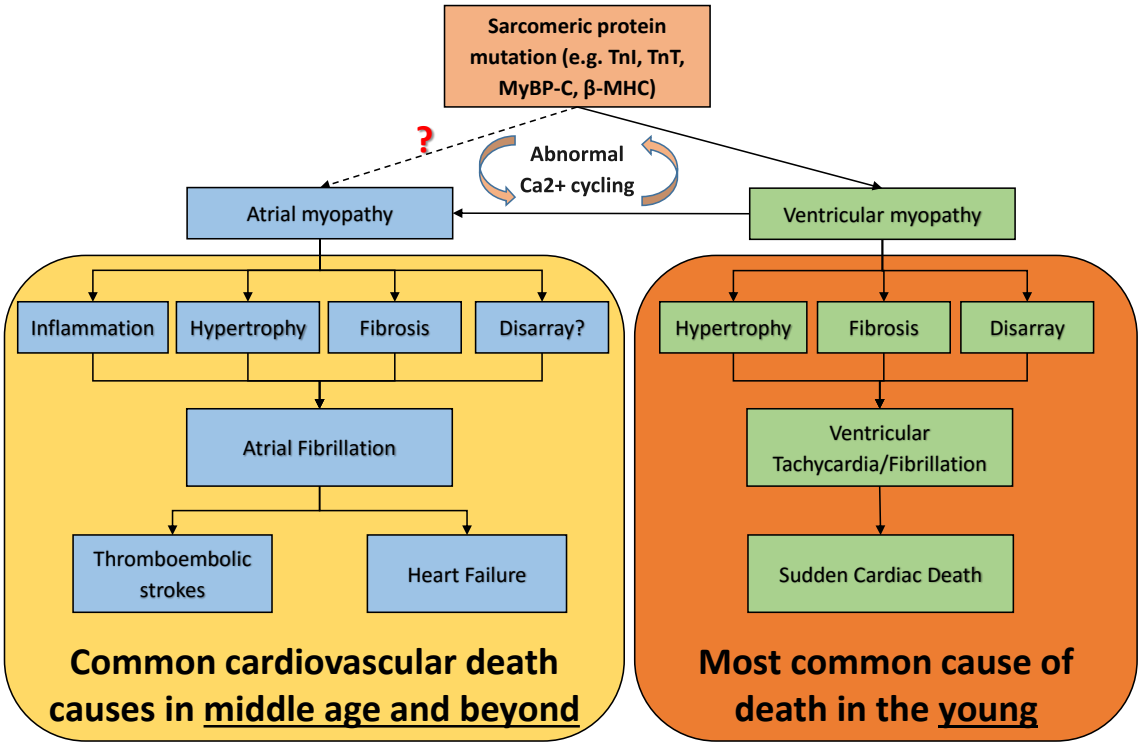


FIGURE 1. Proposed contributors to the pathogenesis of HCM.

To date, many animal models of HCM have been established characterizing the differences in phenotype penetration (in particular ventricular electrophysiology and/or structural remodelling) in a variety of mutations (316,337,349,383,514). However, atrial electrophysiology and structural remodelling has been largely absent (apart from the observations of enlarged atria) despite the importance in establishing their roles in AF risk. Another aspect is myofibrillar disarray in the atria that, unlike the ventricular myocardium, is difficult to demonstrate in small animal models of HCM due to large amounts of cytosol in the atrial myocardium resulting in less compact myofibrillar arrangements (515). However, one may suspect that the same would occur as both atrial and ventricles are subjected to the same malfunctioning mutation, which may otherwise be more prominent in larger animal models and humans although this has not been investigated.

6.1.1. Atrial electrophysiological and structural remodelling in the TnI-203 HCM mouse

In chapter two, we observed bi-atrial enlargement along with increased left ventricular and septal wall thickness indicative of HCM in the missense mutated cardiac troponin I overexpression (TnI-203) mouse. This was not significantly different between 30 and 50 weeks of age, chosen to represent the progression of the cardiac disorder, suggesting that ventricular and atrial hypertrophy was stable between the two ages. Despite similar changes in the macro-structure (namely increased mass) and micro-structural remodelling (increased cardiomyocyte size, fibrosis deposition, and inflammatory infiltration) of the atrial myocardium, our

results demonstrate older age-related increase in atrial refractoriness and conduction slowing, whereas increased conduction heterogeneity and decreased action potential durations (APD) were not affected by aging. This may suggest that histological features occur in the early phase of disease and remain sustained throughout the lifetime of the disorder, thereby providing important contributions to a vulnerable substrate for arrhythmias by increasing conduction heterogeneity and slowing. On the other hand, certain features of atrial electrophysiology are more dependent on aging or chronicity of disease state.

In Ca^{2+} transient studies in ventricular myocytes, Tsoutsman et al. (2006) identified normal Ca^{2+} transient peak and fold increase in the TnI-203 transgenic mice, the same model of HCM in our study, indicating normal sarcoplasmic reticulum (SR) Ca^{2+} release and storage (337). However, Ca^{2+} transient decay constant was increased with cardiomyocytes stimulation and decreased with caffeine-induced SR Ca^{2+} dump in TnI-203 mice, in the presence of unchanged protein expression of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), phospholamban, ryanodine receptors, and the electrogenic Na^{+}-Ca^{2+} exchanger (NCX) (337). The increased Ca2+ transient decay constant and decreased decay constant with caffeine are suggestive of Ca^{2+} cycling abnormalities such that cytosolic Ca^{2+} re-uptake through SERCA is slowed and the extrusion of Ca^{2+} out of the cell is increased with hyper-activity of the NCX respectively. The basis for this hypothesis is largely because the SERCA and NCX play the two most important roles in Ca^{2+} removal from the cytosol with SERCA removing 92%, NCX removing 7% and remaining ~1% by sarcolemma Ca^{2+} ATP-ase and mitochondrial Ca2+ unitransporter in the rat cardiomyocyte (516). A probable explanation for the

sequestration of Ca^{2+} in the cytosol resulting in slowed re-uptake by SERCA may be due to the reduced inhibitory function of the mutated troponin I protein to release Ca^{2+} from the actin-myosin complex at rest. Reduced reactivity of the mutated troponin I with both troponin C and T complexes has been observed in the TnI-203 mouse (337).

Assuming that the same may be applied to atrial myocytes, slowed re-uptake through the SERCA and enhanced NCX Ca^{2+} efflux may explain reduced atrial APD observed in our study. Nonetheless, our results of decreased APD in TnI-203 mice across 20, 50 and 90% of repolarization suggest that other ionic channel expression are altered as well, warranting further studies to identify the type of channels and their functionality using patch-clamp techniques. Interestingly, but perhaps not surprisingly, APD changes can be different depending on the type of mutated sarcomeric protein in HCM. Similar to our findings in the TnI-203 mouse, the troponin T mutant mouse demonstrates shortened APD with slowed Ca2+ transient delay constant (517), whereas the truncated Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) overexpressed transgenic mouse, exerting its effects via CaMKII, develops prolonged APD (514). The similarities in APD and calcium kinetics between the troponin I and T mutant mice may be due their combined involvement in the actin-myosin contractile apparatus (i.e. the troponin-I, -C and -T complex), as opposed to the Ca^{2+} homeostatic effects of CaMKII through L-type Ca^{2+} channels and SR Ca^{2+} uptake and release (518).

In contrast to shortened APD, atrial refractoriness was unchanged in our 30 week old and even increased in 50 week old TnI-203 mice. This disparity of APD

and atrial effective refractory period (ERP) may have been due to the nature of our simultaneous APD-ERP recording where epicardial ERP was recorded with the use of high-density mapping and concurrent endocardial APD was measured at the distant end of stimulation site with an intracellular electrode. The reason for APD recording at distant ends of stimulation site (from the multi-electrode array) was because of the pacing artefact on the recording APD microelectrode that confounds the maximal deflection of the action potential when recordings were made in the region of the pacing stimulus. Our observation of dissimilar APD-ERP may be reflective of the large spatial and transmural heterogeneity in both APD and ERP in the atrium, perhaps due to diffused fibrosis that is known to disrupt cell-to-cell connexin integrity (113). Accordingly, transmural repolarization differences in APD have been demonstrated in mouse ventricular tissue (354), and a lack of correlation between human ventricular APD and ERP when measured at different sites (519), but highly correlated when measuring APD at the immediate vicinity of the pacing stimulus (520). Nonetheless, an increased refractory period in itself has been suggested to be anti-arrhythmic by increasing wavelength travelled by activation wave and thereby increases the chance of the wave running into still-refractory tissue and self-terminate (110). However, wavelength is also influenced by the conduction velocity i.e. slower impulse propagation allows for recovery from tissue refractoriness. Therefore, the complexity of a reduced conduction velocity in concert with increased refractoriness makes it difficult to establish the contribution of either in arrhythmia risk, especially in structural heart disease such as myocardial infarction and congestive heart failure where refractoriness is increased and conduction velocity reduced (521).

A common finding in these diseases is that tissue fibrosis is commonly increased as a result and has implications for conduction slowing (110). Fibrosis can also increase spatial heterogeneity in atrial conduction developing from disrupted cell-to-cell coupling and result in focal areas of conduction slowing and block, thereby contributing to an arrhythmic substrate (113). Furthermore the enlarged atria, as observed in the TnI-203 mice, has been proposed to be an important factor by allowing co-existence of multiple re-entry circuits (110). Further atrial studies are warranted in other models of HCM, to identify phenotypic differences between atrial electrophysiology and structure that may explain why certain mutations in HCM patients are more prone to atrial arrhythmias than others (522,523).

6.1.2. Biomarkers of extracellular matrix remodelling and inflammation in HCM

In recent years, increasing interests have focused on the identification of easily accessible biomarkers to provide insights into the pathophysiological mechanisms in HCM and a potential tool to risk stratify patients (524). In chapter 2, we identified circulating levels of biomarkers involved in extracellular matrix remodelling and inflammatory pathways. Older age in these animals was associated with elevated matrix metalloproteases-2, and -3 (MMP-2, MMP-3), and transforming growth factor-β1 (TGF-β1), whereas pro-MMP-9 and tissue inhibitor of matrix matelloprotease-1 (TIMP-1) were unchanged. It is curious as to why elevations in these biomarkers of extracellular matrix remodelling occurred in the older age group and not the young animals despite similar fibrosis deposition in the atrial myocardium. Notably, several authors have reported alterations in

circulating matrix metalloproteinases and tissue inhibitors of metalloproteinases with age progression in otherwise healthy subjects (525,526). Komosinska-Vassev et al. (2011) demonstrated increased circulating MMP-3, whereas TIMP-1 had a weak but significant increase with age progression (526). Tayebjee et al (2005) observed a trend towards increased circulating MMP-2 levels, whereas TIMP-1 levels decreased with aging (525). Additionally, elevations in circulating MMP-2 was correlated with increasing age in patients with congestive heart failure (527). Our findings of increased MMP-2 and MMP-3 in older animals are in keeping with these studies. Although the reasons for the observed age-dependent variation in biomarkers of extracellular matrix remodelling despite similar histology findings remain to be elucidated, a possible explanation may be that aging reflects the excess synthesis of these biomarkers released into the bloodstream reflective of chronicity of the disorder or secondary to inflammatory pathways contributing to their secretion (527), but functionality of the biomarkers on the myocardium may be limited to their actions on the local tissue and paracrine levels.

TGF-β1 is a potent stimulator of collagen-producing cardiac fibroblasts resulting in myocardial fibrosis, and selective atrial overexpression of TGF-β1 results in increased atrial fibrosis with increased vulnerability of AF (455). Apart from its pro-fibrotic roles, TGF-β1 is also an important regulator of MMP gene expression and function. TGF-β1 promotes MMP-2 and -9 expression in several cell types such as monocytes (528). Furthermore, the observation of increased vascular cell adhesion molecule-1 (VCAM-1) levels in HCM mice in the current study may be partly due to the potent chemotactic activity of TGF-β1, resulting in the recruitment of inflammatory cell infiltration to the atrial myocardium (528).

MMP-2 upregulation in the current study may be due to increased mechanical stress as a result of cardiomyopathy (529), and stability by extracellular matrix components and inhibiting degradation (530). Additionally, MMP-3 function is not limited to digestion of extracellular matrix components such as fibronectin, laminin and some forms of collagen, but is also involved in the activation of precursor form of MMPs (pro-MMPs) into functional MMPs (531).

6.2. SLOWED ATRIAL AND ATRIOVENTRICULAR CONDUCTION IN HYPERTROPHIC CARDIOMYOPATHY: BASED ON ELECTROCARDIOGRAM PARAMETERS IN THE TNI-203 MOUSE

In chapter three, we observed increases in P wave duration and PR interval in the TnI-203 transgenic mouse, indicative of slowed atrial and atrioventricular conduction. The slowed left atrial conduction was also confirmed with high density mapping studies in the isolated atrial preparation which highlighted slowed and heterogeneous intra-atrial conduction in the 50 week old TnI-203 mouse (Chapter 2). Moreover, we observed significantly prolonged P wave duration despite the normal intra-atrial conduction velocity in the 30 week old TnI-203 mouse. As P wave duration represents both intra- and inter-atrial depolarization, an increased P wave duration in the 30 week old TnI-203 mouse may be more suggestive of inter- instead of intra-atrial conduction slowing. Conversely, ventricular depolarization and repolarization (by measure of QRS, QT and QTc intervals) were unaffected in the TnI-203 mouse at both ages of 30- and 50-weeks. Our findings were similar to that in 21 week old TnI-203 mouse as shown by Tsoutsman et al. (2006) that demonstrated prolonged PR intervals, a weak decrease in QRS interval, and unchanged QT and QTc intervals (P wave duration was not investigated in this study) (337). The unchanged ventricular depolarization and repolarization may reflect the findings of unchanged fractional shortening (a measure of ventricular contractility) in the presence of reduced left ventricular end-diastolic diameter and normal end-systolic diameter in the same study (337). This is in contrast with

normal electrocardiogram and echocardiographic parameters in 2 week old TnI-203 mice that have yet to develop hallmarks of HCM (316).

Notably, different mutations in HCM with contrasting electrocardiogram analysis results likely reflect the phenotypic heterogeneity in HCM. The truncated CaMKIV overexpression and the missense mutation alpha myosin heavy chain overexpression (MHC-403) transgenic mice both demonstrate normal atrioventricular conduction with abnormal ventricular repolarization (longer QT intervals despite normal QRS duration) (387,514). Additionally, gender differences exists at least in the MHC-403 mice, where females develop milder cardiomyopathy than males and demonstrate normal electrocardiographic properties (388). On the other hand, homozygous mutations in the truncated myosin binding protein C mouse (MyBP-C) demonstrate normal electrocardiographic properties (383). Lastly, compound mutations such as the TnI-203/MHC-403 double mutant mouse that develops severe cardiomyopathy with premature death demonstrates increased PR, QRS, QT, and QTc intervals (316). Interestingly, none of the previous studies had looked at P wave durations, perhaps suggesting the prior lack of focus on the atrial properties in the scene of sudden death vulnerability in HCM where emphasis in the literature has been centred on the ventricular histopathology.

6.3. AUTONOMIC NEUROPATHY IN HYPERTROPHIC CARDIOMYOPATHY: EVIDENCE FROM HEART RATE VARIABILITY STUDIES IN TNI-203 MICE

HRV is largely recognized as a tool to investigate autonomic imbalance in a host of cardiovascular diseases, and as a predictor of mortality in a variety of disorders (532). Assessments of HRV using time and frequency domains have been used to indicate vagal activity, although the latter has been subjected to ongoing debate over its usefulness as a marker for activity of the individual branches of the autonomic system (as discussed below). The use of time domain indices such as standard deviation of R-R intervals (SDRR), root mean square of successive differences (RMSSD), coefficient of variance in R-R intervals (CVRR) have been suggested as markers of vagal activity (532,533), whereas frequency domain indices of high frequency (HF) power is thought to reflect mainly parasympathetic activity and low frequency (LF) power reflective of both sympathetic and parasympathetic activities (532).

In chapter three, we also observed depressed heart rate variability (HRV), primarily in time domain HRV analyses, demonstrating reduced SDRR, CVRR, and standard deviation of heart rate (SDHR; an index of the degree of variation in heart rate) in the TnI-203 mouse. However, frequency domain parameters of HRV were not significantly altered in this study. These findings are somewhat similar to results demonstrating normal frequency domain parameters of HRV in the MyBP-C mouse model of HCM (383). However, in that study, the authors concluded that

HRV was unchanged in this strain of mouse despite time domain analyses of HRV (apart from unchanged averaged RR intervals and heart rate as per our findings in the TnI-203 mouse) were not investigated. In humans, reduced HRV has been observed in HCM patients and correlated well with the severity of heart failure (394,395,397), although the genetic contributions of differing mutations have not been investigated.

Nonetheless, results from frequency domain analyses of HRV have been controversial. The high frequency (HF) power spectra is often viewed as a marker of vagal tone (399), whereas the low frequency (LF) power spectra, although reflecting a complex interaction of both vagal and sympathetic activity, is often assumed to have a dominant sympathetic component (400). Therefore, it was suggested by Malliani and colleagues that the LF/HF ratio could be an indicator of sympatho-vagal balance (534). However, the value of these frequency domain parameters have been recently challenged, given that evidence suggest that both LF and HF power are not solely attributable to sympathetic and vagal activity but instead a complex interplay of both as well as non-linear physiological interventions (including mechanical influences of respiration and prevailing heart rate) (401). Thus, LF/HF ratio does not accurately account for cardiac autonomic regulation.

On the other hand, time domain analyses are useful in providing information about overall variability of the heart rate but similarly not effective in identifying specific components of sympathetic or vagal neural activities (535). Additionally, the use of the non-linear Poincaré plots enable a graphical display of

variability in successive beats wherein the greater degree of scatter points indicates increasing variability (535). There are other non-linear methods of quantifying HRV such as the fractal scaling (536), multiscale entropy (537), approximate entropy (538), that are based upon the Chaos Theory and fractal mathematics that will not be discussed further in the scope of the current study.

In conclusion, heart rate variability, which quantifies the beat-to-beat fluctuations and provides an indirect measure of autonomic neural regulation (despite current controversies in identification of specific sympathetic and vagal contributions), is reduced in the TnI-203 mouse model for hypertrophic cardiomyopathy. We note that arrhythmias both in the atria (chapter 2) and ventricles were not inducible in this strain of mice (337). Therefore, rather than its association with mortality due to sudden death, the depression in HRV may be related to degree of atrial and ventricular hypertrophy or dysfunction as has been suggested in some human studies (395,539), and increased atrial mass and left ventricular hypertrophy as observed in our studies, and perhaps an indication of worse clinical outcome in heart failure progression.

6.4. ATRIAL REMODELLING IN DIABESITY: IMPLICATIONS FOR ATRIAL FIBRILLATION

Meta-analyses of the literature have highlighted an increased 49% and 40% risk of AF development with obesity, and type 2 DM respectively (45,61). The common link between obesity and diabetes has been suggested to be insulin resistance (472). This begs the question as to how the metabolic state of insulin resistance translates to AF risk. More known for its glucose and lipid metabolism effects, recent evidence also suggest insulin as a novel anti-inflammatory hormone. Insulin treatment for 2 weeks in poor glycaemic controlled type 2 diabetic patients and 24 hours in patients with severe hyperglycaemia associated with increased pro-inflammatory mediators, led to reductions in pro-inflammatory mediators (540,541). Furthermore, separate from their glucose homeostasis effects, insulin reduces the concentrations of pro-inflammatory mediators in animal models of systemic inflammation due to endotoxin-challenge and thermal injury (542,543).

Insulin demonstrates anti-inflammatory and antioxidant properties by suppressing the nuclear factor kappa-B (NF-κB) transcription activity and reactive oxygen species (ROS) generation, as well as reducing circulating levels of intercellular adhesion molecute-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) that are responsible for the recruitment of pro-inflammatory cells (544). Furthermore, insulin inhibits pro-inflammatory transcription factors including activating protein-1 (AP-1) and early growth response gene-1 and -2 (EGR-1, -2) in mononuclear cells, and reduces circulating levels of tissue factor, plasminogen

activator inhibitor-1 (PAI-1), and matrix metalloproteinase-9 (MMP-9) (545-547). Of which, MMP-9 is involved in extracellular remodelling and has been suggested to contribute to fibrosis. Therefore, a lack of insulin function (either due to a lack of functional insulin or its receptor) as per the insulin-resistant states of obesity and type 2 diabetes may be associated with increased inflammation. Conversely, oxidative stress and inflammatory cytokines can also activate stress-sensitive serine/threonine kinases signalling pathways that ultimately inhibit normal insulin signalling and mediate insulin resistance (548). This is at least partially demonstrated by observations of elevated pro-inflammatory tumour necrosis factor alpha (TNF-α) in adipose tissue from a variety of animal models of obesity and obese humans (468). Furthermore, TNF-α ablation in mice is protective against obesity-induced insulin resistance despite maintaining obesity (549), suggestive of direct associations between inflammation and insulin resistance. TNF-α contributes to insulin resistance by resulting in serine phosphorylation of the insulin receptor substrate-1 (IRS-1), thereby acquiring inhibitory activity towards normal tyrosine auto-phosphorylation of the insulin receptor and consequently interferes with downstream insulin signal transduction (550).

Noting that the current evidence suggests that insulin-resistant state in obesity and type 2 diabetes is associated with an inflammatory response and possibly a contributing factor to a majority of diabetic complications, its combined implications on atrial remodelling and ultimately atrial fibrillation risk has not been well characterized.

6.4.1. Diet-induced Obesity, Hyperglycaemia, Leptin, and Adiponectin Alterations in RCS10 Mice

In chapter 4, we highlighted atrial electrophysiological and structural remodelling in the polygenic NONcNZO10/LtJ (RCS10) mouse model of type 2 diabetes and obesity. Ten week old RCS10 mice demonstrate progressive increase in obesity and hyperglycaemia in relation to increased fat content diets. Additionally, circulating leptin levels paralleled the progressive increase in body weight whereas adiponectin levels were unchanged in RCS10 mice compared to diet-resistant SWR/J mice. Our data suggests that adiponectin may not be a suitable biomarker to indicate the severity of obesity in the polygenic model due to its transient peak at 6 weeks of age (431). The lack of a significant decrease in plasma adiponectin may also have been due to less severe obesity as compared to other models such as the db/db mouse that previously demonstrated lower adiponectin levels (551). This may have been demonstrated had we analysed plasma concentrations of adiponectin in the older age animals that demonstrated maturity-onset obesity and hyperglycaemia. However, we did not foresee the need to perform additional tests as there were little significant diet-induced differences among animal characteristics, electrophysiological and structural parameters in the older animals.

6.4.2. Atrial Electrophysiological Remodelling and Structural Remodelling in diabesity

Atrial ERP and APD were progressively greater with increasing fat concentrated diets in the 10 week old RCS10. This parallels the significant weight gains and

hyperglycaemia that was observed in the 10 week old RCS10 mice with diets of increasing fat concentration. In contrast, 30-week old RCS10 mice that demonstrated similar obesity and hyperglycaemia despite the different diets demonstrated unchanged ERP compared to controls. We do not know the exact reasons for the transient increase in refractoriness; however, this normalization over time may be due to adaptation of the atrium with enlargement. We observed an age-related increase in myocyte hypertrophy in the 30 week old RCS10 mice that may account for this. Furthermore, there is ongoing debate with regards to the significance of atrial refractoriness in the context of atrial remodelling and atrial fibrillation, resulting in difficulty of interpretation of those results. ERP prolongation has been observed in some diabetic studies (272), no change in others despite AF vulnerability (271,446-448), and oxidative stress may also contribute to ERP shortening (444).

Significant heterogeneous and slowed conduction was observed in RCS10 mouse in all diets and ages, except for 10-week old RCS10 mice fed normal diet where RCS10 mice had similar body weight and blood glucose to controls. Additionally, progressively slowed and heterogeneous conduction was observed with greater severity observed in the CHOC diet group. This is in parallel with findings of prolonged P wave duration (as an index of atria conduction; Chapter 5) as well as histological findings of greater fibrosis accumulation and inflammatory infiltrates in the CHOC diet group compared to NOM diets (Chapter 4). Interstitial fibrosis has been postulated to disrupt cell-to-cell gap junctions and interfere with impulse propagation.

6.4.3. Alterations in Biomarkers of Extracellular Remodelling and Inflammation

Looking at circulating levels of extracellular remodelling markers, we observed elevations in MMP-2, pro-MMP-9, TGF-β1 and decrease in TIMP-1 concentrations in RCS10 mice compared to controls with progressive changes with higher fat diets. This was in concert with the histological findings of increased areas of fibrosis in the atrial myocardium in the diabesity mouse. Our findings largely agree with a majority of clinical studies in diabetic and/or dyslipidaemia patients (457-460), but not others (461). On the other hand, results on circulating TIMP-1 levels in diabetic and obese subjects have been inconclusive (457,458,461,463). Increase TIMP-1 secretion from macrophages has been associated with adiponectin (464), whereas adiponectin levels were unaltered in our study. Additionally, RCS10 mice are known to develop diabetic nephropathy (466), and decreased TIMP-1 levels have been reported in patients with diabetic nephropathy (465). Noting that TIMP-1 is a potent inhibitor of activated MMPs, an increase in MMP/TIMP ratio may be suggestive of enhanced extracellular matrix turnover and opportunity of collagen deposition, thereby contributing to fibrosis accumulation.

In addition to observations of increased inflammatory infiltration on histological analyses, we observed progressive elevations in circulating ICAM-1, VCAM-1, and TNFα levels in RCS10 mice with increased fat diets. In obese-linked insulin resistant rodent models, pro-inflammatory TNFα expression is increased in adipocytes (468). Furthermore, TNFα is known to promote MMP expression (552), and may explain for the increased MMP concentrations in the diabesity mouse.

Lastly, insulin has a variety of anti-inflammatory effects (544-547), that are likely impaired in insulin resistance due to disrupted insulin signalling. This may possibly explain for an increase in chemotactic factors ICAM-1 and VCAM-1 as well as pro-inflammatory TNFα in the current study.

6.5. SLOWED ATRIAL AND ATRIOVENTRICULAR CONDUCTION IN DIABESITY: BASED ON ELECTROCARDIOGRAM PARAMETERS IN THE NONCNZO10/LTJ MOUSE

In chapter 5, we observed progressive increase in obesity (as measured by body weight) and hyperglycaemia (as measured with unfasted blood glucose) in the RCS10 mouse in response to increasing fat concentrations in diets as compared to the diet-resistant SWR/J mouse. This was similar in both the young 10-week old and the matured 30-week old age groups. On the other hand, urine glucose levels were elevated in these animals regardless of diets and ages, indicative of diabetic nephropathy. In response to the metabolic derangements, increased PR intervals and progressively prolonged P wave duration, indicative of slowed atrioventricular (AV) and atrial conduction in the diabesity mouse were observed. Not surprisingly, prolonged P wave duration was consistent with intra-atrial conduction slowing in the RCS10 mouse (Chapter 4). However, whilst progressively increased P wave duration reflected intra-atrial conduction slowing in the 10-week old age group, this was not replicated in the 30-week old RCS10 mice. This may have been due to differences in animal characteristics in both studies where the 30-week old RCS10 mice in the atrial electrophysiological studies demonstrated similar increased obesity and hyperglycaemia compared to the progressive obesity and hyperglycaemic states in the electrocardiogram studies. The reason for this discrepancy may be due different disease penetrance in these animals at older ages due to the polygenic cause which cannot be controlled for by random sampling in the colony.

It has been observed that connexin expression levels that are involved in impulse propagation, in particular connexin 45 (Cx45), are elevated primarily in the sino-atrial node in diabetic hearts (albeit in a model of type 1 diabetes) (553). However, it is not clear if such connexin expression changes in the AV node and atria may help explain our findings in the RCS10 mouse. The AV node much like the SA node expresses Cx45, however the atrial tissue expresses mainly the atrial-specific connexin 40 (Cx40) and predominantly connexin 43 (Cx43) that is abundant in both atrial and ventricular myocardium (155). Additionally, a dominance of Cx40 decrease and a dominance of Cx43 increase (in Cx40 and Cx43 knock out mouse models) results in increased propagation velocity in cardiomyocytes strands (155). In contrast, decreased expression of Cx40 but unchanged Cx43 was observed in the streptozotocin-induced type 1 diabetic rat atria that demonstrated heterogeneous and slowed conduction (272). For unknown reasons, these results contrast with the prior study which suggested that decreased Cx40 dominance may increase propagation but may have been due to the differences in strain (Wister rats vs. Cx40/43-null mice) and disease state (diabetes vs. non-diabetes). Additionally, evidence suggests that phosphorylated connexins, homomeric or heteromeric expression of connexons, and lateralization of connexins have implications on impulse propagation slowing and heterogeneous conduction (554-556). Interestingly, all of these studies have been conducted in the myocardium of type 1 diabetic animals and not in type 2 diabetes, highlighting the lack of differentiation between the two states of hyperglycaemia of different aetiologies. Further studies are required to identify the roles connexins

play in atrial and atrioventricular conduction slowing observed in the polygenic RCS10 diabesity mouse model.

6.6. HEART RATE VARIABILITY AS A MEASURE OF CARDIAC AUTONOMIC NEUROPATHY IN DIABESITY

The use of HRV as an index of cardiac autonomic balance in a variety of disorders has been discussed before (Chapters 1.5 and 6.3). Experimental studies have linked glucose metabolic pathways and formation of advanced glycation end products to increased oxidative stress and reactive oxygen species that are involved in promoting inflammation and neuronal dysfunction (557). Consequently, diabetic autonomic neuropathy has been associated with increased mortality risk and therapeutic interventions such as tight glycaemic control and pharmacological therapies such as antioxidant treatment have shown some promise in improving neuropathies in humans (558). Existing data on heart rate variability (HRV) in rodent models of type 2 diabetes and/or high fat diet-induced obesity has been summarized in Table 1. Changes in average heart rate in diabetes and/or obesity has been largely inconclusive with studies demonstrating an increase (482,484,493,559), decrease (483,486,500), and even no change in heart rate (500). The differences in these results may be due to the strain differences, age of assessment, and differences in technique of assessment. The techniques used to assess HRV in these models include electrocardiogram (ECG) telemetry, blood pressure telemetry, ECG under anaesthetics, and ECG in restraint animals with needle electrodes. Telemetry recordings are generally considered superior as the measurements are collected from conscious stress-free animals in their natural environment as compared to the use of anaesthetic agents which renders the animal unconscious and restraint which increases stress-induced changes to the

physiologic measurements (560). However, as both the experimental and control animals underwent the same treatment, any significant differences between the two cannot be easily disregarded. Apart from a few studies (483,500), current literature generally highlights that HRV is reduced in diabetic/obese animals (482,484,486,493,559). However, it should be noted that assessment of HRV in these studies have not been standardized, with majority of studies employing frequency domain analysis and others time domain analysis of HRV or both. The implications and controversies of the frequency domain analyses of HRV have been discussed previously in section 6.3 of this chapter.

In Chapter 5, we reported findings of decreased SDRR and RMSSD and trends towards increased HF and decreased LF in the polygenic RCS10 mouse model of diabesity. The difference in age (10 vs. 30 weeks) did not appear to be a major influence in HRV deterioration. Our results were similar to that of early studies by Masaoka et al. (1985) where the authors identified that while age was an important factor accounting for the decrease of HRV in type 1 diabetes, aging in type 2 diabetes played a lesser role (561). Another reason for the lack of age being an important factor to further decrease in HRV may be because 30 weeks of age did not provide additional chronicity of diabetes to illicit a significant difference as compared to 10-week old RCS10 mice. On the other hand despite the significant differences observed in time domain analyses, our findings of a non-significant trend towards increased HF and decreased LF may reflect the contention that LF/HF ratio is not a reliable index of sympatho-vagal balance (401).

In summary, RCS10 mice demonstrate reduced HRV (as observed with time domain indices) regardless of aging (based on the age groups tested) as compared to the diet-resistant to diabetes and obesity SWR/J mouse. Whilst existing HRV studies in other models of diabetes and/or obesity have been listed in Table 1, the RCS10 mouse model differs for a variety of reasons i.e. polygenic vs. monogenic cause (db/db mouse), spontaneously obese vs. lean (Goto-Kakizaki rats), and mild insulin-resistance vs. moderate hyperinsulinemia and hyperglycaemia (HFD rat). As diabetes and obesity are both modifiable risk factors through dietary modification and/or physical activity, it would be interesting to investigate if such interventions are able to rectify and improve heart rate variability dynamics in the RCS10 mouse.

TABLE 1. CURRENT LITERATURE ON HEART RATE VARIABILITY IN RODENT MODELS OF TYPE 2 DIABETES AND DIET-INDUCED OBESITY.

Reference	Diabetes Model	Diabetes Type	Intervention	Method of neuropathy assessment	Time of assessment	Outcome	Effect of treatment
Howarth et al., 2008	Goto-Kakizaki, Rat	Type 2	None	ECG (telemetry) HRV (SDNN)	2, 7, and 15 months of age	↓ HR ↓ HRV (↓SDNN; at 2 & 7 months)	
Goncalves et al., 2009	db/db, Mouse	Type 2	None	BP (telemetry) HRV (LF HR oscillation)	12 weeks	↑ HR ↓HRV (LF HR oscillation)	
Senador et al., 2009	db/db, Mouse	Type 2	Losartan (Ang-II receptor antagonist)	BP (telemetry)	8, 14 weeks	↓ HR ↔ HRV (pulse interval variability, LF, HF)	No effect No effect
VanHoose et al., 2010	ZDF, Rat	Type 2	Treadmill exercise (7 weeks)	ECG (anesthetized) HRV (SDRR)	12 & 19 weeks	↑ HR ↓HRV (SDRR)	No effect No effect
Apaijai et al., 2013	HFD, Rat	Insulin-resistant	Vildagliptin & Sitagliptin (DPP-4 inhibitors)	ECG (restraint) HRV (LF/HF ratio)	12 weeks diet + 3 weeks treatment	↑ HR ↑ LF/HF power ratio	Reversed
Supakul et al., 2013	HFD, Rat	Insulin-resistant	Garlic extract	ECG (restraint) HRV (LF, HF, LF/HF)	12 weeks of diet + 4 weeks of treatment	↑ HR ↑ LF/HF power ratio	Restored Partially restored
Soltysinska et al., 2014	db/db, Mouse	Type 2	None	ECG (anesthetized)	2 months	**Fasted:** ↓ HR ↓ HRV (↑SDARR & pRR6, ↔LF, HF & LF/HF) **Fed:** ↔ HR ↔ HRV	

Chapter Seven

Future Directions

7.1. INTRINSIC VS. SECONDARY ATRIAL MYOPATHY IN HYPERTROPHIC CARDIOMYOPATHY?

Atrial hypertrophy, increased atrial fibrosis, and atrial systolic and/or diastolic dysfunction are collectively known as atrial myopathy. Atrial myopathy is a common presentation in patients with hypertrophic cardiomyopathy (HCM) and the more severe dilated cardiomyopathy (306,562). Components of atrial myopathy such as atrial enlargement and atrial dysfunction are strong predictors of atrial fibrillation (AF) development in these patients (284,303,325). However, whether atrial myopathy occurs primarily as a result of a mutation in the sarcomeric protein (intrinsic atrial myopathy) or a consequence of a progressively deteriorating ventricular function resulting in increased atrial pressure has not been clearly defined. Several studies suggest that atrial myopathy (either through atrial systolic function or atrial fibrosis) does not correlate significantly with left ventricular dysfunction (306,562), thereby suggestive of intrinsic atrial myopathy as opposed to secondary atrial myopathy due to mechanical overload.

The current mutant cardiac troponin I overexpression (TnI-203) mouse model demonstrates age-related differences in atrial refractoriness and slowed conduction heterogeneity despite the similar left ventricular hypertrophy in both

ages, suggesting that at least some electrophysiological parameters linked to atrial function and thereby atrial myopathy may not be entirely related to ventricular dysfunction. Further studies of atrial electrophysiology and histopathology in other mutations related to HCM, such as the little or no ventricular hypertrophy and fibrosis troponin T mutant mouse (517), and the late HCM phenotype developing heterozygous myosin binding protein C mutant mouse (314), will be useful to provide information on the different degrees of atrial myopathy in different mutations but contributions of existing ventricular dysfunction to atrial myopathy would be hard to ignore.

Although the current transgenic mouse models do not completely answer if intrinsic atrial myopathy is a concomitant development of HCM, modern genetic manipulation techniques may help provide some answers. A recent study by Groenke et al. (2014) employed the use of the Cre/loxP recombination system under the control of the atrial-exclusive sarcolipin promoter to selectively knock-out the sodium-calcium exchanger in the atria of transgenic mice (563). This technique presents a useful method to investigate atrial myopathy where by the mutation in a particular sarcomeric protein responsible for HCM (e.g. cardiac-specific TnI-203 mutation) can be selectively knocked out from the atrial of transgenic mouse to provide a model with sole expression of the mutation in the ventricles. Alternative, with the use of a ventricular-selective promoter in conjunction with the Cre/loxP recombinant system, a ventricular-specific knock out mouse model could be generated such that the mutation is solely expressed in the atria of the transgenic mouse. Identifying the cause of atrial myopathy will help to understand the pathogenesis of HCM and improve clinical outcomes with better

targeted AF therapies in the absence of severe ventricular myopathy or fibrillation in HCM.

7.2. REVERSE ATRIAL REMODELLING: TARGETING INFLAMMATION, OXIDATIVE STRESS, AND ATTENUATING FIBROSIS

Increasing evidence suggests the central role of fibrosis, inflammation and oxidative stress to atrial remodelling and ultimately AF. Noting that tachycardia-induced atrial remodelling or "AF begets AF" does not entirely explain the predisposition for the initial AF development in all cardiovascular diseases, increasing number of studies have since identified certain hallmark structural modifications in the atria that are present in a variety of structural heart disease and cardiomyopathies (Chapter 1.4). Figure 1 highlights a schematic diagram on the central role of inflammation, fibrosis and oxidative stress in the promotion of structural and electrical remodelling contributing to AF development in diabetes, obesity, and hypertrophic cardiomyopathy. Notably, the biochemical pathways linking inflammation, fibrosis and oxidative to adverse atrial remodelling is extremely complex and often inter-related (Figure 1). On the other hand, atrial fibrosis itself independent of inflammation, necrosis, hypertrophy, or ventricular fibrosis has also been shown to contribute to AF vulnerability in the transforming growth factor-β1 (TGF-β1) overexpression mouse model (455).

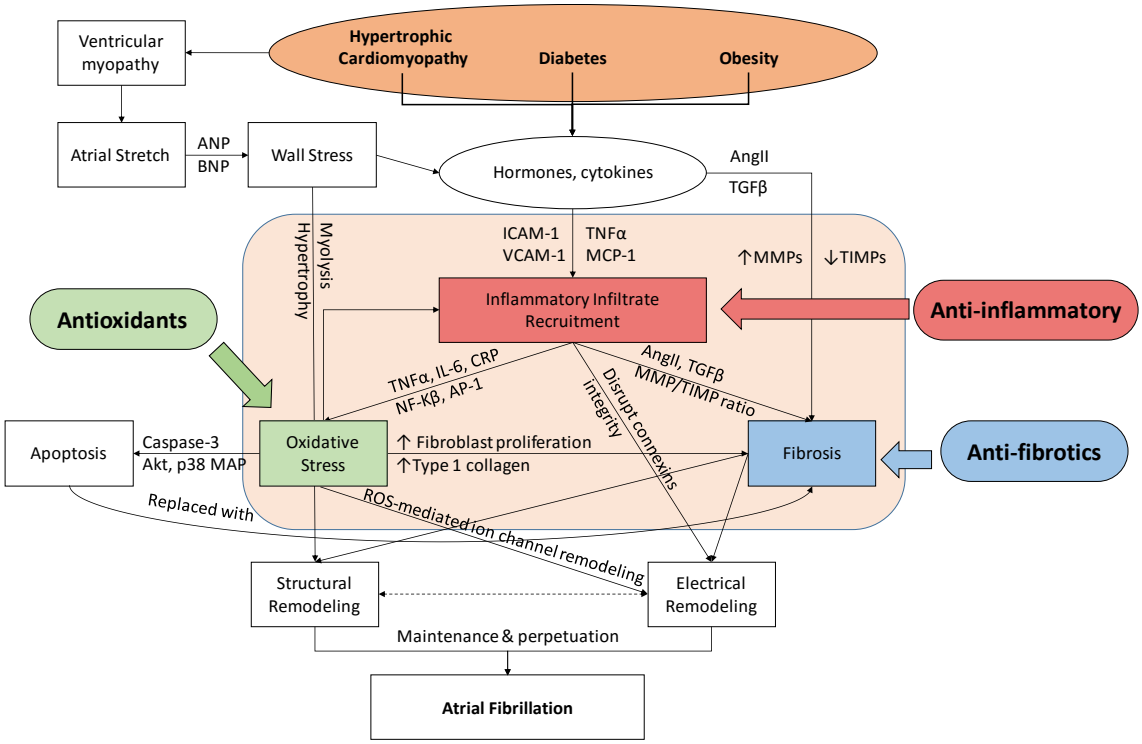


FIGURE 1. The central role of inflammation, fibrosis and oxidative stress as contributors of atrial myopathy and considerations for therapies to counter atrial structural and electrical remodelling.

7.3. LIFESTYLE MODIFICATIONS AND PHARMACOTHERAPIES TO REVERSE ATRIAL REMODELLING IN HYPERTROPHIC AND DIABESITY-INDUCED CARDIOMYOPATHY: ROLE OF INFLAMMATION, OXIDATIVE STRESS, AND FIBROSIS

7.3.1. Lifestyle Changes Through Physical Activity and Diet Modifications

Recent animal studies have also demonstrated that low intensity treadmill exercise (11m/min, 18 mins) provides beneficial cardiac remodelling and reduced myocardial oxidative stress in diabetic rats (564). Similarly, voluntary cage-wheel exercise introduced early prevented the development of HCM hallmarks that includes ventricular fibrosis, myocardial disarray and induction of hypertrophic markers, as well as reversed these established HCM phenotypes (with the exception of fibrosis) in a transgenic mouse model of alpha myosin heavy chain mutation (565). This was primarily due to reduced activation of nuclear factor of activated T-cells (NFAT; a specific marker of pathologic hypertrophy), atrial natriuretic factor, beta myosin heavy chain (dominant myosin heavy chain isoform in foetal development), as well as pro-apoptotic factors. Additionally, voluntary cage wheel exercise performance was the same between non-transgenic and HCM mice as opposed to impaired exercise tolerance by treadmill running (566), presumably due to an increased stress factor in the latter protocol (20m/min, 60 mins). Accordingly, it has been demonstrated that high intensity exercise promotes atrial fibrillation with associated atrial remodelling that is mediated through the actions of the pro-inflammatory cytokine TNFα (567,568). Taken together, the

literature supports that low (but not high) intensity exercise provides cardiovascular benefits in HCM mice. With low intensity exercise demonstrating prominent cardiovascular benefits by improving myocardial function and ventricular pathology through its anti-oxidant effects, similar effects should be observed in the atria although this has not been investigated.

Whilst calorie-rich diets have been known to contribute to obesity-induced electro-anatomical remodelling associated with increased propensity for AF in the sheep model (449), weight reduction has also been shown to reverse obesity-induced structural changes and partially reverse electrophysiological remodelling (344). Based on our current data demonstrating maturity-onset obesity in the RCS10 mouse even with normal chow, it would suggest that switching from a high fat diet to a low fat diet may not substantially alter metabolic characteristics and accordingly may not reverse atrial remodelling in these animals. Hence, low intensity exercise as a method for weight loss should be preferable to test the concept of atrial remodelling reversal in the polygenic diabesity mouse.

Intriguingly, the type of diet composition has been shown to impact on HCM. Using the alpha myosin heavy chain mutant HCM mouse, Stauffer and colleagues demonstrated a more severe HCM pathology in male mice fed a soy-based diet compared to a casein-based diet, whereas female mice were more protected (569). The authors reasoned that females were more tolerant as they were more exposed to phytoestrogens in soy that activates estrogen receptors, as opposed to their male counterparts. It would be interesting to see if the use of a soy diet may

aggravate hypertrophic cardiomyopathy in the cardiac troponin I mutant mouse and perhaps contribute to inducible AF (a physiological outcome of adverse atrial remodelling that was regrettably not observed in our study).

7.3.2. Pharmacotherapies Targeting Inflammation, Oxidative Stress and Fibrosis

Glucose fluctuations in type 1 diabetic rats have been associated with a further increased propensity for AF than uncontrolled diabetes possibly due to substantial atrial fibrosis and reactive oxygen species generation, and well-controlled diabetes with insulin therapy reverses such outcomes (448). The anti-inflammatory and anti-oxidant actions of insulin have been previously discussed in Chapter 6.4. It should be noted that insulin resistance in obesity and type 2 diabetes is associated with initial compensatory pancreatic islet β-cell secretion of excess insulin, followed by the progressive decline in β-cell function resulting in inadequate insulin secretion (472). Therefore, treatment with exogenous insulin may not be an effective therapy in insulin resistant states, especially in the initial phase of hyperinsulinemia. The use of thiazolidinediones may be useful in the alternative treatment of insulin-resistance state in obesity and type 2 diabetes not just for its insulin sensitizing effects, but also for their anti-inflammatory properties (570-572). Notably, obesity and diabetes both result in increased concentrations of asymmetric dimethylarginine (ADMA) responsible for inhibiting nitric oxide synthase activity and consequently reducing nitric oxide bioavailability, thereby limiting the sequestration of superoxide radicals (573,574). Rosiglitazone, belonging to the thiazolidinedione class of anti-diabetic drugs, is known to suppress plasma ADMA concentrations and improve endothelial function in insulin

resistant obese but non-diabetic patients (575). In contrast, although improvements in endothelial function was observed, the beneficial effects of rosiglitazone on ADMA and other markers of oxidative stress was not confirmed in type 2 diabetic patients (576). This suggests of anti-inflammatory effects of rosiglitazone but not supportive of its anti-oxidant role in the setting of diabetes. Further studies are warranted to investigate the biochemical pathways that thiazolidinediones exert their anti-inflammatory effects (direct or indirect actions of insulin sensitization), as well as their potential use to reverse cardiac remodelling and cardio-protective effects.

The use of anti-oxidants such as vitamins, flavonoids and statins has also been demonstrated to reduce oxidative stress and inflammation, paving way for new therapies to reduce cardiac structural and electrophysiological remodelling. Vitamin D supplementation has been shown to demonstrate anti-inflammatory, anti-oxidative, anti-hypertrophic and anti-fibrotic properties in the heart (577-581). Alternatively, flavonoids such as rutin treatment has been shown to reduce cardiac remodelling, left ventricular and myocardial dysfunction in diabetic rats (582). Similarly, apart from their cholesterol lowering effects, statins also possess anti-oxidant and anti-inflammatory properties that are demonstrated with suppression of atrial tachycardia-induced electrical remodelling, attenuation of congestive heart failure-induced atrial fibrosis, and inhibited inflammation and AF development in sterile pericarditis in dogs (583-585).

Lastly, as atrial fibrosis has been a common finding in a variety of heart diseases and arrhythmias (586), anti-fibrotics may provide therapeutic benefits to

reverse structural and electrophysiological remodelling. Over the past two decades, increasing evidence demonstrate the physiological role of the renin-angiotensin-aldosterone system (RAAS) on promoting myocardial fibrosis (172,587). In particular, inhibition of the RAAS in troponin T mutant HCM mice reverses ventricular myocardium fibrosis and also demonstrated to regress ventricular myocardial fibrosis associated with improved ventricular function in hypertensive rats (588-590), whereas this was not confirmed in the atria by Choisy and colleagues where they showed atrial fibrosis was unaffected despite normalization of blood pressures in spontaneously hypertensive rats (591). This may suggest that pathophysiological pathway to fibrosis differs in the atria compared to the ventricles. Perhaps, atrial fibrosis is more dependent on TGF-β1 as evident by selective atrial and not ventricular overexpression of TGF-β1 in transgenic mice despite the use of a cardiac-restricted promoter (455). On the other hand, the use of angiotensin converting enzyme inhibitors, angiotensin II type 1 receptor blockers and aldosterone antagonists were effective in reducing atrial fibrosis in a dog models of congestive heart failure and/or rapid atrial pacing (175,178,592,593), in diabetic rats (594), heart failure in rats after myocardial infarction (595), and rabbits with congestive heart failure (596). These findings collectively suggest that the discrepancy may either be due to the animal model and/or the disease state. Additionally, whilst angiotensin II treatment independently upregulated leptin concentration, increased atrial fibrosis, delay inter-atrial conduction time, and increased AF vulnerability in otherwise normal mice, this was attributed to leptin signalling as leptin-deficient ob/ob obese mice were protected (429,430). This further suggests the need for more research in polygenic models with intact leptin signalling to better reflect human obesity and

diabetes where hyperlipidaemia frequently develops. Alternative anti-fibrotic agents such as tranilast and relaxin have been shown to inhibit myocardial fibrosis and collagen deposition in animal models of hypertension and rapid atrial pacing (597-601), warranting its investigation to reduce cardiac fibrosis in diabetic, obesity, and hypertrophic cardiomyopathy.

Taken together, lifestyle modifications, anti-inflammatory, anti-oxidant, and anti-fibrotic agents are highly probable interventions to attenuate detrimental atrial remodelling in diabetic, obesity and hypertrophic cardiomyopathy where such research is currently lacking. The current studies that have used interventions focus largely on adverse cardiac remodelling with a primary focus on the ventricular myocardium and function. With increasing evidence suggesting that AF is a common and frequent concomitant disorder in a variety of cardiovascular diseases, future research should be undertaken to investigate the molecular pathways of such therapies and their effects to reverse the abnormal atrial substrate that contributes to AF development. These improvements of outcomes may likely improve cardiac autonomic regulation and thus presented with modifications of heart rate variability and if translated in human patients may suggest of improvements to mortality outcomes in these disease states.

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