Inflammation of the Heart in Heart Disease

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Medical and Human Sciences

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ABSTRACT

The University of Manchester
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PhD in Cardiovascular Medicine

Inflammation of the Heart in Heart Disease

Heart failure patients have dysfunction of the cardiac conduction system that contributes to a high burden of arrhythmias including atrial fibrillation and sudden cardiac death. Heart failure has been associated with the inflammatory response, but it is unknown if inflammation is playing a role in the remodelling of the cardiac conduction system in heart failure. Inflammation has been shown to be present in the myocardium from failing hearts and it is known to have detrimental effects on cardiac function, inducing fibrosis, remodelling of ion channels and even arrhythmias. However, the effect of inflammation on the cardiac conduction system has not been investigated. The aims of this study were to determine if there is an increase of pro-inflammatory cytokines and inflammatory cells in the cardiac conduction system in heart failure. In addition, to identify if there is possible inflammation-associated fibrosis and apoptosis in the cardiac conduction system in heart failure.

To test these aims, three models of heart failure were used: a rat model of pulmonary arterial hypertension, a rabbit model of congestive heart failure and a rat model of myocardial infarction. In the rat model of pulmonary arterial hypertension there was a bradycardia, a prolongation of the QT interval, and an increase in the atrioventricular and ventricular refractory periods, suggesting electrical remodelling in these animals. The rats with pulmonary arterial hypertension displayed an increase in pro-inflammatory cytokines such as interleukins 1β and TGFβ in the right side of the heart, including the sinoatrial node and right Purkinje fibres of the cardiac conduction system. In addition, in these areas, there was an increase in components of the extracellular matrix, including fibronectin, collagen I and vimentin. Histology revealed regions of non-myocyte nuclei, only in the right ventricle of the rats with pulmonary arterial hypertension. Immunohistochemistry demonstrated patches of CD68 and vimentin expression (markers for macrophages and fibroblasts, respectively) in the right side of the heart in these animals. TUNEL staining also revealed an increase in apoptosis in the right side of the heart.

In the rabbit model of congestive heart failure, the region most affected by inflammation was the right atrium, while few changes were measured in the ventricles or cardiac conduction system. Although these results are surprising, it is suggested that the atria could be more sensitive to the physical stretch produced in this model. In the rat model of myocardial infarction, there were regions of non-myocyte nuclei in the border zone. This region also had increases in pro-inflammatory and fibrosis markers.

In conclusion, this work has presented the novel finding that there can be inflammation in the cardiac conduction system in heart failure. This could be contributing to the arrhythmias seen in heart failure patients. This could possibly lead the way to anti-inflammatories as a possible novel therapeutic for heart failure patients.
DECLARATION

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACTB</td>
<td>Actin, beta</td>
</tr>
<tr>
<td>AERP</td>
<td>Atrial effective refractory period</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>Ao</td>
<td>Aorta</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>APD</td>
<td>Action potential duration</td>
</tr>
<tr>
<td>AT</td>
<td>Atrial tachycardia</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin type II receptor 1</td>
</tr>
<tr>
<td>ATII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ATP5B</td>
<td>Adenosine triphosphate 5B</td>
</tr>
<tr>
<td>AVERP</td>
<td>Atrioventricular effective refractory period</td>
</tr>
<tr>
<td>AVN</td>
<td>Atrioventricular node</td>
</tr>
<tr>
<td>BBB</td>
<td>Bundle branch block</td>
</tr>
<tr>
<td>CaV1.2</td>
<td>Long lasting type Ca(^{2+}) channels</td>
</tr>
<tr>
<td>CCS</td>
<td>Cardiac conduction system</td>
</tr>
<tr>
<td>CFB</td>
<td>Central fibrous body</td>
</tr>
<tr>
<td>Col1α2</td>
<td>Collagen type I α 2</td>
</tr>
<tr>
<td>Col3α1</td>
<td>Collagen type III α 1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
<td>Coronary sinus</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
</tr>
<tr>
<td>d</td>
<td>Diastole</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERP</td>
<td>Effective refractory period</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FO</td>
<td>Fossa ovalis</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCN4</td>
<td>Hyperpolarisation-activation cyclic nucleotide-gated channel</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>I(_f)</td>
<td>Funny current</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$I_{K,s}$</td>
<td>Slow delayed rectifier current</td>
</tr>
<tr>
<td>$I_{K,r}$</td>
<td>Rapid delayed rectifier current</td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IPAH</td>
<td>Idiopathic pulmonary arterial hypertension</td>
</tr>
<tr>
<td>$I_{to}$</td>
<td>Transient outward $K^+$ current</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>Kir2</td>
<td>Inward rectifier $K^+$ channel</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
</tr>
<tr>
<td>LBBB</td>
<td>Left bundle branch block</td>
</tr>
<tr>
<td>LPF</td>
<td>Left Purkinje fibres</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVAW</td>
<td>Left ventricular anterior wall</td>
</tr>
<tr>
<td>LVID</td>
<td>Left ventricular internal diameter</td>
</tr>
<tr>
<td>LVPW</td>
<td>Left ventricular posterior wall</td>
</tr>
<tr>
<td>LW</td>
<td>Lung weight</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MMP2</td>
<td>Metalloproteinase 2</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Na\textsubscript{v}1.5</td>
<td>Fast Na$^+$ channel</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PA</td>
<td>Pulmonary artery</td>
</tr>
<tr>
<td>PAAT</td>
<td>Pulmonary arterial acceleration time</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulmonary arterial deceleration</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAV</td>
<td>Pulmonary artery velocity</td>
</tr>
<tr>
<td>PAVmax</td>
<td>Maximum pulmonary artery velocity</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PV</td>
<td>Pulmonary vein</td>
</tr>
<tr>
<td>QTc</td>
<td>Corrected QT interval</td>
</tr>
<tr>
<td>RA</td>
<td>Right atrium</td>
</tr>
<tr>
<td>RBBB</td>
<td>Right bundle branch block</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPF</td>
<td>Right Purkinje fibres</td>
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<tr>
<td>RPL13A</td>
<td>60S ribosomal protein L13a</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>RV</td>
<td>Right ventricle</td>
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<tr>
<td>RVEDV</td>
<td>Right ventricular end diastolic volume</td>
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<td>RVID</td>
<td>Right ventricular internal diameter</td>
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<td>RVWT</td>
<td>Right ventricular wall thickness</td>
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<td>RVSV</td>
<td>Right ventricular systolic volume</td>
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<td>RyR</td>
<td>Ryanodine receptor</td>
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<td>s</td>
<td>Systole</td>
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<td>SAN</td>
<td>Sinoatrial node</td>
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<tr>
<td>SERCA2a</td>
<td>Sarcoplasmic reticulum Ca$^{2+}$-ATPase</td>
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<td>Smad2/3</td>
<td>Mothers against decapentaplegic homolog 2/3</td>
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<td>SNRT</td>
<td>Sinoatrial node recovery time</td>
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<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<td>SVC</td>
<td>Superior vena cava</td>
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<tr>
<td>TIMPs 1-4</td>
<td>Tissue inhibitor of metalloproteinases 1-4</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<td>TNFα</td>
<td>Tumour necrosis factor α</td>
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<td>TT</td>
<td>Transitional tissue</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
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<td>TV</td>
<td>Tricuspid valve</td>
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<td>UBC</td>
<td>Ubiquitin C</td>
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<tr>
<td>VERP</td>
<td>Ventricular effective refractory period</td>
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<td>VF</td>
<td>Ventricular fibrillation</td>
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<td>Vim</td>
<td>Vimentin</td>
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<tr>
<td>VT</td>
<td>Ventricular tachycardia</td>
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CHAPTER 1: INTRODUCTION

Inflammation has been associated with severity and progression of heart failure. In heart failure there is dysfunction of the cardiac conduction system, which can lead to disruption of the action potential conduction through the heart and predispose to arrhythmias. However, it is not known if there is inflammation of the cardiac conduction system in heart failure and whether it could be playing a role in the remodelling and dysfunction. The aim of this study is to investigate if inflammation, and associated fibrosis and apoptosis, is occurring in the cardiac conduction system in heart failure.

1.1 THE CARDIAC CONDUCTION SYSTEM

The cardiac conduction system (CCS) can be referred to as the electrical wiring of the heart. The CCS is responsible for the initiation and propagation of the action potential through the heart resulting in synchronised contraction.

1.1.1 Action Potential

To understand the function and electrophysiology of the CCS, an understanding of the action potential is necessary.

1.1.1.1 Action Potential of Working Myocytes

The heart is predominately made up of the working myocardium, the atrial and ventricular muscle. Under normal conditions, the cells of the working myocardium are at rest and require a stimulus to contract. This stimulus is usually the arrival of an action potential from a neighbouring cell. Put simply, the action potential is a voltage change that occurs across the cell membrane as the cell progresses from its resting state to its activated state and then back to its resting state (Figure 1.1). The action potential is made up of 5 phases 0 to 4, and the voltage changes are the result of the movement of electrically charged ions across the cell membrane. The non-spontaneous nature of working myocytes is primarily due to the resting membrane potential, which holds the cells in a polarized state at approximately -85 mV. This is known as diastole or phase 4. The ionic currents mainly responsible for phase 4 are outward potassium currents, mainly the confusingly named inward rectifier K+ current, \( I_{K,1} \), carried by Kir2 channels. Upon activation, the Kir2 channels close and fast sodium channels (\( Na_\text{v}1.5 \)) open, allowing a large and rapid movement of positively charged sodium ions (\( Na^+ \)) into the cell. This results in the membrane potential of the cell changing rapidly from \( \sim -85 \text{ mV} \) to \( \sim +30 \text{ mV} \), which is known as the depolarization phase, or phase 0. This is the upstroke of the action potential which is very rapid in working myocytes, approximately \( >100 \text{ V/s} \). The cell remains in this
positive state before repolarizing rapidly (phase 1). The positive membrane potential inactivates Na_v1.5 and activates transient outward potassium current (I_{to}). The efflux of potassium ions (K^+) causes the cell to repolarize. Long lasting (L-) type calcium channels (Ca_v1.2) are activated, providing an influx of positively charged calcium ions (Ca^{2+}). This influx, together with the efflux of K^+, contributes to the plateau period, phase 2. The influx of Ca^{2+} initiates contraction of the cell via a process known as calcium-induced calcium release (see below). The plateau allows for a period of sustained contraction. This process completes with inactivation of Ca_v1.2 channels while the potassium channels remain active. The continued efflux of K^+, via slow and rapid delayed rectifier channels (I_{K,s} and I_{K,r}, respectively) further repolarizes the membrane, phase 3, until the membrane potential reaches the resting membrane potential of \(~85\) mV. The resting membrane potential is held at this value by I_{K,1} until the next stimulation.

Figure 1.1 Illustration of the different action potential profiles of cells from the working myocardium (atria and ventricles) and the cardiac conduction system (SAN, AVN, His-Purkinje system). Abbreviations: atroventricular node, AV node; left atrium, LA; left ventricle, LV; right atrium, RA; right ventricle, RV; and sinoatrial node, SAN. Taken from Schram et al. (2002).

Calcium-Induced Calcium Release
Contraction in cardiac myocytes is due to a process called calcium-induced calcium release, or excitation-contraction coupling (Figure 1.2).¹ Depolarization of the cell membrane activates opening of the voltage-sensitive L-type Ca^{2+} channels (e.g. Ca_v1.2) that results in an influx of Ca^{2+} into the cell. The Ca^{2+} activates ryanodine receptors
(RyRs) on the sarcoplasmic reticulum (SR) membrane. The SR is the Ca\(^{2+}\) store in the cell cytoplasm. Activation of RyR by Ca\(^{2+}\) causes a much larger release of Ca\(^{2+}\) from the SR, i.e. calcium-induced calcium release. The released Ca\(^{2+}\) binds to and activates troponin C on the cardiac myofilaments and induces mechanical cardiac contraction. During relaxation, the calcium returns to its internal store, the SR, via SR Ca\(^{2+}\)-ATPase (SERCA2a) ready for the next contraction, and some is also extruded from the cytoplasm by the sodium-calcium exchanger (NCX). SERCA2a activity is controlled by the protein, phospholamban.

**Figure 1.2 Schematic diagram illustrating calcium-induced calcium release (excitation-contraction coupling) in cardiac myocytes.** Abbreviations: action potential, AP; adenosine triphosphate, ATP; calcium, Ca; hydrogen, H; sodium, Na; potassium, K; phospholamban, PLB; sodium-calcium exchanger, NCX; ryanodine receptor, RyR; sarcoplasmic reticulum, SR. Taken from Bers (2002).

### 1.1.1.2 Action Potential of Nodal Cells

The CCS is made up of specialised cells, which, unlike working myocytes, do not require a stimulus to trigger an action potential. The cells of the CCS have the ability to produce spontaneous action potentials, i.e. pacemaker activity. The pacemaking potential of the cells is due to their differing ion channel expression from working myocytes, which results in an altered action potential profile (Figure 1.1).\(^{2-5}\) In the case of the nodal cells making up the SA and AV nodes there is no resting potential due to an absence, or very low expression, of Kir2 channels. Instead, during diastole, the nodal cells have a more depolarized, or more positive, membrane potential of approximately -60 mV. The lack of
I_{K,1} and presence of a funny current (I_{f}) produces a pacemaker potential, which causes the nodal cell membrane potential to slowly depolarize and become more positive. I_{f} is an inward current carried by a mixture of Na\(^+\) and K\(^+\), which is unique to the CCS. It is this pacemaker potential that is responsible for the pacemaking properties of the nodal cells. The membrane potential continues to depolarize until it reaches the threshold potential at approximately -40 mV. At the threshold potential, a spontaneous action potential is triggered. Phase 0, the depolarizing upstroke of the action potential, is generated by activated L-type Ca\(^{2+}\) channels (Ca\(_{v}\)1.3 as well as Ca\(_{v}\)1.2 in the nodal cells), as they do not contain Na\(_{v}\)1.5 channels. However, the current produced by Ca\(_{v}\)1 channels is much slower and smaller than that generated by Na\(_{v}\)1.5 channels, and as a result, the depolarizing upstroke is much slower than in the working myocardium. The upstroke velocity is only ~10 V/s in nodal cells, compared to a rapid 100 V/s in working myocytes.\(^6\) Towards the end of phase 0, when the membrane potential reaches a positive value, there is inactivation of the Ca\(_{v}\)1 channels and activation of I_{K,r} and I_{K,s} channels. Unlike working myocytes, nodal cells usually do not have an initial sharp repolarization, phase 1, but instead a prolonged plateau, phase 2. The membrane slowly repolarizes and gives rise to a more prolonged action potential than that of the neighbouring atrial myocytes.

### 1.1.2 Anatomy and Function of the CCS

The CCS is primarily made up of the sinoatrial node (SAN), the atrioventricular node (AVN) and the His-Purkinje system that work together to ensure efficient initiation and conduction of the action potential in the heart.

#### 1.1.2.1 The Sinoatrial Node

One of the main components of the CCS is the SAN. The SAN is the primary pacemaker of the heart and is responsible for the initiation of the action potential. This action potential propagates throughout the heart stimulating contraction of the myocytes. The SAN is a long, thin structure located in the right atrium, beside the crista terminalis and between the superior and inferior vena cava (Figure 1.3),\(^7\) but its exact location and distribution varies slightly in different species.\(^8\),\(^9\) In humans, its size and morphology is found to vary among individuals, highlighting that this is indeed a complex and intricate structure.\(^10\) The SAN is supplied by a relatively large artery and is made up of small, specialised nodal cells positioned within a connective tissue matrix. The SAN is found not to be an isolated structure; it appears to have protrusions into the adjacent atrial muscle.\(^10\)
1.1.2.2 The Atrioventricular Node

The action potential is initiated in the SAN from where it propagates throughout the atria, inducing atrial contraction. A fibrous sheet separates the atria and ventricles and prevents the action potential from propagating from the atria. Under normal conditions, the only pathway for the action potential to travel into the ventricles is via the atrioventricular node (AVN). Similar to the SAN, the AVN is a complex tissue, the structure and orientation of which can vary in different species. The AVN is situated in the triangle of Koch, which is made up of the septal leaflet of the tricuspid valve, the coronary sinus and the tendon of Todaro (Figure 1.4).4 The action potential can propagate along either a fast or slow pathway into the penetrating bundle (Figure 1.4).11, 12 The fast pathway runs from the interatrial septum to the compact node via the transitional zone. On the other hand, the slow pathway runs from the coronary sinus, along the inferior nodal extension to the compact node. From the compact node, the action potential propagates along the penetrating bundle into the ventricles.
The primary function of the AVN is to put a short pause in the conduction of the action potential from the atria to the ventricles. By doing this, the AVN provides a delay between atrial and ventricular contraction. This delay allows for atrial depolarization and contraction, ensuring complete ventricular filling before ventricular contraction.

In addition to providing a conduction pause between the atria and ventricles, the AVN can also act as a back up pacemaker. It has the ability to take over pacemaking and control heart rate if the SAN fails or its rate falls precariously low. In addition, the AVN has the ability to protect the ventricles from excess stimulation during atrial tachycardia. It acts as a buffer, by reducing the frequency of action potentials transmitted into the ventricles and thus preventing ventricular tachycardia. The AVN does this by having a relatively long refractory period. The refractory period is the time when a tissue cannot be stimulated by an action potential; it is inexcitable.

1.1.2.3 The His-Purkinje System
Following propagation through the atria and AVN, the action potential conducts along the next part of the CCS, the His-Purkinje system. The His-Purkinje system is comprised of the bundle of His and Purkinje fibres, both named after their respective discoverers in the 1800’s. The His bundle is also known as the penetrating bundle and is the first part of the conduction pathway of the action potential into the ventricular myocardium. From here, the conduction pathway forks, and the action potential propagates along the left and right bundle branches, which excite the left and right ventricles, respectively. The action potential spreads along the fast conducting Purkinje fibre network and is responsible for
efficient and synchronous excitation, and thereby contraction, of the ventricles. The fibres are complex and have many branches from their source at the bundle branch (Figure 1.5).\textsuperscript{14} To efficiently reach the entire ventricular myocardium, the Purkinje fibres run freely along the endocardium wall as well as penetrating deeper into the sub-endocardium.

\textbf{Figure 1.5 Anatomy of Purkinje fibres in the rabbit left ventricle.} Immunohistochemically labelled neurofilament to identify Purkinje fibres, in brown (A). Location of the different segments of the His-Purkinje system (B). The left bundle branch is highlighted in red, with the branching Purkinje fibres in green. The terminal Purkinje fibres are in blue. Taken from Atkinson \textit{et al.} (2011).
1.2 GAP JUNCTIONS

Gap junctions are responsible for effective electrical coupling between myocytes. Gap junctions are made up of connexins. Gap junctions are non-selective ion channels that allow the movement of small ions and metabolites from the cytoplasm of one cell to the cytoplasm of a neighbouring cell. This transfer of ions is essential for efficient action potential propagation. Each gap junction is made up of 2 connexons, one from each neighbouring cell, and each connexon is made up of 6 connexin proteins. The main connexins in the heart are connexins (Cx) 40, 43 and 45. The connexins are numbered according to their molecular mass and, although they all have similar structures, have slightly different properties. Cx43 is the most abundantly expressed connexin in the working myocardium. It has a relatively large conductance and, therefore, facilitates fast propagation of the action potential through the working myocardium. Cx40 is expressed in the atria, AVN and also the His-Purkinje system. Cx45 is the most abundantly expressed connexin in the nodal tissue. It does not conduct as fast as Cx43 and contributes to the slower propagation of the action potential through the nodal tissues.

1.3 ELECTROCARDIOGRAPHY

Electrocardiography (ECG) is a non-invasive way to record the electrical activity of the heart. It is a useful way to measure the functioning of the CCS. In a single heartbeat there are five main waves that make up the ECG trace (Figure 1.6), P to T. The first wave of the ECG, the P wave, is a result of atrial depolarization and corresponds to atrial contraction following action potential propagation from the SAN through the atria. Under normal conditions, this is a relatively small, positive wave. Following the P wave is the QRS complex, which begins with a small, initial deflection (Q) followed by a large sharp R wave. The QRS complex represents the large and rapid ventricular depolarization and corresponds to the start of ventricular contraction. This is a result of the action potential conducting from the AVN and along the His-Purkinje system. The end of the R wave is followed by a small, negative deflection (S). Finally, the ECG trace is completed by a positive T wave, which corresponds to repolarization and relaxation of the ventricles.
The shape of the ECG trace can give indications of the action potential initiation and conduction through the heart. For example, the R-R interval between 2 adjacent traces is an indication of the cycle length, or heart rate, and therefore a measure of SAN firing rate. Intervals between the different points on the ECG give the time of conduction from one point on the heart to another. The P-R interval is a measure of conduction through the atria to the ventricles and is a measure of AVN function. The Q-T interval is an indication of the depolarization and repolarization of the ventricles and is a measure of action potential duration of the ventricular myocardium. Changes in these intervals, for instance in diseases such as heart failure, can give specific information, for example of abnormalities on conduction or dysfunction of components of the CCS.

1.4 HEART FAILURE

Although there is no widely accepted definition of heart failure,\textsuperscript{20} it is often referred to as the heart’s inability to pump enough blood to meet the body’s demands. It is a complex, progressive syndrome of which there are innumerable causes, but it is often the result of damage to the heart muscle, for example following myocardial infarction, or prolonged hypertension.\textsuperscript{21} The heart continues to weaken with worsening symptoms despite medical treatment. The symptoms include shortness of breath (dyspnoea), oedema, exercise intolerance and fatigue. In the USA, approximately 5.7 million people are diagnosed with heart failure.\textsuperscript{22} Despite advancing therapies, the prognosis for patients with heart failure
remains poor; around half of those diagnosed with heart failure die within 5 years and it is the cause of death in 1 in 9 death certificates.\textsuperscript{22}

1.4.1 Heart Failure and the CCS
Patients with heart failure often have dysfunction of the CCS, which can lead to improper contraction of the heart and predisposition to arrhythmias. Around 40% of fatalities in heart failure are due to sudden cardiac death.\textsuperscript{23} The major cause of sudden cardiac death is ventricular tachycardias, although bradycardias are also a cause.\textsuperscript{24}

1.4.1.1 Dysfunction of the SAN in Heart Failure
It has been found that in heart failure there is dysfunction of the SAN. In a study by Sanders \textit{et al}. of patients with heart failure and no prior history of atrial arrhythmias, there was a remodelling of the SAN that resulted in a bradycardia (as measured by an increase in the intrinsic SAN cycle length).\textsuperscript{25} The patients also had a prolongation of the SAN recovery time, which is a measure of the ‘health’ of the node.\textsuperscript{25} SAN dysfunction has also been demonstrated in animal models, such as a rabbit model of heart failure.\textsuperscript{26}

1.4.1.2 Dysfunction of the AVN in Heart Failure
In heart failure, a slowing of conduction through the AVN resulting in heart block has been noted.\textsuperscript{27} Patients with increased PR interval, also known as first-degree heart block, have been associated with increased risk of heart failure and increased risk of death.\textsuperscript{28-30} In addition to increased mortality, a prolonged PR interval is associated with increased risk of atrial fibrillation and pacemaker implantation.\textsuperscript{29} A longer PR interval is also a predictor of hospitalisation for heart failure.\textsuperscript{30}

1.4.1.3 Dysfunction of the His-Purkinje System in Heart Failure
A fifth of patients with heart failure have a prolonged QRS interval (>120 ms),\textsuperscript{31} which is a measure of action potential conduction through the His-Purkinje system and ventricles. A prolonged QRS complex is often associated with impaired contraction and cardiac output. Heart failure patients with a longer than normal QRS interval have greater systolic dysfunction.\textsuperscript{31} Prolonged QRS interval has been shown to be a predictor of sudden cardiac death and mortality.\textsuperscript{32} A prolonged QRS interval can be an indication of bundle branch block (BBB), or impaired conduction through the Purkinje fibres. Approximately 20% of patients with heart failure have been found to have BBB.\textsuperscript{33} Patients with left BBB (LBBB) have a diminished diastolic function and increased mortality.\textsuperscript{34} Reduced left ventricular function appears to be more detrimental than reduced right ventricular function; patients with LBBB have an increased risk of death than those with right BBB (RBBB).\textsuperscript{32}
1.4.2 Cause of CCS Dysfunction in Heart Failure
The cause of the electrical remodelling in heart failure has been suggested to be ion channel remodelling and fibrosis. As discussed above, ion channels are responsible for the formation and conduction of the action potential. Changes in ionic currents, as a result of altered expression or function of the ion channels, could be responsible for the impaired initiation and conduction of the action potential seen in heart failure.\(^35\)

In a dog model of heart failure, in addition to SAN dysfunction, there is a down regulation of hyperpolarisation-activated cyclic nucleotide-gated (HCN4) channel mRNA and protein in the SAN.\(^36\) HCN channels are responsible for carrying the pacemaking current, \(I_f\). Down regulation of the pacemaking channel could reduce the pacemaking potential of the node. In addition, there was an increase in HCN4 in the right atrium, which could be responsible for increased atrial arrhythmias renowned in heart failure.\(^36\)

In the Purkinje fibres in a dog model of heart failure, a downregulation of ion channels carrying the transient outward \(K^+\) current (\(I_{to}\)) was found at the mRNA and protein level.\(^37\) \(I_{to}\) is partly responsible for repolarization and thus affects the action potential profile and duration. Moreover, there is a reduction in mRNA and protein levels of Cx40 and Cx43 in the Purkinje fibres that would help explain the reduction in conduction velocity in the Purkinje fibres in heart failure.\(^37\)

1.5 ATRIAL FIBRILLATION
Supraventricular tachyarrhythmias, such as atrial fibrillation, are common in heart failure patients. Atrial fibrillation is a disruption of the normal cardiac rhythm caused by uncoordinated electrical activity within the atria.\(^38\) This results in rapid, irregular contraction of the atria and the distinct P wave is lost from the ECG. Atrial fibrillation is the most prevalent arrhythmia, affecting approximately 1% of the UK population.\(^39\) The incidence of atrial fibrillation increases with increasing severity of heart disease and also with age, affecting approximately 9% of over 80 year olds.\(^40\) Although atrial fibrillation can be asymptomatic, it can be detrimental to health. Atrial fibrillation results in reduced cardiac output and its occurrence has been shown to increase mortality.\(^41\) Improper contraction of the atria in atrial fibrillation can cause blood in the atria to become static encouraging thrombosis, resulting in an increased risk of stroke.\(^40, 41\) The prevalence of atrial fibrillation increases with degree of heart failure, from approximately 10% in moderate heart failure to 50% in severe.\(^42\)

Atrial fibrillation is often initially paroxysmal; the arrhythmia starts and terminates spontaneously, usually only lasting around 48 hours. However, this can progress to
chronic persistent atrial fibrillation, which is sustained over long periods of time and often does not terminate spontaneously. This worsening of the arrhythmia is due to a phenomenon of ‘atrial fibrillation begets atrial fibrillation’.\textsuperscript{43} In goats, artificial maintenance of atrial fibrillation increases the duration of paroxysmal atrial fibrillation followed by a progression to persistent atrial fibrillation.\textsuperscript{43} The atrial effective refractory period (AERP) shortens, which increases susceptibility to re-entrant arrhythmias.\textsuperscript{43} Atrial fibrillation is also associated with increased P wave duration that is also known to be arrhythmogenic.\textsuperscript{44, 45} Slowed and reduced conduction predisposes the tissue to re-entrant arrhythmias.\textsuperscript{46, 47}

1.5.1 Dysfunction of the CCS in Atrial Fibrillation

Dysfunction of the SAN has been reported in patients with atrial fibrillation. Patients with lone atrial fibrillation have prolonged SAN recovery times and a reduced AERP.\textsuperscript{48} Even transient episodes of atrial pacing for 10 to 15 min have the ability to cause SAN dysfunction.\textsuperscript{49} Prolonged atrial fibrillation in dogs increases SAN recovery time and causes abnormal automaticity in addition to atrial conduction dysfunction.\textsuperscript{50, 51}

The majority of the literature on CCS function in atrial fibrillation has been focused on SAN. However, AVN ablation is often the treatment of choice for patients with atrial fibrillation. The AVN is overwhelmed by the electrical activity and ablation helps to prevent the tachycardia in the atria inducing ventricular fibrillation. This therapy can relieve some of the symptoms of atrial fibrillation relating to ventricular tachycardia, such as improving ventricular contraction, but it does not alleviate SAN dysfunction or occurrence of atrial fibrillation.\textsuperscript{52}

Contributing Factors to Atrial Fibrillation

Atrial fibrillation is usually due to dysfunction of conduction through the working myocardium and/or CCS. In the healthy myocardium, there is extensive close contact between the myocytes, and via gap junctions, aids efficient conduction of the action potential. In atrial fibrillation, interruption of the action potential conduction, for example by fibrosis or altered connexin expression, is thought to play a role. In patients with atrial fibrillation there is an increase in atrial fibrosis, as measured by an increase in collagen I, and a reduction in Cx43 expression.\textsuperscript{53} However, as reviewed by Nattel et al. there are inconsistencies as to whether connexins are upregulated or downregulated in atrial fibrillation in both patients and animal models.\textsuperscript{35} Nevertheless, a common observation in atrial fibrillation is changes to the localisation and distribution of the connexins.\textsuperscript{35} This heterogeneity of connexin expression would encourage conduction abnormalities and facilitate the conduction of the action potential through alternative, tortuous pathways. This would inevitably aid the generation of re-entrant arrhythmias and AF.
Apoptosis has also been observed in right atrium specimens obtained from patients with atrial fibrillation. Apoptosis is a tightly controlled programmed cell death following activation of intracellular signalling cascades, including cysteine proteases caspases 9 and 3, to destroy unwanted or no longer needed cells. This is in contrast to the process of necrosis, which is cell death induced following insult or injury to the cells. However, defective apoptosis can occur in disease states and during atrial fibrillation. In addition to increased fibrosis, increased apoptosis would also provide a substrate for re-entrant arrhythmias.

The question is, what is instigating the remodelling in heart failure and atrial fibrillation? Inflammation appears to be playing an important role.

1.6 INFLAMMATION

The inflammatory response is a complex cascade of events that, under normal conditions, is an important defence mechanism of the body. It is a tightly regulated process with the aim of restoring tissue homeostasis following injury or infection (Figure 1.7). The presence of pathogens or dead/damaged cells changes the chemical composition of the tissue that triggers release of pro-inflammatory mediators, such as histamine and prostaglandins. Prostaglandins induce vasodilation of the local blood vessels to increase blood flow to the area. This extra blood flow will increase the supply of oxygen to the site of inflammation and aid the transport of inflammatory cells. Endothelial permeability is also increased to aid inflammatory cell infiltration from the blood into the tissue. Clotting factors are activated to provide a protective mesh around the region to help confine the inflammation and prevent it spreading to the rest of the body.
Figure 1.7 Illustration of infiltration of white blood cells (leukocytes) from the blood stream to the site of inflammation. The inflammatory cascade is resolved when all pathogens/damaged cells have been removed from the tissue. Abbreviations: interleukin 1β, IL1β; transforming growth factor-β, TGF-β; and tumour necrosis factor-α, TNF-α.

Histamine and prostaglandins activate pro-inflammatory cytokines, such as interleukins 1β and 6 (IL1β and IL6) and tumour necrosis factor-α (TNF-α). Cytokines are a large family of proteins that control the length and amplitude of the inflammatory cascade. IL-1β and TNF-α stimulate the transcription factor nuclear factor-κB (NF-κB). NF-κB is thought to be one of the critical signalling molecules activated by the pro-inflammatory cytokines. Upon activation, NF-κB translocates to the nucleus where it stimulates gene expression of further inflammatory cytokines. Pro-inflammatory cytokines stimulate the migration of white blood cells, leukocytes, into the area. Neutrophils are one of the first cell types to arrive at the site followed by macrophages, derived from circulating monocytes. Leukocytes travel in the blood stream and attach to the endothelium by adhesion molecules. Attachment and rolling of the leukocytes along the endothelium is mediated by selectins and integrins. The leukocytes roll along the endothelium and migrate into the tissue by either paracellular or transcellular migration. Paracellular migration is the classic method of passage; the leukocyte passes through the endothelium via endothelial cell junctions. Transcellular migration involves the leukocyte passing along a channel through an endothelial cell cytoplasm. Neutrophils and macrophages belong to a group of leukocytes, termed phagocytes. Phagocytes capture the targeted cells between processes of their cell membrane, called pseudopods, and engulf the cell. Inside the phagocyte, toxic chemicals, for example the enzyme myeloperoxidase (MPO) and reactive oxygen species (ROS), are released from their granules and kill the targeted...
cells. However, these chemicals cannot discriminate host from target and therefore, usually host tissue is damaged in the process.

Leukocytes have a short half-life so they also release cytokines to recruit further leukocytes and maintain the inflammatory response. The process continues until all of the pathogens or cell debris are removed from the tissue. Neutrophils undergo programmed cell death, apoptosis, and are safely cleared from the site by macrophages. The inflammatory cascade is then concerned with repair of the tissue, activated by pro-resolution mediators.

*Transforming growth factor-β (TGF-β)*

One of the most prominent mediators in tissue repair is the anti-inflammatory cytokine transforming growth factor-β (TGF-β) that acts via the Smad2/3 pathway (Figure 1.8). TGF-β dimers bind to a TGF-β type-2 receptor located on the cell membrane, which recruits and phosphorylates a TGF-β type-1 receptor. The activated TGF-β type-1 receptor phosphorylates a receptor regulated Smad (abbreviation of mothers against decapentaplegic homolog), for example Smad2 and Smad3. Smad3 binds to a common Smad (coSmad), like Smad4, and forms a heterodimeric complex that can transmigrate into the nucleus and act as a transcription factor and alter gene expression. As a result, TGF-β can upregulate mediators of the inflammatory and fibrotic response involved in scar formation. TGF-β activates fibroblasts, which produce components of the extracellular matrix (ECM; see below), such as collagen. TGF-β stimulates fibroblast differentiation to myofibroblasts. Myofibroblasts express α-smooth muscle actin (α-SMA) and they have the ability to contract the ECM-an important part of scar formation following tissue injury. TGF-β also increases expression of connective tissue growth factor (CTGF), which facilitates the fibrotic response. These together result in production of a collagen scar, which over time is remodelled to produce new healthy tissue.

Ideally, following the inflammatory process, the tissue is restored to its original structure and function. However, in certain conditions such as chronic inflammation, there can be permanent scarring and loss of function of the tissue. In chronic inflammation, for example during a persistent infection or autoimmune disease, the inflammation cascade is not resolved and continues in a vicious cycle (Figure 1.8). Over a long period of time this process can become detrimental and can even result in further damage to the tissue.
Figure 1.8 Schematic diagram of a number of major pathways involved in the inflammatory process. In chronic inflammation, the process is not resolved which can result in remodelling and tissue damage. Abbreviations: angiotensin II, ATII; interleukin 1β, IL1β; mitogen-activated protein kinase, MAPK; nuclear factor-kB, NF-kB; mothers against decapentaplegic homolog 2/3/4, Smad2/3/4; transforming growth factor- β, TGF-β; and tumour necrosis factor- α, TNF-α.

The Extracellular Matrix
The extracellular matrix (ECM) is the support structure of the myocardium. It is a complex scaffold-like structure and its components include collagens I and III, fibronectin, elastin, laminin, integrins and proteoglycans (Figure 1.9). Collagens provide tensile strength and are important for maintaining cardiac structure and pressure during contraction and relaxation. Collagen production is primarily by fibroblasts that also reside in the ECM. Collagen type-I (collagen I) makes up 80% of the collagen in the heart, with collagen type-3 (collagen III) making up a further 10%. Collagen I provides more tensile strength than collagen III, which is more elastic and provides stretch. In addition to providing support, the ECM provides a microenvironment for cell and cytokine interactions. In health, there is a tightly regulated balance of ECM production and degradation to maintain tissue homeostasis. This balance is maintained by matrix metalloproteinases (MMPs), which are enzymes that degrade the ECM, and by tissue inhibitors of metalloproteinases (TIMPs). MMPs can also stimulate TGF-β release, important for ECM synthesis.
1.6.1 Inflammation in Heart Failure

An association between heart failure and inflammation has been known for over twenty years. Heart failure patients have increased serum levels of C-reactive protein (CRP), the level of which is correlated with the severity of heart failure. CRP is an acute phase protein produced in the liver that is present at very low levels under normal conditions but increases rapidly and significantly at the onset of the inflammatory response. CRP levels are higher in patients with higher New York Heart Association (NYHA) class and this increase is also associated with higher mortality rates. Heart failure patients have increased circulating levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, which correlate with the severity of the disease. Infiltration of macrophages are found in the myocardium in heart failure, in addition to increased expression of adhesion molecules. Cell adhesion molecules are a superfamily of receptors that aid the attachment and infiltration of leukocytes through the endothelium to the site of inflammation. Upregulation of cell adhesion molecules has been found to be prognostic in the severity of heart disease. An increased level of cell adhesion molecules also negatively correlates with left ventricular function.

An increase in mediators of the inflammatory response correlates with severity of heart failure suggesting they can be used as biomarkers of disease. However, more than this, inflammation has been found to predict not only survival, but risk of developing heart failure. In asymptomatic subjects, an increase in IL-6 and CRP is able to predict the occurrence of heart failure.
Although there is increased inflammation measured in heart failure, it is not the cause of death in the majority of patients, whose cause of death is usually pump failure or fatal arrhythmia. However, the inflammatory response has been found to be detrimental on the progression of failure and, although it may not be the cause of death, inflammation can itself damage the heart. Inflammation appears to be more than a biomarker of disease progression and is a contributor to the pathogenesis of heart failure. Our hypothesis is that inflammation could be a cause of structural and electrical remodelling in heart failure, for example, by inducing fibrosis and remodelling ion channels.

In mouse models of heart disease, overexpression of CRP causes increased cytokine levels, increased fibrosis and reduced cardiac function. In dogs, infusion of the pro-inflammatory cytokine TNF-\(\alpha\) causes significant reduction in systolic and diastolic left ventricular function. Using transgenic mice, tissue-specific production of TNF-\(\alpha\) by myocytes induces cardiac fibrosis, dysfunction and failure. In addition, TNF-\(\alpha\) has been shown to increase apoptosis. Apoptotic myocytes have been found to increase macrophage infiltration by releasing chemoattractants.

### 1.6.2 Fibrosis in Heart Failure

It has long been established that there is increased fibrosis in the working myocardium in heart failure. In an attempt to maintain systolic output, there is increased expression of fibrosis genes. However, over time this process becomes decompensatory; fibrosis causes the myocardium to become stiff and as a result the myocardium cannot contract as easily or efficiently. In heart failure, there are alterations in ECM turnover, which is thought to contribute to the decompensatory fibrosis. In heart failure patients, increased levels of MMP1 and biomarkers of collagen synthesis and degradation positively correlate with worse outcomes. An increase in collagen I is measured in heart failure. Alterations in the collagen I/III ratios are found, which interferes with the tensile strength and elasticity balance of the myocardium.

In the rat, there is an increase in ventricular stiffness and collagen deposition in heart failure, which is much more than in the preceding stage of hypertrophy. In a different study in rats, there was an increase in gene expression of fibronectin, collagen and TGF-\(\beta\) as heart disease progressed from compensatory hypertrophy to failure. As described above, one of the primary mediators involved in the fibrotic response is the cytokine TGF\(\beta\).

**Angiotensin II**

The renin-angiotensin system is a cascade of hormonal activation that can control blood pressure and fluid homeostasis. A drop in blood pressure or blood volume stimulates the
release of the enzyme renin from the kidneys. Renin cleaves the N-terminal of the peptide angiotensinogen, which is primarily produced by the liver, and forms the inert angiotensin I.\textsuperscript{87} The enzyme angiotensin-converting enzyme (ACE) converts the inert angiotensin I into the active angiotensin II. Angiotensin II binds to and activates angiotensin II type I receptors (AT\textsubscript{1}R) on the cell membrane, which activates downstream signalling to induce vasoconstriction.\textsuperscript{87} AT\textsubscript{1}R is present in most organs of the body, including the heart and vasculature. Two subtypes of AT\textsubscript{1}R have been identified in the rat, AT\textsubscript{1a} and AT\textsubscript{1b},\textsuperscript{88} which have similar binding and signal transduction properties.\textsuperscript{89} Angiotensin II activates a large number of signalling pathways, including mitogen-activated protein kinase pathways (MAPKs) (Figure 1.9).

It is well established that activation of the renin-angiotensin system occurs in heart failure. Patients have a progressive increase in angiotensin II production.\textsuperscript{90} Angiotensin II has the ability to induce inflammation.\textsuperscript{91} In healthy subjects, infusion of angiotensin II induced increased expression of the cytokine IL-6.\textsuperscript{92} However, angiotensin II is best known for its fibrosis inducing characteristics. Angiotensin II infusion in rats causes fibrosis of working myocardium and vessels.\textsuperscript{93} Angiotensin II appears to work alongside the cytokine TGF-β to produce its hypertrophic and fibrotic effects.\textsuperscript{94, 95}

1.6.3 Inflammation and Atrial Fibrillation

Atrial fibrillation has also been shown to be associated with inflammation. Patients with atrial fibrillation have been shown to have 2-fold greater levels of circulating CRP, and its levels appear to correlate with severity of the arrhythmia as patients with persistent atrial fibrillation have much higher levels than those with paroxysmal atrial fibrillation.\textsuperscript{96} The levels of CRP are particularly high within 24 hours of the occurrence of atrial fibrillation.\textsuperscript{96} In addition to being raised during AF, increased CRP levels are also found to be a good predictor of atrial fibrillation risk.\textsuperscript{97, 98} Patients with atrial fibrillation have been noted to have increased infiltration of inflammatory cells in the atria,\textsuperscript{99} including the atrial endocardium.\textsuperscript{100}

In addition to increased CRP levels, patients with atrial fibrillation have increased circulating levels of the neutrophil enzyme MPO, which is also found to be expressed in the right atrium.\textsuperscript{101} In mice, MPO has been shown not only to be a marker of inflammation, but also to induce atrial fibrosis and increase occurrence of atrial fibrillation.\textsuperscript{101}

In humans, there is a high incidence of atrial fibrillation following cardiac surgery,\textsuperscript{102} approximately 28%, which increases with increasing age of the patient; approximately 42% of over 70 year old patients experience atrial fibrillation after cardiac surgery.\textsuperscript{44} CRP levels peak at day 1 following cardiac surgery and then decline.\textsuperscript{102} Patients that receive
anti-inflammatory treatment following cardiac surgery have significantly fewer incidents of post-operative atrial fibrillation than those without treatment.\textsuperscript{103} In addition to an increased mortality risk, patients with post-operative atrial fibrillation incur prolonged hospital admissions.

A study in dogs reported an increase in inflammation following cardiac surgery, which increased with severity of the operation.\textsuperscript{104} The increase in inflammation also correlated with inhomogeneity of conduction of the action potential though the atria and occurrence of atrial fibrillation.\textsuperscript{104} The inhomogeneity of conduction is highly likely to be a consequence of increased fibrosis. Interestingly, MPO levels, atrial fibrillation incidence and conduction inhomogeneity also reduced towards control values following anti-inflammatory treatment.\textsuperscript{104}

1.6.4 Inflammation and Ion Channels

As discussed above, activation of the inflammatory response has been found in heart failure and can be used as a prognosis of severity of the disease. In addition, inflammation has been found to have a detrimental effect on the working myocardium and cardiac function. In part, this is the result of increased fibrosis and apoptosis discussed above. What is less known is the effect of inflammation on the action potential.

Inflammation has been found to alter ion channel function and expression in various organs of the body.\textsuperscript{105} In the heart, induction of inflammation causes a reduction in Cx43 mRNA.\textsuperscript{106} As described above, Cx43 is the most abundant gap junction protein in the working myocardium. Downregulation of Cx43 results in reduced electrical coupling between myocytes. Loss of Cx43 causes a reduction in conduction velocity through the ventricle and increased risk or arrhythmias.\textsuperscript{107}

The pro-inflammatory mediator histamine, which is released at onset on the inflammatory response, was found to increase force of contraction and prolong action potential duration in isolated human papillary muscles.\textsuperscript{108} Voltage clamp experiments on cultured rat ventricular myocytes revealed a reduction of the L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}) following addition of the pro-inflammatory cytokine IL-1\beta.\textsuperscript{109} The reduction in current occurred in a concentration-dependent manner.\textsuperscript{109} I\textsubscript{Ca,L} plays an important role in contraction of the myocytes and reduction would have a negative inotropic effect. In another study, addition of the cytokine TNF-\alpha to isolated rat ventricular cells caused a dose-dependent inhibition of I\textsubscript{Ca,L} and a reduction in the Ca\textsuperscript{2+} transient.\textsuperscript{110}

In agreement with the studies on rat ventricular myocytes, TNF-\alpha causes a reduction in I\textsubscript{Ca,L} and a reduction in Ca\textsuperscript{2+} transients in isolated rabbit pulmonary vein
cardiomyocytes.\textsuperscript{111} TNF-\(\alpha\) alters \(\text{Ca}^{2+}\) handling in the cells; TNF-\(\alpha\) causes a reduction in sarcoplasmic reticulum (SR) \(\text{Ca}^{2+}\) content and a reduction in SR \(\text{Ca}^{2+}\)-ATPase (SERCA2a) protein expression.\textsuperscript{111} Additionally, TNF-\(\alpha\) increases the number of delayed after-depolarisations (DADs), which are known to be arrhythmogenic.\textsuperscript{111}

Altered \(\text{Ca}^{2+}\) handling was also noted in rat ventricular myocytes following addition of a combination of the cytokines TNF-\(\alpha\) and IL-1\(\beta\).\textsuperscript{112} The cytokines reduced SR \(\text{Ca}^{2+}\) content but increased \(\text{Ca}^{2+}\) spark frequency.\textsuperscript{112} Sparks are discrete pockets of \(\text{Ca}^{2+}\) spontaneously extruded from the SR and have been found to be arrhythmogenic.\textsuperscript{113}

In summary, inflammation has been shown to be detrimental in heart failure and can be responsible for dysfunction of the working myocardium and remodelling of ion channels. In addition, inflammation has been found to be arrhythmogenic. However, is inflammation responsible for the CCS dysfunction in heart failure?

1.7 PULMONARY ARTERIAL HYPERTENSION

Up to now when discussing heart failure, the findings described have been related to left-sided heart failure. Left-sided heart failure is more prevalent than right-sided heart failure, and as a result has been investigated in more depth.\textsuperscript{114} The causes of left-sided heart failure are numerous; however, the most common are coronary artery disease and hypertension.\textsuperscript{21} The most common cause of right-sided heart failure is pulmonary arterial hypertension.\textsuperscript{114}

Pulmonary arterial hypertension (PAH) is defined as having a mean pulmonary arterial pressure greater than 25 mmHg at rest. Due to the increased resistance in the lungs in PAH, the blood flow in the pulmonary arteries from the right ventricle to the lungs is under higher pressure. To deal with the increased pressure, the right ventricle becomes hypertrophied and dilated to maintain cardiac output to the lungs.\textsuperscript{114} In a review on PAH, Bogaard et al. explains the hypertrophy as a result of the Laplace relationship (Figure 1.10).\textsuperscript{115} The increased pressure results in right ventricular wall stress and to adapt, the ventricle increases in thickness and becomes more spherical.\textsuperscript{115} Under normal conditions the right ventricle is crescentic in shape due to the larger and higher pressured left ventricle.
Figure 1.10 The ventricles in normal conditions (A) and in IPAH (B). In IPAH, an increase in RV wall thickness ($h$) occurs as a result of increased pressure ($P$), wall stress ($\sigma$), and dilation ($r$). Abbreviations: idiopathic pulmonary arterial hypertension, IPAH; left ventricle, LV; and right ventricle, RV. Taken from Bogaard et al. (2009).

However, over time, this initial compensatory hypertrophy becomes decompensatory and right-sided heart failure ensues. The prognosis for patients with PAH is poor. Patients with idiopathic PAH have a mean survival of only 2.8 years.

A reduction in right ventricular function in PAH is associated with poor prognosis. A reduced right ventricular stroke volume (RVSV), which is the volume of blood pumped out of the ventricle in a contraction, and a large right ventricular end-diastolic volume (RVEDV), the volume of blood at the end of chamber filling, is associated with increased risk of treatment failure and death.

1.7.1 PAH and Cardiac Arrhythmias

PAH patients have altered ECG parameters, indicating altered electrical activity in the heart. In PAH, the ECG can reveal right ventricular dysfunction and predict survival. Patients with severe PAH have a prolonged QTc interval, and in women, the QTc correlates positively to mean pulmonary artery pressure. The QTc is the QT interval corrected for heart rate and, like QT interval, is a measure of ventricular action.
potential duration. Increased QT interval is associated with increased risk of mortality and sudden death.\textsuperscript{122, 123} A prolonged QT interval increases risk of ventricular arrhythmias.\textsuperscript{124} In addition to increased risk of ventricular arrhythmias, patients with PAH have an increased risk of atrial arrhythmias.\textsuperscript{126} PAH patients have a relatively high incidence of supraventricular tachycardias, such as atrial fibrillation and atrial flutter.\textsuperscript{126} 28\% of patients with PAH suffer sudden cardiac death, further demonstrating the high burden of cardiac arrhythmias in these patients.\textsuperscript{127}

An interesting study by Medi \textit{et al.} measured a prolongation of the SAN recovery time in PAH patients, indicating dysfunction of the SAN.\textsuperscript{128} In this study, atrial conduction times were slower in PAH patients; there were also regions of the atria that were electrically silent.\textsuperscript{128} These factors could explain, at least in part, the increased atrial arrhythmias seen in both this cohort of PAH patients and in other studies.\textsuperscript{128}

In a study of a rat model of PAH from Umar \textit{et al.} there is also increased occurrence of spontaneous ventricular fibrillation, preceded by early afterdepolarisations (EADs).\textsuperscript{129} Additionally in this study, 30\% of the animals died as a result of sudden cardiac death.\textsuperscript{129} In a rat model of PAH, there is a downregulation of K\textsuperscript{+} channels and a prolongation of the action potential duration in the right ventricle.\textsuperscript{130} Additionally, the animals have increased occurrence of sustained ventricular tachyarrhythmias.\textsuperscript{130} Disorganisation in the distribution of the gap junction protein Cx43 is found in the rat right ventricle in PAH, which would undoubtedly lead to impaired electrical coupling and be proarrhythmic.\textsuperscript{131} Altered Ca\textsuperscript{2+} handling components, involved in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, are found in PAH.\textsuperscript{132} In the right ventricle, but not left, there is a downregulation of SERCA2a, phospholamban and RyR.\textsuperscript{132}

\textbf{1.7.2 Inflammation in PAH}

Patients with PAH have elevated circulating levels of pro-inflammatory cytokines IL-6 and TNF-\textgreek{a} and the chemokine monocyte chemoattractant protein-1 (MCP-1), which plays a role in activating and attracting macrophages in inflammation.\textsuperscript{133} An increased level of various interleukins in PAH has been found to be a predictor of mortality.\textsuperscript{134}

In a study by Campian \textit{et al.} (2010) using a rat model of PAH, there was inflammation in the right ventricle, measured by increased levels of MPO.\textsuperscript{135} MPO is an enzyme released by neutrophils when they are killing invading/damaged cells and is, therefore, used as a marker of inflammation. Interestingly, the level of inflammation increased as the disease progressed from its initial stages of dilation and hypertrophy, through to the final stages of heart failure.\textsuperscript{135} Furthermore, an increase in gene expression of the pro-inflammatory cytokine TNF\textgreek{a} was measured in the right but not the left ventricle.\textsuperscript{135} Increased
chemokine expression and increased infiltration of both neutrophils and macrophages was found in the right ventricle in rats with pulmonary hypertension following pulmonary embolism.\textsuperscript{136} Myocyte damage and increased immune response was only seen in the right ventricle, and not the left.\textsuperscript{136}

1.7.3 Monocrotaline Model of PAH

One of the most common animal models of PAH is the rat monocrotaline model.\textsuperscript{137} This model has been used extensively for decades and closely resembles idiopathic PAH.\textsuperscript{137-139} To induce PAH, monocrotaline is injected into rats at a concentration of usually 60 mg/kg body weight. Other concentrations have been used depending on the strain and age of model. 30 mg/kg body weight has been used to develop a milder phenotype of dilation but not failure.\textsuperscript{140}

Monocrotaline is an inactive alkaloid derived from the seeds of the tropical plant \textit{crotalaria spectabilis}. In the rat liver, monocrotaline is processed into the toxic metabolite monocrotaline-pyrrole, which selectively targets and injures the vascular endothelium of the lungs.\textsuperscript{138} Although its exact mode of action is unknown, monocrotaline-pyrrole induces medial hypertrophy of the pulmonary vasculature.\textsuperscript{138, 141} This results in increased pulmonary resistance in the pulmonary vessels and thus, pulmonary hypertension. The blood flow from the right ventricle to the lungs is under higher pressure, and similarly to human PAH, produces an increase in right ventricular pressure. Right ventricular hypertrophy and dilation occurs, followed by right ventricular failure, approximately 21-28 days following injection.\textsuperscript{139, 140}

The rat monocrotaline model has been used in this study to mimic idiopathic PAH. It has been widely used and is well characterised. It is easy to produce involving just a single intraperitoneal injection and no surgery. In addition, the model is fairly acute; the animals develop PAH after approximately 3-4 weeks.

1.8 HYPOTHESIS AND AIMS

In conclusion, inflammation is a biomarker of disease progression in heart failure, predicting future cardiac events and even survival. In addition, inflammation has detrimental effects on the working myocardium, inducing fibrosis, remodelling of ion channels and even arrhythmias. Inflammation appears to be playing a major role in the pathogenesis of heart failure in contributing to pump dysfunction. However, the effect of inflammation on the CCS has not been investigated. The CCS is a complex structure, responsible for efficient electrical propagation through the heart, resulting in synchronised
contraction. It is hypothesised that there is increased inflammation and associated fibrosis and apoptosis in the CCS in heart failure.

The aims of the study were:

1. To successfully establish, characterise and validate the rat monocrotaline model of pulmonary arterial hypertension.
2. To determine if there is infiltration of inflammatory cells into the CCS in pulmonary arterial hypertension.
3. To determine if there is an increase in pro-inflammatory cytokines and also inflammation-associated fibrosis and cell death in the CCS in pulmonary arterial hypertension.
4. To determine if there is an increase in pro-inflammatory cytokines and also inflammation-associated fibrosis and cell death in two further animal models of heart disease: the rabbit model of congestive heart failure and the rat model of myocardial infarction.
CHAPTER 2: MATERIALS AND METHODS

2.1 MODELS OF HEART DISEASE

2.1.1 Rat Model of Pulmonary Arterial Hypertension
To model pulmonary arterial hypertension, male Wistar rats weighing approximately 200 g were intraperitoneally injected with monocrotaline at a concentration of 60 mg/kg body weight. Control rats were injected with sterile saline at the same volumes. Rats were purchased from Charles River Laboratories International, Inc. Monocrotaline is derived from the seeds of the tropical plant *crotalaria spectabilis*. In the rat, monocrotaline is metabolised into the toxic compound monocrotaline-pyrrole. For reasons not completely understood, monocrotaline-pyrrole selectively targets the endothelium of pulmonary vessels inducing pulmonary vasculitis and medial hypertrophy. This results in increased pressure and resistance in the pulmonary vessels and thus pulmonary hypertension. The blood flow to the lungs from the right ventricle via the pulmonary artery is under higher pressure, which leads to compensatory right-sided hypertrophy as the right ventricle attempts to maintain cardiac output to the lungs. However, over time this hypertrophy becomes decompensatory and right-sided heart failure ensues. The time-point of development of failure varied slightly between animals but was usually 21 days post-injection. All animal work was carried out under appropriate Home Office regulations.

2.1.1.1 Preparation of Monocrotaline
Monocrotaline was prepared fresh on the day of injection at a working concentration of 20 mg/ml. 2 ml of 1 M of hydrochloric acid (Sigma-Aldrich) was added to 200 mg of monocrotaline (Sigma-Aldrich). 7 ml of sterile saline was added and the pH adjusted to 7.4 using 4 M sodium hydroxide (Sigma-Aldrich). The solution was made up to 10 ml with sterile saline to give a final concentration of 20 mg/ml.

*Calculation of Injection Volume*
1) The dose of injection, in mg, was calculated as 60 multiplied by weight of the rat, in kg.
2) The injection volume, in ml, was calculated as the dose of injection divided by the working concentration (20 mg/ml).

*Injection Day Protocol*
After an acclimatisation period of at least seven days, the rats were anaesthetised and ECG and echocardiography recordings were taken. The rats were then given 60 mg/kg body weight of monocrotaline or of the equivalent volume of saline solution. The animals were then monitored to ensure complete recovery from the anaesthesia.
2.1.1.2 In vivo Electrocardiography and Echocardiography

To measure the ECG and carry out echocardiography, the rats were anaesthetised with 2% isoflurane. The rats were placed in a pre-oxygenated anaesthetic chamber and, after approximately 30 s, isoflurane was started at 1% and increased to 2% until the animal became unconscious. The rats were transferred onto a heat mat to maintain body temperature and onto a facemask to maintain anaesthesia.

Electrocardiography

To record the ECG, two ECG electrodes were inserted into both front limbs and a third earth electrode inserted into one of the hind limbs. The ECG was recorded for 5 min. Immediately following the recording, while the rats were still anaesthetised, echocardiography measurements were taken. When analysing the ECG traces, the beginning and end of the waves was chosen by eye. Corrected QT (QTc) was calculated using Bazett’s formula:

\[
\text{QTc} = \frac{\text{QT}}{\sqrt{\text{RR}}}
\]

Echocardiography

Transthoracic two dimensional echocardiography parameters were recorded in accordance with published methodology. To achieve a clear image of the heart, the fur on the thorax was removed with hair removal cream. A VisualSonics Vevo 770 system and a 14 Hz transducer were used throughout. M-mode through the parasternal short axis was taken at the level of the papillary muscles to measure left ventricular anterior wall thickness (LVAW), left ventricular posterior wall thickness (LVPW) and left ventricular internal diameter (LVID) (Figure 2.1). To measure pulmonary artery velocity (PAV), the transducer was moved more proximal, to the level of the aortic and pulmonary valves (Figure 2.2). On this axis, the velocity of the blood through the valve could be measured using pulse wave Doppler. In control conditions, the profile of the pulmonary arterial velocity is slow and symmetrical. Using pulse wave Doppler the maximum velocity of blood flow through the artery can be measured (PAVmax) (Figure 2.2). In addition, the time taken to reach maximum velocity, pulmonary artery acceleration time (PAAT), and time of the initial reduction in velocity, pulmonary artery deceleration (PAD), can be read from the profile (Figure 2.2). These measurements give an indication of changes to the flow as a result of the altered pulmonary pressure. M-mode at the parasternal long axis, 90° to the short axis, was used to measure right ventricular wall thickness (RVWT) and right ventricular internal diameter (RVID) (Figure 2.3). The same M-mode position was used to measure both the diastolic and systolic chamber wall thicknesses.
Figure 2.1 Echocardiography at the short axis used to measure left ventricle (LV) dimensions. (A) Parasternal short axis through the papillary muscles of the LV enabling measurements to be taken of the LV anterior wall thickness (LVAW), LV posterior wall thickness (LVPW) and LV internal diameter (LVID). (B) A snapshot of the M-mode recording through the LV. Both systolic (s) and diastolic (d) measurements were taken from the same M-mode position.

Figure 2.2 Echocardiography at the short axis used to measure pulmonary artery (PA) velocity. (A) Parasternal short axis at the level of the aortic valve, proximal to axis to measure LV dimensions, revealing the pulmonary valve. (B) A snapshot of pulse wave Doppler used to measure the maximum velocity (PAV) of the blood as it was pumped through the pulmonary valve and into the PA. This method also allowed measurements to be taken of the PA velocity acceleration time (PAAT) and the PA deceleration (PAD).
Figure 2.3 Echocardiography at the long axis used to measure right ventricle (RV) measurements. (A) Parasternal long axis enabling visualisation of the RV wall thickness (RVWT) and the RV internal diameter (RVID). (B) A snapshot of the M-mode recording through the RV. Systolic (s) and diastolic (d) measurements were taken from the same M-mode position.

2.1.1.3 Experimental Endpoints
For 21 days following injection, the animals were weighed at least twice a week and monitored for clinical signs of heart failure. These signs included rapid weight loss (more than 10 g over 48 h), piloerection, tachypnea, cyanosis, oedema and reduced mobility. From day 21, the monitoring was increased and the rats were weighed and checked daily. In accordance with Home Office requirements, the rats were killed humanely by a Schedule 1 method if they showed any of the clinical signs above, or by 28 days following injection. Prior to termination, final ECG and electrocardiography recordings were taken. To prevent blood clotting, approximately 10 min before being culled, 100 units of heparin was administered to the animal via intraperitoneal injection.

2.1.1.4 Langendorff Perfusion Experiments
Immediately following Schedule 1, a thoracotomy was performed and the heart and lungs excised. Excess tissue was trimmed and the wet weight of both the heart and lungs was recorded. The aorta was placed onto the cannula of the Langendorff set up (Figure 2.4) and the heart was retrogradely perfused with Tyrode’s solution. Tyrode’s solution is a roughly balanced salt and glucose solution to simulate physiological conditions in the experiment. Tyrode’s solution comprised of 120 mM NaCl, 4 mM KCl, 1.3 mM MgSO$_4$7H$_2$O, 1.2 mM NaH$_2$PO$_4$2H$_2$O, 1.2 mM CaCl, 25.2 mM NaHCO$_3$ and 5.8 mM glucose. Additionally, the Tyrode’s solution was heated to 37°C using a water bath and bubbled with 95% O$_2$ and 5% CO$_2$. The flow rate of Tyrode’s solution perfusion was set at 6.6 ml/min/g heart weight.
Figure 2.4 Langendorff setup to perfuse the isolated rat heart. Tyrode’s solution was bubbled with 95% O₂, 5% CO₂ and heated to 37°C in a water heated reservoir. The flow rate of Tyrode’s solution was set at 6 ml/min/g heart weight using a peristaltic pump. After passing through a bubble trap, the solution was retrogradely perfused through the cannulated heart. A stimulation electrode and two ECG electrodes were placed on the heart and connected to a stimulation and an amplifier box, respectively. The amplifier was connected to a computer so the ECG could be recorded throughout pacing programmes.

Two ECG electrodes were placed on the heart, one on the left atria and one close to the apex. The electrodes were adjusted until a clear trace was obtained. A pacing electrode was placed on the right atrium, close to where the sinoatrial node (SAN) is predicted to be located. The following pacing protocols were carried out:

1. Atrial Threshold: The threshold is the minimum pacing voltage required to attain 1:1 pacing, i.e. for every pacing pulse (S1), there is stimulation of an action potential (Figure 2.5). This can be picked up by the ECG electrodes and monitored on the computer. The threshold can vary for every heart. To measure threshold, a train of 2 ms pulses was applied to the heart at a cycle length of 200 ms. The amplitude of the voltage was reduced until 1:1 pacing was lost; this is the threshold. This voltage was doubled and used for the pacing pulse for the rest of the experiment.
Figure 2.5 Example of 1:1 pacing at threshold. For every pacing stimulus (bottom trace), there is activation of an action potential as demonstrated by the ECG (top trace).

2. SAN Recovery Time (SNRT): The SNRT protocol, also called the S1S1 protocol, is a measure of how well the SAN is functioning. This was measured using a train of 150 S1 pulses (S1S1) at a cycle length of 200 ms applied to the right atrium. The S1 pulses take over from the SAN and for every S1 there was an action potential produced. When the pacing was stopped after 150 pulses, the SAN resumed pacing the heart. The time taken for the SAN to recover, in other words, the time taken for a non-stimulated action potential, is the SNRT (Figure 2.6). In a dysfunctional SAN, it may take longer for the node to resume pacing. For each heart, this experiment was carried out three times. The SNRT was measured as the time taken from the last pacing stimulus until the first intrinsic P wave.
Figure 2.6. Measurement of the SAN recovery time (SNRT). Immediately following a S1S1 pacing protocol, the SNRT was measured as the interval from the final S1 pacing stimulus to the beginning of the next intrinsic P wave.

3. Wenckebach Cycle Length: The Wenckebach cycle length is a measure of atrioventricular (AVN) function. A train of 100 S1 pulses was applied to the right atrium, at a cycle length of 200 ms. This was repeated three times with a 2 s gap between each train. After three trains, the cycle length between the S1 pulses was reduced by 10 ms, i.e. to 190 ms and then 180 ms etc. This was continued until there was no longer 1:1 pacing, indicating AVN block (Figure 2.7). The largest S1S1 cycle length that resulted in AVN block was measured. The protocol was repeated, but this time the S1S1 cycle length was reduced by 1 ms. This provided a more accurate Wenckebach cycle length.
Figure 2.7 Measurement of the Wenckebach cycle length. To measure the Wenckebach cycle length, an S1S1 pacing train of reducing cycle lengths was applied until there was a dropped beat or AVN block. The S1S1 cycle length responsible for this block is termed the Wenckebach cycle length.

4. Effective Refractory Period (ERP): The ERP is the period of time for which a new action potential cannot be instigated; the heart tissue is unexcitable. An increase in ERP can be an indication of an increase in action potential duration, because the ERP is normally about the same duration as the action potential itself.

   a. AVN ERP (AVERP): To measure the ERP, a S1S2 protocol was used. Eight S1 pulses (200 ms cycle length) were followed by an additional S2 pulse 190 ms later. This was repeated, but each time the cycle length between the final S1 pulse and the S2 pulse was reduced by a further 10 ms. This was continued until the S2 stimulus produced an ECG recording with a dropped QRS wave, indicating conduction block through the AVN. The interval between the final S1 pulse and the S2 pulse is the ERP for the AVN. Similar to the Wenckebach cycle length, the protocol was repeated, reducing the S1S2 interval by 1 ms each time to get a more precise measurement.

   b. Atrial ERP (AERP): To measure AERP, the protocol was continued until the S2 cycle length was reduced so much that there was no longer a P wave. The protocol was repeated, reducing the S1S2 interval by 1 ms each time to get a more precise measurement.
5. Atrial fibrillation (AF) Threshold: To measure increased vulnerability to AF, as often seen in heart disease, AF threshold was measured. The lower the threshold, the greater the vulnerability to arrhythmias. To do this, trains of S1S1 pulses were applied to the atria for 3 s. The cycle length between the pulses was reduced from 100 ms to 10 ms, in 10 ms intervals in different runs. The largest cycle length at which AF was elicited was taken as the threshold.

6. Ventricular Threshold: The stimulating electrode was moved from the right atrium to the right ventricle, approximately half way between the apex and base. The protocol described for the atria was repeated.

7. Ventricular ERP (VERP): The protocol used for AVERP and AERP was repeated in the ventricle. When the ventricle was paced it produced an ECG trace without a discrete P wave (there is expected to be retrograde conduction through the AVN and atrial activation, but this may be lost among the ventricular QRS and T waves). In the case of VERP, the protocol was continued until the interval between S1 and S2 was reduced to the extent that it no longer produced a QRS complex (Figure 2.8).

![Figure 2.8 Measurement of the ventricular ERP (VERP).](image)

Figure 2.8 Measurement of the ventricular ERP (VERP). The S1S2 pacing protocol used to measure AVERP and AERP was also used to measure ventricular ERP (VERP), after the pacing electrode was moved from the right atria to the ventricles. The protocol continued until there was a dropped QRS complex.
8. VF Threshold: To test vulnerability to VF, a protocol from the literature was used. The ventricle was first paced at 6 Hz (166 ms cycle length) for 20 s and then burst paced at 50 Hz (20 ms cycle length) for 1 s (Figure 2.9). This was repeated three times. If VF was not induced, the protocol was repeated, but the intensity of the stimulus was increased by increasing the voltage.

![Figure 2.9 An example of burst pacing being applied to the ventricle to test vulnerability to ventricular tachycardia. The ventricle was burst paced at 50 Hz for 1 s. In this example, VF was not induced.](image)

The pacing protocols were performed and recorded using Spike2 software (Cambridge Electronic Design) and analysis was carried out using LabChart7 software (AD Instruments).

2.1.1.5 Sample Collection
Immediately following the functional experiments (which lasted approximately 45 min) the heart was removed from the set up and it was either snap frozen whole or samples were taken from the SAN, the AVN, the right and left Purkinje Fibres, the right and left atria, and the right and left ventricles. Samples were snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C.

Dissection of the SAN
The heart was placed on its dorsal side on silicon and held in place with insect pins. The superior vena cava, aorta and right atrium were identified and pinned. The apex of the heart was removed to allow access into the ventricles. The right ventricle was cut longitudinally along the lateral side, away from the septum, from the apex towards the base of the heart. The dorsal side of the right ventricle was pinned in place. At the base of the right ventricle, cuts were made into the right atrium through the tricuspid valve. The
right atrium was cut along its lateral edges from the tricuspid valve towards the superior vena cava. The right atrium could now be opened up like a book, revealing the endocardium of the chamber. The SAN is located to the anatomical left of the crista terminalis, between the superior and inferior vena cava (the pectinate muscles lie to the anatomical right of the crista terminalis). The node was also identified by the presence of the SAN artery and also by its appearance; it is thinner and more translucent than the surrounding myocardium. A 2 mm biopsy punch was used to sample the centre of the SAN and to minimise contamination from the atrial muscle.

Dissection of the AVN
Following dissection and sampling of the SAN, the AVN preparation was isolated and frozen by Dr. Ian Temple.

Dissection of Purkinje Fibres
The apex of the heart (removed during SAN dissection) was pinned onto silicon. Both ventricles were cut longitudinally along their lateral wall, furthest away from the septum. The chambers were carefully opened slightly to reveal the Purkinje network. The fibres were grasped by forceps and cut way from the endocardium before being collected in a liquid nitrogen cooled Eppendorf tube. The ventricles were continued to be opened further and the fibres collected until no more could be seen.

2.1.2 Rabbit Model of Congestive Heart Failure
To induce congestive heart failure, a rabbit model of pressure and volume overload was used.\textsuperscript{143, 144} This work was completed by collaborators at the University of Liverpool, under the supervision of Professor George Hart. Three month old male New Zealand rabbits (weighing approximately 3 kg) underwent two surgical procedures. The first step was to destroy the aortic valve with a catheter to induce volume overload on the left side of the heart. This was confirmed by an increased pulse pressure in the aorta. The second procedure to induce pressure overload was carried out three weeks later. Partial constriction of the abdominal aorta was achieved by banding the vessel just proximal to the renal arteries. Regular echocardiography recordings were taken to monitor disease development. Five weeks following the final surgical procedure, the animals were in volume and pressure overload induced congestive heart failure and killed humanely by a Home Office Schedule 1 method. Sham-operated rabbits were used as controls. Samples of the working myocardium (right and left atria and ventricles) and of the CCS (SAN and Purkinje fibres) were dissected and frozen as described for the rat model of pulmonary arterial hypertension.
2.1.3 Rat Model of Myocardial Infarction
A rat model of myocardial infarction, donated by Dr. Michal Mączewski, Medical Centre of Postgraduate Education (Warsaw) was also used, as previously described by Yanni et al.\textsuperscript{145} In 12 week old Sprague-Dawley rats, a large infarct of the left ventricle was induced by partial constriction of the proximal left coronary artery. 12 weeks following surgery the animals were in left-sided heart failure, as confirmed by ECG and echocardiography. Sham-operated rats were used as controls. Tissue was collected and frozen as previously described.

2.2 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.2.1 Cryosectoning
Whole hearts were removed from the -80°C freezer and cut into two along the short axis, just below the base of the ventricles. The proximal half (containing the atria, SAN and AVN) was mounted dorsal side down onto a cryostat chuck using optimal cutting temperature medium (OCT). The tissue was cut 10 μm thick using a cryostat (Leica CM30505, Germany) along the long axis in a ventral to dorsal direction. The distal half of the hearts was cryosectioned along the short axis in a proximal to distal direction. The sections were collected onto Superfrost Plus microscope slides (Thermo Scientific) and were stored at -80°C.

2.2.2 Masson's Trichrome Staining
Sections were fixed in Bouin’s solution (Sigma-Aldrich) overnight. Bouin’s solution, which contains picric acid, formaldehyde and acetic acid, preserves the tissue architecture. All stains were from Sigma-Aldrich and were filtered before each use. The next day, the following staining protocol was carried out:
- Three washes in 70% ethanol, 10 min each wash
- Stained in Celestine blue for 1.5 min
- Rinsed in distilled water for 15 min
- Stained in haematoxylin for 4 min
- Rinsed in tap water for 15 min
- Stained in acid fuchsin for 1.5 min
- Rinsed in distilled water for 15 min
- Stained in phosphomolybdic acid for 3 min
- Drain
- Stained in methyl blue for 1 min
- Rinsed in distilled water for 30 min
- Treated with 1% acetic acid for 2 min
- Dehydrated though alcohols:
Cell nuclei were stained a dark blue/black, myocytes were stained pink/purple and collagen was stained blue by Masson’s trichrome.

2.2.3 Haematoxylin and Eosin (H&E) Staining
Sections were fixed in the preservative formalin for 1 h and then placed in Harris’s haematoxylin (Sigma-Aldrich) for 15 min. The slides were washed in tap water for 5 min and then treated in 1% acid alcohol (1% hydrochloric acid in 70% ethanol) for 10 s. The slides were washed for a further 15 min in tap water and then stained in eosin Y for 4 min. Following a final 15 min wash in tap water, the sections were dehydrated through increasing concentrations of alcohol: 70% ethanol for 1 min, 90% ethanol for 1 min and then twice in 100% ethanol, 2 min each. The sections were cleared in Histo-Clear (National Diagnostics) twice, 5 min each. Finally, the slides were mounted with DPX mountant (Sigma-Aldrich).

2.2.4 Light Microscopy
Histology sections were scanned using a Pannoramic Slidescanner and imaged using 3D Histec Pannoramic Viewer software.

2.2.5 Immunohistochemistry
Sections were fixed in formalin for 30 min. Formalin fixes the tissue, but does not inhibit the binding of antibodies to proteins. The slides were placed on a rocker and washed 3 times in phosphate buffered saline (PBS, Sigma-Aldrich) for 10 min each wash. The sections were treated with 0.01% Triton-X100 (Sigma-Aldrich) in PBS for 30 min. Triton-X100 permeabilises the cell membrane to allow the antibodies access to the antigens. The slides were washed a further three times in PBS, 10 min for each wash. A PAP pen (Sigma-Aldrich) was used to draw a thin hydrophobic ring around the tissue to localise solutions to the section. To block non-specific antigen sites, a blocking serum consisting of 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, was added to each section for 1 h. If either a donkey or goat secondary antibody was to be used, 10% of either donkey or goat serum (Sigma-Aldrich), respectively, was added to the 1% BSA solution to provide a better block. The primary antibody was applied to the tissue and left overnight in a humidified chamber at 4°C (Table 2.1). The next day, the sections were washed three times in PBS and the secondary antibody applied. The secondary antibody was
conjugated with a fluorescent dye, either green fluorescein isothiocyanate (FITC) or red cyanine 3 (Cy3), Figure 2.10 and Table 2.1. The sections were allowed to incubate at room temperature for 90 min and then were washed in PBS (three 10 min washes). The slides were mounted with VectaMount (Vector Labs) and sealed with clear polish. The slide were covered to prevent bleaching of the antibodies and stored at 4°C.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Manufacturer</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
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<td>Mouse</td>
<td>Serotec</td>
<td>MCA5709</td>
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<tr>
<td>Connexin 43</td>
<td>Mouse</td>
<td>Millipore</td>
<td>MAB3068</td>
</tr>
<tr>
<td>HCN4</td>
<td>Rabbit</td>
<td>Alomone Labs</td>
<td>APC-052</td>
</tr>
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<td>TGFβ</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>Sc-146</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Guinea pig</td>
<td>Progen Biotechnik</td>
<td>GP53</td>
</tr>
<tr>
<td>Donkey anti-goat FITC</td>
<td>Donkey</td>
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<td>Donkey anti-rabbit FITC</td>
<td>Donkey</td>
<td>Millipore</td>
<td>AP182F</td>
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</tbody>
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Table 2.1 List of primary and secondary antibodies used for immunohistochemistry.

![Figure 2.10 Schematic diagram illustrating the immunohistochemistry method. Secondary antibodies were conjugated with a FITC or Cy3 dye and imaged using a confocal laser microscope.](image-url)
2.2.6 TUNEL Assay
To investigate programmed cell death, apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was carried out on sister cryosections to those used for immunohistochemistry. TUNEL assay stains the fragmented DNA strands that are produced as a result of the apoptosis process. The assay is incorporated with a green fluorescent dye to visualise the location of the apoptosis.
Sections were fixed in formalin for 30 min then washed 3 times in PBS (10 min for each wash). The sections were treated with 0.01% Triton-X100 for 3 min on ice then washed again in PBS (3 times, 10 min each wash). A hydrophobic ring was drawn around each section and 1% BSA added for 30 min. 50 μl of TUNEL reaction mixture was then applied to each section and incubated at 37°C in a covered humidified chamber for an hour. Sections were washed three times in PBS (10 min for each wash) then mounted with VectaMount. The slides were sealed with clear nail polish and stored at 4°C.

2.2.7 Confocal Laser Microscopy
Immunohistochemistry and TUNEL sections were imaged using a Zeiss LSM 5 Pascal confocal microscope using Pascal software. Confocal laser microscopy provides higher resolution images than conventional widefield microscopy. Put very simply, light with particular wavelength is emitted from a laser and excites a tissue section that has been tagged with fluorescent secondary antibodies (fluorophores, Figure 2.10). The fluorophores absorb the light and emits light with a lower energy and longer wavelength. This emitted light can then be detected by the microscope and relayed to the computer. Two types of fluorophores were used for immunohistochemistry, FITC and Cy3. FITC has an approximate peak excitation of 492 nm and peak emission of 520 nm (in the green part of the spectrum), while Cy3 has a peak excitation of 550 nm and peak emission of 570 nm (in the red part of the spectrum).

2.2.8 Analysis of Immunohistochemistry and TUNEL
Semi-quantitative analysis was carried out using ImageJ software. A threshold of staining was set and the area of positive staining was automatically calculated. The percentage area of staining was calculated in regards to tissue area.

2.3 mRNA ANALYSIS

2.3.1 RNA Isolation
To isolate total RNA, two different kits were used. For the rabbit and rat MI samples, a RNeasy mini kit (Qiagen) was used and, for the rat PAH samples, a mirVanna miRNA isolation kit (Life Technologies) was used. Before all protocols, the lab bench and equipment were treated with RNaseZap (Life Technologies) to remove any ribonuclease
that would degrade RNA. All steps were carried out at room temperature, unless specified otherwise. 15 cryosections, 20 μm thick, were cut from atrial and ventricular samples. Due to their small size, this was not necessary for the SAN biopsies and Purkinje fibre samples.

**RNeasy Mini Kit**

To isolate mRNA from the rabbit model and rat MI model, the RNeasy mini kit (Qiagen) was used. Following the manufacturer’s protocol, 10 μl of β-mercaptoethanol (β-ME) and 35 μl of 4 ng/μl carrier RNA was added to 1 ml RLT buffer and the mixture was warmed to 55°C in a water bath. 155 μl of the warmed β-ME/carrier RNA/RLT buffer mixture was added to each sample and immediately homogenised for 2 min using a handheld Polytron homogeniser. 295 μl of RNase free water was pipetted over the tip of the homogeniser and into the tube to collect any residual tissue. To digest any protein present, 5 μl of proteinase K was added and the sample placed in the water bath at 55°C for at least 10 min until the sample was clear. The samples were centrifuged for 5 min at 13,000 rpm and the supernatant transferred to a new Eppendorf tube. Half the volume of 100% ethanol was added and mixed by pipetting. The solution was transferred to an RNeasy spin column placed in a collection tube and centrifuged for 15 s at 10,000 rpm. The columns contain a silicon gel that collects the RNA and allows the waste solution to pass through (this was discarded). In accordance with manufacturer’s protocol, 350 μl of buffer RW1 was added to the column and the tube was spun as before. To degrade any contaminating genomic DNA, as suggested by the manufacturers, 80 μl of RDD/DNase I (70 μl of RDD and 10 μl DNase I) was added to the centre of the silicon gel and the samples were left for 15 min at room temperature. A further 350 μl of buffer RW1 was added and the samples centrifuged. Following addition of 500 μl RPE buffer and centrifugation, 500 μl of 80% ethanol was added. The tubes were centrifuged for 2 min at 10,000 rpm and then the columns were transferred to new collection tubes. To dry the columns, the tubes were spun at 13,000 rpm for 5 min. To collect the RNA, 14 μl of RNase free water was pipetted onto the centre of the silicon gel and allowed to stand for 1 min before being centrifuged for 1 min at 13,000 rpm. The eluate was pipetted back onto the gel and centrifuged for a final time to maximise the amount of RNA collected.

**mirVanna miRNA Isolation**

mRNA was isolated from samples from the rat model of PAH using a mirVanna miRNA isolation kit (life Technologies). To breakdown the cell membranes, 100 μl of lysis/binding buffer was added to each sample and immediately homogenised for 4 min using a handheld Polytron homogeniser. Towards the end of the 4 min, the remaining 200 μl of lysis/binding buffer was added to the Eppendorf tube. 30 μl of miRNA homogenate additive was added, the sample mixed by vortexing and then placed on ice for 10 min. To
extract RNA, 300 μl of acid-phenol:chloroform was added and the solution vortexed for 1 min to ensure it was mixed well. To separate the organic and aqueous phases, the samples were centrifuged for 5 min at 10,000 rpm. The upper aqueous layer was carefully pipetted into a new Eppendorf tube (without removing any of the bottom layer) and 1.25 volumes of 100% ethanol added. The ethanol mixture was added onto a filter cartridge placed onto a collection tube and was centrifuged for 15 s at 10,000 rpm. The filter cartridge collected the extracted RNA and allowed the waste solution to pass though into the collection tube, which was then discarded. 700 μl of miRNA wash solution 1 was added to the filter cartridge and was centrifuged for 10 s as before. The solution that passed through the filter was again discarded and 500 μl of wash solution 2/3 was applied to the filter. Following centrifugation, the solution that passed through was discarded and a further 500 μl of wash solution 2/3 added. After the tube was centrifuged and the solution discarded, the tube was centrifuged for a further minute to ensure that the filter was dry and all excess solution has been removed. To extract the RNA from the filter cartridge, 50 μl of elution solution, preheated to 95°C, was applied to the centre of the filter and the samples centrifuged for 30 s at maximum speed. Due to small sample size, 20-30 μl of elution solution was used for SAN samples, and 11-20 μl for Purkinje fibres. The eluate was replaced on the filter and centrifuged for a second and final time, to ensure maximum yield of RNA.

### 2.3.2 Measurement of RNA Concentration

To analyse the concentration of isolated RNA, a NanoDrop 1000 spectrophotometer (Thermo Scientific) was used. 1 μl of each sample was pipetted onto the measurement pedestal and the top arm closed down on top to produce a 1 mm liquid column. A beam of light is passed through the column and the absorbance is measured by the detector. The absorbance is converted to concentration according to the Beer-Lambert equation that has been modified for nucleic acids:

\[ c = \frac{a \times e}{b} \]

Where \( c \) is the nucleic acid concentration (ng/μl), \( a \) is the absorbance (arbitrary units, AU), \( e \) is the wavelength-dependent extinction coefficient (ng-cm/μl) and \( b \) is the path length (cm). For RNA, \( e \) is 40 ng-cm/μl.

**Calibrator Sample**

From each group, 1 μl from the sample with the largest concentration of RNA was pooled together to form a calibrator sample. This sample was run in all the qPCR plates as a check that the reaction was successful.
2.3.3 Reverse Transcription

Two different kits were used to reverse transcribe the isolated RNA into complementary DNA (cDNA). For the rabbit and rat MI sections, the SuperScript III first-strand synthesis system (Invitrogen) was used, and for the rat PAH samples the high capacity RNA-to-cDNA kit (Applied Biosystems) was used. The rabbit and rat MI reverse transcriptions were carried out at the beginning of this project and used the reverse transcription kit that had been successfully established by the laboratory. On progression of the thesis, a new kit from Applied Biosystems was introduced. Following initial trail experiments, the kit was found to be as effective as the older Invitrogen kit, yet it was more cost effective. As a result, the rat PAH sections, which were carried out at a later time point, were reverse transcribed using the new Applied Biosystems kit. The both protocols, all sections in each animal model were diluted so that they had the same starting concentration of RNA.

SuperScript III First-Strand Synthesis System

To each sample, 1 μl of random hexamers (50 ng/μl) and 1 μl of 10 mM dNTP mix was added and the mixture heated to 65°C for 10 min. The samples were then put on ice for at least 3 min to denature the RNA. A mastermix consisting of, per sample, 2 μl of 10X buffer (200 mM Tris-HCl, 500 mM KCl), 4 μl of 25 mM MgCl₂, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT and 1 μl of SuperScript III RT was added. The samples were placed in a PCR machine and heated to 25°C for 10 min for annealing, 50°C for 50 min for cDNA synthesis and then 85°C for 15 min to terminate the reaction.

High Capacity RNA-to-cDNA Kit

On ice, 10 μl of 2X RT buffer and 1 μl of 20X enzyme mix were added to each sample. The tubes were placed in a PCR machine and the following conditions were run: 37°C for 60 min; 95°C for 5 min to stop the reaction; cooled to 4°C to finish.

On completion, the cDNA was diluted 1:10 for qPCR and kept at 4°C for short-term or -20°C for long-term storage.

2.3.4 Quantitative PCR

Quantitative polymerase chain reaction (qPCR) is used to analyse levels of messenger RNA (mRNA). Very simplistically, the central dogma of molecular biology is that single stranded mRNA, transcribed from double-stranded DNA, can be translated into protein. Although changes in mRNA expression cannot tell us about the amount of viable protein being produced, it is an indication of the changes in gene expression. As the cDNA produced during reverse transcription is copied from the mRNA isolated from the samples, this can then be used as a measure of the mRNA. The basic principal of qPCR is that addition of primers with the genetic sequence of a particular gene of interest, will bind to
cDNA with identical sequence. The reaction occurs in a thermal cycler that provides the exact temperatures for copying DNA:

1. 95°C to unwind the double stranded cDNA into single strands.
2. 60°C to optimise binding of primer to the single strands of cDNA.

This cycle is repeated 40 times. After each cycle, each gene is copied exponentially, ending up with millions of copies. To detect the number of copies produced, a dye (for example SYBR green) is included in the reaction. SYBR green binds to double-stranded DNA, altering its structure and generating a fluorescent product. After each cycle, a beam of blue light is passed through the wells of the PCR plate, exciting the fluorescent SYBR green-DNA PCR product, which emits green light. The threshold for detection, in other words the cycle from which the green light can be picked up by the detector (the threshold cycle, Ct), corresponds with the amount of primer-cDNA interactions. The lower the threshold of detection, the greater amount of cDNA present.

Two types of primer were used. The first was in-house designed primers from Eurofins that came in two tubes, one containing the forward primer and one containing the reverse primer. The second was commercially available primers that came in one tube that contained both the forward and reverse primers. Slightly different volumes of primer and master mix were used for each type. For the in-house designed primers the following was added to each well of a 96 well PCR plate (StarLab): 5 μl Power SYBR green master mix (Life Technologies), 0.4 μl forward primer, 0.4 μl reverse primer and 3.2 μl nuclease free water. For the commercially available primer in each well: 5 μl Power SYBR green master mix, 1 μl primer and 3 μl nuclease free water. To each well 1 μl of 1:10 diluted cDNA was added and mixed by pipetting. The plates were covered with clear adhesion film (Applied Biosystems) and centrifuged for 2 min at 12,000 rpm, ensuring no bubbles were present at the bottom of the wells as these could interfere with detection of the fluorescence produced during the reaction. The plates were run on an Applied Biosystems 7900HT PCR machine using SDS 2.4 software. All samples were run in triplicate.

2.3.4.1 Design of Rabbit Primers
Unlike the rat, the full genomic sequence for the rabbit has not been established and, therefore, few commercially available primers are available. Gene sequences for the inflammatory and fibrosis targets of interest were found using the search engines on Pubmed (www.ncbi.nlm.nih.gov/pubmed) and Ensembl (www.ensembl.org) websites. Primers were designed for the chosen sequences using Primer3 Input software (version 0.4.0) and tested using Netprimer (www.premierbiosoft.com) and BLAST (http://blast.ncbi.nlm.nih.gov) search tools. These rated the primers and checked for possible primer dimers. DNA folding of the primers was investigated using Mfold software (http://mfold.rna.albany.edu) that showed the location and positioning of the primer on the
gene. This also gave the thermodynamic details of the primer and identification of folds or helices. Primers that were deemed satisfactory following the analysis were manufactured by Eurofins (www.eurofinsdna.com). To test the specificity and optimal concentrations of the new primers, PCR reactions were carried out using three primer concentrations: 50 nM, 300 nM and 900 nM.

DNA Gel
Following the initial PCR reactions to test the specificity of the primers and their optimal concentration, the in-house designed rabbit primers were further investigated using DNA gels. The PCR product was run on agarose gels to confirm that the weight of the product corresponded with the weight of the target of interest and also to check for any primer dimers produced. The gel was prepared by adding 2 g of agarose powder (Sigma-Aldrich) to 100 ml of 0.5% Tris-acetate-EDTA (TAE) buffer and swirled to mix. TAE stock was prepared by adding 54 ml of Tris base to 27.5 ml of boric acid and 20 ml ethylenediaminetetraacetic acid (EDTA), made up to 1 l with water and adjusted to pH 8. The gel was heated using a microwave oven until clear. 7 μl of ethidium bromide was added to the warm (but slightly cooled) gel and swirled to mix. The warm gel was poured into an electrophoresis rig that contained a comb to form wells. Once the gel had set, the comb was removed and 0.5% TAE buffer was added to completely submerse the gel. 5 μl of HyperLadderIV (Bioline) was pipetted into the first and last wells. The HyperLadderIV contains bands of known molecular weight and runs along either side of the samples. 3 μl of blue loading buffer was added to the 10 μl PCR product in the PCR plate and mixed by pipetting. The total volume (13 μl) was pipetted into a new well. This was repeated for the remaining samples. A negative electrode was connected to the rig at the sample end of the electrophoresis rig, and a positive electrode was connected to the opposite end. An electric current was passed through the gel at 130 V. As both DNA and ethidium bromide are negatively charged, they will run from the negative end towards the positive end. Heavier fragments of DNA travel slower through the gel matrix than shorter fragments. This can be seen as blue bands running through the gel. The current was continued until the bands reached the positive terminal. As the loading dye tends to leech out into the gel, the bands are more clearly visualised by imaging the gel under UV light. Ethidium bromide fluoresces when it binds to DNA fragments and is imaged using a digital camera. The DNA bands were compared to the position of the bands of the HyperLadderIV so that their weight could be determined.

2.3.4.2 Reference Gene Analysis
An important part of the qPCR analysis is normalising the target gene expression to a reference gene(s) expression. A reference gene should be stably and equally expressed across all the samples and all experimental conditions. The most appropriate gene can
vary in different tissues, species, models and conditions and, therefore, analysis must be carried out for separate experimental sets. To do this, geNorm kits (PrimerDesign) containing primers for six common reference genes were used. In a particular model, the expression of the six genes was analysed in all samples and the Ct values investigated using qBase software (Biogazelle). The programme uses mathematical formulae to find which reference gene, or which combination of genes, is the most stable throughout the samples. The qBase analysis is displayed in two graphs. The first is a line graph demonstrating a ‘geNorm M’ score on the y-axis, for each of the genes analysed, on the x-axis. The geNorm M score is a measure of the stability of the genes expression; the lower the score, the more stable the expression. The second graph displays a ‘geNorm V’ score on the y-axis for the optimal number of reference genes on the x-axis. The higher the geNorm V score, the better that combination of reference genes.

GeNorm kits were used only for the rat models, as a kit specific for rabbit samples was not available. For the rabbit model, only two in-house designed reference genes were analysed, 28S and GAPDH. The Ct values were analysed with StatMiner software (Integromics). Similar to the qBase software, following analysis of the stability of the genes, a stability score was produced; the lower the score, the more stable the reference gene.

2.3.5 qPCR Analysis

The SDS software produces two graphs of particular interest. The first is an amplification plot that displays the cycle number on the x-axis and the fluorescence (amount of PCR product) on the y-axis. The amount of PCR product increases exponentially with increasing cycle number, and plateaus when either all the PCR product or all the dye has been depleted (Figure 2.11). The Ct is fixed along the linear phase of the graph. The second graph is the melt curve (or dissociation curve), which displays the rate of change of fluorescence, on the y-axis, over time, on the x-axis (Figure 2.12). This plot is useful for checking the specificity of the reaction. This is particularly important when using SYBR green dye as it non-specifically binds to all double-stranded DNA. Ideally, the melt curve consists of a single, smooth, narrow and symmetrical peak at round 80-95°C. However, if there are primer dimers or genomic DNA contamination, a distortion of the graph or second peak can be produced (Figure 2.13).
Figure 2.11 Representative image of an amplification plot. The PCR product increases exponentially with increasing cycle number, until all the product or dye is depleted (the plateau phase).
Figure 2.12 Representative image of a melt curve. Ideally in specific PCR reactions, the melt curve consists of a single, smooth, narrow and symmetrical peak at round 80-95°C.
**Figure 2.13 Example of a primer dimer.** The melt curve can be used to identify primer dimers, often displayed as a double peak instead of a large, single peak.

**Efficiency**

Theoretically, in a perfect PCR reaction, each amplified sequence should produce two identical copies. However, this is often not the case and the number produced is less than two. Therefore, the efficiency of the primer was calculated using one of two methods. For both methods, the efficiency was calculated using raw amplification data calculated by the SDS software. This data consisted of individual fluorescent values detected at each cycle for all the samples. The first method calculated the efficiency using the equation:

\[
\text{efficiency} = 2\sqrt{\frac{a}{b}}
\]

Where \(a\) is the amount of fluorescence detected at the threshold cycle number (the Ct) and \(b\) is the amount of fluorescence detected two cycles following the Ct. If the primer was 100% efficient, it produced an efficiency of 2, however, a value of 1.7 or above was considered acceptable.
The second method calculated efficiency using LinRegPCR software (developed by the Academic Medical Centre, Amsterdam). The programme uploads the raw amplification data and calculated the efficiencies using various mathematical formulae.  

The efficiency of all individual samples was averaged to calculate the mean primer efficiency. As the efficiency can change in different PCR runs, primer efficiency was calculated for each PCR plate.

**Relative Abundance of mRNA**

To measure the relative abundance of mRNA, \( \Delta C_t \) was calculated using the equation:

\[
\Delta C_t = \text{efficiency} C_t
\]

The \( \Delta C_t \) of the sample was then normalised to the reference gene \( \Delta C_t \):

\[
\text{relative abundance} = \frac{\text{reference gene } \Delta C_t \text{ / sample } \Delta C_t}
\]

**Outlier Analysis**

Anomalies can occur for a number of reasons including pipetting error and improper amplification of the PCR product. In order to prevent these results from distorting the true differences in mRNA levels, outlier analysis was preformed. The median of absolute deviation (MAD test) was used to calculate any outliers. Essentially, the MAD test measures the variability of an individual value from the median of the group. A threshold is set, and if the value exceeds this threshold it is considered an outlier and was excluded from further analysis.

**2.4 STATISTICAL ANALYSIS**

Student’s t-tests, Mann-Whitney Rank Sum tests and one way ANOVA tests were carried out using SigmaPlot software (Systat Software Inc). P values less than 0.05 were considered statistically significant.
CHAPTER 3: RAT PULMONARY ARTERIAL HYPERTENSION MODEL; DEVELOPMENT AND FUNCTIONAL STUDIES

3.1 INTRODUCTION

Although the rat monocrotaline-induced pulmonary arterial hypertension (PAH) model is a well-established model,\textsuperscript{137-139} it had not been previously used at the University of Manchester. The model was developed along with Dr. Ian Temple, a British Heart Foundation Clinical Research Fellow. All work shown in chapter three was carried out along with Dr. Temple (I did half of all work). Initial experiments failed to result in the required phenotype of PAH. There were no alterations to ECG recordings and echocardiography revealed no changes in pulmonary arterial velocity or right ventricular dimensions. In addition, no differences in weights or symptoms of heart failure were noted. With assistance from Professor Ed White at the University of Leeds, who has successfully established the model in his group, changes to the experimental setup were applied. The changes included using Wistar rats instead of Sprague Dawley rats, using younger rats of 200 g as opposed to 300 g, and dissolving the monocrotaline powder in hydrochloric acid instead of saline. Additionally, the final monocrotaline solution was given by intraperitoneal injection instead of subcutaneous injection. Following the modifications, the animals developed the symptoms and echocardiography changes of PAH as described previously in the literature.

The effect of monocrotaline on the rats was not consistent; the severity and time point of development of PAH varied between rats. Therefore, there had to be careful monitoring of the animals, particularly after day 21 following monocrotaline injection. In addition, although the majority of rats developed the disease, a small number (3 out of 26 injected with monocrotaline) did not show signs of PAH by day 28.

3.2 RESULTS: DEVELOPMENT OF THE PULMONARY ARTERIAL HYPERTENSION MODEL

3.2.1 Body Weights

The rats were weighed prior to injection to establish the injection volume of either 60 mg/kg body weight monocrotaline or saline. The body weights were normally distributed at the start of the experiment. Following this, the rats’ weights were recorded at least once a week. In the final week, from days 21 to 28, the rats were weighed daily to monitor weight gain or loss. In accordance with Home Office regulations, rats were culled if they lost more than 10 g over 48 hours. The experiment was terminated at day 28 even if they did not show signs of heart failure or weight loss.
All rats weighed approximately 200 g when injected with either monocrotaline or saline solution at day 0 (Figure 3.1). In both groups, the body weights continued to increase following injection, although the monocrotaline-injected rats increased at a slower rate (Figure 3.1). Two sets of results were analysed (Figure 3.1). The first set compares control rats and all rats injected with monocrotaline and includes the three rats that were injected but did not show signs of PAH or heart failure (Figure 3.1A). The second set compares control rats to the monocrotaline-injected rats that developed PAH, i.e. excluding the three rats that were non-symptomatic by day 28 (Figure 3.1B).

The body weights of both control and monocrotaline-injected rats (all and excluding non-PAH animals) peaked at day 26 (Figure 3.1). When non-PAH animals were excluded from the monocrotaline group, there was a small reduction in body weight on days 27 and 28 that was also seen in the control animals. (Figure 3.1B) The drop in body weight of the monocrotaline-injected rats with PAH is one of the clinical symptoms of PAH and right-sided heart failure. However, the weight loss of the control animals cannot be explained. None of the control rats developed signs of heart failure or dropped more than 10 g.
Figure 3.1 Time course of mean body weights. The body weights of the control and monocrotaline-injected rats from day 0 (injection day) towards day 28 (termination day) are shown. (A) All data including three monocrotaline-injected rats that did not develop PAH. (B) Selected data. Only monocrotaline-injected rats that developed signs of PAH are included. Control, n=25; monocrotaline-injected, n=23.
Figure 3.2 The change in body weight over time. The baseline body weight at time of injection (day 0) is taken as zero. Body weights at later times are with respect to this. (A) All data including three monocrotaline-injected rats that did not develop PAH. (B) Selected data. Only monocrotaline-injected rats that developed signs of PAH are included. Control, n=25; monocrotaline-injected, n=23.
If body weights are shown with respect to the initial body weight at the time of injection, the increase in body weight from this point onwards is shown (Figure 3.2)

As the project is concerned with the disease of PAH, only the rats that developed PAH were used for further investigation.

![Graph showing pre-termination body weights](image)

**Figure 3.3 Pre-termination body weights.** The graph shows mean ± SEMs for control (n=25) and monocrotaline-injected (n=23) animals. Mann-Whitney Rank Sum test; normality passed, equal variance failed. Asterisk, significantly different from control group (P<0.001).

At the time of termination, the monocrotaline-injected rats had a lower body weight (265 ± 4.9 g) than the control rats (310 ± 6.9 g). This was significantly different (P<0.001; Figure 3.3.)
3.2.2 Heart and Lung Weights

Immediately following excision of the heart and lungs, their wet weights were recorded. Figure 3.4 shows the wet weights as well as the heart:body weight and lung:body weight ratios.

![Figure 3.4 Heart and lung weights recorded immediately after excision.](image)

**Figure 3.4 Heart and lung weights recorded immediately after excision.** (A) The graph shows mean heart and lung weights ± SEMs for control and monocrotaline-injected animals (n=20). (B) Heart:body weight and lung:body weight ratios. Mann-Whitney Rank Sum test was used as either normality or equal variance failed. Asterisk, significantly different from the corresponding control group (P<0.005).

The monocrotaline-injected animals had a significantly increased heart weight, 1.60 ± 0.08 g, compared to the control animals, 1.28 ± 0.08 g (P=0.005; Figure 3.4A). This shows hypertrophy of the heart in the monocrotaline-injected animals. Lung weight was also significantly increased in the monocrotaline-injected animals; 2.49 ± 0.11 g compared to only 1.36 ± 0.04 g in the control animals (P<0.001; Figure 3.4A). An increase in lung weight indicates congestion and is therefore an indication of heart failure.

The enlarged heart and lungs of the monocrotaline-injected animals was further demonstrated by comparing the heart and lung weights to the body weights. The monocrotaline-injected rats had significantly increased heart weight:body weight ratio, 0.59 ± 0.03 %, compared to the controls at 0.41 ± 0.03 % (P<0.001; Figure 3.4B). The increased lung weight:body weight ratio of the monocrotaline-injected rats, 0.93 ± 0.005 %, was significantly different to that of the controls, 0.44 ± 0.02 % (P<0.001) (Figure 3.4B).
3.2.3 In Vivo ECG

Three lead ECG recordings were taken prior to termination to investigate electrophysiological changes in the heart. The rats were anaesthetised with 2% isoflurane and an ECG was recorded for 5 min. Typical recordings are shown in Figure 3.5.

Figure 3.5 In vivo ECG recordings in anaesthetised animals. (A and B) Representative pre-termination ECG traces from (A) control and (B) monocrotaline-injected rats. Traces shown are averaged data.
Figure 3.6 Mean ECG measurements calculated from in vivo recordings in anaesthetised rats. The graphs show the means ± SEMs for control (n=25) and monocrotaline-injected (n=22) animals. For RR, HR, PR and QRS intervals, statistical differences between groups were determined using Student’s t-test. For P duration, QT and QTc Mann-Whitney Rank Sum test was used (Normality test failed). Asterisk, significantly different from the corresponding control group (P<0.005). Abbreviations: beats per minute, bpm; heart rate, HR; corrected QT interval, QTc.
In vivo ECG recordings revealed a significantly increased RR interval in the monocrotaline-injected rats, 159 ± 2 ms, compared to 144 ± 2 ms in the control rats (P<0.001; Figure 3.6A). This slowing of sinoatrial node (SAN) activity was also demonstrated as a significant reduction in heart rate; the monocrotaline-injected rats had a heart rate of 379 ± 6 beats per minute (bpm) compared to 419 ± 5 bpm in the control rats (P<0.001; Figure 3.6B).

P wave duration is an indication of action potential conduction through the atria. In the monocrotaline-injected rats, the mean P wave duration was 14.1 ± 0.9 ms, which was not significantly different to that of control rats, 16.1 ± 0.7 ms (P=0.082; Figure 3.6C). PR interval is a measure of the conduction of the action potential from its initiation at the SAN through the atroventricular node (AVN) to the ventricles. There was no significant difference between the mean PR interval of the monocrotaline-injected rats, 46.8 ± 0.8 ms, and the control rats, 45.9 ± 0.8 ms (P=0.470; Figure 3.6D).

The QRS complex duration is an indication of action potential conduction through the ventricles, and can give an indication of Purkinje fibre function. The mean QRS duration of the monocrotaline-injected rats was 14.6 ± 0.5 ms, which was not significantly different to the mean duration in control rats, 15.6 ± 0.5 ms (P=0.202; Figure 3.6E). The QT interval is a representation of the depolarization and subsequent repolarization of the ventricle. In the monocrotaline-injected rats, the QT interval was prolonged, which can be seen in the representative ECG traces (Figure 3.5). The mean QT interval for the monocrotaline-injected rats was 106.1 ± 2.5 ms, which was twice the mean interval for control rats, 50.9 ± 1.6 ms (P<0.001; Figure 3.6F). Similarly, the corrected QT interval (QTc), which has been corrected for heart rate, was 266.0 ± 4.8 ms in the monocrotaline-injected rats, almost twice that of the control rats, 134.1 ± 3.7 ms (P< 0.001; Figure 3.6G).
3.2.4 In Vivo Echocardiography

While the animals were anaesthetised for in vivo ECG recordings, echocardiography measurements were also taken to investigate heart function.

3.2.4.1 Left Ventricular Measurements

Figure 3.7 Representative parasternal short axis echocardiography images taken pre-termination from control (A) and monocrotaline-injected (B) animals. Abbreviations: left ventricle, LV; and right ventricle, RV.
To measure left ventricular dimensions, parasternal short axis images were taken (Figure 3.7). From this view, the large circular left ventricle could be identified, along with the crescentic right ventricle, as demonstrated in the control example (Figure 3.7A). Pre-termination, the monocrotaline-injected rats had a much larger right ventricle than the controls. The chamber was so dilated that it bulged into the left ventricle (Figure 3.7B).

Figure 3.8 Summary of pre-termination echocardiography left ventricular measurements. A-C, changes measured during diastole (d) and systole (s) of left ventricular anterior wall thickness (LVAW; A), left ventricular posterior wall thickness (LVPW; B) and left ventricular internal diameter (LVID; C). The graphs show the means ± SEMs for control (n=14) and monocrotaline-injected (n=12) animals. Statistical differences between groups were determined using Student’s t-test (normality and equal variance tests passed). Asterisk, significantly different from the corresponding control group (P<0.005).
At the parasternal short axis (Figure 3.8), M-mode was used to measure left ventricular dimensions. The same M-mode position was used for both diastolic and systolic measurements. There were no significant changes in left ventricular anterior wall thickness (LVAW) between the two groups. In diastole, the mean LVAWd for control animals was $0.162 \pm 0.008$ cm and the mean for monocrotaline-injected animals was $0.186 \pm 0.012$ cm ($P=0.113$; Figure 3.8A). In systole, the mean LVAWs for control animals was $0.277 \pm 0.017$ cm and the mean for monocrotaline-injected animals was $0.287 \pm 0.013$ cm ($P=0.664$; Figure 3.8A).

Correspondingly, there were no significant differences in the measurements of the left ventricular posterior wall thickness (LVPW). In diastole, the mean LVPWd for control animals was $0.189 \pm 0.015$ cm and the mean for monocrotaline-injected animals was $0.194 \pm 0.011$ cm ($P=0.796$; Figure 3.8B). In systole, the mean LVPWs for control animals was $0.280 \pm 0.012$ cm and the mean for monocrotaline-injected animals was $0.285 \pm 0.023$ cm ($P=0.850$; Figure 3.8B).

Due to the increased size of the right ventricle, there was a reduction in the internal diameter of the left ventricle (LVID; Figures 3.7B and 3.8C). In diastole, the mean LVIDs for control animals was $0.673 \pm 0.023$ cm, compared to a mean of only $0.444 \pm 0.033$ cm for monocrotaline-injected animals ($P<0.001$; Figure 3.8C). In systole, the mean LVIDd for control animals was $0.322 \pm 0.024$ cm, compared to only $0.189 \pm 0.021$ cm for monocrotaline-injected animals ($P<0.001$; Figure 3.8C).
3.2.4.2 Pulmonary Artery Measurements

To investigate the pulmonary artery, the echocardiography transducer was moved proximal to the level of the aortic valve. Here, pulse wave Doppler was used to obtain a profile of the velocity of the blood through the pulmonary valve (Figure 3.9).

![Figure 3.9](image)

**Figure 3.9** Representative pulse wave Doppler profiles of pulmonary artery velocity at pre-termination from control (A) and monocrotaline-injected (B) animals. Abbreviations: maximum pulmonary artery velocity, PVmax; pulmonary artery acceleration, PAA; pulmonary artery deceleration, PAD.

Under normal conditions, the pulmonary artery velocity profile is large, slow and symmetrical, as seen in the image from a control animal in Figure 3.9A. However, when there is high pulmonary pressure, the profile changes. As a result of increased pulmonary pressure, there is a reduction in the peak pulmonary velocity as the blood is being pumped against a greater resistance. The profile becomes smaller and sharper (shorter duration). When the monocrotaline-injected rats developed pulmonary arterial hypertension, this change in profile was seen (Figure 3.9B).
Figure 3.10 Mean pre-termination measurements of maximum pulmonary velocity (PVmax; A), pulmonary artery acceleration time (PAA; B) and pulmonary artery deceleration (PAD; C). The graphs show the means ± SEMs for control (n=14) and monocrotaline-injected (n=12) animals. For PVmax, statistical differences between groups were determined using Student’s t-test. For PAA and PAD Mann-Whitney Rank Sum test was used (equal variance test failed). Asterisk, significantly different from the corresponding control group (P<0.005).

The mean maximum pulmonary artery velocity (PVmax) of the monocrotaline-injected rats was 0.646 ± 0.068 m/s, which was significantly less than the mean PVmax of control rats of 1.073 ± 0.035 m/s (P<0.001; Figure 3.10A). The monocrotaline-injected rats also displayed a significant reduction in the pulmonary artery acceleration time, 14.6 ± 0.6 ms, compared to 31.3 ± 2.4 ms of the control rats (P<0.001; Figure 3.10B). The monocrotaline-injected rats had an increased pulmonary artery deceleration (PAD), 35.6 ± 2.8 m/s², which was significantly greater than the value of 15.4 ± 1.4 m/s² for the control rats (P<0.001; Figure 3.10C). This reduction in maximum velocity, reduction in acceleration time and increase in deceleration is in keeping with pulmonary arterial hypertension.¹⁴²
3.2.4.3 Right Ventricular Measurements

The right ventricle was investigated using the parasternal long axis view.

Figure 3.11 Representative parasternal long axis echocardiography images taken pre-termination from a control rat heart (A) and a monocrotaline-injected rat heart (B). Abbreviations: right ventricle, RV; and left ventricle, LV.
In control hearts, the large left ventricle could be easily identified with the smaller, narrow right ventricle above it (Figure 3.11A). Pre-termination, the right ventricle of the monocrotaline-injected rats was greatly dilated and protruded into the left ventricle. Consequentially, the right ventricle could be easily identified, whereas the left ventricle could not (Figure 3.11B).

Figure 3.12 Representative M-mode images through the parasternal long axis of the right ventricle used to measure right ventricular wall thickness (RVWT) and right ventricular internal diameter (RVID). A and B, images of control (A) and monocrotaline-injected (B) rat hearts taken pre-termination.

M-mode was used to measure right ventricular dimensions through the parasternal long axis. As with the left ventricle measurements, the same M-mode position was used for both diastole and systole. In the control rat hearts, the contraction and relaxation of the ventricles during systole and diastole, respectively, could be easily seen (Figure 3.12A). However, the dilated right ventricle in the monocrotaline-injected rat did not contract efficiently during systole; Figure 3.12B shows little moment of the right ventricle free wall in systole. This is expected to result in a reduced fractional shortening in the monocrotaline-injected rats.
Figure 3.13 Mean pre-termination measurements of right ventricular dimensions in control and monocrotaline-injected rats. A, right ventricular wall thickness (RVWT). B, right ventricular internal diameter (RVID). M-mode though the parasternal long axis was used to make measurements in diastole (d) and systole (s). The graphs show the means ± SEMs for control (n=14) and monocrotaline-injected (n=12) animals. For RVWTd and RVIDd, statistical differences between groups were determined using Student’s t-test. For RVWTs and RVIDs Mann-Whitney Rank Sum test was used (equal variance test failed). Asterisk, significantly different from the corresponding control group (P<0.005). ‘a’, significant difference between RVIDd and RVIDs in controls; ‘b’, significant difference between RVIDd and RVIDs in monocrotaline-injected; and ‘c’, significant difference between RVWTd and RVWTs in controls (all three carried out using Student’s t-test).

In control rats there was a significant increase in right ventricular wall thickness (RVWT) and a significant decrease in right ventricular internal diameter (RVID) from diastole to systole (P<0.001 for both; Figure 3.13). In the monocrotaline-injected rats there was also a significant decrease in RVID from diastole to systole (P=0.035), however, there was no significant difference in RVWT from diastole to systole (P=0.506).

Right ventricular wall thickness in diastole (RVWTd) in monocrotaline-injected rats was 0.14 ± 0.01 cm and was significantly greater than that of the control rats, 0.09 ± 0.01 cm (P<0.001; Figure 3.13A). The right ventricular wall thickness in systole (RVWTs) in monocrotaline-injected rats was 0.15 ± 0.01 cm, which was also greater than the control value, 0.12 ± 0.01 cm, but was not significantly different (P=0.083; Figure 3.13A). The right ventricle in the monocrotaline-injected rats was dilated as demonstrated by an increase in right ventricular internal diameter (RVID). In diastole, the mean RVIDd was 0.38 ± 0.04 cm in monocrotaline-injected rats, which was significantly greater than 0.28 ±
0.02 cm measured in control rats (P=0.023; Figure 3.13B). In systole, the mean RVIDs was 0.27 ± 0.03 cm in monocrotaline-injected rats, which was more than double the mean RVIDs in control rats, 0.12 ± 0.01 cm (P<0.001; Figure 3.13B).

![Figure 3.14 Percentage change in left ventricular internal diameter (LVID, A) and right ventricular internal diameter (RVID, B) from diastole to systole. Blue dots represent individual control percentage changes and red dots represents individual monocrotaline-injected percentage changes. Grey dots indicate the mean ± SEMs. Statistical differences between groups were determined using Student's t-test (normality and equal variance were passed). Asterisk, significantly different from corresponding control group (P<0.001).](image)

The data were analysed to determine the percentage change in internal diameter of the ventricles from diastole to systole (Figure 3.14). There was no significant difference between the percentage change in the left ventricular internal diameter (LVID) from diastole to systole in control and monocrotaline-injected rats: in control rats the LVID reduced by 52.6% ± 2.5, and in the monocrotaline-injected rats the LVID reduced by 56.9% ± 4.9 (Figure 3.14A; P=0.426). However, the monocrotaline-injected rats had a significantly reduced percentage change in right ventricular internal diameter (RVID) from diastole to systole, 30.21% ± 4.3, compared to 55.8% ± 3.4 in the control rats (Figure 3.14B; P<0.001).

This data suggest that the right ventricle of the monocrotaline-injected rats did not contract as effectively as that seen in control rats. This is further illustrated in the representative M-mode image through the right ventricle (Figure 3.12B). A reduction in the efficiency of right ventricular contraction suggests a reduction in fractional shortening in these animals.
On the other hand, there was not a significant difference in the percentage change in LVID from diastole to systole between the two groups (Figure 3.14A). This indicates that there is little or no dysfunction or reduction in fractional shortening of the left ventricle in the monocrotaline-injected animals.

3.3 RESULTS: *EX VIVO FUNCTIONAL EXPERIMENTS*

After the heart was excised and weighed, it was immediately cannulated and connected to a Langendorff setup to carry out further electrophysiological experiments. As the heart was separated from the autonomic nervous system, intrinsic measurements could be obtained.
3.3.1 *Ex Vivo* ECG

Figure 3.15 Mean ECG measurements from *ex vivo* recordings from Langendorff-perfused rat hearts. The graphs show the means ± SEMs for control (n=11) and monocrotaline-injected (n=12) animals. Statistical differences between groups were determined using Student’s t-tests, with the exception of QRS interval (Mann-Whitney Rank Sum tests was used; normality test failed). Asterisk, significantly different from the corresponding control group (P<0.005). Abbreviations: beats per minute, bpm; heart rate, HR; corrected QT interval, QTc.
Two ECG electrodes were placed on the surface of the heart, one at the apex of the ventricles and the other on the right atrium. ECG recordings were taken for 5 min. In contrast to the in vivo recordings, the ex vivo Langendorff-perfused monocrotaline-injected hearts did not demonstrate a bradycardia (Figure 3.15A and B). The mean RR interval of monocrotaline-injected rats was 242 ± 7 ms, which was not significantly different to the mean RR interval of control rats 238 ± 13 ms (P=0.767; Figure 3.15A). The heart rates of the two groups were also similar; the mean heart rate of the monocrotaline-injected rats was 255 ± 12 bpm and the mean heart rate of the control rats was 255 ± 8 bpm (P=0.997; Figure 3.15B).

As in the case of the in vivo measurements, there were no significant differences in either the P wave duration or the PR interval (Figure 3.15C and D). The mean P wave duration of the monocrotaline-injected rats was 15.9 ± 1.1 ms and the mean P wave duration of the control rats was 13.6 ± 1.0 ms (P=0.143; Figure 3.15C). The mean PR interval of the monocrotaline-injected rats was 43.9 ± 1.4 ms and the mean PR interval of the control rats was 42.6 ± 1.3 ms (P=0.522; Figure 3.15D).

The QRS, QT and QTc interval changes were in accordance with those seen in the in vivo recordings. The mean QRS interval was 15.7 ± 2.5 ms in the monocrotaline-injected rats and was not significantly different from the mean value of 14.0 ± 1.1 ms in the control rats (P=0.735; Figure 3.15E). There was a significant increase in the mean QT interval of the monocrotaline-injected rats, 136.0 ± 8.7 ms, compared to 59.5 ± 4.8 ms in the control rats (P<0.001; Figure 3.15F). The mean QTc interval in the monocrotaline-injected rats was 277.7 ± 16.9 ms, which was more than double the interval of 122.2 ± 9.5 ms in the control rats (P<0.001; Figure 3.15G).

3.3.2 Langendorff Perfusion Experiments

To investigate the electrophysiological properties of the hearts further, pacing protocols were carried out on the Langendorff-perfused hearts. A stimulation electrode was placed on the right atrium, close to where the SAN is expected to be.
Figure 3.16 Electrophysiological measurements from Langendorff-perfused rat hearts. The graphs show the means ± SEMs for control (n=11) and monocrotaline-injected (n=12) animals. For Wenckebach, AERP and VERP, statistical differences between groups were determined using Student’s t-test. For the remaining measurements Mann-Whitney Rank Sum test was used (normality test failed). Asterisk, significantly different from the corresponding control group (P<0.005). Abbreviations: sinoatrial node recovery time, SNRT; atrial effective refractory period, AERP; atrioventricular node effective refractory period, AVERP; and ventricular effective refractory period, VERP.
Initially, the atrial threshold for pacing was found for each heart. The threshold is the minimum point at which 1:1 pacing is established; in other words, for every pacing stimulus there was an action potential produced. The mean atrial threshold for the monocrotaline-injected rats was $3.9 \pm 0.7$ V, which was not significantly different to the threshold of $3.5 \pm 0.8$ V for the control rats ($P=0.878$; Figure 3.16A).

The SAN recovery time (SNRT) is a measure of SAN function. Following burst pacing, the time taken for the first intrinsic P wave is regarded as the SNRT. The SNRT was similar for both groups; the mean SNRT of the monocrotaline-injected rats was $304.6 \pm 19.8$ ms, compared to $305.1 \pm 18.3$ ms for the control rats ($P=0.667$; Figure 3.16B).

The atrial effective refractory period (AERP) was measured. The ERP is the period of time in which a new action potential cannot be produced in that tissue; it is inexcitable. The mean AERP of the monocrotaline-injected hearts was $42.6 \pm 4.9$ ms, which was greater than the mean value of $35.9 \pm 2.2$ ms of the control hearts, however they were not significantly different ($P=0.216$; Figure 3.16C). The ERP of the AVN, the AVERP, was also measured as a way of investigating AVN function. There was a significant difference in the AVERP between the monocrotaline-injected rats, $93.7 \pm 2.5$ ms, and the control rats, $87.0 \pm 1.2$ ms ($P=0.035$; Figure 3.16D).

A further measure of AVN conduction is the Wenckebach cycle length. The hearts were paced with reducing stimulus intervals until a QRS complex was dropped, indicating that AVN conduction failed. The largest pacing interval that induced this block is the Wenckebach cycle length. The mean Wenckebach cycle length measured in the monocrotaline-injected rats was $116.3 \pm 2.8$ ms and was not significantly different to the mean Wenckebach cycle length in the control rats, $111.6 \pm 2.5$ ms ($P=0.230$; Figure 3.16E).

To investigate the electrophysiological properties of the ventricles, the stimulating electrode was moved to the ventricles. As for the atria, the threshold was found for each heart. Like the atrial threshold, although the mean ventricular threshold of the monocrotaline-injected rats, $3.0 \pm 1.3$ V, was higher than the mean threshold of the control rats, $2.4 \pm 1.4$ V, it was not significantly different ($P=0.534$; Figure 3.16F). Also noted was that the ventricles of three out of the nine monocrotaline-injected hearts stimulated failed to capture, even at the maximum voltage of 99 V. The data was reanalysed, replacing the three rats with non-capturing ventricles with threshold values of 99 V (Figure 3.16G). The mean ventricular threshold for the monocrotaline-injected rats increased to $35.0 \pm 16.0$ V and, although the P value was reduced, it was still not significantly different to the control threshold of $2.4 \pm 1.4$ V ($P=0.122$; Figure 3.16G)
The ventricular ERP (VERP) was calculated using a similar pacing protocol to AERP and AVERP. The VERP of the monocrotaline-injected hearts was 97.6 ± 8.5 ms and this was significantly different to the value of 43.1 ± 3.6 ms for the control hearts (P<0.001; Figure 3.15H).

Pacing protocols were also carried out to investigate vulnerability to arrhythmias. Briefly, the atria and ventricles were burst paced to stress them and reveal any vulnerability to arrhythmias. To measure AF threshold, trains of S1S1 burst pacing were applied to the atria for 3 s. Each train consisted of a reducing gap between the S1S1 pulses from 100 ms to 10 ms, in 10 ms intervals. To measure ventricular fibrillation (VF) / ventricular tachycardia (VT) threshold, the ventricle was first paced at 6 Hz for 20 s (166 ms interval between each S1 pulse) and then paced at 50 Hz for 1 s (20 ms interval between each S1 pulse). This was repeated three times. If VF or VT was not induced, the protocol was repeated, but the intensity of the stimulus was increased by increasing the voltage.

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<th>Control rats</th>
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<tr>
<td>AF</td>
<td>7/7 (100%)</td>
<td>6/9 (66.7%)</td>
</tr>
<tr>
<td>Mean number of episodes</td>
<td>5.5</td>
<td>2.6</td>
</tr>
<tr>
<td>VF/VT</td>
<td>3/7 (42.9%)</td>
<td>1/6 (16.7%)</td>
</tr>
<tr>
<td>Mean number of episodes</td>
<td>2.3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of the number of animals that demonstrated arrhythmias following stimulation protocols. Abbreviations: atrial fibrillation, AF; ventricular fibrillation, VF; and ventricular tachycardia, VT.

Surprisingly, the hearts of monocrotaline-injected rats did not show any increased susceptibility to arrhythmias (Table 3.1). All of the hearts from control rats went into atrial fibrillation (AF) following stimulation protocols, and only six of the nine monocrotaline-injected hearts (66.7%) went into AF (Figure 3.17). The mean number of episodes per animal was 5.5 for the control animals, twice that of the monocrotaline-injected animals (2.6 episodes). Three out of seven hearts from control rats (42.9%) went into ventricular fibrillation (VF) or ventricular tachycardia (VT), compared to only one of the six hearts from monocrotaline-injected rats (16.7%; Figure 3.18). The mean number of episodes was 2.3 and 2 for control and monocrotaline-injected rats, respectively.
Figure 3.17 Example of atrial fibrillation, following burst pacing, from a control rat.

Figure 3.18 Example of ventricular fibrillation, following burst pacing, from a monocrotaline-injected rat.
3.4 Summary

Following an intraperitoneal injection of 60 mg/kg body weight monocrotaline, the majority of rats developed PAH and subsequent right-sided heart failure within 28 days following injection. The monocrotaline-injected rats had a lower pre-termination body weight, in addition to increased heart and lung weights. Echocardiography confirmed development of PAH in the monocrotaline-injected rats by reduced pulmonary artery maximum velocity and changes to the pulmonary artery velocity profile. Echocardiography also demonstrated hypertrophy and dilation of the right ventricle in these animals. Pre-termination *in vivo* ECG showed a bradycardia and a prolongation of the QT interval in the monocrotaline-injected animals, compared to the control animals.

*Ex vivo* ECG recordings, which removes autonomic nervous system innervation, did not show a bradycardia in the monocrotaline-injected rats, but only a prolonged QT interval. Further *ex vivo* functional analysis revealed a significantly increased AVERP and VERP in the monocrotaline-injected rats, compared to control rats. The ventricles in three out of the nine monocrotaline-injected hearts stimulated failed to capture at maximum stimulus amplitude. Surprisingly, an increased vulnerability to atrial or ventricular arrhythmias was not found in the monocrotaline-injected rats, compared to control rats.

3.5 Discussion

The monocrotaline-injected rat model of PAH has been used extensively for decades and it remains a popular choice of model for investigating this disease. The protocol consists of a single injection of monocrotaline, usually 60 mg/kg body weight, and is non-invasive and relatively cheap to produce.

Due to the numerous studies investigating PAH using the monocrotaline-induced model, the characteristics of the model have been widely reported. Monocrotaline appears to directly target the vasculature of the lungs, causing increased muscularisation and hypertrophy of the pulmonary vessels.\textsuperscript{141, 147} This results in increased pulmonary resistance, and causes the blood flow from the right ventricle to the lungs, via the pulmonary artery, to be under higher pressure. To cope with the increased pressure, the right ventricle must adapt and remodel to maintain the cardiac output to the lungs. Like the left ventricle in left-sided heart failure, the right-ventricle dilates and becomes hypertrophied.\textsuperscript{139, 147, 148} In a previous study, right ventricular hypertrophy correlated with the increase in pulmonary artery pressure.\textsuperscript{147} For a time, this remodelling appears to work as the ventricle can compensate for the increased pressure and resistance. However, as the pressure in the right ventricle is not alleviated, over time the chamber begins to fail.
The remodelling that was initially compensatory becomes decompensatory as the ventricle struggles to cope.

3.5.1 Heart and Lung Weight
An increase in heart weight, an indicator of hypertrophy, was found in the monocrotaline-injected animals. This has been shown in other studies as well.\textsuperscript{130, 141, 149} Specifically, an increase in ‘dry’ right ventricular weight has been noted in other studies, which is possibly a more accurate measure of mass because it excludes any possible water gain as a result of oedema.\textsuperscript{139} Increased lung weight was also measured in monocrotaline-injected rats compared to control rats. This is indicative of pulmonary remodelling as has also been shown in other investigations.\textsuperscript{130, 141, 149, 150}

3.5.2 Echocardiography
Echocardiography is a useful and accurate method to investigate changes to both the pulmonary atrial blood flow and right ventricular dimensions in the rat monocrotaline-induced PAH model.\textsuperscript{129, 142} In this study, echocardiography showed a reduction in maximum velocity through the pulmonary artery in the monocrotaline-injected rats, which was used as an indication of increased resistance in the vessel. Alterations to the profile of the pulmonary artery velocity, measured by pulse-wave Doppler, is indicative of PAH.\textsuperscript{129, 142} In controls, the pulmonary arterial blood velocity is high and the profile of the pulmonary arterial blood velocity is symmetrical and relatively long-lasting. However, due to the increased resistance of the pulmonary vasculature in PAH, the profile becomes smaller, sharper and shorter.\textsuperscript{129, 142, 151} This change in profile is measured as a reduction in pulmonary artery acceleration time and an increase in the pulmonary artery deceleration, and in addition, these alterations are found to correlate with the severity of PAH.\textsuperscript{142}

Echocardiography also demonstrated the development of right ventricular hypertrophy and dilatation in the monocrotaline-injected rats. Particularly in the later stages of the disease development, the increased dimensions of the right ventricle were clear in comparison to the controls. Echocardiography confirmed that there were no differences in wall thickness of the left ventricle in monocrotaline-injected rats, compared to control rats. This has also been reported in another study using this model.\textsuperscript{139} There have been conflicting reports on whether there is dysfunction of the left side of the heart in this model. A previous study found no changes to the left ventricular pressure in the monocrotaline-injected animals.\textsuperscript{129} Conversely, a study by Correai-Pinto et al. reported dysfunction of the left ventricle in the monocrotaline-injected rats; however, this was six weeks following administration of the drug.\textsuperscript{152} No changes in left ventricular function were noted at four weeks following monocrotaline injection in the same study.\textsuperscript{152} Hardziyenka et
also saw a reduction in left ventricular function, as measured by a reduced cardiac output and ejection fraction, in monocrotaline-injected rats.\textsuperscript{153}

3.5.3 Time Course of Changes
The time of onset of right ventricular heart failure was variable among rats. The experimental end points were used as a prediction of deterioration of the animals' health. In the later part of the experiment, from around day 21 to day 28, the animals were closely monitored, as sudden cardiac death has been widely reported in this model.\textsuperscript{139, 141} The deterioration of health, onset of clinical symptoms of heart failure and/or weight loss usually occurred relatively suddenly; an animal could appear healthy one day and be ill the next. Previous studies have noted that spontaneous death of rats had followed weight loss.\textsuperscript{139, 141}

The development of reduced maximum pulmonary artery velocity and right ventricular dilatation and hypertrophy in the monocrotaline-injected animals as detected by echocardiography often occurred prior to onset of clinical symptoms. This supports the concept that the initial alterations to the right ventricle are compensatory before eventually becoming decompensatory.

Previously published data have shown that soon following injection of the monocrotaline, the medial hypertrophy of the pulmonary vessels occurs just prior to the increase in the pulmonary pressure and right ventricular hypertrophy.\textsuperscript{147} The vascular changes occur rapidly following injection and are more intense than the increase in pulmonary pressure, which is more gradual.\textsuperscript{147} This suggests that the increased pulmonary artery pressure and changes to the right ventricle are secondary to the monocrotaline insult on the endothelium of the pulmonary vessels.

3.5.4 Alterations in Electrical Activity
ECG analysis revealed alterations to the electrical activity of the heart in the monocrotaline-injected animals. In vivo ECG recordings revealed a bradycardia (Figure 3.6). Inconsistent changes in heart rate in this model have been reported. Some studies agree with the present study and reported a reduction in heart rate in monocrotaline-injected rats.\textsuperscript{148, 154} In a study by Benoist et al. a small reduction in heart rate was recorded in monocrotaline-injected rats, compared to control rats, but this was not significantly different.\textsuperscript{130} Other studies have shown no significant difference in heart rate.\textsuperscript{155, 156}

In vivo and ex vivo recordings did not show any significant differences in the P wave duration between the control and monocrotaline-injected rats. The P wave is a measure of
electrical conduction from the right atrium to the left atrium and is representation of atrial depolarization.\textsuperscript{17}

In this study, a prolongation of the QT interval and corrected QT interval (QTc) was found in the monocrotaline-injected rats. The QT interval is a measure of ventricular action potential duration. An increase in QT interval has also been seen in another study.\textsuperscript{149} In agreement with this work, Lee \textit{et al.} reported a prolongation of the QT and QTc intervals and no change in PR interval.\textsuperscript{149} However, they measured a reduction in QRS interval, which not seen in this study.\textsuperscript{149}

A prolongation of the QT interval has been seen in patients with PAH and has been associated with increased risk of ventricular arrhythmias.\textsuperscript{121, 124, 125} An increased QT interval is also linked to an increased risk of mortality and sudden death.\textsuperscript{122, 123}

\subsection*{3.5.5 Arrhythmias}

In this study, the monocrotaline-injected rats did not have a reduced threshold of initiation of either atrial or ventricular arrhythmias (Table 3.1). In addition, the monocrotaline-injected rats had less instances of arrhythmia than the control rats (Table 3.1). This is in contrast to previous data. A study by Umar \textit{et al.} found increased occurrence of VF in the monocrotaline-injected rats compared to the control rats.\textsuperscript{129} VT was often preceded by early-afterdepolarizations (EADs), which degenerated into VF.\textsuperscript{129} This group also found that the EADs emerged from the right ventricle and preceded left ventricular activation, suggesting it was the right ventricle responsible for the EAD-triggered activation of VF.\textsuperscript{129}

Increased episodes of VF in monocrotaline-injected rats were also found in a study by Benoist \textit{et al.}\textsuperscript{130} They reported that VF was induced at lower stimulus intensities in monocrotaline-injected rats than control rats, and once induced, the VF was much prolonged and more likely to be sustained.\textsuperscript{130} In this study, the same approach was applied to induce arrhythmias, but no susceptibility to VF was found in the monocrotaline-injected rats (Table 3.1).

It has previously been reported that isolated myocytes from the right ventricle, but not the left ventricle, of monocrotaline-injected rats have increased action potential duration compared to the control right ventricular myocytes.\textsuperscript{129, 151, 157} Benoist \textit{et al.} found, in addition to a prolongation of action potential in the right ventricle of monocrotaline-injected rats, also found a prolongation of the action potential in the left ventricle.\textsuperscript{130} However, the prolongation of the action potential in the left ventricle was not as great as that measured in the right ventricle.\textsuperscript{130}
The prolongation of the action potential could be explained, in part, by a downregulation of K⁺ channels, which are responsible for the repolarization phase of the action potential (see Introduction – Chapter 1 for more details). A reduction in mRNA levels of K⁺ channels (namely Kv1.2, Kv1.5, Kv4.2 and Kv4.3) has been found in the right ventricle of monocrotaline-injected rats.¹²⁹,¹³⁰,¹⁴⁹

Prolongation of the action potential has been extensively reported in left- and right-sided failure in both human and animal studies.¹³⁰,¹⁵⁷,¹⁵⁸ Action potential prolongation is associated with alterations to Ca²⁺ handling, including an increase in the intracellular Ca²⁺ transient.¹⁵⁷,¹⁵⁸ It is hypothesised that prolongation of the action potential and increased Ca²⁺ release are part of the adaptation process with onset of heart failure.¹⁵⁸ However, these modifications become decompensatory as the failure ensues.

### 3.5.6 Effective Refractory Period

Langendorff-perfusion revealed an increase in effective refractory period (ERP) of the AVN and ventricles of the monocrotaline-injected rats. The ERP is the period of time immediately after the start of an action potential during which a cell cannot be stimulated and is inexcitable. Under normal conditions, the ERP is a protective mechanism by controlling the timing of the next action potential. By preventing the action potential from occurring too soon or too frequently after the previous one, it protects against hyperactivity of the cell, which is proarrhythmic. The ERP is approximately the same as the action potential duration. Under normal conditions, there is a positive correlation between ERP and action potential duration.¹⁵⁹,¹⁶⁰ Alterations to the ERP/action potential duration ratio indicate changes to the repolarization and excitability of the tissue and can predispose to arrhythmias.¹⁶¹ However, the monocrotaline-injected rats in this study did not have a predisposition to arrhythmias.

A study by Hardziyenka et al. found that in the right ventricle of monocrotaline-injected rats, in addition to increased action potential duration, there is also a prolonged ERP.¹⁵³ The ERP increases in the left ventricle also, but not as much as measured in the right ventricle.¹⁵³ Hardziyenka et al. also reported that the right ventricle of the monocrotaline-injected rat also has increased conduction velocity.¹⁵³ Interestingly, in agreement with this study, an increase in ventricular fibrillation or ventricular tachycardia was not found.¹⁵³ The lack of arrhythmias seen in the monocrotaline-injected rats could partly be explained by the equation:

\[
\text{Wavelength} = \text{refractory period} \times \text{conduction velocity}
\]

Although conduction velocity was not measured in this study, if it was increased as seen by Hardziyenka et al. this would result in an increased wavelength. An increase in
wavelength reduces the occurrence of arrhythmias.\textsuperscript{162} If the wavelength is reduced, the wave propagation can rotate around an area of conduction block and reexcite the tissue. The smaller the wavelength, the greater number of wavelets that can circulate.\textsuperscript{162, 163} Therefore, longer wavelength reduces the number of wavelets and reduces the risk of re-entrant arrhythmias.

All control rats stimulated with burst pacing of the atria went into atrial fibrillation (Table 3.1). A reason for this unexpected occurrence could be a result of the short AERP measured in these animals (Figure 3.16C). In this study, the controls had an AERP of only $35.9 \pm 2.2$ ms, which is smaller than noted in previous studies.\textsuperscript{164-166} As discussed above, a shortened ERP would result in a smaller wavelength and would facilitate the occurrence of re-entrant arrhythmias, such as atrial fibrillation.
CHAPTER 4: RAT PULMONARY ARTERIAL HYPERTENSION MODEL; HISTOLOGY AND MOLECULAR STUDIES

4.1 INTRODUCTION

Chapter 3 describes the development of the rat monocrotaline-induced model of pulmonary arterial hypertension, and it also demonstrates the functional and electrophysiological characteristics of the model. In this chapter, the possibility of inflammation in the model is explored. This was investigated by histology, immunohistochemistry, TUNEL assay and quantitative PCR.

4.2 RESULTS: MASSON’S TRICROME STAINING

4.2.1 Atria

To investigate changes in tissue architecture in the model of pulmonary arterial hypertension, Masson’s trichrome staining was carried out. This staining technique stains myocytes pink/purple, nuclei dark blue/black and collagen blue. 10 μm thick sections, approximately 450 μm apart, were stained and imaged. To investigate the atria, long axis sections were used.

![Images of Masson's trichrome stained right atrium from control (A and C) and monocrotaline-injected animals (B and D). Sections were 10 μm thick and imaged at 40x magnification. Scale bar represents 50 μm. Arrows highlight examples of non-myocyte nuclei.](image-url)
Two out of the three right atria investigated from monocrotaline-injected rats contained small areas of cells that did not stain pink-purple, suggesting they are non-myocytes (Figure 4.1B and D). These areas were few and small in number and were not noted in the remaining right atrium from a monocrotaline-injected rat. The rest of the atria in these animals appeared normal, and was similar to that seen in three control animals (Figure 4.1A and C). No increase in collagen, identified by blue staining, was found in the right atria of the monocrotaline-injected animals.

![Figure 4.2 Representative images of the left atrium taken from control (A) and monocrotaline-injected (B) animals. Sections were 10 μm thick and imaged at 40x magnification. Scale bar equals 50 μm.](image)

The left atria of all three monocrotaline-injected animals were found to be similar to that of three control animals (Figure 4.2). Unlike the right atrium, no areas of non-myocyte nuclei were noted. Similarly, no increase in collagen was found.
4.2.2 Ventricles

To investigate histology of the ventricles, short axis sections were taken. The sections were approximately half way between the base and apex of the ventricles.

Masson’s trichrome staining of the right ventricle revealed proliferation of non-myocyte nuclei in all five monocrotaline-injected animals, similar to that seen in the right atria. However, the amount and distribution of these cells was much greater than that seen in the atria. In the right ventricle, the non-myocyte cells were distributed in one of two ways. Firstly, the cells were aggregated in pockets that appeared to be surrounded by ‘normal’ myocytes (Figure 4.3B). The second way was to be distributed between the strands of myocytes in a more random and less tightly packed manner (Figure 4.3D). This proliferation of non-myocytes was not seen in the right ventricle of five control animals (Figure 4.3A and C).
Figure 4.4 Representative image of a cross section through the ventricles of a monocrotaline-injected rat stained using the Masson’s trichrome method. (A) Low power image taken at 1.5x magnification; scale bar represents 1000 μm. (B, C and D) Three high power images of the right ventricle taken at 40x magnification; scale bar represents 50 μm. The location of these areas are labelled as ‘b’, ‘c’ and ‘d’, respectively, on the 1.5x magnification image (A). Arrows highlight examples of non-myocyte nuclei.

The areas of the non-myocyte nuclei in the monocrotaline-injected animals were located throughout the right ventricle (Figure 4.4). Examples of their locations include at the epicardium of the free wall of the right ventricle (Figure 4.4B and area ‘b’ of Figure 4.4A), the septal wall (Figure 4.4C and area ‘c’ of Figure 4.4A) and the mid-ventricular free wall (Figure 4.4D and area ‘d’ of Figure 4.4A). The amount and distribution of the non-myocyte nuclei varied between the different monocrotaline-injected animals (n = 5). Masson’s trichrome staining revealed no increase in collagen deposition in the right ventricle of the monocrotaline-injected rats.

Figure 4.5. Representative images of the left ventricle stained with the Masson’s trichrome technique from control (A) and monocrotaline-injected (B) animals. Images taken at 40x magnification; scale bar represents 50 μm.
The histology of the left ventricle was very similar in both five control and five monocrotaline-injected animals (Figure 4.5). Unlike the right ventricle, no proliferation of non-myocyte nuclei was found in the left ventricle. No difference in collagen was identified in the left ventricle.

4.3 RESULTS: HAEMATOXYLIN AND EOSIN (H&E) STAINING

Ventricular sister sections were also stained using an alternative stain, haematoxylin and eosin (H&E). H&E has been used by other investigators and was carried out to see if the results obtained using Masson’s trichrome are also applicable with this staining method.

![Figure 4.6. Examples of right ventricular sections stained with haematoxylin and eosin from control (A and C) and monocrotaline-injected (B and D) animals. Images taken at 40x magnification; scale bar represents 50 μm. Arrows highlight examples of non-myocyte nuclei.](image)

The H&E staining echoed what was seen with Masson’s trichrome staining; there was proliferation of non-myocyte cells in the right ventricle of five monocrotaline-injected rats (Figure 4.6). The non-myocyte cells were either in discrete patches surrounded by
myocytes (Figure 4.6B) or they were located between the strands of myocytes (Figure 4.6D). No non-myoce cells were observed in the right ventricle of five control animals.

Figure 4.7 Representative images of left ventricular sections stained with haematoxylin and eosin from control (A) and monocrotaline-injected (B) animals. Images taken at 40x magnification; scale bar represents 50 μm.

In keeping with the Masson’s trichrome staining, there were no differences noted between H&E stained left ventricles from five control and five monocrotaline-injected animals (Figure 4.7).
4.4 RESULTS: IMMUNOHISTOCHEMISTRY

4.4.1 Identification of SAN and AVN

In addition to studying the working myocardium, Masson’s trichrome staining was also used in conjunction with immunohistochemistry to identify the SAN and AVN.

Figure 4.8 Example of identification of the SAN in a control atrium. Masson’s trichrome staining was used (A and C) in conjunction with immunohistochemistry for HCN4 and Cx43 (B and D). HCN4 expression is in green and Cx43 expression is in red. (A and B) Mosaic of 3 x 3 images at 20x magnification; scale bar represents 200 μm. (C and D) Images taken at 20x magnification; scale bar represents 100 μm. Red dotted line highlights boundary of the SAN.

Masson’s trichrome stained tissue was compared to sister sections stained for HCN4 and Cx43 (Figure 4.8). HCN4 is the gene coding for the funny current (an important pacemaker current) and Cx43 is a gap junction abundantly expressed in the working myocardium (see Introduction – Chapter 1). With Masson’s trichrome stain, the nodal cells of the SAN and AVN stain paler than the surrounding atrium, which stains dark pink-
purple. In addition, the CCS stains positive for HCN4 and negative for Cx43; the working myocardium is negative for HCN4 and positive for Cx43.

4.4.2 CD68

Histology revealed the presence of non-myocyte cells in the monocrotaline-injected hearts, but what are these cells? They could be invading inflammatory cells, such as macrophages or neutrophils, or possibly fibroblasts. To investigate this further, immunohistochemistry was carried out.

CD68 is a glycoprotein expressed by macrophages. Protein expression of CD68 was labelled using immunohistochemistry in six regions of the heart; this included four regions of the working myocardium (left and right atrium and ventricles) and two regions of the CCS (SAN and AVN). This was carried out on 5 sections from three monocrotaline-injected animals and two control animals. A mouse anti-CD68 primary antibody was applied, followed by an anti-mouse secondary antibody conjugated with green fluorescein isothiocyanate (FITC) tag. Expression was visualised using confocal fluorescence microscopy and semi-quantified using ImageJ software. From each animal, 5-18 sections were analysed for each region (not all sections contained all the regions of interest). The mean of all sections in each group was calculated.

With the exception of the left atrium and left ventricle, the monocrotaline-injected animals had greater expression of CD68 than the controls in all regions (Figures 4.9 and 4.10). This was particularly apparent in the right ventricle and was significantly different to CD68 expression in the control animals ($P<0.001$). Although there could be staining in the control animals, the staining in the monocrotaline-injected animals was more intense and could be in clumps or patches. The expression pattern of CD68 (Figure 4.9 and 4.10) was the same as the distribution of the non-myocyte cells as seen in the Masson’s trichrome and H&E stained sections (Figures 4.1, 4.3, 4.4 and 4.6). This suggests that at least some of these cells are macrophages.

There was also a significant difference in CD68 expression in right atrium and AVN in the monocrotaline-injected animals compared to the control animals ($P<0.001$ and $P=0.041$, respectively). There was a trend for an increase in CD68 expression in the SAN of the monocrotaline-injected animals; however, it failed to reach significance ($P=0.159$). There was very little staining found in the left ventricle in both groups (Figure 4.10). Similarly, only small amounts of staining were measured in the left atrium (Figure 4.11).
Figure 4.9 Representative images of CD68 expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Regions stained were sinoatrial node, SAN (A and B); right atrium (C and D) and left atrium (E and F). SAN image were taken at 20x magnification; scale bar represents 100 μm. Atrial images are mosaics of 3x3 images taken at 20x magnification.
Figure 4.10 Representative images of CD68 expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Regions stained were atrioventricular node, AVN (A and B); right ventricle (C and D) and left ventricle (E and F). AVN images were taken at 20x magnification; scale bar represents 100 µm. Ventricular images are mosaics of 3x3 images taken at 20x magnification.
Figure 4.11. Semi-quantitative measurement of CD68 expression in various regions of the heart. The mean expression of all sections in each group was calculated. The graph shows the mean ± SEMs of the means for control (n=5-12 sections; 2 animals) and monocrotaline-injected animals (n=10-18 sections; 3 animals). For the LA, statistical differences between groups were determined using Student’s t-test. For the remaining regions, Mann-Whitney Rank Sum test was used (normality test failed). Asterisk, significantly different from the corresponding control groups. Abbreviations: atrioventricular node, AVN; left atrium, LA; left ventricle, LV; right atrium, RA; right ventricle, RV; sinoatrial node, SAN.
4.4.3 TGFβ
Transforming growth factor β (TGFβ) is a cytokine involved in inflammatory and fibrosis pathways (see Introduction – Chapter 1). As for CD68, protein expression was evaluated using immunohistochemistry and semi-quantified using ImageJ analysis to help identify the nature of the non-myocyte cells. Proteins were labelled using a goat anti-TGFβ primary antibody and an anti-goat secondary antibody conjugated with green FITC dye. Sections were co-stained with a mouse anti-Cx43 antibody and an anti-mouse secondary antibody conjugated with red cyanine 3 (Cy3) dye. Cx43 was used to aid identification of the SAN and AVN. Sections from the SAN, AVN, left and right atrium, and left and right ventricle were investigated. Two control and three monocrotaline-injected animals were studied. From each animal, 6-75 sections of each region were analysed and the mean calculated. The mean of all sections in each group was calculated.

TGFβ expression was significantly greater in the right atrium and ventricle of the monocrotaline-injected animals than in the controls (P<0.001 for both; Figures 4.12-4.14). In addition, there was a trend for an increased expression of TGFβ in the SAN and AVN of the monocrotaline-injected rats compared to the control rats, however, this was not significantly different (P=0.078 and P=0.526, respectively). No obvious differences were noted in the left atrium between the two groups. Similarly, only small amounts of TGFβ expression were found in the left ventricle, but there was significantly more in the monocrotaline-injected animals than in the control animals (P=0.008). However, in comparison to the other regions, the expression in the left ventricle was very small (Figure 4.14).

The distribution of TGFβ was generally in discrete patches, similar to that of the non-myocyte nuclei. This suggests that the non-myocyte nuclei could possibly be expressing TGFβ. The level of staining of TGFβ was not as great or as intense as that of the CD68 expression.
Figure 4.12 Representative images of transforming growth factor β (TGFβ) expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Sections were co-stained with the myocyte marker Cx43, in red. Regions stained: sinoatrial node, SAN (A and B); right atrium, RA (C and D) and left atrium, LA (E and F). Images were taken at 20x magnification; scale bar represents 100 μm.
Figure 4.13 Representative images of transforming growth factor β (TGFβ) expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Sections were co-stained with the myocyte marker Cx43, in red. Regions stained: atrioventricular node, AVN (A and B); right ventricle, RV (C and D) and left ventricle, LV (E and F). Images were taken at 20x magnification; scale bar represents 100 μm.
Figure 4.14 Semi-quantitative measurement of transforming growth factor β (TGFβ) expression in various regions of the heart. The mean expression for each animal was calculated. The graph shows the mean ± SEMs of the means for control (n=6-54 sections; 1/2 animals) and monocrotaline-injected animals (n=11-75 sections; 2/3 animals). For the SAN, statistical differences between groups were determined using Student’s t-test. For the remaining regions, Mann-Whitney Rank Sum test was used (normality test failed). Abbreviations: atrioventricular node, AVN; left atrium, LA; left ventricle, LV; right atrium, RA; right ventricle, RV; sinoatrial node, SAN.
4.4.4 Vimentin

An anti-vimentin antibody was used to identify fibroblast expression. Vimentin is a protein expressed by the undifferentiated mesenchymal cells that gives rise to the connective tissue. As fibroblasts originate from mesenchymal cells, it can be used as a marker of fibroblasts. Sections from the SAN, AVN, left and right atrium, and left and right ventricle were investigated. Two control and three monocrotaline-injected animals were studied. From each animal, two to six sections of each region were stained. Vimentin expression was labelled using a guinea pig anti-vimentin primary antibody and an anti-guinea pig secondary antibody that was conjugated with green FITC dye. As for TGFβ, the right and left atrium, SAN and AVN were co-stained with a mouse anti-Cx43 antibody and an anti-mouse secondary antibody conjugated with red Cy3 dye. The right and left ventricles were co-stained with phalloidin conjugated with red Cy3 to identify working myocardial cells. Phalloidin is a toxin that binds to f-actin of myocytes.

No differences in the level or distribution of the vimentin expression was seen in the atrium, left and right, between control and monocrotaline-injected animals (Figure 4.15). There appeared to be more staining in the SAN and AVN of the monocrotaline-injected animals than in the controls (Figure 4.13A, B, G and H). There were patches of vimentin staining found in the right ventricle of the monocrotaline-injected animals, which contained little or no myocytes as identified by Cx43 expression (Figure 4.16). This was in a similar pattern to that seen in the right ventricle following Masson’s trichrome staining (Figures 4.3 and 4.4). These patches were not seen in the right ventricle of the control animals (Figure 4.16), or in the left ventricle of either control or monocrotaline-injected animals (Figure 4.16). It could be concluded from this that fibroblasts could comprise some of the non-myocyte cells seen in the right ventricle of the monocrotaline-injected animals.
Figure 4.15 Representative images of vimentin expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Sections were co-stained with the myocyte marker Cx43, in red. Regions stained: sinoatrial node, SAN (A and B); right atrium, RA (C and D) and left atrium, LA (E and F). Images were taken at 20x magnification; scale bar represents 100 μm.
Figure 4.16 Representative images of vimentin expression, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Regions stained: atrioventricular node, AVN (A and B); right ventricle, RV (C and D) and left ventricle, LV (E and F). AVN sections were co-stained with Cx43, while RV and LV sections were co-stained with phalloidin, both co-stains are in red. AVN images were taken at 20x magnification; scale bar represents 100 μm. RV and LV images were taken at 40x magnification; scale bar represents 20 μm.
4.4.5 TUNEL Assay

Activation of the inflammatory pathway can result in apoptosis. To investigate apoptosis, terminal deoxynucleotidyl transferase mediated nick end labelling (TUNEL) assays were carried out on sister cryosections to those used for immunohistochemistry. The TUNEL assay stains the ends of fragments of DNA that occur as a result of the apoptotic process.

Sections from the SAN, AVN, left and right atrium, and left and right ventricle were investigated. Two control and three monocrotaline-injected animals were studied. From each animal, two to six sections of each region were stained. Similar to the secondary antibodies for the proteins of interest in the previous immunohistochemistry, the TUNEL assay was conjugated with green FITC dye. A mouse anti-Cx43 primary antibody was also applied followed by an anti-mouse secondary antibody conjugated with red Cy3. Cx43 expression was used as a co-stain for myocytes.

There appeared to be more TUNEL staining in the right and left atrium and right ventricle of the monocrotaline-injected animals than the control animals (Figure 4.17 and 4.18). The density of the staining varied throughout the myocardium, from regions with little or none, to regions with a lot. There tended to be more TUNEL staining in the SAN and AVN in the monocrotaline-injected animals (Figure 4.17 and 4.18). No apparent differences in TUNEL expression were seen in the left ventricle between control and monocrotaline-injected animals (Figure 4.17). It could be concluded that the increased inflammatory cell and fibroblast infiltration (as indicated by increased CD68 and vimentin expression, respectively) could be causing an increase in apoptosing cells (as indicated by an increase in TUNEL staining). This was particularly apparent in the right ventricle of the monocrotaline-injected animals, and to a lesser extent in the atria, SAN and AVN.
Figure 4.17 Representative images of TUNEL assay, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Sections were co-stained with Cx43, in red. Regions stained: sinoatrial node, SAN (A and B); right atrium, RA (C and D) and left atrium, LA (E and F). Images were taken at 20x magnification; scale bar represents 100 μm.
Figure 4.18 Representative images of TUNEL assay, in green, from control (A, C and E) and monocrotaline-injected (B, D and F) animals. Sections were co-stained with Cx43, in red. Regions stained: atrioventricular node, AVN (A and B); right ventricle, RV (C and D) and left ventricle, LV (E and F). Images were taken at 20x magnification; scale bar represents 100 μm.
4.5 RESULTS: QUANTITATIVE PCR

4.5.1 Reference Gene Analysis
mRNA was isolated from the working myocardium (right and left atria and ventricles; n=7-10) and from the CCS (SAN; n=7-10). To select the most appropriate reference gene(s), samples were run against common reference genes, as part of a geNorm kit (Primer Design). qBase software (Biogazelle) was used to calculate the most stable genes.

![Figure 4.19](image)

**Figure 4.19** qBase (Biogazelle) analysis of stability of individual reference genes. The stability score (geNorm M) was calculated for six common reference genes.

qBase software (Biogazelle) calculated the stability of each gene across all samples (Figure 4.19). The geNorm M score is a measure of the stability of each gene, the more stable the gene, the lower the score. Therefore, for this data set, GAPDH was the most stable and ACTB was the least (Figure 4.19).
Figure 4.20 qBase (Biogazelle) analysis of number of reference genes. The stability score (geNorm V) was calculated for various numbers of reference genes.

The number of reference genes required to give maximum stability was calculated according to the geNorm V score. The lower the geNorm V score the greater the stability for that number of genes. The greatest stability for the data set was achieved by using a combination of three or four of the most stable reference genes (Figure 4.20). Therefore, taking together the geNorm M and V scores, a combination of the reference genes GAPDH, RPL13A and 28S was used for this data set.

mRNA was isolated from Purkinje fibres at a different time point from the working myocardium and SAN and AVN. Therefore, geNorm analysis for the most suitable reference genes for the Purkinje fibres was calculated separately (Figures 4.21 and 4.22). As for the previous data set, all samples isolated were analysed.
Figure 4.21 qBase (Biogazelle) analysis of stability of reference genes for Purkinje Fibre samples. The stability score (geNorm M) was calculated for seven common reference genes.

Figure 4.22 qBase (Biogazelle) analysis of number of reference genes for Purkinje Fibre samples. The stability score (geNorm V) was calculated for various numbers of reference genes.

Using qBase software, for the Purkinje fibres, the most stable reference tested was ATP5B as it had the lowest geNorm M score, while the least stable was ACTB (Figure 4.21). The best number of reference genes was six or seven, as calculated by the lowest geNorm V score (Figure 4.22). Therefore, for the Purkinje fibres, a combination of the genes ATP5B, GAPDH, UBC, RLP13A, 28S and 18S was used.
4.5.2 qPCR Analysis

Quantitative PCR was used to measure changes in expression of a number of inflammatory and fibrosis targets that have been shown to be important in the inflammatory response. Each sample was run in triplicate. The results are presented in two figures: figure 4.24 shows data for the SAN and the two atria and Figure 4.25 shows data for the right Purkinje fibres, and the two ventricles. Results are displayed as changes in gene expression compared to control expression. There was not sufficient mRNA isolated from the left Purkinje fibres therefore gene expression could only be analysed in the right Purkinje fibres.

There were changes in pro-inflammatory and fibrotic gene expression in the monocrotaline-injected animals compared to the control animals, particularly in the right ventricle (Figures 4.23 and 4.24). There was significantly increased mRNA levels of the pro-inflammatory cytokine interleukin 1β (IL1β) and fibrotic cytokine transforming growth factor β (TGFβ) in all areas of the right side of the heart, including the working myocardium (right atria and ventricle) and CCS (SAN and right Purkinje fibres) in the monocrotaline-injected animals compared to control animals (Figures 4.23 and 4.24). In addition, in these areas there was also significantly increased mRNA levels of components of the extracellular matrix (ECM): collagen Iα2 (Col1α2), connective tissue growth factor (CTGF), elastin, fibronectin (FN1) and tissue inhibitor of metalloproteinase 1 (TIMP1) in the monocrotaline-injected animals (Figures 4.23 and 4.24). Collagen IIIα1 (Col3α1) increased in all areas of the right side of the heart in the monocrotaline-injected animals, except for in the SAN (Figure 4.23), and vimentin (Vim) increased in all areas except the right Purkinje fibres (Figure 4.24).

In the left ventricle, there was a modest significant increase in mRNA levels of IL1β, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the monocrotaline-injected animals (Figure 4.24). The left ventricle of the monocrotaline-injected rats also had a significant increase in the ECM components FN1, Vim and CTGF, but this was much smaller than that seen in the right ventricle (Figure 4.24). In the left atria, there were no significant increases in mRNA levels but a large number of decreases (Figure 4.23). In the left atria, NF-κB, Col1α, Col3α1, CTGF, elastin, FN1, TIMP2 and TIMP3 were significantly reduced in monocrotaline-injected animals compared to control animals (Figure 4.23).
Figure 4.23 Relative abundance of pro-inflammatory and fibrosis markers in different regions of the heart. The graphs show mean ± SEMs for control (n=10) and monocrotaline-injected animals (n=7/8). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise the Mann-Whitney Rank Sum test was used). Asterisk indicates significantly different from the corresponding control group. Gene expression was measured in the sinoatrial node, SAN (A and B), the right atrium (C and D) and left atrium (E and F). Abbreviations: angiotensin II receptor 1, ATR1; collagen Iα2, Col1α2; collagen IIIα1, Col3α1; connective tissue growth factor, CTGF; fibronectin, FN1; interleukin 1β, IL1β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; metalloproteinase 2, MMP2; mothers against decapentaplegic homolog 2/3, Smad 2/3; tissue inhibitor of metalloproteinases 1-4, TIMPs 1-4; transforming growth factor β, TGFβ; tumour necrosis factor α, TNFα; vimentin, Vim.
Figure 4.24 Relative abundance of pro-inflammatory and fibrosis markers in different regions of the heart. The graphs show mean ± SEMs for control (n=5-10) and monocrotaline-injected animals (n=7/8). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise the Mann-Whitney Rank Sum test was used). Asterisk indicates significantly different from the corresponding control group. Gene expression was measured in the right Purkinje fibres (A and B), the right ventricle (C and D) and left ventricle (E and F). Abbreviations: angiotensin II receptor 1, ATR1; collagen Iα2, Col1α2; collagen IIIα1, Col3α1; connective tissue growth factor, CTGF; fibronectin, FN1; interleukin 1β, IL1β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; metalloproteinase 2, MMP2; mothers against decapentaplegic homolog 2/3, Smad 2/3; tissue inhibitor of metalloproteinases 1-4, TIMPs 1-4; transforming growth factor β, TGFβ; tumour necrosis factor α, TNFα; vimentin, Vim.
4.6 SUMMARY

Patches of non-myocyte nuclei were found in the right atria and ventricles of the monocrotaline-injected rats, which were not seen in the left atrium and ventricle or in any region of the heart from control rats. In addition, there was an increase in expression of the macrophage marker CD68, the cytokine TGFβ and TUNEL staining in the monocrotaline-injected rats, which tended to be greater in the right side of the heart including the SAN and AVN. Vimentin expression did not differ greatly between the two groups of animals, except for the right ventricle where large patches of vimentin expression were found in only the monocrotaline-injected animals.

mRNA levels of a number of pro-inflammatory and fibrotic markers increased in the monocrotaline-injected animals, compared to control animals, particularly in the SAN, right atrium, right Purkinje fibres, and right ventricle (AVN not investigated). In the left atrium, there were reductions in the expression of pro-inflammatory and fibrotic markers in the monocrotaline-injected animals, compared to the control animals.

4.7 Discussion

4.7.1 Inflammation

Using qPCR analysis, changes in mRNA levels of components of the pro-inflammatory and fibrotic pathways in various regions of the CCS and working myocardium in PAH were identified. An increase in the pro-inflammatory cytokine interleukin 1β (IL1β) was found in all areas of the right side of the heart in the monocrotaline-injected rats, including the SAN and the right Purkinje fibres (Figures 4.23 and 4.24).

The increase in inflammation on the right side of the heart in monocrotaline-injected animals was also shown by an increase in CD68 expression (Figures 4.9, 4.10 and 4.11). CD68 is a glycoprotein expressed by macrophages. In hearts from monocrotaline-injected animals, CD68 was expressed in very dense patches, particularly in the right ventricle, suggesting areas with a large infiltration of macrophages (Figure 4.10). This was seen also in the right atrium, SAN and AVN, but it was not as great as in the right ventricle (Figures 4.9, 4.10 and 4.11). A minor increase was seen in the left side of the heart, but not to the same degree as in the right side (Figures 4.9, 4.10 and 4.11). In accordance with this work, a study by Handoko et al. found an increase in CD45 expression in the right ventricle, but not the left ventricle, of monocrotaline-injected animals. CD45 is expressed by a number of leukocytes, while CD68 is primarily expressed by macrophages. Also, Campian et al. found increased expression of myeloperoxidase.
(MPO; a toxin expressed by activated neutrophils) activity only in the right ventricle, but not the left, in monocrotaline-injected rats.\textsuperscript{135}

An alternative model of right ventricular dysfunction is following pulmonary embolism. Similar to the monocrotaline-injected model, right sided dysfunction is induced following right ventricular overload, as a result of pulmonary arterial hypertension. In a rat model of pulmonary embolism, an increase in right ventricular inflammation was found.\textsuperscript{136} The authors saw an increase in MPO activity and an increase in CD68 expression in the right ventricle following pulmonary embolism.\textsuperscript{136} Only a small increase in MPO activity was found in the left ventricle.\textsuperscript{136} This study also showed accumulation of macrophages in clusters, which was similar to the pattern seen here, and the clusters of macrophages were close to areas of damaged myocardium.\textsuperscript{136}

There was little change in the mRNA levels of the pro-inflammatory cytokine TNF\(\alpha\) in the monocrotaline-injected rats (Figures 4.23 and 4.24). An increase in TNF\(\alpha\) expression was measured in the right Purkinje fibres, while a decrease was measured in the right atrium (Figures 4.23 and 4.24). No change in TNF\(\alpha\) expression was found in the right ventricle of the monocrotaline-injected animals, although this was significantly increased in a previous study using this model.\textsuperscript{135}

4.7.2 Fibrosis

Compared to the control animals, there was a significant increase in mRNA levels of the cytokine TGF\(\beta\) in the right side of the heart, including the SAN and the right Purkinje fibres (Figures 4.23 and 4.24). As described in the Introduction (Chapter 1), TGF\(\beta\) is activated by a number of cells, including inflammatory cells, and it plays a role in the fibrosis process by regulating the expression of a number of components of the extracellular matrix. In humans, TGF\(\beta\) levels correlate with deposition of collagen in the heart.\textsuperscript{169} In the monocrotaline-injected animals, on the right side of the heart there was an increase in the mRNA levels of collagen 1\(\alpha\)2 (Col1\(\alpha\)2), connective tissue growth factor (CTGF), elastin, fibronectin (FN1) and tissue inhibitor of metalloproteinase 1 (TIMP1) (Figures 4.23 and 4.24). Collagen 3\(\alpha\)1 (Col3\(\alpha\)1) was increased in all areas on the right side of the heart except for the SAN, and vimentin (Vim) was increased in all areas of the right side except for the right Purkinje fibres (Figures 4.23 and 4.24).

Under normal conditions there is a balance of metalloproteinases (MMPs) and TIMPs to help maintain extracellular matrix homeostasis.\textsuperscript{170} MMPs are enzymes that degrade the extracellular matrix as part of normal tissue remodelling. The equilibrium of tissue repair is controlled in part by TIMPs, which inhibit the MMPs. In heart failure, there is disruption to the extracellular matrix turnover and an alteration in the expression of MMPs and
TIMPs. In this study, no significant changes in mRNA level of MMP2 were measured in any region of the heart in the monocrotaline-injected animals (Figures 4.23 and 4.24). However, in the right side of the heart of the monocrotaline-injected animals, there was a significant increase in TIMP1 and a significant decrease in TIMP4 (with the exception of the right Purkinje fibres; TIMP4 was not detected in either group) (Figures 4.23 and 4.24). In animal models of left-sided heart failure, an increase in TIMPs 1 and 2, and a decrease in TIMP4 was found in the failing ventricle. In heart failure patients, an increase in plasma levels of TIMP1 correlates with left ventricular dysfunction and increased risk of mortality. However, the interaction and balance between MMPs and TIMPs appears complex, with discrepancies among different studies and animal models.

Tissue structure was investigated using Masson’s trichrome staining. Using this technique myocytes are stained pink/purple, nuclei are stained blue/back and collagen stained blue. An increase in fibrosis, as measured as an increase in collagen deposition, was not seen in any region of the heart from the monocrotaline-injected animals (Figures 4.1, 4.2, 4.3, 4.4 and 4.5). An increase in fibrosis in the right ventricle of monocrotaline-injected rats has been seen in previously published data. The level of interstitial collagen increases from hypertrophy and dilatation, and peaks at the later stage of disease development when the animals are in failure. However, in agreement with this study, Hessel et al. also reported no increase in fibrosis in the right ventricle of the monocrotaline-injected rats at four weeks following injection.

Two studies that observed right ventricular fibrosis had an experimental end point of day 42 post-injection, and one study 36-40 days. These were at least a week (up to two weeks) longer than the experimental end point of this study. The disease progression in the previous data could be more severe and could account for the differences seen. It is possible that the fibrosis occurs in a later stage of heart failure (as indicated by Campian et al.) and in this study only the initial states of inflammation and apoptosis are seen. A large number of increases in fibrotic genes were seen in the right side of the heart in monocrotaline-injected animals, which was not reflected at the protein level as an increase in fibrosis. It could be hypothesised that if the end point of the model was to be continued for a further week or two then an increase in the amount of the fibrosis would have been measured. This was not possible in this study due to Home Office licensing restrictions as a result of the increased mortality rate seen beyond 28 days.

The absence of fibrosis seen in this study could be a factor in the inability to induce ventricular tachycardia or ventricular fibrillation in the monocrotaline-injected animals. An increase in fibrosis, particularly interstitial fibrosis, in heart disease is associated with
arrhythmias. Fibrosis interferes with electrical coupling between the cells and causes conduction heterogeneity, which produces a substrate for arrhythmias.

4.7.3 Apoptosis

In this study, an increase in TUNEL staining was found in the right ventricle of the monocrotaline-injected animals (Figure 4.18). This was also seen in a study by Campian et al. who also reported that the level of apoptosis peaks at the earlier stages of hypertrophy and dilation before declining at heart failure. The study of Campian et al. suggests that the apoptotic response occurs earlier in the disease progression, with fibrosis occurring later when the animals are in failure. Increased immunolabelling for caspase 3 (a protein involved in the apoptotic pathway) has also been seen in the right ventricle, and not the left ventricle, of monocrotaline-injected hearts.

In addition to increased TUNEL staining in the right ventricle, there also appeared to be increased levels in the left and right atria (Figure 4.17). In addition, a moderate increase in TUNEL stain was seen in the SAN and AVN (Figures 4.17 and 4.18).

4.7.4 Histology and Immunohistochemistry

Masson’s trichrome and haematoxylin and eosin (H&E) staining was used to assess tissue architecture. In the right atria, and particularly in the right ventricle, there were patches of ‘non-myocyte’ nuclei (Figures 4.1, 4.3, 4.4 and 4.6). The cells in these areas did not stain pink, indicating they are not part of the working myocardium (Figures 4.1, 4.3, 4.4 and 4.6). It is hypothesised that these patches contain either inflammatory cells or fibroblasts, or both. In the right ventricle there was positive staining for both CD68 and vimentin, markers of macrophages and fibroblasts, respectively (Figures 4.10, 4.11 and 4.16). However, caution must be taken when interpreting vimentin staining as it has also been shown to be expressed by macrophages. Although the CD68 and vimentin staining was diffusely expressed in the right ventricle, there were also patches of dense expression (Figures 4.10, 4.11 and 4.16). These patches of CD68 and vimentin expression could correspond to the patches of non-myocyte nuclei seen with Masson’s trichrome and H&E staining.

Histology revealed that the regions of non-myocyte cells also contained granules, which are not normally seen in the healthy myocardium (Figures 4.3 and 4.4). It is possible that these granules could be cell debris as a result of either cell damage from the pressure overload on the ventricle wall, or cell damage as a result of the inflammatory process. TUNEL staining increased in the right ventricle indicting that there was an increase in the number of apoptosing cells (Figure 4.18).
Using histology, no regions of non-myocyte nuclei were found in the left atrium or left ventricle of the monocrotaline-injected hearts, or in any region of the control hearts (Figures 4.2 and 4.5). Additionally, patches of CD68 or vimentin staining were not found (Figures 4.9, 4.10, 4.11, 4.15 and 4.16).

Immunohistochemistry also revealed increased expression of the cytokine TGFβ in the right side of the heart in the monocrotaline-injected animals, including the SAN, right Purkinje fibres, right atrium and right ventricle (Figures 4.12, 4.13 and 4.14). This is consistent with the mRNA levels, which were shown to be significantly increased in these areas (Figures 4.23 and 4.24).

4.7.5 Changes in Gene Expression in the CCS AVN

In this model, the AVN was primarily investigated by Dr. Ian Temple. Dr. Temple was investigating electrophysiological properties of the isolated AVN and changes in mRNA expression. Because of the complex anatomy of the AVN, sampling of tissue from the AVN for qPCR analysis was beyond the scope of the current study; this is why it was undertaken by Dr. Temple.

At the time of writing, Dr. Temple was assessing changes in mRNA levels in the AVN, which included a number of the inflammatory and fibrosis targets of interest to this study. Preliminary results from Dr. Temple suggest that there is also an increase in inflammation in the AVN in monocrotaline-injected rats. Six regions were investigated: the atrial and ventricular septa, transitional tissue, inferior nodal extension, compact node and penetrating bundle. There was a trend for an increase in mRNA level of the cytokines IL1β and TGFβ in almost all the regions of the monocrotaline-injected rats (Figure 4.25). There was a significant increase in the atrial and ventricular septa and in the transitional tissue (Figure 4.25). In this study, immunohistochemistry of the AVN revealed an increase in TGFβ expression in the monocrotaline-injected rats (Figures 4.13 and 4.14), which correlates with the changes in gene expression (Figure 4.25B). In addition, this study has demonstrated that there also appears to be more CD68 staining in the AVN (Figures 4.10 and 4.11).
**Figure 4.25** Relative abundance of interleukin 1β (IL1β) and transforming growth factor β (TGFβ). The graphs show mean ± SEMs for control (n=7-8) and monocrotaline-injected (n=6-8) animals. Statistical differences were calculated by Limma tests. Gene expression was measured in the atrium, ventricle, transitional tissue (TT), inferior nodal extension (INE), compact node and penetrating bundle (PB). Asterisk indicates significantly different from corresponding control. From Dr. Ian Temple (unpublished data).

**SAN and Purkinje Fibres**

In addition to increases in IL1β and TGFβ in the AVN in the monocrotaline-injected animals, there was also increases in the other regions of the CCS investigated. mRNA levels of IL1β and TGFβ were significantly increased in both the SAN and right Purkinje fibres (Figures 4.23 and 4.24). Additionally in the monocrotaline-injected animals, there was an increase in the extracellular components Col1α2, CTGF, elastin, FN1 and TIMP1 in the SAN and right Purkinje fibres (Figures 4.23 and 4.24). Immunohistochemistry revealed an increase in expression of CD68, TGFβ and vimentin in the SAN of the monocrotaline animals. To my knowledge, this is the first time that inflammation and fibrosis has been investigated in the CCS in this model.

**4.7.6 Left Side of the Heart**

In addition to the right side of the heart, the left ventricle of the monocrotaline-injected rats saw an increase in the mRNA levels of the cytokine IL1β and the extracellular components CTGF, fibronectin and vimentin (Figures 4.23 and 4.24). However, the magnitude of these changes was much less than that measured in the right side of the heart. Changes to gene expression in the left ventricle is not surprising. In the monocrotaline-injected rats, the right ventricle is under great pressure and is significantly hypertrophied and dilated. Right pressure overload has been shown to impact left ventricle diastolic function. However, the changes in mRNA levels did not translate into
changes in protein level. There was no increase in CD68 or vimentin staining in the left ventricle of the monocrotaline-injected rats.

The left atrium of the monocrotaline-injected rats displayed a large number of significant decreases in mRNA levels of components of the extracellular matrix, suggesting atrophy could be occurring. Dysfunction of left ventricular function studies (a reduction in stroke volume and cardiac output) was seen in some previous, which could possibly have an effect on left atrial function and structure.\textsuperscript{153} The atrium, being thinner walled, could feasibly be more sensitive to change in pressure than the more muscular and thicker walled ventricle.

4.8 Conclusions

It is concluded that there is inflammation of the right side of the heart, including the SAN, AVN and right Purkinje fibres, in PAH. This is the first report of inflammation of the CCS in heart disease.
CHAPTER 5: RABBIT MODEL OF CONGESTIVE HEART FAILURE

5.1 INTRODUCTION

Inflammation and fibrosis was also assessed in a rabbit model of congestive heart failure. Other models of heart failure were included in this study to see if the results obtained from the rat PAH model are generally applicable. The rabbit model of congestive heart failure was developed and produced by collaborators at the University of Liverpool, under supervision of Professor George Hart. Left-sided heart failure was induced by two surgical steps. First, volume overload was produced by destruction of the aortic valve, followed three weeks later by pressure overload caused by partial constriction of the abdominal aorta (see Methods - Chapter 2 - for more details). This study was less comprehensive than the study of PAH, with the primary aim being to investigate changes in gene expression.

Tissue was isolated from the working myocardium (right atrium, RA; left and right ventricles, LV and RV, respectively) and the CCS (sinoatrial node, SAN; left and right Purkinje fibres, LPF and RPF, respectively). The tissue was then generously donated to me and I carried out all the molecular work shown in this chapter. mRNA was isolated and quantitative PCR (qPCR) was carried out to investigate mRNA levels of pro-inflammatory and fibrotic genes.

5.2 RESULTS: QUANTITATIVE PCR

5.2.1 Primer Design

Due to incomplete sequencing of the rabbit genome, there are few rabbit primers commercially available. Primers for the same pro-inflammatory and fibrotic targets of interest used for qPCR analysis in the rat monocrotaline-induced pulmonary arterial hypertension (PAH) model were designed as described in the methods section (Chapter 2). Briefly, the sequence for the gene of interest was found using the search engines on Pubmed and Ensembl websites. The primer sequence was tested and checked for folding using appropriate software, and if satisfactory, the primers were manufactured by Eurofins. The primers were used in test qPCR reactions at various concentrations, and the product run on DNA gels. Only the primers deemed acceptable, including neat amplification plots and absence of primer dimers, were used for further analysis. Table 5.1 summarises the successful left and right primer sequences, along with the corresponding reference sequence. The primers were used for qPCR analysis of the samples obtained from the rabbit left-sided heart failure model.
e qPCR results were analysed by StatMiner software and stability of the two reference genes compared (Figure 5.1). 28S had a lower stability score than GAPDH and was, therefore, more stable (Figure 5.1). In addition, 28S alone was more stable than using a combination of 28S and GAPDH (Figure 5.1).

### Table 5.1 Left and right primer sequences for rabbit pro-inflammatory and fibrotic genes of interest
The primers were used for qPCR analysis for the rabbit model of left-sided heart failure.

<table>
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<th>Gene</th>
<th>Primer</th>
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<th>Reference Sequence</th>
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<td>Right</td>
<td>CCACCGTGGAAGCCATCTCT</td>
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<td>TNF</td>
<td>Left</td>
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<td>NM_001082263.1</td>
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<td></td>
<td>Right</td>
<td>AGGGTTGACTGAGGAGTTGTTT</td>
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<td></td>
<td>Right</td>
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<tr>
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</tr>
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<td>NM_001195690.1</td>
</tr>
<tr>
<td></td>
<td>Right</td>
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</tbody>
</table>

5.2.2 Reference Gene Analysis
As in the case of the rat model of PAH, experiments were carried out to establish the most appropriate reference gene for the samples. However, due to limitations in availability of primers, only 28S and GAPDH genes were tested. The qPCR results were analysed by StatMiner software and stability of the two reference genes compared (Figure 5.1). 28S had a lower stability score than GAPDH and was, therefore, more stable (Figure 5.1). In addition, 28S alone was more stable than using a combination of 28S and GAPDH (Figure 5.1).
5.2.3 qPCR Analysis

Each sample was run in triplicate. Results are displayed as changes in gene expression compared to control expression.

qPCR analysis revealed surprisingly few significant changes in the left-side of the heart in heart failure; the only significant differences were a reduction in the mRNA level of a tissue inhibitor of metalloproteinase (TIMP4) and angiotensin II receptor 1 (AT1R) in the left Purkinje fibres (Figure 5.3B). No significant changes were seen in the left ventricle in heart failure (LV, Figure 5.3E and F).

The extracellular matrix component fibronectin was significantly increased in the SAN, right Purkinje fibres and right ventricle in heart failure (Figure 5.2B and F, and 5.3D). In heart failure, TIMP3 was reduced in the SAN and TIMP1 was increased in the right ventricle (Figure 5.2B and D). The majority of changes were found in the right atrium; there were increases in mRNA levels in all targets, but the significant increases were interleukin 1β (IL1β), tumour necrosis factor α (TNFα), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), AT1R, metalloproteinase 2 (MMP2), and TIMPs 1 and 2 (Figure 5.2C and D).
Figure 5.2 Relative abundance of pro-inflammatory and fibrosis markers in different regions of the heart. The graphs show mean ± SEMs for control (n=4-8) and heart failure animals (n=7). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum test was used). Asterisk indicates significantly different from the corresponding control group. Gene expression was measured in the sinoatrial node, SAN (A and B); the right atrium, RA (C and D) and right Purkinje fibres, RFP (E and F). Abbreviations: angiotensin II receptor 1, ATR1; collagen Iα2, Col1α2; collagen IIIα1, Col3α1; fibronectin, FN1; interleukin 1β, IL1β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; metalloproteinase 2, MMP2; tissue inhibitor of metalloproteinases 1-4, TIMPs 1-4; tumour necrosis factor α, TNFα; vimentin, Vim.
Figure 5.3 Relative abundance of pro-inflammatory and fibrosis markers in different regions of the heart. The graphs show mean ± SEMs for control (n=5-8) and heart failure animals (n=6/7). Statistical differences between groups were determined using Student's t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum test was used). Asterisk indicates significantly different from the corresponding control group. Gene expression was measured in the left Purkinje fibres, LPF (A and B); the right ventricle, RV (C and D) and left ventricle, LV (E and F). Abbreviations: angiotensin II receptor 1, ATR1; collagen Iα2, Col1α2; collagen IIIα1, Col3α1; fibronectin, FN1; interleukin 1β, IL1β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; metalloproteinase 2, MMP2; tissue inhibitor of metalloproteinases 1-4, TIMPs 1-4; tumour necrosis factor α, TNFα; vimentin, Vim.
5.3 SUMMARY

Primers were designed and manufactured for the majority of the pro-inflammatory and fibrotic targets of interest, with the successful ones being used for qPCR analysis. Surprisingly, relatively few gene expression changes were measured in the left-side of the heart in the heart failure animals, compared to control animals, with the majority of changes found in the right atrium. The left atrium was not available for study.

5.4 DISCUSSION

5.4.1 Model of Congestive Heart Failure

The rabbit model of congestive heart failure was generated by Professor George Hart at the University of Liverpool. It was induced following two surgical steps of pressure and volume overload. As the insult was predominantly on the left side of the heart, it can be looked at as a model of left-sided heart failure. However, ascites (accumulation of fluid in the abdominal cavity) and pericardial effusion (accumulation of fluid in the pericardial cavity that surrounds the heart) have been noted in this model, which are evidence of right-sided heart failure (secondary to the left-sided heart failure). Sham-operated rabbits were used as controls.

Pre-termination echocardiography was used to confirm heart failure eight weeks following the first surgical step. The heart failure rabbits had a significantly increased left ventricular internal diameter in diastole compared to control rabbits, indicating left ventricular dilatation (Figure 5.4A). Left ventricular dysfunction in the heart failure rabbits was demonstrated by a significant reduction in fractional shortening (Figure 5.4B).

![Graph A: Left ventricular internal diameter in diastole (LVIDd) for control and heart failure rabbits.](image1)

![Graph B: Fractional shortening (FS) for control and heart failure rabbits.](image2)

**Figure 5.4 Pre-termination echocardiography measurements for control (in yellow) and heart failure (HF; in red) rabbits.** (A) Left ventricular internal diameter in diastole, LVIDd. (B) Fractional shortening, FS. Statistical differences between groups were determined using unpaired t-tests. Asterisk indicates significantly different from corresponding control group. From Professor G. Hart (unpublished data).
ECG recordings were also taken pre-termination to investigate electrical activity. 1.2 mg of atropine and 3 mg of propranolol were applied to block autonomic innervation of the heart. In the heart failure rabbits, an intrinsic bradycardia was measured (an increased cycle length), in addition to prolonged PR and QRS intervals (Figure 5.5). This is evidence of dysfunction of the SAN, AVN and Purkinje fibers.

Figure 5.5 Pre-termination ECG measurements from control (in yellow) and heart failure (HF; in red) rabbits. (A-C) The cycle length (A), PR interval (B) and QRS interval (C) measured at baseline and following administration of atropine and propranolol.

The rabbit is electrophysiologically and structurally closer to humans than are rodents, and is, therefore, a preferred model. However, the rabbit genome has yet to be fully sequenced and, therefore, it is more difficult to investigate at the gene level. There are fewer primers and antibodies specific for rabbit targets making mRNA and protein analysis more challenging. In this study, not all the targets of interest could be investigated due to unsuccessful primer design. Additionally, initial immunohistochemistry for a number of targets of interest did not work, and resulted in non-specific and high background staining.

In the rat model of PAH, ex vivo experiments were carried out using the Langendorff-perfusion technique to investigate electrophysiological properties of the working myocardium and CCS. Unfortunately, due to partial destruction of the aortic valve as part of the surgical step to induce volume overload, Langendorff-perfusion experiments were
not possible. The Langendorff-perfusion technique involves retrogradely perfusing the heart via cannulation of the aorta and was not successful in early attempts.

5.4.2 mRNA Analysis

The major insult in the model of rabbit heart failure is on the left-side of the heart and, therefore, the greatest number of changes in gene expression would have been expected to be on the left. However, in this study no significant changes were measured in the left ventricle in heart failure rabbits. In the left Purkinje fibres only two significant differences were seen: a reduction in angiotensin II receptor 1 (AT1R) and a reduction in tissue inhibitor of metalloproteinase 4 (TIMP4). Perhaps this could result in fibrosis of the left Purkinje fibres in the heart failure rabbits, but it is unclear why there were not significant changes in other fibrosis genes, e.g. collagens 1 and 3, fibronectin and vimentin. An increase in fibrosis interferes with action potential conduction and could partly be responsible for the prolongation of the QRS interval seen in the heart failure rabbits.\(^{180}\) Fibrosis impairs electrical coupling between cells and slows conduction.\(^{181, 182}\) There was also an increase in mRNA levels of fibronectin in the right ventricle and right Purkinje fibres in the heart failure rabbits, in addition to an increase (rather than decrease) in TIMP1 in the right ventricle. Could this lead to remodelling and fibrosis? In the SAN, there was an increase in fibronectin and a decrease in TIMP3 and angiotensin II receptor 1 (AT1R) in the heart failure rabbit compared to the control rabbit.

The majority of changes in mRNA levels were measured in the right atrium. In the right atria of the heart failure rabbits there was significant increase in the pro-inflammatory cytokines interleukin 1β (IL1β) and tumour necrosis factor α (TNFα), and the downstream mediator nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Additionally, there was a trend for increases in all of the components measured relating to fibrosis. Those that were significantly different were AT1R, metalloproteinase 2 (MMP2) and TIMPs 1 and 2. Unfortunately the primer for rabbit transforming growth factor (TGFβ) was not successful; it would be interesting to see if TGFβ was a possible mediator of the increased fibrotic gene expression in this rabbit model of heart failure. The reason for the large number of changes in mRNA levels in the right atria in heart failure, compared to the rest of the heart, is not clear. It is proposed that due to its thin walls it is not able to cope with the changing haemodynamics during heart failure. The pressure and volume overload was severe and resulted in substantial dilation of the left ventricle. It is possible that the effects on the left side of the heart were having secondary effects on the right side of the heart. As mentioned above, some of the heart failure rabbits displayed symptoms of right-sided heart failure. The secondary effects could cause remodelling of the right side of the heart and particularly of the atria, which could be more susceptible to the insults. A study by Burstein et al. found that the atrium is more susceptible to fibrosis than
In addition to different gene profiles, atrial fibroblasts are more sensitive to stimulation by fibrotic mediators such as TGFβ and angiotensin II. A difference in chamber sensitivity was also demonstrated by Nakajima et al. In mice over-expressing myocardial TGFβ, although there was equal amounts of active TGFβ throughout the heart, the atria had significantly more fibrosis than the ventricles.

5.5 Conclusions

In conclusion, in a rabbit model of congestive heart failure inflammation was evident, but only in the right atrium (left atrium not investigated). In this model, there was little evidence of inflammation of the CCS (SAN and left and right Purkinje fibres).
CHAPTER 6: RAT MODEL OF MYOCARDIAL INFARCTION

6.1 INTRODUCTION

Early in this study, I investigated the ventricular border zone of a rat model of myocardial infarction as a 'positive control' – inflammation is expected at this site of myocardial damage and loss. The rat model of myocardial infarction was developed by collaborators at the Medical Centre of Postgraduate Education, Warsaw, under supervision of Dr. Michal Maćzewski. Briefly, the left coronary artery was partially constricted, inducing a large infarct of the left ventricle (see Methods – chapter 2 for more details). Sham-operated rats were used as controls. Tissue samples were taken from the border region of the infarct zone and a remote region of the left ventricle, which was normal. The samples were used for histology and qPCR analysis.

6.2 RESULTS: MASSON’S TRICHROME STAINING

6.2.1 Remote Region

As in the case of the rat PAH model, 10 μm thick sections were stained using the Masson’s trichrome technique to investigate tissue architecture. The staining method stains myocytes pink/purple, nuclei dark blue/black and collagen blue.

Figure 6.1 Masson’s trichrome staining of the remote region of a control rat. (A) Whole section of remote region taken at 1.5x magnification. (B and C) High power images of the remote region taken at 40x magnification; scale bar represents 50 μm. The location of these areas are labelled ‘b’ and ‘c’, respectively, on the 1.5x magnified image (A).
The remote region was sectioned and stained from six control and five myocardial infarction remote animals. Two out of the control rats had an area of non-myocyte nuclei that also contained blue stained tissue, which is indicative of fibrosis. An example of a fibrotic area is shown in Figure 6.1C. The remaining tissue of the remote region in these animals appeared healthy with no fibrosis or non-myocyte nuclei, as demonstrated in Figure 6.1B. The remaining four control sections were similar to that seen in Figure 6.1B.

Figure 6.2 Masson’s trichrome staining of the remote region of a myocardial infarction rat. (A) Whole section of remote region taken at 1.5x magnification. (B and C) High power images of the remote region taken at 40x magnification; scale bar represents 50 μm. The location of these areas are labelled ‘b’ and ‘c’, respectively, on the 1.5x magnified image (A).

Two out of the five myocardial infarction stained sections also contained areas of fibrosis with non-myocyte nuclei. An example is shown in Figure 6.2B. As with the control rats, the areas away from the regions of fibrosis appeared normal (Figure 6.2C). The remaining three sections of remote region from myocardial infarction rats had no evidence of fibrosis and were similar to that seen in Figure 6.3C.
6.2.2 Border Zone

Figure 6.3 Masson’s trichrome staining of the equivalent region to the border zone in a control rat. (A) Whole section of remote region taken at 1.5x magnification. (B) High power image of the remote region taken at 40x magnification; scale bar represents 50 μm. The location of the area is labelled as ‘b’ on the 1.5x magnified image (A).

A representative image of the equivalent region to the border zone in a control rat is shown in Figure 6.3. None of the six control rats had any regions of fibrosis or proliferation of non-myocyte nuclei and appeared to consist of healthy myocardium.

Two out of the five myocardial infarction rats had regions of non-myocyte nuclei in the border zone. Figure 6.4C is a high power image of an area of the border zone in the myocardial infarction rat with the most pronounced region of non-myocyte proliferation (the region in the second rat covered a smaller area). The tissue appears disorganised and contains granules that could possibly be cell debris. The area had a purple hue, as opposed to a blue hue colour of regions of non-myocyte nuclei seen in the remote region (Figure 6.2B and 6.4C). The tissue adjacent appeared healthy and was similar to that seen in the equivalent border zone of the control rats (Figure 6.4B). The two different types of tissue have been separated by a yellow dotted line on the image (Figure 6.4). The border zones from the remaining three myocardial infarction rats did not contain regions of non-myocyte proliferation and appeared to consist of healthy myocardium. This was surprising and could be hypothesised that perhaps these sections are not true border zones from myocardial infarction animals.
Figure 6.4 Masson’s trichrome staining of the border zone of a myocardial infarction rat. (A) Whole section of remote region taken at 1.5x magnification. (B and C) High power images of the remote region taken at 40x magnification; scale bar represents 50 μm. The location of these areas are labelled as ‘b’ and ‘c’, respectively, on the 1.5x magnified image (A).
Figure 6.5 Quantification of fibrosis. The amount of fibrosis in the tissue was calculated as a percentage of the tissue area in the remote region (A) and the border zone (B) from control and (blue dots) and myocardial infarction (red dots) animals. The grey dot represents the mean ± SEMs for control (n=6) and myocardial infarction animals (n=5).

The amount of fibrosis present in the tissue sample was quantified as a percentage of the total area (Figure 6.5). Although there was a trend for an increase in the percentage of fibrosis in the myocardial infarction animals compared to the control animals, this was not significantly different. Mann-Whitney Rank Sum test revealed that there was no significant difference in the amount of fibrosis in the the remote region of the control and myocardial infarction animals, or in the border region of the control and monocrotaline-injected animals (P=0.792 and P=0.329, respectively. Normality test failed).

<table>
<thead>
<tr>
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<th>B</th>
<th>Border zone</th>
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<tr>
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</tr>
<tr>
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<td>4</td>
<td>2</td>
<td>Control</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>3</td>
<td>2</td>
<td>Myocardial infarction</td>
</tr>
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</table>

Table 6.1 Summary of incidence of fibrosis in the remote region (A) and border zone (B) from control and myocardial infarction animals.

The incidence of fibrosis in the remote region and border zone from control and myocardial infarction animals is summarised in Table 6.1. Two out of the four remote
regions from control animals and two out of the five remote regions from myocardial infarction animals displayed regions of fibrosis. As expected, Fisher’s exact analysis calculated that there was no significant difference in the incidence of fibrosis between the two groups (P=1.000). No regions of fibrosis were found in the border zone of control animals, however, fibrosis was seen in two out of the five border zones from myocardial infarction animals. Although the incidence of fibrosis was greater in the border zone of the myocardial infarction animals, it was not significantly different from control animals (P=0.182; Fisher’s exact test).

6.3 RESULTS: QUANTITATIVE PCR

6.3.1 Reference Gene Analysis

mRNA was isolated from the remote regions and border zones from both the control and monocrotaline-injected rats. As in the case of the rat PAH model, the most appropriate reference gene(s) for the samples was tested using a geNorm kit (Primer Design). qBase software (Biogazelle) was used to calculate the stability of the reference genes.

![Figure 6.6 qBase (Biogazelle) analysis of stability of individual reference genes.](image)

The stability score (geNorm M) was calculated for six common reference genes.

The stability of each individual reference gene was calculated according to a geNorm M score; the lower the M score, the more stable the gene. For the rat myocardial infarction model, the most stable was 18S, while ATP5B was the least stable (Figure 6.6).
Figure 6.7 qBase (Biogazelle) analysis of number of reference genes. The stability score (geNorm V) was calculated for various numbers of reference genes.

The qBase software also calculates the optimum number of reference genes using a geNorm V score. Similar to the M score, the lower the V score the more stable that combination of reference genes. For the rat myocardial infarction model, a combination of two or three of the most stable reference genes was best (Figure 6.7). Therefore, for these samples, a combination of 18S, 28S and GAPDH genes was used as a reference.

The reference gene analysis has indicated different reference genes for each of the models. In addition, the analysis has revealed different optimal number of reference genes, for example, six genes were used for mRNA analysis of Purkinje fibres from the rat model of pulmonary arterial hypertension while only three were used in this rat model of myocardial infarction. In all four sets of analyses, 28S was chosen as one of the genes, further emphasising this as an important reference. However, different genes fared well in both the different models and in different data sets from the same model, highlighting the importance of carrying out reference gene analysis for each individual population of samples.

6.3.2 qPCR Analysis
Quantitative PCR was carried out to investigate changes in gene expression in components from the inflammatory and fibrosis pathways. The same targets studied in the rat pulmonary arterial hypertension model were investigated in this study of myocardial infarction. Each sample was run in triplicate. Results are displayed as changes in gene expression compared to control expression.
Figure 6.8 Relative abundance of pro-inflammatory and fibrosis markers in the remote region or border zone of the left ventricle. The graphs show mean ± SEMs for control (n=6) and myocardial infarction rats (n=5). Figures A-D: statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum test was used). Gene expression was measured in the remote region (A and B) and the border zone (C and D). Asterisk indicates significantly different from the
Figures E and F: data were reanalysed to compare the border zone of myocardial infarction animals with no non-myocyte nuclei and those with non-myocyte nuclei. For AT1Ra, Col3α1 and TIMP4, statistical differences between groups were determined using One Way ANOVA followed by Bonferoni’s post-test. For the remaining genes, One Way ANOVA on Ranks was used (equal variance test failed). ‘b’ indicates significantly different from corresponding control group, ‘c’ indicates significant differences between myocardial infarction border with no non-myocytes and those with non-myocytes. Abbreviations: angiotensin II receptor 1, ATR1; collagen Iα2, Col1α2; collagen IIIα1, Col3α1; connective tissue growth factor, CTGF; fibronectin, FN1; interleukin 1β, IL1β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; metalloproteinase 2, MMP2; mothers against decapentaplegic homolog 2/3, Smad 2/3; tissue inhibitors of metalloproteinases 1-4, TIMPs 1-4; transforming growth factor β, TGFβ; tumour necrosis factor α, TNFα, vimentin, Vim.

There were relatively few changes in mRNA levels measured in the remote region of the myocardial infarction rats, compared to the control rats (Figure 6.8A and B). The myocardial infarction rats had a significantly increased mRNA level of collagen IIIα1 (Col3α1) and connective tissue growth factor (CTGF), compared to control rats (Figure 6.8B). There was an increase in angiotensin II receptor 1b (AT1Rb) expression in the myocardial infarction rats, but it did not reach significance (Figure 6.8A).

There were a greater number of significant differences in mRNA levels in the border zone than the remote region in the myocardial infarction rats (Figure 6.8). Compared to the control rats, the myocardial infarction rats had greater expression of the cytokines interleukin 1β (IL1β), tumour necrosis factor α (TNFα) and transforming growth factor β (TGFβ), and two of the downstream targets, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mothers against decapentaplegic homolog 3 (Smad 3) (Figure 6.8C).

The myocardial infarction rats also had increased expression of a number of components of the extracellular matrix compared to the control rats including collagen Iα2 (Col1α2), CTGF, fibronectin (FN1) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP1 and 2) (Figure 6.8D). In addition to increased numbers of genes that were significantly different in the border zone of myocardial infarction rats compared to the control, the magnitude of the changes was larger (Figure 6.8). For example, the mRNA level of FN1 in the border zone of the myocardial infarction rats was more than 30 times the expression measured in the control rats, and TIMP1 was more than 20 times (Figure 6.8D).

However, are the changes in gene expression in the border zone of the myocardial infarction rats a reflection of all sections investigated? Only two out of the five border zone sections displayed regions of non-myocyte nuclei. The border zone from the myocardial
infarction rats was reanalysed to compare the animals that appeared healthy with no signs of non-myocyte nuclei, and those that appeared more abnormal with the regions of non-myocyte nuclei. Due to small n numbers a lot of the differences were not significant, although a trend in changes could be seen (Figure 6.8E and F). In the border zone of the myocardial infarction rats that had regions of non-myocyte nuclei, there tended to be an increase in gene expression in the inflammatory and fibrosis markers, in comparison to the myocardial infarction border zones without any regions of non-myocyte nuclei (Figure 6.8E and F). There was a significant difference for IL1β, NFκB, AT1Ra, Col1α2, Col3α1, CTGF and TIMP2 (P<0.05). There was also a trend for an increase in elastin, fibronectin and vimentin in the border zone with non-myocyte nuclei; however, they were not significantly different (Figure 6.8E and F). It could be proposed that these genes are playing a role in the genesis of these non-myocyte cells. TNFα was unchanged, and even slightly less, in the border zone with non-myocyte nuclei suggesting that perhaps this gene was not as important in the formation of these cells (Figure 6.8E).

6.4 Summary

Although there was some fibrosis and non-myocyte proliferation found in the remote region of two out of the six control rats, there appeared to be a greater amount in the remote zone of the myocardial infarction rats. However, like the control rats, it was not consistent among all myocardial infarction animals, with only two out of the five rats displaying this pattern.

All control rats appeared to have healthy myocardium in the equivalent ‘border zone,’ with no evidence of fibrosis or non-myocyte proliferation. Two out of the five myocardial infarction rats had regions of non-myocyte nuclei. This was particularly extensive in one of the myocardial infarction rats, with areas that also appeared disorganised.

qPCR analysis revealed only few changes in mRNA levels of pro-inflammatory and fibrotic genes in the remote region of the myocardial infarction rats, compared to the control rats. There was a greater number of genes with significantly different expression levels in the border zone of the myocardial infarction animals compared to the controls. The magnitude of changes in gene expression in the border zone of the myocardial infarction rats was also much greater than in the remote region. When the data were reanalysed to compare differences in gene expression of the border zone in myocardial infarction rats with regions of non-myocyte nuclei and those without the non-myocyte nuclei, a lot of the changes in gene expression remained. However, there were some differences between the two groups; for example, TNFα was not upregulated in the border zone of the
myocardial infarction rats with non-myocyte nuclei, suggesting that perhaps it is not playing a role in their genesis.

6.5 Discussion

The rat model of myocardial infarction was studied as a positive control early in this study, because inflammation is known to occur in this model.

6.5.1 Myocardial Infarction

In the USA, there are almost a million people with myocardial infarction and the related hospital costs are $31 billion. Myocardial infarction is the consequence of a blockage of one of the coronary vessels by a thrombus, reducing blood supply to an area of myocardium. The infarct starves the tissue in that region of oxygen and nutrients and as a result there is an increase in apoptosis and necrosis. In the infarct zone there is an increase in pro-inflammatory cytokines and influx of inflammatory cells, such as macrophages and neutrophils. Over time the infarct remodels: extracellular matrix is deposited and scar tissue is produced. Due to the mass of fibrosis present and dysfunctional myocytes, the infarct region is incapable of contracting efficiently, and depending on the size of the infarct, ventricular dysfunction and failure can ensue.

It is widely recognised that there is activation of the inflammatory response in myocardial infarction. Following myocardial infarction, patients have a massive increase in the acute phase protein CRP, which is a well-known inflammatory marker. There is also an increase in circulating plasma levels of the pro-inflammatory cytokine interleukin 1β (IL1β), which is followed by an increase in IL6.

Three regions of myocardium have been classified in myocardial infarction: the infarct region, the border zone and the remote region. The border region is defined as the region of viable tissue immediately next to the infarcted tissue. A study by Jackson et al. found that in a sheep model of myocardial infarction the region classified as border zone enlarges as the infarct scar remodels. The border region consists of tissue that does not contract as efficiently as the remote parts of the ventricle, although it appears to be just as well perfused. The border zone contains high levels of fibrosis, which would undoubtedly contribute to its reduced contractility. The remote region is a part of the ventricle distinct from the infarct and can be classified as contractile with an unaffected blood supply.
6.5.2 Rat model of Myocardial Infarction

The rat myocardial infarction model has been widely used for decades.\cite{192} The model is induced by partial ligation of the proximal left coronary artery, which produces a large infarct of the left ventricle. Approximately 12 weeks following surgery, the rats are in failure.

In this study, tissue samples were obtained from the border zone of the infarct and from a remote region. The largest number of significant differences in gene expression was measured at the border zone. Here, the myocardial infarction rats had increased mRNA levels of the pro-inflammatory cytokines IL1β and tumour necrosis factor α (TNFα) and the down stream mediator nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). In humans and animal studies an increase in TNFα expression is found in the border zone, and is greater than in the infarct zone.\cite{193,194} In this study, no differences were seen in the IL1β, TNFα or NF-κB in the remote region of the myocardial infarction rats compared to control rats.

There was a trend of increased gene expression for the majority of the components of the extracellular matrix investigated in the border zone of the myocardial infarction rats compared to control rats. Those that were significantly increased were collagen Iα2 (Col1α2), connective tissue growth factor (CTGF), fibronectin (FN1) and tissue inhibitor of metalloproteinases (TIMPs) 1 and 2. The increase in gene expression of the inflammatory cytokines and extracellular matrix components could help explain the proliferation of non-myocyte nuclei seen in the border zone of the myocardial infarction rats. It is possible that these non-myocytes were leukocytes and/or fibroblasts. Further investigations using immunohistochemistry would need to be used to identify the type of cells present.

However, only two out of the five border zones from myocardial infarction rats contained regions of non-myocytes, three did not. The differences seen in the border zone in this group are not understood. Possibly the regions with no non-myocytes were not true border zone, or perhaps the myocardial infarction in these animals was not as severe. This leads to the question, are the mRNA changes seen in the border zone of the myocardial infarction rats a reflection of all five sections? The data from the border zone from the myocardial infarction rats were reanalysed so that instead of one data set there were two. The mRNA expression of the border zone of the myocardial infarction rats that contained non-myocytes was compared to the mRNA expression from the border zone of myocardial infarction rats that did not have regions of non-myocytes. Although there was a trend for an increase in the inflammatory and fibrosis markers in both groups compared to the control rats, it was more pronounced in the border zone of the myocardial infarction rats with non-myocytes. This suggests that the genes upregulated in the non-myocyte
border zone could be playing a role in the genesis of the non-myocytes. However, TNFα was not upregulated in the border zone with non-myocytes, which implies that this is not playing as much of a role. Immunohistochemistry could help establish which cytokines or fibrosis markers are expressed locally in the patches of non-myocytes.

In the regions of non-myocyte nuclei there was evidence of granules and cell disorganisation. It is possible that these areas also contained cell debris and apoptosing cells as a result of the increased inflammatory response and extracellular matrix remodelling. An increase in apoptosis has been found in the border zone of infarcts in humans and in animal models.\textsuperscript{193,195} 

The remote region sampled can vary in different studies.\textsuperscript{190} Although the remote region is far from the infarct and the primary insult, it can still be affected by the altered haemodynamics as a result of the myocardial infarct.\textsuperscript{190} In this study, for example, the myocardial infarction rats’ hearts are hypertrophied and the rats have left ventricular dysfunction. The hypertrophy and dilation is likely to affect the remote tissue in these hearts and could, in part, explain the minor changes in gene expression seen in the remote region of the myocardial infarction hearts.

In the rat, an increase in collagen has been found both in the infarct and non-infarcted tissue.\textsuperscript{196} A study by McCormick \textit{et al.} reported a large increase in collagen in the infarct, a smaller increase in the border zone and even smaller increase in the remote region.\textsuperscript{197} However, in all regions, including the remote region, collagen was significantly increased compared to control rats.\textsuperscript{197} 

Apoptosis has also been found in remote regions of the ventricle of myocardial infarction rats, and although it is not as great as in the border zone, is significantly greater than in control rats.\textsuperscript{195} In addition, the apoptosis of the remote region increases over time post-myocardial infarction and correlates with the developing left ventricular enlargement.\textsuperscript{195} This highlights the detrimental effect myocardial infarction has on the entire ventricle, especially as heart failure ensues.
CHAPTER 7: GENERAL DISCUSSION

7.1 Rat model of Pulmonary Arterial Hypertension

7.1.1 Validation of Model
The rat monocrotaline model is a well-established and popular model of pulmonary arterial hypertension (PAH). It is easy and cheap to produce, a single injection of 60 mg/kg body weight, and relatively quick to induce disease - the rats develop PAH and right ventricular dysfunction within three to four weeks following injection. One of the major criticisms of this model is that monocrotaline itself could directly injure the right side of the heart inducing right ventricular dysfunction. However, there is evidence that this is not the case, and the dysfunction is a result of pressure overload following PAH.

Lack of Structural Changes in Left Side of Heart
The first argument in defence of the monocrotaline model is that there are no structural changes seen in the left side of the heart. Echocardiography revealed no changes in anterior or posterior wall thickness of the monocrotaline-injected rats compared to control rats (Figure 3.8). In addition, no difference in the architecture of the left ventricle of the monocrotaline-injected rats was seen using both Masson’s trichrome and H&E staining (Figure 4.5). Using immunohistochemistry, there were no increases in CD68, vimentin or TGFβ expression (although there was a significant difference in the expression of CD68 expression in the left atria between the control and monocrotaline rats, the mean increase was small in comparison to that in the right side of the heart; Figures 4.9, 4.10, 4.11, 4.12, 4.13, 4.14, 4.15 and 4.16). No increases in apoptosis, as measured by TUNEL staining were seen in the left side of the heart in the monocrotaline-injected animals (Figures 4.17 and 4.18).

Selectivity of Monocrotaline
In the rat, the injected monocrotaline is inactive. In the liver, the monocrotaline is metabolised to the toxic monocrotaline-pyrrole, which selectively targets and injures the pulmonary vasculature. Regardless of the selectivity of the monocrotaline-pyrrole, the first vascular bed after the liver is the pulmonary vasculature. Here, the monocrotaline-pyrrole damages the vessels, inducing muscularisation and medial hypertrophy. In this study, the lungs and mesenteric vascular bed from control and monocrotaline-injected rats were donated to Dr. Basma Eid who was investigating the contractility of the vessels. To investigate the histology of the lungs, Dr. Eid stained sections from the cranial lobe using H&E (Figure 7.1). There was a significant increase in the medial thickness of the pulmonary arteries from the monocrotaline-injected animals compared to control animals (Figure 7.1).
Lung sections were also stained with aldehyde fuchsin to investigate elastin fibres. Normal, healthy vessels have a single elastic lamina. There was an increase in the number of vessels with a double elastic lamina, indicative of arterial muscularisation, in the monocrotaline-injected rats compared to control rats (Figure 7.2). This has been documented in previous studies of monocrotaline-induced PAH.\textsuperscript{198}
In this study, vessel structure in the ventricles was investigated using Masson’s trichrome staining. There were no differences seen in both the larger and smaller vessels of the right ventricle (and indeed left ventricle) in the monocrotaline-injected and control hearts (Figure 7.3). Unlike the pulmonary vessels, there was no muscularisation or hypertrophy of the ventricular vessels in the monocrotaline-injected rats. The remodelling of the pulmonary arteries and preservation of the vessels in the ventricles in the monocrotaline-injected animals demonstrates the selectivity of the toxin to the pulmonary vasculature.
Figure 7.3 Representative images of vessels in the right ventricle from control (A and C) and monocrotaline-injected animals (B and D). (A to D) Examples of larger (A and B) and smaller (C and D) vessels in the ventricles. Arrows indicate location of small vessels. Images taken at 20x magnification; scale bar represents 100 μm.

Systemic Vascular Effects
To further investigate the effect of monocrotaline, the contractility of the vessels were also measured by Dr. Eid. Following initial incubation with 50 nM potassium chloride (KCl) to test maximal contraction, application of the Kv7 blocker (XE991) to control pulmonary arteries caused contraction at 1 μM (Figure 7.4A). In comparison, in monocrotaline-injected animals, contraction of the pulmonary arteries occurred at 100 nM, resulting in a leftward shift in the concentration-response curve (Figure 7.4). These results suggest that the pulmonary arteries in the monocrotaline-injected animals are more contractile than the pulmonary arteries in control animals. This hypercontractility could possibly contribute to the pulmonary hypertension seen in vivo.
The experiment was repeated using mesenteric vessels. In comparison to the pulmonary arteries, the mesenteric arteries from both the control and monocrotaline-injected animals were insensitive to XE991 (Figure 7.5). This suggests that, unlike in the lungs, monocrotaline did not alter the contractile properties of the mesenteric arteries and this supports the hypothesis that the drug acts specifically on the pulmonary vasculature.

Figure 7.4 Effect of the vasoconstrictor XE991 on pulmonary arteries from control (A, top trace) and monocrotaline-injected (A, bottom trace) animals. (A) Typical traces. Following maximal constriction of the vessels with 50 nM KCl, increasing concentrations of XE991 were applied to the vessel and changes in tension measured. (B) Concentration-response curves for XE991 on pulmonary arteries from monocrotaline-injected and control animals. n=4. **p<0.01; ***p<0.001 (two-way ANOVA). Abbreviations: potassium chloride, KCl; monocrotaline-injected animals, MCT. From Dr. Basma Eid (unpublished data).
Figure 7.5 Effect of the vasoconstrictor XE991 on mesenteric arteries from control (A, top trace) and monocrotaline-injected (A, bottom trace) animals. (A) Typical traces. Following maximal constriction of the vessels with 50 nM KCl, increasing concentrations of XE991 were applied to the vessel and changes in tension measured. (B) Concentration-response curves for XE991 on pulmonary arteries from monocrotaline-injected and control animals. n=4. **p<0.01; ***p<0.001 (two-way ANOVA). Abbreviations: potassium chloride, KCl; monocrotaline-injected animals, MCT. From Dr. Basma Eid (unpublished data).
7.1.2 Therapeutic Implications of PAH

PAH is devastating illness, and despite advances in health care and treatments, the outlook still remains poor. Currently, treatment for PAH involves targeting the vascular hypertension. Some of the pharmacological agents used include endothelin-1 receptor antagonists, phosphodiesterase (PDE) type 5 inhibitors and prostaglandins. Endothelin-1 and PDE5 are potent vasoconstrictors, while prostaglandins are vasodilators. The treatments work by alleviating the increased pressure and resistance in the pulmonary arteries in PAH. Although these pharmacological therapeutics improve survival and clinical outcomes, the prognosis still remains poor for patients with PAH.

The major cause of death in patients with PAH is right-sided failure. In addition to current therapy to treat the hypertension in the pulmonary vasculature, cardio-specific therapeutics to target and treat the secondary right-sided heart failure would undoubtedly improve clinical outcomes and reduce morbidity and mortality. Cardio-specific treatments to prevent or slow the adverse remodelling would directly aid the failing heart. This study has shown that there is inflammation in both the working myocardium and CCS in PAH. As reviewed in the Introduction (Chapter 1), inflammation has been shown to be detrimental in the heart and is capable of inducing structural and electrical remodelling. A therapeutic target to interfere with the inflammatory process could be predicted to prevent, if not reverse, the cardiac remodelling.

7.2 Major Findings of the Study

Three animal models have been investigated in this study: the rat model of PAH, the rabbit model of pressure and volume overload (congestive heart failure) and the rat model of myocardial infarction. In all three models, there was evidence of cardiac dysfunction and failure. Additionally, all models showed an increase in gene expression of inflammatory and fibrosis targets.

Figures 7.6, 7.7 and 7.8 compares the changes in gene expression in heart disease across the three models. In all models there appears to be an upregulation of fibronectin (Figure 7.7). In the rat PAH and myocardial infarction models there is a general upregulation of the cytokines IL1β and TNFα and the extracellular matrix components collagens I and III, connective tissue growth factor (CTGF) and TIMP1 (Figures 7.6, 7.7 and 7.8). The cytokine TNFα appears to be important in the rabbit model of congestive heart failure and the rat model of myocardial infarction, while few changes were measured in the rat model of PAH (Figure 7.6). Interestingly, fibronectin and TIMP1 were often greatly increased; the magnitude of change was much greater then the other genes measured.
Figure 7.6 Comparison of changes in gene expression in the different models. The graphs show mean ± SEMs for control (in blue; n=4-8) and heart failure animals (in red; n=7). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum). Abbreviations: angiotensin II receptor 1 types a and b, ATR1a and ATR1b; border zone, BZ; left atrium, LA; left ventricle, LV; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; right atrium, RA; right Purkinje fibres, RPF; remote region, RR; right ventricle, RV; sinoatrial node, SAN; mothers against decapentaplegic homolog 2/3, Smad 2/3; transforming growth factor β, TGFβ; tumour necrosis factor α, TNFα.
Figure 7.7 Comparison of changes in gene expression in the different models. The graphs show mean ± SEMs for control (in blue; n=4-8) and heart failure animals (in red; n=7). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum). Abbreviations: border zone, BZ; connective tissue growth factor, CTGF; left atrium, LA; left ventricle, LV; right atrium, metalloproteinase 2, MMP2; RA; right Purkinje fibres, RPF; remote region, RR; right ventricle, RV; sinoatrial node, SAN; tissue inhibitor of metalloproteinase 1, TIMP 1.
Figure 7.8 Comparison of changes in gene expression in the different models. The graphs show mean ± SEMs for control (in blue; n=4-8) and heart failure animals (in red; n=7). Statistical differences between groups were determined using Student’s t-test (if normality and equal variance tests were passed; otherwise Mann-Whitney Rank Sum Abbreviations: border zone, BZ; left atrium, LA; left ventricle, LV; right atrium, RA; right Purkinje fibres, RPF; remote region, RR; right ventricle, RV; sinoatrial node, SAN; tissue inhibitor of metalloproteinases 2-4, TIMPs 2-4.

By comparing the changes in gene expression across the three models investigated, it can be seen that some genes, like fibronectin and TIMP1, appear to be modulated in the different types of heart disease, irrespective of the primary insult. However, some genes appear to be more important in some models than others. These findings suggest that the inflammatory and fibrosis processes are complex and are possibly disease, or animal, specific.

In this study, the rat model of PAH was examined in most detail and was the main focus of the research. Following development of the model, the animals were shown to have acquired right-sided heart failure and electrical remodelling (Chapter 3). Post-mortem examination of the animals revealed increased mRNA and protein expression of inflammatory and associated fibrotic and apoptosis mediators in the right side of the heart.

A novel feature of this study was that different regions of the heart were examined and compared. This included the working myocardium (the atria and ventricles) and the CCS
(SAN, AVN and Purkinje fibres). Most other investigators have only studied the ventricles. The heart is a complex, heterogeneous organ, with no two regions the same. For instance, the atria and ventricles are structurally and functionally different. They respond differently to different stimuli and insults. For example, as discussed in chapter five, the atria are more susceptible to fibrosis by growth factors. There are also differences between the different sides of the heart. The left side of the heart is responsible for pumping blood to the entire body, and as a result is muscular and thick walled to generate the required pressure. On the other hand, the right side of the heart only perfuses the nearby low resistance pulmonary vasculature, which does not require the same level of pressure, and is consequently much thinner walled. Additionally, the CCS is distinctive to working myocardium. The CCS expresses different ion channels that give it its unique conducting and pacemaking properties. The different regions of the heart would inevitably react differently in disease states.

7.2.1 Further Work
As in many studies, the work here is not complete and further research is required to obtain a comprehensive picture of the role of inflammation in remodelling of the CCS in heart failure. It is likely that the inflammation is responsible for the upregulation of fibrosis genes in the three models because of the association of the two in the current work (Chapters 4 to 6) and also the known link between inflammation and fibrosis. However there is also remodelling of ion channels and related molecules. Is inflammation responsible for this?

In the rabbit model of congestive heart failure, it would be interesting to repeat the study to investigate if there is inflammation and associated fibrosis in the left atrium. The left atrium was unfortunately not available for study in this project. Surprisingly, the majority of mRNA changes were measured in the right atrium and it is possible that changes in the left atrium are more pronounced, given that it is a left-sided pressure and overload heart failure model.

It will be important to distinguish which cell types are responsible for the increase in inflammatory cytokines. The majority of cells of the heart are capable of secreting cytokines, including leukocytes, fibroblasts and even myocytes. In this study, in the PAH model, histology has shown patches of non-myocytes and histology revealed patches of cells that were positively labelled for vimentin. Vimentin is a classic fibroblast marker but it has also been shown to be expressed by macrophages. It can be suggested at this stage that either macrophages or fibroblasts, or a combination of the two, are responsible for the cytokine expression. However, double labelling with more specific macrophage or fibroblast markers might help to obtain a clearer picture.
7.3 Therapeutic Potential

Heart failure is a devastating syndrome, affecting millions of people worldwide. While the current treatments available improve patients’ morbidity and mortality, the prognosis for heart failure patients still remains poor. There is a need for novel therapeutics. Could anti-inflammatories be the answer?

There is a well-established association between heart failure and activation of the inflammatory response. Systemic inflammation is capable of predicting severity and progression of heart failure. Animal models have shown that activation of the inflammatory response is detrimental to the heart's function. Inflammation activates fibrosis pathways, remodels the extracellular matrix and alters ion channel expression. If chronic, as in heart failure, this normally protective and crucial process can become destructive and exacerbate an already diseased heart.

Inflammation and associated fibrosis and apoptosis have been shown to occur in the failing myocardium. In this study, it is shown that in addition to the myocardium, there is inflammation of the CCS in heart failure. However, although this study identified activation of inflammation, it was unable to decipher if inflammation was the cause of CCS dysfunction in heart failure. Further studies need to be carried out to find the exact role inflammation is playing in the remodelling of the CCS. To do this, two approaches could be applied. Firstly, the effect of inducing inflammation, for example, by activating an inflammatory pathway(s) and CCS function could be studied. Secondly, the effect of inhibiting one or more inflammatory pathways on the remodelling of the CCS could be studied. This could reveal which pathways and cytokines are the main contributors to dysfunction and identify possible targets of intervention.

7.3.1 Previous Clinical Trials

As a result of the overwhelming evidence that inflammation plays a role in the pathogenesis of heart failure, a number of clinical trials targeting the inflammatory response have taken place. The most well known of these are the RENAISSANCE, RECOVER and ATTACH trials.

The RENAISSANCE (Randomised Etanercept North American Strategy to Study Antagonism of Cytokines) and RECOVER (Research into Etanercept Cytokine Antagonism in Ventricular Dysfunction) trials were parallel studies investigating etanercept (Enbrel), a fusion protein that inhibits the bioactivity of TNFα in heart failure. Over 900 patients were enrolled in each trial. The RENAISSANCE patients received 25 mg etanercept either twice or three times a week, while the RECOVER patients received 25
mg once or twice a week. Control patients in both trials received a placebo. Despite the success of previous preliminary studies, the RENAISSANCE and RECOVER trials were prematurely terminated by the Data Monitoring Safety Board (DMSB) as it was found that etanercept treatment did not demonstrate a benefit.202

Around the same time, another clinical trial, ATTACH (the Anti-TNFα Therapy Against Congestive Heart Failure) was being carried out. This was a phase II trial, and like the RENAISSANCE and RECOVER trials, the ATTACH trial was also targeting TNFα.203 ATTACH investigated the use of infliximab (Remicade), a monoclonal IgG1 antibody for TNFα which had previously been shown to be beneficial in the autoimmune conditions Crohn’s disease and rheumatoid arthritis. The ATTACH trial recruited 150 patients who were given either 5 mg/kg or 10 mg/kg infliximab, or a placebo. However, as with etanercept, infliximab did not improve clinical outcomes in heart failure.203

Given the well-established association between inflammation and heart failure, which includes the involvement of the pro-inflammatory cytokine TNFα, the results of the RENAISSANCE, RECOVER and ATTACH trials are surprising. The failure of TNFα inhibition to alleviate conditions in heart failure has yet to be explained. However, there are some hypotheses as to why this could be the case. It has been proposed that infliximab antibody is toxic to cells that express TNFα on their cell membranes.200 As the failing myocytes express TNFα, infliximab would be expected to be toxic and could contribute to worsening outcomes.200

The TNFα therapies used in the trials could have been too severe. Inflammation is an essential process required for tissue homeostasis and is involved in general repair and maintenance. If the inflammation is completely abolished or impaired, the benefits of the process will be compromised. To target chronic inflammation, as seen in heart failure, the inflammation should not be completely abolished and a physiological level should be retained. The maintenance of a physiological inflammation would be complex and would undoubtedly involve careful monitoring to ensure a careful balance was obtained. In addition, very high doses of both etanercept and infliximab were administered to patients.204 In the clinical trials, patients given the lower dose of both drugs fared better, but it has been suggested that even these were too high.204

In these trials, the cause of heart failure was not taken into consideration. Heart failure is a complex and progressive syndrome, of which there are innumerable causes. For instance, myocardial infarction, PAH and valve disease all produce heart failure, but as a result of different primary insults. It is feasible that the different types of heart failure activate different components of the inflammatory response. In this study, there are differences in
cytokine activation between the different animal models. In PAH, there was activation of interleukin 1β (IL1β) on the right side of the heart, while TNFα was not (Figures 4.24 and 4.25). In contrast, in the myocardial infarction model, in the border zone, both IL1β and TNFα were activated (Figure 6.7).

Additionally, in these trials a systemic inhibition of TNFα was induced. Perhaps a more cardiac-specific therapy would have been more beneficial, or even a heart region-specific therapy. This study has demonstrated, in the PAH model and myocardial infarction model, there was an increase in inflammation in the regions of the heart expected to receive the primary insult (i.e. the right side of the heart in PAH and the border zone in myocardial infarction). However, in the rabbit model of congestive heart failure, the story is less straightforward. Although the primary insult was on the left side of the heart, inflammation was primarily seen in the right atrium, possibly due to this chamber being more sensitive to insult than the other regions investigated. This suggests more research should be applied to determine which regions are most affected by inflammation and would be most appropriately targeted. This could depend on the cause of heart failure. However, as no two individual cases of heart disease are identical it could be a complex story.

In summary, despite initial clinical trials being unsuccessful, anti-inflammatory therapy should not be ruled out as a novel treatment for heart failure. Research has shown that inflammation is involved in the pathogenesis of heart failure. However, both heart failure and the inflammatory process are complex and further research is required to fully understand how they are interacting. Only then will it be feasible to produce disease, and possibly cardiac, specific therapeutics.
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