High fat diet alters left atrial electrophysiology and predisposes murine hearts to arrythmias.

By

Katie Tompkins

A thesis submitted to the University of Birmingham for the degree of

MASTER OF SCIENCE BY RESEARCH

Department of Medical and Dental Sciences

25.09.2024

Supervisors – Dr Davor Pavlovic and Dr Christoper O'Shea

ACKNOWLEDGEMENTS

I would like to offer my sincere gratitude to my supervisors, Dr Davor Pavlovic and Dr Christopher O'Shea for all their support throughout this project, and without whom this project would not have been possible. I would like to extend my thanks to Dr O'Shea for completing the experiments carried out within the project, so I could complete the analysis for my thesis.

I would also like to acknowledge and thank my laboratory group, including Abbie, Olivia, Rina and Daniyah, who continually supported me and constantly had patience to answer my questions.

Contents

ABBREVIATIONS

The International Systems of Units was used throughout	
AF	Atrial Fibrillation
ANS	Autonomic nervous system
APD	Action Potential Duration
AV Node	Atrioventricular Node
Ca^{2+}	Calcium channel
cm/s	Centimetre per second
CV	Conduction Velocity
DI	Diastolic Interval
EADs	Early afterdepolarisations
EAT	Epicardial adipose tissue
HFD	High fat diet
IVC	Inferior vena cavae
K^{+}	Potassium channel
KHB	Krebs-Henseleit Bicarbonate
KO	Knockout
LA	Left Atria
L-type	long opening
LV	Left Ventricle
mg/ml	Milligrams per millilitre
mM	Millimolar
ms	Millisecond
Na^{+}	Sodium channel
nM	Nanomolar
°C	Degree Celsius
Pitx2	Paired-like Homeodomain 2
RA	Right Atria
ROS	Reactive oxygen species
RV	Right ventricle
SA Node	Sinoatrial Node
SVC	Super vena cavae
TTP	Time-to-peak
WT	Wild type
µM	Micromolar

1. ABSTRACT

Atrial fibrillation is the most common form of cardiac arrythmia. High fat diet significantly increases atrial fibrillation directly, and indirectly, through related cardiac conditions, like hypertension. High fat diet- driven electrical remodelling contributes to atrial fibrillation through ion channel alterations, inflammation, autonomic dysfunction and oxidative stress which can all lead to alterations in action potential duration, conduction velocity, diastolic interval, time to peak and more. A genetic deficiency in Pitx2 has been closely linked to atrial fibrillation, and the gene is a crucial factor for the development and function of the left atrium. A deficiency in Pitx2 can disrupt the ion channel regulation and electrical conduction, which leads to an imbalance within the electrical signalling across the atria. This creates a substrate that is prone to re-entrant circuits and ectopic activity, leading onto arrythmias.

The studies primary aim was to investigate whether a high fat diet induces electrophysiological remodelling in the murine left atria specifically, and whether the response is further modulated by altered expression of Pitx2. Additionally, the study aimed to see whether proteomic experiments can be conducted on dyed, optically mapped murine left atria.This study optically mapped the left atria only, from wild type and Pitx2 deficient mice, who both obtained a control group and a high fat diet group. Langendorff-perfused left atria were paced at different cycle lengths and recorded using optical mapping. Two-way ANOVA and one-way ANOVA statistical analyses revealed a significant difference in action potential duration, showing a prolongation in the high fat diet group against control. However, the results identified a shortening in action potential duration within the Pitx2 deficient murine hearts, versus wild type. There were no other significant differences established in the study.

The study also assessed whether optically mapped, dyed murine hearts can undergo proteomic analysis. The results found that the optically mapped hearts can successfully endure proteomic analysis, with no significant differences found between the two groups. There were a similar number of contaminants identified, all under 5 each and each heart expressed over 400 proteins.

Further experiments including a larger sample size, longer study period and using both male and female mice is essential to gain a deeper insight into the underlying mechanisms of high fat diet altering left atrial electrophysiology. Additionally, more proteomic analysis on the optically mapped, high fat diet and Pitx2 deficient hearts is crucial to conduct, to identify any metabolic changes with protein expression within the hearts.

2. INTRODUCTION

Arrythmias are classified as a chaotic, irregular heart rhythm or rate. The most prevalent form of arrythmia is known as atrial fibrillation (AF), which is defined as a rapid and disorganised activation of the atria, leading to impaired atrial function (Wyndham, 2000). With the aging population and demographic changes, AF has risen to become a pandemic with the global prevalence being approximately 37.5 million people, with a predicted 60% increase in this number by 2050 (Lippi et al., 2021). In the UK, approximately 1.4 million members of the population have been diagnosed with AF.

AF can present itself in a variety of forms, including paroxysmal (erratic episodes which stop within seven days), persistent (episodes lasting more than seven days and may require intervention) and permanent (continuous AF) (Dilaveris and Kennedy, 2017). AF is a growing concern due to its close association to severe health complications and high mortality rates from stroke and heart failure (Essa, Hill and Lip. 2021). For example, there is a five-fold increase in suffering from a stroke if the individual has AF, due to the formation of blood clots which can travel to the brain (Essa, Hill and Lip, 2021).

The aetiology of AF is multifactorial, involving a large variety of risk factors which include hypertension, genetic predisposition, obesity, diabetes, hyperthyroidism and coronary artery disease (Lau et al., 2017). In addition, lifestyle factors contribute to

increased risk, such as smoking, excessive alcohol, and stress all exacerbate the cardiac condition (Lau et al., 2017).

Clinically, an individual may present AF asymptomatically, however symptoms may include heart palpitations, shortness of breath, dizziness and fatigue (Heidt et al., 2016). Individuals are commonly diagnosed through an electrocardiogram (ECG) which provides a tracing of the heart rhythm. Typically, an AF tracing does not demonstrate a P-wave and obtains a chaotic QRS complex rhythm (Nuzhat and Yong, 2020). Further diagnostic tools may be required, such as echocardiogram, Holter monitoring and blood tests.

Obesity and genetic predisposition are both significant factors linked to the development of AF. The global prevalence of obesity has dramatically risen over recent years, with approximately 25.9% of adults in England being obese, and a further 37.9% classed as overweight (Keaver et al., 2020), coinciding with an increased high-fat diet consumption. This dietary shift has been consistently linked to adverse events, particularly a range of cardiovascular diseases (Keaver et al., 2020). Effects on the electrophysiological properties of the heart are less clear, particularly the link to the left atrium and how high-fat diet may lead to AF. Electrophysiological remodelling in the left atrium is maladaptive, thus any alterations may predispose individuals to arrhythmias (Abed et al., 2013).

Developing evidence from animal models suggests high-fat diet-induced obesity results in significant changes in cardiac structure and function. High fat diets are associated to increased left atrial size, fibrosis and inflammation, promoting arrhythmogenic conditions (Suffee et al., 2021). The structural remodelling of the heart involves alterations in the cellular and extracellular properties in heart tissue. Structural

remodelling is commonly supplemented with electrical remodelling, which refers to alterations in the ion channel expression, action potential characteristics and electrical conduction of the heart tissue and cells (Suffee et al., 2021).

There is and established genetic component to AF. Mutations near the gene encoding for Pitx2 develop and maintain the individuality and function of the left atrium. Furthermore, it regulates the expression of multiple genes involved with calcium handling, ion channel expression and the architecture of cells (Syeda, Kirchhof and Fabritz, 2017). Mutations in the vicinity of Pitx2 genes have been associated with AF, and previous research has shown that a reduction in Pitx2 can drive cardiac electrical remodelling, increasing susceptibility to arrythmias (Syeda, Kirchhof and Fabritz, 2017). However, the mechanism behind this research remains unclear.

Management of AF is comprehensive, due to potential disparities between individuals' severity, symptoms and any underlying conditions. Essentially, management is aiming to restore sinus rhythm, control heart rate and prevent stroke. Treatment options can include beta blockers, calcium channel blockers, sodium channel blockers, potassium channel blockers and anticoagulants. Furthermore, pharmacological interventions may include cardioversion or catheter ablation (Sha et al., 2024).

In this study, we investigate the effects of high-fat diet on the electrophysiological properties of the left atrium in murine models, subsequently identifying whether the Pitx2 gene contributes to further cardiac remodelling.

2.1. Anatomy and Function

Newly oxygenated blood enters the left atrium (LA) from the lung, via the pulmonary veins and is further pumped into the left ventricle (LV) where it travels through the body. Once the blood has travelled through the systemic circulation, the de-

oxygenated blood enters the right atrium (RA) from the superior vena cave (SVC) and inferior vena cave (IVC), which pumps blood into the right ventricle (RV). From here, the blood enters the pulmonary circulation to be re-oxygenate via the pulmonary artery (Buckberg et al., 2018). This process is collectively known as the cardiac cycle and maintains blood pressure.

The heart is made up primarily (by volume) of cardiomyocytes, which are cardiac muscle cells. Cardiomyocytes are electrically and mechanically coupled together by intercalated discs. These discs contain gap junctions for electrical communication, and adherence proteins for dynamic mechanical coupling. The junctions are located at cell ends and permit cell-to-cell electrical and mechanical impulses (Goodenough and Paul, 2009).

Cardiomyocyte contraction is initiated by electrical excitation of the cell. During sinus rhythm, action potentials begin in the sinoatrial node (SA Node) located in the RA, containing pacemaker cells which are specialised and exhibit automaticity, and able to depolarise without a neighbouring cell impulse. These pacemaker cells set the electrical impulse conduction, which propagate throughout the myocardium (Mikawa and Hurtado, 2007).

If a neighbouring cell is less depolarised, the current can flow into the electrically coupled cell, meaning the transmembrane voltage will increase until threshold potential is reached. At this point, voltage gated sodium channels (Na^+) will open, which causes a large influx of sodium to allow initiation of action potentials (Mikawa and Hurtado, 2007).

Cardiomyocytes can be categorised into three groups.

1. Working cardiomyocytes: the cells form a majority of the myocardium layer and are connected into the electrical and mechanical function gap junctions and adherence proteins. The sarcolemma of cardiomyocytes contains t-tubules, these permit a greater ion transfer into the cells during action potential initiation (Woodcock and Matkovich, 2005)
2. Pacemaker cardiomyocytes: as previously mentioned. These cells create the SA node and AV node, obtaining automaticity. They achieve spontaneous depolarisation due to specialised ion channels which slowly conduct Na^+. Pacemaker cells produce a 'funny current' (I_f), which depolarises the membrane to initiate action potential activation. Pacemaker cells are smaller than working, they obtain little contraction and are prone to regulate cardiac function through the autonomic nervous system (Woodcock and Matkovich, 2005).
3. Conduction cardiomyocytes: these cells are in the bundle of his and Purkinje fibres. They obtain similarities to working cardiomyocytes but are larger and less contraction (Woodcock and Matkovich, 2005).

2.2. Action Potential Morphology

The cardiac action potential is initiated and controlled by ion movements, predominantly sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) – movement in and out of both intracellular and extracellular environments (Ottolia et al., 2021). There are multiple phases to the action potential.

Phase 0- Depolarisation

The transmembrane voltage rises from the resting membrane potential to a threshold value. This value triggers the opening of voltage gated Na^+ channels, which increases the membrane conductivity of Na^+ and produces a depolarising influx of Na^+ ions (I_{Na}).

Furthermore, the transmembrane voltage depolarises to approximately +20mV to +30mV, leading to Na^+ channels deactivating due to a short lifetime (Ravens and Cerbai, 2008).

Phase 1 – Initial Repolarisation

The transmembrane begins to repolarise quick due to transient outward potassium current (I_{to}). I_{to} allow K^+ ions to flow out the cell along the electrochemical pathway. The rise in intracellular Na^+ also leads to an increase in sodium-potassium pump activity, increasing the repolarising current of the action potential (Varro et al., 2021).

Phase 2 - Plateau

The quick depolarising cell initiates the long opening (L-type) Ca^{2+} channels, these channels carry a depolarising current (I_{CAL}), which permits Ca^{2+} ions into the cell. Comparing L-type to fast sodium channels, the L-type open and inactivate much slower which causes a plateau phase due to prolongation of the action potential (Grant, 2009). I_{CAL} obtains similarities to outward K^+ channel, which is further reduced during the plateau phase due to a blockade of inward rectifier channels (I_{Kir}).

As the L-type Ca^{2+} channels close, the late phase of the plateau is maintained by a Na^+-Ca^{2+} exchanger. The influx of Ca^{2+} into the sarcoplasm from both the L-type Ca^{2+} channels and calcium-induced-calcium-release from sarcoplasmic reticulum induces the Na^+-Ca^{2+} exchanger (Grant, 2009).

Phase 3 – Repolarisation

When the action potential reaches the late phase of the plateau, the K^+ conductance increases because of activation of the slow rectifier K^+ channels (I_{KR} and I_{KS}). Like Na^+

and Ca^{2+} channels, I_{KR} and I_{KS} obtain slow activation – which drives the action potential in repolarisation during late phase.

Phase 4 – Resting Membrane Potential

The action potential reaches the resting membrane potential, at a baseline electrical charge due to no active signal. The potential rests around -85mV and is maintained from the sodium-potassium pump, restoring the balance.

3. REVIEW OF THE LITERATURE

3.1. Mechanisms driving atrial fibrillation

3.1.1. Ectopic Activity

Ectopic activity is defined as minor changes to a heartbeat (Voigt and Dobrev, 2012). Ectopic foci are characterized as an excitable group of cells which cause a premature heartbeat, located outside the SA node (Lin et al., 2000).

In relation to AF, it is common for multiple ectopic foci to be located around the pulmonary veins. When the ectopic foci produce a premature beat in the atria, it can initiate a wave of electrical activity across the whole heart, thus disrupting sinus rhythm (Gong et al., 2007). Underlying conditions may include hypertension, coronary artery disease or a range of structural changes, leading onto the evidence showing that repeated occurrences of ectopic activity can lead to structural changes, for instance fibrosis (Gong et al., 2007).

The potential for an ectopic beat to trigger AF can be further related to haemodynamic stress factors, for instance an increase in intra-atrial pressure leads to atrial electrical and structural remodelling (Yamazaki et al., 2012). Thus, this concept leads onto atrial

stretch, myocardial ischemia, and pre-existent intra-atrial conduction inhomogeneity which all predispose individuals to AF (Yamazaki et al., 2012).

A study by Waktare et al., (2001) identified that characteristics of atrial ectopic activity which precedes the onset of AF, differ from ectopic activity at different times through the heart. This is further supported by De Vries et al., (2018), who related the coupling interval (distance between onset of preceding sinus QRS and premature beat) of the ectopic activity that initiated AF, to a shortening in activity when comparing to ectopic activity unrelated to AF (Maupoil et al., 2007). The study by Waktare et al., (2001) distinguished the key differences being the atrial ectopic activity is more frequent and significantly more premature (Waktare et al., 2001).

Looking into the structure of ectopic beats, a study by Lenis, Baas and Dossel (2013) found that there was a consistency in the morphology of ectopic beats across all study subjects. Additionally, the study identified two potential mechanisms to reason behind the association of ectopic beats and AF. Firstly, atrial focus may act as a trace of individual ectopic beats which trigger the AF. Secondly, atrial focus may repetitively be active as a form of atrial tachycardia – which enhances fibrillatory conduction, thus, AF (Lenis, Baas and Dossel, 2013).

In addition, Pitx2 regulates multiple ion channels and genes involved in the heart's electrical activity, when Pitx2 is deficient, it leads to imbalances in electrical properties of the cardiac cells. This causes abnormal repolarisation and depolarisation which creates favourable conditions for ectopic activity (Bai et al., 2021).

Furthermore, Pitx2 deficiency unsettles the normal development of the myocardium and associated pulmonary veins. The pulmonary veins are essential in maintaining the electrical stability, thus improper development will lead to generating abnormal

electrical impulses. The SA node initiates the electrical impulses which regulates heart beats, however if Pitx2 is absent, an ectopic pacemaker activity will develop in external regions from the SA node, resulting in ectopic heartbeats (Bai et al., 2021).

Obesity initiates ectopic heartbeats in various ways. As obesity causes cardiac hypertrophy, particularly in the left ventricle, it imposes extra workload onto the heart. The enlargement and wall thickening can disrupt the heart's normal electrical conduction pathway, which makes the heart prone to ectopic activity (Verheule and Schotten, 2021). Similarly, left atrial enlargement alters the electrical properties of the atrial cells and can cause ectopic heartbeats.

Furthermore, obesity leads to abnormal electrolyte levels, particularly causing low potassium and magnesium levels. Potassium in the heart is responsible for the resting membrane potential of cardiac cells, therefore when obesity causes low potassium levels it decreases the potassium gradient which makes the resting membrane potential hyperpolarised and negative (Weiss, Qu and Shivkumar, 2017). This can lead to the cardiac cells becoming more excitable and prone to impulsive depolarisation, which can result in ectopic heartbeats. Low potassium levels also contribute to increased automaticity of the ectopic pacemaker cells, which leads to abnormal signals in the tissue to cause premature beats (Weiss, Qu and Skivkumar, 2017).

Magnesium is essential for stability in the heart, by modulating calcium and potassium ion channels. Obesity can cause low magnesium levels which can lead to increased intracellular calcium, enhancing the excitability of cardiac cells resulting in premature ectopic activity. Furthermore, magnesium helps to prevent early and delayed afterdepolarisations, which are abnormal electrical impulses – therefore, low

magnesium levels would increase the chance of these depolarisations can lead to ectopic beats (Negru et al., 2022).

3.1.2. Re-entry Circuits

Re-entry circuits occur when a propagating impulse fails to die after normal heart activation (Atienza and Jalife, 2007). Furthermore, the impulse travels continuously in a loop circuit manner to re-excite the heart after the refractory period (Atienza and Jalife, 2007). To note, the refractory period is a timeframe where a cell is incapable of initiating an action potential (Carlson, 2007).

Within the heart's electrical circuit, there is a factor called 'wavelength'. This is a product of impulse conduction velocity and the tissue refractory period, which further describes the distance of a wavefront during the refractory period (Ciaccio et al., 2023). The wavelength of a circuit is critical when understanding how re-entry is established. Research by Carrick et al., (2021) identified a re-entry wavelength obtains no excitable gap and continuously rotates with the wavefront encroaching onto tissue which had recovered from excitability (Carrick et al., 2021). Additionally, a smaller circuit is not able to maintain re-entry, as the circulating wavefront will encounter refractory tissue which inevitably blocks and terminates the circuit (De Coster et al., 2023). On the other hand, a larger circuit can sustain re-entry with an excitable gap (De Coster et al., 2023).

Obesity is known to increase adipose tissue, particularly epicardial adipose tissue (EAT). Increased fatty infiltration leads to atrial fibrosis and remodelling of gap junctions, alongside reducing conduction velocity causing conduction heterogeneity (Limpitikul and Das, 2023). These factors combined promote re-entrant circuits. Within healthy hearts, the electrical impulses would die due to cells completing excitation and

maintaining in the refractory period, preparing to excite again. However, within obese hearts, a slowed conduction velocity allows extensive time for certain regions of the heart to recover and become re-excited by re-entrant impulses (Limpitikul and Das, 2023).

Obesity alters this by typically shortening the refractory period, providing time for electrical impulses to circle back and re-excite tissue early (Veeraraghavan, Gourdie and Poelzing, 2014). Furthermore, a shortened refractory period from obesity contributes to electrical homogeneity so regions of the atria obtain varying refractory periods (Veeraraghavan, Gourdie and Poelzing, 2014). This non-consistency contributes to the initiation and maintenance of re-entrant circuits, due to certain areas conducting electrical impulses quicker than others, permitting abnormal propagation of electrical activity, resulting in re-entrant circuits and inducing AF (Antzelevitch and Burashnikov, 2011).

Pitx2 develops left-to-right asymmetry in embryonic development, therefore when the gene is deficient, it causes differential effects in the atria, with Pitx2 being expressed more in the left atria compared to the right. The effects result in uncoordinated electrical activity and contributes to re-entry activity in the atria, as similarly to the obesity-mediated re-entry, parts of the atria will be ready to re-excite cells before other regions. Furthermore, Pitx2 deficiency leads to altered calcium handling in atrial myocytes, which disrupts the calcium homeostasis and promotes afterdepolarisations (Ramos-Mondragon et al., 2023).

A study by Tarifa et al., (2023) established the connection between Pitx2 deficiency and alterations in afterdepolarisations. Early after-depolarisations (EADs) occur within phase 2 and phase 3 of the action potential cycle, if the EADs reaches its threshold, it

will initiate a premature beat. The extra beat can interrupt normal sinus rhythm and create an opportunity for re-entry to occur, if an impulse finds excitable tissue to propagate through. Similarly, this same theory is linked to delayed after-depolarisations (DADs), however it occurs in phase 4 of the action potential cycle (Tarifa et al., 2023).

3.1.3. Autonomic Dysfunction

The autonomic nervous system (ANS) is designed to maintain homeostasis (Rafanelli et al., 2019). The system regulates key functions like blood pressure, heart rate, respiration and digestion. Autonomic dysfunction is defined as a condition where the nerves within the ANS have become damaged (Rafanelli et al., 2017). There are three common cardiac system dysfunctions that have been linked to autonomic dysfunction, these are postural tachycardiac syndrome (POTs), orthostatic hypotension with supine hypertension and reflex cardiovascular syndromes (Rafanelli et al.,2017).

When relating ANS to the cardiovascular system, it is well-known that heart rate is a common baseline reading for cardiovascular health. Furthermore, heart rate variability provides insight into the ANS-mediated modulation of heart rate (Chen et al., 2014). Both divisions of ANS (parasympathetic and sympathetic) interact with the underlying atrial substrates and play an important role in atrial activity maintenance, and initiation of AF (Chen et al., 2014).

A study by Coumel (2014) suggested that the sympathetic nerve primarily drives AF, however their results indicated the parasympathetic nerve contributed mainly within young individuals with AF. Sheng et al., (2011) identified the sympathetic system may promote arrythmias by increasing the Ca^{2+} transient. In relation, the activated β-adrenergic signal pathways increase Ca^{2+} entry, and further spontaneous release of

ca^{2+} from sarcoplasmic reticulum (Francis, 1988). However, the results from Oberhauser et al., (2001) showed vagal stimulation or acetylcholine (ACh) perfusion contributed to the development of AF by miscellaneous action potential duration and refractory period shortening.

The parasympathetic system has been long associated with increased proclivity to AF. The increased onset of paroxysmal AF is surpassed by evidence of increased vagal tone, particularly in individuals who obtain AF with normal structural modelling of the heart (Agarwal et al., 2017). The vagal effects within AF are closely related to shortened action potential duration aspect discussed associated to Ach, alongside increased spatial heterogeneity. In addition, research showed that vagal stimulation increases risk of delays in conduction (Agarwal et al., 2017).

Obesity heightens the sympathetic activity of the ANS, which puts the body into a continual ‘fight or flight’ response, this can lead to constant elevated heart rate, blood pressure and cardiovascular stress (Scott, Melhorn and Sakai, 2012). Furthermore, obesity increases adipose tissue which releases inflammatory cytokines, for instance TNF-Alpha and Interleukin-6, which stimulates and leads to persistent sympathetic activation. On the other hand, the parasympathetic system is often suppressed in obese individuals, which is the system that promotes ‘rest and digest’, meaning the body cannot relax (Gibbons, 2019). This imbalance through obesity leads to chronic autonomic dysfunction, can lead to parameters like atrial tachycardia and promotes AF.

Pitx2 deficiency obtains a similar concept to how obesity affects the autonomic nervous system. As Pitx2 is important in the development and function of the ANS, a

deficiency in the gene leads to hyperactivity in the sympathetic system alongside a reduction in the parasympathetic system (Tran and Kioussi, 2021).

3.1.4. Fibrosis

Fibrosis is defined as the hardening and/or scarring of tissue, it is attributed to excessive deposition of extracellular matrix components, including collagen (Wynn, 2007). Fibrosis is the end results of chronic inflammatory reactions, which tend to be induced by different stimuli, for instance, persistent infections, allergies, autoimmune reactions, tissue injury and more (Wynn, 2007). A key cellular mechanism of fibrosis are myofibroblasts, which are a collagen producing cell. These are generated from mesenchymal, endothelial and epithelial cells – together known as the epithelial/endothelial-mesenchymal transition.

Within healthy hearts, fibroblasts are arranged into sheets and strands which appear as flat, elongated cells across the cardiac myocytes – the form the stability for the heart to hold together during contraction (Baum and Duffy, 2011). Fibroblasts are directly coupled to one another through junctions, and they pass electrical signals when externally stimulated, however, they are not electrically excitable cells and cannot maintain action potential propagation (Baum and Duffy, 2011).

On the other hand, when the cardiac tissue undergoes an injury, it develops myofibroblasts – these are large cells with highly active endoplasmic reticulum. Myofibroblasts are not part of healthy hearts, and they are distinguishable from fibroblasts due to the high levels of exocytotic vesicles and stress fibres (Pellman, Zhang and Sheikh, 2016). Upon cardiac injury, fibroblasts are stimulated both mechanically, by altered activation patterns and chemically, by inflammatory mediators – resulting into the process known as fibroblast-to-myofibroblast transition (Baum and

Duffy, 2011). Myofibroblasts migrate and respond quickly to inflammatory chemokines which are released from the injury site, but also, myofibroblasts commonly produce and secrete cytokines themselves which maintains the inflammatory response to cardiac injury (Pellman, Zhang and Sheikh, 2016).

Inflammation is a common cause of fibrosis. AF is common is patients with explicit inflammatory states in cardiac conditions, for instance, myocarditis, pericarditis and non-cardiac conditions, for instance; pneumonia and irritable bowel disease (Lajiness and Conway, 2012). Regardless of whether AF is a cause of consequence of inflammatory processes, the concept is related to oxidative stress, which is maintained by myocardial infiltration with macrophages, this is accompanied by release of reactive oxygen species (ROS) (Lajiness and Conway, 2012). Inflammation in fibrosis is further exacerbated by activation of the renin-angiotensin-aldosterone system (RAAS), followed by Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Both these processes trigger TGF Beta-1 protein signalling which is linked to structural and electrical remodelling (Ramos-Mondragon, Galindo and Avila, 2008).

Obesity influences fibrosis strongly, through many mechanisms. Firstly, obesity instigates low-grade inflammation, which is characterised through elevated levels of cytokines including, tumour necrosis factor- alpha and interleukin-6, which activate the fibroblast-to-myofibroblast transition process (Barron, Fabre and De, 2024). Furthermore, these cytokines are responsible for producing extracellular matrix (ECM) which increase collagen synthesis, thus fibrosis. Additionally, excess adipose tissue secretes adipokines, including leptin, resistin and adiponectin, which are pro-inflammatory molecules and contribute to fibrosis by increasing the fibroblast-to-myofibroblast activation and collagen synthesis (Barron, Fabre and De, 2024).

Obesity further increases the production of reactive oxygen species (ROS), which leads to oxidative stress. Oxidative stress damages the cardiac cells, triggering signal pathways and myofibroblasts and resulting in fibrosis (Manna and Jain, 2015). Furthermore, obesity upregulates pro-fibrotic pathways, such as transforming growth factor -β (TGF-β), which is a potent, pro-inflammatory factor and stimulates fibroblasts to differentiate into myofibroblasts (Manna and Jain, 2015).

Research has established Pitx2 downregulates the anti-fibrotic genes, whilst regulating the genes that suppress fibrosis. Thus, when Pitx2 deficiency occurs, the pro-fibrotic pathways obtain the opportunity to dominate. Furthermore, a Pitx2 deficiency upregulates pro-fibrotic expression, for instance, collagen, connective tissue growth factor and fibronectin, which all contribute to ECM accumulation and fibrosis (Dai, Kesaraju, and Weber, 2021).

3.2. Lifestyle risks for AF

3.2.1 Type 2 Diabetes Mellitus

Types 2 diabetes mellitus has a well-known connection to increasing cardiac diseases. There is a strong, independent correlation between type 2 diabetes and AF, despite minor limitations in studies, the association is exceptionally valid and reliable (Mozaffarian et al., 2008). Additionally, a study on type 2 diabetic rabbits and mice acknowledged substantial occurrences of electrical remodelling, which were identified as specific changes in the sodium current within the atrial myocytes (Gallego et al., 2021). Likewise, the expression of connexin 40 – which is a major gap junction protein, was shown to be downregulated in a type 2 diabetic rat model. These results indicated slower conduction velocity and heterogeneous conduction patterns (Sun et al., 2014).

Animal analysis in type 2 diabetic mice showed significant structural remodelling of atrial dilatation and interstitial fibrosis, which both contribute as major AF triggers within type 2 diabetes (Leopoulou et al., 2023). These factors can lead to ion channel remodelling which increases the exposure of atrial tissue, this prompts inter-atrial conduction delay and subsequently the initiation and maintenance of AF (Leopoulou et al., 2023). The interstitial fibrosis aspect is caused by oxidative stress, leading to inflammation, increased creation of glycation end products, more expression of growth factor-β and downregulated expression of gap junction proteins – as discussed by Sun et al., (2014) (Nowotny et al., 2015).

A diabetic mouse is more susceptible to the development of AF after controlled sympathetic stimulation (Grisanti, 2018). Results from electrophysiological testing identified a shortened atrial effective refractory period in type 2 diabetics, promoting the onset of re-entry AF. Furthermore, there was an increase in dispersion time, which is the difference in time between longest and shortest repolarization time (Sicouri et al., 2010). Conversely, there were no differences established in the controlled parasympathetic stimulation within type 2 diabetic mice, acknowledging no effects on atrial refractory period or AF occurrence (Grisanti, 2018).

3.2.2. Obesity and atrial fibrillation

Obesity promotes the onset of adipose tissue. Adipose tissue cells are derived from mesenchymal stem cells and can be broadly categorized as white, brown or beige. White adipose tissue is widely distributed around the body through either subcutaneous or visceral fat. Its main function is storing energy; hence its adipocytes consist of single lipid droplet of triglycerides. White tissue secretes hormones, cytokines, and growth factors which obtain both paracrine and endocrine effects on

adjacent tissues (Ibrahim, 2010). On the other hand, brown adipose tissue is commonly located deep in the abdominal regions and dissipates energy through thermogenesis (Rina et al., 2024).

Obesity-mediated atrial remodelling involves multiple interrelated mechanisms, including structural changes, electrophysiology modulations and neurohormonal activation. Adipose tissue deposition increases around the myocardium due to adiposity, specifically in the pericardial and epicardial regions (Song et al., 2023). The adipose tissue can infiltrate the atria, causing disarrays structurally and fibrosis, disrupting the normal electrical conduction pathways and heterogeneity of atrial conduction (Song et al., 2023).

When looking into how obesity affects the ion channels of the heart, a study by McCauley et al., (2020) established obesity played significant role in downregulating Nav1.5, which is important in rapid depolarisation, maintaining action potential duration and propagating impulses through the heart. In addition, L-type calcium current (I_{NA}) was downregulated through obesity, which reduces contractility, prolongs action potential duration and impairs electrical conduction, thus increasing the risk of heart failure. The study looked further into the mechanisms behind these alterations and established that an increase in the protein kinase enzymes (PKC-a and PKC-O) and NADPH Oxidase 2 (NOX2) were responsible for the alterations of Nav1.5 and I_{NA} (McCauley et al., 2020).

Relating to potassium currents, obesity downregulates the delayed rectifier potassium channels (for instance IK_R and IK_S) which are fundamental in repolarisation. These alterations are often demonstrated through prolonged QT interval and strong mechanism in arrythmias (Aromolaran and Boutjdir, 2017). Additionally, obesity alters

the inward rectifier potassium channels (for instance, I_{K1}), which helps to maintain the heart's resting membrane potential and contributes to the final phase of repolarisation. Alterations in this ion channel leads to abnormal cardiac excitability and arrhythmias (Wei, Yohannan and Richards, 2023).

Additionally, obesity is associated with systemic inflammation. This is characterized by elevated levels of pro-inflammatory cytokines, for instance interleukin 6. These inflammatory facilitators promote atrial fibrosis and alters the ion channel expression – both leading to changes in action potential duration and refractoriness (Goudis et al., 2015). Obtaining these alterations increases the susceptibility of the atria to abnormal electrical impulses, facilitating the maintenance and initiation of AF.

Epicardial Adipose Tissue (EAT) is a form of visceral tissue, located between the visceral pericardium and myocardium (Sacks and Fain, 2007). EAT primarily consists of adipocytes, but it also contains ganglionic plexi, fibroblasts, smooth muscle, stem cells, immune cells and endothelial cells.

In normal individuals, EAT is cardioprotective and emits adipokines to regulate oxidative stress and inflammation (Sacks and Fain, 2007). However, high volume of EAT has been independently associated with the occurrence and severity of AF – providing a substantial link between obesity and AF (Sha et al., 2021). Obese people naturally have higher volumes of EAT, particularly in the left atrium. This has been independently correlated with fibrosis and dilatation of the left atrium (Sha et al., 2021). In agreement with this study, Chahine et al., (2022) researched obese individuals with AF and identified increased EAT around the atrioventricular groove and posterior left atrium, furthermore, the results acknowledged the increased proinflammatory, profibrotic and proarrhythmic substances.

As discussed, interstitial fibrosis obtains a significant link to AF. EAT promotes atrial fibroblast activation and interstitial fibrosis as it releases a multitude of profibrotic adipocytokines, which include any in transforming growth factor- β (TGF- β) (Woo et al., 2021). Furthermore, EAT was associated with higher ROS which can cause damage to multiple cellular processes, alongside impairing autophagy (Sovari, 2016). Autophagy is defined as a maintained eukaryotic recycling process, and impairment means the ROS is not reduced and an inability to recycle mitochondria occurs, this can lead to cellular stress and possibly damage DNA, proteins and mitochondria (Sovari, 2016).

A dilated left atrium is an atrial dysfunction that is a consequence of cardiomyopathy, hypertension and hypertrophy, which is commonly linked to AF recurrence and chronic disease (Bhupathi, Chalasani and Rokey, 2011). Atrial dilatation, and accordingly disfigured electroanatomic shape, leads to slowed conduction speed, and increased conduction heterogeneity, which are hallmarks of proarrhythmic activity (Bhupathi, Chalasani and Rokey, 2011).

As stated, obesity increases the volume of EAT, and this results in excess amounts of fatty tissue entering the heart, known as fatty myocardial infiltration (Ouwens et al., 2010), alongside fat depot increase, which results in the adipocytes secreting pro-inflammatory adipocytokines. EAT volumes are greater in patients with AF, versus patients with sinus rhythm, independent of age, sex, hypertension, BMI and diabetes (Conte et al., 2022). This is supported by Wong et al., (2016) who conducted a meta-analysis of 63 observational studies (352,275 participants). The study identified EAT volume obtained 2.6-fold higher risk of AF and 2.5-fold higher risk of an individual having persistent AF over paroxysmal AF.

Hight fat diet (HFD) fed mice have consistently shown atrial electrophysiological changes, particularly shortened PR interval, prolonged sinoatrial recovery time and reduced conduction velocity (Zhang et al., 2016). Nav1.5 (sub-unit protein in sodium channel) is downregulated, and this is supported by Huang et al., (2012) who established a markedly reduced Nav1.5 expression in the sinoatrial node cells, in both mice and rats, which leads to bradycardia. Furthermore, mice are more prone to AF from alteration of sodium channels modulating shortened APD. On the other hand, shortened APD may be related to the modulation of potassium repolarizing currents through upregulation (Huang et al., 2012).

Additionally, obesity-mediated oxidative stress leads to further electrical remodelling. Excess adipose tissue promotes the increased production of ROS which leads to cellular structural damage and modified ion channel function through oxidative modification. These modifications alter the gating properties of sodium and calcium channels, affecting atrial conduction velocity and excitability (Rivaud, Delmar and Remme, 2020).

As stated, the ANS is linked to AF and obesity promotes dysregulation of the ANS – this is characterized by increased sympathetic activity and decreased parasympathetic activity (Canale et al., 2013). The imbalance exacerbates electrical instability in the atria, which promote adrenergic stimulation, thus enhancing calcium influx and increases the likelihood of afterdepolarizations and related triggered activity (Canale et al., 2013). Furthermore, the strengthened sympathetic drive may lead to atrial tachycardia and formation of ectopic foci, predisposing individuals to AF.

3.2.3. Genetics and atrial fibrillation

Inherited DNA sequences play a role in promoting risk of cardiac disease. In the general population, the history of premature atherosclerotic-related cardiovascular disease within parents confers a 3-fold increase in passing cardiac disease to their children (Kathiresan and Srivastava, 20212). However, the magnitude of inheritance role varies by disease, and other factors including age, onset and subtype of disease. Some forms of cardiac disease exhibit simple patterns from inheritance, which can be suggestive of single causal gene conferring a large effect on an individual (Kathiresan and Srivastava, 2012). Although, most cardiac traits show complex interactions, which is suggestive of an interplay between numerous genes and non-genetic aspects.

Potential associations have been identified between connexins and AF, but the clear mechanisms underlying this remain weak. Connexins are major gap junction proteins, which are key in the cell-to-cell electrical communication (Wang, Hu and Yang, 2015). Thibodeau et al., (2010) identified when gap junction protein alpha-1 is mutated, loss of function occurs. This consequently leads to intracellular retention of protein, reduced gap junction conduction and failure of electrical coupling of atrial cells. In addition, gap junction protein alpha-5 also loses function when mutated, which results in impaired connexin transport and assembly of gap junctions at the cell, alongside failure of electrical coupling between the cells themselves (Thibodeau et al., 2010). Gollab et al., (2006) identified significant somatic mutations from gap junction alpha 5, which were specifically found in atrial biopsies rather than isolated DNA in blood.

Atrial natriuretic peptide (ANP) is a circulating hormone produced within the atria, it regulates blood pressure and stimulates vasodilation. ANP is encoded by natriuretic peptide A (NPPA) which is a mutation commonly linked to familial AF (Hodgson-

Zingman et al., 2008). Evidence shows the heterozygous shift mutation in the NPPA gene leads to shortened APD and effective refractory period, whilst increasing ANP production (Hodgson-Zingman et al., 2008). Abraham et al., (2010) discovered a link between mutations in potassium voltage-gated channel subfamily Q member-1 (KCNQ1) and NPPA genes, gaining function of slow delayed rectifier K+ current (Iks), shortening APD and altered Ca^{2+} current. In addition to KCNQ1, mutations have been shown in K^+ channel genes including KNA5, KCND3 and KCNJ2 (Ravens and Cerbai, 2008). These mutations are linked to prolonged atrial action potentials, caused by loss of function in the K^+ channels which lead to early afterdepolarizations and AF (mutations have been shown in K^+ channel genes including KNA5, KCND3 and KCNJ2 (Ravens and Cerbai, 2008).

Mutations in sodium voltage-gated channel alpha subunit-5 (SCN5A) also impact the sodium currents and promote long QT syndrome, dilated cardiomyopathy, AF, impaired automaticity and conduction delay (Olson et al.,). The voltage-gated sodium channel – Nav1.8 is encoded by sodium voltage-gated channel alpha-10 (SCN10A) and is primarily related mediating pain. However, the expression of SCN10A has been detected within cardiomyocytes and intracardiac neurons, particularly in the conduction system (Park and Fishman, 2014) and loss of function in mutation has been commonly linked to AF.

A potassium voltage-gate channel modifier subfamily S member-1 (KNNS1) encodes calcium-activating K^+ SK3 channel and is active during the repolarization phase, as the channel is responsible for beat-to-beat activity (Zhang et al., 2021). Research suggests blocking the SK3 channel reverses APD shortening within rabbits, at a burst-pacing cycle, linking to AF (Christopheresen and Ellinor, 2016). Moreover, a hyperpolarised activated cyclic nucleotide-gated potassium channe-4 (HCN4) gene,

encodes the hyperpolarization-activated cyclic nucleotide-gated channel which carries the funny current. It is responsible for the SA-node pace making in the left atrium, however mutations in HCN4 have been related to sinus node dysfunction, tachy-brady syndrome and AF (Christopheresen and Ellinor, 2016).

3.2.3.1 Pitx2 and AF

This study focused on the transcription factor, paired-like homeodomain 2 (Pitx2). The Pitx2 gene is located on chromosome 4, and expressed in three isoforms: Pitx2a, Pitx2b and Pitx2c (Franco, Sedmera and Lozano-Velsaco, 2017). The focus of this study was primarily Pitx2c, which is the cardiac isoform and is expressed within the cardiomyocytes and skeletal muscle. Pitx2c is regulated by its individualised intergenic system and expressed asymmetrically, being only on the left side of an embryo (Franco, Sedmera and Lozano-Velsaco, 2017). Pitx2a and Pitx2b are not involved with left-to-right asymmetry. Reduced expressions of Pitx2c at neonatal age can result in developmental defects and increase the susceptibility of AF within adulthood. Moreover, during the ageing process, Pitx2c levels decline, which in parallel leads to increased AF risk.

Evidence has shown Pitx2 plays an important role during embryonic development and maintenance of electrophysiology within adult atriums (Qiu et al., 2014). Additionally, Pitx2c regulated left-to-right asymmetry and develops the pulmonary vein, myocardium and sinoatrial node (Qiu et al., 2014). Deficiency of Pitx2 has shown severe cardiac impairment, including malformation in valves and right pulmonary isomerism (Yuan et al., 2013). Thus, over recent years there has been numerous studies utilising Pitx2 knock out and these studies agree that Pitx2 deficiency accentuates AF risk.

As Pitx2c plays a critical role in left-to-right asymmetry within the heart, that loss of the transcription factor may cause incomplete suppression of the left atrial pacemaker (Dai, Kesaraju and Weber, 2021). Wang et al., (2015) identified Pitx2 knockout mice obtained higher susceptibility to inducible atrial arrythmias – this statement is further supported by Chinchilla et al., (2011) and Kirchhof et al., (2011). Furthermore, all studies acknowledged a link between Pitx2 reduction with shortened APD, which facilitated the re-entry phenomenon.

Syeda, Kirchhof and Fabritz (2017) showed Pitx2 knockout mice obtained an irregular rest heart rate, when compared to their control group whilst showing low amplitude p waves associated to AF. The results also identified structural remodelling and cell junction damage to intercalated discs, this would lead to the cardiac muscles being unable to contract properly meaning blood cannot travel to essential organs. Additionally, upregulation of bone morphogenetic protein-10 (BMP10) alongside mediated Pitx2 increases the cell proliferation creating larger atrial chambers (Chinchilla et al., 2011). This concept is a putative mechanism for triggering the onset and maintenance of arrhythmogenic processes.

Atrial deletion of Pitx2 has been linked to ion channel remodelling. Wang et al., (2015) reported that Pitx2 obtains a key role in inhibiting the sinoatrial pacemaker in the left atrial, which can result in atrial arrythmias. Furthermore, atrial specific deletion of Pitx2 displays conductive disturbances, particularly atrioventricular block (AV block), meaning the speed of electrical messages is disrupted which can lead to a slower heartbeat (Syeda et al., 2016). Curiously, analysis into the morphology characteristics of SA node and ventricular conduction system appeared to be unaltered, yet there appeared to be reduced protection of AV node and bundle of his (Syeda et al., 2016).

Thus, AV block may be caused by Pitx2 deficiency, leading to sodium channel impairment and AF.

There is conclusive evidence that in severe Pitx2 models, through homozygous knockout, there is a rise in the SCN5a gene linking to impaired expression of I_{NA} and I_{K1}, , within only the atria and not the ventricles (Syeda et al., 2016). This is further consistent with the theory of altering the electrophysiology properties in only the left side of the heart, not right.

3.3. Treatment and therapies within AF

There are a variety of methods utilised clinically to treat AF, primarily medications and surgical procedures. Medications are split into different classes of anti-arrhythmic drugs from class 1 to class 5, and the primary surgical procedures conducted are cardioversion and catheter ablation.

Firstly, Flecainide is a popular, class 1c anti-arrhythmic drug which is used to restore AF back to, and maintain, sinus rhythm. This class 1c drug modifies the electrical signals which control the heartbeat, specifically targeting the erratic electrical signals that cause AF. Flecainide obtains a primary effect of blocking sodium channels within the cells, whereby these channels are essential for conducting and initiating electrical signals across the myocardium (Lavalle et al., 2021). By Flecainide reducing sodium influx into the cells, it correspondingly decreases the velocity of electrical impulses travelling through the atria – this enables prevention of chaotic electrical activity that generates AF (Lavalle et al., 2021). Flecainide affects the effective refractory period by prolongation, which reduces the opportunities for premature or ectopic electrical impulses to be re-excited and trigger additional heartbeats (Lavalle et al., 2021).

Similarly, Mexiletine is a class 1b anti-arrythmic drug used to stabilise the electrical activity across the heart. Mexiletine exerts its benefits by blocking sodium channels, and with the same concept as Flecainide, it slows down the influx of sodium ions entering the cells. This also leads to a reduction in the velocity of electrical activity and allow control of the abnormal heart rhythm (Inoue et al., 1988). It has been shown in HFD murine hearts that the late sodium current is increased, which can lead to prolonged APD, dispersed repolarisation and risk of arrythmias. Therefore, the therapeutic effect of mexiletine in HFD murine heart is suggestive of mediating a decrease in late sodium current, leading to reduced risk of AF (Liu et al., 2017).

Contrary to the other two classes, class III anti-arrhythmic drugs contribute to blocking potassium channels which results in increased APD and effective refractory period. Class III drugs include a commonly used one called Amiodarone, which obtains a high lipophilicity and accumulates primarily within the adipose tissue (Singh et al., 2000).

3.4. Optical Mapping

For coordinated contraction of the heart, transmission of electrical impulses is vital. An optical mapping technique was introduced 40 years ago as a method to analyse pre-clinical electrophysiology. Optical mapping utilises high speed cameras with fluorescent markers to identify electrical activation, calcium handling and repolarisation – which can all be imaged with spatial resolutions across the whole myocardium, isolated cells, cardiac slices and cellular monolayers (O'Shea et al., 2020).

3.4.1. Fluorescent Dyes

There are three key components involved in optical mapping, 1.) preparation of cardiac samples loaded with fluorescent dyes, 2.) optical setup for the dye excitation and collection of fluorescent outputs and 3.) imaging detector (O'Shea et al., 2020). When analysing whole heart, the fluorescent dyes are inputted into the coronary arteries through Langendorff perfusion. Commonly, potentiometric dyes are used to super fuse the atria and coronary slices. These potentiometric dyes embed the cell membrane and obtain a fluorescent output to react to transmembrane voltage which permits the recording of the action potentials (Lipovsky et al., 2018). Simultaneously to action potential recording, optical mapping recording may use intracellular calcium indicators (Lipovsky et al., 2018).

As stated, potentiometric dyes are commonly used in optical mapping. Specifically small molecular-based styryl dyes known as Di-4-ANEPPS or rh-237 are the most used (Laughner et al., 2012). Despite the fluorescent dyes being useful, they come with limitations. Di-4-ANEPPS is commonly used in whole heart analysis but requires blue-green light excitation. This lighting is absorbed by chromophores within the blood and tissue; therefore, signals are effectively taken from the samples surface only (Laughner et al., 2012). Within isolated cell, Di-4-ANEPPS is still used however crystalloid solutions are essential for strong signal quality in cells. Consequently, crystalloid solutions struggle to meet the oxygen and metabolic demand in free beating samples, meaning hearts may experience hypoxia and ischaemia (Mullenbroich et al., 2021).

3.4.2. Mapping of the heart

There are proposed challenges when performing the mapping of freely beating hearts. As the tissue moves, the region corresponding to a particular pixel will change which will result in distorting images. To conquer this side effect, excitation-contraction uncouplers, like Blebbistatin, are commonly used. These molecules inhibit myosin II which reduces cardiomyocyte contraction, but not the electrical excitation, therefore permitting the mapping of the heart without distorted signals (Barrick and Greenberg, 2021). Uncoupled hearts possess reduced oxygen and energy demand, whilst missing the bi-directional communication between the mechanical and electrical movement, therefore they cannot be utilised within in-vivo experiments (Garrott et al., 2017).

Conversely, a study by Fedorov et al., (2007) provided evidence on potential indirect side effects from utilising excitation-contraction uncouplers. The results from their study established excitation-contraction uncouplers may affect the propagation of the heart tissue which could change the transmission of electrical signals through the heart. Furthermore, Blebbistatin obtains fluorescence properties which may interfere with the optical mapping technique, as it can emit light which overlaps the wavelengths utilised in voltage or calcium sensitive dyes (Fedorov et al.,2007).

3.4.3. Optical Mapping and Atrial Fibrillation

Action Potential Duration (APD)

The ability to study the repolarisation of myocardial tissue is a key mechanism in atrial fibrillation. Optical mapping permits this as changes in the fluorescence intensity are corresponding to changes in the transmembrane voltage, whilst the values are calculated by optical signals (Attin and Clusin, 2009). Commonly, studies analyse APD at 30,50 and 80% - meaning the duration between activation time and time at 30,50

or 80% of repolarisation. Using multiple measurements across the electrical pathway allows a more accurate description of the optical action potential and overall shape (Lee et al., 2012).

Conduction Velocity

Optical mapping provides a generation of local conduction paths, which enables quantification of local conduction velocity, alongside description of myocardium activation (Lee et al., 2019). One common method used through optical mapping is to compare activation times between a particular pixel and its immediate neighbour. A large difference identifies fast conduction velocity, and a small difference shows a slower conduction velocity – these are calculated via gradient vectors per pixel (Lee et al., 2019).

3.5. Proteomics

Underlying mechanisms are unclear within AF. Proteomics profiling permits analysis of a large-scale study of proteins, offering a strong approach to elucidate mechanisms by identifying and quantifying the proteins involved in AF pathogenesis – which can lead to therapeutic targets and biomarkers (Al-Amrani et al., 2021).

There are a variety of proteomics analysis used within AF research, which involves the application of advanced techniques including mass spectrometry (MS), two-dimensional gel electrophoresis (2D-GE) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) – which all investigate the protein composition of atrial tissue, blood or other necessary biological samples in AF patients (Zhan, Huang and Long, 2018).

AF has been associated with alterations in the proteome of atrial cardiomyocytes, including changes in metabolic enzymes, ion channel proteins and mitochondrial dysfunction (Rennison et al., 2021). These adaptations reflect the metabolic stress and structural remodelling which may occur during AF, on the other hand, proteomics enables identification of protein expression alterations in relation to calcium handling and contractility within the atrial tissue, which permits a deeper understanding of the electrophysiological adaptations in AF (Rennison et al., 2021).

Compared to the genome, the proteome is closely related to disease phenotype and stronger at predicting the manifestation of unravelling cardiac diseases. Proteomic studies have identified between 5,240 – 11,000 proteins and a transcriptome analysis showed 68% of human proteins are expressed in the heart. Whereas animal models have identified between 3,700 and 7,300 proteins within mice hearts (Edwards et al., 2023). In mice models of AF, proteomic studies have identified several key proteins that are frequently associated with the progression of the disease.

Connexin 43 (Cx43) is a protein within gap junctions, which enables electrical coupling between neighbouring cardiomyocytes. Alterations in Cx43 lead to disrupted electrical conduction by blocking electrical signals that pass through each cardiomyocyte, meaning the heart will abnormally beat and induce arrythmias (Rodriquez-Sinovas et al., 2021). Furthermore, voltage-gated sodium channel (NaV 1.5) is essential in the transmission and initiation of action potentials. NaV 1.5 is the main protein responsible for the inward sodium current (I_{NA}) which induces fast depolarisation to initiate the action potential (Iqbal and Lemmens-Gruber, 2019). If alterations occur with NaV 1.5, it can increase the opportunity for late sodium currents which also may contribute to arrythmias (Iqbal and Lemmens-Guber, 2019).

The potassium channel proteins are critical for progression of repolarisation in cardiac cells. Dysregulation or mutations may lead to abnormal repolarisation, inducing arrythmias. Studies have identified key proteins associated with the potassium channels to enable the repolarisation process allowing cells return to their resting state after-depolarisation (Kim and Nimigean, 2016). These key proteins identified were KV7.1, KCNE1, KV11.1, KCNE2, Kir2.1, KV1.5, KV4.2, KV4.3, TASK-1, Kv7.4 and TWIK-1.

The results showed proteins like HERG, KV1.5, KV4.2 and KV4.3 all hold a form of responsibility for conducting various potassium currents at different phases of the action potential cycle, specifically the fast delayed rectifier K^+ current, transient outward K^+ current and inward rectifier K^+ channel (Jeevaratnam et al., 2018). Mutations of these proteins will impact the flow of electrical signals through the heart and delay repolarisation, meaning the heart cannot be ready for the following beat. Additionally, proteins KV7.4, TWIK-1, TASK-1 and Kir2.1 all help to regulate the resting membrane potential of the heart, regulate cardiac repolarisation and overall electrical stability (Reilly and Eckhardt, 2021). By ensuring the resting membrane is regulated appropriately, it helps maintain the heart's regular contractions to allow blood supply to body's organs (Reilly and Eckhardt, 2021).

Ryanodine receptor type-2 (RyR2) and Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase 2a (SERCA2a) are both channel proteins and are involved in the excitation-contraction coupling, however abnormal RyR2 and SERCA2a activity have been linked to calcium dysregulation which is significantly linked to AF (Belevych et al., 2013). Furthermore, a decrease in SERCA2a activity may cause endoplasmic reticulum stress which can impair protein-to-protein interactions and promote spontaneous pro-arrhythmic waves (Belevych et al., 2013).

Despite the thousands of proteins identified through proteomics analysis, the differentially expressed proteins allow the opportunity for further investigation into the mechanisms initiating AF. Furthermore, recognising consistent protein alterations permits a deeper understanding into exact roles of proteins during AF.

4. AIMS AND HYPOTHESES

4.1 Aims

The studies primary aim is to investigate whether high fat diet induces electrophysiological remodelling in the murine left atria, and whether the response is modulated by altered expression of Pitx2

Further, we aimed to evaluate whether proteomic experiments can be conducted on dyed, optically mapped hearts.

4.2. Hypotheses

- High fat diet does alter left atrial electrophysiology, in both wild type and Pitx2 deficient mice.
- There is a disparity in electrophysiological alterations between the wild type and pit2x mice.
- Optical mapping does not alter results from proteomic analyses of the murine heart.

5. METHODS AND MATERIALS

5.1. Mice and Ethical Approval

All animal procedures were conducted in accordance with ethical guideline set out by the UK animals (Scientific procedures) Act 1986 and directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Studies conformed to the Guide of the Care and Use of Laboratory Animals by the U.S. National Institutes of Health under assurance number A5634-01. Experiments were approved by the home office (mouse: PP: 30/2967 and PFDAAF77F) and the institutional review board at University of Birmingham. All animal scientific procedures within this study were conducted by Dr Christopher O'Shea.

5.2. Optical Mapping

The wild type and Pitx2null male-only mice were categorised into two groups, control who were fed a normal chow diet and high fat diet (HFD) who were fed "SDS Western" diet for 20 weeks. This food and water were available ad libitium. The western diet consisted of 21% fat and 20% protein, with a supplement of 15% fructose water. Alongside HFD, age-matched mice were given their normal chow diet. This diet consisted of 2.5% fat and 14% protein, with normal drinking water. Both diets began at the ages of 6-12 weeks, meaning mice were 26-32 weeks at the time of the experiments. For both control and HFD mice, food and water was provided as desired for the mice. All mice were monitored weekly for any adverse health complications and measured weight gain, or loss.

5.2.1. Isolated left atrial and perfusion

A bicarbonate buffered krebs-henseliet (KHB) solution was utilised for the optical mapping experiments. The ingredients for the KHB included (in mM): NaCI 118; KCI 3.52; MgSO4 0.83; KH2PO4 1.18; NaHCO3 24.9; Glucose 18.0 and CaCI2 1.8. The solution was heated between 36-37°c and equilibrated at 95% O2 and 5% CO2, this was to maintain a pH level of 7.4. All chemicals were purchased from Sigma-Aldrich.

Mice were placed in an anaesthetisation chamber which was filled with 4% isoflurane, in oxygen (O_2) for 2-minutes. The flow was 2-3L/min, with gas being continuously delivered. The mice were placed in the surgical bed and placed in a supine positioning; they were further supplied with continuous 4% in O_2. The pedal reflex test was performed to ensure deep anaesthetisation had occurred, once confirmed, the limbs were taped down. An initial cut was made below the rib cage and the rib cage was cut bilaterally, the diaphragm was then pierced, and the rib cage moved back to identify the heart and lungs. The heart was lifted from behind using curved forceps and vessels were used to remove the whole heart. Death was via exsanguination.

The isolated heart was placed in KHB solution, in a petri dish at 4°c to temporally stop contraction, furthermore, Heparin (an anticoagulant) was added to the dish to prevent blood clots in the isolated heart. The heart was then placed into a petri dish, containing KHB solution to be ready for cannulation of the ascending aorta. The dish was then placed on a custom stand for 2mL syringe with a 1mm cannula being attached – the syringe contained 2mL of KHB solution. All air bubbles were removed before cannulation, where the ascending aorta was cannulated above the aortic valve. Sutures were used to tie the heart to the cannula, with KHB perfused through the aorta to clear blood.

The heart was cannulated vertically through a Langendorff system, with 37°c KHB solution. The initial flow rate was 4mL/min but was reduced to 2mL/min for dye load, the hearts were allowed to equilibrate until beating was observed.

5.2.2. Pacing in Optical Mapping

Potentiometric dye, Di-4-ANEPSS, was stored at 4°c at a concentration of 5mg/ml in Dimethyl sulfoxide (DMSO) in 25µl aliquots. The dye was then removed from storage and thawed for 5-10 minutes, then it was transferred to be dissolved into 1mL of 37°c KHB solution and injected into the perfusion line of the Langendorff system through a 3-way trap before the solution enters the heart. Dye loading was performed via a bolus injection across 2-3 minutes. During the loading, the solution which passed the heart was collected and reapplied to the epicardial surface to permit maximum dye loading. Once the heart was loaded with the fluorescent dues, the heart was removed from Langendorff and placed into a petri dish in 37°c KHB solution.

The left atria (LA) were dissected and placed into the optical mapping super fusion chamber. Then the LA was pinned to a black silicone surface of the chamber using fine, entomology pins – it was pinned with the anterior facing upwards. Two more pins were utilised at the edge of the issues for two reasons, 1.) so the pins did not block the camera; 2.) so the stimulus electrodes could be placed near where the LA attached to the septal wall.

Four light-emitting diodes (LEDs) provided an illumination at 530nm directly onto the cardiac tissue, which excites the DI-4-ANEPPS and emits fluorescent photons – this was achieved through 1-minute recordings at a sampling rate of 0.987kHz. The fluorescent productions were imaged utilising an ORCA flash 4.0 camera at a frame rate of 1kHz and 200x2048 pixel resolution.

There were two pacing protocols used for the analysis. The first protocol obtained a holding cycle length (CL) of 1000ms (1Hz) and was primarily aimed to analyse adaptation of the heart between CL and beat-to-beat variability. During recordings, the atria were paced at a CL of 120ms, 100ms and 80ms (8.33Hz, 10Hz and 12.5Hz) for 100 beats. Additionally, this protocol included a burst of 20 pulses at a CL of 50ms. There was a 5 second gap between each CL to ensure the heart had enough time to adapt to each CL. The second pacing protocol obtained a holding CL of 333ms (3Hz). At the start, the CL was reduced to 150ms or 120ms (6.67Hz or 8.33Hz) for 100 beats. The CL was further reduced by 10ms for every 20 beats until the CL was 50ms (20Hz). This protocol was to identify restitution properties.

5.3. ElectroMap Software

All optical mapping images shown in the results were analysed using a high-throughput software called ElectroMap (O'Shea et al., 2019). The primary electrophysiological parameters assessed were action potential duration (APD), conduction velocity (CV), diastolic interval (DI) and time to peak (TTP).

- APD (ms) was defined as the time taken starting from the maximal upstroke to 30%, 50% or 80% respectively of repolarisation. To calculate APD30, APD50 and APD80
- DI (ms) was defined as the duration from APD50 to the following activation time.
- TTP (ms) was defined as being between 10% to 90% of depolarisation of the action potential.
- CV (cm/s) was defined as the propagation of electrical activity across the LA, taking speed and direction into account.

- Heterogeneity was defined as the intra-atria variability and calculated through (X95 – X5)/X50.

Data was exported from WinFluor in an uncompressed, tagged image file format (TIFF) and all analysed using a matlab built-in software called ElectroMap. The images were downloaded onto the ElectroMap software, and a region of interest was drawn in the LA image, to distinguish the specific region to be analysed. Not only does this provide more specific analysis, but it also permits the reduction of motion artefacts and improves the accuracy for results. The images were spatial filtered using 3x3 gaussian filter, alongside temporal filtered using savitzky-golay to enable smooth data. Furthermore, any non-physiological alterations within baseline fluorescence were corrected utilizing a top-hat filter with Kernel length of 100ms length.

The last 20 beats of each 100 stimuli were averaged to improve data quality, across each three pacing cycles (80ms, 100ms and 120ms) were identified and analysis proceeded for each length.

5.4. Statistical Analysis

All analysis of the data and statistical testing was performed blinded, and the identity of each group was revealed after analysis had been completed to reduced biased. The results from ElectroMap were exported and inputted into GraphPad 9.1.0 to perform statistical analyses and produce necessary graphs.

A 2-way ANOVA statistical test was used to detect any significant relationship between all pacing cycle lengths, whereby a 1-way ANOVA statistical test was used when analysing the data in only one specific pacing cycle length. Additionally, the Alpha was set to 0.05 throughout all analysis and the p-value were identified from the Tukey's multiple comparisons test, in both 2-way and 1-way ANOVA.

3.5. Proteomics

3.5.1. Extraction, reduction, and alkylation

Alongside the optical mapping, proteomics testing was performed to assess whether there were any protein alterations between the control and HFD group.

5mg of whole left atria samples were added to 100µL of lysis solution, then disrupted with homogenizer. Once homogenized, samples were centrifuged at 16,000 x g for 10 minutes. The protein concentration was established using a Pierce™ BCA Protein Assay Kit. Between 10-100µg of protein was transferred into a new microcentrifuge tube and adjusted to a final volume of 100µL using lysis solution. 50µL of reduction solution was added and gently mixed, then 50µL of alkylation solution was added and gently mixed. The sample was then incubated at 95°c using a heat block for 10 minutes to reduce and alkylate the sample. After incubation is complete, the sample was removed from the heat block to cool within room temperature.

3.5.2. Digestion

500µL of enzyme reconstitution solution was added, this was to 1 vial of trypsin/Lys-C protease mix. 50µL of the reconstitution enzyme solution was added to the reduced and alkylated protein sample. The sample was then incubated at 37°C for 1-3 hours to digest the protein sample. From here, the next phase of labelling is performed using the TMT reagent labelling kit. Once the incubation is complete, 50µL of digestion stop solution was added and gently mixed.

3.5.3. TMT Reagent Labelling Kit

40µL of TMT reagent was added and dissolved in 100% acetonitrile to each buffered peptide sample and incubated for 30-60 minutes at room temperature. 50µL of 5%

hydroxylamine, 20% formic acid solution to each labelling reaction to acidify. The pH was verified as <4 using pH paper.

3.5.4. Clean-up Peptides

The white cap was removed at the base of the peptide clean-up column, with the green cap loosened and placed into a 2mL microcentrifuge tube. Then the samples were centrifuged at 3000 x g for 2 minutes, to remove all liquid from the column. The digested protein sample was transferred into the dry peptide clean-up column and centrifuged at 1,500 x g for 2 minutes, discarding the flowthrough. 300µL of wash solution A was added and centrifuged at 1,500 x g for 2 minutes, discarding the flowthrough. Then the sample was washed twice with wash solution B and centrifuged at 1,500 x g for 2 minutes. The steps were repeated one more time. The peptide clean-up column was transferred into a new 2mL microcentrifuge tube. Then 300µL of elution solution was added, this was centrifuged at 1,500 x g for 2 minutes to collect the clean peptide sample.

6. RESULTS

The aim of the study was to identify whether a HFD altered left atrial electrophysiology, alongside seeing whether a Pitx2 deficiency caused any further alterations. All results were mapped and analysed on ElectroMap, alongside the graphs and statistical results developed in GraphPad.

6.1 High fat diet prolongs action potential duration, with a shortening in Pitx2 groups.

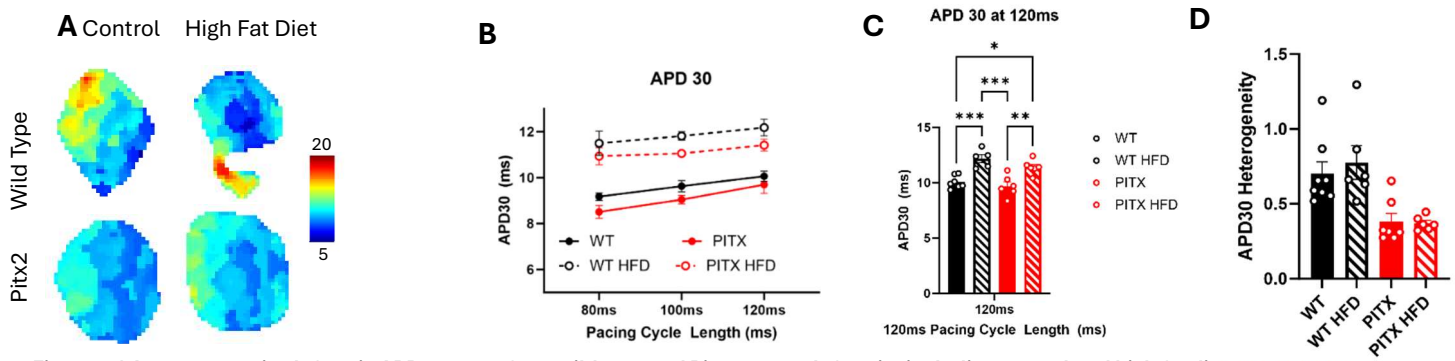

Figure 1a) A representative left atria APD30 maps from wild type and Pitx2 mouse left atria, including control and high fat diet. 1b) APD30 as a function of all three pacing cycle lengths, analysed using 1-way ANOVA test. 1c) Grouped APD30 data 120ms cycle length. 1d) Grouped APD30 Heterogeneities, both C and D analysed using a 2-way ANOVA test WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

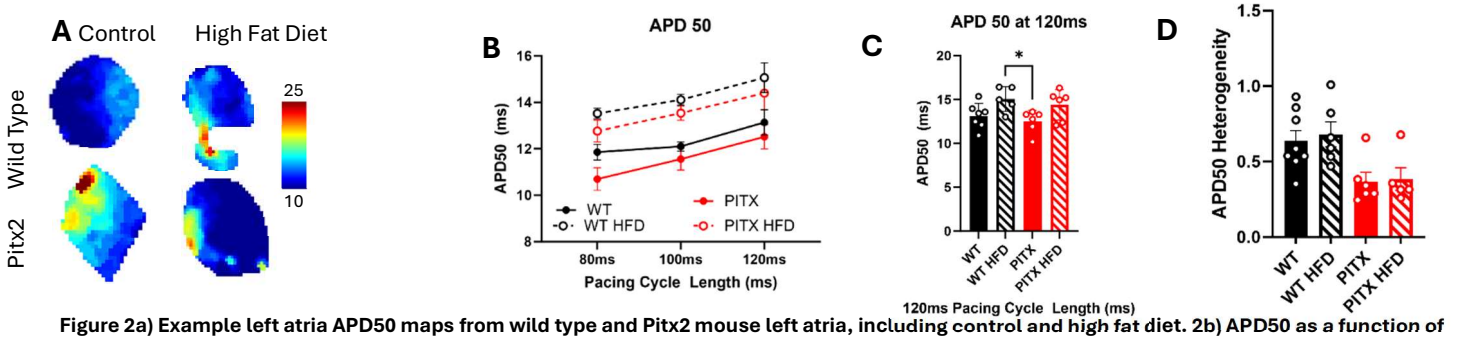

Figure 2a) Example left atria APD50 maps from wild type and Pitx2 mouse left atria, including control and high fat diet. 2b) APD50 as a function of all three pacing cycle lengths, analysed using 1-way ANOVA test. 2c) Grouped APD50 data 120ms cycle length. 2d) Grouped APD50 heterogeneities, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

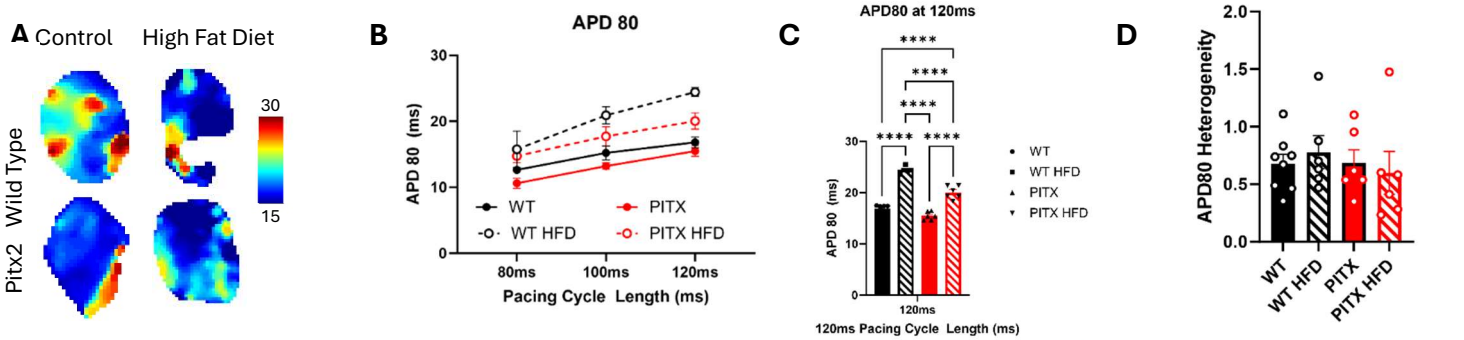

Figure 3a) A representative left atria APD80 maps from wild type and Pitx2 mouse left atria, including control and high fat diet. 3b) APD80 as a function of all three pacing cycle lengths, analysed using a 1-way ANOVA test. 3c) Grouped APD80 data 120ms cycle length. 1d) Grouped APD80 heterogeneity, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

Figures 1a (APD30), 2a (APD50) and 3a (APD80) all identify representative optical mapping recordings for mouse left atria, showing the corresponding groups of wild type control and high diet, alongside Pitx2 deficient control and high fat diet. Figure 2a (APD30), 2b (APD50), and 2c (APD80) identify all groups across each pacing cycle length (80ms, 100ms, and 120ms) and figure 3a (APD30), 3b (APD50), and 3c (APD80) identify grouped APD data at 120ms alone. Figure 1d, 2d, 3d and 4d identify all the grouped APD heterogeneity data to show the variability in each group.

The results showed a strong association in HFD prolonging APD, across all pacing cycle lengths. APD30 results identified significant association between HFD and APD alteration in wt vs wt hfd (p <0.0001), wt vs Pitx2 hfd (p=<0.0001), wt hfd vs Pitx2 (p=<0.0001) and Pitx2 vs Pitx2 hfd (p=<0.0001). Furthermore, analysing 120ms cycle length alone showed significant change in wt vs wt hfd (10.06±1.51 vs 12.18±2.11, p=0.0005), wt vs Pitx2 hfd (10.06±1.51 vs 11.41±1.26**,** p=0.0816), wt hfd vs Pitx2 (12.18±2.11 vs 9.69±1.73, p=0.0001) and Pitx2 vs Pitx2 hfd (9.69±1.73 vs 11.41±1.26**,** p=0.0036).

Similarly, results showed prolongation in APD50 from HFD. Across all cycle lengths, significant data was shown in wt vs wt hfd (p=<0.0001), wt vs Pitx2 hfd (p=0.0067), wt hfd vs Pitx2 (p=<0.0001) and Pitx2 vs Pitx2 hfd (p=<0.0001). However, 120ms cycle length alone only showed wt hfd vs Pitx2 (15.06±2.81 vs 12.51±2.50, p=0.0463) to be significant.

Finally, results also showed a strong association between HFD and alterations in APD80. Across all cycle lengths, significant data was shown in wt vs wt hfd (p<0.0001), wt vs Pitx2 (p<0.0001), wt vs Pitx2 hfd (p=<0.0005), wt hfd vs Pitx2 (p=<0.0001), Pitx2 vs Pitx2 hfd (p=<0.0001) and wt hfd vs Pitx2 hfd (p=<0.0001). Furthermore, results in

120ms cycle length alone shown significant data in wt vs wt hfd (16.81±3.44 vs 24.43±4.02, *p*=<0.0001), wt vs Pitx2 hfd (16.81±3.44 vs 20.04±2.51. *p*=<0.0001), wt hfd vs Pitx2 (24.43±4.02 vs 15.49±3.54, *p*=<0.0001), wt hfd vs Pitx2 hfd (24.43±4.02 vs 20.04±2.51, *p*=<0.0001) and Pitx2 vs Pitx2 hfd (15.49±3.54 vs 20.04±2.51, *p*=<0.0001).

6.2. High fat diet does not alter conduction velocity

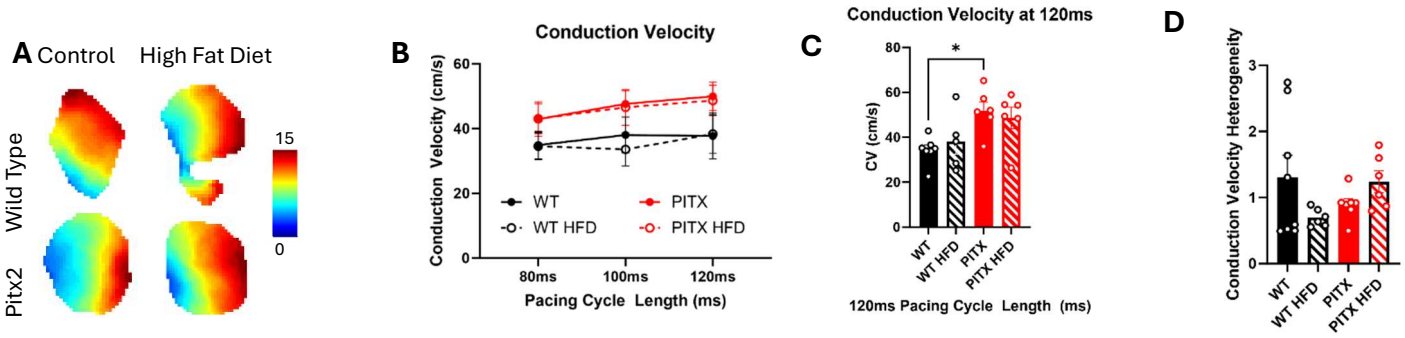

Figure 4a) example activation maps of WT and HFD groups, 4b) conduction velocity functioned at each cycle length, analysed using a 1-way ANOVA test. 4c) group conduction velocity at 120ms alone. 4d) Grouped conduction velocity heterogeneities, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

Figure 4a identifies example activation maps including both WT and Pitx2 groups in control and HFD. Figure 4b identifies conduction velocity across all pacing cycle lengths, whereas figure 4c identifies grouped conduction velocity at 120ms.

Results showed minimal significant alterations of HFD and Pitx2 deficiency affecting conduction velocity. Across all group's analysis, significant differences were established in wt vs Pitx2 (*p*=0.0162) and wt hfd vs Pitx2 (*p*0.0066). Furthermore, within the 120ms only analysis, wt vs Pitx2 (34.31±48.85 vs 51.77±19.53, *p*=0.0254) showed significant differences, providing a correlation between Pitx2 deficiency altering conduction velocity but HFD providing no effect.

6.3. High fat diet exhibits potential evidence of altering diastolic interval

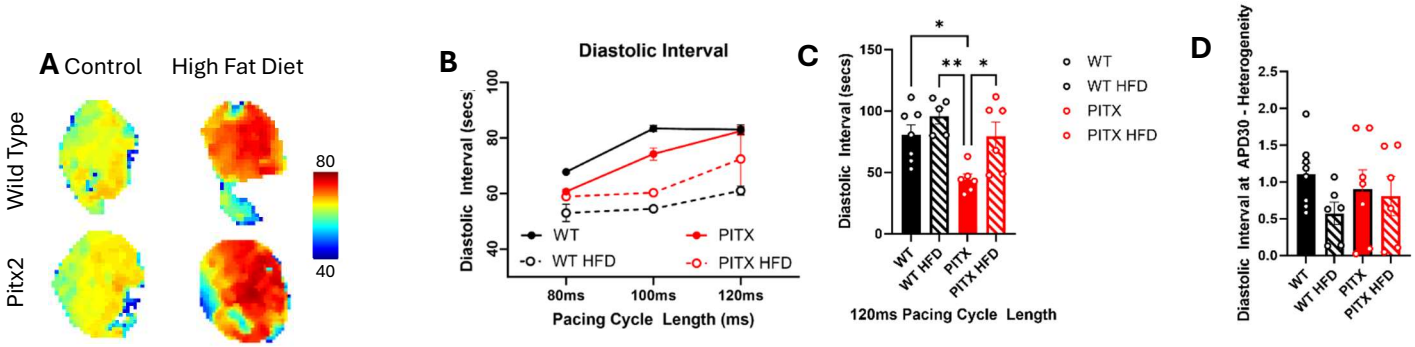

Figure 5a) example diastolic interval (DI) maps at APD30 at 120ms, 5b) DI at all pacing cycle lengths, analysed using a 1-way ANOVA test. 5c) grouped DI at 120ms. 5d) Grouped DI heterogeneity data, at APD30, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

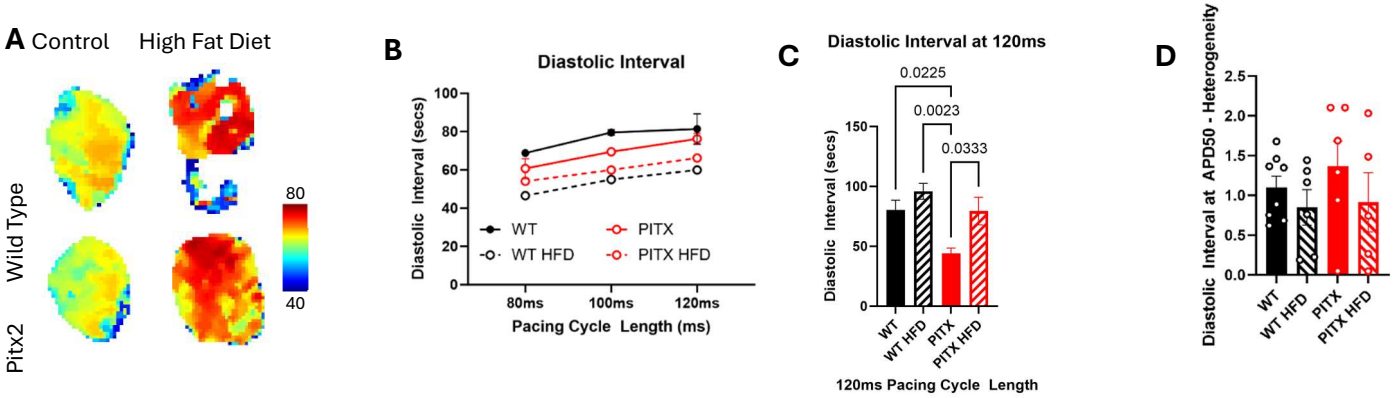

Figure 6a) example DI maps at APD50 at 120ms, 6b) DI across all cycle lengths, analysed using a 1-way ANOVA test. 6c) grouped DI at 120ms. 6d) Grouped DI heterogeneity data, at APD50, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

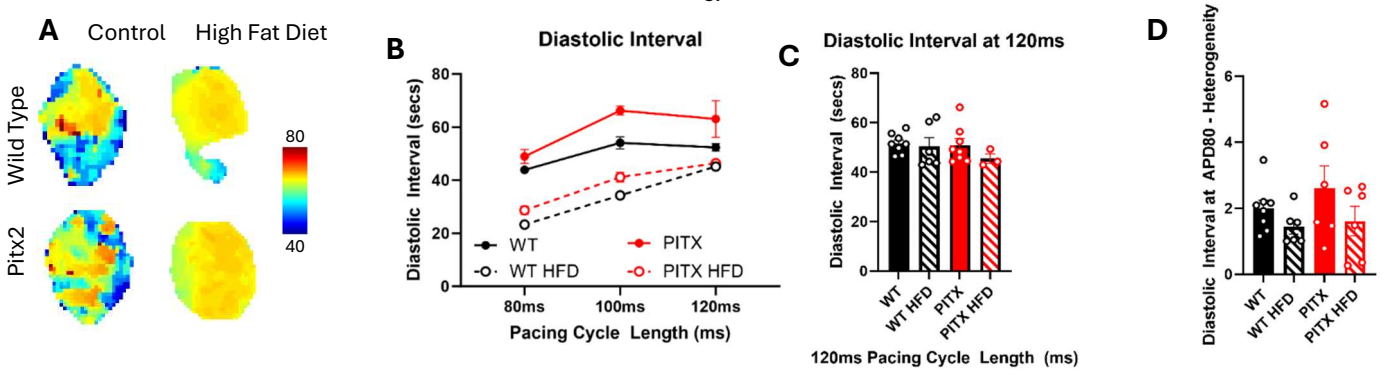

Figure 7a) example DI maps at APD80 at 120ms, 7b) DI across all pacing cycle lengths, analysed using a 1- way ANOVA test. 7c) grouped DI data at 120ms 7d) Grouped DI heterogeneities, at APD80, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

Diastolic Interval (DI) is the duration from APD30/50/80 to the following activation time, units in milliseconds (ms). Figure 5a, 6a and 7a show example DI maps at the corresponding APD30,50 and 80 at a pacing length of 120ms. Figure 5b,6b and 7b all show DI data across all pacing cycle lengths and figures 7a,7b and 7c show grouped DI data at 120ms.

Results from DI at APD30 showed some significant associations between HFD and Pitx2 deficiency on altering diastolic interval. Wt vs wt hfd (p=<0.0001), wt vs Pitx2 (p=0.0220), wt vs Pitx2 hfd (p=<0.0001), wt hfd vs Pitx2 (p=<0.0001) and Pitx2 vs Pitx2 hfd (p=0.0115). Whereas when analysing 120ms cycle length alone, results showed wt vs Pitx2 (80.25±48.58 vs 51.77±30.43, p=0.0225), wt hfd vs Pitx2 (38.20±23.34 vs 51.77±30.43, p=0.0023) and Pitx2x vs Pitx2 hfd (51.77±30.43 vs 48.65±26.08, p=0.0333).

Results from DI at APD50 showed significant associations between wt vs wt hfd (p=<0.0001), wt vs Pitx2 hfd (p=<0.0001), wt hfd vs Pitx2 (p=<0.0001) and Pitx2 vs Pitx2 hfd (p=0.0288). On the other hand, there were no significant differences when analysing data at 120ms alone.

Results from DI at APD80 showed significant differences between wt vs wt hfd (p=<0.0001), wt vs Pitx2 (p=0.0001), wt vs Pitx2 hfd (p=<0.0001), wt hfd vs Pitx2 (p=0.0001), Pitx2 vs Pitx2 hfd (p=0.002). Furthermore, at 120ms pacing cycle length results showed, wt hfd vs Pitx2 (45.14±28.54 vs 63.07±28.38, p=0.0154) and Pitx2 vs Pitx2 hfd (63.07±28.38 vs 46.04±19.99, p=0.0185) were both statistically significant.

6.4. High fat diet and Pitx2 deficiency do not alter time to peak

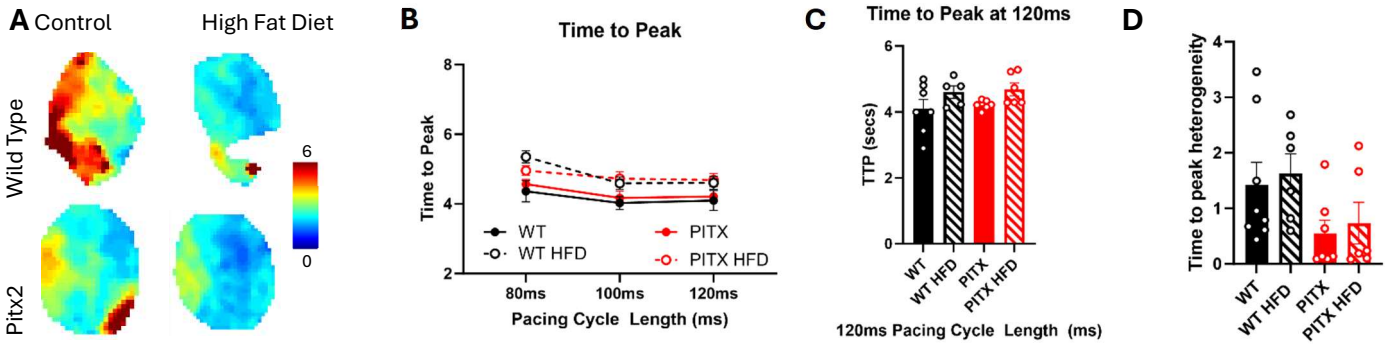

Figure 8a) example time to peak (TTP) maps at 120ms, 8b) TTP across all pacing cycle lengths, analysed using a 1-way ANOVA test. 8c) grouped TTP data at 120ms. 8d) Grouped TTP heterogeneity data, both analysed using a 2-way ANOVA test. WT, n = 7, WT HFD, n = 6, Pitx2, n = 6 and Pitx2 HFD, n = 5.

Time to peak (TTP) is defined as the time between 10% and 90% of depolarisation in the action potential cycle. Figure 8a identities example images of TTP across the LA, figure 8b shows the TTP across all pacing cycle lengths and figure 8c shows grouped TTP data at 120ms. From the two-way and one-way anova analyses, it was established that there were no significant differences in either HFD or Pitx2 deficiency causing alterations in the LA. However, figure 8c does show a longer TTP in both HFD groups.

6.5. Optical mapping permits proteomics analysis, but with less protein expressions

An optimisation experiment was performed to identify whether optically mapped hearts can undergo proteomic analysis. The experiment used two optically mapped murine hearts and two controlled, non-optically mapped murine hearts.

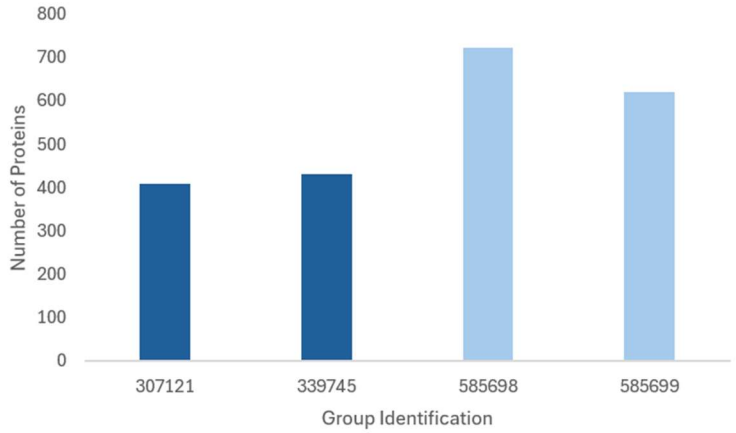

Figure 9: data showing number of proteins expressed per group. Dark blue (307121 and 339745): optically mapped hearts. Light blue (585698 and 585699): controlled hearts.

The results identify that although optically mapped hearts express proteins still through proteomics analysis, the controlled murine hearts expressed a higher amount. A one-way anova test established no significant differences within the data. When combining the groups, there were 1,344 proteins expressed in the combined controlled groups and 842 proteins expressed in the combined optically mapped groups, producing a 2,186 difference. The mean proteins expressed in optically mapped hearts was 421 and the mean within the controlled groups was 672.

Table 1: Data showing number of peptides expressed, per key protein identified.

Protein Expressed	307121	339745	585698	585699
Myosin-binding protein- cardiac	49	35	37	51
Tropomyosin alpha-1	64	57	55	62
Sarcoplasmic/endoplasmic reticulum calcium ATPase-2	47	49	48	48
Sodium/potassium-transporting ATPase subunit- alpha	19	11	8	17
Natriuretic Peptide – A	52	42	42	42
Gap Junction Protein	2	5	n/a	3

Despite the hundreds of proteins expressed in all four hearts, table 1 identifies six key proteins acknowledged through analysis that contribute to heart function. Furthermore, the table identifies the abundance of proteins by showing the number of peptides per protein, which can help determine any changes in protein expression from the dye loading, optical mapping or perfusion procedure.

Overall, the results show the optically mapped hearts expressed more peptides through the proteins identified than the controlled murine hearts. Across all the identified six proteins, optically mapped hearts expressed 432 peptides, compared to the controlled groups which expressed 423 peptides. The mean for optically mapped hearts was 216 peptides and the mean for controlled hearts was 212 peptides.

6.6. Fluorescent dyes produce no significant difference in number of contaminants expressed during proteomics analysis.

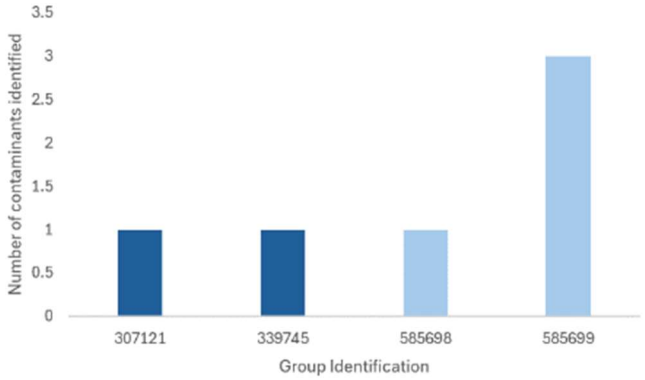

Figure 10: Data showing number of contaminants identified through proteomics analysis. Dark blue (307121 and 339745): optically mapped. Light blue (585698 and 585699): controlled hearts.

Figure 10 shows the number of contaminants identified through both optically mapped and controlled murine hearts. As shown, both optically mapped hearts only obtained one contaminant, whereas within the controlled hearts, one sample contained one contaminant, and one sample contained 3 contaminants. When assessing the overall group results, there were only two contaminants identified in optical mapping, and four identified in controlled – producing a difference of 2 overall.

7. DISCUSSION

This study utilised optical mapping to investigate the effects of HFD on left atrial electrophysiology, whilst further analysing Pitx2 deficient mice to see if any further alterations occurred. The study showed HFD prolonged action potential duration at each pacing cycle length, showing potential mechanisms for arrythmias. Furthermore, there was indication Pitx2 deficiency causes a shortened APD, when comparing to wild type mice. Additionally, results showed Pitx2 deficiency alters conduction velocity whereas HFD had no effect.

7.1. High fat diet prolongs action potential duration

Prolongation of APD is commonly associated with the incidence of both delayed after depolarisations (DADs) and early after depolarisations (EADs). Both DADs and EADs can produce ectopic activity, so act as triggers for arrythmias (Song et al., 2015). Further, changes in repolarisation dynamics across the cardiac tissue, especially if they introduce heterogeneity, can act as a substrate for arrythmias such as atrial fibrillation.

When understanding why prolongation of APD occurs, Wickenden et al., (1997) identified the decreased density of the transient outward potassium current (I_{to}) with obesity from a HFD, obtains a prominent role in inducing APD prolongation, in both animal and human models. This is further supported by Carmeliet (2006) who identified that HFD prolonged APD. The study showed that HFD increases the risk of EADs, and the identified that this was caused by reduced outward currents - I_{to}, I_{kr} and I_{ks}. Alongside this, the research by Carmeliet (2006) showed an increase in inward current late I_{na}, through a HFD, which was established to be carried by Na^+ through tetrodotoxin (TTX) sensitive pathways and primarily within the late I_{na} phase. To note,

TTX is a toxin which binds to voltage-gated sodium channels and blocks the Na^+ flow across cell membranes (Bucciarelli et al., 2021). Additionally, Houser et al., (1981) identified HFD altered the I_{to} current, which was associated to cardiac hypertrophy and APD prolongation. Alongside this, the inward rectifying potassium current (I_{k1}) which primarily contributes within the late repolarisation phase and setting the resting membrane potential (Houser et al., 1981).

Previous research has shown that along with HFD reducing potassium currents and playing a role in APD prolongation, HFD also causes an increase in calcium currents which also partakes in the concept. Shah et al., (2022) identified that if there is an increase in calcium influx within the L-type calcium channels, it causes a sustained prolonged depolarisation – which inevitably causes prolongation in the action potential's plateau phase. In relation, an increase in the late I_{NA} causes a depolarising current during the plateau phase which further triggers EADs. The results from Maier (2009) identified this theory linked to HFD can cause torsade's de pointes arrythmias, which are characterised as an ECG showing a twisting pattern of the QRS complex. Alongside torsade's de pointes arrythmias, late I_{NA} can further contribute to re-entrant arrythmias by causing prolonged refractoriness, which produces a unidirectional block and initiates re-entry (Maier, 2009).

A study by Peterson et al., (2009) identified within a healthy heart and a normal action potential, the L-type calcium channels undergo voltage- and Ca^{2+}- dependent inactivation which limits Ca^{2+} influx. As the APD progresses, it permits the L-type calcium channels to recover from the inactivation, which generates inward currents which lead to early after-depolarisations (Shu et al., 2023). Furthermore, the study results identified early after-depolarisations are a key concept in the APD prolongation. However, results from the study are more transparent as they identified key atrial

structural remodelling factors, mainly consisting of fibrosis, atrial enlargement and changes in cardiomyocyte ultrastructure which are all mechanistic in AF (Shu et al., 2023).

Interestingly, the findings here contradict others which suggest HFD shortens atrial APD. Many theories have been related to the shortening concept and more research studies have provided evidence of the associations compared to prolongation.

On the contrary to prolongation of APD, previous studies have established that an increase in certain voltage-gated potassium channels ($I_{K,}$ I_{KR} and I_{KS}), shorten the APD. Potassium is a key application in the action potential process due to the responsibility for restoring negative charge within the cell. Additionally, potassium efflux is responsible for repolarisation after the action potential has fired, thus, an increase in potassium current will shorten the time that the cell remains depolarised (Jeevaratnam et al., 2018).

Studies have identified that HFD can reduce calcium influx within the L-type calcium channels, this consequently shortens the plateau phase of the action potential which causes faster repolarisation and an overall shortened APD (Lee et al., 2016). Furthermore, the enhanced activity of the sodium-to-calcium (na^{+}-ca^{2+}) exchanger can help to remove Ca^{2+} rapidly and efficiently, which also speeds the repolarisation process and shortens APD. Furthermore, this can reduce the calcium influx within the L-type calcium channels, thus consequently shortens the plateau phase of the action potential which also causes faster repolarisation and an overall shortened APD (Lee et al., 2016).

Aromolan et al., (2016) studied guinea pigs consuming a HFD and found an increase in the delayed rectifier potassium current which results in APD shortening.

Furthermore, their results showed that ion channel remodelling alterations through HFD resulted in decreased peak sodium current, reduced long-type calcium currents alongside increased ultrarapid potassium currents (McCauley et al., 2020). All these alterations together are strong mechanisms in the provocation of APD shortening and reduced conduction velocity.

From this study's results, it demonstrates that the mice within the HFD groups within both wild type and Pitx2 deficient groups potentially obtained reduced potassium efflux and increased calcium influx. However, the study did not measure ion channel results, therefore the theory is based solely on previous research.

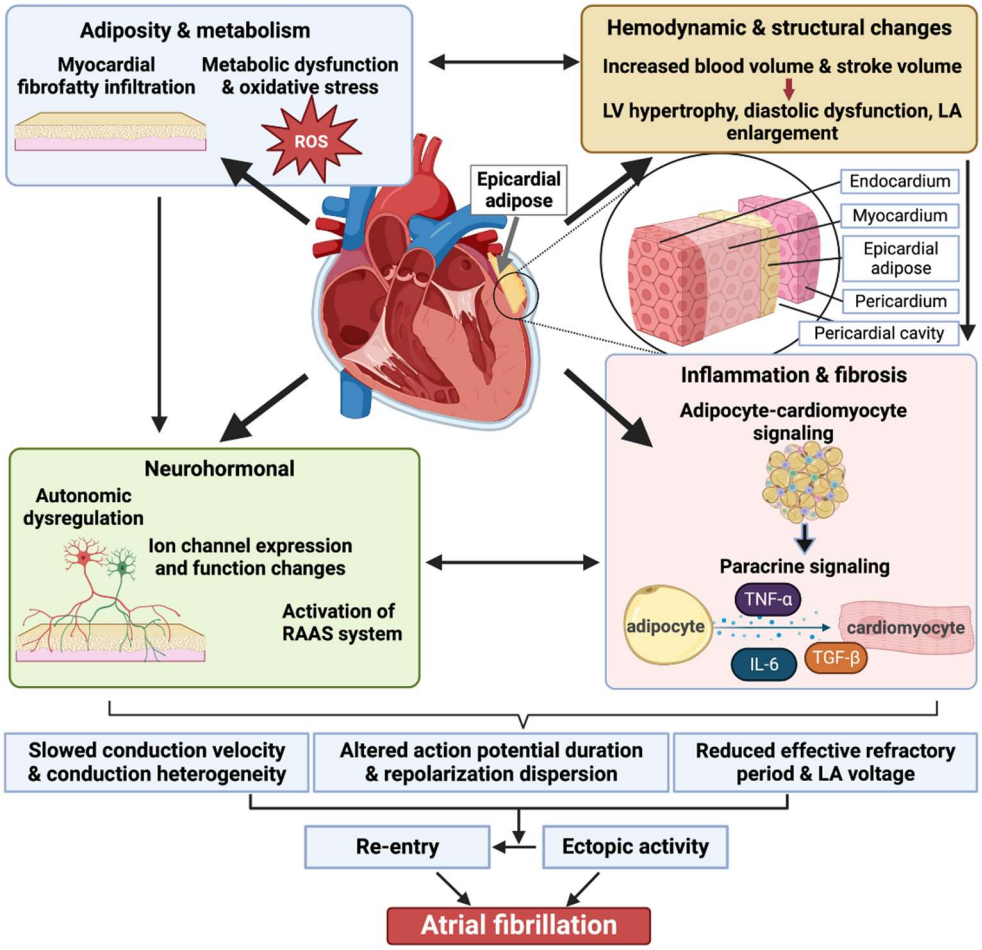

Figure 11: Illustration summary of effects HFD on the increased susceptibility to arrythmias (Sha et al., 2024).

7.2. Pitx2 deficient mice showed shortening of APD compared to wild type mice

This study identified despite the prolongation within the two HFD groups, when initiating a sub-group analysis of wild type mice versus Pitx2 deficient mice, the results identified a shortening of APD in both Pitx2 deficient groups, against the wild type.

Kirchhof et al., (2011) researched into how reducing Pitx2 promotes AF and any complex changes associated with the gene expression. The results agreed with this study, where they identified shortened APD in Pitx2 deficient mice when comparing against the wild type mice. Interestingly, the study further acknowledged Kcna3, Kcnc4 and Kcnk5 were also all involved in the genetic expression changes. From here, the study's results identified the effective refractory period was shortened during a Pitx2 deficient expression, meaning the heart is going to be stimulated before its ready (Kirchhof et al., 2011). Additionally, Tsai et al., (2008) established a shortened effective refractory period within Pitx2 deficient mice which they stated could be associated to re-entrant arrhythmias, due to a shorter effective refractory period permitting the tissue to recover and become excitable again more rapidly, creating a common situation when the electrical impulse could re-enter a recently excitable tissue.

Pitx2 regulates the expression of ion channels, therefore over-expression and under-expression of the transcription gene can lead to immediate alterations in the electrophysiology process in the heart. Chen et al., (2003) specifically established Pitx2 regulates the rapid and slow delayed rectifier K^+ channels (I_{kr} and I_{ks}). Furthermore, when performing the Pitx2 knockout aspect in mice it showed an upregulation of the K^+ channels which caused rapid depolarisation during the plateau phase, thus shortening APD (Chen et al., 2003).

Interestingly, Kirchhof et al., (2011) identified no changes in left or right, atrial sizes, alongside finding a normal sinoatrial node pattern and normally appearing pulmonary veins when analysing Pitx2 deficient mice. Furthermore, despite subtle abnormalities identified structurally, there was no evidence of any abnormal automaticity or triggered activity being potential arrhythmogenic factors – however, as stated previously, more research is needed within this area.

Moreover, Pitx2 reduction was found to have changed the calcium ion binding with both the cell junctions and adhesions (Tao et al., 2014). Interestingly, the deficiency of the gene showed a possible association to increased melanocortin (a group of peptide hormones and neuropeptides, which regulate physiological functions), which was further linked to stimulation of interleukin expressions, increasing inflammation and altering general electrophysiological alterations (Tao et al., 2014). Additionally, Kirchhof et al., (2011) identified a possible alteration in the sarcoplasmic reticulum during Pitx2 knockout, which effects the calcium binding genes. The study established that spontaneous AF is rare within Pitx2 deficient mouse models and contractile function was normal, however this is contradictory to various evidence of differences in intracellular calcium homeostasis is intrinsically linked to contractility alterations (Torrado et al., 2014).

Syeda et al., (2016) identified Pitx2 deficiency reduced the resting membrane potential by 2mV, from -79.9mV to -77.9mV, with the prorenin receptor being enhanced. Meaning there is more of a key role in the proteins which modulate cardiovascular homeostasis. Furthermore, considering the reduced resting membrane potential, the study identified a decrease in action potential duration as a consequence of this, compared to wild type.

From the results of the study and previous research conducted, it was established that more research is essential to understand more between Pitx2 deficiency and how it affects parameters associated with AF.

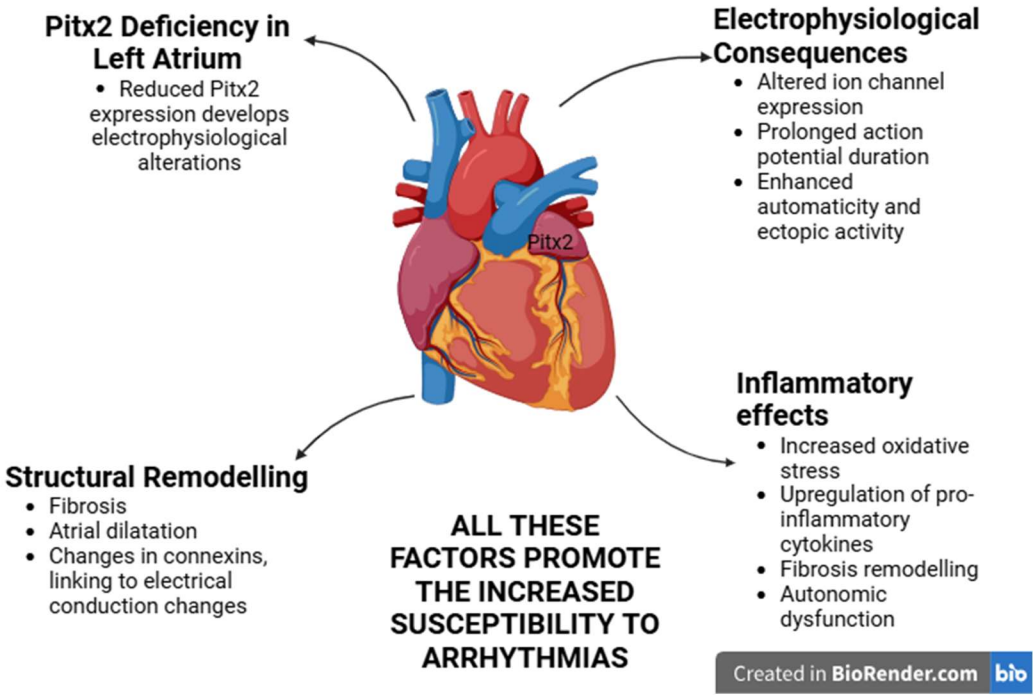

Figure 12: summary illustration of how Pitx2 deficiency may induce arrythmias. Image created on biorender.com.

7.3. HFD does not alter conduction velocity

The study identified no alterations in the conduction velocity within the HFD group, however, the results could suggest slight increase in the Pitx2 deficient murine hearts compare to wild type. Although, no significant differences were established the concept provides an interesting take.

Munger et al., (2012) agreed with the study's results, as they also identified the LA conduction velocity did not differ between the wild type and HFD groups. Interestingly, these results occurred despite the HFD murine hearts obtaining the presence of

haemodynamic stress including, enlarged LA volume, low LA function and impaired diastolic function. Additionally, the study's main findings showed overall there was no significant difference between the two groups. However, further analysis showed the HFD group obtained a shorter conduction velocity from the LA entering the pulmonary vein and a shorter effective refractory period. These two factors combined are common mechanisms in re-entrant arrythmias (Munger et al., 2012).

On the contrary, Axelsen et al., (2015) identified a slowed conduction velocity within rats who consumed an HFD. The study results identified through a 6-week HFD, they developed QRS prolongation, decreased conduction velocity and increased arrhythmogenesis during perfusion. These outcomes were all related to altered gap junction activity, where the analysis predicted this outcome to occur from three possible mechanisms; 1.) the study established there was a possible deficiency in the Cx43 protein, 2.) possible closure or reduced conductance in the gap junction and 3.) loss of gap junction due to intercalated discs (Axelsen et al., 2015).

Their study focused on the loss of Cx43 protein, which is a key protein used in maintain electrical function within the heart. Cx43 ensures the electrical impulses are transmitted across the tissue uniformly, therefore in the absence of the protein, the heart will experience heterogenous conduction – meaning impulses may travel faster or slower, through specific localised regions compared to others. Thus, contributing to conduction block, re-entrant circuits and AF (Axelsen et al., 2015).

Maintained LA conduction velocity and no established conduction heterogeneity in the HFD murine hearts suggested no present pro-fibrotic activity in the left atrium. This was suggested through analysis, as areas of fibrotic activity promotes conduction slowing, reduced overall conduction velocity and promote conduction heterogeneity

between wild type and HFD hearts. The results from this study contradict previous findings, due to no alteration, but clear AP morphology changes.

Contradictory to these findings, Okumara et al., (2015) analysed 8-week-old pigs and established evidence of atrial and pulmonary vein stretch. These findings associated atrial stretch to reduced effective refractory period, reduced APD and reduced conduction velocity, primarily due to reduced cellular-excitability through the opening of stretch-activated channels. Stretch-activated channels identify a type of ion channel which opens when a cell membrane is mechanically deformed, leading to changes in the concentration of intracellular calcium and transmembrane potentials (Sachs, 2010).

In addition, Abed et al., (2013) analysed thirty sheep who followed an 8-month HFD. The study provided interesting results, due to following the sheep across a long-term period, compared to this short-term study. Their results identified long-term used of HFD drastically alters the electrophysiology of the heart, particularly conduction velocity. Furthermore, the study established increased weight of the HFD sheep was accompanied by increased LA volume, fibrosis, inflammatory infiltrates, lipidosis – alongside, reduced conduction velocity, increased conduction heterogeneity but no changes to the effective refractory period (Abed et al., 2013).

Further analysis showed the reduced conduction velocity was shown through both isochronal crowding (where there is local deceleration of propagation in a region within scar with bunching isochrones, >2 isochrones within 1cm diameter), alongside delayed activation. Furthermore, the study adjusted the results according to haemodynamic changes and identified maintained slow conduction velocity (Abed et al., 2013).

7.4. Optically mapped hearts can undergo proteomic analysis

As shown through previous research and within the results of this study, both optical mapping and proteomics are essential techniques within cardiovascular research. Merging both techniques offers a strong approach by providing both functional, and molecular, insights into cardiovascular diseases. Optical mapping permits the visualisation of electrical activity across the heart, elaborating how arrythmias or other electrical disturbances can develop. Whereas proteomics dives into biochemical changes that induce the electrical abnormalities, identifying possibilities of ion channel dysfunction or alterations in signal pathways.

Being able to combine optical mapping and proteomic analysis on hearts would provide cardiovascular researchers with a comprehensive approach to thorough identification of electrical abnormalities in the heart, and any related structural differences in control and diseased hearts.

Presently, while little research exists on combining optical mapping and proteomics on the same hearts, one of the study's aims was to address this gap by utilising optically mapped, and controlled, hearts throughout a proteomic analysis. The results from this study identified that optically mapped murine hearts can successfully undergo proteomic experiments, with no significant differences between the two groups. Furthermore, the results showed that a substantial number of proteins were still expressed despite hearts undergoing perfusion of fluorescent dyes previously. Considerable amounts of research are needed to confirm the consistency and reliability of combining both techniques, but the data from this study produced a positive outlook on the topic.

As the results show, hundreds of proteins were expressed in the analysis from all four hearts. Whilst comparing this to previous research, on just proteomic data, six expressed proteins became apparent, which were 1.) myosin-binding protein-cardiac, 2.) tropomyosin alpha-1, 3.) sarcoplasmic/endoplasmic reticulum calcium ATPase-2, 4.) sodium/potassium-transporting ATPase subunit- alpha, 5.) natriuretic peptide-A and 6.) gap junction protein. All six proteins are essential in cardiac function, electrical signals and pathways.

To elaborate on these proteins, firstly, myosin-binding protein-cardiac is a key regulator in the actin-myosin interactions affecting the contractile function. Mutations of this protein may lead to disorganised sarcomeres, and hypertrophic cardiomyopathy contributing to abnormal conduction and instability to promote arrythmias (Flashman et al., 2004). Similarly, tropomyosin alpha-1 mutations lead to distinct structural and functional changes within the tropomyosin (which also regulates contraction), promoting both inherited hypertrophic cardiomyopathy and dilated cardiomyopathy (Redwood and Robinson, 2013). Thirdly, the cardiac isoform of Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) is a calcium ion pump, and when expressed differently, leads to abnormal Ca^{2+} handling and contractility deficiency, promoting arrythmias (Kawase and Hajjar, 2008).

The sodium/potassium transporting ATPase subunit- alpha actively transports sodium out, and potassium into the myocyte. Disruptions in this process has been linked to increasing susceptibility to early- and delayed- afterdepolarisation, both linked to AF (Schwinger et al., 2003). The natriuretic peptide-A protein is a key regulator in functioning the response to stretch and pressure overload within the atrial myocyte. When this function is disrupted, it increases susceptibility to the atria over-stretching, which can lead to electrical and structural remodelling (Kuwahara, 2021). Finally, the

gap junction protein is essential for electrical conduction through the heart, as gap junctions allow direct passing for ions and electrical impulses between cardiomyocytes, enabling the action potential propagation. Therefore, disruptions in this protein will cause slower conduction velocity, alter action potential duration and increasing the risk of re-entrant arrythmias (Kanno and Saffitz, 2001).

Therefore, if future research, for instance future HFD studies, can visualise electrical abnormalities or alterations through the optical mapping and identify key protein expression changes between HFD group and controlled – it permits a deeper understanding into the underlying mechanisms and risk of AF or related cardiac diseases.

7.5. Limitations and Future Directions

One limitation from the study was there was no analysis of structural remodelling, only electrical. Gaining structural remodelling results, like identifying fibrotic activity, provides a greater insight into the overall mechanistic process of the cardiac remodelling. To elaborate, certain structural remodelling of the hearts in circumstances of changes in heart size, shape and composition is important, as these factors elaborate from diseases like hypertension and myocardial infarction. Whereas, identifying electrical remodelling involves change within ion channels, conduction pathways and overall electrical activity which promotes arrythmias. As electrical remodelling is often a consequence of certain structural remodelling, obtaining structural data would have provided this study with a deeper understanding on some concepts, for instance how APD prolongation occurred, and so forth.

Some other limitations identified in the study was the sample size, study length and the methodological approach of only using male mice. By obtaining a larger sample

size, it promotes more consistent and reliable data - and by increasing the length of the study, it would produce more long-term results for future research. This study only used male mice to reduce any confounding factors within the study and allow focus on single sex changes within Pitx2 deficiency, HFD and arrythmias. However,using only male mice restricts data as from these results, it is only subjected to males. Whereas research has shown that females and males can obtain varied results in both structural and electrical remodelling of the heart. For instance, research has shown females have more advanced atrial remodelling than males which is characterised by lower activation and slower conduction velocity (Martin and Leinwand, 2024).

In terms of future directions from this study, it would be useful for studies to incorporate metabolic techniques to assess protein expression changes, genetic expression changes and related aspects alongside the optical mapping arm of a study. This would permit cardiac researchers to acknowledge both aspects of disease and gain a deeper understanding of the mechanisms, function and structure of the developing heart disease. Furthermore, it permits a greater understanding of whether over and under expression of certain proteins and genes, may contribute to promoting the risk of cardiovascular diseases. Additionally, as stated in the limitations, it would be beneficial for research to be conducted into males versus females and how each sex cardiac structure and function differ or develop from diseases.

8. CONCLUSION

To conclude, the study's aims were to see whether HFD induces electrophysiological remodelling in the murine LA, and whether the response is regulated by altered expression of Pitx2 gene. Further, we aimed to evaluate whether proteomic experiments can be conducted on dyed, optically mapped hearts.

Through a comprehensive analysis using data from optical mapping technique, the research demonstrated that HFD does alter the electrophysiological remodelling in the murine LA. The results particularly highlighted the prolongation in the action potential duration within the HFD groups, and how this is a mechanism associated with re-entrant arrythmias. Furthermore, the analysis acknowledged despite the prolongation in APD, the Pitx2 deficiency caused a shortening of APD when comparing to the wild type murine hearts. This led to the possibility of Pitx2 deficiency developing alterations in ion channel remodelling or causing structural remodelling associated fibrosis or atrial enlargement. However, as stated in the limitations, this study did not analyse structural remodelling, so it is not evident to conclude this theory fully.

On the topic of electrical remodelling, contradictory to other findings, this study's results established no alteration in conduction velocity. These results suggested no-fibrotic activity within the HFD hearts, as recent studies have shown that fibrosis slows conduction velocity overall and causes heterogeneity in results (Okumara et al., 2015). Furthermore, the study found no alterations in diastolic interval or time to peak.

The second part of the study was an optimisation sub-study to identify whether dyed, optically mapped hearts could undergo proteomic analysis. The results from this study suggest the dyed hearts can be used for proteome analysis, meaning future research on metabolic and electrical changes can be completed.

This study has highlighted the necessity for research to be conducted in the association between HFD and LA electrophysiological changes. Furthermore, more research could be conducted to establish a clearer insight into how Pitx2 deficiency alters ion channels and structural remodelling. With continued research, it would enable a reduction in the statistics of people with arrhythmias and other related diseases, and help researchers understand the mechanisms underlying HFD-induced arrythmias, alongside Pitx2 deficient associations.

9. LIST OF REFERENCES

Abed, H.S., Samuel, C.S., Lau, D.H., Kelly, D.J., Royce, S.G., Alasady, M., Mahajan, R., Kuklik, P., Zhang, Y., Brooks, A.G., Nelson, A.J., Worthley, S.G., Abhayaratna, W.P., Kalman, J.M., Wittert, G.A., Sanders, P. (2013) 'Obesity results in progressive atrial structural and electrical remodelling: Implications for atrial fibrillation.' *Heart Rhythm,* 10(1), pp. 90-100.Al-Amrani, S., Al-Jabri, Z., Al-Zaabi, A., Alshekaili, J., and Al-Khabori, M. (2021) 'Proteomics: Concepts and applications in human medicine.' *World Journal of Biological Chemistry,* 12(5), pp. 57-69.

Anter, E., Tschabrunn, C.M., Buxton, A.E., and Josephson, M.E. (2016) 'High resolution mapping of postinfarction re-entrant ventricular tachycardia.' *Circulation,* 134(4), pp. 314-327,Antzelevitch, C. (2007) 'Role of spatial dispersion of repolarisation in inherited and acquired sudden cardiac death syndromes.' *American Journal of Physiology- Heart and Circulatory Physiology,* 293(4), pp. 2024-2038.

Antzelevitch, C., and Burashinov, A. (2011) 'Overview of basic mechanisms of cardiac arrythmias.' *Cardiac Electrophysiology Clinical,* 3(1), pp. 23-45.

Aromolaran, A.S., and Boutjdir, M. (2017) 'Cardiac ion channel regulation in obesity and metabolic syndrome: Relevance to long QT syndrome and atrial fibrillation.' *Frontiers in Physiology,* 8, pp. 421.

Argarwal, S.K., Norby, F.L., Whitsel, E.A., Soliman, E.Z., Chen, L.Y., Loehr, L.R., Fuster, V., Heiss, G., Coresh, J., and Alonso, A. (2017) 'Cardiac autonomic dysfunction and incidence of atrial fibrillation.' *Journal of the American College of Cardiology,* 69(3), pp. 291-299.

Atienza, F., Almendral, J., Moreno, J., Vaidyanathan, R., Talkachou, A., Kalifa, J., Arenal, A., Villacastin, J.P., Torrecilla, E.G., Sanchez, A., Polutz-Snyder, R., Jalife, J and Berenfield, O. (2006). 'Activation of inward rectifier potassium channels accelerates atrial fibrillation in humans.' *Circulation,* 23, pp. 2343-2442.

Atienza, F., and Jalife, J. (2007) 'Reentry and atrial fibrillation.' *Heart Rhythm,* 4(3), pp. 13-16.

Attin, M., and Clusin, W.T. (2009) 'Basic concepts of optical mapping techniques in cardiac electrophysiology.' *Biological Research in Nursing,* 11(2), pp. 195-207

Barron, A., Fabre, T., and De, S. (2024) 'Distinct fibroblast functions associated with fibrotic and immune-mediated inflammatory disease and their implications for therapeutic development.' *F1000 Research,* 13, pp. 54.

Baum, J., and Duffy, H.S. (2011) 'Fibroblasts and myofibroblasts: What are we talking about?" *Journal of Cardiovascular Pharmacology,* 57(4), pp. 376-379.

Belevych, A.E., Radwanski, P.B., Carnes, C.A., and Gyorke, S. (2013) 'Ryanopathy: Causes and manifestations of RyR2 dysfunction in heart failure.' *Cardiovascular Research,* 98(2), pp. 240-247.

Bhupathi, S.S. Chalasani, S., and Rokey, R. (2011) 'Stiff heart syndrome.' *Clinical Medical Research,* 9(2), pp. 92-99.

Bosch, R.F., and Nattel, S. (2002) 'Cellular electrophysiology of atrial fibrillation.' *Cardiovascular Research,* 54(2), pp. 259-269.

Boyden, P.A., Tilley, L.P., Pham, T.D., Liu, S.K., Fenoglic, J.J., and Wit, A.L. (1982) 'Effects of left atrial enlargement on atrial transmembrane potentials and structure in dogs with mitral valve fibrosis.' *American Journal of Cardiology,* 49(8), pp. 1896-1908.

Brooks, A.G., Rangnekar, G., Ganesan, A.N., Salna, I., Middeldorp, M.E., Kuklik, P., Baumert, M., Roberts-Thomson, K.C., and Sanders, P. (2012) 'Characteristics of ectopic triggers associated with paroxysmal and persistent atrial fibrillation: Evidence for a changing role.' *Heart Rhythm,* 9(9), pp. 1367-1374.Bucciarelli, G.M., Lechner, M., Fontes, A., Kats, L.B., Eisthen, H.L., and Shaffer, H.B. (2021) 'From poison to promise: The evolution of tetrodotoxin and its potential as a therapeutic.' *Toxins,* 13(8).

Buckberg, G.D., Nanda, N.C., Nguyen, C., and Kocica, M.J. (2018) 'What is the heart? Anatomy, function, pathophysiology and misconceptions.' *Journal of Cardiovascular Development and Disease,* 5(2), pp. 33.

Canale, M.P., Manca di Villahermosa, S., Martino, G., Rovello, V., Noce, A., De Lorenzo, A., and Di Daniele, N. (2013) 'Obesity-related metabolic syndrome: Mechanisms of sympathetic overactivity.' *International Journal of Endocrinology,* pp. 865-895.

Carnagarin, R., Kiuchi, M.G., Ho, J.K., Matthews, V.B., and Schlaich, M.P. (2018) 'Sympathetic nervous system activation and its modulation: Role in atrial fibrillation.' *Frontiers in Neuroscience,* 12, pp. 1058.

Carlson, A.J. (2007) 'On the mechanism of the refractory period in the heart.' *American Journal of Physiology,* 18(1), pp. 71-88.

Carrick, R.T., Benson, B.E., Bates, O.R.J., and Spector, P.S. (2021) 'Competitive drivers of atrial fibrillation: The interplay between focal drivers and multiwavelet reentry.' *Frontiers in Physiology,* 12, pp. 16-21.

Chahine, T.A., Atapour, B., Kwan, K.T., Anderson, C.A., Macheret, F., Afroze, T., Bifulco, S.F., Cham, M.D., Ordovas, K., Boyle, P.M. and Akoum, N. (2022) 'Epicardial

adipose tissue is associated with left atrial volume and fibrosis in patients with atrial fibrillation.' *Frontiers in Cardiovascular Medicine,* 9, pp. 21-29.

Chang Yi, L., Zhang, J.R., Wan Ning, H., and Song Nan, L. (2021) 'Atrial fibrosis underlying atrial fibrillation.' *International Journal of Molecular Medicine,* 47(3), pp. 9.

Chen, P.S., Chen, L.S., Fishbein, M.C., Lin, S.F., and Nattel, S. (2014) 'Role of the autonomic nervous system in atrial fibrillation: Pathophysiology and therapy.' *Circulation Research,* 114(9), pp. 1500-1515.

Chen, Y.H., Xu, S.J., Bendahhou, S., Wang, X.L., Wang, Y., Xu, W.Y., Jin, H.W., Sun, H., Zhuang, Q.N., Yang, Y.Q., Li, Y.B., Xu, H.J., Li, X.F., Ma, N., Mou, C.P., Chen, Z., Barhanin, J., and Huang, W. (2003) 'KCNQ1 gain-of-function mutation in familial atrial fibrillation.' *Science,* 299(5604), pp. 251-254.

Chinchilla, A., Daimi, H., Lozano-Velasco, E., Dominguez, J.N., Cabellero, R., Delpon, E., Tamargo, J., Cinca, J., Hove-Madsen, L., Aranega, A.E., and Franco, D. (2011) 'Pitx2 insufficiency leads to atrial electrical and structural remodelling linked to arrhythmogenesis.' *Circulation: Cardiovascular Genetics,* 4(3), pp. 269-279.

Ciaccio, E.J., Coromilas, J., Wan, E.Y., Yarmohammadi, H., Saluja, D.S., Peters, N.S., Garan, H., and Biviano, A.B. (2023) 'Correlation relationships of the reentrant ventricular tachycardia circuit.' *Computer Methods Programmes in Biomedics,* 241, pp. 107-117.

Conte, M., Petraglia, L., Cabaro, S., Valerio, V., Poggio, P., Pilato, E., Attena, E., Russo, V., Ferro, A., Formisano, P., Leosco, D., and Parisi, V. (2022) 'Epicardial adipose tissue and cardiac arrythmias: Focus on artrial fibrillation.' *Frontiers in Cardiovascular Medicine,* 9, pp. 932-962.

Coumel, P. (2004) ‘Paroxysmal atrial fibrillation: A disorder of autonomic tone?’ *European Heart Journal,* 15, pp. 9-16.

Cox, J.L. (2003) ‘Atrial fibrillation: A new classification system.’ *The Journal of Thoracic and Cardiovascular Surgery,* 126(6), pp. 1686-1692.

Dai, W., Kesaraju, S., and Weber, C.R. (2021) ‘Transcriptional factors in calcium mishandling and atrial fibrillation development.’ *Journal of Physiology,* 473(8), pp. 1177-1197.

Dilaveris, P.E., and Kennedy, H.L. (2017) ‘Silent atrial fibrillation: Epidemiology, diagnosis and clinical impact.’ *Clinical Cardiology,* 40(6), pp. 413-418.

De Coster, T., Teplenin, A.S., Feola, I., Bart, C.I., Ramkisoensing, A.A. Den Ouden, B.L., Ypey, D.L., Trines, S.A., Panfilov, A.V., Zeppenfeld, K., De Vries, A.F., and Pijnappels, D.A. (2023) ‘Trapped re-entry as a source of acute focal atrial arrythmias.’ *Cardiovascular Research,* 120(3), pp. 249-261.

De Vries, L.J., Martirosyan, M., Van Domburg, R.T., Wijchers, S.A., Geczy, T., and Szili-Torok, T. (2018) ‘Coupling interval variability of premature ventricular contractions in patients with different underlying pathology: An insight into the arrythmia mechanism.’ *Journal of Interventional Cardiac Electrophysiology,* 51(1), pp. 25-33.

Dzeshka, M.S., Lip, M.Y.H., Snezhitskiy, V., and Shantsila, E. (2015) ‘Cardiac fibrosis in patients with atrial fibrillation.’ *Journal of the American College of Cardiology,* 66(8), pp. 943-959.

Edwards, W., Greco, T.M., Miner, G.E., Barker, N.K., Herring, L., Cohen, S., Cristea, I.M., and Conlon, F.L. (2023) ‘Quantitative proteomic profiling identifies global protein

network dynamics in murine embryonic heart development.' *Development of Cells,* 58(12), pp. 1087-1105.

Essa, H., Hill, A.M., and Lip, G.Y.H. (2021) 'Atrial fibrillation and stroke.' *Elsevier,* 13(1), pp. 243-255.

Fedorov, V.V., Lozinsky, I.T., Sosunov., E.A., Anyukhovsky, E.P., Rosen, M.R., Balke, C.W., and Efimov, I.R. (2007) 'Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts.' *Heart Rhythm,* 4(5), pp. 619-626.

Flashman, E., Redwood, C., Moolman-Smook, J., and Watkins, H. (2004) 'Cardiac myosin binding protein C: Its role in physiology and disease.' *Circulation Research,* 94(10), pp. 12-14.

Francis, G.S. (1988) 'Modulation of peripheral sympathetic nerve transmission.' *Journal of the American College of Cardiology,* 12(1), pp. 250-254.

Gallego, M., Zayas-Arrabal, J., Alquiza, A., Apellaniz, B., and Casis, O. (2021) 'Electrical features of the diabetic myocardium. Arrhythmic and cardiovascular safety considerations in diabetes.' *Frontiers of Pharmacology,* 12, pp. 687-690.

Garrott, K., Kuzmiak-Glancy, S., Wengrowski, A., Zhang, H., Rogers, J., and Kay, M.W. (2017) 'KATP channels inhibition blunts electromechanical decline during hypoxia in left ventricular working rabbit hearts.' *The Journal of Physiology,* 595(12), pp. 3799-3813.

Gibbons, C.H. (2019) *Chapter 27- Basics of autonomic nervous system function*.' 160, pp. 407-418.

Goodenough, D.A., and Paul, D.L. (2009) ‘Gap junctions.’ *Cold Spring Harbour and Perspective Biology,* 1(1), pp. 25-29.

Gong, Y., Xie, F., Stein, K.M., Garfinkel, A., Calianu, C.A., Lerman, B.B., and Christini, D.J. (2007) ‘Mechanism underlying initiation of paroxysmal atrial flutter/atrial fibrillation by ectopic foci.’ *Circulation,* 115(16), pp. 2094-2102.

Goudis, C.A., Korantzopoulous, P., Ntalas, I.V., Kallergis, E.M., and Ketikoglou, D.G. (2015) ‘Obesity and atrial fibrillation: A comprehensive review of the pathophysiological mechanisms and links.’ *Journal of Cardiology,* 66(5), pp. 361-369.

Grant, A.O. (2009) ‘Cardiac ion channels.’ *Circulation: Arrythmias and Electrophysiology,* 2(2), pp. 185-194.

Gristani, L.A. (2018) ‘Diabetes and arrythmias: Pathophysiology, mechanisms and therapeutic outcomes.’ *Frontiers of Physiology,* 9, pp. 1669.

Gupta, V., Munjal, J.S., Jhajj, P., Jhajj, S., and Jain, R. (2022) ‘Obesity and atrial fibrillation: A narrative review.’ *Cureus,* 14(11), pp. 32-39.

Han, T.S., Lean, M.E.J. (2015) ‘Metabolic Syndrome.’ *Medicine,* 43(2), pp. 80-87.

Heidt, S.T., Kratz, A., Najarian, K., Hassett, A.L., Oral, H., Gonzalez, R., Nallmothu, B.K., Clauw, D., and Ghanbari, H. (2016) ‘Symptoms in atrial fibrillation: A contemporary review and future directions.’ *Journal of Atrial Fibrillation,* 9(1), pp. 1422.

Heron, L., O’Neill, C., McAneney, H., Kee, F., and Tully, F.A. (2019) ‘Direct healthcare costs of sedentary behaviour in the UK.’ *Journal of Epidemiology and Community Health,* 73(7), pp. 625-629.

Houser, S.R., Freeman, A.R., Jaeger, J.M., Breisch, E.A., Coulson, R.L., Carey, R., and Spann, J.F. (1981) 'Resting potential changes associated with Na-K pump in failing heart muscle.' *American Journal of Physiology,* 240(2), pp. 168-176.

Huang, C.L., Lei, L., Matthews, G.D., Zhang, Y., and Lei, M. (2012) 'Pathophysiological mechanisms of sino-atrial dysfunction and ventricular conduction disease associated with SCN5A deficiency: Insights from mouse models.' *Frontiers of Physiology,* 3, pp. 234.

Ibrahim, M.M. (2010) 'Subcutaneous and visceral adipose tissue: Structural and functional differences.' *Obesity Reviews,* 11(1), pp. 1-18.

Inoue, D., Shirayama, T., Omori, I., Katsume, H., and Nakagawa, M. (1988) 'Mexiletine for paroxysmal atrial fibrillation.' *Elsevier,* 94(6), pp. 1317.

Iqbal, S.M., and Lemmens-Gruber, R. (2019) 'Phosphorylation of cardiac voltage-gated sodium channel: Potential players with multiple dimensions.' *Acta Physiology,* 225(3), pp. 13-21.

Jansen, H.J., Bohne, L.J., Gillis, A.M., Rose, R.A. (2020) 'Atrial remodelling and atrial fibrillation in acquired forms of cardiovascular disease.' *Heart Rhythm,* 1(2), pp. 147-159.

Jeevaratnam, K., Chadda, K.R., Huang, C.L., and Camm, A.J. (2018) 'Cardiac potassium channels: Physiological insights for targeted therapy.' *Journal of Cardiovascular Pharmacological Therapies,* 23(2), pp. 119-129.

Kanno, S., and Saffitz, J.E. (2001) 'The role of myocardial gap junctions in electrical conduction and arrhythmogenesis.' *Cardiovascular Pathology,* 10(4), pp. 169-177.

Kawase, Y., and Hajjar, R.J., (2008) ‘The cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase: A potent target for cardiovascular disease.’ *Nature Reviews Cardiology,* 5, pp. 554-565.

Keaver, L., Xu, B., Jaccard, A., and Webber, L. (2020) ‘Morbid obesity in the UK: A modelling projection study to 2035.’ *Scandinavian Journal of Public Health,* 48(4), pp. 422-427.

Kim, D.M., and Nimigean, C.M. (2016) ‘Voltage-gated potassium channels: A structural examination of selectivity and gating.’ *Cold Harbour SpringPerspective Biology,* 8(5), pp. 201-206.

Kirchhof, P., Kahr, P.C., Kaese, S., Piccini, I., Vokshi, I., Scheld, H-H., Rotering, H., Fortmeuller, L., Laakmann, S., Verheule, S., Schotten, U., Fabritz, L., and Brown, N.A. (2011) ‘Pitx2 is expressed in the adult left atrium, and reducing Pitx2 expression promotes atrial fibrillation inducibility and complex changes in gene expression.’ *Circulation: Cardiovascular Genetics,* 4(2), pp. 123-133.

Lajiness, J.D., and Conway, S.J. (2012) ‘The dynamic role of cardiac fibroblast in development and disease.’ *Journal of Cardiovascular Translational Research,* 5(6), pp. 739-748.

Laughner, J.I., Ng, F.S., Sulkin, M.S., Arthur, R.M., and Efimov, I.R. (2012) ‘Processing and analysis of cardiac optical mapping data obtained with potentiometric dyes.’ *American Journal of Physiology: Heart and Circulatory Physiology,* 303(7), pp. 753-765.

Lau, D.H., Nattel, S., Kalman, J.M., and Sanders, P. (2017) ‘Modifiable risk factors and atrial fibrillation.’ *Circulation,* 136(6), pp. 583-596.

Lavalle, C., Magnocavallo, M., Straito, M., Santini, L., Forleo, G.B., Grimaldi, M., Badagliacca, R., Lanata, L., and Ricci, R.P. (2021) 'Flecainide how and when: A practical guide in supraventricular arrythmias.' *Journal of Clinical Medicine,* 10(7).

Lee, D.C., Pate, R.R., Lavie, C.J., Sui, X., Church, T.S., Blair, S.N. (2014) 'Leisure-time running reduces all-cause and cardiovascular mortality risk.' *Journal of American College of Cardiology,* 64(5), pp. 472-481.

Lee, H.A., Hyun, S.A., Park, S.G., Kim, K.S., and Kim, S.J. (2016) 'Comparison of electrophysiological effects of calcium channels blockers on cardiac repolarisation.' *Korean Journal of Physiological Pharmacology,* 20(1), pp. 119-127.

Lee, P., Yan, P., Ewart, P., Kohl, P., Loew, L.M., and Bollensdorff, C. (2012) 'Simultaneous measurement and modulation of multiple physiological parameters in the isolated heart using optical techniques.' *European Journal of Physiology,* 464(4), pp. 403-414.

Lee, P., Quintanilla, J.G., Alfonso-Almazan, J.M., Galan-Arriola, C., Yan, P., Sanchez-Gonzalez, J., Perez-Castellano, N., Perez-Villacastin, J., Ibanez, B., Loew, L.M., and Filgueiras-Rama, D. (2019) 'In vivo ratiometric optical mapping enables high-resolution cardiac electrophysiology in pig models.' *Cardiovascular Research,* 115(11), pp. 1659-1671.

Lee, S-H., Tai, C-T., Hsieh, M-H., Tsao, H-M., Lin, Y-J., Chang, S-L., Huang, J-L., Lee, K-T., Chen, Y-J., Cheng, J-J., and Chen, S-A. (2005) 'Predictors of non-pulmonary vein ectopic beats initiating paroxysmal atrial fibrillation.' *Journal of the American College of Cardiology,* 46(6), pp. 1054-1059.

Leopoulou, M., Theofilis, P., Kordalis, A., Papageorgiou, N., Sagris, M., Oikonomou, E., and Tousoulis, D. (2023) 'Diabetes mellitus and atrial fibrillation – from pathophysiology to treatment.' *World Journal of Diabetes,* 14(5), pp. 512-527.

Lenis, G., Baas, T., Dossel, O. (2013) ‘Ectopic beats and their influence on the morphology of subsequent waves in the electrocardiogram.’ *Biomedical Engineering,* 58(2), pp. 109-119.

Limpitikul, W.B., and Das, S. (2023) ‘Obesity-related atrial fibrillation: Cardiac manifestation of a systemic disease.’ *Journal of Cardiovascular Development and Disease,* 10(8), pp. 323.

Lin, W-S., Prakash, V.S., Tai, C-T., Hsieh, M-H., Tsai, C-F., Yu, W-C., Lin, Y-K., Ding, Y-A., Chang, M-S., and Chen, S-A. (2000) ‘Pulmonary vein morphology in patients with paroxysmal atrial fibrillation initiated by ectopic beats originating from the pulmonary veins.’ *Circulation,* 101(11), pp. 1274-1281.

Lipovsky, C.E., Brumback, B.D., Khandekar, A., and Rentschler, S.L. (2018) ‘multi-scale assessments of cardiac electrophysiology reveal regional heterogeneity in health and disease.’ *Journal of Cardiovascular Development Disease,* 5(1), pp.201-208.

Lippi, G., Sanchis-Gomar, F., and Cervellin, G. (2021) ‘Global epidemiology of atrial fibrillation: An increasing epidemic and public health challenge.’ *Internation Journal of Stroke,* 16(2), pp. 217-221.

Liu, T., Xiong, F., Qi, X.Y., Xiao, J., Villeneuve, L., Abu-Taha, I., Dobrev, D., Huang, C., Nattel, S. (2020) ‘Altered calcium handling produces re-entry-promoting action potential alternans in atrial fibrillation-remodelled hearts.’ *JCI Insight,* 5(8), pp. 23-29.

Liu, G., Xue, X., Gao, C., Huang, J., Qi, D., Zhang, Y., Dong, J-Z., Ma, C-S., and Yan, G-X. (2017) ‘Synergistic effect of dofetilide and mexiletine on prevention of atrial fibrillation.’ *Journal of the American Heart Association,* 6(5), pp. 54-82.

Maier, L.S. (2009) ‘A novel mechanism for the treatment of angina, arrythmias and diastolic dysfunction: Inhibition if late I_{NA} using ranolazine.’ *Journal of Cardiovascular Pharmacology,* 54(4), pp. 279-286.

Manna, P., and Jain, S.K. (2015) ‘Obesity, oxidative stress, adipose tissue dysfunction and the associated health risks: Causes and therapeutic strategies.’ *Metabolic Syndrome Related Disorders,* 13(10), pp. 423-444.

Markides, V., and Schilling, R.J. (2003) ‘Atrial fibrillation: Classification, pathophysiology, mechanisms and drug treatment.’ *Heart,* 89(8), pp. 939-943.

Martin, T.G., and Leinwand, L.A. (2024) ‘Hearts apart: Sex differences in cardiac remodelling in health and disease.’ *Journal of Clinical Investigation,* 134(13), pp.40-47.

McCauley, M.D., Hong, L., Sridhar, A., Menon, A., Perike, S., Zhang, M., Da Silva, I.B., Yan, J., Bonini, M., Ai, X., Rehman, J., and Darbar, D. (2020) ‘Ion channel and structural remodelling in obesity- mediated atrial fibrillation.’ *Circulatory Arrythmia Electrophysiology,* 13(8), pp. 82-96.

Mikawa, T., and Hurtado, R. (2007) ‘Development of the cardiac conduction system.’ *Seminars in Cell and Developmental Biology,* 18(1), pp. 90-100.

Mullenbroich, M.C., Kelly, A., Acker, C., Bub, G., Bruegmann, T., Di Bona, A., Entcheva, C., Kohl, P., Lenhart, S.E., Mongillo, M., Parmeggiani, C., Richter, C., Sasse, P., Zaglia, T., Sacconi, L., and Smith, G.L. (2021) ‘Novel optics-based approaches for cardiac electrophysiology: A review.’ *Frontier in Physiology,* 12, pp. 769-786.

Nowotny, K., Jung, T., Hohn, A., Weber, D., and Grune, T. (2015) ‘Advanced glycation end products and oxidative stress in type 2 diabetes mellitus.’ *Biomolecules,* 5(1), pp. 194-222.

Nuzhat, A., and Yong, Z. (2020) ‘Early detection of atrial fibrillation based on ECG signals.’ *Bioengineering,* 7(1), pp. 16.

Oberhauser, V., Schwertfeger, E., Rutz, T., Beyersdorf, F., Rump, L.C. (2001) ‘Acetylcholine release in human heart atrium.’ *Circulation,* 103(12), pp. 1638-1643.

Okumura, Y., Watanabe, I., Nagashima, K., Sonoda, K., Sasaki, N., Kogawa, R., Takahasi, K., Iso, K., Ohkubo, K., Nakai, T., Takahashi, R., Taniguchi, Y., Mitsumata, M., Nikaido, M., and Hirayama, A. (2015) ‘Effects of a high-fat diet on the electrical properties of porcine atria.’ *Journal of Arrythmia,* 31(6), pp. 352-358.

Ottolia, M., John, S., Hazan, A., and Goldhaber, J.J. (2021) ‘The cardiac Na^+ and Ca^{2+} exchanger: From structure to function.’ *Computer Physiology,* 12(1), pp. 2681-2717.

O’Shea, C., Holmes, A.P., Yu, T.Y., Winter, J., Wells, S.P., Correia, J., Boukens, B.J., De Groot, J.R., Chu, G.S., Li, X., Ng, A., Kirchhof, P., Fabritz, L., Rajpoot, K., and Pavlovic, D. (2019) ‘Electromap: High- throughput open-source software for analysis and mapping of cardiac electrophysiology.’ *Scientific Reports,* 9(1389).

O’Shea, C., Kabir, S.N., Holmes, A.P., Lei, M., Fabritz, L., Rajpoot, K., and Pavlovic, D. (2020) ‘Cardiac optical mapping – State of the art and future challenges.’ *The International Journal of Biochemistry and Cell Biology,* 126, pp. 105.

Ouwens, D.M., Sell, H., Greulich, S., and Eckel, J. (2010) ‘The role of epicardial and perivascular adipose tissue in the pathophysiology of cardiovascular disease.’ *Journal of Cellular and Molecular Medicine,* 14(9), pp. 2223-2234.

Patterson, E., Lazzara, R., Szabo, B., Liu, H., Tang, D., Li, Y.H., Scherlag, B.J., and Po, S.S. (2006) ‘Sodium-calcium exchange initiated by ca2^{+} transient: An arrythmia trigger within pulmonary veins.’ *Journal of American College of Cardiology,* 47(6), pp. 1196-1206.

Pellman, J., and Sheikh, F. (2001) ‘Atrial fibrillation: Mechanisms, therapeutics and future directions.’ *Computer Physiology,* 5(2), pp. 649-665.

Pellman, J., Zhang, J., and Sheikh, F. (2016) ‘Myocyte-fibroblast communication in cardiac fibrosis and arrythmias: Mechanisms and model systems.’ *Journal of Molecular and Cellular Cardiology,* 94, pp. 22-31.

Polontchouk, L., Haefliger, J-A., Elbelt, B., Schaefer, T., Stuhlmann, D., Mehlhorn, U., Kuhn-Regnier, F., De Vivie, E.R., and Dhein, S. (2001) ‘Effects of chronic atrial fibrillation on gap junction distribution in human and rat atria.’ *Journal of American College of Cardiology,* 38(3), pp. 883-891.

Rafanelli, M., Walsh, K., Hamdan, M.H., Buyan-Dent, L. (2017) ‘Autonomic dysfunction: Diagnosis and management.’ *Handbook of Clinical Neurology,* 167, pp. 123-137.

Ramos-Mondragon, R., Galindo, C.A., and Avila, G. (2008) ‘Role of TGF-beta on cardiac structural and electrical remodelling.’ *Vascular Health Risk Management,* 4(6), pp. 1289-1300.

Ramos-Mondragon, R., Lozhkin, A., Vendrov, A.E., Runge, M.S., Isom, L.L., Madamanchi, N.R. (2023) ‘NADPH oxidases and oxidative stress in the pathogenesis of atrial fibrillation.’ *Antioxidants,* 12(10), pp. 20-23.

Redwood, C., and Robinson, P. (2013) ‘Alpha-tropomyosin mutations in inherited cardiomyopathies.’ *Journal of Muscle Research and Cell Motility,* 34, pp. 285-294.

Reilly, L., and Eckhardt, L.L. (2021) ‘Cardiac potassium inward rectifier Kir2: Review of structure, regulation, pharmacology and arrhythmogenesis.’ *Heart Rhythm,* 18(8), pp. 1423 – 1434.

Rennison, J.H., Li, L., Lin, C.R., Lovano, B.S., Castel, L., Wass, S.Y., Cantale, C.C., McHale, M., Gillinov, A.M., Mehra, R., Willard, B.B., Smith, J.D., Chung, M.K., Barnard, J., and Van Wagoner, D.R. (2021) ‘Atrial fibrillation rhythm is associated with marked changes in metabolic and myofibrillar protein expression in left atrial appendage.’ *World Journal of Physiology,* 473(3), pp. 461-475.

Rivaud, M.R., Delmar, M., and Remme, C.A. (2020) ‘Heritable arrythmia syndromes associated with abnormal cardiac sodium channel function: Ionic and non-ionic mechanisms.’ *Cardiovascular Research,* 116(9), pp.1557 – 1570.

Rodriquez-Sinovas, A., Sanchez, J.A., Valls-Lacalle, L., Consegal, M., and Ferreira-Gonzalez, I. (2021) ‘Connexins in the heart: Regulation, function and involvement in cardiac disease.’ *International Journal of Molecular Sciences,* 22(9), pp. 202-210.

Sachs, F. (2010) ‘Stretch-activated channels: What are they?’ *Physiology,* 25(1), pp. 50-56.

Sacks, H.S., and Fain,J.N. (2007) ‘Human epicardial adipose tissue: A review.’ *American Heart Journal,* 153(6), pp. 907-917.

Schwinger, R.H.G., Bundgaard, H., Muller-Ehmsen, J., and Kjeldsen, K. (2003) ‘The Na, K-ATPase in the failing human heart.’ *Cardiovascular Research,* 57(4), pp. 913-920.

Scott, K.A., Melhorn, S.J., and Sakai, R.R. (2012) ‘Effects of chronic social stress on obesity.’ *Current Obesity Reports,* 1(1), pp. 16-25.

Sha, R., Baines, O., Hayes, A., Tompkins, K., Kalla, M., Holmes, A.P., O’Shea, C., and Pavlovic, D. (2024) ‘Impact of obesity on atrial fibrillation pathogenesis and treatment options.’ *Journal of American Heart Association,* 13(1), pp. 322.

Sha, R., Han, W., Lin, M., and Zhong, J. (2021) ‘Is epicardial adipose tissue associated with atrial fibrillation following cardiac surgery? A systematic review and meta-analysis.’ *The Heart Surgery Forum,* 24(5), pp. 801-807.

Shah, K., Seeley, S., Schulz, C., Fisher, J., and Gururaja, R.S. (2022) ‘Calcium channels in the heart: Disease states and drugs.’ *Cells,* 11(6).

Sheng, X., Scherlag, B.J., Yu, L., Li, S., Ali, R., Zhang, Y., Fu, G., Nakagawa, H., Jackman, W.M., Lazzara, R., and Po, S.S. (2011) ‘Prevention and reversal of atrial fibrillation inducibility and autonomic remodelling by low-level vagosympathetic nerve stimulation.’ *Journal of American College of Cardiology,* 57(5), pp. 563-571.

Shu, J., Cheng, J., Li, N., Zhang, Z., Nie, J., Peng, Y., Wang, Y., Wang, D-W., and Zhou, N. (2023) ‘Obesity and atrial fibrillation: A narrative review from arrhythmogenic mechanisms to clinical significance.’ *Cardiovascular Diabetology,* 22(1), pp. 192.

Sicouri, S., Glass, A., Ferreiro, M., and Antzelevitch, C. (2010) ‘Transseptal dispersion of repolarization and its role in the development of torsade de pointes arrythmias.’ *Journal of Cardiovascular Electrophysiology,* 21(4), pp. 441-447.

Singh, S., Zoble, R.G., Yellen, L., Brodsky, M.A., Feld, G.K., Berk, M., and Billing, C.B. (2000) ‘Efficacy and safety of oral dofetilide in converting to and maintaining sinus

rhythm in patients with chronic atrial fibrillation or atrial flutter.' *Circulation,* 102(19), pp. 2385-2390.

Song, Y., Tan, Y., Deng, M., Shan, W., Zheng, W., Zhang, C., Cui, J., Feng, K., Shi, L., Zhang, M., Liu, Y., Sun, Y., and Yi, W. (2023) 'Epicardial adipose tissue, metabolic disorders and cardiovascular diseases: Recent advances classified by research methodologies.' *Medical Communications,* 4(6), pp. 413-424.

Song, Z., Ko, C.Y., Nivala, M., Weiss, J.N., and Qu, Z. (2015) 'Calcium-voltage coupling in the genesis of early and delayed afterdepolarisations in cardiac myoctes.' *Biophysics Journal,* 108(8), pp. 1908-1921.

Sovari, A.A. (2016) 'Cellular and molecular mechanisms of arrythmia by oxidative stress.' *Cardiology Research and Practice,* 1, pp. 965-978.

Staerk, L., Sherer, J.A., Ko, D., Benjamin, E.J., and Helm, R.H. (2017) 'Atrial fibrillation: Epidemiology, pathophysiology, and clinical outcomes.' *Circulation Research,* 120(9), pp. 1501-1517.

Suffee, N., Baptista, E., Piquereau, J., Ponnaiah, M., Doisne, N., Ichou, F., Lhomme, M., Pichard, C., Galand, V., Mougenot, N., Dilanian, G., Lucats, L., Balse, E., Mericksay, M., Le Goff, W., and Hatem, S.N. (2021) 'Impacts of high-fat diet on the metabolic profile and the phenotype of atrial myocardium in mice.' *Cardiovascular Research,* 118(15), pp. 3126-3139.

Sun, Y., Tong, X., Chen. H., Huang, T., Shao, Q., Huang, W., Laird, D.W., and Bai, D. (2014) 'An atrial-fibrillation-linked connexin40 mutant is retained in the endoplasmic reticulum and impairs the function of atrial gap-junction channels.' *Disease Models and Mechanisms,* 7(5), pp. 561-569.

Syeda, F., Kirchhof, P., and Fabritz, L., (2017) ‘Pitx2-dependent gene regulation in atrial fibrillation and rhythm control.’ *The Journal of Physiology,* 595(12), pp. 4019-4026.

Syeda, F., Holmes, A.P., Yu, T.Y., Tull, S., Kuhlmann, S.M., Pavlovic, D., Betney, D., Riley, G., Kucera, J.P., Jousset, F., De Groot, J.R., Rohr, S., Brown, N.A., Fabritz, L., and Kirchhof, P. (2016) ‘Pitx2 modulates atrial membrane potential and the antiarrhythmic effect of sodium channel blockers.’ *Journal of the American College of Cardiology,* 68(17), pp. 1881-1894.

Tao, Y., Zhang, M., Li, L., Bai, Y., Zhou, Y., Moon, A.M., Kaminski, H.J., and Martin, J.F. (2014) ‘Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes.’ *Circulation Cardiovascular Genetics,* 7(1), pp. 23-32.

Tarifa, C., Serra, S.A., Herraiz-Martinez, A., Lozano-Velasco, E., Benitez, R., Aranega, A., Franco, D., and Hove-Madsen, L. (2023) ‘Pitx2 deficiency confers cellular electrophysiological hallmarks of atrial fibrillation to isolated atrial myocytes.’ *Biomedicine and Pharmacotherapy,* 162, pp. 114-117.

Thijssen, V., Ausma, J., and Borgers, M. (2001) ‘Structural remodelling during chronic atrial fibrillation: Act of programmed cell survival.’ *Cardiovascular Research,* 52(1), pp. 14-24.

Tomasek, J.J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R.A. (2003) ‘Myofibroblasts and mechano-regulation of connective tissue remodelling.’ *Nature Reviews Molecular Cell Biology,* 3(5), pp. 349-363.

Tran, T.Q., and Kioussi, C. (2021) ‘Pitx genes in development and disease.’ *Cellular and Molecular Life Sciences,* 78(11), pp. 4921-4938.

Varro, A., Tomek, J., Nagy, N., Virag, L., Passini, E., Rodriguez, B., and Baczko, I. (2021) ‘Cardiac transmembrane ion channels and action potentials: Cellular physiology and arrhythmogenic behaviour.’ *Physiological Reviews,* 101(3), pp. 1083-1176.

Tsai, C-T., Lai, L-P., Hwang, J-J., Lin, J-L., and Chiang, F-T. (2008) ‘Molecular genetics of atrial fibrillation.’ *Journal of the American College of Cardiology,* 52(4), pp. 241-250.

Veeraraghavan, R., Gourdie, R.G., and Poelzing, S. (2014) ‘Mechanisms of cardiac conduction: A history of revisions.’ *American Journal of Physiology Heart and Circulatory Physiology,* 306(5), pp. 619-627.

Viskin, S., Fish, R., Zeltser, D., Belhassen, B., Heller, K., Brosh, D., Laniado, S., Barron, H.V. (2000) ‘Arrythmias in the congenital long QT syndrome: How often is torsades de pointes pause dependent?’ *Heart,* 83(6), pp. 661-666.

Voigt, N., and Dobrev, D. (2012) ‘Cellular and molecular correlates of ectopic activity in patients with atrial fibrillation.’ *Electrophysiology Europace,* 14(5), pp. 97-105.

Vonck, K.E.J., and Larsen, L.E. (2018) ‘*Chapter 18- Vagus nerve stimulation: Mechanism of action.’* Belgium: Academic Press

Waktare, J.E., Hnatkova, K., Sopher, S.M., Murgatroyd, F.D., Guo, X., Camm, A.J., and Malik, M. (2001) ‘The role of ectopics in initiating paroxysmal atrial fibrillation.’ *European Heart Journal,* 22(4), pp. 333-339.

Weber, K.T., Sun, Y., and Diez, J. (2008) ‘Fibrosis.’ *Journal of the American College of Cardiology,* 52(24), pp. 2029-2031.

Wei, X., Yohannan, S., and Richards, J.R. (2023) ‘*Physiology, cardiac repolarisation, dispersion and reserve.*’ Treasure Island: Stats Pearls.

Weiss, J.N., Qu, Z., and Shivkumar, K. (2017) ‘Electrophysiology of hypokalaemia and hyperkalaemia’. *Circulatory Arrhythmic Electrophysiology,* 10(3), pp. 46-67.

Woo, J., Koziol-White, C., Panettieri, R.J., and Jude, J. (2021) ‘TGF-B: The missing link in obesity-associated airway diseases?’ *Current Research in Pharmacological Drug Discovery,* 2, pp. 100-116.

Wong, C., Sun, M.T., Odutayo, A., Emdin, C., Mahajan, R., Lau, D., Pathak, R., Wong, D., Selvanayagam, J., Sanders, P., and Clarke, R. (2016) ‘Associations of epicardial, abdominal, and overall adiposity with atrial fibrillation.’ *Circulation: Arrythmia and electrophysiology,* 9, pp. 43-78.

Woodcock, E.A., and Matkovich, S.J. (2005) ‘Cardiomyocytes structure, function and associated pathologies.’ *The International Journal of Biochemistry and Cell Biology,* 37(9), pp. 1746-1751.

Wyndham, C. (2000) ‘Atrial fibrillation: The most common arrythmia.’ *Texas Heart Institute Journal,* 27(3), pp. 257-267.

Wynn, T.A. (2007) ‘Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases.’ *Journal of Clinical Investigations,* 117(3), pp. 524-529.

Yamazaki, M., Filgueiras-Rama, D., Berenfeld, O., and Kalifa, J. (2012) ‘Ectopic and re-entrant activation patterns in the posterior left atrium during stretch-related atrial fibrillation.’ *Progress in Biophysics and Molecular Biology,* 110(2-3), pp. 269-277.

Zhan, X., Huang, Y., and Long, Y. (2018) ‘Two-dimensional gel electrophoresis coupled with mass spectrometry methods for an analysis of human pituitary adenoma tissue proteome.’ *Journal of Visualised Experiments,* 134, pp. 201-210.

Zhang, F., Hartnett, S., Sample, A., Schnack, S., and Li, Y. (2016) ‘High fat diet induced alterations of atrial electrical activities in mice.’ *American Journal of Cardiovascular Disease,* 6(1), pp. 1-9.