

Improved Outcome Prediction in Tetralogy of Fallot

Thesis submitted for the degree of *Doctor of Philosophy*

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For my parents

&

To Julian and Peter

Statement of Originality

I certify that this thesis, and the research to which it refers, represents my own work except where referenced or acknowledged otherwise in accordance with the standard referencing practices of the discipline.

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Abstract

Successful advances in cardiac surgery have led to a paradigm shift in the management of an expanding population of repaired tetralogy of Fallot (rTOF) patients. However, late morbidity and mortality have not been abolished, with patients vulnerable to arrhythmia and sudden death. Outcome prediction remains challenging, mandating the identification of novel sensitive and specific non-invasive biomarkers. Cardiac fibrosis in rTOF has been shown to correlate to adverse clinical features, and therefore merits further study, particularly with regards to interstitial fibrosis.

Cardiac remodelling following surgical pulmonary valve replacement in patients with rTOF was investigated. Structural reverse remodelling was observed to occur immediately after surgery, followed by gradual biological remodelling. A proactive surgical approach before right ventricular (RV) end-systolic indexed volumes exceed 82ml/m^2 confers optimal postoperative RV normalisation.

Novel cardiovascular magnetic resonance T1 mapping techniques were developed and tested to improve identification of RV interstitial cardiac fibrosis in rTOF. Multi-echo imaging to separate fat from myocardium, combined with blood signal suppression is promising as a feasible method in saturation-recovery T1 mapping, but requires further technical study prior to clinical application and validation.

The genomic signatures of the pathological RV in rTOF were investigated by next generation RNA sequencing. Differential gene expression was evident, and potential molecular determinants of fibrotic and restrictive phenotypes were ascertained. Ubiquitin C may have important functional implications as a 'network hub' gene in rTOF.

Finally, the longitudinal predictive role of neurohormone expression in patients with rTOF was examined. Neurohormonal activation was confirmed in rTOF, with serum brain natriuretic peptide being prognostic for mortality and sustained arrhythmias during extended follow-up.

In conclusion, this work reflects the complex interplay of candidate biomarkers in influencing clinical outcomes. Myocardial fibrosis in rTOF remains a key diagnostic and therapeutic target for improving risk stratification and ameliorating morbidity in the lifelong care of these individuals.

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Table of Contents

Abstract.....	5
Acknowledgements.....	6
List of Figures	15
List of Tables	22
List of Abbreviations	25
Preamble	29

Chapter 1 INTRODUCTION.....31

1.1 Tetralogy of Fallot.....	31
1.1.1 Background	31
1.1.2 Epidemiology and survival in repaired tetralogy of Fallot	31
1.1.3 The repaired tetralogy of Fallot patient.....	32
1.1.4 The pathological right ventricle in tetralogy of Fallot.....	32
1.1.5 Pulmonary Valve Replacement (PVR)	33
<i>Indications and current practice for surgical pulmonary valve replacement</i>	<i>33</i>
<i>Percutaneous Pulmonary Valve Replacement</i>	<i>34</i>
1.1.6 Restrictive phenotype in tetralogy of Fallot	35
1.1.7 Risk stratification	35
1.2 Cardiovascular Magnetic Resonance	37
1.2.1 Background	38
1.2.2 Basic Physics of CMR	39
<i>Specific considerations for CMR</i>	<i>41</i>
1.2.3 T1 and T2 Relaxation	41
1.2.4 CMR Sequences	43
<i>Gradient echo bright blood imaging.....</i>	<i>44</i>
<i>Spin echo black blood imaging.....</i>	<i>45</i>
1.2.5 Late Gadolinium Enhancement Cardiovascular Magnetic Resonance.....	46
1.3 Myocardial Fibrosis	47
1.3.1 The pathophysiology of myocardial fibrosis	47
1.3.2 Circulating biomarkers of myocardial fibrosis.....	50
1.3.3 Myocardial Fibrosis in tetralogy of Fallot.....	50

1.4	CMR T1 Mapping in Congenital Heart Disease	51
1.4.1	CMR T1 Mapping: Background	51
1.4.2	CMR T1 Mapping Sequences.....	54
	<i>Modified Look-Locker Inversion Recovery (MOLLI).....</i>	<i>54</i>
	<i>Saturation Recovery Single-shot Acquisition (SASHA)</i>	<i>55</i>
1.4.3	Accuracy and Precision	56
1.4.4	T1 mapping in Adult Congenital Heart Disease	58
1.4.5	Considerations in RV T1 Mapping	59
	<i>RV Epicardial and Intramyocardial Fat.....</i>	<i>59</i>
	<i>Partial Volume Effects.....</i>	<i>60</i>
1.4.6	Approaches to RV T1 Mapping.....	60
1.5	Genomics in tetralogy of Fallot	71
1.5.1	Genomics in adult congenital heart disease	71
1.5.2	Genetic determinants of tetralogy of Fallot	72
1.5.2.1	<i>Chromosomal abnormalities and gene mutations in TOF</i>	<i>72</i>
1.5.2.2	<i>Differential Gene Expression in TOF</i>	<i>73</i>
1.5.2.3	<i>The impact of hypoxia on gene expression in TOF</i>	<i>74</i>
1.5.2.4	<i>Altered signalling pathways in TOF</i>	<i>75</i>
1.5.2.5	<i>Copy Number Variants (CNVs) in TOF.....</i>	<i>75</i>
1.5.2.6	<i>Genome Wide Association Studies (GWAS) in TOF.....</i>	<i>75</i>
1.5.3	Clinical applications of genomics in congenital heart disease	76
1.6	Neurohormonal biomarkers in tetralogy of Fallot	78
	Specific Aims of the Thesis	80
	Chapter 2 METHODS	81
2.1	Patient Identification.....	81
2.2	Cardiovascular Magnetic Resonance	81
2.2.1	The CMR examination.....	81
	<i>Patient Preparation</i>	<i>81</i>
	<i>Inclusion Criteria</i>	<i>82</i>

<i>Exclusion Criteria</i>	82
2.2.2 CMR data acquisition protocol	82
<i>Pilot Scouts</i>	83
<i>Cine Imaging</i>	84
<i>Flow Imaging</i>	87
<i>Late Gadolinium Enhancement (LGE) Imaging</i>	88
2.2.3 CMR image analysis	90
<i>Volumetric & functional analysis</i>	90
<i>Flow quantification: pulmonary valve peak velocity and regurgitant fraction</i>	92
<i>Definitions of CMR derived indices</i>	92
<i>Late Gadolinium Enhancement Analysis</i>	93
<i>Reproducibility Assessment of CMR measurements</i>	94
2.2.4 T1 mapping in repaired tetralogy of Fallot	95
2.3 Endomyocardial biopsy and histological analysis	96
2.4 RNA Sequencing	97
2.5 Non-invasive Investigations	97
2.5.1 Venous blood sampling for neurohormonal analysis	98
2.5.2 Echocardiography	98
2.5.3 Cardiopulmonary exercise testing	99
2.6 Statistical Methods	100
RESULTS	101
 Chapter 3 REPRODUCIBILITY OF RIGHT VENTRICULAR VOLUMETRIC AND FUNCTIONAL ASSESSMENT	 102
3.1 Introduction	102
3.2 Methods	102
3.2.1 CMR Acquisition – TOF Protocol	102
3.2.2 CMR Image Analysis	103
3.2.3 Statistical Analysis	103
3.3 Results	104

4.4.4	Evidence of left ventricular reverse remodelling.....	127
4.4.5	Limitations	128
4.5	Conclusions	128

Chapter 5 QUANTIFICATION OF RIGHT VENTRICULAR DIFFUSE FIBROSIS IN REPAIRED TETRALOGY OF FALLOT 130

5.1	Aims and Methods	130
5.1.1	Phantom preparation	130
5.1.2	Short-term phantom stability	132
	<i>Phantoms: Same-day repeatability and 10-week stability results and discussion</i>	<i>133</i>
5.1.3	Long-term phantom stability	134
	<i>Phantoms: One-year weekly stability results and discussion.....</i>	<i>135</i>
5.1.4	RV T1 mapping technical development	137
5.1.4.1	Modifications to the MOLLI sequence.....	138
5.1.4.2	Respiratory-navigator gated, fat-water separated segmented Saturation Recovery Single-Shot Acquisition (SASHA)	141
5.2	Results and Discussion	150
	Right ventricular T1 mapping by free-breathing fat-water separated dark blood single shot saturation-recovery imaging	150
5.2.1	Dark blood motion-sensitized preparation (MSPrep)	150
5.2.2	Optimising saturation efficiency	152
5.2.3	Optimising saturation recovery imaging	154
5.2.4	RV T1 Mapping Scan Protocol	157
5.2.5	RV T1 Mapping Reproducibility	163
5.2.6	Limitations	167
5.2.7	Future work	167
5.3	Conclusions	168

Chapter 6 GENOMIC SIGNATURES IN REPAIRED TETRALOGY OF FALLOT: MYOCARDIAL FIBROSIS AND IMPLICATIONS FOR CARDIAC REMODELLING 169

6.1	Aims and Methods	169
------------	-------------------------------	------------

6.1.1	Patient Population	169
6.1.2	RNA Sequencing.....	170
6.1.3	Data Processing	170
6.1.4	RV data analyses	174
6.1.4.1	<i>Differential gene expression testing</i>	174
6.1.4.2	<i>Relating gene expression to fibrosis burden in rTOF</i>	174
	<i>Functional Gene Set Enrichment Analysis (GSEA) for fibrosis in rTOF</i>	174
6.1.4.3	<i>Genomic network analysis in rTOF</i>	174
6.1.5	Statistical Analysis.....	175
6.2	Results.....	175
6.2.1	Baseline Characteristics	175
6.2.2	Differential Gene Expression in rTOF	177
6.2.3	Genomic signatures of fibrosis in rTOF	184
6.2.4	Genomic signatures of the restrictive rTOF phenotype	188
6.2.5	Ubiquitin C (UBC) as a gene network hub in rTOF	190
6.3	Discussion.....	193
6.3.1	Altered expression of the extracellular matrix and collagens.....	193
6.3.2	Oxidative phosphorylation in rTOF	194
6.3.3	Neurohormonal gene expression in TOF	195
6.3.4	TGF β in rTOF	197
6.3.5	The restrictive RV phenotype and fibrosis	197
6.3.6	Ubiquitin in rTOF	198
6.3.7	Limitations	200
6.3.8	Future Work	201
6.4	Conclusions	201

Chapter 7 NEUROHORMONAL ACTIVATION AND ITS RELATION TO OUTCOMES LATE AFTER REPAIR OF TETRALOGY OF FALLOT

.....**202**

7.1	Aims and Methods	202
7.1.1	Patient Population and Study Design	202
	<i>CMR Assessment</i>	203
	<i>Neurohormone Assessment</i>	203

<i>Study Endpoints</i>	204
7.1.2 Statistical Analysis.....	204
7.2 Results	204
7.2.1 Baseline characteristics.....	204
7.2.2 Neurohormonal dysregulation in rTOF.....	207
7.2.3 Outcomes.....	210
<i>Primary endpoint: Mortality</i>	210
<i>Secondary endpoint: Sustained atrial or ventricular tachy-arrhythmia</i>	213
7.3 Discussion	213
7.3.1 The prognostic value of BNP	213
7.3.2 Dysregulation of ET-1 in rTOF	214
7.3.3 Applicability of neurohormone measurement to clinical practice	215
7.3.4 Limitations	216
7.4 Conclusion	216
Chapter 8 CONCLUSIONS	217
8.1 Summary of Findings	217
8.2 Future Work	220
8.2.1 Longitudinal predictive value of focal fibrosis in rTOF	221
8.2.2 Technical development for RV T1 mapping by MRI	221
8.2.3 Clinical validation study of diffuse fibrosis in rTOF	221
8.2.4 Advancing genomics in patients with rTOF	223
8.2.5 CMR detection of myocardial disarray in rTOF	223
8.2.6 Diastolic dysfunction in rTOF by DENSE CMR imaging.....	224
8.3 Concluding remarks	225
REFERENCES	226
APPENDICES	260
Abstracts and Publications List Related to Thesis	260
Publications	260
Abstracts & Presentations	260

List of Figures

1.1: System components of an MRI scanner with upper image illustrating positions of main magnet coils, RF transmitter and receiver coils relative to patient and lower image showing typical arrangement of cylindrical bore MR system with reference coordinate axes in which the static B_0 field is aligned with the horizontal z axis. Reproduced from Ridgway et al.⁷⁶

1.2: Random orientation of protons outside the magnet on the left, with subsequent alignment of a small excess majority following application of a magnetic field (blue arrow).

1.3: T1 relaxation process illustrated following application of a 90° RF pulse, with M_z (z component of net magnetisation) reduced to zero and exponentially recovering with time back to equilibrium. (From Ridgway, J Cardiovasc Magn Reson 2010)⁷⁶

1.4: T2 decay illustrated similarly following application of 90° RF pulse, with M_{xy} decaying with time after RF excitation into the transverse plane to zero.

1.5: Flow diagram of myocardial fibrosis cascade in which myocardial injury triggers local cell activation, leading to altered ECM expression that culminates in myocardial fibrosis.

1.6: Sampling of the IR curve at multiple inversion-recovery times at the same cardiac phase of multiple cycles. The vertical black lines each represent a single-shot bSSFP image acquisition. There are 3 images after the first inversion pulse, followed by 3 cycles recovery, followed by another inversion with 3 images (slightly incremented T1), another 3 cycles of recovery, and a final inversion with 5 images. This scheme is written as 3(3)3(3)5. Reproduced from Kellman et al²¹⁴ based on the original 3(3)3(3)5 MOLLI protocol diagram by Messroghli.²⁰²

1.7: Sampling scheme for SASHA (reproduced from Kellman et al)²¹⁴

1.8: Fat-water separated free-breathing LGE phase sensitive inversion recovery (PSIR) images in LVOT view from patient with rTOF. From left to right – magnitude water image illustrating area of hyper-enhancement of the RV (arrowed), middle panel fat only image illustrating significant epicardial fat surrounding the heart, right panel superimposed fat-water image with surrounding epicardial fat highlighted. The epicardial fat layer would have introduced substantial error to T1 measurements made if not identified and avoided in ROI selection.

2.1: Siemens 3T Skyra scanner at the Royal Brompton Hospital (image courtesy of Ms Julia Coffey, Cardiovascular BRU public engagement coordinator).

2.2: Transaxial HASTE **(A)** with corresponding transaxial TruFISP slice **(B)** and representative coronal **(C)** and sagittal **(D)** TruFISP scouts in a patient with rTOF. Note RV dilatation visible in all illustrated planes.

2.3: Representative cine frames acquired in a patient with rTOF in: RVOT sagittal **(A)**, RVOT transaxial **(B)**, LV vertical long axis **(C)**, right and left branch pulmonary arteries **(D to F)**, four chamber **(G)**, LVOT transaxial and coronal views **(H and I)**, RV inflow and outflow **(J)**, RV oblique long axis **(K)** and RV long axis **(L)** views. The akinetic RVOT region is demarcated by the yellow arrow in Panel **A**.

2.4: Short-axis cines from base to apex (top left hand panel to bottom right panel) with RV dilatation in patient with rTOF. AV: aortic valve, LV: left ventricle, RV: right ventricle.

2.5: Flow sequence magnitude image aligned with RVOT in sagittal projection in patient with rTOF **(A)**, with in-plane velocity encoding along the head-foot image axis (displayed vertically on page) so that corresponding velocity maps show forward flow as indicated by black arrow **(B)** and severely regurgitant jet across an ineffective pulmonary valve, indicated by white arrow **(C)**. Through-plane phase contrast velocity mapping demonstrates the pulmonary artery (PA) in cross-section in the magnitude image **(D)**, with forward flow appearing bright in systole **(E)** and a dark appearance of a broad area of severe regurgitation in diastole **(F)** in velocity maps (yellow arrows).

2.6: LGE images of adult patient with rTOF in **(A)** SAX **(B)** LVOT transaxial **(C)** RV inflow and outflow as well as **(D)** RV oblique views. There is RV LGE of the RVOT (indicated by solid white arrows) seen in all panels, which extends to the endocardium of the anterior RV free wall in Panel A. Further LGE of the VSD patch surgical sites (marked with asterisk *) as well as LGE of RV trabeculations (indicted by dotted white arrows in Panels A and C) are present. LV apical LGE consistent with an apical vent from previous surgery is seen in Panel B. Reproduced from Heng et al, Tetralogy of Fallot In: Cardiac CT and MR for Adult Congenital Heart Disease, Springer 2014⁸² with permission of Springer.

2.7: Measurement of biventricular volumes and function with CMRtools. Screenshots from the semi-automated model illustrates **(A)** demarcation of epicardial and endocardial borders of both ventricles, such that the borders of myocardial mass is shaded in yellow, LV blood shaded in orange and RV blood shaded in blue. **(B)** Manual thresholding of the blood pool excludes LV papillary muscle and RV trabeculations from the blood pool to generate 3D models of biventricular volumes **(C)** in long axis (with RV on left and LV on right of image) and **(D)** in short axis in a similar image plane to panels A and B.

2.8: **(A)** Right atrial area delineated in four chamber cine. Akinetic length of RVOT bordered by white arrows in RVOT cine frame (sagittal view) in panels **(B)** and **(C)** illustrating variation in RVOT akinesia.

2.9: Segmental system for scoring RV LGE in rTOF (reproduced from Babu-Narayan *et al*⁶¹ with permission from Wolters Kluwer Health) The RV is divided into 7 segments in slices aligned with the RVOT, the LV outflow tract, and the LV short axis, with maximum LGE score per segment in brackets. Each segment was graded according to the linear extent of enhanced myocardium as follows: 0 - no enhancement, 1 - up to 2 cm LGE in length, 2 - 2 to 3 cm LGE in length, and 3 - more than 3 cm LGE in length. Enhancement seen in trabeculations, including the moderator band, was graded as 0 - no enhancement, 1- enhancement of 1 trabeculation, 2 - enhancement of 2 to 4 trabeculations, and 3 - enhancement of more than 4 trabeculations.

2.10: Continuous wave Doppler of RV outflow tract in patient with repaired tetralogy of Fallot illustrating 'a' wave (white arrow) of RV restrictive physiology. Reproduced from Orwat *et al*, Eur Heart J Cardiovasc Imag, 2014³⁵⁸ by permission of Oxford University Press.

3.1: Bland-Altman plots of intra-observer mean differences for LV and RV parameters. The horizontal red line plots the mean difference and the dashed blue lines demarcate the limits of agreement (= 2 standard deviations) for each parameter.

4.1: Right heart CMR parameters at study time points (red – pre-PVR, yellow – immediate post-PVR within 14 days, green – at midterm follow-up post-PVR).

4.2: Receiver-operating characteristic curves illustrating threshold value of **(A)** pre-PVR RVEDVi of 158ml/m² for normalisation of RVEDVi **(B)** pre-PVR RVESVi of 82ml/m² for normalisation of RVESVi. The 95% confidence intervals for area under curve are quoted within the parentheses

5.1: Phantom experiment images illustrating derived T1 and T2 maps (B&D) generated.

5.2: Variation of T1 from high-resolution protocol at RR 800ms over ten weeks with temperature corrected values shown in black (T=20 to 22.5°C). Tubes A to D are as defined in *Table 5.1*. Reproduced from Heng *et al*.

5.3: Longitudinal trend in T1 and ECV values over a one year timeframe, illustrating gradual positive drift in native blood T1 values whilst native myocardial T1, post- contrast T1 values and ECV remained more stable in comparison. Reproduced from Vassiliou *et al*, J Cardiovasc Magn Reson 2016.³⁹⁶

5.4: T1 maps derived from MOLLI images acquired of systemic RV patient for pilot study.

5.5: Representative MOLLIs in SAX slice in systemic RV patient in top row with clearly visible RV myocardium in contrast to second row in patient with rTOF in whom the thinner subpulmonary RV myocardium cannot be clearly delineated without corruption by adjoining blood and fat.

5.6: MOLLI acquisition in normal subject with limited visualisation of thin RV myocardium despite systolic and prone imaging.

5.7: Schematic of pulse sequence in which $N=2$ echoes and $M=12$ (comprising 6 segments with 2 interleaves with each interleave collecting data for 2 echo points). Reproduced from Kellman.⁴²⁰

The IR was replaced with a saturation preparation pre-pulse, and four different echo times were acquired by two-fold interleaving of 2-echo readouts (the faster fat-water phase accumulation at 3T than 1.5T is a disadvantage).

5.8: Water images of navigator RV T1 in the SAX orientation in a normal volunteer with dark blood acquisition, followed by stepwise increasing TIs and a final anchor image. The dark-blood acquisition in this study was solely in order to support ROI delineation at the final stage of image analysis onto the thin RV wall. This blood suppression was achieved by the “double-inversion-recovery” approach which is, unfortunately, fundamentally incompatible with T1 mapping. The dark-blood image was not an input to the T1 calculation.

5.9: Increasing dephasing and loss of signal in the RV myocardium in patient with previous cardiac surgery caused by sternal wire artefact (arrowed) with higher TEs across images from left panel to right.

5.10: Results of inter-scan reproducibility of RV myocardial and blood, as well as LV septal T1 measurements.

5.11: Images from normal volunteer illustrating respiratory ghosting artefact in the ‘anchor’ image panel, in which the RV free wall myocardium cannot be discerned.

5.12: Increasing FOS values from left to right in panel showing progressively weaker blood suppression, most evident in the superior aspect of the RV in this SAX slice in a normal volunteer. The images have been acquired at identical cardiac diastasis timings, with more bright blood signal visible in the RV cavity in the FOS 45 image compared to FOS 20.

5.13: BIR4 performance for myocardial tissue ($T_1=1100\text{ms}$, $T_2=45\text{ms}$) with design range of FA and off-resonance variation denoted by dotted box. Pulse evaluation at NIH provided suppression to $<0.5\%$ of M_0 over 70-100% FA range (image courtesy of Dr Peter Kellman).

5.14: Myocardial T1 measurement by multi-echo fat/water separated saturation-recovery single-shot acquisition (SASHA) with blood signal suppression. Image courtesy of Dr Peter Gatehouse.

5.15: Schematic of RV T1 mapping scan protocol.

5.16: Sequential outline of RV T1 mapping in normal volunteer. The same RV SAX slice is selected throughout. MSPrep optimisation images demonstrate that an image start time of 615ms in this instance provides the best myocardial definition when compared to 565ms and 665ms (while the RV is not unusable at those times lest any drift should occur, a loss of RV signal at either of those times would have ruled out proceeding with the use of 615ms at 40cm/s FOS and would have required further test scans). Typically 6 to 12 test scans for MSPREP must be expected.

Diastolic imaging was therefore carried out at 615ms with a FOS 40cm/s to produce representative images of free-breathing, fat-water separated saturation recovery imaging and the corresponding pixelwise T1 map in the bottom panel.

5.17: Typical variation of FOS and cardiac timing (in ms) to determine optimal MSPrep parameters in a normal volunteer. This enabled selection of imaging timing of 650ms and FOS 50cm/s as the optimal parameters for this subject.

5.18: Representative short-axis images from subjects scanned by fat-water separated, MsPrep dark blood imaging for RV T1 mapping. Images for five subjects with rTOF and five healthy volunteers are shown (one subject per column). *Top row* – MoCo averaged water-only image at Ts600ms, *Second row* – MoCo averaged water-only anchor image at same window/level, *Third row* – MoCo averaged fat only image, *Bottom row* – T1 map generated from registration and 2-parameter fit of the six images per sampling scheme.

The different image orientation for most rTOF compared to normals was not considered likely to be relevant: the phase-encode direction was not changed. This orientation is simply a display convention and the angle of this plane was near to 45 degrees in some axis used by the scanner reconstruction that governed the displayed orientation.

6.1: Log2 FPKM values of the genes in the RV cohort, before (light purple) and after (dark purple) filtering out genes expressed at low levels. The filtering criterion applied was FPKM=1 in at least 5% of the cohort samples (i.e. in at least two samples).

6.2: Distribution of the filtered VST gene counts in the RV data cohort.

6.3: Boxplot of global gene expression after VST transformation and filtering, in which each boxplot represents each RV sample (rTOF patients denoted by case numbers, whilst controls denoted by 'DRV' prefix).

6.4: Heat map of VST transformed expression counts levels for selected sex associated genes.⁴³⁵ In the top bar, samples are colour coded by gender (*black*: male, *orange*: female and *white*: sample with missing gender).

6.5: Variable extent of RV myocardial fibrosis seen with picrosirius red stain, ranging from predominantly normal cardiomyocyte structure with pale yellow appearance in panel **(A)** to increasing extents of fibrosis from panels **(B)** to **(D)** with progressive intensity of red stain. Destruction of normal myocyte architecture with extensive fibrosis is seen in panel **(D)**.

6.6: GO hierarchical analysis of cellular components gene expression in restrictive rTOF phenotype, illustrating the dominant role of the extracellular matrix in this phenotype.

6.7: Network analysis of rTOF restrictive phenotype (seeded from top 25 genes differentially expressed in restrictive rTOFs, ranked according to the nominal p value). Protein-protein interactions are clustered around fibrosis-related genes that include TGF β , TIMP1, EGFR, MMP1 and SMADs.

6.8: Protein-protein interactions of differentially expressed genes in rTOF with UBC as centralised seed.

6.9: Protein-protein interactions of gene expression correlated to fibrosis burden in study subjects with rTOF. Of note, UBC again features centrally in the schema.

6.10: Interactions of UBC with differentially expressed, fibrosis-related genes in rTOF **(A)** with TRAF6 **(B)** with CHEK1 and **(C)** with MYL12B.

6.11: Protein-protein interactions of NPR3. Of note, there are important connections with natriuretic peptides (A to C), but also Ubiquitin C (UBC).

6.12: Expanded 25 protein interactants of NPR3 with centralized role of NPR3 and UBC.

6.13: Protein degradation by UPS takes place through protein conjugation to ubiquitin (*Ub*) by ubiquitin activating enzyme (*E1*) through an ATP-dependent activation process. Activated ubiquitin is then transferred to ubiquitin conjugating enzyme (*E2*) with linkage to the lysine residue in proteins. This reaction is catalyzed by ubiquitin ligases (*E3*) repeatedly to form an ubiquitin chain. The ubiquitin-conjugated proteins will then be recognized by the 19S subunit of the proteasome and degraded into peptides by the 20S core proteasome, before being released and degraded to amino acids by peptidases in the cytoplasm.

7.1: Neurohormonal and clinical variable interactions in rTOF. Reproduced from Heng *et al*, Heart 101(6):447-54, 2015⁷¹ with permission from BMJ Publishing Group Ltd.

7.2: (A) Kaplan-Meier survival curve (dotted lines represent 95% confidence intervals) at BNP threshold of 15pmol/litre **(B)** ROC survival analysis for BNP cutoff at 14.9pmol/litre (AUC = Area under curve). Reproduced from Heng *et al*, Heart 101(6):447-54, 2015⁷¹ with permission from BMJ Publishing Group Ltd.

8.1: Proposed study protocol schematic for Remodelling after pulmonary valve replacement for rTOF, a clinical validation study for the proposed novel CMR RV diffuse fibrosis T1 (ECV) mapping technique.

List of Tables

1.1: Clinical parameters associated with arrhythmias and/or mortality in rTOF in published studies. *ICD*: Implantable cardioverter defibrillator, *LVEDP*: left ventricular end-diastolic pressure, *NSVT*: Non-sustained ventricular tachycardia, *PAP*: pulmonary artery pressure, *Peak VO₂*: peak oxygen uptake, *RVOT*: right ventricular outflow tract, *VT*: ventricular tachycardia

1.2: In vivo studies of T1 mapping in healthy normals.^{191-197,234} *FCH*: four chamber, *HB*: heartbeat, *LL*: Look-Locker (a cine inversion-recovery sequence), *SAX*: short-axis

1.3: In vivo studies of T1 mapping in disease and health by MOLLI (studies listed in chronology of publication). *ARVD*: Arrhythmogenic Right Ventricular Dysplasia, *AS*: Aortic Stenosis, *CM*: Cardiomyopathy, *DCM*: Dilated Cardiomyopathy, *HF*: Heart Failure, *HCM*: Hypertrophic Cardiomyopathy, *LVH*: Left Ventricular Hypertrophy, *MD*: Myotonic Dystrophy, *T2DM*: Type 2 Diabetes Mellitus

1.4: In vivo studies of T1 mapping in disease and health by ShMOLLI and saturation recovery. *AFD*: Anderson-Fabry Disease, *AS*: Aortic stenosis, *CM*: Cardiomyopathy, *DCM*: Dilated Cardiomyopathy, *HCM*: Hypertrophic Cardiomyopathy, *HT*: Hypertension, *LVH*: Left Ventricular Hypertrophy

1.5: In vivo studies by other T1 mapping methods

1.6: Available range of CMR sequences for T1 mapping.

1.7: Factors influencing accuracy of T1 quantification of MOLLI versus SASHA.

1.8: High-resolution T1 mapping options

1.9: Developmental syndromes associated with TOF. AD – autosomal dominant, OMIM – Online Mendelian Inheritance in Man. Adapted and modified from Fahed *et al* (Circ Res 2013)²⁸⁶

1.10: Gene mutations associated with TOF. OMIM - Online Mendelian Inheritance in Man. Adapted and modified from Fahed *et al* (Circ Res 2013)²⁸⁶

3.1: Results of intra-observer and inter-observer variability analysis.

3.2: Coefficients of variation in current study compared to selected publications assessing the right ventricle in congenital heart disease

3.3: Reported ranges of RV reproducibility in normal subjects. SDD = standard deviation of the difference

3.4: Reported range of reproducibility in patients with congenital heart disease.

4.1: Baseline characteristics and PVR surgical details.

4.2: ECG, cardiothoracic ratio, echo and CMR data at baseline and after PVR.

4.3: Year on year comparison of study participants with remaining patients with rTOF who underwent PVR 2005-2009 demonstrating lack of recruitment bias.

5.1: Phantom composition for derivation of reference T1 and T2 values.

5.2: T1 values from 11-cycle 8-image fast MOLLI protocols: pre-Gd 5(3)3 and post-Gd 4(1)3(1)2. These were repeated in high resolution (75bpm) and low resolution (100bpm) versions without temperature correction. T2 values from spin-echo imaging are also illustrated. CoV = Coefficient of Variation.

5.3: Variability over 52 week period for T1, T2 values and phantom ECV measurements (calculated with haematocrit of 0.43).

5.4: Technical factors relevant to RV T1 mapping.

5.5: Pilot study patient demographics and ECV calculations. Pilot study patients were of similar age with larger systemic RVs in comparison to published data by Plymen et al.²²¹ The derived RV ECV cannot be directly compared due to differences in sequence methodology and ROI delineation.

5.6: Results of navigator T1 pilot in patients with rTOF versus healthy controls. CoV refers to the intra-session precision between consecutive runs 1 and 2.

5.7: Results of navigator RV T1 inter-observer and inter-scan reproducibility assessment.

5.8: Study subject demographics and T1 mapping results reported as mean $\pm$ standard deviation. Note that MOLLI was used for LV values as the new method was targeted on the RV wall.

5.9: Results of inter-observer variability analysis.

6.1: Demographics of study subjects with rTOF.

6.2(a): Collagen network genes with differential gene expression in rTOF.

6.2(b): Extracellular matrix genes differentially expressed in rTOF.

6.2(c): TGF and TNF pathway genes differentially expressed in rTOF.

6.2(d): Interleukins differentially expressed in rTOF.

6.2(e): Neurohormones differentially expressed in rTOF.

6.2(f): Differential gene expression in rTOF.

6.3(a): Upregulated gene expression sets in rTOF.

6.3(b): Downregulated gene expression sets in rTOF.

6.4: Differentially expressed genes correlated to fibrosis burden in rTOF.

6.5(a): GSEA gene sets upregulated with fibrosis extent in rTOF.

6.5(b): GSEA gene sets downregulated with fibrosis in rTOF.

6.6: Genes upregulated in restrictive rTOF phenotype.

7.1: Baseline demographics of rTOF patients vs controls (mean $\pm$ SD or median (IQR) as appropriate)

******Nine patients (10%) had pulmonary atresia subtype, 1 patient had absent pulmonary valve and 1 double outlet right ventricle.

7.2: Neurohormonal activation and symptoms in patients with TOF.

7.3: Correlation coefficients of neurohormones in rTOF.

7.4: Characteristics of follow-up: survival and deceased rTOF patients.

7.5: Univariate predictors of outcome **(A)** Mortality **(B)** Sustained arrhythmia. Parameters not listed did not reach statistical significance.

Abbreviations

2D	Two dimensional
3D	Three dimensional
ACHD	Adult congenital heart disease
ANP	Atrial natriuretic peptide
ARVC/ ARVD	Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia
ATP	Adenosine triphosphate
BH	Breath hold
BMP	Bone morphogenic protein
BNP	Brain natriuretic peptide
BSA	Body surface area
bSSFP	Balanced steady state free precession
CHD	Congenital Heart Disease
CI	Confidence interval
CITP	Carboxyl-terminal telopeptide of collagen type I
CMR	Cardiovascular Magnetic Resonance
CNP	C-type natriuretic peptide
CNR	Contrast-to-noise ratio
CNVs	Copy number variations
CoV	Coefficient of Variation
CTR	Cardiothoracic ratio
DCM	Dilated cardiomyopathy
DEGs	Differentially expressed genes
DENSE	Displacement Encoding with Stimulated Echoes
DNA	Deoxyribonucleic acid
ECE2	Endothelin converting enzyme 2
ECG	Electrocardiogram
ECM	Extracellular matrix
ECV	Extracellular volume fraction
EDN2	Endothelin-2
EDV	End-diastolic volume
EDTA	Ethylenediaminetetraacetic acid
EDVi	Indexed end-diastolic volume
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
NT-proBNP	N-terminal pro-B-type natriuretic peptide

ELISA	Enzyme-linked immunosorbent assay
ESV	End-systolic volume
ESVi	Indexed end-systolic volume
ET	Endothelin
FB	Free breathing
FDR	False discovery rate
FE	Frequency encode
FID	Free induction decay
FLASH	Fast Low Angle Shot
FOS	Field-of-speed
FOV	Field of View
FPKM	Fragments per kilobase of transcript per million mapped reads
Gd/Gd-DTPA	Gadolinium contrast
GO	Gene ontology
GSEA	Gene set enrichment analysis
GWAS	Genome wide association study
HIF1A	Hypoxia-induced transcription factor 1 α
ICD	Implantable cardioverter defibrillator
IQR	Interquartile range
LAAi	Indexed left atrial area
LGE	Late gadolinium enhancement
LV	Left ventricle
LVEDV(i)	Left ventricular end-diastolic volume (indexed)
LVESV(i)	Left ventricular end-systolic volume (indexed)
LVOT	Left ventricular outflow tract
LVSV	Left ventricular stroke volume
MAPK	Mitogen-activated protein kinase
Mi	Indexed mass
MMP	Matrix metalloproteinases
MoCo	Motion correction
MOLLI	Modified Look Locker Inversion Recovery
MRI	Magnetic resonance imaging
MTORC	Mammalian target of rapamycin complex
NGS	Next Generation Sequencing
NMR	Nuclear magnetic resonance
NPP	Natriuretic peptide precursor (A to C)
NPR	Natriuretic peptide receptor

NYHA	New York Heart Association
PA	Pulmonary artery
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Peak VO₂	Maximum oxygen uptake
PE	Phase encode
PET	Positron emission tomography
PICP	Carboxy-terminal propeptide of type I procollagen
PIINP	Amino-terminal propeptide of type III procollagen
PR	Pulmonary regurgitation
PRF	Pulmonary regurgitant fraction
PSIR	Phase Sensitive Inversion Recovery
PV	Pulmonary valve
PPVR	Percutaneous pulmonary valve replacement
PSIR	Phase sensitive inversion recovery
PVR	Pulmonary valve replacement
RA	Right atrium/atrial
RAAi	Indexed right atrial area
RER	Peak respiratory quotient
RF	Radiofrequency
RNA	Ribonucleic acid
ROC	Receiver-operating curve
ROI	Region of interest
rTOF	Repaired tetralogy of Fallot
RV	Right ventricle
RVEDV(i)	Right ventricular end-diastolic volume (indexed)
RVEF	Right ventricular ejection fraction
RVESV(i)	Right ventricular end-systolic volume (indexed)
RVMi	Right ventricular mass (indexed)
RVOT	Right ventricular outflow tract
RVSV	Right ventricular stroke volume
SASHA	Saturation Recovery Single-Shot Acquisition
SAX	Short axis
SCD	Sudden cardiac death
SD	Standard deviation
ShMOLLI	Shortened Modified Look Locker Inversion Recovery

SNP	Single nucleotide polymorphism
SNR	Signal-to-noise ratio
SV	Stroke volume
TE(s)	Time to echo(es)/echo times
TGF	Transforming growth factor
TGFβ	Transforming growth factor beta
TI	Inversion time
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
TOF	Tetralogy of Fallot
TSE	Turbo spin echo
TR	Repetition time
UBC	Ubiquitin C
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
VENC	Velocity encoding
VE/VCO₂	Ventilatory equivalent ratio for oxygen and carbon dioxide
VLA	Vertical long axis
VSD	Ventricular septal defect
VST	Variance stabilising transformation
VT	Ventricular tachycardia

Preamble

This thesis seeks to examine potential biomarkers to improve outcome prediction in adult patients with repaired tetralogy of Fallot. Cardiovascular magnetic resonance, next generation sequencing and peripheral blood biomarkers are investigated to delineate the roles of cardiac remodelling and myocardial fibrosis in influencing clinical outcomes in this patient population.

Chapter 1 reviews the background and current clinical landscape for managing patients with rTOF. The contributory roles of myocardial fibrosis, genomics and neurohormonal activation are explored. An overview is provided on the basic physics of CMR, as well as focal and interstitial fibrosis imaging by CMR.

The specific aims and objectives of the thesis are outlined on Page 80.

Chapter 2 describes the methods and materials applied in this thesis.

Chapter 3 examines the reproducibility of CMR derived cardiac volumetric and functional data, which is subsequently utilised to assess cardiac remodelling following surgical pulmonary valve replacement in patients with rTOF in **Chapter 4** where CMR derived parameters for predicting outcomes are surveyed.

In **Chapter 5**, approaches to developing and testing novel T1 mapping based techniques for quantifying RV diffuse fibrosis and extracellular volume fraction in patients with rTOF are described. Some supporting work on the development and validation of T1 “gel” phantoms as a quality assurance metric is included at the start of this chapter.

Potential insights to genotype-phenotype associations in patients with rTOF are investigated in **Chapter 6**, in which the results of RNA sequencing of the pathological RV in rTOF are presented. The clinical relevance of the findings is contextualised where possible.

The prognostic utility of neurohormone expression in rTOF is currently unknown. The findings from a longitudinal assessment of the clinical implications of neurohormonal activation are detailed in **Chapter 7**.

The overall conclusions derived from each of the separate studies are reiterated and summarised in **Chapter 8**. The scope for future work to address research questions and hypotheses arising from this thesis are discussed.

Chapter 1

Introduction

1.1 Tetralogy of Fallot

1.1.1 Background

The anatomical features of Tetralogy of Fallot (TOF) were first described by Etienne-Louis Fallot in 1888, comprising ventricular septal defect (VSD), right ventricular hypertrophy, pulmonary stenosis and an overriding aorta. This ‘tetrad’ malformation arises due to anterocephalad deviation of the outlet septum, and is the most common cyanotic congenital heart disease (CHD). Anatomical variants include pulmonary atresia with or without major aortopulmonary collaterals, absent pulmonary valve and double outlet right ventricle (RV) subtypes. Associated lesions may be present, such as atrial septal defect (that is, “pentalogy” of Fallot), atrioventricular septal defects (typically in trisomy 21 patients), patent ductus arteriosus or further muscular ventricular septal defects. Coronary artery anomalies may be present in 3-5% of patients with TOF, including single coronary artery variants.

There is a growing population of adult patients with rTOF requiring lifelong follow-up, with correspondingly increasing numbers of patients at risk of adverse outcomes including ventricular dysfunction, arrhythmias and sudden cardiac death (SCD). Concurrent left heart sequelae of left ventricular (LV) dysfunction, aortic root dilatation and associated aortic regurgitation may occur. New diagnostic techniques are therefore needed to refine the management of late complications of repaired tetralogy of Fallot (rTOF) such as pulmonary regurgitation (PR), RV failure and arrhythmia.¹⁻³ The clinical management of patients with rTOF necessitates judicious clinical decisions for timing pulmonary valve replacements (PVRs) and risk stratification.

1.1.2 Epidemiology and survival in repaired tetralogy of Fallot

CHD is the most common birth defect with an estimated 2800 adults per 1 million of the population in 2000.⁴ TOF accounts for 10% of patients with CHD and occurs in approximately 1 in 3600 births.^{4,5} Although palliative and subsequent reparative surgery have revolutionised survival prospects leading to an expanding population of adult patients with rTOF (~1000 at our institution at present), there remains a long-term estimated 3.7% to 8.3% risk of SCD^{1, 2, 6-9} and up to 11.9% risk of ventricular tachycardia (VT).⁹

Survival beyond 30 years of age is greater than 90% for patients with rTOF, with 32- and 36-year survival of 86% and 85% reported respectively.^{1, 2} Mortality nearly quadruples in the third decade after surgical repair, from 0.24%/year to 0.94%/year in a series of 490 patients

with rTOF, where SCD and congestive cardiac failure were found to be the main causes of mortality in this patient group.² The low but tangible event rates drive the impetus towards improved risk stratification to minimise the risk of unexpected death in a young individual.

1.1.3 The repaired tetralogy of Fallot patient

The natural history of unrepaired TOF portends a poor prognosis with high infant mortality. Historical reports quote 25% mortality by the age of 1 year in the presence of severe pulmonary stenosis and 70% by age 10 years.^{10, 11} Cardiac surgery has therefore radically changed the outlook for these patients.

Surgical palliation was initially achieved by means of a Blalock-Taussig shunt in 1944 (subclavian artery to ipsilateral pulmonary artery connection via end-to-end anastomosis or interposition of a Goretex graft in contemporary practice), before primary surgical repair evolved in the 1950s with the advent of cardiopulmonary bypass. Surgical repair of TOF was first performed by Lillehei in 1954,¹² in which patch closure of the VSD and intervention to the RV outflow tract (RVOT) was undertaken to improve pulmonary blood flow. RV muscle bundles were typically resected, with the same technical principles having perpetuated in current surgical practice. The size of the outflow tract, pulmonary valve and pulmonary arteries influence decision making for the insertion of transannular, RVOT and/or pulmonary artery patches. In the variants with anomalous coronary arteries or pulmonary atresia-type Fallot, a RV to pulmonary artery (RV-PA) conduit may be used.

Surgical treatment of TOF has advanced over the past 50 years. The early surgical era was characterised by staged surgery of a preceding shunt, followed by extensive right ventriculotomies, RVOT resection and extending incisions across the pulmonary annulus for generous transannular patches. This resulted in obligatory PR and large akinetic regions in the RVOT, with its now recognised late deleterious effects on ventricular function and triggering arrhythmias. Therefore, surgical practice has gradually shifted to the less invasive, early repair transatrial-transpulmonary approaches of the modern era in a bid to preserve RV function.¹³

1.1.4 The pathological right ventricle in tetralogy of Fallot

The RV has historically been the 'neglected' ventricle, with a firm focus on LV form and function as a valued, major determinant of clinical status and outcomes, especially in acquired heart disease. However, this perception is gradually evolving, particularly for congenital heart diseases such as TOF in which pathology is centred instead on the RV.

RV performance should be given due consideration in its own right, as it differs substantially from the LV in its structure, morphology and function. The RV should therefore not be regarded merely as an ‘extension’ to the adjacent LV. The RV and LV have separate embryological origins, with the RV arising from the secondary heart field, whilst the LV develops from the primary heart field.¹⁴ These disparate origins result in different ventricular responses to abnormal haemodynamic loading conditions.¹⁵ The triangular, trabeculated and thin-walled RV is geometrically complex and generates low pressures as blood is channelled into the low-impedance pulmonary circulation. This is in stark contrast to the thick-walled, bullet-shaped LV, which generates high pressures for systemic distribution of oxygenated blood. These structural differences translate to predominantly longitudinal motion of the RV as it contracts sequentially from inlet to outlet with a ‘bellowing’ motion of the RV free wall towards the septum, whereas the LV contracts with mainly circumferential and radial forces.

^{16, 17}

Together with the growing importance of the RV, there has been an increasing awareness of an interdependence between LV and RV function that was first reported by Bernheim in 1910.¹⁸ This arises due to shared myofibres, septum, coronary supply and pericardial space of both ventricles.¹⁹ Myocardial cross-talk has been demonstrated *in vivo*, as well as in experimental models in which >50% of normal RV mechanical work was reported to be generated by LV contraction.^{20, 21} This phenomenon is of particular relevance in patients with rTOF, in whom the presence of RV-PA conduit stenosis has been observed to prolong RV contraction resulting in septal shift, which in turn reduces LV filling. This adverse effect on LV mechanics was effectively reversed when conduit stenosis was relieved.²² Ventricular-ventricular interaction has been demonstrated in rTOF,²³ and is also evident from the beneficial effects of PVR on LVEF reported by various groups.^{24, 25}

1.1.5 Pulmonary Valve Replacement (PVR)

The majority of adult patients with rTOF remain largely asymptomatic and lead unrestricted lives, but may subsequently present with late symptoms of exertional breathlessness, palpitations, syncope or SCD. As surgery is not curative, patients should have lifelong surveillance for electrical, haemodynamic and anatomical complications, which may require reintervention.

Indications and current practice for surgical pulmonary valve replacement

PR is an almost inevitable consequence of TOF repair during the adult years, which is initially well tolerated before progressive RV dilatation and ventricular dysfunction ensues. It arises as generous transannular patching during primary surgical repair to relieve RVOT

obstruction compromises native pulmonary valve integrity, which predisposes to valvular incompetence and RVOT akinetic regions.²⁶⁻²⁸

PVR is contemplated in patients with severe PR and RV dilatation, and is regarded as highly effective with corresponding symptomatic benefit.²⁹⁻³⁴ PVR reduces RV size and stabilises QRS duration, although its prognostic impact on arrhythmia and death is uncertain.^{3, 6} The optimal timing of PVR remains equivocal, especially as patients may remain asymptomatic for years. Up to 40% reduction in RV end-diastolic and end-systolic volumes have been observed within a year post surgical PVR,¹⁹ but there is scarce data on early cardiac remodelling post-operatively.

Studies have shown persistently elevated RV volumes post-surgical PVR when the pre-operative indexed RV end-diastolic volumes (RVEDVi) exceed 160-170ml/m² or indexed RV end-systolic volumes (RVESVi) exceed 82-85ml/m².^{32, 35} This implies that timely intervention could potentially have prevented chronic RV dilatation, and Buechel et al have identified that RVEDVi<150ml/m² is the threshold below which RV volumes return to the normal range.³⁶ Pre-operative RVESVi<90ml/m² and QRS duration <140ms have also been shown to independently predict normalisation of RV volumes and function post-operatively.³⁰ Our institution currently applies a cutoff threshold of RVEDVi≥150ml/m² for considering surgical PVR in asymptomatic patients, together with the results of clinical variables such as exercise capacity, ventricular function and serum brain natriuretic peptide (BNP) levels but further work is required to ascertain if optimal thresholds for surgical decision making are in use.

Percutaneous Pulmonary Valve Replacement

Percutaneous pulmonary valve replacement (PPVR) was introduced clinically in 2000 as an alternative, minimally invasive option for treating pulmonary valve dysfunction in patients with rTOF.³⁷⁻⁴² Stented conduit valves are selectively placed during surgical PVR to facilitate subsequent PPVR when conduit dysfunction develops. The percutaneous PV implant consists of a bovine native valve derived from the internal jugular vein mounted within a platinum stent (Melody valve, Medtronic, USA), which is advanced under fluoroscopy and inflated with precise placement into an operator selected location within the RVOT conduit. The device has been adopted into global clinical practice with no peri-procedural mortality and encouraging early to midterm results.

Careful patient selection is paramount to avoid coronary artery compression during valve insertion, and to exclude patients with unsuitable anatomy (such as those with grossly dilated native outflow tracts) which would predispose to poor valve apposition or

displacement. As with any invasive procedure, late complications have been reported which includes stent fracture (most common),⁴³ and valve failure,⁴⁴ as well as a presently perceived increased incidence of infective endocarditis that is the focus of further study.^{45, 46}

1.1.6 Restrictive phenotype in tetralogy of Fallot

RV restrictive physiology is defined by antegrade late diastolic flow in the pulmonary artery during atrial systole throughout the respiratory cycle, during which there is little or no true RV filling with reduced diastolic compliance.^{47, 48}

The reported prevalence of RV restrictive physiology varies between 28% and 67%.⁴⁹⁻⁵² RV restriction is well documented and associated with varied clinical responses at different stages of follow-up in patients with rTOF.^{50, 53} Primary RV restrictive physiology presents acutely with a prolonged post-operative course at the time of reparative surgery due to intra-operative injury with presumed RV fibrotic response.^{49, 54} In childhood and adolescence, RV restriction appears to confer superior exercise performance⁵⁵ and a lower risk of malignant arrhythmias. However, RV restrictive physiology has been recently shown in late adulthood to relate to right atrial enlargement and consequently atrial arrhythmias,⁵⁶ which causes significant morbidity.⁵⁷ In addition, it is a predictor of sustained ventricular tachycardia and death. 'Primary' restriction is presumed to reflect a primary myocardial phenomenon, in contrast to 'secondary' restriction, in which chronic volume overload in an initially compliant (non-restrictive) RV may lead to reduced compliance and late restriction, with detrimental consequences for the patient.^{53, 58, 59}

The mechanism and aetiology of restrictive filling in rTOF patients are not known. Pressure-conductance studies suggest an increased diastolic stiffness of the RV in restrictive rTOF patients.⁶⁰ Associations of restrictive physiology with age at surgical repair, timing and type of surgery, severity of pre-operative pulmonary stenosis and post-operative PR have been reported.^{49-51, 53} Early work speculated on a combination of RV hypertrophy and hypoxia as possible substrates for driving restriction.⁵¹ RV fibrosis has more recently been associated with restriction as assessed by echo and cardiovascular magnetic resonance, with fibrosis proposed to impair RV compliance (in keeping with 'secondary' restriction).^{61, 62} There is therefore continued clinical interest to better define restrictive phenotypes, which could then allow tailored risk assessment and clinical care.

1.1.7 Risk stratification

Risk assessment in TOF is necessary to mitigate against the low but pertinent incidence of SCD. The conservative annual incidence of SCD in the order of 0.15% per year⁷ renders risk

factor identification challenging, particularly as an individual's risk of SCD is dynamic and changes with time as disease progresses. Various clinical parameters such as impairment of biventricular function, QRS prolongation, older age at repair, prior palliative shunt, renal dysfunction,⁶³ cardiothoracic ratio, impaired exercise capacity, elevated BNP, residual pulmonary regurgitation and right atrial dilatation⁶⁴ have all been linked to adverse outcomes in patients with rTOF (*Table 1.1*). In addition, the combination of moderate to severe LV dysfunction with QRS duration $\geq 180\text{ms}$ has a high positive and negative predictive value for SCD in TOF.⁶⁵ However, data is lacking as to the relative contribution of each of these risk factors to best identify and target preventative treatment of high risk individuals.

When contemplating risk stratification in rTOF, conventional risk factors that have been identified thus far can be broadly categorised under three major themes of:

1. Patient factors – older age at repair, history of syncope
2. Electrophysiological markers – QRS duration $\geq 180\text{ms}$, history of sustained VT
3. Haemodynamic parameters – Degree of biventricular dysfunction and RV dilatation

Parameter	Outcomes	Publications
LV ejection fraction	Sudden cardiac death Atrial arrhythmias Ventricular arrhythmias	Ghai et al, <i>J Am Coll Cardiol</i> , 2002 Khairy et al, <i>Circulation</i> , 2010 Knauth et al, <i>Heart</i> , 2008 Diller et al, <i>Circulation</i> , 2012 Valente et al, <i>Heart</i> , 2014
ECG QRS duration$\geq$180ms	Sudden cardiac death Ventricular arrhythmias	Gatzoulis et al, <i>Circulation</i> , 1995 Gatzoulis et al, <i>Lancet</i> , 2000 Ghai et al, <i>J Am Coll Cardiol</i> , 2002 Khairy et al, <i>Circulation</i> , 2010
Peak VO₂ on exercise testing	Mortality after PVR	Babu-Narayan et al, <i>Circulation</i> , 2013
Brain natriuretic peptide	All cause mortality Arrhythmias	Heng et al, <i>Heart</i> , 2015
Patient factors: Patient age and age at surgical repair Hypertension	Mortality Atrial arrhythmias	Katz et al, <i>Circulation</i> , 1982 Gatzoulis et al, <i>Lancet</i> , 2000 Khairy et al, <i>Circulation</i> , 2010
Anatomical and haemodynamic factors: Pulmonary regurgitation Right ventricular hypertrophy Inducible sustained VT or NSVT Right atrial dilatation Mean PAP and LVEDP	Death and ventricular arrhythmias Appropriate ICD shocks	Ghai et al, <i>J Am Coll Cardiol</i> , 2002 Khairy et al, <i>Circulation</i> , 2008 Khairy et al, <i>Circulation</i> , 2010 Valente et al, <i>Heart</i> , 2014
Surgical features: Prior palliative shunt Ventriculotomy incision Transannular or RVOT patch Number of prior cardiac surgeries	Atrial and ventricular arrhythmias Appropriate ICD shocks	Katz et al, <i>Circulation</i> , 1982 Nollert et al, <i>J Am Coll Cardiol</i> , 1997 Khairy et al, <i>Circulation</i> , 2008 Khairy et al, <i>Circulation</i> , 2010

Table 1.1: Clinical parameters associated with arrhythmias and/or mortality in rTOF in published studies.^{8, 9, 57, 65-72} ICD: Implantable cardioverter defibrillator, LVEDP: left ventricular end-diastolic pressure, NSVT: Non-sustained ventricular tachycardia, PAP: pulmonary artery pressure, Peak VO₂: peak oxygen uptake, RVOT: right ventricular outflow tract, VT: ventricular tachycardia.

Risk scoring algorithms have been proposed to aid in decision making for the insertion of implantable cardioverter defibrillators (ICDs) as protection against SCD.⁷³ The precise indications for primary or secondary prevention with ICD insertion remain incompletely defined and individualised therapy should be prescribed. Patients with TOF represent the largest subgroup of ICD recipients with CHD.^{72, 74}

1.2 Cardiovascular Magnetic Resonance

Cardiovascular magnetic resonance (CMR) is a versatile imaging modality that allows visualisation of the cardiovascular system in any plane non-invasively, and without exposure to ionising radiation. As a technique, it excels at soft tissue characterisation, and provides vast programming flexibility to sense various aspects of tissue components such as water and fat content, rigidity and motion. Since it is not limited by acoustic windows, it is

particularly well-suited for imaging patients with CHD, who require regular surveillance as they progress through somatic growth from childhood right through their adult life.

As CMR has been applied as the primary imaging modality across all studies within this thesis, it is imperative to glean an understanding of its principles and capabilities. CMR has an expanding role in diagnostic evaluation in congenital heart disease, and is recognised as the gold standard for the assessment of ventricular volumes, mass and function.⁷⁵

1.2.1 Background

A magnetic resonance imaging (MRI) system comprises three main electromagnetic components, namely main magnet coils, three gradient coils, a radiofrequency transmitter and receiver coils (*Figure 1.1*). The main magnet coils generate a strong, constant field, for which the strength of the field (B_0) is measured in Tesla units. The commonest field strength for cardiac clinical imaging is 1.5T. The magnetic field direction is typically defined using a reference coordinate system of x, y and z axes, with B_0 parallel to the z axis (*Figure 1.1*). The gradient magnetic fields are generated by gradient coils switching on and off rapidly as required by a “pulse” sequence, combined with pulsed application of the radiofrequency (RF) field (that is, B_1 field) by the RF transmitter coil within the gradient coil. In the following section, a brief description is given of how the static magnetic field and RF fields combine to generate MR signals, and how these support imaging through spatial localisation by gradient magnetic field encoding of position.

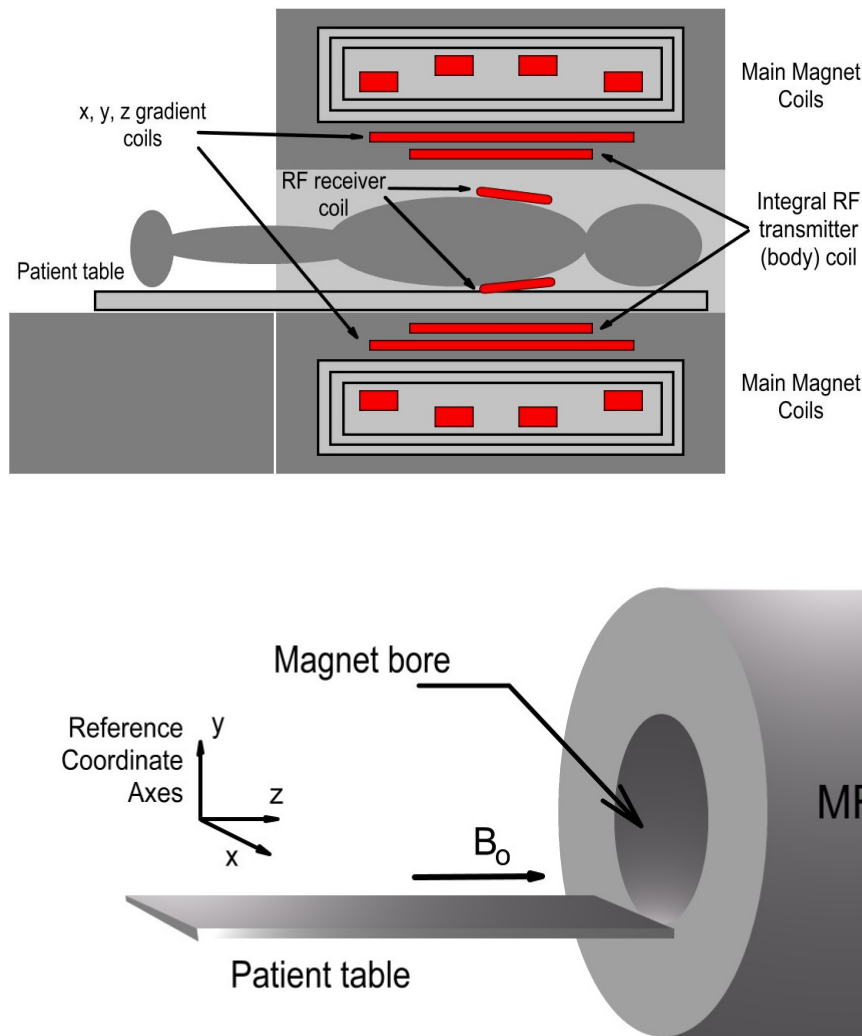


Figure 1.1 System components of an MRI scanner with upper image illustrating positions of main magnet coils, RF transmitter and receiver coils relative to patient and lower image showing typical arrangement of cylindrical bore MR system with reference coordinate axes in which the static B_0 field is aligned with the horizontal z axis. Reproduced from Ridgway et al.⁷⁶

1.2.2 Basic Physics of CMR

Nuclear magnetic resonance (NMR) describes the response of atomic nuclei placed in a magnetic field to radiofrequency waves. CMR studies are of hydrogen nuclei (protons), mainly in water but also in lipids. (This description of NMR involves a mixture of quantum mechanical and classical physics, and contains some approximations as occur in any non-mathematical description). Each hydrogen nucleus possesses electrical charge and its own nuclear spin, such that each hydrogen nucleus has a tiny magnetic field termed the nuclear magnetic moment. Protons are randomly oriented in their resting state outside the magnet. Within an applied B_0 field, protons align toward or against the applied field (*Figure 1.2*), with a small excess population aligned with B_0 , which make the net nuclear magnetisation (M_0).

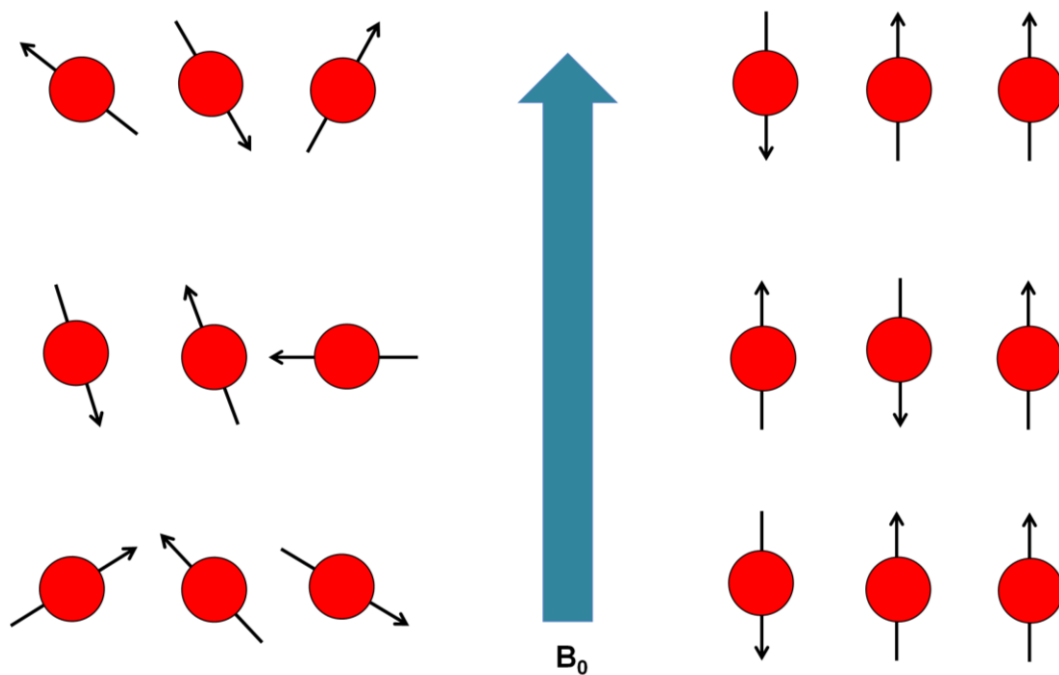


Figure 1.2: Random orientation of protons outside the magnet on the left, with subsequent alignment of a small excess majority following application of a magnetic field (blue arrow).

The application of radio waves at the “resonant frequency” (Larmor frequency) corresponding to the energy difference between aligned and opposite states excites some protons to a higher energy anti-parallel orientation. The MRI physics used in imaging is usually modelled at a large-scale (“macroscopic” or “crowd behaviour”) where the total magnetisation of a sufficiently large number of protons, still far smaller than an imaging voxel is called the “net magnetisation vector” (formally known as a “spin isochromat”).

The applied RF field rotates (“nutates”) the net magnetisation vector away from its equilibrium alignment along the main magnetic field. By applying the RF pulse with a “slice selection” gradient, only the magnetisation at the required slice is affected because other locations up and down the gradient have such different resonant frequencies that the RF pulse does not normally affect them significantly.

After the RF pulse, the magnetisation vector can be modelled “classically” as precessing around the direction of the main field, rather like a spinning top precesses in a gravitational field.

The precessing magnetisation vector relaxes back to the equilibrium alignment after the excitation RF pulse is switched off. Energy is emitted during this process, which can be detected by the receiver coils as a decaying oscillating radio-frequency magnetic field on the precession frequency (that is, “free induction decay” or FID). The key to imaging is the impact of in-plane magnetic field gradients on the frequency of precession during the FID, which is amplified and plotted to produce a cross-sectional image of the tissue source as follows.⁷⁶

Following slice-selective excitation, image formation is achieved through application of short gradient pulses along the two in-plane axes, followed by Fourier transformation of the acquired signal, in effect extracting its frequency spectrum and thereby its distribution of magnetisation over position. The two in-plane gradients are known as frequency and phase-encoded gradient directions. A more detailed description is provided by Ridgway *et al* in their comprehensive review.⁷⁶

Specific considerations for CMR

Cardiovascular imaging is more complex than general MRI imaging. The heart has regular motion, originating both from cardiac contraction and from respiratory motion which constantly alters its position in the thorax. Specific techniques are required to overcome these sources of motion. For most cardiac imaging, the sequences are gated to the patient’s electrocardiogram (ECG), with the heart being imaged at nearly the same point within the cardiac cycle each time. Patients are instructed to hold their breath in end-expiration, which aims to keep the heart stationary during image acquisition. End-expiration breathholds are generally preferred as they are more reproducible and stable than end-inspiratory breathholds. When long CMR sequences are required, imaging may be gated to both diaphragmatic movement and ECG to optimise timing of imaging at the same point in the cardiac cycle and in a respiratory phase where the diaphragm is relatively stationary.

1.2.3 T1 and T2 Relaxation

A unique strength of CMR is its ability for non-invasive tissue characterisation, which is based on the relaxation properties of hydrogen protons within a magnetic field. The relaxation of the hydrogen nucleus proton affects the pixel signal intensity (among numerous other factors that must be separated carefully) and is characterised by two specific magnetic resonance relaxation parameters:

- T1 or spin-lattice (or longitudinal) relaxation time: recovery of the z-component of net magnetisation along the longitudinal (z) axis to its original value at equilibrium. It is the time decay constant when the z-component of the magnetisation recovers

approximately 63% of its longitudinal magnetisation equilibrium value, and involves the release of energy from the nuclear magnetisation to other compartments of energy (as a negligible addition to such as molecular vibrational energy). The shorter the T_1 time constant, the faster the relaxation process allowing the return to equilibrium (*Figure 1.3*).

- T_2 or spin-spin (or transverse) relaxation time: responsible for the decay of the in-plane precessing (M_{xy}) component of net magnetisation, as it rotates about the z axis, causing a corresponding decay of the observed MR signal. It is the specific time when transverse magnetisation decays to approximately 37% of its original starting value (*Figure 1.4*). T_2 is shortened when water can fleetingly interact with collagen or other large molecules on a picosecond timescale (and T_2 becomes very short if water is bound tightly to such molecules), but is longer when water is free. Although T_1 and T_2 occur simultaneously, T_2 relaxation is always $< T_1$.

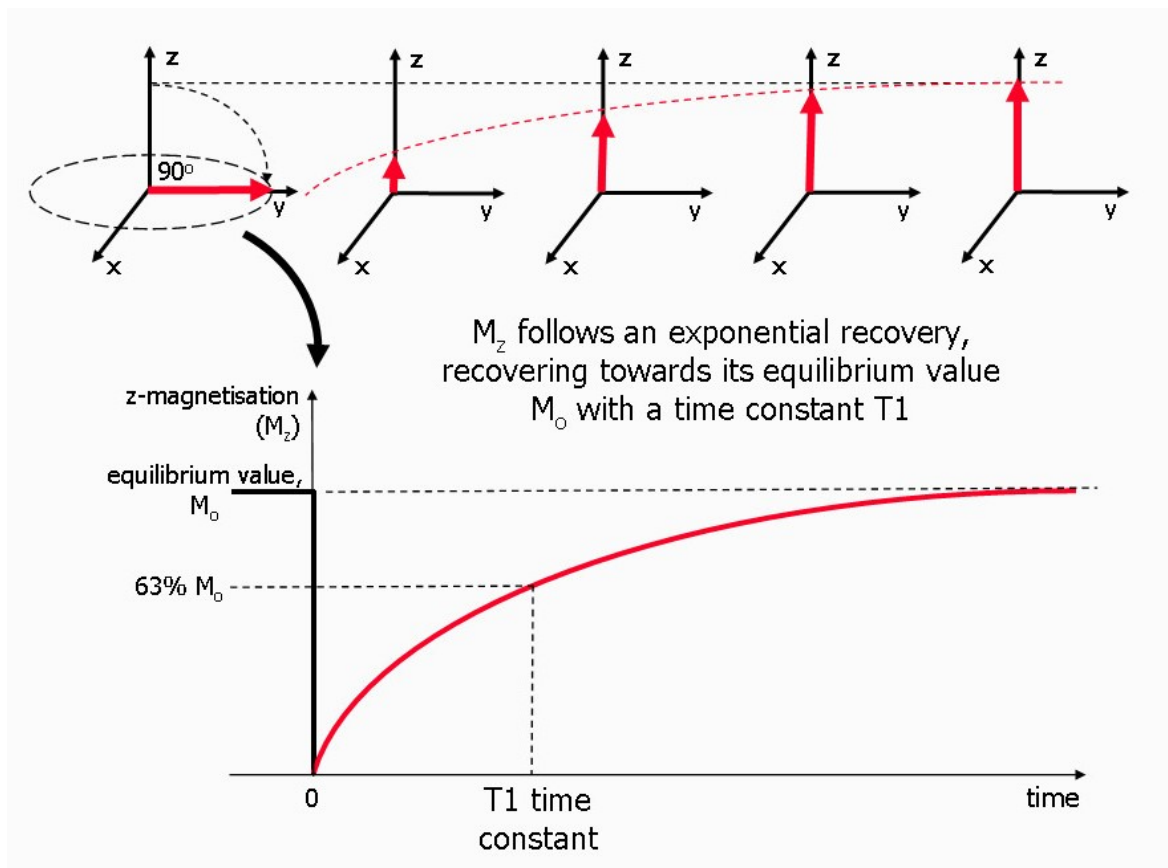


Figure 1.3: T_1 relaxation process illustrated following application of a 90° RF pulse, with M_z (z component of net magnetisation) reduced to zero and exponentially recovering with time back to equilibrium. (From Ridgway, J Cardiovasc Magn Reson 2010)⁷⁶

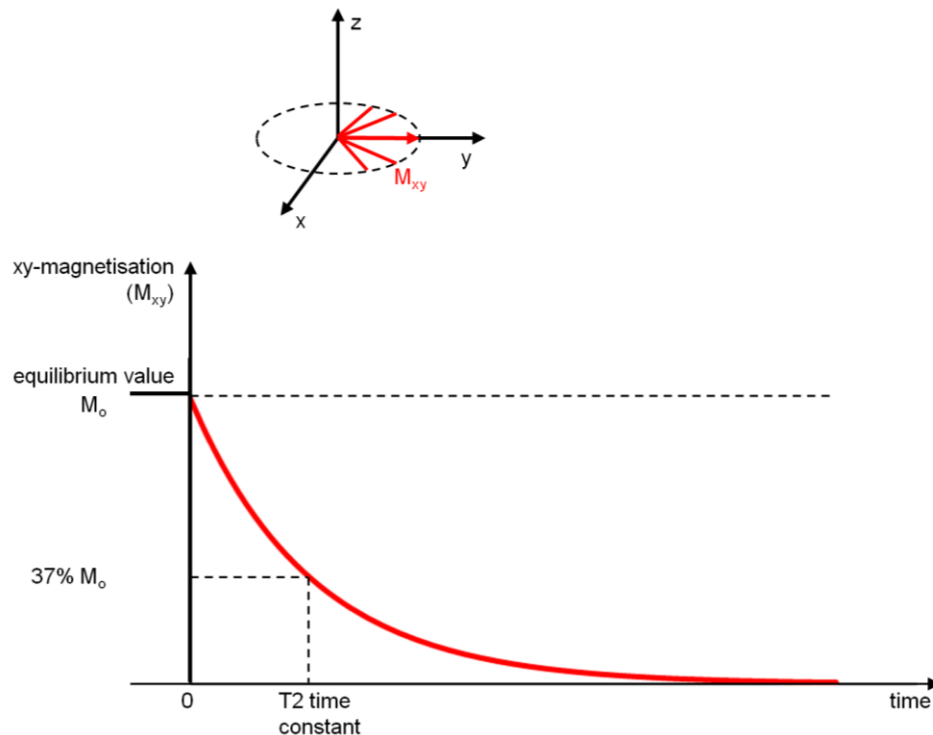


Figure 1.4: T2 decay illustrated similarly following application of 90° RF pulse, with M_{xy} decaying with time after RF excitation into the transverse plane to zero.

Both T1 and T2 relaxation times are increased with greater water content of the tissue, and vary not only between different tissue types (such as fat and myocardium), but within the same tissue. The pathophysiological status of a single tissue entity can be characterised by the presence of inflammation, oedema or fibrosis.⁷⁷ These inherent tissue differences can be enhanced by the use of extracellular contrast agents as described later in *Section 1.2.5*.

1.2.4 CMR Sequences

A pulse sequence is a pre-defined series of RF and gradient pulses modulating the received RF signal so that the desired tissue location and properties are obtained by MRI image reconstruction. Scanning sequences are highly flexible and should be carefully tailored to the question at hand, with components comprising preparation and excitation RF and gradient pulses (generating tissue contrast and localising excitation areas), with further magnetic field gradient pulses (for image formation) followed by signal read-outs for data collection.

From the basic physics references,^{76, 78} TR refers to the repetition time, and is formally defined as the time between RF excitation pulses applied to collect the image data (as opposed to other types of RF pulse applied, for example to invert the magnetisation).

There are two basic categories of pulse sequences in cardiac MRI:

Gradient echo bright blood imaging

Gradient echoes are signals generated by controlled application of bipolar magnetic field gradients, which change the field strength and hence, Larmor frequency along a specified direction. Gradient “echoes” are created after equal and opposite gradient areas are applied, essential to maintain the signal strength by re-phasing the impact of the frequency dispersion along the gradient.

The two main types of gradient echo pulse sequences used for cardiac cine imaging are spoiled gradient echo and balanced steady state free precession (generic name bSSFP). While the latter gives more reliable blood:myocardium contrast and high signal-to-noise ratio (SNR), some applications such as imaging of blood flow velocity or imaging near metallic implants still necessitate the spoiled gradient echo approach.

The main sequences used on our Siemens platform include:

Spoiled gradient echo imaging (also named Fast Low Angle Shot (FLASH)) utilises short TR values (<10ms) and low flip angles to generate images which are mainly T2* weighted with relatively weak T1 sensitivity when applied continuously for cine imaging without any “preparation” of magnetisation, such as inversion. Image contrast between blood and myocardium is largely reliant on fully magnetised blood flowing into the image slice. This sequence has limited use for cine assessment of cardiac function, but can be combined with other methods such as velocity mapping (below) or myocardial tagging.

True Fast Imaging with Steady Precession (TruFISP) is the Siemens term for the generically named bSSFP sequence for cine imaging, which confers improved image SNR and particularly image contrast between blood and myocardium. These advantages are delivered through more efficient utilisation of the available magnetisation, in effect reusing rather than discarding it (“balanced” steady state imaging vs “spoiled” gradient echo), but is more susceptible to dark band artefacts arising from magnetic field inhomogeneities.⁷⁹ These artefacts particularly distort the appearance of fast-moving blood⁸⁰ but can usually be minimised at 1.5T.

Flow Imaging: Phase Shift Velocity Mapping

Phase-shift velocity mapping enables accurate and versatile flow assessment by tracking frequency changes experienced by moving nuclei relative to applied magnetic gradients. Image contrast in the velocity map is produced in linear proportion to blood flow velocities, as two-dimensional images from which peak flow and velocity measurements can be derived in both directions up to the peak velocity range selected before scanning. Velocities can be encoded in or through the image plane of interest by selecting the velocity-encoding direction and velocity sensitivity. Typically this is run as a cine through the cardiac cycle enabling plotting of velocity at each image pixel as a function of time.^{81, 82} Volume flow curves are produced by measuring the luminal cross-sectional area and multiplying it by mean velocity within the area, again throughout the cardiac cycle, or summed over the cycle for volume of blood flow per beat. Computation of systolic forward flow and diastolic flow reversal is done via the separate integrations of the velocities along opposing directions through the vessel area.

Phase-contrast velocity mapping utilises segmented, spoiled gradient-echo sequences with velocity compensation, short echo times (TEs) and low flip angles to generate magnitude and phase images. Velocity maps are produced by subtracting the phase images of two acquisitions, one with an added velocity-encoding gradient pulse along the direction of interest. Velocity mapping as a technique is inherently susceptible to background offset or baseline errors, arising from any other difference caused by the added velocity-encoding gradient. This results in stationary tissue displaying an apparent non-zero velocity due to unintended phase differences. While some sources of velocity error can be computed and removed (Maxwell errors), velocity offset errors caused by eddy currents can only be 'corrected' by using stationary tissue as a reference or by phase contrast imaging of static gelatin or water based phantoms.⁸³⁻⁸⁷

Applying velocity mapping allows the calculation of cardiac output and shunt, peak velocity across stenotic valves, as well as the severity of aortic and pulmonary regurgitation. Velocities are encoded either in- or through the plane of interest as selected by the operator. Specific to rTOF, through-plane velocity mapping can delineate shunts such as ventricular septal defects (VSDs), or assess branch pulmonary flow, in addition to valvular assessment.

Spin echo black blood imaging

Spin echoes are generated by the application of an 180° refocusing RF pulse at time $TE/2$ after the 90° excitation pulse, with signal read-out by a gradient echo also formed at TE. The amplitude of spin echo signals are generally greater than gradient echo, because the 180°

refocusing pulse removes dephasing (usually denoted “T2 prime”) caused by main magnetic field inhomogeneities. In addition, spin echo is less affected by the presence of metals (such as sternal wires and valve components) which exacerbate field inhomogeneities. Spin echo imaging is therefore preferred when less artefacts and high signal-to-noise ratio images are desirable, whereas fast gradient echo imaging is needed in other acquisitions requiring blood signal or cine acquisitions.

Spin echo images have greater flexibility than most gradient echo methods regarding image contrast, and may be T1-weighted, T2-weighted or proton density weighted (image contrast not influenced by T1 nor T2 differences). The repetition time (TR) and time to echo (TE) can be adjusted to control the image contrast. Some caution is necessary to avoid a mixture of T1 and T2 effects, but manipulating TR affects T1 weighting, while TE determines the T2 weighting.^{76, 78}

1.2.5 Late Gadolinium Enhancement Cardiovascular Magnetic Resonance

Delayed or late gadolinium enhancement (LGE) CMR identifies focal areas of myocardial fibrosis or infarction by exploiting the extracellular properties of gadolinium chelates, which are unable to penetrate intact myocyte cell membranes.⁸⁸⁻⁹⁰ LGE reflects the relative differences in volume of distribution of gadolinium contrast between abnormal and normal myocardium. The gadolinium chelate (usually written as “gadolinium”) washes out relatively quickly in normal myocardium (~10 minutes), but lingers in the extracellular space when it is expanded by fibrosis, oedema or infiltrative processes. Myocardial volume of distribution increases in these pathological states, resulting in gadolinium leakage and accumulation in tissues that manifests as the characteristic bright signal with short T1s on delayed T1-weighted CMR. The pattern of LGE distribution can provide diagnostic information of the causative cardiac aetiology. LGE CMR has been validated histologically in animal models and *in vivo*,⁹¹⁻⁹⁵ with its prognostic value demonstrated across a range of pathologies and cardiomyopathies.⁹⁵⁻⁹⁷

Gadolinium (Gd) based chelated complex-ions are the most common MR contrast agent used, which reduce T1 and T2 relaxation times through its paramagnetic properties. Gadolinium (in the Gd³⁺ state but as a complex-chelate to limit its toxicity) possesses 7 unpaired electrons in its outer shell, which creates paramagnetism in an externally applied magnetic field that generates short range local magnetic fields. Local magnetic interactions between the paramagnetic Gd ions and hydrogen nuclei in water molecules shorten T1 and T2 of the surrounding tissues. Chelation of Gd renders it safe for *in vivo* use,⁹⁸ apart from in patients with renal impairment due to the risk of nephrogenic systemic fibrosis.⁹⁹⁻¹⁰¹

Following administration of a Gd-DTPA bolus, a segmented inversion recovery, fast (or turbo) gradient echo sequence is performed, with increments of the inversion time (TI) set manually by the operator to null 'normal' remote myocardium. The TI is adjusted to maximise the image intensity differences between the 'nulled' black remote myocardium and the bright infarcted or fibrotic tissue.¹⁰² LGE imaging can alternatively be acquired with Phase Sensitive Inversion Recovery (PSIR) based approaches,^{103, 104} which increases the dynamic range of IR signal intensities, reducing the reliance on operators to achieve nulling. In recent years, the emergence of three-dimensional LGE offers additional advantages over 2D imaging in terms of higher SNR, with continuous coverage of the heart and thinner slices to improve image resolution. 3D LGE imaging is performed free-breathing using diaphragmatic navigators to control respiratory motion whilst optimising image quality through different imaging sampling strategies such as continuously adaptive windowing strategy (CLAWS).^{105, 106} Volumetric scar quantification and segmentation with 3D LGE datasets may be of clinical utility for risk prognostication and providing a therapeutic roadmap to guide ablation procedures in patients with rTOF.¹⁰⁷

1.3 Myocardial Fibrosis

1.3.1 The pathophysiology of myocardial fibrosis

Myocardial fibrosis is defined as a significant increase in the collagen volume fraction of cardiac tissue, with two main subtypes of interstitial and focal fibrosis representing a continuum of fibrotic stages.^{108, 109} Focal, replacement or scarring fibrosis is caused by plexiform replacement of myocytes after cell damage or necrosis, predominantly by type I collagen. This established, irreversible fibrosis is preceded by interstitial (diffuse) fibrosis, which can be modulated with specific therapies.¹¹⁰⁻¹¹² Collagen regression has been documented after treatment with angiotensin-converting enzyme inhibitors,¹¹³ angiotensin receptor blockers¹¹⁰ and mineralocorticoid antagonists.¹¹⁴ Interstitial fibrosis is a potential therapeutic target, as it has been implicated in mediating cardiac dysfunction and is a substrate of electrical instability in arrhythmias. Interstitial fibrosis arises with an increase in collagen synthesis by myofibroblasts, and takes place either with a diffuse distribution or clustered in perivascular regions.^{77, 115} It has been described as part of the physiological ageing process, but is also accelerated in conditions affecting the LV such as hypertension, dilated cardiomyopathy, and aortic valve dysfunction (with pressure-loading in aortic stenosis and volume overloading with aortic regurgitation).¹¹⁶⁻¹¹⁹

Myocardial fibrosis is implicated in virtually all pathological end stages of the failing heart, and contributes towards ventricular systolic and diastolic dysfunction, abnormal cardiac

remodelling and arrhythmias.¹²⁰⁻¹²⁵ Its role as an independent predictor of mortality and adverse clinical outcomes across a range of LV cardiac pathology has also been elucidated.^{96, 126-128} Fibrotic responses are regulated by complex interactions of angiotensin II, endothelin-1, transforming growth factor-beta (TGF β) and platelet-derived growth factor (PDGF) in combination to activate interstitial fibroblasts and induce altered expression of extracellular matrix (ECM) components.¹²⁹ Fibrotic tissue exerts its deleterious effects on the myocardium by altering the biophysical properties of collagens type I and III, realigning collagen fibres relative to cardiomyocytes which then impairs force transmission causing increased stiffness and myocardial dysfunction.

The ECM is a dynamic collection of collagen and non-collagenous proteins maintained in a delicate equilibrium as scaffolding for myocytes, fibroblasts, endothelial cells and vessels. It constitutes approximately 6% of the normal heart, and facilitates mechanical, chemical and electrical signalling between cells.^{130, 131} The scaffold comprises mainly of type I (80%) and type III (11%) collagen.^{132, 133} At birth, right and left ventricular collagen concentrations are approximately equal,¹³⁴ before higher collagen concentrations in the adult RV compared to the LV are seen, due to regression in RV myocyte size with an increase in LV mass.¹³⁵

When myofibroblasts are subjected to stress in disease states, cell necrosis or apoptosis releases growth factors and cytokines which attract fibroblasts. ECM remodelling is initiated with upregulation of higher-tensile type I collagen,¹³⁶ together with increased fibre cross-linkage. A key pathway in this process involves angiotensin II, which induces fibrosis by stimulating TGF β production, thereby triggering fibroblast proliferation and differentiation into collagen-secreting myofibroblasts.¹³⁷ Upregulation of procollagen I and III is effected, and when these molecules are cleaved, the carboxyterminal is released as carboxy-terminal propeptide of type I procollagen (PICP) and amino-terminal propeptide of type III procollagen (PIIINP) into the circulation.¹³⁸ Myofibroblast differentiation and apoptosis eventually culminates in myocardial fibrosis. Fibrosis alters myocardial function (initially diastole) and conduction, resulting in ventricular dysfunction and arrhythmias (*Figure 1.5*).^{139, 140} Collagen turnover and ECM remodelling is intricately regulated by matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), with MMPs formed by a family of proteolytic enzymes implicated in fibrotic remodelling by regulating degradation in response to their own tissue inhibitors.¹⁴¹

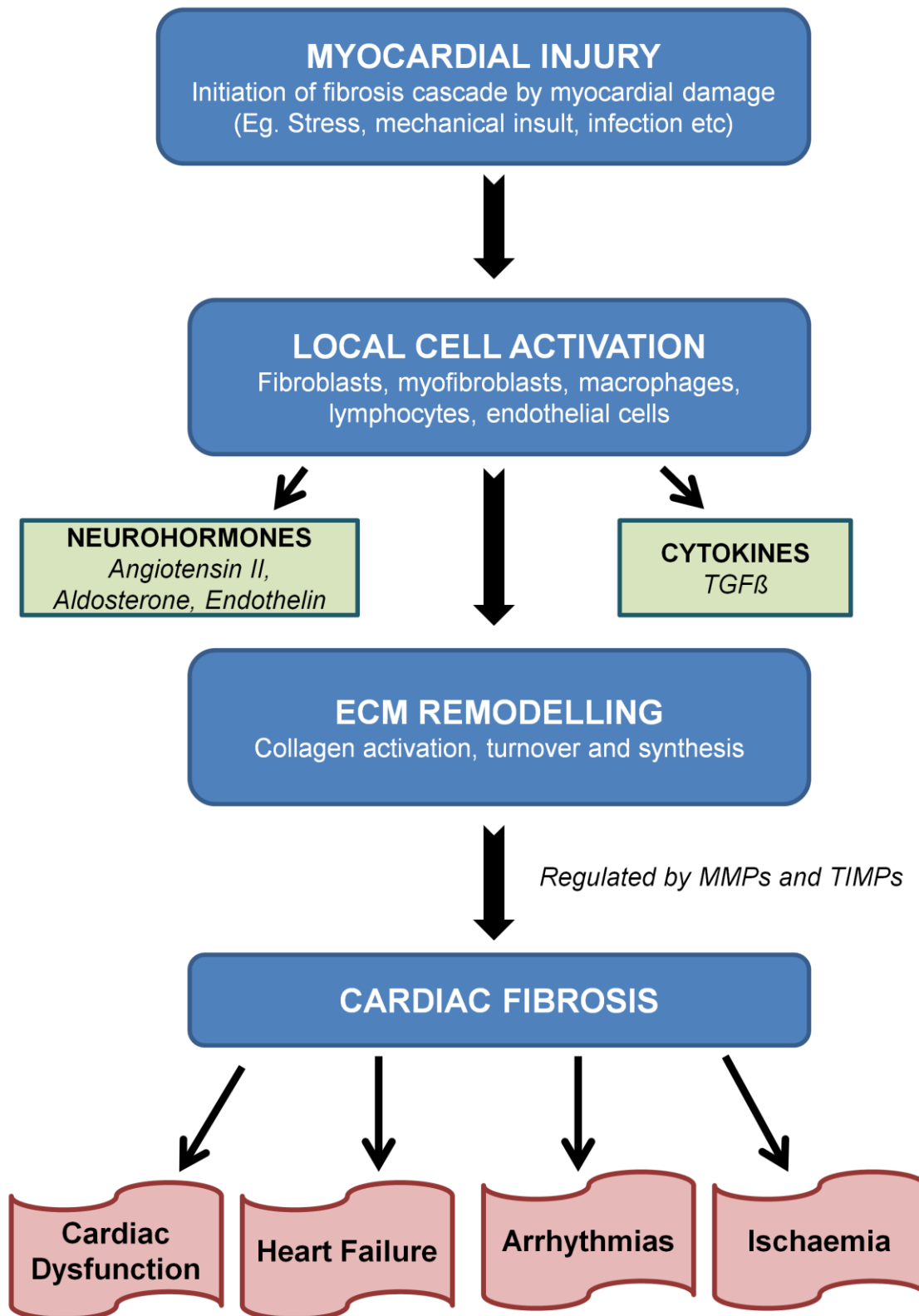


Figure 1.5: Flow diagram of myocardial fibrosis cascade in which myocardial injury triggers local cell activation, leading to altered ECM expression that culminates in myocardial fibrosis.

To investigate the extent of myocardial fibrosis in various pathological states, non-invasive quantification of diffuse myocardial fibrosis has been attempted by a variety of imaging

modalities that includes CMR, calibrated integrated backscatter signal echocardiographic measurements, positron emission tomography (PET) assessment of the perfusable tissue index (PTI) and molecular imaging.¹⁴²⁻¹⁴⁴ Within MRI, endogenous contrast based (that is, direct) detection of cardiac fibrosis with T1_{rho} mapping, magnetisation transfer imaging and T2 mapping are being studied.¹⁴⁵

1.3.2 Circulating biomarkers of myocardial fibrosis

Histopathological analysis of myocardial tissue is the gold standard for assessing and quantifying myocardial fibrosis, but is invasive and impractical to apply across large patient populations. Numerous candidate peripheral biomarkers for myocardial fibrosis have been proposed and investigated, but only a scant few have demonstrated associations between the biomarker in question and histological collagen volume fraction. PICP and PIIINP are collagen-derived serum peptides, for which direct associations with myocardial fibrosis have been demonstrated.^{111, 114, 136, 146-148} Elevated serum PICP levels have been found to precede the fibrotic phenotype in hypertrophic cardiomyopathy, supporting its potential use as a biomarker.¹⁴⁹ Other candidate peripheral biomarkers include TGF β , galectin-3, MMPs and their tissue inhibitors (TIMPs), all of which lack conclusive evidence of molecular circulating levels being associated with histologically proven myocardial fibrosis.¹⁵⁰

1.3.3 Myocardial Fibrosis in tetralogy of Fallot

Diffuse and focal cardiac “sclerosis” (that is, fibrosis) has been recognised in patients with TOF as early as 1965, with the study author purporting that diffuse sclerosis occurs in the most hypertrophied sections of myocardium with focal sclerosis present in other sections.¹⁵¹ Myocardial fibrosis has since been demonstrated in unrepaired TOF patients, and also implicated in the pathophysiology of SCD in rTOF.¹⁵¹⁻¹⁵³

Interstitial fibrosis has been demonstrated histologically in 65% of cyanotic patients undergoing repair of Fallot’s tetralogy (n=183, aged 12 months to 42 years, mean 107 $\pm$ 79 months); with moderate to severe endocardial thickening and perivascular fibrosis in all patients with RV restrictive physiology in the series.¹⁵² RV dysfunction was found to relate to moderate-to-severe myocyte degeneration and interstitial fibrosis within the same study cohort. Fibrous endocardial thickening of the RV infundibulum in native TOF has been found to be predictive of diastolic dysfunction after surgical repair.¹⁵⁴

Histological myocardial disarray has been demonstrated in the RV infundibulum in rTOF in association with cardiac fibrosis.¹⁵⁵ The extent of myocardial disarray has also been linked to cyanosis, with greater extents of disarray seen in children with TOF and hypercyanotic

spells.¹⁵⁶ Disarray has been postulated to arise as a direct consequence of concurrent fibrosis, which produces cell separation and culminates in diverse myofibril orientation.

Focal fibrosis in rTOF has been demonstrated non-invasively by late gadolinium enhancement (LGE) cardiovascular magnetic resonance (CMR) and in separate histological reports.^{61, 153, 157, 158} LGE CMR has demonstrated focal fibrosis in both ventricles in rTOF patients. In particular, focal fibrosis in the RV relating to surgical sites and also in areas remote from surgical scars has been shown in a cross-sectional study to correlate to adverse risk factors including ventricular dysfunction, exercise intolerance, neurohormonal activation and arrhythmias.⁶¹ However, the long-term impact of focal fibrosis on clinical outcomes is unknown. More extensive RV focal fibrosis correlates with restrictive RV physiology in adults and children.^{62, 157}

Although LGE CMR has suggested the presence of focal ventricular fibrosis in TOF, it is unable to diagnose interstitial fibrosis (that is, because diffuse fibrosis does not permit identification of remote myocardium in the standard or PSIR LGE methods). Interstitial (diffuse) fibrosis has been imaged by a recently described novel CMR T1 relaxation “mapping” technique and validated for the LV.¹⁵⁹ Extracellular matrix expansion can be quantified by extracellular volume fraction (ECV) derived from pre and post contrast T1 mapping CMR techniques, and it is hoped that this could have important clinical applications prior to the development of established, irreversible focal fibrosis.

1.4 CMR T1 Mapping in Congenital Heart Disease

1.4.1 CMR T1 Mapping: Background

T1 mapping has gained momentum in the past decade as a clinically relevant, non-invasive prognostic marker of interstitial (diffuse) fibrosis burden for a range of LV pathologies.¹⁶⁰⁻¹⁶² The technique has been validated against whole-heart histological findings in dilated and ischaemic cardiomyopathies,^{163, 164} in addition to endomyocardial biopsies in patients with aortic stenosis, hypertrophic cardiomyopathy, dilated and infiltrative cardiomyopathies.^{159, 165-168} Supporting histological data has been accompanied by an expanding body of observational clinical studies illustrating separation (albeit often with overlap) of myocardial T1 and ECV values between pathology and health (see *Tables 1.2 to 1.5*).

A myocardial T1 map is a reconstructed image, in which the signal intensity of each pixel corresponds to the myocardial voxel T1 relaxation time (also called a “parametric” map of T1). T1 mapping is performed during the magnetisation recovery of each tissue after either

an inversion or saturation procedure (or combination of both) causing disruption of longitudinal magnetisation. T1 maps can be generated pre-contrast for derivation of “native” T1 values, and also post-contrast to enable calculation of the extracellular volume fraction (ECV) by the formula: ^{159, 169}

$$\text{Extracellular volume fraction (ECV)} = \frac{(1 - \text{haematocrit}) \times (1/T1_{\text{postGd myo}} - 1/T1_{\text{preGd myo}})}{(1/T1_{\text{postGd blood}} - 1/T1_{\text{preGd blood}})}$$

This relationship is built on several assumptions, mainly that the plasma concentration of Gd = the interstitial concentration of Gd (valid long enough post injection in most myocardial tissues) and that the “water exchange” effectively transporting the impact of Gd into myocytes and blood cells is fast compared to T1 (considered valid for any clinically safe doses of Gd late enough after the high peak concentrations of first-pass). It is also subject to the “relaxivity” (that is, relaxation ‘power’ of the Gd type used) being the same or at least well known in blood and myocardium at the field strength being used. This description is just one of several factors that can render ECV rather dependent on the individual site’s acquisition set-up.¹⁷⁰

Native T1 inherently encompasses signals from the myocardial cells and interstitium,¹⁷¹ and is prolonged by the increased interstitial water space associated with collagen deposition in fibrosis,¹⁵⁹ by oedema,¹⁷²⁻¹⁷⁴ and by infiltration in amyloidosis.^{175, 176} It is conversely shortened by the presence of iron,¹⁷⁷ fat^{178, 179} and haemorrhage.¹⁸⁰ Post-contrast T1 measurements have been reported to be shortened in fibrosis across a range of pathologies and arrhythmias,^{144, 165, 168, 176, 179, 181-187} but post-contrast T1 clinical applications are limited by the susceptibility to variable gadolinium kinetics, in addition to patient specific factors such as heart rate, renal function and haematocrit.^{77, 88, 188} In order to address the variability of Gd washout, earlier work used the steady-state of a slow infusion of Gd, while more recently ECVs derived 15-20 minutes post-Gd bolus injection have demonstrated comparable measurements in pathologies in which ECV<0.40.^{164, 168, 189} CMR measures of ECV typically over-estimate histological collagen volume fraction, and it has been suggested that ECV derived from T1 mapping only partially reflects interstitial fibrosis, also being affected by an expansion of other components of the extracellular matrix.¹⁹⁰

There are a variety of CMR sequences available for T1 mapping (*Table 1.6*), each with their own institution-specific normative reference ranges and variable susceptibility to errors and artefacts. Native T1 values in normal healthy adults range from ~900-1200ms at 1.5T and ~1050-1350ms at 3T depending on the T1 mapping sequence utilised, whilst ECVs range

from 18-30% at 1.5T and 24-30% at 3T.¹⁹¹⁻¹⁹⁸ Native myocardial T1 values are prolonged whilst post-contrast myocardial T1s are shortened in the presence of fibrosis.¹⁹⁹ The presence of LGE has to be taken into consideration when assessing native myocardial T1, and excluded from measurements to better reflect interstitial fibrosis load. This can be a pitfall when examining references as sometimes this is not excluded. Inversion recovery sequences (MOLLI and ShMOLLI) have been shown to deliver superior precision but inferior accuracy to saturation recovery sequences (SASHA and SAPHIRE).²⁰⁰

Pulse	Sequence	Reference Paper & Summary of technique
Inversion Recovery	Multiple breath-hold FLASH	<i>Iles et al (J Am Coll Card, 2008)</i> 10 images acquired over different Tis applying inversion recovery 2D fast gradient-echo sequence with temporal sampling of k-space (VAST)
	MOLLI	<i>Messroghli et al (MRM, 2004; JMRI, 2007)</i> Comprises 3 successive inversion recovery experiments with different TIs, from which pixelwise T1 maps are generated from 3-parameter curve fitting.
	ShMOLLI	<i>Piechnik et al (JCMR, 2010)</i> Shortened alternative to MOLLI, in which 5(1)1(1)1 sampling takes place over 9 heartbeats, without full recovery of magnetisation between inversion pulses. Conditional data analysis is incorporated.
	ANGIE	<i>Mehta et al (MRM, 2014)</i> Accelerated and navigator-gated look-locker imaging for cardiac T1 estimation with segmented readout, compressed sensing and parallel imaging.
Saturation Recovery *	SASHA	<i>Chow et al (MRM, 2014)</i> 10 single shot bSSFP images acquired in a breathhold, with the first image acquired without magnetisation preparation before imaging a range of saturation recovery times.
	MLLSR	<i>Song et al (MRM, 2012)</i> Modified Look-Locker using Saturation Recovery with bSSFP readout.
Combined inversion and saturation	SAPHIRE	<i>Weingartner et al (MRM, 2013)</i> Novel pre-pulse of Saturation pulse prepared heart-rate-independent inversion recovery. T1 mapping performed with 9 single-shot images with a SAPHIRE pre-pulse.

*Also includes CLAIR and SMART₁MAP

Table 1.6: Available range of CMR sequences for T1 mapping.^{122, 198, 201-206}

Although T1 mapping theoretically allows for tissue characterisation at pixel-wise level, its T1 sensitivity is affected by several random and systematic sources of errors that necessitate rigorous imaging protocols and conditions to improve its accuracy for cross-sectional comparisons.²⁰⁷ Despite the brisk adoption of T1 mapping techniques across the CMR community, its clinical applications have been hampered by the significant overlap that exists between normal and disease states, particularly for subtle and early pathology.^{178, 208} Moreover, lack of standardisation of imaging and post-processing protocols between vendors leads to the need for centre specific normative reference values. This therefore limits inter-institutional comparisons of absolute T1 mapping derived measurements unless identical setups are carefully performed.^{196, 209, 210} Calibration bottles with known T1 values

(“phantoms”) have therefore been recommended as a quality assurance metric within the field.²¹⁰⁻²¹²

1.4.2 CMR T1 Mapping Sequences

The main sequences (most commonly applied) for inversion and saturation recovery T1 mapping are described in brief:

Modified Look-Locker Inversion Recovery (MOLLI)

Each MOLLI acquisition consists of merged data from a series of ECG-triggered inversion recovery experiments with different inversion-recovery times typically acquired during diastole within a single breathhold (*Figure 1.6*).^{201, 202} Unperturbed magnetisation recovery occurs between each sub-series of images during recovery periods, based on the number of cardiac cycles or fixed timings (in seconds). Multiple different configurations of the acquisition readouts and recovery periods have been applied (please see definition in *Figure 1.5* legend), ranging from the original 17-heartbeat MOLLI 3(3)3(3)5 single-shot bSSFP readouts to the more commonly applied shorter breath-hold 11-heartbeat 5(3)3 pre-contrast and 4(1)3(1)2 post-contrast varieties.^{189, 191} The differing distributions of images acquired after each inversion pulse and the recovery periods were optimised to suit longer native and shorter post-Gd T1 values.

T1 is then calculated in each image pixel from a 3-parameter curve fit to the corresponding pixels of each inversion-recovery bSSFP shot by applying the equation:

$$T_1 = T_1^* \cdot ((B/A) - 1)$$

where T_1^* is the apparently shortened T_1 originating from the Look-Locker experiment.²¹³ This equation accounts for the saturating impact of each single-shot image acquisition on the recovery (notably, omitted or unclear in many publications such as *Figure 1.6*).²¹⁴

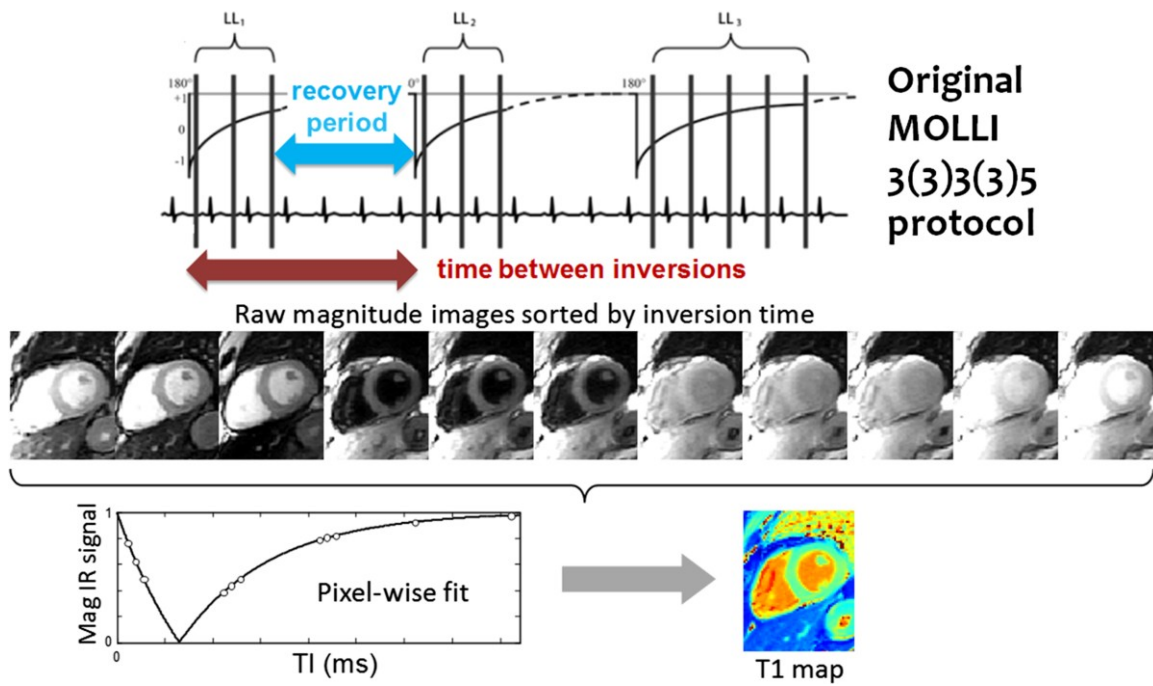


Figure 1.5: Sampling of the IR curve at multiple inversion-recovery times at the same cardiac phase of multiple cycles. The vertical black lines each represent a single-shot bSSFP image acquisition. There are 3 images after the first inversion pulse, followed by 3 cycles recovery, followed by another inversion with 3 images (slightly incremented T1), another 3 cycles of recovery, and a final inversion with 5 images. This scheme is written as 3(3)3(3)5. Reproduced from Kellman et al²¹⁴ based on the original 3(3)3(3)5 MOLLI protocol diagram by Messroghli.²⁰²

Motion correction reconstruction has been incorporated to reduce the impact of poor breath-holds on T1 maps.^{104, 215} T1 quantification by MOLLI results in systematic underestimation of T1 for a range of factors still subject to debate (such as “magnetisation transfer” effects between protons embedded in large molecules and those imaged which are mainly water in myocardium); therefore derived values such as ECV should be interpreted accordingly.

Saturation Recovery Single-shot Acquisition (SASHA)

SASHA consists of typically 10 ECG-triggered single-shot bSSFP images acquired over consecutive heartbeats in a breath-hold (*Figure 1.7*), which is reported to provide good SNR and blood-tissue contrast. The first image is acquired without magnetisation preparation followed by variable saturation recovery time (T_s) images that uniformly span the RR interval.

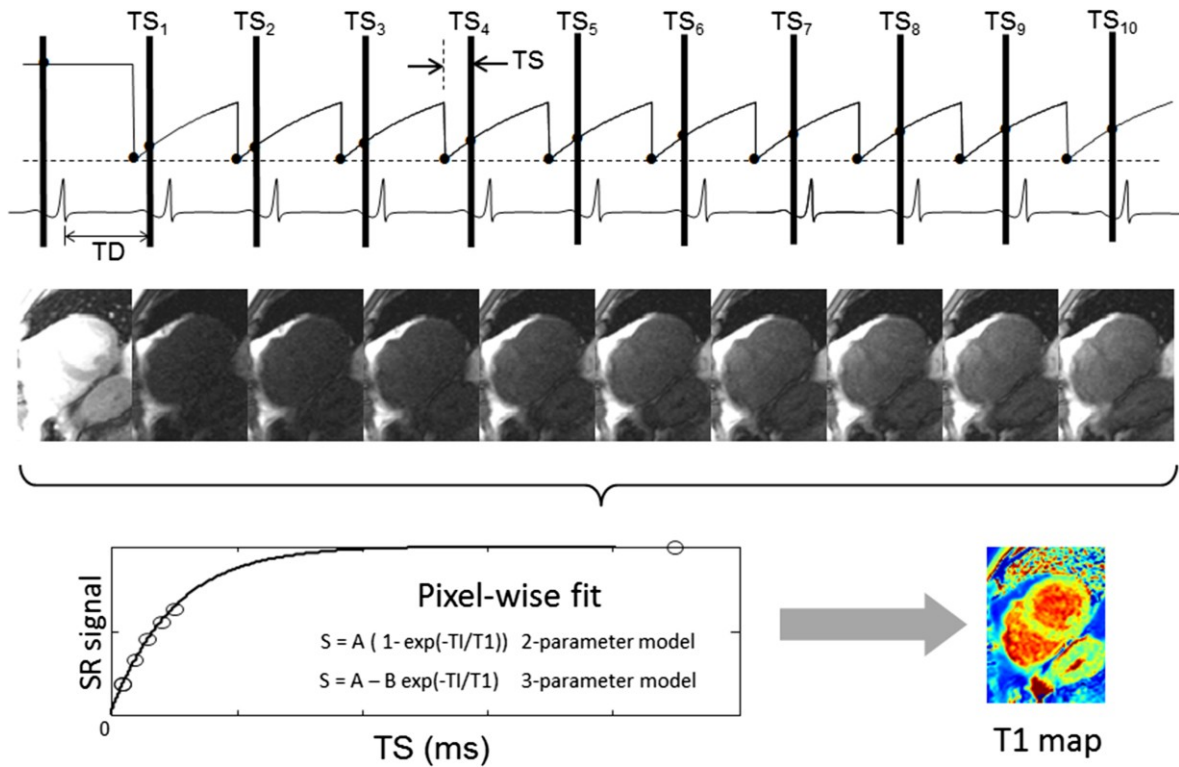


Figure 1.6: Sampling scheme for SASHA (reproduced from Kellman et al)²¹⁴

SASHA has been validated by simulation and phantom experiments, with its accuracy assessed in health and heart failure. SASHA derived T1 values were found to be independent of heart rate, flip angle and off-resonance frequencies up to $\approx \pm 100\text{Hz}$ (approximately ± 1.5 parts per million of main field strength at 1.5T which is still of concern around the myocardium near the relatively empty lungs).¹⁹⁸ It is also insensitive to magnetisation transfer effects, which accounts for some of the under-estimation of MOLLI T1s in comparison to SASHA.^{201, 202, 204, 211, 216} Optimised sampling strategies for SASHA T1 mapping have been validated, in which fixed saturation delays are set for pixelwise T1 quantification (optimised Ts 591ms pre-contrast and Ts 193ms post contrast).²¹⁷

1.4.3 Accuracy and Precision

It is necessary to consider the errors in T1 quantification, which can be categorised into “accuracy” and “precision”. Accuracy refers to systematic bias errors, whereas precision is determined by the extent of random errors such as electrical noise adding “speckle” to MRI image pixels.^{214, 218} Accuracy is influenced by numerous factors as summarised in *Table 1.7*.

Parameter	MOLLI	SASHA
Absolute accuracy	-	++ (3p fit) + (2p fit)
<u>Protocol parameters</u>		
- Matrix size (for spatial resolution and minimising partial volume effects)	••	••
<u>Sequence design</u>		
- T2 sensitivity	•••	• (2p fit)
- Non-ideal adiabatic pulse efficiency	••	••
<u>Influence of off Resonance</u>		
- Shim	•••	• (3p fit) • (2p fit)
- B1+ effects/ flip angle	••	•
<u>Tissue characteristics</u>		
- Blood flow	••	•
- Magnetisation transfer	•••	• (2p fit) • (3p fit)
<u>Patient factors</u>		
- Heart rate	••	•
Precision	++	+ (2p fit) - (3p fit)

Table 1.7: Factors influencing accuracy of T1 quantification of MOLLI versus SASHA.

• susceptibility to parameter, • independence from parameter, ++ good, + fair, - poor.

Off-resonance errors in particular may cause apparent regional T1 variations over the myocardium that is artifactual, for which MOLLI is more susceptible than SASHA. The off-resonance inaccuracies can be minimised by reducing the imaging flip angle,²¹⁴ albeit at the expense of SNR which equates to a loss of precision. Magnetisation transfer effects can be considerable, resulting in ~15% lower IR-based T1 measurements when compared to SR sampling.²¹⁶ MOLLI has higher precision than SASHA due to the larger range of the inversion-recovery change in the signal, compared to saturation-recovery, combined with MOLLI's more distributed measurement timings along the recovery curve than SASHA, in which measurements are clustered in the early part of recovery especially for fast heart-rates. The precision loss of SASHA is 35% (2-parameter fit) and 125% (3-parameter fit) compared to 5(3)3 MOLLI with 3-parameter fitting.²¹⁴ To some extent, these considerations of accuracy and precision are different post-contrast when compared to pre-contrast.

It must be remembered that mapping is usually based on single-shot images, which inherently have coarser image resolution than "segmented" imaging over multiple cycles, and also have longer shot-duration for the single-shot leading to risk of cardiac motion blurring of even this coarser resolution. This leads to blood and fat contamination of myocardial T1 values, this latter problem may often not be immediately obvious on viewing the images. Careful timing of the single-shot acquisition into a diastolic stationary window

using prescan cines is vital to consistently good quality of mapping, even in the LV let alone the RV as described below in *Section 1.4.5*.

The extremely wide range of methods and their results in health and disease, furthermore across 1.5T and 3T, and also with differing Gd types and doses, are listed comprehensively in *Tables 1.2 to 1.5*, and demonstrate the difficulties of the current situation with little prospect of any consensus in the near future on a clear standard method. Some of the studies in *Tables 1.3 and 1.4* include active myocarditis, in which the pathology may predominantly be due to oedema and inflammation increasing native T1 and also ECV, rather than interstitial fibrosis.

1.4.4 T1 mapping in Adult Congenital Heart Disease

Whilst there has been enthused uptake of T1 mapping across the spectrum of acquired heart disease and cardiomyopathies with a predominant LV focus, there has been a comparative lack of T1 mapping applied to adult and paediatric patients with CHD. This may, at least in part, be due to the need for sequence adjustments to account for higher heart rates, increased motion artefacts as well as higher spatial resolution required for smaller cardiac anatomy in the paediatric population. There has been similar scarcity of T1 mapping clinical studies in the adult congenital heart disease (ACHD) population.

To date, Broberg *et al* have identified diffuse myocardial fibrosis in the systemic ventricle of ACHD patients (17 of 50 patients studied had LV assessment in rTOF, 11 of 50 with systemic RV) which correlated with ventricular dysfunction.²¹⁹ They also reported on elevated LV ECVs in a prospective cohort of 52 patients with rTOF, which correlated with age at surgical repair, QRS duration on ECG, 6-minute walk distances, bi-atrial volumes and serum BNP levels. LV ECV $\geq 30\%$ was found to relate to an increased risk of atrial arrhythmias and cardiovascular death (albeit with a very small number of events).²²⁰ Both studies acknowledged the intrinsic challenges of applying the technique to the RV.

There have been only 2 other reported studies where T1 mapping has been applied to the RV in CHD. Equilibrium-contrast CMR in adults (n=14) with systemic RVs late after atrial redirection surgery for transposition of the great arteries had elevated septal ECVs.²²¹ Post-contrast T1 values of both ventricles have also been reported to be shorter in children and adolescents (n=18) with rTOF compared to controls,²²² although there are significant methodological issues which may have created systematic bias in this study's results.

There therefore remains an unmet clinical need within ACHD for T1 mapping of the RV, as fibrosis is recognised not only as a byproduct of adverse ventricular loading but also appears to contribute to adverse clinical outcomes.²²³ Non-invasive detection of interstitial fibrosis by CMR may potentially offer better tracking of RV disease than currently utilised volumes and ejection fraction in rTOF. Application of interstitial imaging to the thinner RV in rTOF mandates CMR sequence development as in the experimental work of this thesis in Chapter 5.

1.4.5 Considerations in RV T1 Mapping

The RV is distinctly different from the LV in its morphology, physiology and function.^{15, 224} Its complex 3D geometry, trabeculated myoarchitecture and predominant longitudinal motion during contraction hamper the direct application of LV mapping techniques. Consequently, there have been limited CMR T1 measurements of the RV reported in congenital heart disease. In these studies, existing LV sequences have been applied to the RV without histological validation.^{219, 221, 222} The RV structure mandates a tailored technical approach to achieve high spatial resolution, whilst concurrently addressing errors introduced by epicardial and intramyocardial fat, as well as partial volume effects where the blood signal in the RV contaminates the thin adjoining layer of pixels of RV myocardium.

RV Epicardial and Intramyocardial Fat

Physiological myocardial fat has been described in the anterolateral and apical RV walls of normal subjects (*Figure 1.7*),²²⁵⁻²²⁷ and as a result, RV myocardial fat introduces error into both native and post-contrast T1 measurements. The error does not cancel out and therefore impacts severely on the derived myocardial ECV.²²⁸ The native T1 of fat is short (~250ms at 1.5T) compared to the much longer myocardial native T1. Furthermore, there is presumed differential T1 shortening with gadolinium administration of lipomatous tissue when compared to myocardial tissue.

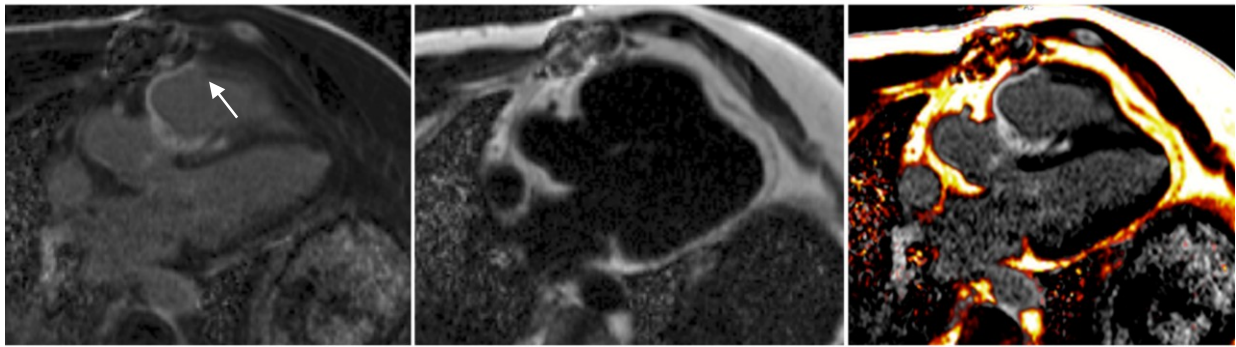


Figure 1.7: Fat-water separated free-breathing LGE phase sensitive inversion recovery (PSIR) images in left ventricular outflow tract (LVOT) view from patient with rTOF. From left to right – magnitude water image illustrating area of hyper-enhancement of the RV (arrowed), middle panel fat only image illustrating significant epicardial fat surrounding the heart, right panel superimposed fat-water image with surrounding epicardial fat highlighted. The epicardial fat layer would have introduced substantial error to T1 measurements made if not identified and avoided in region of interest (ROI) selection.

Partial Volume Effects

Partial volume effects are particularly important in T1 mapping, and arise due to limited in-plane spatial resolution, in addition to through-plane pitfalls of a thick imaging slice.²¹⁴ As these effects are most marked at borders between myocardial tissue and blood within a voxel, the trabeculated architecture of the thin RV invariably results in pixel contamination. This is likely to produce a falsely elevated ECV in healthy myocardium, whereas in disease states with RV hypertrophy, there is likely to be less partial volume effect, which can produce a more accurate ECV that may appear paradoxically low compared to potentially falsely elevated values in normals. Further degradation of myocardial resolution may occur due to cardiac motion, all of which may introduce bias in ECV quantification. Both these factors can potentially be tackled with higher in-plane resolution combined with a very short acquisition time span during the cardiac cycle, but still requires stringent care to ensure that the RV wall is aligned perpendicular to the slice selected and that image acquisition occurs as far as possible during diastasis.

1.4.6 Approaches to RV T1 Mapping

Various research groups have sought to develop high-resolution approaches to T1 mapping, some targeted towards RV imaging. These broadly include 2 and 3-dimensional respiratory-motion gated (“diaphragm navigator”) and accelerated imaging by “compressed sensing” (sampling incomplete rawdata, with some assumptions allowing computation of omitted rawdata) methods as summarised in *Table 1.8*. These methods have yet to be applied to a CHD cohort to date. We therefore sought to develop and validate a novel RV T1 mapping

technique for application in patients with CHD (specifically rTOF) as described in *Chapter 5*. In support of this development, some initial work produced and maintained T1 MRI test object “phantoms” (*Chapter 5*).

	CLAIR	hrMOLLI	Kt-SENSE-SLR	Fast-MOLLI	3D free-breathing	ANGIE
Research Group	Pagano et al University of Alberta, Canada (SCMR 2012)	Pastor et al Kings College London (SCMR 2012)	Chen et al University of Virginia (ISMRM 2013)	Semaan et al Northwestern University, Chicago (EuroCMR 2013)	Weingartner et al Heidelberg University, Germany (MRM 2014)	Mehta et al University of Virginia (MRM 2014)
Method	•Saturated recovery •Segmented cine SSFP •FOV 300x256	•FOV 320x320 •TR/TE 3.2/1.57ms	• k-t acceleration •Compressed sensing	•5(3)3 sequence •Image acquisition pre-contrast, 10 & 25 mins post contrast	•Navigator gating •Image acquisition 5-30 mins post contrast	•Navigator •Compressed sensing •Adaptive sampling
Platform		3T	1.5T Avanto	3T	1.5T Achieva	1.5T Avanto
Acquisition window	50ms		160ms (17 heartbeats)		100ms (scan time 11 mins)	68±18 seconds per slice
Resolution	8mm slice	0.9x0.9x8mm ³ Interpolated	1.2x1.2x8mm ³	1x1x8mm ³	1.5x1.5x4-10mm ³	
Acceleration rate		-	4	-	-	2.3 – 3.5
ROIs	Septal	Septal				

Table 1.8: High-resolution T1 mapping options^{203, 206, 229-233}

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)	Comments
Messroghli et al Radiology 2006	15 normals	MOLLI 3(3)5 Study 1 +3: mid-SAX Study 2: SAX x3	1.5T Philips	Single centre	980±53		@15min: 494±23 @20min: 510±23			
Nacif et al JMRI 2011	Normals: 168 MOLLIs 165 LL	Look Locker vs 3(3)3(3)5 MOLLIs SAX x1 +FCH	1.5T Siemens	Single centre	LL: 1005±120, MOLLI: 1034±53		LL: 487±70, MOLLI: 418±70			
Lee et al JCMR 2011	11 normals	MOLLI 3(3)3(3)5 vs 3(3)5 SAX x1	3T Siemens	Single centre	1315±39	2020±129	@18.5min 17HB: 622±40 11HB: 619±48	@18.5min 17HB: 433±42 11HB: 429±39	@18.5min 17HB: 26.1±3.4 11HB: 26.7±3.9	No diff btwn 11 vs 17 HB MOLLIs
Kawel et al JCMR 2012	24 normals	MOLLI 3(3)5 SAX x1	3T Siemens	Single centre	1286±59	2074±139	(Values in Figure 2)			Gd-DTPA vs Gd-BOPTA Pre-Gd values corrected for HR
Von Knobelsdorff et al JCMR 2013	60 normals	MOLLI 3(3)3(3)5 SAX x3	3T Siemens	Single centre	Base:1157.1 Mid: 1158.7 Apex: 1180.6		Base: 427.3 Mid: 411.2 Apex: 399.7			Segmental diff T1 noted. Diff with age also.
Dabir et al JCMR 2014	102 normals (only 66 had contrast), 113 low risk subjects	3(3)3(3)5 MOLLIs Single mid-SAX plane	1.5 & 3T Philips	Multicentre (x4)	1.5T: 950±21 3T: 1052±23	1.5T:1551±115 3T:1736 ± 139	1.5T: 415±113 3T: 421±131	1.5T: 291±122 3T: 277±106	1.5T:0.25±0.04 3T: 0.26±0.04	No effect of age, gender
Piechnik et al SCMR 2012, Poster	231 normals	ShMOLLI SAX x3	1.5T Siemens	3 centres	961±26 Males: 947±20, females 974±25					Difference between gender and age
Piechnik et al JCMR 2013	342 normals	ShMOLLI SAX x3	1.5T Siemens	3 centres	962±25 Midwall ShMOLLI 953±23	LV: 1535±76 RV: 1515±91				Females longer T1

Table 1.2: In vivo studies of T1 mapping in healthy normals.^{191-197, 234} *FCH*: four chamber, *HB*: heartbeat, *LL*: Look-Locker (a cine inversion-recovery sequence), *SAX*: short-axis

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)
Sibley et al Radiology 2012	47 HF patients (13 DCM, 11 myocarditis, 22 infiltrative CM, 1 ARVD) 13 normals	Look Locker FCH	1.5T Siemens	Single centre			HF: 351±76 (LGE -ve 406±59 vs LGE +ve 303±53) Normals: 466±14		Shorter T1 correlates to greater fibrosis. Histo – RV septal biopsy
Mongeon et al; JACC Card Imag 2012	38 patients infiltrative (17 amyloid, 20 cardiomyopathy, 1 lysosomal storage), 9 normals	Look Locker SAX x1	3T Siemens	Single centre					Median ECVs Amyloid 0.49 Highly probable amyloid 0.41 Cardiomyo 0.33 Normal 0.24 ECV correlates to LGE
Turkbey et al Heart Rhythm 2012	33 myotonic dystrophy, 13 normals	Look Locker FCH	1.5T Siemens	Single centre			MD: 394.5±57.6 Normals: 441.4±32.0		
Rao et al Am J Cardiol 2013	53 T2DM without CAD 10 normals	Look Locker SAX x1	3T Siemens	Single centre					DM 0.36±0.05 Normals 0.27±0.03
Ho et al Circ Cardiovasc Imag 2013	HCM 37 G+/LVH+, 29 G+/LVH -, 11 G-, 11 normals	Look Locker SAX x3	3T Siemens	Single centre					G+/LVH+ 0.36±0.01 G+/LVH - 0.33±0.01 Normals 0.27±0.01
Yingchoncharoen et al Circulation 2013 (abstract)	103 DCM	Look Locker	(not stated)	Single centre			415±98 (LGE+ve 381±104 vs LGE -ve 440±87)		
Dusenbury et al JACC 2014	35 cong AS, 27 normals	Look Locker SAX x1	1.5T Philips	Single centre					AS: 0.27 (0.22-0.42), Normals: 0.25 (0.18-0.27) ECV assoc with diastolic function
Yi et al Int J Cardiovasc Imag 2014	52 DCM	Look Locker FCH view	1.5T Siemens				468±106 (@2 yr FU 434±82)		

Table 1.3: In vivo studies of T1 mapping in disease and health by MOLLI (studies listed in chronology of publication).^{165, 181, 182, 235-238}

ARVD: Arrhythmogenic Right Ventricular Dysplasia, AS: Aortic Stenosis, CM: Cardiomyopathy, DCM: Dilated Cardiomyopathy, HF: Heart Failure, HCM: Hypertrophic Cardiomyopathy, LVH: Left Ventricular Hypertrophy, MD: Myotonic Dystrophy, T2DM: Type 2 Diabetes Mellitus

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)
Sparrow et al Am J Roentgenol 2006	8 patients with AR, 15 normals	MOLLI 3(3)5 SAX x1	1.5T Philips	Single centre	Normals ~1000ms (from figure). No diff in AR.				Diff T1 in segments with RWMAs
Messroghli et al MRM 2007	24 acute MI	MOLLI 3(3)5 SAX x3	1.5T Philips	Single centre	Chronic MI Myo LGE+ve 1060±61 LGE -ve 987±34 Acute MI LGE +ve 1197±96 LGE -ve 1011±66				
Flett et al EHJ 2012	63 severe AS (+42 post AVR) 30 normals	EQ-CMR (FLASH IR) Basal septum imaged	1.5T Siemens	Single centre					Vd AS: 18±8, normals: 13.4±7 Histo
Wong et al Circulation 2012	793 patients, 9 normals	MOLLI PreGd: 5(3)2 PostGd: 4(1)3(1)2 SAX x2	1.5T Siemens	Single centre					Patients: 21.0-45.8 Normals: 21.7 -26.2 Patients = CAD, arrhythmia, cardiomyopathy
Kellman et al JCMR 2012	33 MI, 33 HCM, 15 DCM, 7 myocarditis, 4 amyloid, 2 systemic capillary leak syndrome, 62 normals	MOLLI 3(3)3(3)5 → 5(3)3(3)3 SAX x1 + FCH	1.5T Siemens	Single centre	Normals: 965±35 (range 893-1035)				MI LGE +: 68.5±8.6 HCM: 35.7±2.9 DCM: 38.1±1.9 (in 4 of 16) Amyloid: 46±12 SCLS: 40-41 Myocarditis STIR +ve regions: 44±6, STIR -ve 26.4±3 Normals: 25.4±2.5 (range 20.4-30.4)
Ugander et al Eur Heart J 2012	126 patients suspected/kn own CAD	MOLLI 3(3)5 SAX x1 + FCH	1.5T Siemens	Single centre					MI patients LGE +: 51±8, LGE -: 27±3 DCM LGE +: 37±6, LGE -: 26±3 No segmental ECV variation. ECV increases with age
Puntmann et al JACC Cardiovasc Imag 2013	25 HCM, 27 DCM, 30 normals	MOLLI 3(3)3(3)5 Single mid-SAX plane	3T Phillips	Single centre	HCM: 1254±43 DCM: 1239±57 N: 1070±55	HCM: 1869±92 DCM: 1901±92 N: 1871±89	(@20min) HCM: 363±63 DCM: 355±44 N: 440±58	(@20min) HCM: 299±36 DCM: 297±33 N: 294±32	HCM: 0.41±0.12 DCM: 0.40±0.09 N: 0.27±0.09

Table 1.3 (Continued): In vivo studies of T1 mapping in disease and health by MOLLI (studies listed in chronology of publication).^{160, 172, 184, 188, 239-241}

AR: Aortic Regurgitation, AS: Aortic Stenosis, AVR: Aortic Valve Replacement, CAD: Coronary Artery Disease, DCM: Dilated Cardiomyopathy, FLASH IR: cine inversion-recovery sequence as the Look-Locker sequence in Tables 1.8 and 1.9, HCM: Hypertrophic Cardiomyopathy, LVH: Left Ventricular Hypertrophy, MI: Myocardial Infarction, Vd: Volume of Distribution

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)
Puntmann et al JACC Cardiovasc Imag 2013	33 SLE, 21 normals	MOLLI 3(3)3(3)5 SAX x1	3T Philips	Single centre	SLE: 1152±461 Normals: 1056±27		SLE: 411±62 Normals: 454±53		SLE: 30±6 Normals: 26±5 Assoc with strain
Miller et al Circ Cardiovasc Imag 2013	6 heart transplant pts, 30 normals	MOLLI 3(3)3(3)5 SAX x3	1.5T Siemens	Single centre	Pre-Tx pts 1187±163 Normals 1045±46				Pre-Tx pts 41.4±5.0 Normals 25.5±2.6 Segmental diff ECV reported. Histo
Rogers et al JCMR 2013	25 LVH, 43 DCM, 38 normals	MOLLI 3(3)3(3)5 SAX x1 (Septal vs. whole SAX ROI)	1.5 & 3T Philips	Single centre	Septal 1.5T:976±37 3T: 1108±67 Lateral 1.5T:943±45; 3T 1087±60				Regional diff T1s (all pts grouped together)
Beinart et al Heart Rhythm 2013	51 patients with AF, 16 normals	MOLLI (17HB) VLA	1.5T Siemens	Single centre			AF: 387 (IQR 364-428) Normals: 459 (418-532)		T1 shorter in AF pts with prev ablation
Brooks et al JMIR 2013	5 amyloid, 8 normals	MOLLI 3(3)5 SAX x1	1.5T Siemens	Single centre	Amyloid: 1144±49 Normals: 963±42	Amyloid: 1665±133 Normals: 1509±85	Amyloid: 503±61 Normals: 544±36	Amyloid: 486±18 Normals: 408±42	
Wong et al Eur Heart J 2014	1176 patients (231 DM)	MOLLI PreGd: 5(3)2 PostGd: 4(1)3(1)2 SAX x2	1.5T Siemens	Single centre					DM: 30.2 (IQR 26.9-32.7) Non-DM: 28.1 (IQR 25.9-31.0) ECV predicts adverse events in DM patients
Su et al JACC Cardiovasc Imag 2014	40 systolic HF, 62 HFpEF, 22 no HF	MOLLI SAX x5 (base to apex)	3T Siemens	Single centre	SHF: 1209 (1153-1251) HFpEF: 1201 (1159-1238) Non-HF: 1185 (1170-1214)		SHF: 545 (52-612) HFpEF: 613 (568-655) Non-HF: 649 (615-677)		SHF: 31.2 (29.0-34.1) HFpEF: 28.9 (27.8-31.3) Non-HF: 27.9 (26.2-29.4) In HFpEF: ECV correlates to peak filling rate (diastolic function)

Table 1.3 (Continued): In vivo studies of T1 mapping in disease and health by MOLLI (studies listed in chronology of publication).^{161, 164, 176, 183, 209, 242, 243}

AF: Atrial Fibrillation, DM: Diabetes Mellitus, HF: Heart Failure, pEF: Preserved ejection fraction, LVH: Left Ventricular Hypertrophy, SLE: Systemic Lupus Erythematosus

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)	
Puntmann et al Hypertension 2014	173 DCM, 47 normals	MOLLI 3(3)5 SAX x1	3T Philips	Single centre	Septal IHD 1114±48 NICM 1145±37 Normals 1055±22 SAX IHD 1091±84 NICM 1102±72 Normals 1035±47					T1 associated with diastolic function
Edwards et al Am J Cardiol 2015	129 patients (43 CKD, 43 HT, 43 normals)	MOLLI 3(3)5 SAX x2	1.5T Siemens	Single centre	CKD 986±37 HT 956±31 Normals 955±30		CKD 419±52 HT 497±37 Normals 465±50		CKD 0.28±0.04 HT 0.25±0.02 Normals 0.25±0.03	Correlates to EF and strain
Singh et al EHJ Card Imag 2015	50 patients with AS, 22 normals (age matched)	MOLLI 3(3)3(3)5 SAX x1	3T Siemens	Single centre	AS: 1103±33 Normals: 1092±34				AS: 0.24±0.02 Normals: 0.25±0.03	No diff in T1 & ECV btwn mod vs severe AS
Hong et al Int J Card Imag 2015	41 DCM, 10 normals	MOLLI 3(3)3(3)5 SAX x3	3T Siemens	Single centre	DCM 1248±67 (LGE+ 1256±76; LGE- 1238±55) Normals 1205±37				DCM 30.7±5.9 (LGE + 31.2±5.6, LGE – 29.9±6.2) Normals 25.6±3.2	ECV correlates to LVEF
Lee et al Radiology 2015	80 mod/ severe aortic stenosis, 15 normals	MOLLI 3(3)5 SAX x1	3T Siemens	Single centre	AS 1208±45, Normals 1169±21					Native T1 correlates to strain, e' and LA volume Histo
Kuruvilla et al JACC Cardiovasc Imag 2015	20 HT LVH+, 23 HT LVH- 22 normals	MOLLI 3(3)5 SAX x2	1.5T Siemens	Single centre	HTN LVH+ 996±32 HT LVH- 974±34 Normals 967±35				HTN LVH+ 0.29±0.03 HTN LVH- 0.27±0.02 Normals 0.26±0.02	ECV correlates to strain

Table 1.3 (Continued): In vivo studies of T1 mapping in disease and health by MOLLI (studies listed in chronology of publication).^{185, 244-248}

AS: Aortic Stenosis, CKD: Chronic Kidney Disease, DCM: Dilated Cardiomyopathy, HT: Hypertension, IHD: Ischaemic Heart Disease, LVH: Left Ventricular Hypertrophy, NICM: Non-ischaemic cardiomyopathy

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)	
Aus dem Siepen et al EHJ Card Imag 2015	45 early DCM, 29 DCM, 56 normals	MOLLI 3(3)5 SAX x1	1.5T Philips	Single centre	Early DCM 1019±47, DCM 1056±62, Normals 1020±40	Early DCM 1600±124, DCM 1596±130, Normals 1558±79	Early DCM 431±40, DCM 420±45, Normals 442±43	Early DCM 265±40, DCM 265±39, Normals 262±38	Early DCM 25±4, DCM 27±4, Normals 23±3	Histo
Collins et al Circ Cardiovasc Imag 2015	31 non- ischaemic CM	MOLLI 3(3)5 SAX x3	1.5T Siemens	Single centre	All 1002±52, LVEF<50% 1004±44, LVEF≥50% 1001±57				All 0.29±0.08, LVEF<50%: 0.28±0.06, LVEF≥50%: 0.30±0.09	Increased ECV correlates to reduced LV velocities
Hinojar et al JACC Card Imag 2015	165 viral myocarditis, 40 normals	MOLLI 3(3)5 SAX x1	1.5T and 3T Philips	2 centres	<u>1.5T</u> Acute myo 1064±37 Inactive myo 995±19 Normals 940±20 <u>3T</u> Acute myo 1189±52 Inactive myo 1099±22 Normals 1045±23		<u>1.5T</u> Acute myo 373±42 Inactive myo 383±43 Normals 422±68 <u>3T</u> Acute myo 397±62 Inactive myo 426±73 Normals 422±68		<u>Λ @ 1.5T</u> Acute myo 50±7 Inactive myo 46±9 Normals 42±4 <u>Λ @ 3T</u> Acute myo 53±8 Inactive myo 45±8 Normals 44±5	
Piechnik et al JCMR 2010	4 MI patients 10 normals	MOLLI ShMOLLI SAX x3	1.5 & 3T Siemens	Single centre	<u>MOLLI</u> Normals: 1.5T 976±46 3T 1169±45 <u>ShMOLLI</u> Normals: 1.5T 966±48 3T 1166±60 MI LGE+: 3T 1432±32					

Table 1.3 (Continued): In vivo studies of T1 mapping in disease and health by MOLLI. ^{204, 249-251}

CM: Cardiomyopathy, DCM: Dilated Cardiomyopathy, HF: Heart Failure, HCM: Hypertrophic Cardiomyopathy, LVEF: Left Ventricular Ejection Fraction, LVH: Left Ventricular Hypertrophy, MI: Myocardial Infarction

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)
Dass et al Circ Cardiovasc Imag 2012	28 HCM 18 DCM 23 normals	ShMOLLI SAX x3	3T Siemens	Single centre	HCM 1209±28 DCM 1225±42 Normals 1178±13				T1 higher in LGE +ve segments. Correlates with LGE & strain
Fontana et al JCMR 2012	12 HCM, 18 severe AS 20 amyloid 50 normals	ShMOLLI EQ-CMR (FLASH-IR)	1.5T Siemens	Single centre					Normals: EQ-CMR 26±3, ShMOLLI 27±3 HCM: EQ 28±4, ShMOLLI 30±3 AS: EQ 27±6, ShMOLLI 31±5 Amyloid: EQ 48±6, ShMOLLI 52±7 Histo
Bull et al Heart 2012	109 AS (mod/severe) 33 normals	ShMOLLI	1.5T Siemens	2 centres	Asymp AS: 972±33 Symp AS: 1014±38 Normals: 944±16				Histo
Sado et al Circ Cardiovasc Imag 2013	44 AFD, 41 HT, 34 HCM, 21 severe AS, 20 amyloid , 67 normals	ShMOLLI SAX x2	1.5T Siemens	Single centre	AFD 882±47, normals 968±32 Others 1018±74				Male<female T1 in AFD. AFD pts on Rx had higher T1s.
White et al JACC Cardiovasc Imag 2013	25 HCM 22 severe AS 30 MI 20 amyloid 50 normals	ShMOLLI (EQ-CMR vs bolus) FCH	1.5T Siemens	Single centre	HCM 997 Sev AS 997 Amyloid:1137 Normals 968	HCM1519 Sev AS 1558 Amyloid 1597 Normals: 1530	HCM: EQ 592 bol 589 AS: EQ 575 bol 603 Amyloid: EQ 509 bol 525 Normals: EQ 613 bol 600		If ECV>0.4, bolus produces higher ECV than EQ-CMR Histo
Karamitsos et al JACC Cardiovasc Imag 2013	53 AL amyloid 17 AS 36 normals	ShMOLLI SAX x1 + FCH	1.5T Siemens	2 centres	Amyloid 1140± 61, AS 979± 51, Normals 958±20				
Sado et al JMIR 2014	88 iron overload 67 normals	ShMOLLI MOLLI 6(3)3 in subset n=35 SAX x1	1.5T Siemens	Single centre	High Fe 827±135, Normals 968±32				Good correlation ShMOLLI v T2* and v MOLLI

Table 1.4: In vivo studies of T1 mapping in disease and health by ShMOLLI and saturation recovery. ^{166-168, 175, 177, 178, 252}

AFD: Anderson-Fabry Disease, AS: Aortic Stenosis, CM: Cardiomyopathy, DCM: Dilated Cardiomyopathy, HCM: Hypertrophic Cardiomyopathy, HT: Hypertension, LVH: Left Ventricular Hypertrophy

Authors	Sample size (N)	Sequence/ Slice	Platform	Population	Pre-Gd Myo T1	Pre-Gd Bld T1	Post-Gd Myo T1	Post-Gd Bld T1	ECV (%)
Ferreira et al JCMR 2014	60 (query) myocarditis, 50 normals	ShMOLLI SAX	1.5T Siemens	2 centres	Myocarditis 1011±64, Normals 946±23				
Pica et al JCMR 2014	63 AFD, 63 normals	ShMOLLI MOLLI 5(3s)3	1.5T Siemens	Single centre	AFD +LVH 853±50, AFD no LVH 904±46, Normals 968±32				Male<female T1 in AFD also.
Mahmod et al JCMR 2014	26 severe AS, 16 normals	ShMOLLI SAX x3	3T Siemens	Single centre	AS: 1196±47 (post-AVR 1167±43) N: 1168±27				Stress perfusion done. Myocardial T1 recovers towards normal post AVR
Banyersad et al Eur Heart J 2015	100 amyloid, 54 normals	ShMOLLI FCH	1.5T Siemens	Single centre	Amyloid 1080±87 Normals 954±34				Amyloid EQ-CMR 0.44±0.12 Bolus 0.44±0.12 Normals 0.25±0.02 ECV>0.45 carries 3.84 hazard ratio for death
Tham et al JCMR 2013	30 children post anthracycline chemo	SASHA	1.5T Siemens	Single centre	1155.3±56.5	1149±215	652.1±53.6	390.5±51.6	20.7±3.6 T1 & ECV correlates to cumulative chemo dose
Thompson et al Circ Cardiovasc Imag 2013	31 Fabrys (FD), 21 LVH, 23 normals	SASHA SAX x2	1.5T Siemens	Single centre	FD 1070±50 LVH 1207±33 Normals 1177±27	FD 1620±192 LVH 1643±164 Normals 1624±96	FD 541±42 LVH 537±45 Normals 559±51	FD 314±51 LVH 294±42 Normals 331±45	FD 21.7±3.0 LVH 21.8±3.9 Normals 22.2±3.1 Lipid content inversely related to native T1. Males had lower T1 and ECVs.
Jellis et al Circ Cardiovasc Imag 2011	67 T2DM	MLLSR SAX x3	1.5T GE	Single centre	830±168 (range 600-1569)		434±64 (range 388-472)		Segmental T1s given (Figure 3) Correlates to diastolic f(x)
Song et al MRM 2012	9 patients (LGE -ve, Dx?), 8 patients LGE +ve, 24 normals	MLLSR SAX x1	1.5T GE	Single centre	All subjects 910±93		@10-15min Normal myo 435±36 LGE +ve myo 336±57		

Table 1.4 (Continued): In vivo studies of T1 mapping in disease and health by ShMOLLI and saturation recovery. ^{144, 162, 173, 179, 205, 253-255}

AFD: Anderson-Fabry Disease, AS: Aortic Stenosis, LVH: Left Ventricular Hypertrophy, T2DM: Type 2 Diabetes Mellitus

Authors	Sample size (N)	Sequence	Platform	Population	Results
Messroghli et al JCMR 2003	8 acute MI	IR fast gradient echo sequence with varying TIs	1.5T GE	Single centre	Pre-Gd Myo: LGE +ve 849±60 LGE -ve/remote 721±37 Post-Gd Myo: LGE +ve 262±19 LGE -ve/remote: 362±27
Maceira et al Circulation 2005	22 amyloid, 16 HT (as controls)	FISP cine with incremental T1 per frame	1.5T Siemens	Single centre	Post-Gd 4min: subendocardial T1 amyloid 427±73, HT pts 579±75
Iles et al JACC 2008	25 heart failure 9 heart transplants 20 normals,	VAST SAX x3	1.5T GE	Single centre	Post-Gd Myo T1: HF 383±17, normals 564±23 Post-Gd Blood T1: HF 272±12, normals 286±7 Post-contrast T1 related to systolic & diastolic function (*restriction) Histo (RV biopsy)
Amano et al JMIR 2009	23 HCM, 7 control (= 5 chronic MI + 2 pericardial effusion)	Look Locker SAX x1	1.5T Philips	Single centre	Null points of LGE+ve HCM shorter than normal blood, and myocardium
Broberg et al Circ Cardiovasc Imag 2010	50 ACHD pts 14 normals	Look Locker	1.5 or 3T Philips	Single centre	Fibrosis Index: Normals: 24.8±2.0 Systemic RV (n=11): 35.0±5.8 LV in Tetralogy (n=17): 29.2±3.4 Cyanosis (n=10): 33.7±5.6 Other ACHD (n=12): 31.4±3.2 Acquired heart disease (n=4): 36.2±5.8
Ling et al Circ Arr Elec 2013	33 atrial tachy patients 20 normals	Look Locker SAX x3	1.5T GE	Single centre	Global corrected T1 (post-Gd): AT-normal EF 480±76 AT-low EF 442±52 Normals 506±61
Ellims et al JACC 2014	20 cardiac Tx pts	VAST SAX x1	1.5T GE	Single centre	Pre-Gd Myo T1: 937±158 blood T1: 1435±335 Post-Gd Myo T1: 380±82 Blood 229±34 ECV 26.8±8.5% Post-Gd T1 & ECV correlates to LV stiffness
McLellan et al Circ Arrhythm Electrophysiol 2014	103 AF patients (68 PAF, 35 persistent)	VAST SAX x1	1.5T GE	Single centre	Post-Gd Myo T1: PAF 415±87, persistent 385±99. No AF 366±73, recurrence 429±90. Post-Gd Blood T1: PAF 245±37, persistent 247±34. No AF 248±39, recurrence 239±26
Barison et al EHJ Card Imag 2015	30 systemic sclerosis pts 10 normals	Modified-cine IR (MCine-IR) SAX x1	1.5T GE	Single centre	Native myo T1: SSc 790±84, Normals 811±89 Native blood T1: SSc 1495±130, Normals 1488±161 Post-Gd myo T1: SSc 383±45, Normals 390±36 Post-Gd blood T1: SSc 304±53, Normals 290±47 Myo ECV: SSc 30±4, Normals 28±4%

Table 1.5: In vivo studies by other T1 mapping methods.^{122, 123, 199, 219, 256-260}

ACHD: Adult Congenital Heart Disease, AF: Atrial Fibrillation, HCM: Hypertrophic Cardiomyopathy, HT: Hypertension, LVH: Left Ventricular Hypertrophy, MI: Myocardial Infarction, Tx: Transplant

1.5 Genomics in tetralogy of Fallot

In support of the experimental work in Chapter 6 in which unbiased genome wide analysis of the RV in patients with rTOF is planned, an overview of the field of integrative genomics pertaining to CHD is summarised in the following section. There have been no specific, dedicated studies to date examining the genomic signatures of cardiac fibrosis in rTOF, with limited published applications of genomics in ACHD, emphasizing the relevance and significance of our proposed genomic premise.

1.5.1 Genomics in adult congenital heart disease

Congenital heart disease arises from dysregulation of cardiac development in utero, which is mediated by a complex interplay of genetic, stochastic and environmental factors.^{261, 262} The aetiology of the majority of CHD is still unknown. The contributory components of each factor towards the spectrum of CHD remain poorly understood; and in spite of the rapid growth of integrative genomics in the past decade, only 13% of CHD has been attributable to genetic chromosomal variations to date.²⁶¹ Most of CHD is regarded as polygenic disorders, with only a minority caused by single-gene defects. Genetic studies have elucidated that interaction of transcription-factors, signalling pathway defects, copy number variations (CNVs) and micro-RNA dysfunction are implicated in the pathogenesis of CHD.^{263, 264}

Genomics is the study of the sequence, structure and biology of genome-level data sets of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).²⁶⁵ An accelerated era of genomic discovery is afoot in CHD and cardiovascular medicine, aided by the shift to Next Generation Sequencing (NGS)²⁶⁶ techniques from Sanger DNA sequencing.²⁶⁷ NGS has sharply increased the throughput by gene sequencing in parallel at lower costs per base. NGS encompasses multiple approaches for probing the genetic spectrum of an individual, spanning targeted techniques (of exome sequencing or disease-specific gene panels) or unselective comprehensive analysis (whole genome sequencing). Direct RNA sequencing utilises high resolution transcriptome analysis to achieve complete quantitative assessment of transcript expression, splicing and sequence variation. The derived results are individual specific, with rapid simultaneous interrogation of multiple gene sets being possible. It also permits the discovery of novel variants and disease genes. Complex data analysis and data storage are some of the technical issues currently limiting translation of RNA sequencing into the clinical arena. Large scale genetic studies have recently become feasible due to the formation of research consortia to achieve adequate study power.^{268, 269}

Genetic testing across the range of CHD can be targeted or unselective. Specific deletion disorders such as 22q11 can be diagnosed by chromosomal karyotyping or microarray

analysis, with higher sensitivity achieved by the microarray approach. This, however, has led to the detection of small deletions or duplications of uncertain clinical significance.²⁷⁰ Gene sequencing with gene panels can identify genetic variation at the nucleotide level, and if a pathogenic mutation is not found, second-line testing can be extended to exome or whole genome sequencing. Although genome sequencing is unbiased and permits the discovery of novel variants and disease genes, the sheer complexity of the dataset may uncover gene variants of doubtful clinical significance or links to unrelated medical conditions. This in turn can have potentially deleterious psychosocial, employment and health insurance implications.

1.5.2 Genetic determinants of tetralogy of Fallot

Recurrence risks in CHD are often difficult to quantify, as disease phenotypes are complicated by reduced penetrance and variable expressivity, such that significantly different phenotypes can be exhibited by patients with identical mutations, even within a single family. Higher recurrence rates of CHD are recognised in TOF,²⁷¹ with various approaches in human and animal studies undertaken to examine the genetic contributors to TOF. The overall recurrence risk in tetralogy is believed to be in the region of 2.5-3.0%, with a higher recurrence risk in an affected mother. There is also some suggestion that affected families without a 22q11 deletion may exhibit an autosomal recessive pattern.²⁷²⁻²⁷⁴ Research groups have chosen to examine comparative phenotypes of TOF, which have included: native versus repaired, cyanotic versus acyanotic, as well as pressure versus volume loaded haemodynamics. Other studies have chosen to examine the functional significance of specific pathways and genes.

1.5.2.1 Chromosomal abnormalities and gene mutations in TOF

Chromosomal syndromes account for approximately 20% of all CHD. DiGeorge syndrome is the most common cause of syndromic TOF, with a microdeletion of 22q11.2 reported in between 4-35% of cases.²⁷⁵⁻²⁷⁸ The ICD-10 classification reports a prevalence of 8% of 22q11 deletion in patients with TOF.²⁷⁷ The TBX1 mutation has been identified as an important determinant of DiGeorge syndrome.^{279, 280} Other recognised mutations associated with TOF include Jagged-1 gene (in 1-2%) in familial cases and Alagille syndrome,²⁸¹⁻²⁸³ in addition to NKX2.5 mutations (in 4%)^{284, 285} Other developmental syndromes and point mutations associated with TOF are listed in *Tables 1.9 and 1.10*.

Syndrome	Defect	Locus	Causal Gene	Inheritance	OMIM
22q11 deletion	Deletion	22p11.2	TBX1	De novo, AD	188400
Cri-Du-Chat	Deletion	5p15.2	CTNND2	De novo	123450
Cat Eye	Inversion duplication	22q11	Unknown	De novo, AD	115470
<i>Single gene mutations</i>					
Alagille syndrome	Single gene	20p12, 1p12	JAG1, NOTCH2	AD	118450
CHARGE	Single gene	8p12, 7q21.11	CHD7, SEMA3E	AD	214800
Duane-radial ray syndrome	Single gene	20q13.2	SALL4	AD	607343
Kabuki syndrome	Single gene	12q13.12	MLL2	De novo, AD	147920

Table 1.9: Developmental syndromes associated with TOF. AD – autosomal dominant, OMIM – Online Mendelian Inheritance in Man. Adapted and modified from Fahed *et al* (Circ Res 2013)²⁸⁶

Gene	Protein	OMIM
<i>Transcription factors and cofactors</i>		
FOG2/ZFPM2	Friend of GATA	603693
GATA4	GATA4 transcription factor	600576
GATA6	GATA6 transcription factor	601656
HAND2	Helix-loop-helix transcription factor	602407
NKX2-5/NKX2.5	Homeobox containing transcription factor	600584
TBX1	T-box 1 transcription factor	602054
ALDH1A2	Retinaldehyde dehydrogenase	603687
CFC1/CRYPTIC	Cryptic protein	605194
FOXH1	Forkhead activin signal transducer	603621
GDF1	Growth differentiation factor 1	602880
JAG1	Jagged-1 ligand	601920
NODAL	Nodal homolog (TGFβ superfamily)	601265
TDGF1	Teratocarcinoma derived growth factor 1	187395
VEGF	Vascular endothelial growth factor	192240

Table 1.10: Gene mutations associated with TOF. OMIM - Online Mendelian Inheritance in Man. Adapted and modified from Fahed *et al* (Circ Res 2013)²⁸⁶

1.5.2.2 Differential Gene Expression in TOF

Differential gene expression has been demonstrated in TOF versus normal controls.²⁸⁷⁻²⁹⁰ DNA microarray analysis has been performed on RV biopsy samples from 20 patients (12 native TOF in childhood and 8 adult rTOFs) with age-matched controls (n=16) to demonstrate altered gene expression of 236 genes. There was upregulation of ECM proteins (particularly collagen types I and III, fibronectin) and myocardial angiogenesis genes (VEGF-A to D, flk-1, TNFα, TGFβ and PDGF), whilst TIMP-1 and TIMP-4 were downregulated. Matrix metalloproteinase (MMP) expression remained unchanged or diminished.²⁹¹ A smaller series of native TOF children (n=12) compared to age-matched controls (n=6) conversely reported decreased expression of TGFβ in tandem with the enhanced expression

of angiogenic factors (VEGF, TNF and PDGF).²⁹² A further study additionally identified dysregulation of oxidative phosphorylation pathways in unrepaired children with TOF.²⁸⁹

1.5.2.3 The impact of hypoxia on gene expression in TOF

Hypoxic states prompt the activation of hypoxia-induced transcription factors, which promotes the synthesis of erythropoietin, VEGF and glycolytic enzymes to increase oxygen delivery and energy production via hypoxia-induced transcription factor 1 α (HIF1A).^{293, 294} Whilst HIF1A is cardioprotective in acute hypoxia,²⁹⁵ chronic activation of HIF1A is recognised to promote TGF β 1-mediated fibrosis.^{296, 297}

When considering cyanotic (n=11) and acyanotic (n=9) children with native TOF prior to surgical repair, RNA microarray analysis revealed 795 differentially expressed genes (≥ 1.8 fold).²⁹⁸ There was downregulation of myocardial contractility and function genes, with upregulation of transcripts encoding apoptosis, cell movement, morphogenesis and remodelling in cyanotic hearts. In particular, there was upregulation of growth-signalling factors FGF7, TGFBR2, EGFR and insulin-like growth factor binding protein 7 (IGFBP7). Transcription factors GATA4, STAT3 and JUN were downregulated, together with a reduction of MAPK signalling genes MAPK1, MAPK14, MAP3K2, MAP3K3 and AKT2. These results were postulated to be predominantly related to cyanosis in the absence of significant RV hypertrophy in the study cohort. In a separate analysis of 23 children with TOF (13 cyanotic, 10 acyanotic) by real-time PCR, cyanosis was found to confer downregulation of VEGF, glycolytic enzymes and glutathione peroxidase. There was concurrent increased expression of collagen (in particular procollagen type 1 α 1). Gene expression was found to correlate inversely with the degree of cyanosis and pre-operative saturations.²⁹⁹

It has also been suggested that the adaptive response to hypoxia in children with TOF may influence RV phenotype. When children with TOF were genotyped for single-nucleotide polymorphisms of HIF1A, patients with more functioning HIF1A alleles were found to have an early phenotype of higher TGF β 1 expression and therefore more fibrosis at the time of surgical repair, in comparison to age-matched children undergoing VSD repair. The presence of more functioning HIF1A alleles appeared to result in a beneficial late phenotype of less RV dilatation, preservation of RV function and greater freedom from RV intervention over a follow-up period of 9.0 ± 3.5 years.³⁰⁰

1.5.2.4 Altered signalling pathways in TOF

VEGF has been identified as a gene modifier of TOF, in which polymorphisms in the VEGF promoter region lower expression levels of VEGF. VEGF mutations and polymorphisms have been shown to increase the risk of TOF.³⁰¹⁻³⁰⁴

Altered cardiomyocyte differentiation via bone morphogenic protein (BMP) has been reported in TOFs compared to normal controls, with over-expression of natriuretic peptide precursors A and B (NPPA and NPPB). Suppression of WNT and Notch signalling pathways was also evident.³⁰⁵ These pathways undoubtedly participate in a complex interplay with other regulatory genes in rTOF, and therefore individual signalling molecule dysregulation cannot be interpreted in isolation when considering the pathogenesis of TOF.

1.5.2.5 Copy Number Variants (CNVs) in TOF

CNVs are structural genomic variations (typically ≥ 1000 base pairs) that can contain multiple contiguous genes, which lead to gain or loss of chromosomal segments through duplication or deletion.³⁰⁶ Pathogenic CNVs represent a growing cause of CHD, occurring in 3-25% of patients with extra-cardiac abnormalities and 3-10% of patients with isolated cardiac defects.³⁰⁷ CNVs represent a major source of genetic variation that contributes to disease, with limited insight for its relevance to the aetiology of TOF to date.³⁰⁸ Up to 10% of a series of 114 patients with TOF studied were found to harbour rare de novo CNVs.³⁰⁹

Three recent studies have identified CNVs in large cohorts of non-syndromic TOF patients, with a single locus that overlapped between the studies at chromosome 1q21.1. GJA5 at this locus has been postulated as a potential candidate gene for TOF.³⁰⁹⁻³¹¹ Copy number gains have also been described in a small study of 8 patients in three genes – namely ISL1, NOTCH1 and PRODH.³¹²

1.5.2.6 Genome Wide Association Studies (GWAS) in TOF

GWAS are based on the principle of genotyping large numbers of genetic variants across the entire genome, through a systematic approach for comparing allele distributions between cases and controls for a chosen disease. It enables novel molecular pathway identification, even of low-penetrance susceptibility loci for complex diseases.^{313, 314} The uptake of GWAS in CHD has been limited by the relatively low prevalence of CHD, in contrast to the vast cohort sizes required to power GWAS adequately for typical effect size of single nucleotide polymorphisms (SNPs). Furthermore, such studies are designed to detect common genetic variants, begging the theoretical question as to whether such studies are suitable for the heterogeneous nature of CHD.

To investigate relationships between common SNPs and the risk of TOF, a number of GWAS have been conducted. Genetic variants in chromosome 12q24 have been found to be associated with TOF, with tyrosine-protein phosphatase non-receptor type 11 (PTPN11) emerging as the strongest candidate gene.^{315, 316} PTPN11 regulates the Ras/MAPK signalling pathway, with mutations reported to cause Noonan's syndrome.³¹⁷ SNPs in the glypican 5 gene on chromosome 13q32, chromosomes 10p14 and 10p11.2 are also implicated in TOF.^{315, 318}

The findings from these various studies are challenging to interpret in a clinical context as mixed cohorts of patients were often included, encompassing a range of CHD and even different phenotypes within the single entity of TOF (for example, when native and rTOF patients were included within the same analysis group). Nonetheless, there have been encouraging developments to translate these findings into the clinical environment.

1.5.3 Clinical applications of genomics in congenital heart disease

The application of genomics-driven diagnostics, prognostics and therapeutics is experiencing rapid expansion due to decreasing costs of integrative genomics for genotype-phenotype identification. Individual specific genetic information may improve patient care through personalising medical treatment, guiding further investigations and management, as well as informing targeted follow-up. Genetic assessment may also define inheritance patterns to allow recurrence risk prediction, thereby informing cascade screening of family members and family planning.

In CHD, there is increasing relevance of genetic testing with the American Heart Association recommending that genetic evaluation is undertaken for all patients with CHDs accompanied by extracardiac manifestations or neurodevelopmental abnormalities.³¹⁹ For example, genetic testing should be undertaken routinely for all children with supra-valvular aortic stenosis or pulmonary stenosis for Williams syndrome, while screening for 22q11 deletion should be performed in those with an interrupted aortic arch, aortic arch anomalies, conotruncal defects (truncus arteriosus, TOF), or disconnected branch pulmonary arteries.²⁶¹ Knowledge of genetic mutations can facilitate tailoring of surveillance and intervention for mutation-related complications. An example includes the NKX 2.5 mutation in the setting of an atrial septal defect, which increases the risk of heart block and progressive ventricular dysfunction.³²⁰ Proactive and timely monitoring for pacemaker insertion when required can then be instituted. Genotyping has also been associated with post-operative outcomes. In patients with TOF, the presence of 22q11 deletion predicts longer cardiopulmonary bypass

times intra-operatively as well as longer intensive care stays post-operatively.³²¹ Such information could be utilised to manage patient expectations and better inform the consent process.

Fetal genetic screening for CHD is also an expanding arena, as microscopic chromosomal deletions and CNVs can be detected^{322, 323} for the diagnosis of trisomies 21, 18 and 13, in addition to microdeletion syndromes including DiGeorge syndrome. Pre-implantation diagnosis of Holt-Oram³²⁴ and Marfan syndromes is already feasible, and may identify fetuses at risk for CHD earlier than fetal cardiac imaging. Fetal genetic screening options consequently raise ethical dilemmas over the role of therapeutic abortions and challenge the ambiguities inherent in the clinical interpretation of prenatal screening results. There are no clear directives on how to account for false positive tests, and there is insufficient current know-how to accurately predict disease severity and phenotypic penetrance of some of these conditions.

Other potential genomic applications include gene therapy, genome editing and pharmacogenetics, through which personalised genotype-based treatments may become a future clinical reality. Gene therapy involves the substitution of a mutated gene for a healthy copy, inactivation of a faulty gene or introducing a new gene as treatment. Whilst it represents an ideal therapeutic milieu of tailored medicine, it remains very much in the clinical trial research domain at present.^{325, 326} Genetic determinants of cardiovascular drug responses may be of increasing relevance to direct the choice and dose of cardiovascular medications taken by CHD patients. For example, variants of CYP2D6 regulate the metabolism of beta-blockers and flecainide,^{319, 327} whilst CYP2C9 variant testing may improve time in therapeutic window for patients on warfarin anticoagulation.³²⁸⁻³³⁰

As discussed, there is immense scope for potential clinical applications of genomic data in TOF. We therefore undertook RNA sequencing in a relatively large cohort of patients with rTOF to examine the principal molecular genetic mechanisms and pathways as outlined in *Chapter 6*.

1.6 Neurohormonal biomarkers in tetralogy of Fallot

Biomarkers are defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention”.³³¹ Biomarkers are widely prevalent throughout the medical arena, especially so in CHD where limited patient numbers, low event rates amongst a predominantly asymptomatic population precludes larger scale randomised controlled trials to elicit clinical outcomes or therapeutic responses. The notion of reproducible and reliable non-invasive biomarkers is therefore intrinsically desirable. There is emerging evidence that neurohormonal biomarkers may be of clinical relevance in CHD, particularly in view of its simplicity of assessment.

The diagnostic and prognostic value of neurohormones is firmly established in chronic heart failure, with well documented morbidity and mortality associations. BNP and its precursor, N-terminal pro-B-type natriuretic peptide (NT-proBNP) in particular, have emerged as relevant peripheral biomarkers, which have been incorporated into clinical care for disease monitoring and to assess pharmacological treatment responses.^{332, 333} BNP in heart failure patients is associated with functional capacity, LV dysfunction and mortality. In contrast, the role of neurohormonal activation in CHD remains ill-defined, with limited longitudinal data.

Neurohormonal activation has been reported in complex CHD, even amongst asymptomatic patients.^{334, 335} Elevated levels of natriuretic peptides (ANP, BNP), endothelin-1, norepinephrine, renin, aldosterone and PIIINP have been documented in heterogenous cohorts of adult patients with CHD.^{334, 336, 337}

Specific to patients with rTOF, cross-sectional studies have shown elevated BNP levels, which correlates with RV end-diastolic dimensions and the severity of PR.^{335, 338, 339} In addition, elevated levels of circulating PICP and PIIINP have been reported in small cohorts of patients with rTOF, and may be implicated in the pathogenesis of LV mechanical dyssynchrony.^{337, 340}

A systematic review of twenty publications encompassing 770 rTOF patients verified significantly elevated BNP levels in rTOF patients compared to controls, which in turn correlated to New York Heart Association (NYHA) class.³³⁵ The relationship between BNP with RV volumes and function was overall inconsistent, with non-significant correlations in some studies, but also evidence of positive correlations between BNP, RV volumes and the severity of pulmonary regurgitation in others.^{335, 341}

The use of biomarkers to determine prognosis in rTOF is not clearly delineated,^{334-336, 342} and there remains a relative paucity of sensitive early biomarkers to detect adverse outcomes prior to the onset of overt clinical signs and symptoms. In view of this, we sought to investigate neurohormonal activation and the prognostic value of serum BNP levels in patients with rTOF (*Chapter 7*).

Specific Aims of the Thesis

Overall Aim:

To investigate the role of non-invasive biomarkers (including CMR) for outcome prediction in patients with repaired tetralogy of Fallot.

Thesis Aim 1:

To examine non-invasive quantification of diffuse and focal myocardial fibrosis in rTOF and its relation to clinical outcomes.

Thesis Aim 2:

To investigate the genomic signatures of the pathological right ventricle in rTOF.

Towards addressing these aims, specific hypotheses were generated for each sub-study included within the thesis. These include:

Hypothesis 1:

Diffuse fibrosis assessment (by means of CMR T1 mapping) can be applied to the right ventricle and correlates with other markers of adverse clinical outcome.

Hypothesis 2:

Integrative genomics (through RNA sequencing) can identify the extent of right ventricular fibrosis and restrictive phenotypes in rTOF.

Hypothesis 3:

Cardiac remodelling as assessed by CMR may have prognostic implications in rTOF.

Hypothesis 4:

Neurohormonal activation may predict clinical outcomes in rTOF.

Chapter 2 Methods

This thesis comprises of four major studies involving CMR as the primary imaging modality in patients with rToF. Specific methods are detailed in each of the *Results* chapters with an overview outlined within this chapter. For clarity, the main studies include:

- Cardiac remodelling following pulmonary valve replacement in adults with rToF
- Right ventricular T1 mapping in rToF
- Genomic signatures in patients with rToF
- Neurohormonal activation and its relation to outcomes late after rToF

2.1 Patient Identification

Patients with rToF over 16 years of age (without contraindications to CMR) were identified from the Adult Congenital Heart Disease service at the Royal Brompton and Harefield NHS Foundation Trust. Healthy, normal subjects were recruited as controls for the RV T1 mapping and neurohormones studies from members of staff working at the Royal Brompton and Harefield NHS Foundation Trust.

Study specific ethics approval was obtained prior to patient and volunteer recruitment, with written informed consent obtained from each study participant. All eligible participants were sent study information and given at least 24 hours to consider study enrolment prior to signed consent if they were agreeable.

2.2 Cardiovascular Magnetic Resonance

2.2.1 *The CMR examination*

Patient Preparation

All patients completed a safety questionnaire prior to entry into the scanner to assess for contraindications to CMR and the use of gadolinium intravenous contrast. Patients were cannulated (20G or larger calibre) with 0.5ml of venous blood drawn to measure whole blood creatinine for the calculation of estimated glomerular filtration rate (eGFR). If renal function was satisfactory with $eGFR > 30 \text{ ml/min/1.73m}^2$, gadolinium contrast would be injected peripherally at the appropriate timing during the scan. Data were acquired in a single examination which took approximately 60 minutes.

Inclusion Criteria

Patients with rTOF without contraindications to CMR were considered for study recruitment. Specifically, patients referred for surgical PVR were invited to participate in the cardiac remodelling and RV T1 mapping studies. Volunteers were age and gender-matched and considered for inclusion in the absence of a history of cardiovascular disease and being a lifelong non-smoker.

Exclusion Criteria

Patients with contraindication to CMR including an ICD or non-CMR conditional pacemaker in situ, claustrophobia, metal fragments in the eyes or a eGFR of less than 30 ml/min/1.73m² precluding administering gadolinium were excluded.

2.2.2 CMR data acquisition protocol

An overview of the main CMR sequences was delineated in *Section 1.2.4*.

All datasets (except for some sections of sequence development work for RV T1 mapping) were acquired on a 1.5T MR scanner (Avanto, Siemens Healthcare, Erlangen, Germany) with an anterior phased array 6-channel surface coil and 6-9 channels of a posterior array as the top surface of the patients' bed ("spine coil"). Dedicated segments of RV T1 mapping sequence development was carried out on a 3.0T MR scanner (Magnetom Skyra, Siemens Healthcare, Erlangen, Germany) with an anterior 18 element matrix coil and 8-12 elements of a matrix spine coil. The large number of coils distributed around the patient is engineered to assist fast imaging by using coil signal strengths to supply additional position information in "parallel imaging", accelerating the conventional phase-encoding process in MRI.³⁴³

Care was taken with patient placement within the scanner bore in addition to optimal positioning of ECG leads for good quality capture of the ECG trace. All images were acquired with retrospective ECG gating unless otherwise specified. Patient position was adjusted in the head-foot direction to centre the heart as near as possible to the magnet iso-centre prior to image acquisition, as several types of error in MRI increases with distance from the iso-centre.



Figure 2.1: Siemens 3T Skyra scanner at the Royal Brompton Hospital (image courtesy of Ms Julia Coffey, Cardiovascular BRU public engagement coordinator).

Patients with rTOF underwent a comprehensive scan in accordance with our institution's suggested, published protocol:⁸²

Pilot Scouts

A breath-hold bSSFP multi-slice single-shot sequence was acquired in three orthogonal planes (transaxial, coronal and sagittal) to confirm that the patient was positioned optimally with the heart at isocentre with selection of suitable receiver coils around the heart. Adjustments to patient position were made as necessary (*Figure 2.2*).

This was followed by multislice HASTE (half-Fourier acquisition single-shot turbo spin-echo) imaging in the transaxial plane (30 slices over two breathholds) followed by multislice TruFISP (fast imaging steady state free precession) imaging in transaxial, coronal and sagittal orientations (30 slices).

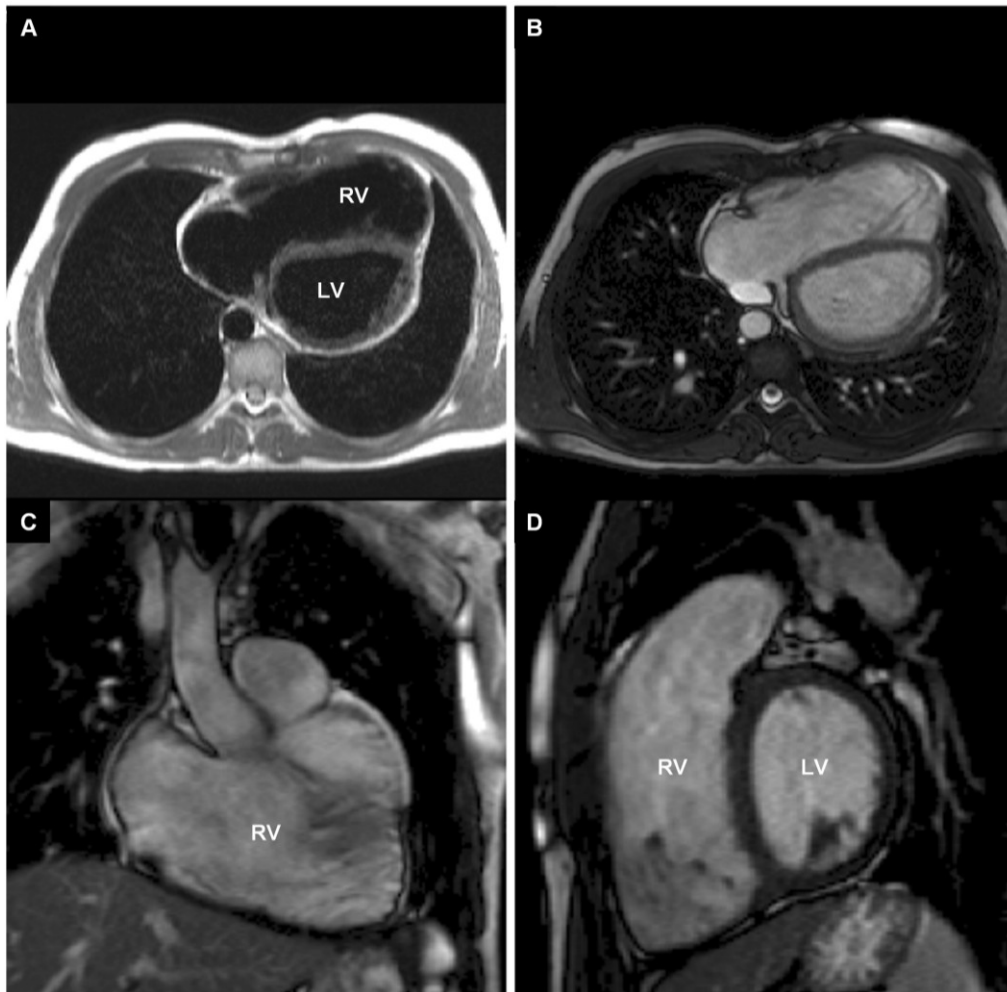


Figure 2.2: Transaxial HASTE (**A**) with corresponding transaxial TruFISP slice (**B**) and representative coronal (**C**) and sagittal (**D**) TruFISP scouts in a patient with rTOF. Note RV dilatation visible in all illustrated planes.

Cine Imaging

ECG-triggered and breath-hold balanced SSFP cines encompassing the ventricular long and short axes were acquired in the following order (*Figure 2.3*): RV outflow tract sagittal and transaxial views, right and left pulmonary artery aligned coronal cine views and repeated to produce a well-aligned bifurcation cine, LV vertical long axis (VLA), basal short axis scout, followed by a four chamber view aligned through the centre of the mitral, tricuspid valves and LV apex. A LVOT view was planned through the centre of the mitral valve, aortic valve and LV apex. A coronal LVOT cine was also acquired, in addition to an 'en face' view of the aortic valve.

Additional RV long axis views were carried out to complete cine imaging to include: RV inflow and outflow view, RV oblique and RV vertical long axis planes in oblique sagittal and oblique coronal orientations.

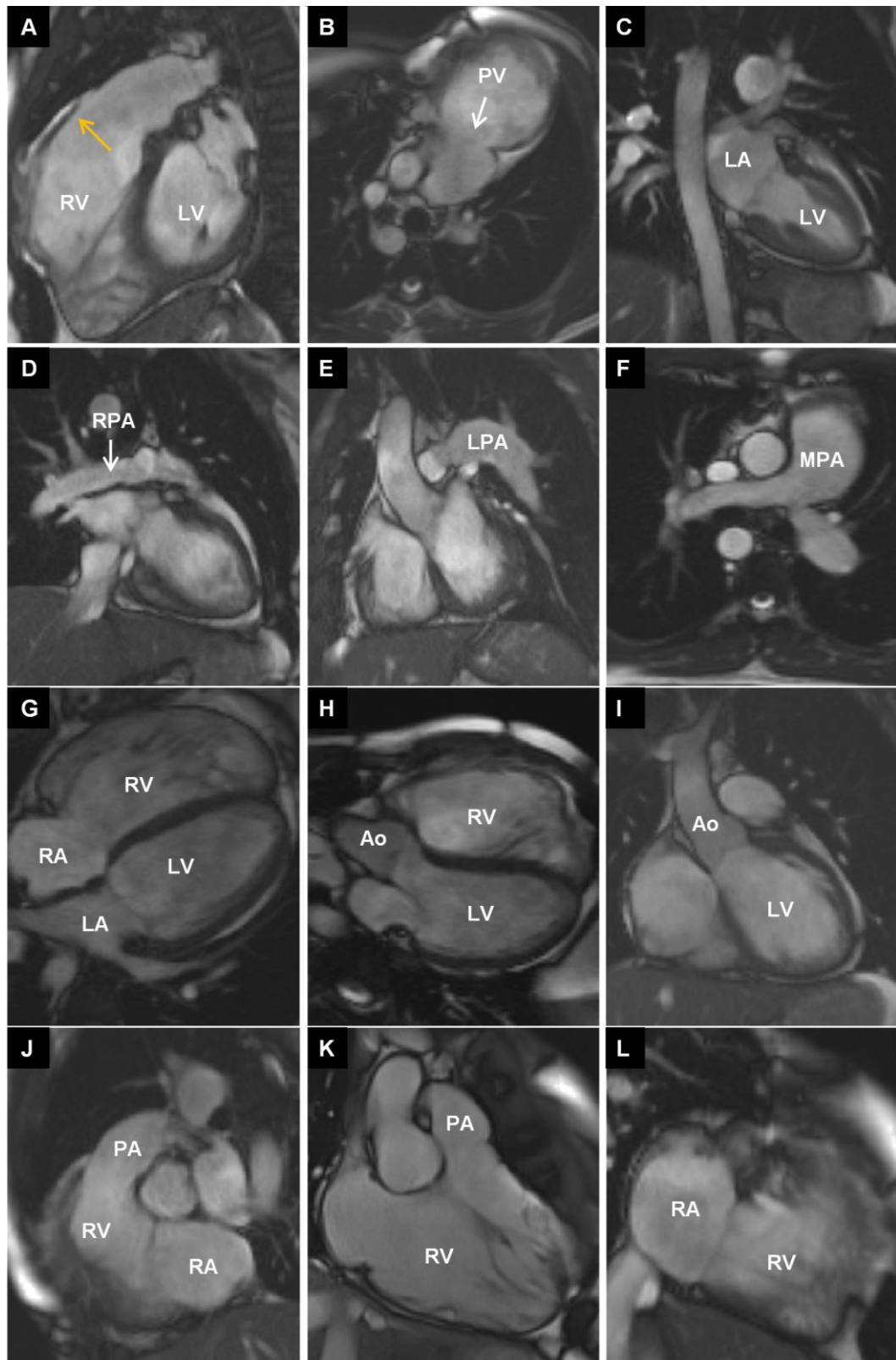


Figure 2.3: Representative cine frames acquired in a patient with rTOF in: RVOT sagittal (**A**), RVOT transaxial (**B**), LV vertical long axis (**C**), right and left branch pulmonary arteries (**D to F**), four chamber (**G**), LVOT transaxial and coronal views (**H and I**), RV inflow and outflow (**J**), RV oblique long axis (**K**) and RV long axis (**L**) views. The akinetic RVOT region is demarcated by the yellow arrow in Panel **A**.

A short axis (SAX) stack was prescribed at 10mm equidistant intervals (7mm slice thickness, 3mm gap) from base to apex until complete biventricular coverage was achieved, typically encompassing 10-12 cines (*Figure 2.4*). The basal slice was specifically aligned using both the VLA and four chamber cines to contain ventricular myocardium in the end-diastolic frame.

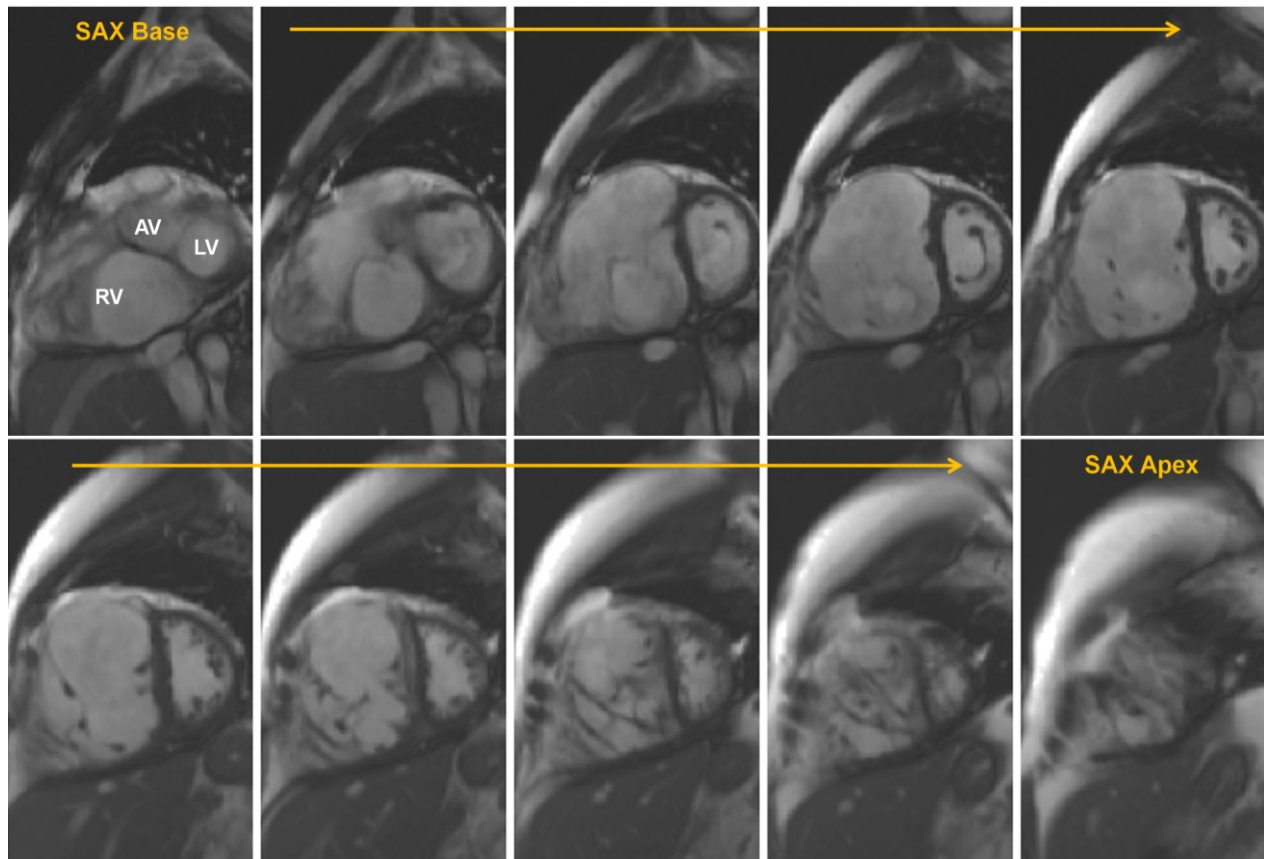


Figure 2.4: Short-axis cines from base to apex (top left hand panel to bottom right panel) with RV dilatation in patient with rTOF. AV: aortic valve, LV: left ventricle, RV: right ventricle.

Typical imaging parameters for bSSFP cine acquisitions were: Acquired resolution 1.7x1.7mm by 7mm slice thickness, approx 320mm x 300mm (PE “oversampled” to avoid wraparound over many oblique slices used) field of view, continuous “retrospectively” ECG gated acquisition time per acquired cine frame 41ms, reconstructed as 25 frames presented over the cardiac cycle. Typically 15 rawdata lines per acquired for each cine frame per cardiac cycle. Breath-holding for 9-10 cardiac cycles was typically required with this protocol, using parallel imaging acceleration (rate 2, with 23 extra reference lines during the breath-hold to obtain the supporting coil profile information). Although parallel imaging is well-known to reduce SNR, the bSSFP cine sequence has ample SNR to support this acceleration. The time between bSSFP RF pulses (TR) was 2.7ms, TE=1.15ms (asymmetric echo) with

930Hz/pixel sampling bandwidth. The flip angle was adapted depending on patient heating (specific absorption rate of RF energy as heat) but typically 60-70°. Images were reconstructed straightforwardly as combined magnitudes of all the coils with no additional filtering or processing, except “normalisation” correction of the drop in image brightness with increasing depth from the coils.

Flow Imaging

Breath-hold velocity mapping was run in-plane to the RVOT, and through-plane across the pulmonary trunk and infundibular region, to obtain peak pulmonary artery velocity, pulmonary regurgitant fraction and sub-valvular regurgitant fractions respectively (*Figure 2.5*). Branch pulmonary artery velocity mapping was carried out where applicable if there was suspicion or evidence of branch pulmonary artery stenosis. A through-plane velocity map was also obtained at the sinotubular junction of the aorta to derive cardiac output, LV stroke volume and aortic regurgitant fraction (if present).

Typical imaging parameters for velocity mapping sequences were: Acquired resolution 1.3(FE)x2.5(PE)mm* by 8mm slice thickness, at approx 320mm x 240mm field of view (adapted to patient), continuous “retrospectively” ECG gated acquisition time per acquired cine frame 60ms, reconstructed as 30 frames presented over the cardiac cycle. Breath-holding for 16-20 cardiac cycles was typically required with this protocol, collecting 5 rawdata lines into each cine image per cardiac cycle. Parallel imaging was not usually applied because of the low SNR of spoiled gradient echo imaging. The time between RF pulses (TR) was 6.1ms, TE=3.1ms with 391Hz/pixel sampling bandwidth. The flip angle was typically 25°. The velocity encoding range (VENC) was adapted to each patient sometimes requiring multiple scans to avoid excessive velocity aliasing. Velocity maps were generated as described above.

* FE: frequency encode, PE: phase encode

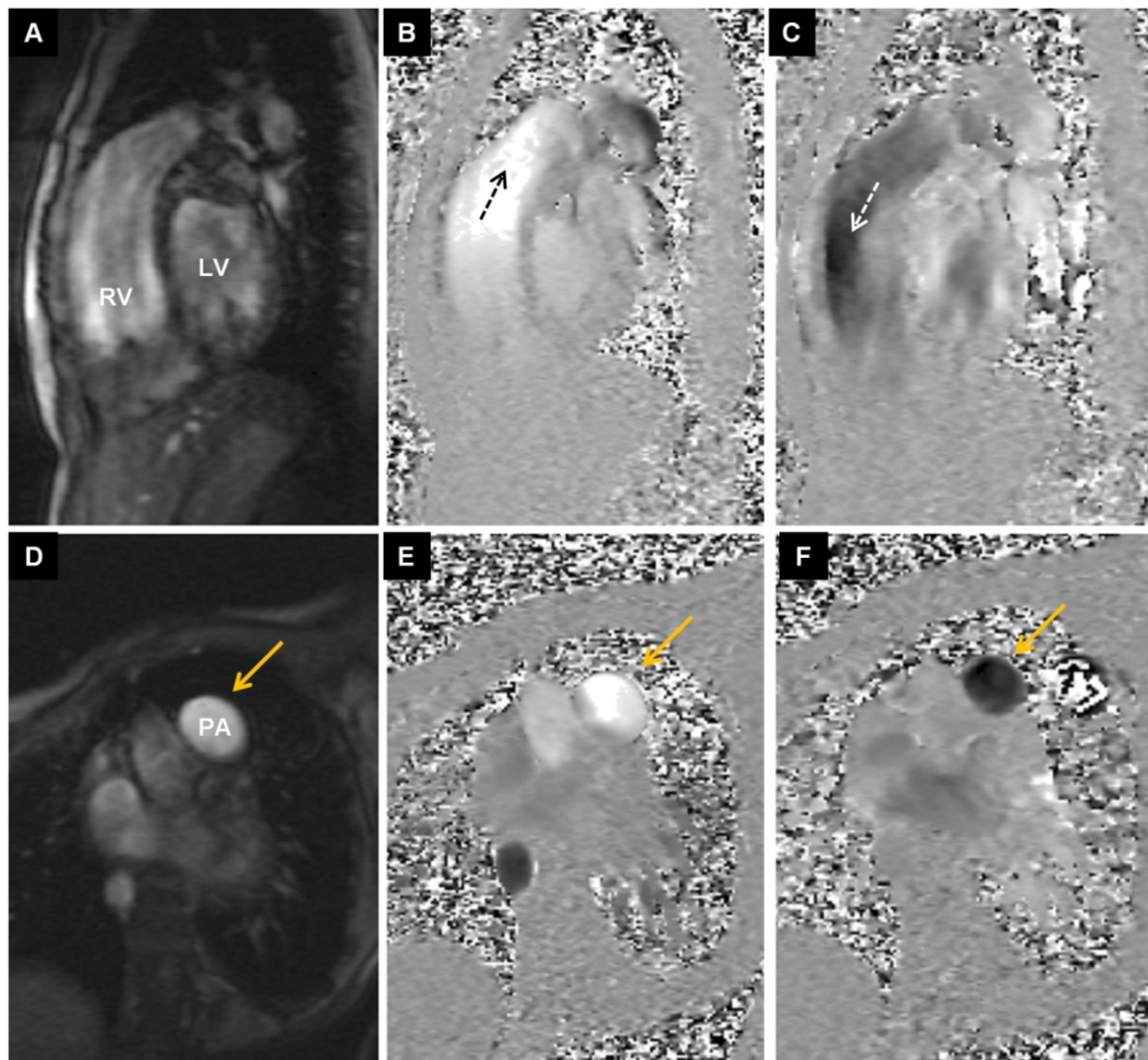


Figure 2.5: Flow sequence magnitude image aligned with RVOT in sagittal projection in patient with rTOF (**A**), with in-plane velocity encoding along the head-foot image axis (displayed vertically on page) so that corresponding velocity maps show forward flow as indicated by black arrow (**B**) and severely regurgitant jet across an ineffective pulmonary valve, indicated by white arrow (**C**). Through-plane phase contrast velocity mapping demonstrates the pulmonary artery (PA) in cross-section in the magnitude image (**D**), with forward flow appearing bright in systole (**E**) and a dark appearance of a broad area of severe regurgitation in diastole (**F**) in velocity maps (yellow arrows).

Late Gadolinium Enhancement (LGE) Imaging

Tissue characterisation by LGE CMR was performed after anatomical and functional assessment in accordance with established protocols.^{19, 61, 75, 344} All patients with rTOF without contraindications were intravenously administered gadolinium chelate 0.1mmol/kg (Gadovist, Bayer Schering, Berlin, Germany), followed by a bolus of 10ml 0.9% normal saline flush.

Early gadolinium enhancement images of RV and LV long-axis planes were acquired with an expiratory breath-hold ECG-gated segmented inversion recovery prepared turbo-FLASH sequence, with TI set to 440ms.³⁴⁵ From ten minutes following the injection of gadolinium, LGE imaging was acquired using 2D segmented fast low-angle shot inversion recovery sequence optimised for RV imaging (*Figure 2.6*). Imaging parameters were optimised to maintain nulling of healthy myocardium, typically by incrementing TI tracking Gd washout starting from TI ~270ms dependent on image feedback to the operator, whilst minimising artefacts and ensuring adequate biventricular coverage (including repeated RV and LV long-axis in addition to short axis planes). Cross-cuts of suspected enhanced myocardial regions indicative of focal fibrosis were acquired when deemed appropriate. Phase swaps (of orthogonal phase encode directions – right to left and head to foot) were routinely acquired in order to eliminate some imaging artefacts associated with the phase-encode axis (such as poorer resolution) but also to maintain multiple “washout” stages of the same tissue to enhance confidence, together with systolic imaging where necessary to define subtle RV LGE. Three-dimensional LGE was acquired to enhance image resolution when feasible.¹⁰⁶

Typical imaging parameters were as follows: Acquired resolution 1.3(FE)x2.2(PE)mm by 8mm slice thickness, at approx 340mm x 250mm field of view (adapted to each individual patient). Breath-holding for 10 cardiac cycles was typically required with this protocol, as non-selective inversion-recovery was performed only on alternate cardiac cycles so that recovery in between was near complete (for fast heartrates every 3rd cycle was sometimes used), collecting usually 23 rawdata lines per acquired cycle. Again parallel imaging was not usually applied because of the low SNR of spoiled gradient echo imaging. The time between RF pulses (TR) was 6.9ms, TE=3.2ms with 230Hz/pixel sampling bandwidth (low because of low SNR inherent in the LGE technique but very high CNR between enhanced and properly nulled normal myocardium). The flip angle was typically 25°.

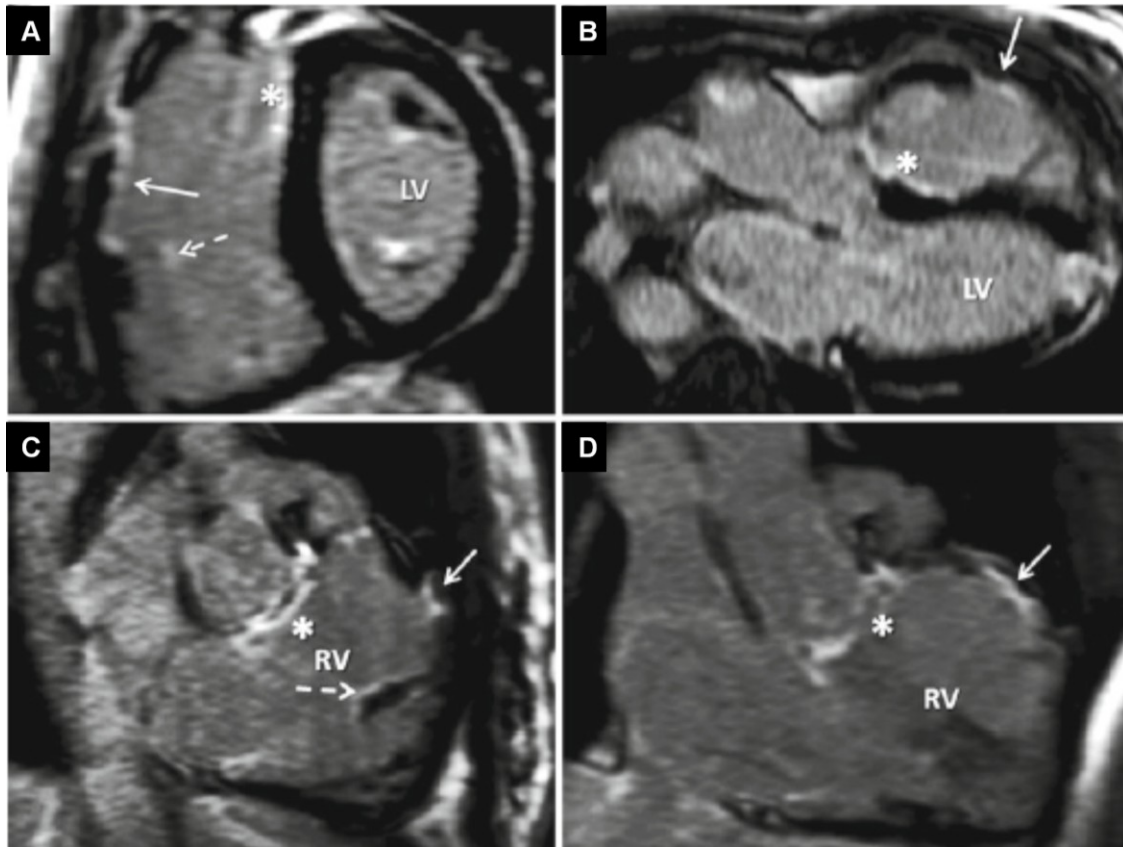


Figure 2.6: LGE images of adult patient with rTOF in (A) SAX (B) LVOT transaxial (C) RV inflow and outflow as well as (D) RV oblique views. There is RV LGE of the RVOT (indicated by solid white arrows) seen in all panels, which extends to the endocardium of the anterior RV free wall in Panel A. Further LGE of the VSD patch surgical sites (marked with asterisk *) as well as LGE of RV trabeculations (indicted by dotted white arrows in Panels A and C) are present. LV apical LGE consistent with an apical vent from previous surgery is seen in Panel B. Reproduced from Heng et al, Tetralogy of Fallot In: Cardiac CT and MR for Adult Congenital Heart Disease, Springer 2014⁸² with permission of Springer.

2.2.3 CMR image analysis

Volumetric & functional analysis

Biventricular volumes, ejection fraction and mass were assessed using a semi-automated, threshold-based algorithm using dedicated analysis software (CMRtools, Cardiovascular Imaging Solutions, London, UK). The 2 chamber, 4 chamber, RVOT and short axis stacks were manually selected. The mitral, tricuspid, aortic and pulmonary valve planes were defined in systole and diastole on the long axis views. The endocardial and epicardial borders were manually traced in diastole and systole. Thresholding software was used to manually adjust the blood pool image to exclude the LV papillary muscles and major trabeculations of the RV from the blood pool, such that these were finally included in the myocardial mass (*Figure 2.7*).

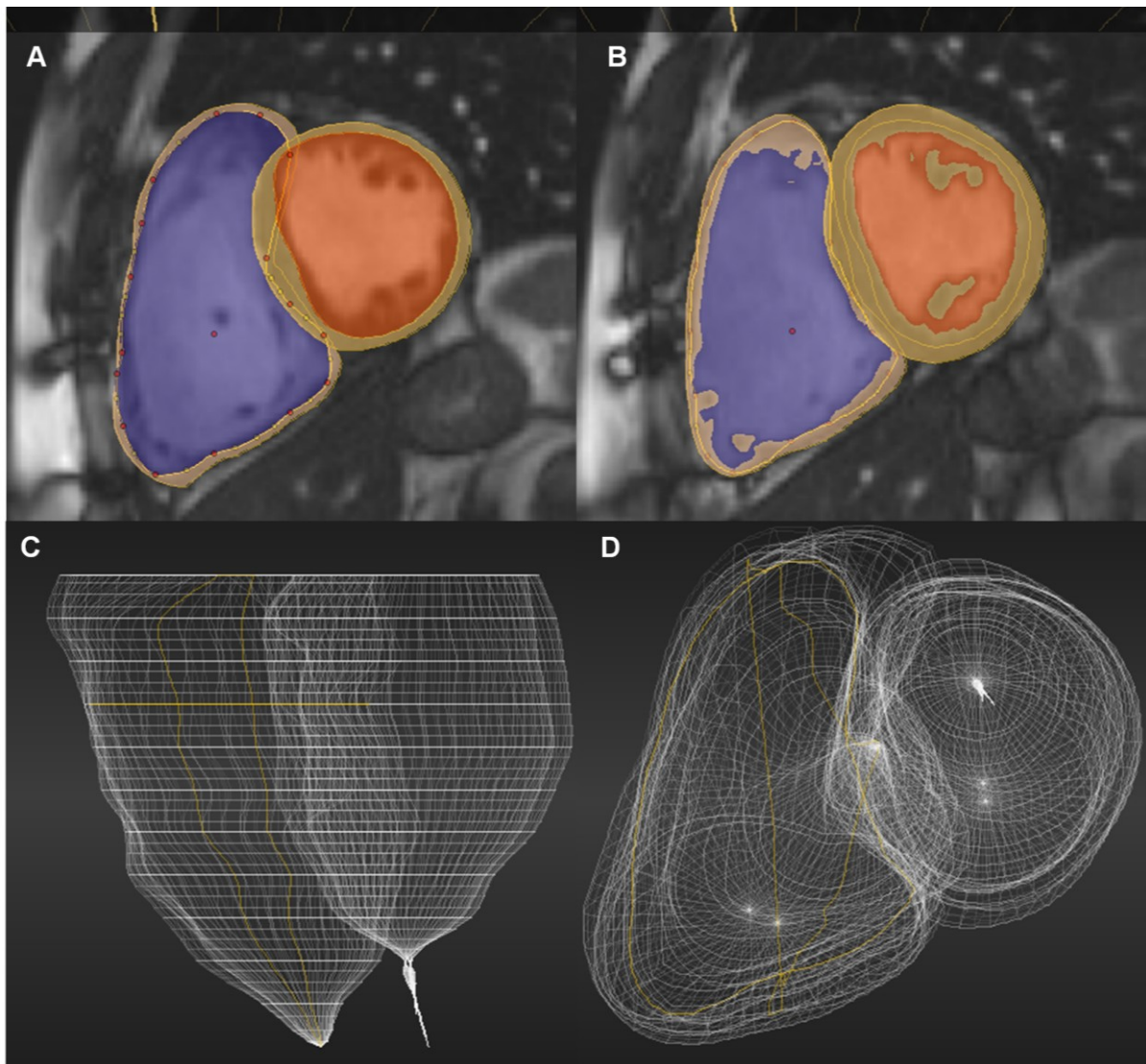


Figure 2.7: Measurement of biventricular volumes and function with CMRtools. Screenshots from the semi-automated model illustrates **(A)** demarcation of epicardial and endocardial borders of both ventricles, such that the borders of myocardial mass is shaded in yellow, LV blood shaded in orange and RV blood shaded in blue. **(B)** Manual thresholding of the blood pool excludes LV papillary muscle and RV trabeculations from the blood pool to generate 3D models of biventricular volumes **(C)** in long axis (with RV on left and LV on right of image) and **(D)** in short axis in a similar image plane to panels A and B.

The end-diastolic volume (EDV) and end-systolic volume (ESV) were calculated using summation of the volume of blood pool for the contiguous slices of the ventricle in diastole and systole respectively. This technique does not make any assumptions regarding ventricular geometry and is therefore considered the gold standard for volumetric assessment. Ventricular stroke volumes were calculated as follows:

$$\text{Left ventricular stroke volume (LVSV)} = \text{LVEDV} - \text{LVESV}$$

$$\text{Right ventricular stroke volume (RVSV)} = \text{RVEDV} - \text{RVESV}$$

The ejection fractions were defined as:

$$\text{Left ventricular (LV) EF} = \text{LVSV} / \text{LVEDV}$$

$$\text{Right ventricular (RV) EF} = \text{RVSV} / \text{RVEDV}$$

The LV and RV masses were calculated in end-diastole. The respective ventricular mass was calculated as the volume of ventricle delineated by the epicardial border minus the EDV of that ventricle.

Biventricular volumes and mass were then indexed to the body surface area (BSA) using the Du Bois equation:³⁴⁶

$$\text{Body surface area (m}^2\text{)} = 0.20247 \times \text{height in metres}^{0.725} \times \text{weight in kg}^{0.425}$$

Flow quantification: pulmonary valve peak velocity and regurgitant fraction

The peak velocity through the pulmonary valve was measured using flow velocity mapping. Pulmonary regurgitant fraction was calculated from phase velocity mapping in a plane transecting the main pulmonary trunk. The regurgitant volume was calculated as the difference between the RV stroke volume and the volume of blood per beat moving forwards into the main pulmonary artery. The regurgitant fraction is therefore subsequently expressed as a percentage of the stroke volume where:

$$\text{Pulmonary regurgitant fraction (\%)} = 100 \times (\text{Total reverse pulmonary flow} / \text{Total forward pulmonary flow})$$

Definitions of CMR derived indices

- Measurement of right atrial area

The maximal right atrial area was manually contoured on a four chamber view on a cine frame at the time, just prior to atrioventricular valve opening, excluding the atrial appendage and caval veins (*Figure 2.8A*); then indexed to body surface area. Right atrial dilatation was defined as indexed right atrial area (RAAi) $\geq 15\text{cm}^2/\text{m}^2$ measured in a four chamber view.

- RVOT akinesia

This was defined as non-contractile RV myocardium which did not thicken in systole, as visualized in sagittal and transaxial cine views of the RVOT (akinetetic region delineated by white arrow in *Figure 2.8B and C*).



Figure 2.8: (A) Right atrial area delineated in four chamber cine. Akinetic length of RVOT bordered by white arrows in RVOT cine frame (sagittal view) in panels (B) and (C) illustrating variation in RVOT akinesia.

Late Gadolinium Enhancement Analysis

LGE was analysed with semi-quantitative assessment: LGE analysis was based on a standard 17-segment model³⁴⁷ for the LV on a 5-point scale.⁶¹ For the RV, a previously described segmentation and RV LGE scoring (maximum score 18) was applied (*Figure 2.9*).⁶¹ LGE was scored as present if bright signal within myocardium was visualised with good quality images in the same region on phase swap or crosscuts. The maximum linear extent of RV outflow tract (RVOT) LGE was measured. The intra-observer reproducibility of LGE scoring was assessed by re-scoring a subset of the scans blind to previous analysis (with minimum 6 month interval).

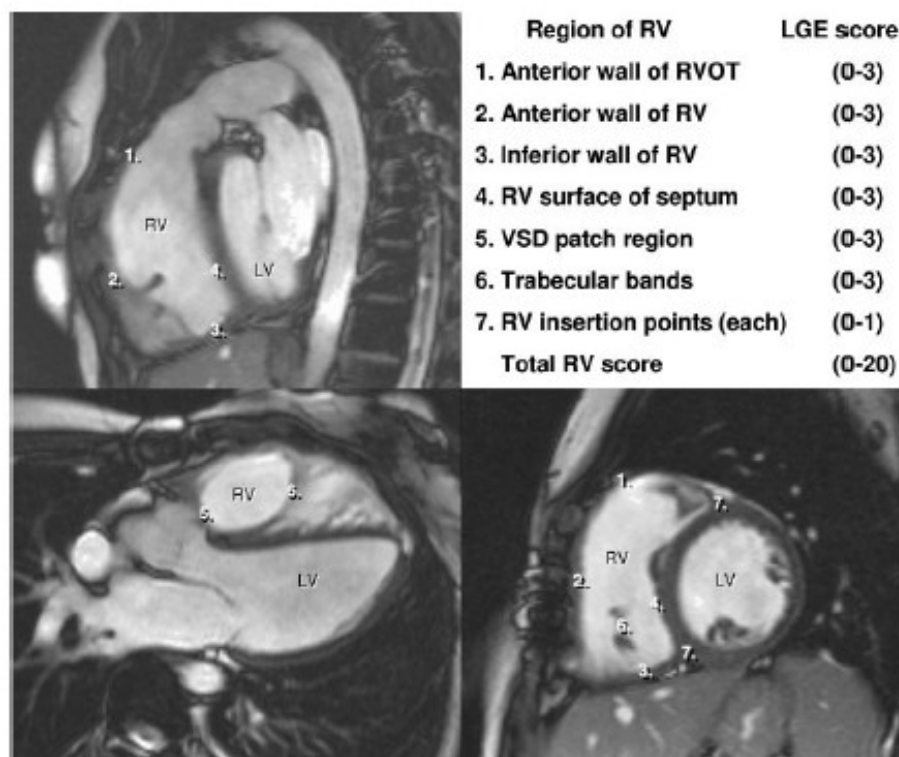


Figure 2.9: Segmental system for scoring RV LGE in rTOF (reproduced from Babu-Narayan et al ⁶¹ with permission from Wolters Kluwer Health) The RV is divided into 7 segments in slices aligned with the RVOT, the LV outflow tract, and the LV short axis, with maximum LGE score per segment in brackets. Each segment was graded according to the linear extent of enhanced myocardium as follows: 0 - no enhancement, 1 - up to 2 cm LGE in length, 2 - 2 to 3 cm LGE in length, and 3 - more than 3 cm LGE in length. Enhancement seen in trabeculations, including the moderator band, was graded as 0 - no enhancement, 1- enhancement of 1 trabeculation, 2 - enhancement of 2 to 4 trabeculations, and 3 - enhancement of more than 4 trabeculations.

Reproducibility Assessment of CMR measurements

For the assessment of intra- and inter-observer reproducibility, variability was expressed as the coefficient of variation (%), derived from the within-subject standard deviation divided by the mean, multiplied by 100%. The within-subject standard deviation is $\sqrt{\sum (\text{difference between paired measurements})^2 / 2n}$. Bland-Altman plots were utilised to assess potential bias between observers or observations. Intraclass correlation coefficients were also assessed.

2.2.4 T1 mapping in repaired tetralogy of Fallot

For the RV T1 technical work described within this thesis, physics sequence development was undertaken based on and compared to existing clinical T1 mapping sequences. These included the Modified Look-Locker Inversion Recovery (MOLLI) sequence^{201, 202} and Saturation Recovery Single-shot Acquisition (SASHA).¹⁹⁸ The basic experimental parameters of these sequences primarily used in clinical research for diffuse fibrosis were described in *Section 1.4.2*. Basic aspects of the MOLLI method are outlined in this section, and further specific RV modifications to the sequences are described in detail as part of the study results in *Chapter 5*.

At the time of venous cannulation prior to the scan, patients and volunteers who provided specific consent for T1 mapping had additional blood sampled to check their haematocrit via a laboratory analysed full blood count sample. T1 measurements by previously established clinical MOLLI sequences³⁴⁸ were applied at 15 minutes after the administration of gadolinium contrast with pre-Gd 5(3)3 MOLLI and post-Gd 4(1)3(1)2 MOLLI sequences run. The arrangement of inversion-recovery times for pre-Gd and post-Gd scans was designed so that the range of the most accurate and precise T1 estimation suited the *in vivo* blood and myocardium values;²¹⁴ that is, long pre-Gd T1 (900-1300ms by 5(3)3) and short post-Gd 300-600ms by 4(1)3(1)2).

Typical imaging parameters for the 5(3)3 MOLLI were as follows: Eight different inversion recovery times were acquired in 11 cardiac cycles. TR 2.6ms, TE 1.1ms, flip angle 35°; field of view: FOV 360mm (FE) x 307mm (PE); slice thickness 8mm; acquired voxel size 1.4mm (FE) x 2.1mm (PE) x 8mm (slice) in each 195ms single-shot image; bandwidth 1085Hz/pixel; magnetisation preparation = inversion recovery by 5(3)3 scheme, TI=120ms +1 through 4 subsequent RR intervals with pause of 3 cycles, then 200ms, + 1 and 2 subsequent RR intervals; phase-encode partial Fourier 7/8 (ie. zero-filled 1/8th); k-space trajectory linear-order Cartesian; parallel imaging mode: GRAPPA with acceleration factor 2; magnetisation preparation timing after R-wave was adjusted so that each single-shot image was acquired in the diastolic pause. Parallel imaging and somewhat coarse in plane resolution are necessary so that each T1-weight single-shot image can be completed within diastasis of each cycle.

Typical imaging parameters for the 4(1)3(1)2 MOLLI were as above except for the inversion recovery times: Nine different inversion recovery times were acquired in 11 cardiac cycles. The magnetisation preparation was inversion-recovery, with time by the 4(1)3(1)2 scheme, TI =120ms, +1 through 3 subsequent RR intervals with pause for 1 cycle, then 200ms, +1

and 2 subsequent RR intervals with further pause for 1 cycle; then 280ms, +1 subsequent RR interval.

A slightly modified set of parameters were used if the heart rate was faster than 90bpm, because the diastolic pause could in that situation be too short for the single-shot image duration of 194ms to be relatively free of cardiac motion. The modified parameters acquired a single-shot image in 159ms with 1.9 (FE) x 2.4 mm (PE) in-plane acquired resolutions. However, the T1 estimation by this version of the mapping had been carefully evaluated to match that of the higher resolution protocol, bearing in mind increased risk of contamination by adjoining tissues.

The study design also incorporated planned histological validation of our proposed RV optimised T1 mapping technique against RV histology sampled at the time of surgery in patients with rTOF undergoing PVR, taking histology as gold standard validation (*Section 2.3*). Data collected was anonymised for blinded CMR and histological analysis. Derived ECV values were compared to prospectively recruited age and gender-matched controls (though this may be more relevant for the LV as the RV is so thin).

Image analysis for derivation of native, post-contrast T1s and ECVs was carried out with dedicated custom-written software in MATLAB (Version 2014A, The MathWorks, Natick, Massachusetts, USA), CMR42 (Circle Cardiovascular Imaging, Calgary, Canada) and CMRtools (Cardiovascular Imaging Solutions, London, UK). Care was taken for ROI selection to minimise potential measurement errors by blood pixel contamination.

2.3 Endomyocardial biopsy and histological analysis

Histopathological assessment of myocardial fibrosis by means of endomyocardial biopsies remains the gold standard for detecting and determining the extent of myocardial fibrosis. Written informed consent was obtained from patients with rTOF undergoing surgical PVR for the donation of myocardial biopsies taken at the time of open heart surgery. Surgical biopsies were collected when technically feasible as judged by the surgical expertise of the consultant cardiac surgeon. Biopsies were obtained from sites of incision or of immediate access to the surgeon, usually from the right atrium, aortic cardiopulmonary bypass incision site and the body of the RV. The biopsy process under direct vision is regarded as safe with minimal risk,^{349, 350} with no reported complications from previous similar practice for research studies at our institution.

Myocardial biopsy samples were fixed in 10% buffered formalin, processed, embedded in paraffin and 5-micron sections stained with picrosirius red.^{351, 352} Qualitative and quantitative assessment was then performed by 2 independent observers with image analysis software (ImageJ) with high power magnification (250x) for 20 fields per biopsy using a Leica Qwin video image analysis system under polarised light. All fields analysed were at least one field away from the specimen edge. The collagen volume fraction of the sample was measured, which fundamentally applies a 'thresholding' option to reflect the myocardial collagen content.³⁵³ If discrepancies in the analysis were present, the specimen was re-evaluated by both observers with final consensus reached by both observers together with a third experienced observer.

2.4 RNA Sequencing

Myocardial biopsies from patients with rTOF were promptly snap-frozen in liquid nitrogen immediately after tissue collection (*Section 2.3*), and stored in controlled conditions at -80°C until the samples could be analysed. RV control tissue was obtained from heart transplant donor hearts, for which access was granted via the Cardiovascular Biomedical Research Unit Biobank of the Royal Brompton & Harefield NHS Foundation Trust.

RNA was extracted from frozen RV samples using TRIzol (Thermo Fisher Scientific) as per the manufacturer's protocol, and quantified by ultraviolet spectrophotometry. RNA quality was ascertained prior to library preparation with Illumina TruSeq RNA sample preparation kits (Illumina, San Diego, USA). Further details of library assembly are outlined in *Chapter 6*. Paired-end 100 base-pair sequencing was then carried out on the Illumina Hi-Seq platform at the Medical Research Council Clinical Sciences Centre, Hammersmith Hospital, Imperial College London.

Details of RNA sequencing data analysis and bioinformatics are also presented within *Chapter 6*.

2.5 Non-invasive Investigations

Clinical data obtained as part of routine clinical care or pre-PVR surgical work-up were collated. These included records of baseline observations of height, weight, heart rate, blood pressure and oxygen saturations. Electrocardiography was performed in each patient, from which the rhythm, rate and QRS duration was manually measured by a blinded experienced observer. Posteroanterior chest radiographs were manually analysed for the cardiothoracic ratio (CTR), which was derived from ratio of the maximal transverse diameter of the cardiac

silhouette to the distance between internal rib margins at the level of the right hemidiaphragm (expressed as a percentage).^{354, 355}

2.5.1 Venous blood sampling for neurohormonal analysis

Patients with rTOF (aged ≥ 16 years) attending tertiary adult congenital heart disease outpatient care at the Royal Brompton Hospital were recruited at the time of referral for CMR.

Peripheral blood samples were obtained for neurohormonal quantification from all patients, taken on the morning of the CMR after 20 minutes of rest. Blood was sampled to fill three tubes: with EDTA (Ethylenediaminetetraacetic acid) anticoagulant, EDTA with aprotinin a third tube with no additives. The blood samples were centrifuged at 4°C for 15 minutes at 3000rpm into their constituent plasma and serum aliquots. These were stored at -80°C with minimal freeze-thaws until analysis.

Atrial natriuretic peptide (ANP), BNP and active renin were quantified with immunoradiometric assays (with BNP 1 pmol/L=3.472 pg/mL). Serum aldosterone was determined by radioimmunoassay (EuroDPC) and endothelin-1 (ET-1) was measured by enzyme-linked immunosorbent assay (ELISA) (Bachem). Full blood count, renal function and liver function were assayed by certified quality-controlled clinical biochemistry at the Royal Brompton Hospital.

2.5.2 Echocardiography

Two dimensional (2D) transthoracic echocardiography was performed using a Hewlett Packard Sonos 5500 (Andover, Massachusetts, USA) according to the British Society of Echocardiography guidelines,³⁵⁶ encompassing M-mode, 2D and Doppler acquisitions. Chamber dimensions, LV ejection fraction and RV fractional area change were measured³⁵⁷ as part of the clinical scanning protocol. A single blinded experienced echocardiographer acquired all echocardiography data. Pulsed and continuous wave Doppler pulmonary flow velocities were digitally recorded. RV restrictive physiology was defined as laminar antegrade flow in the pulmonary artery in late diastole coinciding with atrial contraction and present throughout the respiratory cycle (ie, the 'a' wave illustrated in *Figure 2.10*).^{50, 55}

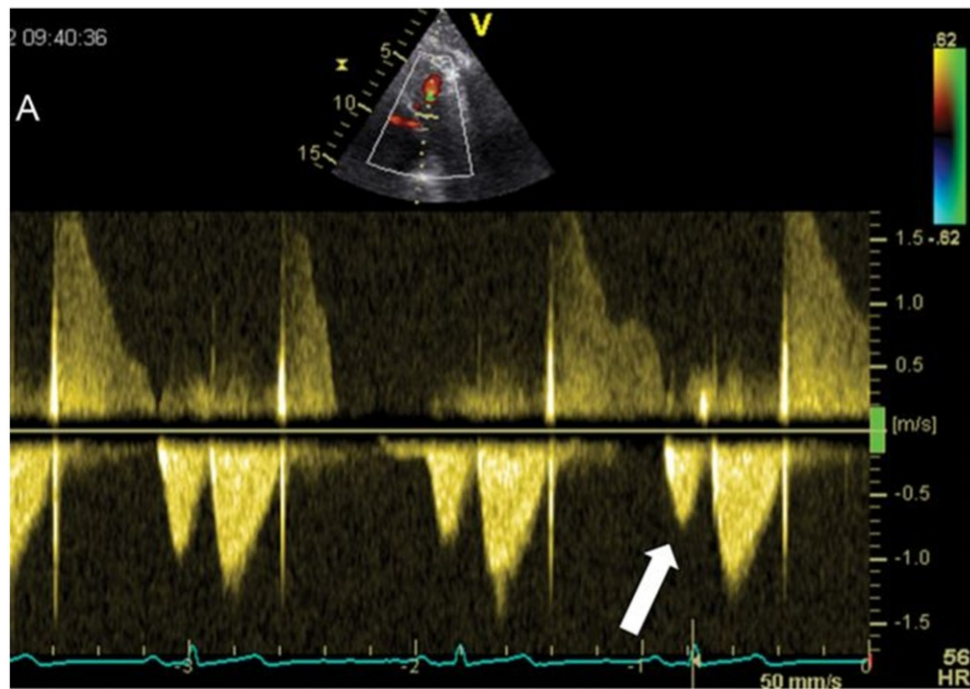


Figure 2.10: Continuous wave Doppler of RV outflow tract in patient with repaired tetralogy of Fallot illustrating 'a' wave (white arrow) of RV restrictive physiology. Reproduced from Orwat *et al*³⁵⁸, Eur Heart J Cardiovasc Imag, 2014 by permission of Oxford University Press.

2.5.3 Cardiopulmonary exercise testing

Cardiopulmonary exercise testing was carried out with the modified Bruce protocol to symptom-limitation. Patients were instructed to exercise while using a specialized mouthpiece that recorded expired oxygen and carbon-dioxide levels. Continuous ECG monitoring was performed and the expired oxygen and carbon dioxide levels were sampled every few seconds during exercise. The modified Bruce protocol starts at a low level of exercise, walking slowly and progressively increases levels of exercise in a standardized fashion. Patients exercise until limited by symptoms and the specific symptoms causing termination of exercise were recorded. In our patient cohort, this was typically shortness of breath.

Peak oxygen uptake (peak VO₂), ratio of minute ventilation to carbon dioxide production (VE/VCO₂) and anaerobic threshold were assessed. Patients who did not reach anaerobic respiration (a respiratory quotient value <1) were excluded from analysis as the values recorded would not accurately reflect exercise capacity.

2.6 Statistical Methods

Data analysis was carried out blinded to patient demographic information. Data normality was assessed by Kolmogorov-Smirnov analysis. Normally distributed continuous variables were expressed as means $\pm$ standard deviation, and non-parametric continuous variables reported with medians and interquartile ranges (IQR). Comparisons between groups were assessed by paired Student t test, Mann Whitney U-test or Wilcoxon test as appropriate. Categorical data were presented as frequencies and percentages, with differences assessed by Chi-squared or Fisher's exact tests as appropriate. Correlations were sought with Pearson's or Spearman's rank correlations.

Relevant statistical methods specific to each sub-study of this thesis will be outlined in each of the Results chapters. All statistical analysis was performed with SPSS (IBM Statistics V.22) and/or R software (v.3.1.3). A p value <0.05 was considered statistically significant

RESULTS

The results presented in the following chapters are grouped according to thematic factors that influence may clinical outcomes in repaired tetralogy of Fallot. Chapters 3 and 4 are focussed on **Cardiac Remodelling following Surgical Pulmonary Valve Replacement (PVR)**. Chapter 3 examines the reproducibility of CMR derived RV volumetric and functional measures, while Chapter 4 examines early and midterm cardiac remodelling following surgical PVR in adults with repaired tetralogy of Fallot.

Chapters 5 and 6 will examine **myocardial fibrosis in repaired tetralogy of Fallot**, firstly by exploring novel CMR biomarkers of diffuse fibrosis by means of T1 mapping of the RV before penultimately moving onto investigating the genomic signatures of cardiac fibrosis in rTOF.

Chapter 7 will report on the role of peripheral neurohormonal biomarkers for predicting outcomes in patients with rTOF.

Chapter 3 Reproducibility of CMR Right

Ventricular Volumetric and Functional Assessment

3.1 Introduction

Cardiovascular Magnetic Resonance (CMR) is considered the reference standard for the assessment of right ventricular (RV) volumes and systolic function in patients with repaired tetralogy of Fallot (rTOF).³⁵⁹⁻³⁶¹ CMR plays an ever increasing role in the management of this patient cohort, from providing serial surveillance of residual haemodynamic lesions such as pulmonary regurgitation, influencing the clinical decision making process for timing of PVR, to monitoring the cardiac remodelling response to surgical or transcatheter interventions. The reproducibility of volumetric and functional analysis is therefore of utmost importance.

Whilst the reproducibility of CMR left ventricular measurements have been reported more widely across a range of pathologies, there have been comparatively modest reports of intra- and inter-observer reproducibility of the RV. Although reassuring levels of agreement have been shown in small series for RV volumes and ejection fraction in healthy volunteers and congenital heart disease, RV mass measurements have shown higher variability across multiple studies.³⁶²⁻³⁶⁴ We therefore sought to prospectively determine our reproducibility at intra-observer and inter-observer levels.

3.2 Methods

CMR scans for 12 patients with rTOF were acquired by a single experienced operator as part of a prospectively recruited study (see section 4.2) and randomly selected for inclusion towards reproducibility assessment.

3.2.1 CMR Acquisition – TOF Protocol

Datasets were obtained in accordance with the TOF CMR acquisition protocol outlined in the *Methods* chapter. Briefly, balanced SSFP cines were acquired following localisers in planes including vertical long axis, four chamber, RV in and outflow, in addition to the short axis stack. The basal slice of the short axis stack was carefully aligned to contain ventricular myocardium in the end diastolic frame in both VLA and four chamber views. Cines were then acquired at 10mm intervals (7mm slice thickness, 3mm gap) until complete ventricular coverage to apex was included (typically 10-12 cines).

3.2.2 CMR Image Analysis

Individual datasets were anonymised before image analysis was performed with semi-automated software (CMRtools, Cardiovascular Imaging Solutions). Biventricular volume, mass and function were quantified on all 12 acquisitions by two independent, experienced observers blinded to previous analysis of the same patient and each other. Intra-observer variability was assessed by re-measuring anonymised scan sessions by the same first experienced observer with a minimum 6-month interval.

Ventricular analysis incorporated short-axis stack cines and 3-4 LV and RV long axis cines in total with visualisation of atrioventricular and outflow tract valves. The analysis encompassed three principle steps each applied for the LV and RV: delineation of the ventricular endocardial and epicardial borders in all slices in all cardiac phases, accounting for systolic descent and twist of the atrioventricular valve of the respective ventricle by tracking the valve motion on long axis cines (to correct for loss of systolic LV volume due to descent of the atrioventricular ring); and lastly blood pool thresholding. LV papillary muscles and major trabeculae (such as septal and septomarginal trabeculae) of the RV were excluded from the blood pool by means of threshold segmentation software, and were therefore considered part of myocardial mass. Ventricular mass was calculated from end-diastolic frames. End-diastolic (EDV) and end-systolic (ESV) volumes were obtained from ventricular volume/time curves generated from all frames of all slice slices acquired. Stroke volume (SV) was calculated as the difference between EDV and ESV, with ejection fraction (EF) calculated as SV/EDV .^{359, 360}

3.2.3 Statistical Analysis

Continuous variables are expressed as mean +/- standard deviation. The intra-observer reproducibility was determined as the mean +/- standard deviation of the signed differences between the repeated measurements of the same scan, whilst inter-observer reproducibility was expressed as the mean +/- standard deviation of the signed differences between both observers. The variability of volumetric, mass and function measurements were expressed as coefficient of variation (CoV) which was calculated by:

$$\text{CoV (\%)} = (\text{within-subject standard deviation/mean}) \times 100\%$$

in which within-subject standard deviation is $\sqrt{\sum (\text{difference between paired measurements})^2 / 2n}$.³⁶¹ The agreement within and between observers was also assessed and visualised with Bland-Altman plots.³⁶² Statistical analysis was performed with SPSS (IBM Statistics V.22). A two-sided p value <0.05 was considered statistically significant.

3.3 Results

Patients with rTOF included for reproducibility consisted of 7 males/5 females with mean age 34.8 ± 8.7 years. Patients had dilated right ventricles, normal left ventricular volumes with preserved biventricular ejection fractions: mean RVEDVi $127.1 \pm 34.1 \text{ ml/m}^2$, RVESVi $58.9 \pm 19.4 \text{ ml/m}^2$, RVSVi $68.2 \pm 17.3 \text{ ml/m}^2$, right ventricular ejection fraction (RVEF) $54.2 \pm 5.1\%$, RV indexed mass $55.3 \pm 14.4 \text{ g/m}^2$, LVEDVi $78.9 \pm 20.0 \text{ ml/m}^2$, LVESVi $29.2 \pm 11.9 \text{ ml/m}^2$, LVSVi $49.9 \pm 9.5 \text{ ml/m}^2$, LVEF $63.9 \pm 5.9\%$ and LV indexed mass $63.0 \pm 15.4 \text{ g/m}^2$.

The intra- and inter-observer reproducibility data are shown in *Table 3.1*. There was excellent intra-observer reproducibility of biventricular volumes, ejection fraction and mass with CoV ranging between 0.9 - 3.4%. As illustrated in the Bland-Altman plots, measures of ventricular volumes, mass and function were highly repeatable with narrow limits of agreement (*Figure 3.1*). There were good levels of agreement between observers for biventricular measures of volume and function, but greater variability was seen for quantification of ventricular mass.

		Right ventricle		Left ventricle	
		<u>Intra-observer</u>	<u>Inter-observer</u>	<u>Intra-observer</u>	<u>Inter-observer</u>
EDVi , ml/m^2	Mean $\pm$ SD	0.12 ± 1.52	0.86 ± 5.58	-0.10 ± 1.24	3.00 ± 2.93
	CoV (%)	0.9	3.1	1.1	3.8
ESVi , ml/m^2	Mean $\pm$ SD	-0.29 ± 2.49	-4.51 ± 5.23	0.04 ± 1.12	-1.20 ± 2.81
	CoV (%)	2.8	7.9	3.3	6.7
SVi , ml/m^2	Mean $\pm$ SD	0.22 ± 3.21	5.35 ± 6.70	0.08 ± 1.33	4.38 ± 2.52
	CoV (%)	3.4	8.5	1.6	7.4
EF , %	Mean $\pm$ SD	0.42 ± 2.19	4.00 ± 3.88	-0.08 ± 1.44	3.08 ± 2.97
	CoV (%)	2.9	7.4	1.5	5.0
Indexed mass , g/m^2	Mean $\pm$ SD	-0.11 ± 2.99	11.59 ± 7.92	0.49 ± 1.96	8.43 ± 6.02
	CoV (%)	3.2	20.8	2.1	11.7

Table 3.1: Results of intra-observer and inter-observer variability analysis.

When compared to previously published series in patients with rTOF, our findings are consistent in terms of greater inter-observer variability compared intra-observer variability (*Table 3.2*).

	Current Study Heng et al	Mooij et al		Winter et al	Luijnenburg et al	Walsh et al	Lu et al
No of subjects	12	60	20	29	35	40	53
Subjects grouped by diagnosis	12 rTOF	20 rTOF *	20 rTOF	Systemic RV	8 rTOF **	30 rTOF ***	53 rTOF
No of reproducibility subjects	12	60	20	20	35	40	29
CMR cine technique	SSFP	SSFP	SSFP	SSFP	SSFP	SSFP	SSFP
Inclusion of papillary muscles/trabeculations	×	Not stated	Not stated	✓ vs ×	×	×	Not stated
<u>Intra-observer CoV, %</u>							
<i>Right ventricle</i>							
EDVi/ EDV	0.9			6.1 vs 15.2	3.0	3.2	2.4
ESVi/ ESV	2.8			6.7 vs 7.5	3.9		3.8
SVi/SV	3.4			9.4 vs 14.4	4.8		
EFi/ EF	2.9			5.8 vs 7.7	3.5	2.9	7.2
Indexed mass/ mass	3.2				5.7		8.0/9.9 [†]
<i>Left ventricle</i>							
EDVi/ EDV	1.1				2.9		
ESVi/ ESV	3.3				6.8		
SVi/SV	1.6				4.1		
EFi/ EF	1.5				3.7		
Indexed mass/ mass	2.1				3.3		
<u>Inter-observer CoV, %</u>							
<i>Right ventricle</i>							
EDVi/ EDV	3.1	6.4	4.8	10.0 vs 13.5	4.0	3.3	12.1
ESVi/ ESV	7.9	13.0	10.9	12.7 vs 18.5	7.7		11.4
SVi/SV	8.5	11.8	10.6	12.2 vs 16.8	5.5		9.5
EFi/ EF	7.4	8.0	8.0	7.5 vs 12.8	5.5	3.5	11.1/13.0 [†]
Indexed mass/ mass	20.8	11.3	10.1		6.2		
<i>Left ventricle</i>							
EDVi/ EDV	3.8	3.6	3.1		4.3		
ESVi/ ESV	6.7	10.5	7.8		10.2		
SVi/SV	7.4	6.6	4.9		5.1		
EFi/ EF	5.0	5.8	4.8		3.9		
Indexed mass/ mass	11.7	5.3	4.9		6.0		

* Mixed cohort of 20 rTOF, 20 ASD/PAPVD, 20 normals ** Mixed cohort of 8 rTOF, 10 Fontan, 5 aortic stenosis, 4 systemic RV, 3 ASD/VSD, 3 pulmonary atresia, 1 pulmonary stenosis, 1 DORV *** Mixed cohort of 30 rTOF, 4ASD, 1 DORV, 3 pulmonary stenosis, 2 TAPVD/PAPVD[†] Diastole/systole

Table 3.2: Coefficients of variation in current study compared to selected publications assessing the right ventricle in congenital heart disease

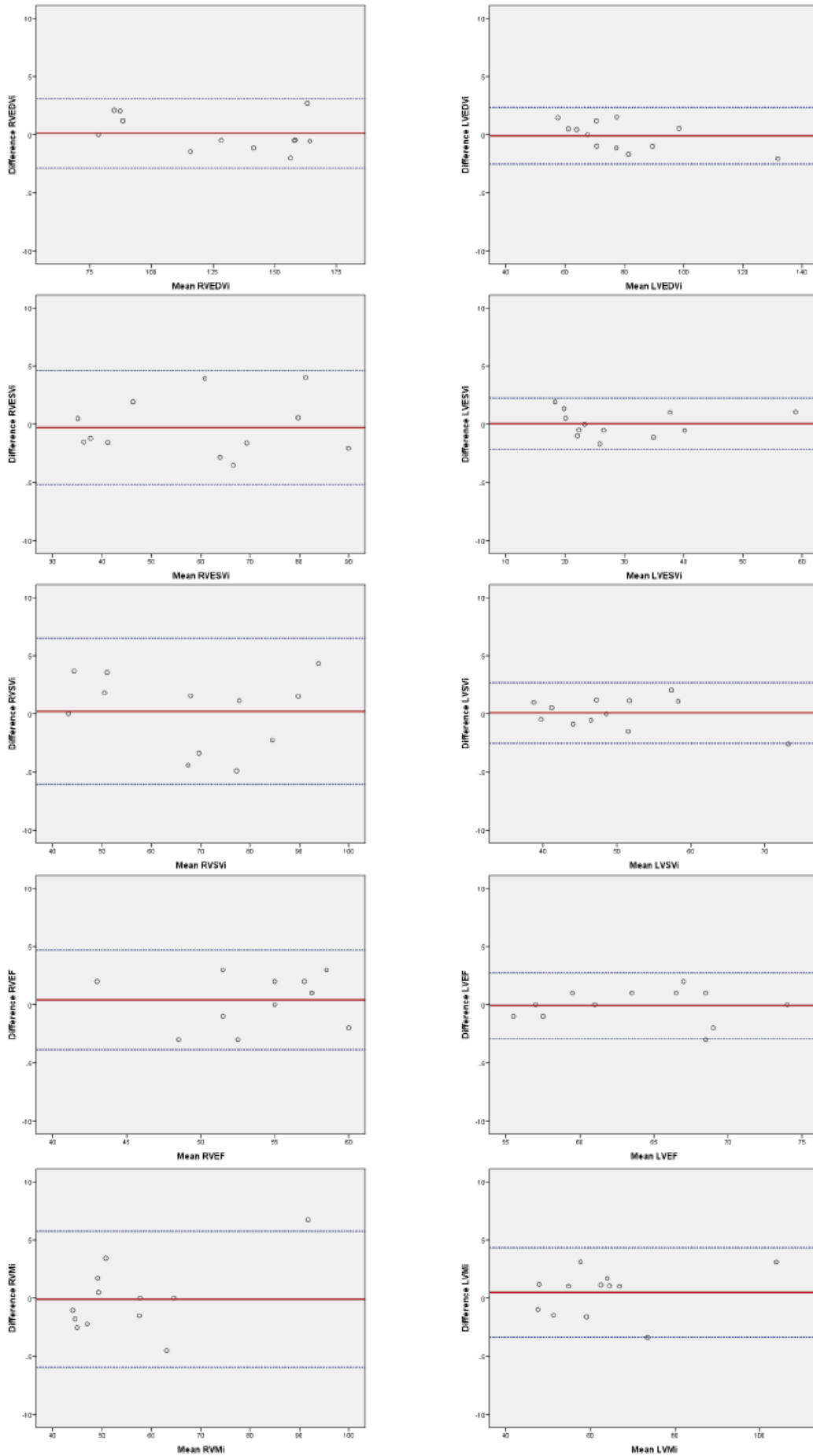


Figure 3.1: Bland-Altman plots of intra-observer mean differences for LV and RV parameters. The horizontal red line plots the mean difference and the dashed blue lines demarcate the limits of agreement ($= 2$ standard deviations) for each parameter.

3.4 Discussion

Our study demonstrates robust volumetric, mass and functional assessment of both ventricles with CMR, particularly when measured by the same observer. When considering measurements between observers, good levels of agreement were evident. The reproducibility of RV measurements did not differ significantly from LV measurements, albeit with greater variability of derived RV mass. Understanding the reproducibility of CMR measurements are pertinent, as they allow the clinician to distinguish between technique and operator-associated measurement differences in contrast to disease-related progression or change.

3.4.1 Right ventricular volumetric and functional analysis

Wider limits of agreement are recognised for CMR RV measurements between observers when compared to intra-observer repeated measures in normal, healthy volunteers (*Table 3.3*) and across a range of congenital heart disease pathology including rTOF (*Table 3.4*).^{363, 364} Our inter-observer reproducibility data shows the smallest variation in RVEDVi accompanied by larger variations in systolic related parameters of RVESVi, RVSVi and RVEF. These findings are in keeping with other groups,^{360, 364, 365} which may relate to smaller absolute values of ESV resulting in higher inter-observer differences when expressed as a percentage compared to EDV.

There are numerous variables that contribute towards measurement inaccuracies, which include image acquisition and post-processing factors. Targeting image acquisition parameters such as imaging planes and choice of CMR sequence has been undertaken as a means of reducing inter-observer variability. Various groups have explored alternative imaging planes to conventional short-axis slices for the quantification of ventricular volumes and function. Transaxial plane acquisitions and measurements in normal subjects were found to yield lower end-diastolic volumes and variability, but do not permit ventricular mass quantification because of poorly defined epicardial and endocardial myocardium.^{363, 366} A modified short-axis stack aligned to the RVOT has also been investigated to minimise variability.³⁶⁷ Different CMR sequences applied will contribute to discrepancies in measurements as gradient echo imaging underestimates volumes and overestimates ventricular mass when compared to balanced SSFP imaging. Lower intra- and inter-observer variability was evident with balanced SSFP imaging, in which there is clearer delineation of myocardial-blood interfaces.³⁶⁸ This should therefore be taken into account when interpreting RV measurements for clinical patient management and findings of clinical studies.

When considering post-processing issues, variations can arise due to indistinct borders between the RVOT and main pulmonary artery, as well as RA and RV at the atrioventricular junction. Inconsistencies in delineating ventricular inflow and outflow boundaries can result in errors. Measurement guidelines can be implemented to minimise subjectivity in identifying the pulmonary valve annular region, such as ascribing to the hypothetical annular line perpendicular to the long axis of the RVOT, utilising the aortic intercoronary commissure which always faces the native pulmonary annulus, and designating the artefact edge as the annular line of a pulmonary valve prosthesis. The atrioventricular and outflow tract valve planes should be defined in two orthogonal long-axis cines to minimise observer error.³⁶⁹

3.4.2 Right ventricular mass measurements

Right ventricular mass quantification is of emerging relevance in patients with rTOF, with RV mass-to-volume ratio ($\geq 0.3\text{g/ml}$) predicting adverse outcomes of death and sustained ventricular tachycardia.⁶⁸ Diastolic right ventricular indexed mass (RVMi) has been found to be associated with decreased general health scores, with elevated RVMi suggested as a marker of adverse remodelling secondary to residual valvular lesions, ventricular dilatation or cardiac dysfunction.³⁷⁰ Within our study, the mean RVMi was $55.3 \pm 14.4\text{g/m}^2$, with inter-observer CoV 3.2% and inter-observer CoV 20.8%. Our limits of agreement for RV mass analysis are comparable to previous published series. The comparatively wide variation in RVMi highlights potential pitfalls of interpreting mass-related outcomes from clinical studies, and may mandate institution specific thresholds for RV mass-related outcome measures, such as RV mass-to-volume ratio. We note that our RVMi indices are higher than those previously reported, but in line with our institution's normative reference values.^{68, 360, 370} This may be due to the software analysis packaged used, in addition to careful and explicit inclusion of RV trabeculae into myocardial mass.

The accuracy of RV mass measurements will be affected by suboptimal visualisation of the thin sub-pulmonary RV wall in diastole, which may be limited by image resolution and partial volume effects. In theory, clearer RV myocardial borders may be seen in systole, which could improve contour reliability. However, diastolic versus systolic measures of RVMi have not conclusively favoured one approach over the other.^{364, 370, 371} Although the principle of conservation of mass means systolic and diastolic mass measurements should be identical, differences have been documented. Diastolic quantification may over-estimate RV mass, whereas systolic quantification may pose difficulties in demarcating the blood-endocardial border during wall thickening.

3.4.3 Influence of right ventricular trabeculae

To date, there has been no real consensus within the CMR community with regards to standardising the inclusion or exclusion of RV trabeculae and papillary muscles in CMR image analysis. Indeed, numerous clinical studies with CMR-based outcome measures in rTOF patients have not explicitly outlined their approach in RV volume measurements.^{32, 35, 372} Whilst the impact of trabeculae is potentially minimal in normal subjects,³⁷³ the exclusion of RV trabeculae from the blood volume in patients with systemic RV and rTOF have been shown to result in significant differences in RV measurements, with lower RVEDVi together with increased RVESVi, RVEF and RVMi.³⁷⁴⁻³⁷⁶

The impact of excluding trabeculae appears to vary with different congenital heart pathologies and the extent of RV hypertrophy present. Delineating smaller trabeculae may be more challenging in end-diastole when they are less 'compacted' and therefore less visible than in end-systole. This could therefore introduce inadvertent inaccuracies. Narrower limits of agreement have been demonstrated for inter-observer reproducibility of RV parameters when trabeculae are excluded from the blood volume in patients with rTOF,^{375, 376} whereas better reproducibility was seen in patients with systemic RV when trabeculae are included.³⁷⁴ Furthermore, there are institutional differences in defining the extent to which trabeculae are included or excluded from the blood volume,^{369, 375} which may be overcome by consensus training.³⁷⁷ An additional source of variation to measurements may arise from the image analysis methodology. Manual planimetry of RV endocardial borders in a series of patients with systemic RVs has been shown to be less reliable than semi-automatic software segmentation.³⁷⁴

3.4.4 Limitations

The conservative sample size may limit generalizability of our findings, although our results are comparable with previous reproducibility data from our institution. Interscan reproducibility was not assessed as part of this study as it has been undertaken previously.³⁷⁸ There are additional sources of potential variability which cannot be fully addressed that include patient factors such as poor breath-holds, arrhythmia, and patient motion; as well as technical issues, each of which may contribute further to error. In vivo validation of RV mass measurements was not possible in the absence of pathology specimens.

3.5 Conclusions

CMR is an accurate and reliable method for serial assessment of biventricular volumes and function. It remains the reference method for ventricular quantitative assessment in patients with rTOF and congenital heart disease. We report superior intra-observer reproducibility

when compared to inter-observer findings, which suggests that experienced single observer measurements would be preferable for CMR-based research or patient outcomes, particularly with more challenging ventricular morphologies. This would not only improve measurement precision, but also importantly reduce the sample size needed for otherwise often under-powered congenital heart disease research. Reference normative ranges should be specific to institutions, applying a standardised approach to LV papillary muscles and RV trabeculae in image analysis to contextualise CMR scan findings. Variations within and between observers remain potentially problematic for RV mass measurements, which should be taken into consideration for data interpretation.

Authors	No of normals	RV trabs (Included/ Excluded/ Not stated)	Intra RVEDV CoV	Intra RVESV CoV	Intra RVEF CoV	Intra RV Mass CoV	Inter RVEDV CoV	Inter RVESV CoV	Inter RVEF CoV	Inter RVMi CoV
Pattynama et al <i>MRI 1995</i>	40 scans in 2 normal males	Included 10 spin echo, 10 cine-MR	Variance component analysis performed, with intra-observer variability contributing towards the majority of observed variance.							
Lorenz et al <i>JCMR 1999</i>	75	Excluded SSFP	Pooled inter-observer data mean difference 0.7±5.9%							
Alfakih et al <i>JMRI 2003</i>	20 (axial vs. SAX)	LV papillary excluded, trabs included SSFP	Axial SDD 5.5 SAX SDD 6.9	Axial SDD 4.2 SAX SDD 6.5	Axial SDD 2.8 SAX SDD 4.4	Not measured	Axial SDD 8.0 SAX SDD 9.2	Axial SDD 4.8 SAX SDD 8.3	Axial SDD 2.9 SAX SDD 5.5	Not measured
Beygui et al <i>Int J Card Imaging 2004</i>	15 (+ 3 AS and 7 MI patients)	Excluded GRE	6.6	8.1	6.7	8.1	7.9	9.6	8.3	8.9
Grothues et al <i>Am Heart J 2004</i>	20 (Total cohort of 60, with further 20 HF, 20 LVH)	Papillary excluded FLASH Interstudy repro assessed	6.2 (4.2- 7.8) N: 4.2, HF 7.8, LVH 6.2	14.1 (8.1- 18.1) N: 8.1, HF 14.5, LVH 18.1	8.3 (4.3- 10.4) N: 4.3, HF 10.4, LVH 10	8.7 (7.8- 9.4) N: 7.8, HF 9.0, LVH 9.4				
Hudsmith et al <i>JCMR 2005</i>	108	Papillary excluded SSFP	9.0		5.3		9.6		10.7	
Maceira et al <i>EHJ 2006</i>	120 (10 for repro)	Excluded	3.6	6.5	4.0	5.7				

Table 3.3: Reported ranges of RV reproducibility in normal subjects. SDD = standard deviation of the difference^{360, 363, 379-383}

Authors	N	Patient Group	RV trabs (Included/ Excluded/ Not stated)	Intra RVEDV CoV	Intra RVESV CoV	Intra RVEF CoV	Intra RV Mass CoV	Inter RVEDV CoV	Inter RVESV CoV	Inter RVEF CoV	Inter RVMi CoV
Helbing et al <i>Am Heart J</i> 1995	43	8 rTOF, 6 ASD, 7 Senning, 22 normals	Excluded GRE Transaxial	% Error							
				N: 3.5±1.2 CHD: 4.2±2.4	N: 4.8±5.6 CHD: 7.0±6.9	N: 4.3±2.1 CHD: 4.5±4.3		N: 5.0±4.5 CHD: 4.2±2.7	N: 10.7±7.8 CHD: 9.3±11.2	N: 3.4±3.0 CHD: 4.9±6.1	
Mooij et al <i>JMRI</i> 2008	60	20 normals, 20 ASD +/- PAPVD, 20 rTOF	Not stated SSFP SAX					ALL: 6.4 N: 6.6 ASD: 6.4 TOF: 4.8	ALL: 13.0 N: 16.3 ASD: 12.1 TOF: 10.9	ALL: 8.0 N: 8.3 ASD: 6.2 TOF: 8.0	ALL: 11.3 N:13.4 ASD: 9.0 TOF: 10.1
Fratz et al <i>Am J Card</i> 2009	46	rTOF Axial vs SAX	Excluded SSFP SAX	Bland-Altman differences, limits of agreement and variance quoted. Smaller intra and inter-observer variance of biventricular volumes with axial datasets compared to SAX datasets.							
Winter et al <i>JCMR</i> 2008	29	Systemic RV	Included vs excluded SSFP SAX	6.1	6.7	5.8		10			
Luijnenburg et al <i>Int J Card Imag</i> 2010	35	8 rTOF †	Excluded SSFP SAX	3	3.9	3.5	5.7	4.0	7.7	5.5	6.2

† Includes further 5 aortic stenosis, 1 ASD, 2 VSDs, 10 Fontan, 4 systemic RVs, 1 DORV, 3 pulmonary atresia with VSD, 1 pulmonary stenosis

Table 3.4: Reported range of reproducibility in patients with congenital heart disease.^{364-366, 374, 384}

Authors	N	Patient Group	RV trabs, CMR sequence, slice orientation	Intra RVEDV CoV	Intra RVESV CoV	Intra RVEF CoV	Intra RV Mass CoV	Inter RVEDV CoV	Inter RVESV CoV	Inter RVEF CoV	Inter RVMI CoV
Walsh et al <i>Pediatr Radiol</i> 2011	40	30 rTOF, 4 ASD, 1 DORV, 3 PS, 2 TAPVD/PAPVD	Partially excluded ‡ SSFP SAX	3.2		2.9		3.3		3.5	
Blalock et al <i>JMRI</i> 2013	30	rTOF (x2 scans/pt)	SSFP SAX					ICC 0.986	ICC 0.928	ICC 0.784	ICC 0.576
Freling et al <i>Int J Cardiovasc Imag</i> 2013	65	rTOF (25 studies for repro)	Include vs exclude SSFP SAX	Coefficients of agreement calculated for both methods: High observer agreement for all measurements except RV mass. Higher inter-observer agreement with exclusion of trabeculae compared to inclusion.							
Driessen et al <i>Int J Cardiovasc Imag</i> 2014	80	20 rTOF, 20 PHT, 20 art switch, 20 atrial switch, 20 controls (25 scans for repro)	Include vs exclude SSFP SAX	ICC 0.990/ 0.985	ICC 0.982/ 0.969	ICC 0.965/ 0.954	ICC 0.983/ 0.990	ICC 0.981/ 0.987	ICC 0.970/ 0.974	ICC 0.934/ 0.934	ICC 0.965/ 0.992
Lu et al <i>Am J Cardiol</i> 2014	53	rTOF (29 for inter-observer repro)	Not stated SSFP SAX	2.4	3.8	7.2	Diastole 8.0 Systole 9.9	12.1	11.4	9.5	Diastole 11.1 Systole 13.0

‡ 'Floating myocardium' as well as moderator band included in EDV.

Table 3.4 (Continued): Reported range of reproducibility in patients with congenital heart disease.^{369-371, 375, 376}

Chapter 4 Early and Midterm Cardiac Remodelling Following Surgical Pulmonary Valve Replacement in Adults with Repaired Tetralogy of Fallot: A Prospective Cardiovascular Magnetic Resonance and Clinical Study

For the cardiac remodelling work presented in this chapter, an introduction and literature review were presented in the Introduction *section 1.1.5*. Each of the Chapter 4 conclusions is reiterated in the overall setting of the thesis Conclusions (Chapter 8).

Prospective study recruitment had taken place between January 2005 and December 2009. My exact role in the study was to organise midterm follow-up and study protocol investigations for patients who had yet to complete study follow-up from October 2012 – October 2013. I participated in CMR data acquisition for follow-up patients, before collating study data from all three time-points for each patient. I measured clinically relevant parameters from the study investigations where applicable, collected follow-up outcomes and subsequently analysed the data. I drafted and then made manuscript revisions of the study findings, which have been submitted for consideration of peer-reviewed publication.

4.1 Introduction

Relief of right ventricular outflow tract obstruction at the time of surgical repair of tetralogy of Fallot frequently leads to pulmonary regurgitation (PR) with its lifelong adverse consequences. Though PR is generally well-tolerated in the short-term; with time, it leads to progressive right ventricular (RV) dilatation, clinical decline (exercise intolerance, arrhythmia, and heart failure), and an increased risk of sudden cardiac death.^{1, 6, 9, 19, 385}

Pulmonary valve replacement (PVR) is undertaken to mitigate these late effects, with reported symptomatic benefits, in conjunction with improved RV volumes and left ventricular (LV) function.^{19, 386, 387} Whilst the overall haemodynamic benefits of PVR are evident with broad consensus to propose surgery before clinical deterioration and/or overt symptoms develop, uncertainties remain regarding the optimal timing of PVR, as well as the timing and nature of reverse ventricular remodelling.

The study aim was to assess prospectively immediate and midterm cardiac remodelling following surgical PVR using cardiovascular magnetic resonance (CMR).

4.2 Methods

4.2.1 Patient population and study design

Patients with repaired tetralogy of Fallot (rTOF) scheduled for elective PVR aged ≥ 16 years with no contra-indications to CMR and estimated glomerular filtration rate ≥ 30 ml/min under the care of the Adult Congenital Heart Disease Centre, Royal Brompton Hospital, UK were approached for study inclusion between January 2005 and December 2009. Individualised recommendations for PVR were made after multi-disciplinary reviews. During this period, our group started applying published preoperative RV threshold volume cutoffs of $RVEDVi \geq 150 \text{ ml/m}^2$ and $RVESVi \geq 80 \text{ ml/m}^2$ amongst other clinical parameters (onset of arrhythmia, impaired exercise capacity, or other symptoms) for referral to PVR. The study complies with the declaration of Helsinki, with local ethics committee approval. All participants provided study-specific written informed consent.

CMR (1.5T Siemens) was performed at three time-points: (1) pre-operatively (2) immediate post-operatively prior to hospital discharge (3) midterm post-operatively (minimum 12 months) after surgery, the latter, as per routine clinical follow-up. Baseline demographics of study participants and clinical observations were obtained pre-operatively and at midterm follow-up. Echocardiography was performed by a single operator for assessment of RV restrictive physiology with laminar anterograde flow in the pulmonary artery in late diastole present throughout the respiratory cycle.⁵⁵ Cardiopulmonary exercise testing for measurements including peak oxygen uptake (peak VO_2) and ratio of minute ventilation to carbon dioxide production (VE/VCO_2) was carried out with the modified Bruce protocol to symptom-limitation. Tests were excluded from analysis if the respiratory quotient value was < 1 .

Reverse RV remodelling was defined as the reduction in non-invasively measured CMR RV volumes post PVR, widely regarded as a beneficial positive outcome of surgery. The primary endpoint was normalisation of RV volumes at midterm follow-up as defined by $RVEDVi \leq 108 \text{ ml/m}^2$ and $RVESVi \leq 47 \text{ ml/m}^2$ (normal RV volume range established in our institution employing the same method).³⁶⁰ Secondary endpoints were percentage change in $RVEDVi$ and $RVESVi$, as well as myocardial fibrosis burden assessed by late gadolinium enhancement (LGE) CMR as a predictor of post-surgical remodelling.

4.2.2 CMR protocol

All participants underwent standardised full CMR assessment, including short-axis steady-state free-precession cine stack from the atrioventricular ring to the apex. Biventricular volumes, mass and function were derived with semi-automated software (CMRtools, Cardiovascular Imaging Solutions). A single corelab observer completed all volumetric measurements on anonymised scans. Twelve study CMR scans were randomly selected and re-measured by the same experienced observer (with minimum 6-month interval) as well as a second blinded experienced observer for ventricular volume reproducibility assessment. The LV papillary muscles and major trabeculations of the RV were carefully excluded from the blood pool and considered part of the myocardial mass.³⁷⁵ The maximal right atrial area index (RAAi) was recorded from a four chamber view. Right atrial dilatation is defined as $RAAi \geq 15 \text{ cm}^2/\text{m}^2$.³⁸⁸ Akinesia was defined as non-contractile myocardium which did not thicken in systole. $RAAi > 16 \text{ cm}^2/\text{m}^2$ and RVOT akinetic length $> 30 \text{ mm}$ were also recorded given their predictive value.⁶⁴ Pulmonary regurgitant fraction was calculated from phase-velocity mapping in a plane transecting the main pulmonary trunk. Corrected RVEF was calculated by dividing the net pulmonary flow (pulmonary forward flow minus the regurgitant flow) by the RVEDV.³⁴

LGE imaging was acquired by a single operator at pre-operative and midterm post-operative time points as previously described.^{61, 389} Non-specific LGE at RV/LV insertion points (present in 98%) was excluded.⁶¹ LGE was present if bright signal within myocardium was visualised with good quality images in the same region on phase swap or crosscuts. The maximum linear extent of RV outflow tract (RVOT) LGE was measured. The LGE findings of 12 scans were re-scored blind to previous analysis (with minimum 6-month interval).⁶¹

4.2.3 Quantification of RV resected during RVOT reconstruction

Study participants donated resected tissue necessarily removed at the time of surgery (typically in the RVOT). The area of resected RVOT tissue was measured against carefully aligned linear scales to include maximum specimen length and height to derive total tissue area prior to fixation.

4.2.4 Statistical Analysis

Data normality was assessed by Kolmogorov-Smirnov analysis. Results are reported as mean $\pm$ standard deviation (SD) or median (IQR) according to data distribution. Groups were assessed by paired Student t tests or Wilcoxon test as appropriate. Categorical variables were compared by Fisher's exact test. Correlations were assessed by Pearson's and

Spearman correlation tests. In view of the range of time intervals at midterm follow-up, a mixed effects model was applied to adjust for the effects of time on derived CMR parameters. Receiver-operating characteristic (ROC) curves were calculated to determine cutoff values for normalisation of RV volumes. For the assessment of intra- and inter-observer reproducibility, variability was expressed as the coefficient of variation (%), derived from the within-subject standard deviation divided by the mean, multiplied by 100%. The within-subject standard deviation is $\sqrt{\sum (\text{difference between paired measurements})^2 / 2n}$.³⁶¹ Intraclass correlation coefficients were also assessed. Statistical analysis was performed with SPSS (IBM Statistics V.22) and R (version 3.1.2). A two-sided p value <0.05 was considered statistically significant.

4.3 Results

4.3.1 Patient Characteristics

Fifty-seven patients with rTOF were prospectively recruited (age 35.8 ± 10.1 years, age at repair $4.0[0.3-36.5]$ years, 38 male) with baseline clinical and surgical characteristics as listed (*Table 4.1*).

4.3.2 Clinical data, Exercise tolerance, QRS duration and Cardiothoracic ratio

There were two early post-operative deaths (both from RV failure at day 3) and one sudden death at 34 days. Patients who died had pre-operative RVEDVi $153.0 \pm 24.3 \text{ ml/m}^2$, RVESVi $78.2 \pm 6.8 \text{ ml/m}^2$, RVEF $47.0 \pm 7.8\%$, LVEF $58.7 \pm 8.1\%$, PRF $39.7 \pm 3.8\%$, RV LGE score 7.3 ± 1.2 and RVOT scar length $45.3 \pm 9.5 \text{ mm}$. Their exercise capacity was impaired with peak VO_2 $16.2 \pm 10.5 \text{ ml/kg/min}$ ($50.7 \pm 10.7\%$ predicted).

Seventy-four percent of patients were symptomatic at baseline (predominant symptoms of dyspnoea/fatigue 56%, documented arrhythmia 11%, palpitations 5% and endocarditis 2%), but only 2% at midterm follow-up after PVR. Peak and percent predicted VO_2 ($n=27$ paired data with $\text{RER} > 1$) remained unchanged (pre-PVR 23.7 ± 5.8 vs post-PVR $24.3 \pm 7.8 \text{ ml/kg/min}$, $p=0.67$ and 73.1 ± 17.9 vs $73.4 \pm 16.7\%$, $p = 0.93$); as did VE/VCO_2 slope (pre-PVR 34.3 ± 13.2 vs post-PVR 34.0 ± 6.9 , $p = 0.92$). Right bundle branch block morphology on ECGs with baseline QRS duration of $155 \pm 21 \text{ ms}$ did not change post-PVR ($p=0.73$). Cardiomegaly ($\text{CTR} \geq 0.50$) was noted in 81% (46 patients) preoperatively, and reduced significantly post-PVR ($p < 0.01$, *Table 4.2*). Restrictive physiology was documented in 27% of patients pre-PVR and 6% of patients at midterm follow-up (*Table 4.2*).

4.3.3 Cardiovascular Magnetic Resonance

Immediate post-operative CMRs were performed at 6 days (IQR 5-7.5, n=46) and midterm CMRs at 3.0 years (IQR 1.0-4.1, n=47) after index PVR. Post-PVR CMRs were not performed due to implantable cardioverter defibrillators (n=6), death (n=3) and move overseas (n=1).

Patient Population (n=57)

Age, years	35.8 ± 10.1
Gender (Male/Female)	38/19
rTOF with associated lesions *	5

Previous palliative operations, n (%) 24 patients with 27 procedures (42)

Blalock-Taussig shunt	17 (63)
Waterston shunt	6 (22)
PDA ligation	1 (4)
Brock procedure	2 (7)
Open pulmonary valvotomy	1 (4)
Restrictive RV physiology at echo, n (%)	21 (37%)

Characteristics of initial repair

Age, years	5.5 ± 5.6
Transannular patch (%)	27 (47)
RVOT patch (%)	17 (30)
RV to PA conduit (%)	7 (12)
Surgical details not known (%)	6 (11)

Pre-operative clinical status

Heart rate, bpm	72 ± 9
Systolic blood pressure, mmHg	116 ± 13
Diastolic blood pressure, mmHg	71 ± 9

Surgical PVR characteristics

Predominant lesion for PVR - PR/PS/Both	53/3/1
Time from repair to PVR, years	31.2 (23.7-36.4)
Length of hospital stay, days	10.5 ± 11.8
Beating heart procedures, n (%)	28 (49)
Bypass time, min	117 ± 62
Cross-clamp time, min	36 ± 42
Temperature, °C	31 ± 3

Prosthesis

Homografts, n (%)	11 (19) pulmonary, 18 (32) aortic
Homograft size, mm (range)	24 ± 1.4 (22-27)
Xenografts, n (%)	28 (49)
Porcine	
• Mosaic	15 (26)
• Hancock	7 (12)
Bovine pericardial	
• Carpentier-Edwards Perimount	5 (9)
• Mitroflow	1 (2)
Xenograft size, mm (range)	25 ± 1.5 (23-29)

Concomitant procedures at PVR, n(%)

Branch pulmonary augmentation	4 (7)
Pulmonary artery patch	16 (28)
RVOT patch	23 (40)
RVOT muscle resection	19 (33)
RVOT plication	21 (37)
Residual VSD closure	3 (5)
Tricuspid valve surgery	9 (16)
Aortic valve replacement	1 (2)
Atrial septal defect/patent foramen ovale closure	1 (2)
Surgical ablation	2 (4)
Additional surgical procedures **	4 (7)

Table 4.1: Baseline characteristics and PVR surgical details.

* 1 pulmonary atresia, 2 absent pulmonary valve, 1 atrioventricular septal defect, 1 patent ductus arteriosus

** Additional surgical procedures included repair of RCA fistula (n=1), repair of perforated right coronary cusp of aortic valve (n=1), right atrial plication (n=1), and the insertion of ventricular assist device and establishment of extracorporeal membrane oxygenation (n=1)

† RV/LV insertion points not included

EDVi, indexed end-diastolic volume; EF, ejection fraction; ESVi, indexed end-systolic volume; PA, pulmonary artery; LAAi, indexed left atrial area; LGE, late gadolinium enhancement; LV, left ventricle; Mi, indexed mass; PDA, patent ductus arteriosus; PR, pulmonary regurgitation; PS, pulmonary stenosis; PVR, pulmonary valve replacement; RAAi, indexed right atrial area; RV, right ventricle; RVOT, right ventricular outflow tract; SV, stroke volume

	Pre-PVR	Immediate Post-PVR	Midterm Post-PVR	p value	p value	p value
	(1)	(2)	(3)	(1) vs (2)	(2) vs (3)	(1) vs (3)
NYHA Class I/II/III/IV, n	15/40/2/0		43/1/0/0 *			<0.01
ECG QRS duration, ms [†]	155±21		154±21			0.73
Cardiothoracic ratio [‡]	0.55±0.06		0.53±0.06			<0.01
CMR						
RVEDVi, ml/m ²	156.1±41.9	104.9±28.4	104.2±34.4	<0.01	0.65	<0.01
RVESVi, ml/m ²	74.9±26.2	57.4±22.7	50.5±21.7	<0.01	<0.01	<0.01
RVSVi, ml/m ²	81.1±21.6	47.6±11.0	53.6±15.6	<0.01	<0.01	<0.01
RVEF, %	52.9±7.7	46.4±8.8	52.4±7.0	<0.01	<0.01	0.72
cRVEF, %	26.9±7.1	40.5±11.3	47.5±12.6	<0.01	<0.01	<0.01
RVMi, g/m ²	64.2±13.8	55.4±15.2	50.9±11.5	<0.01	<0.01	<0.01
RV mass/volume, g/ml	0.42±0.08	0.56±0.11	0.51±0.10	<0.01	<0.01	<0.01
LVEDVi, ml/m ²	80.2±18.5	74.7±17.3	84.2±19.4	<0.01	<0.01	0.03
LVESVi, ml/m ²	33.3±11.7	29.1±11.3	32.6±11.4	<0.01	0.03	0.51
LVSVi, ml/m ²	46.9±10.3	45.6±9.6	51.6±10.9	0.21	<0.01	<0.01
LVEF, %	59.4±7.6	61.4±8.4	61.9±6.8	0.35	0.39	<0.01
LVMi, g/m ²	68.6±15.1	65.3±16.4	67.9±15.1	0.15	0.59	0.49
Pulmonary regurgitant fraction, %	41.3±11.3	2.4±4.5	3.8±6.8	<0.01	0.04	<0.01
RAAi, cm ² /m ²	15.2±3.4	13.2±3.2	13.8±3.6	<0.01	0.02	<0.01
LAAi, cm ² /m ²	10.4±2.4	9.1±2.2	10.2±2.5	<0.01	<0.01	0.64
Akinetic RVOT length, mm	40.7±12.8	30.2±11.0	28.8±11.0	<0.01	0.68	<0.01
RV LGE score (no insertion points)	5.3±2.6		5.5±2.7			0.10
RVOT LGE score	3.3±1.5		3.2±1.3			0.35
RVOT scar length, mm	34.0±18.4		30.5±13.2			0.12

Echo	(n=48)	(n=53)	(n=54)			
RVOT gradient, m/s	2.18±1.00	2.04±0.51	2.06±0.60	0.87	0.76	0.44
Estimated RVSP, mmHg	45.0±25.3	35.6±10.9	39.2±9.9	0.08	0.29	0.18
Grade of Pulmonary Regurgitation						
None/mild (%)	1(2)	52(98)	53(98)			
Moderate/severe (%)	47(98)	1(2)	1(2)			
Grade of Tricuspid Regurgitation						
None/mild (%)	38(79)	50(94)	44(81)			
Moderate/severe (%)	10(21)	3(6)	10(19)			
Restrictive physiology (%)	14(29)	7(13)	8(15)	<0.01	1.00	<0.01

Table 4.2: ECG, cardiothoracic ratio, echo and CMR data at baseline and after PVR.

* 3 patients deceased [†] QRS≥180ms: n=7 pre-PVR, n=7 post-PVR [‡] CTR≥0.50: n=46 pre-PVR, n=34 post-PVR

**Patients with extensive LGE (n=13 top quartile RV LGE ≥ 7, range 7-13) were older (top quartile 43.1 ± 8.5 vs 33.6 ± 9.5 years, p<0.01), more likely to have restrictive RV physiology (top quartile n=7, 54% vs n=7, 16%; p=0.02), had worse RVEF (top quartile 49 ± 6 vs 53 ± 8%; p=0.04), larger indexed right atrial areas (top quartile 17 ± 3 vs 15 ± 3 cm²/m²; p=0.01) and longer RVOT akinetic lengths (top quartile 47 ± 13 vs 38 ± 12mm; p=0.03).

EDVi, indexed end-diastolic volume; EF, ejection fraction; ESVi, indexed end-systolic volume; LAAi, indexed left atrial area; LGE, late gadolinium enhancement; LV, left ventricle; Mi, indexed mass; NYHA, New York Heart Association; RAAi, indexed right atrial area; RV, right ventricle; RVOT, right ventricular outflow tract; SV, stroke volume

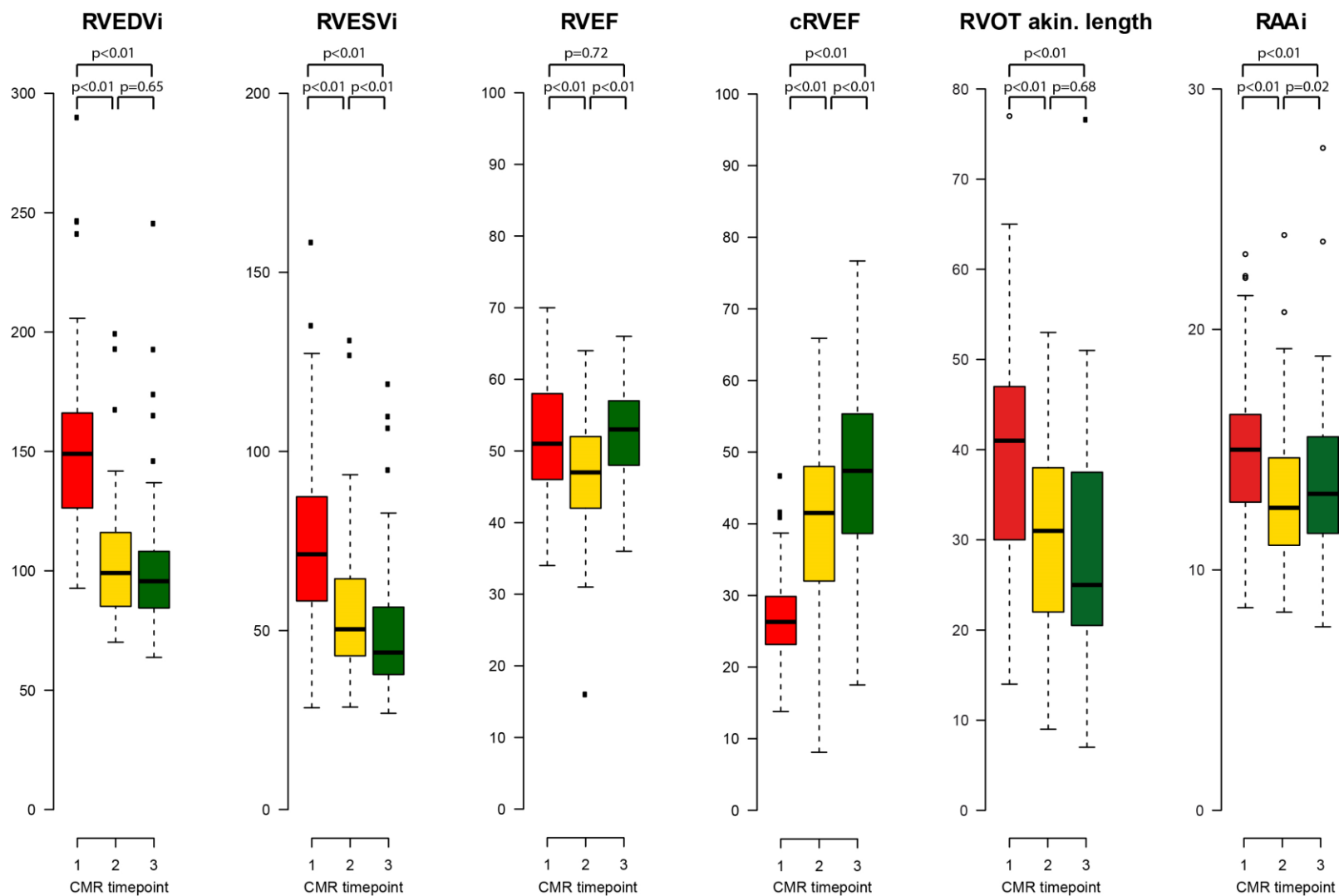


Figure 4.1: Right heart CMR parameters at study time points (red – pre-PVR, yellow – immediate post-PVR within 14 days, green – at midterm follow-up post-PVR).

4.3.4 Right ventricular and atrial reverse remodelling

Right ventricular volumetric and right atrial reverse remodelling data are summarised in *Table 4.2* and *Figure 4.1*. In the immediate post-PVR period (median 6 days) there was a 32% reduction in RVEDVi ($p<0.01$), which was sustained at midterm follow-up. RVESVi promptly decreased by 23% ($p<0.01$) and decreased further by midterm follow-up ($p<0.01$) to 32% lower than the baseline (*Table 4.2* and *Figure 4.1*). Normalisation of RV volumes (both RVEDVi and RVESVi) was achieved in 33 patients (70%) at midterm follow-up; cut-off predictive values for normalisation were preoperative RVEDVi 158ml/m² (sensitivity 86%, specificity 79%) and RVESVi 82ml/m² (sensitivity 86%, specificity 88%), (*Figure 4.2*).

RVEF decreased immediately by 12% following PVR but returned back to baseline, pre-operative levels by midterm follow-up. RV volumetric changes were accompanied by a regression of indexed RV mass (RVMi) at both post-PVR time points ($p<0.01$). RAAi decreased post-PVR with corresponding decrease in the prevalence of right atrial dilatation ($p<0.01$, for both *Table 4.2* and *Figure 4.1*).

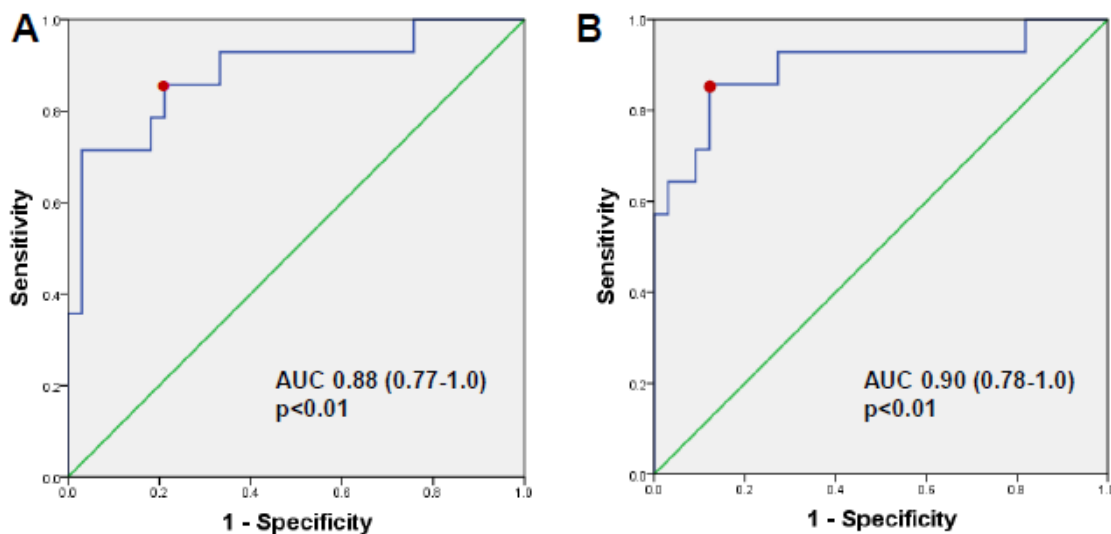


Figure 4.2: Receiver-operating characteristic curves illustrating threshold value of (A) pre-PVR RVEDVi of 158ml/m² for normalisation of RVEDVi (B) pre-PVR RVESVi of 82ml/m² for normalisation of RVESVi. The 95% confidence intervals for area under curve are quoted within the parentheses.

4.3.5 RVOT akinetic regions and RV LGE extent

The area of surgically resected RVOT quantified on linear scales did not correlate with reduction in RV volumes or functional change at midterm follow-up (linear mixed effects model; $p>0.05$). In contrast, RVOT akinetic length on CMR decreased immediately post-PVR and was sustained at midterm post-PVR ($p<0.01$, *Table 4.2* and *Figure 4.1*). Pre-PVR RVOT

akinetic length was modestly associated with change in QRS duration after PVR ($r = 0.44$, $p < 0.01$).

All patients had RV LGE (median RV score 5, range 1-13) at sites of previous surgical intervention (RVOT, ventricular septal defect regions), whereas RV LGE “remote” from surgical sites was present in 13 (23%) patients only. Four patients had non-apical vent related LV LGE (median LV LGE score 9.5, range 7-20). Total RV LGE scores did not change post PVR ($p = 0.10$ and $p = 0.35$), nor did linear extent of RVOT scar ($p = 0.12$, *Table 4.2*). There was an inverse relationship between pre-PVR linear RVOT scar length and change in biventricular ejection fractions, (that is $\Delta EF = \text{midterm post-PVR EF} - \text{pre-PVR EF}$) with $\Delta RVEF$ $r = -0.40$, $p < 0.01$ and $\Delta LVEF$ $r = -0.41$, $p < 0.01$.

4.3.6 Left ventricular volumetric data

Although surgical intervention was confined to the right heart, changes in LV volumes and function were evident (see *Table 4.2*). LVEDVi and LVESVi reduced immediately post-PVR and increased thereafter with LVEDVi increasing above its preoperative value. LVEF increased post-PVR above pre-PVR baseline (*Table 4.2*). There was no overall change to mean LAAi with surgery.

4.3.7 Variability in CMR measurements

Intra- and inter-observer reproducibility of indexed RV measurements was as follows: EDV/ESV/SV/EF/Mass 0.9%/3.1%, 2.8%/7.9%, 3.4%/8.5%, 2.9%/7.4%, and 3.2%/20.8%, respectively. The intraclass correlation coefficients for LGE scores were 0.94 for the RV and 1.0 for the LV, comparable with the high reproducibility previously reported.⁶¹

4.4 Discussion

Our data demonstrates for the first time that the majority of improvement in RV volumes after surgical PVR, previously shown at ≥ 6 months after surgery, takes place very early within days from restoring pulmonary valve competency and is sustained thereafter. The immediate effect of PVR on RV volumes is related to the acute reduction in volume overload. Furthermore, in the evidence presented here, RVESVi continues to decrease suggesting ongoing functional RV recovery and underscores RVESVi as a marker of myocardial function. Increases in LVEDVi and LVEF at midterm follow-up, whilst modest are indicative nevertheless of a positive ventricular interdependence effect after PVR and improved left heart haemodynamics. Changes in biventricular volumes and function in the immediate post-PVR were also observed, which could reflect the effects of cardiopulmonary bypass.

4.4.1 Evidence of time dependent reverse right ventricular systolic volume remodelling

Most of the decrease in RVEDVi took place in the immediate postoperative period (32%) compared to a mere further 2% reduction at midterm follow-up. There was also a rapid reduction of RVESVi in the immediate post-PVR period (23%); but this was followed by a further 9% reduction to midterm. Whilst the changes seen in the days after surgical PVR are likely to represent the effects of cardiopulmonary bypass on the RV, the latter may reflect extended recovery of RV function with time. Our data suggest that the changes of RVEDVi observed are likely to reflect acute mechanical remodelling from volume reduction, whereas the later and progressive improvement of RVESVi may represent time-dependent biological remodelling, that is, continued improvement of load independent RV function. RVESVi has been suggested as a valid estimate of intrinsic, load independent RV function in rToF.³⁹⁰ Our data therefore underscores the value of RVESVi as a parameter of RV myocardial function. We suggest that future studies on the timing of PVR should focus on the presence and degree of any continued reduction in RVESVi during serial post PVR follow-up, as changes in RVESVi are independent from ventricular loading conditions.

Postoperative reduction of RV volumes into the normal range, that is “normalisation”, considered as optimal PVR outcome took place in the majority of our patients (70%). Our cut-offs for RV volumes that predicted RV volume normalisation ($\text{RVEDVi} \leq 158 \text{ ml/m}^2$, $\text{RVESVi} \leq 82 \text{ ml/m}^2$) are in close agreement with previously reported preoperative thresholds of RVEDVi 150-163 ml/m^2 and RVESVi 80-85 ml/m^2 29, 30, 32, 35, 372, 391 Higher pre-operative RVESVi has been identified as an important predictor of post-PVR RVEF, and it has therefore been suggested that greater emphasis should be placed on RVESVi values during decision making towards timing PVR.^{31, 372} Indeed, the identified preoperative RVESVi cutoff was equally sensitive and more specific (sensitivity 86%, specificity 88%) for normalisation of RV volumes than the identified RVEDVi cutoff (sensitivity 86%, specificity 79%).

4.4.2 RV outflow tract and PVR in rTOF

We quantified the area of resected RVOT tissue as a measure (albeit coarse) of accounting for the extent of RVOT surgical remodelling carried out, as judged appropriate by the operating surgeon. However, tissue area measurements did not appear to influence the extent of RV volumetric or functional reverse remodelling seen post PVR. This is in keeping with the findings of a randomised trial of PVR with and without RV remodelling surgery.³⁰ It may also reflect intrinsic measurement bias from 2D area quantification of irregular shaped masses of resected tissue or variations in practice amongst different surgeons.

Postoperative CMRs showed significant reduction in the length of the RVOT akinetic area. Increased preoperative RVOT akinetic lengths correlated with greater reduction in postoperative QRS duration, presumably due to surgical resection of slow-conducting, fibrotic/patch tissue in RVOT akinetic regions.³⁹² We previously showed that larger CMR RVOT akinetic length predicts sustained ventricular arrhythmia⁶⁴ and in the present study that it predicts postoperative reduction in QRS duration. QRS duration is a recognised sudden death risk factor in rTOF,^{19, 61, 69, 392} Longer preoperative LGE CMR- defined RVOT scar length was associated with reduced postoperative improvement in both RV and LVEF, suggesting more extensive preoperative RV LGE may not be amenable to surgical remodelling and may predict suboptimal PVR outcomes. Reassuringly, worsening of overall RV LGE scores did not occur.

Technological advances in CMR enabling enhanced spatial resolution and three-dimensional comprehensive coverage may improve detection and quantification of RV focal fibrosis,^{106, 107} or enable RV T1 mapping before and after PVR to ascertain potentially reversible changes in RV interstitial fibrosis burden not detectable with LGE CMR,^{77, 152, 219, 393} However, to date, useful application of T1 mapping to the RV, avoiding measuring blood pooling in its trabeculated myocardium, remains elusive.

4.4.3 Right atrial reverse remodelling

Indexed RA areas were mildly dilated in our study cohort and decreased post PVR. The fate of the RA during post-operative cardiac remodelling is of importance as RA enlargement is predictive of atrial tachyarrhythmia, especially in patients with RV restrictive physiology.^{57, 64} The positive effect of PVR on RA size merits further work, focusing on RA function and its relationship to outcomes after PVR.

4.4.4 Evidence of left ventricular reverse remodelling

While the focus of PVR outcomes has historically centred on the RV, LV remodelling also occurs. Our immediate post-PVR LV data demonstrates a reduction in both LVEDVi and LVESVi, likely representing the transient and early impact of cardiopulmonary bypass. Previous studies have described the association between significant pulmonary regurgitation and LV systolic dysfunction, and the prognostic importance of LV function in rTOF.^{65, 385} Even though PVR directly exerts right heart alterations, our study like others, demonstrated increased LV volumes^{29, 30} and improved LV function at midterm follow-up.^{24, 35} In addition to any 'reversed Bernheim effect', in which RV geometrical and functional adaptations lead to LV dysfunction,¹⁹ increased LV filling may be achieved due to improved efficiency of RV

cardiac output when a competent pulmonary valve is implanted. This larger preload translates to an increase in LVEDV and function. This may contribute to patients reporting improved exercise tolerance, although we found no objective improvement of peak VO_2 on cardiopulmonary exercise testing in our study. This was anticipated given PR was the predominant lesion in our study cohort and improvements may be due to recovery kinetics after exercise versus peak oxygen uptake.^{394, 395} As we and others have previously reported, nonetheless, patients in this study had marked improvement in symptoms at midterm follow-up.

4.4.5 Limitations

As the study late CMR follow-up took place at the time of routine clinical follow-up, there was variation in the timeframe that this occurred. However, we sought to address any confounding through a time-adjusted linear effects model. Patients with implanted electronic devices were excluded and there was necessary attrition of study participants who had pacemakers or implantable cardioverter defibrillators inserted after study recruitment. This may have introduced bias by excluding patients at the extreme end of the clinical spectrum with significant ventricular dysfunction or other adverse risk markers, who mandated primary or secondary prevention against malignant arrhythmias. Patients who were recruited to the study were not otherwise different from all patients undergoing surgical PVR in the study period (*Table 4.3*).

4.5 Conclusions

In this single-centre prospective study, we have demonstrated pertinent and novel immediate changes after surgical PVR, directly reflecting bi-ventricular and right atrial remodelling. Normalisation of RV volumes, an objective after PVR, was achieved in 70% of study patients and correlated with baseline RVEDVi/RVESVi. While this early and desirable response to volume offloading was sustained at midterm follow-up, we observed additionally a continuing reduction in RVESVi with a concomitant midterm recovery in RVEF, suggesting time-related biological remodelling of the load independent RV function after PVR. A preoperative RVESVi cutoff of $\leq 82\text{ml/m}^2$ was equally sensitive and more specific for normalisation of RV volumes than our preoperative RVEDVi threshold of $\leq 158\text{ml/m}^2$ justifying using RVESVi for clinical timing of PVR to achieve and measure optimal reverse remodelling.

Patients with rTOF	2005			2006			2007			2008			2009		
	n = 19			n = 19			n = 26			n = 25			n = 27		
	Study	Non-Study	p	Study	Non-Study	p	Study	Non-Study	p	Study	Non-Study	p	Study	Non-Study	p
Age, years	38.3±8.5	34.2±16.6	0.63	32.1±11.5	31.0±10.0	0.85	34.1±7.9	30.3±10.3	0.32	34.3±13.0	27.3±8.8	0.12	38.2±9.9	30.8±9.2	0.06
Gender, male/female	6/8	2/3	1.00	5/9	3/2	0.60	9/1	7/9	0.04	6/3	10/6	1.00	8/3	7/9	0.24
Symptoms (NYHA≥2), n	9	2	1.00	9	2	1.00	8	12	0.56	5	6	1.00	5	4	1.00
QRS duration, ms	149±29	113±21	0.06	160±22	178±2	0.28	148±26	147±32	0.92	160±13	162±25	0.84	165±9	144±32	0.07
<i>CMR</i>	n = 14	n=3		n = 14	n = 3		n = 10	n = 12		n = 9	n = 11		n = 11	n=12	
RVEDVi, ml/m ²	161±57	218±158	0.60	157±37	165±71	0.87	163±47	140±65	0.36	153±25	168±61	0.48	148±30	163±28	0.24
RVESVi, ml/m ²	91±38	134±120	0.60	76±19	85±39	0.55	90±30	81±47	0.62	85±17	101±50	0.37	81±25	81±18	0.99
RVSVi, ml/m ²	71±21	84±45	0.67	75±23	79±32	0.79	73±26	58±23	0.17	68±23	67±23	0.98	67±15	81±13	0.02
RVEF, %	45±5	42±19	0.84	48±10	48±3	0.99	45±8	43±9	0.64	44±11	41±9	0.52	46±8	50±5	0.1
LVEDVi, ml/m ²	73±16	55±53	0.62	76±22	91±19	0.30	77±21	69±11	0.28	82±19	77±23	0.64	74±21	82±18	0.37
LVESVi, ml/m ²	29±11	20±22	0.31	34±19	34±10	0.94	33±13	30±10	0.55	38±13	34±13	0.46	32±18	31±11	0.91
LVSVi, ml/m ²	45±8	35±31	0.63	43±11	57±10	0.05	44±10	36±12	0.12	45±13	44±14	0.84	44±10	51±10	0.12
LVEF, %	62±7	66±11	0.54	61±8	63±4	0.64	58±8	58±8	0.93	55±12	57±8	0.64	61±11	62±7	0.68
LVMi, g/m ²	63±15	81±47	0.68	60±14	72±26	0.25	78±16	59±16	0.02	72±20	60±15	0.15	67±16	58±11	0.14
Pulmonary regurgitant fraction, %	38±9	50±6	0.05	45±10	31±19	0.09	39±7	39±10	0.97	36±9	40±16	0.55	37±17	42±5	0.34

Table 4.3: Year on year comparison of study participants with remaining patients with rTOF who underwent PVR 2005-2009 demonstrating lack of recruitment bias.

Chapter 5 Quantification of Right Ventricular Diffuse Fibrosis in Repaired Tetralogy of Fallot

For the RVT1 work in this chapter, an introduction and literature review were presented in the Introduction *section 1.4*. Within Chapter 5, the initial aims of each section and its methods are all presented for each topic, followed by all of their results and discussion incorporating initial conclusions. Each of these Chapter 5 conclusions is reiterated in the overall setting of the thesis Conclusions (Chapter 8).

The experimental work encompassed within this chapter were designed and performed from October 2012 to June 2015. The findings from the phantom work presented within this chapter form part of the results in our publication on 'Magnetic resonance imaging phantoms for quality-control of myocardial T1 and ECV mapping: specific formulation, long-term stability and variation with heart rate and temperature' in the *Journal of Cardiovascular Magnetic Resonance*, 2016.³⁹⁶

5.1 Aims and Methods

Aim: To develop and evaluate non-invasive RV diffuse fibrosis quantification with a novel CMR T1 mapping technique in patients with rTOF.

5.1.1 Phantom preparation

As there is significant overlap in derived ECV between normal and pathological states, the well-known dependence of ECV on scanner and sequence parameters has led to an emergent role for T1 phantoms in quality assurance.^{202, 211, 212} Phantoms are recommended for systematic and regular assessment of T1 accuracy and precision, in addition to validation of new pulse sequences.²¹⁰ There is limited data on the accuracy of phantom measurements for quality assurance of T1 and ECV measurements, with no published phantom studies to date assessing the long-term precision of T1 measurements.^{193, 211, 212,}
³⁹⁷

Phantoms should ideally possess properties that include: independently variable T1 and T2 relaxation times over a range of values to enable simulation of physiological ranges found in vivo in tissue and fluids; longterm stability of relaxation parameters; preparation ease and reproducibility; in addition to minimal susceptibility of relaxation parameters to temperature and frequency changes.^{398, 399}

Phantoms were therefore assembled to study the impact of variables such as heart rate, arrhythmia, temperature, off-resonance and respiration.⁴⁰⁰⁻⁴⁰² Published T1 values were referenced⁴⁰³ to create phantoms with targeted T1 values at 1.5T and 37°C of: pre-Gd myocardial T1 1000ms, T2 50ms; pre-Gd blood T1 1450ms, T2 250ms; post-Gd myocardial T1 500ms, T2 45ms and post-Gd blood T1 380ms, T2 140ms.

Nickel-chloride (NiCl₂) agarose gel phantoms were made by a reproducible lab procedure, calculated to model T1 and T2 of myocardium and blood, native and post-contrast. Nickel doped gels were chosen to vary proton T1 rates, whilst T2 rates were determined by the concentration of agarose used as the gelling agent.^{398, 401} NiCl₂ stock was made, and mixed with agarose and distilled water in specified quantities to derive the selected T1 and T2 values. The specified mixtures were brought to boil until the agarose completely dissolved and a transparent solution was obtained. The solutions were then methodically transferred into 60ml glass narrow-neck sealed thick-wall bottles filled with minimal gaps from gel contraction while cooling. Particular care was taken to minimise air entrapment and formation of cracks through cooling the phantoms in a shallow cold water bath. As an important quality control step in manufacture, multi-slice data (results not shown) demonstrated minimal variation with location in the gels when the boiling agarose mixtures had been stirred adequately before pouring into the phantom tubes for setting.

Upon completion of cooling, the phantoms were imaged with long TR (4.5s) spin-echo sequences. Multiple iterations of phantoms were subsequently made with adjustment of reagent concentrations to derive the targeted T1 and T2 values as tabulated (*Table 5.1*).

	[Nickel] (mM)	Agarose (%)	T1 (ms)	T2 (ms)
Pre-Gd myocardium (Mixture A)	0.9	2.1	1000	55
Pre-Gd blood (Mixture B)	0.48	0.45	1500	225
Post-Gd myocardium (Mixture C)	2.35	2.5	510	44
Post-Gd blood (Mixture D)	3.7	0.45	365	125

Table 5.1: Phantom composition for derivation of reference T1 and T2 values.

The composition of the final phantoms was obtained by combining:

- (A) 0.9mM Ni²⁺ 2.1% 3.150g agarose, 1mM stock 0.9x150/1 = 135ml, add 15 ml water
- (B) 0.48mM Ni²⁺ 0.45% 0.675g agarose, 1mM stock 0.48x150/1 = 72ml, add 78ml water
- (C) 2.35mM Ni²⁺ 2.5% 3.750g agarose, 3mM stock 2.35x150/3 = 117.5ml, add 32.5ml water
- (D) 3.7mM Ni²⁺ 0.45% 0.675g agarose, 6mM stock 3.7x150/6 = 92.5ml, add 57.5ml water

5.1.2 Short-term phantom stability

The short-term temporal stability of gel relaxation-time phantoms for quality control of T1 and ECV measurements was initially assessed over 10 consecutive weeks against 10 same day repeats with repositioning and scanner re-adjustments.

The phantoms were kept in the MRI room (Siemens, Avanto 1.5T) with a temperature logger, and imaged weekly using consistent coil and phantom arrangements, with 11-RR MOLLI: high-resolution (at heart-rate 75bpm) and low-resolution (at 100bpm) versions, with pre-contrast 5(3)3(3)3 and post-contrast 4(1)3(1)2 variants. Image parameters were identical weekly except automatic calibrations of B1 and reference frequency using an identical adjustment volume each week. Spin-echo imaging for T2 was also acquired. Typical imaging parameters were: (1) MOLLI FOV 360 x 306mm, slice thickness 8mm, TR/TE 315/1.12ms, flip angle 35 degrees, acceleration factor 2. (2) T2 spin-echo imaging FOV 230 x 108mm, slice thickness 10mm, TR/TE 4500/22-264ms, flip angle 180 degrees, interpolated.

Mean T1 and T2 values were taken in ROIs on pixelwise maps (*Figure 5.1*), and examined for drift. Phantom ECV was calculated with a fixed haematocrit of 0.43. Temperature corrections were estimated at each weekly scan time using the MRI room temperature recording, as there is a recognised effect of temperature variation on recorded T1 values.^{398,}

⁴⁰⁴ An approximate temperature correction was applied based on the relatively stable T1 relaxivity of Ni²⁺ with temperature when compared to other ions such as Cu²⁺.³⁹⁸ Taking the relaxation time of pure agarose in distilled water at 20°C = 2 seconds, with the +1% change in T1 per degree Celsius of water, then $R1_{\text{native}}(T) = R1_{\text{Gel}} \cdot (100 - (T - 20)) / 100$. For nickel-doped gels, the impact of nickel relaxivity was then added to this temperature-sensitive baseline. For the shorter T1 gels containing more Ni²⁺ (ie, the post-Gd gel phantoms), there was little sensitivity to temperature. Neither accuracy data nor magnetisation transfer was assessed, as this experiment focuses on long-term precision.

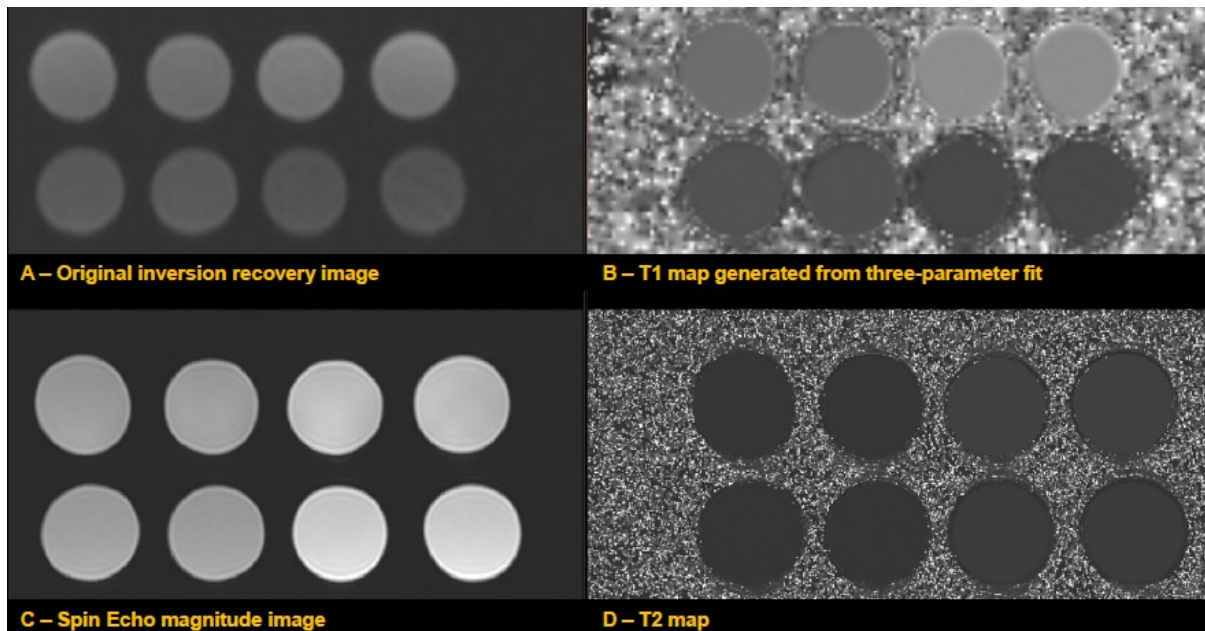


Figure 5.1: Phantom experiment images illustrating derived T1 and T2 maps (B&D) generated.

Phantoms: Same-day repeatability and 10-week stability results and discussion

From the experiments described in *Section 5.1.2*, the T1 and T2 values showed greater variation over 10 weeks (T1 CoV 0.65% across all measurements, Table 5.2) as compared to the 10 same-day measurements (T1 CoV 0.2%). The longer T1 relaxation times demonstrated a larger correlation with temperature fluctuations (after correction T1 CoV 1.0%) as shown in *Figure 5.2*. No consistent direction of drift was observed over the 10 week period. Heart rate and temperature variability were also investigated in the same phantoms, with higher heart rates causing a corresponding increase in ECV of 0.13% for every 10bpm increase. Elevations in temperature were again found to produce an increase in longer T1 values preferentially over shorter T1s.⁴⁰⁵

For long-term validity, phantoms would need to be markedly more stable than the $\sim 1/10^{\text{th}}$ change in native T1 and ECV found in *in vivo* applications. The 10-week native-T1 phantom CoV was $\sim 1.1\%$, while the “phantom ECV” predictably showed smaller CoV $\sim 0.8\%$. This was smaller as anticipated because of the less pronounced temperature effects on short-T1 phantoms. Therefore, these results showed adequate stability of phantoms over ten weeks to support their continued use in ascertaining the long-term precision of T1 mapping.

	T1						T2		
	<u>High resolution protocol</u>			<u>Low resolution protocol</u>			<u>Spin-echo protocol</u>		
	Mean	SD	CoV (%)	Mean	SD	CoV (%)	Mean	SD	CoV (%)
'Native Myocardium' (A), ms	958	6.2	0.7	962	5.8	0.6	56	0.5	0.9
'Native Blood' (B), ms	1595	17.0	1.1	1572	17.0	1.1	235	3.2	1.4
'Post-Gd Myocardium' (C), ms	499	1.9	0.4	498	2.0	0.4	48	0.6	1.2
'Post-Gd Blood' (D), ms	373	2.1	0.6	372	2.1	0.6	150	1.8	1.2

Table 5.2: T1 values from 11-cycle 8-image fast MOLLI protocols: pre-Gd 5(3)3 and post-Gd 4(1)3(1)2. These were repeated in high resolution (75bpm) and low resolution (100bpm) versions without temperature correction. T2 values from spin-echo imaging are also illustrated. CoV = Coefficient of Variation.

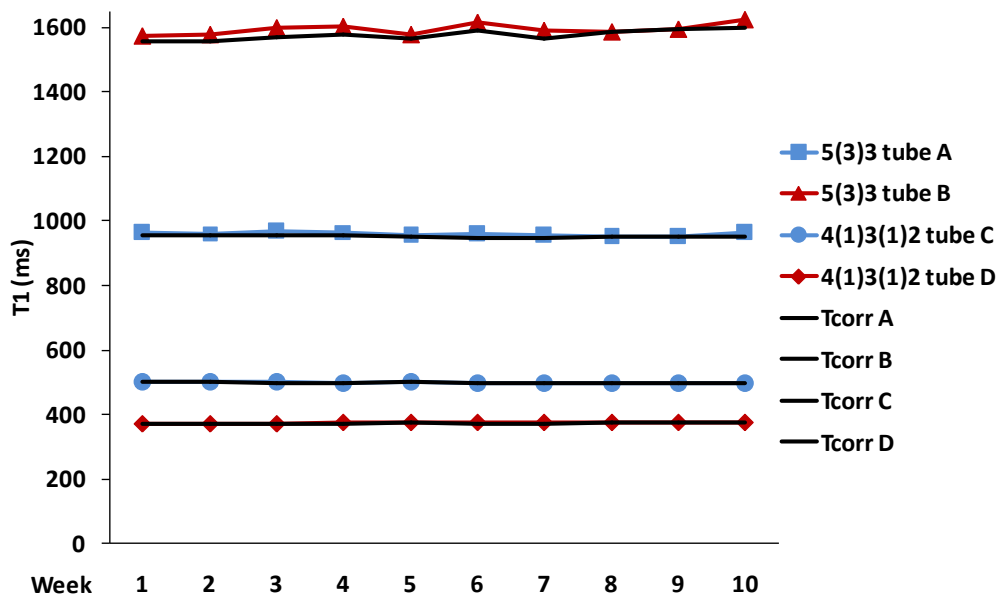


Figure 5.2: Variation of T1 from high-resolution protocol at RR 800ms over ten weeks with temperature corrected values shown in black (T=20 to 22.5°C). Tubes A to D are as defined in Table 5.1. Reproduced from Heng et al.⁴⁰⁶

5.1.3 Long-term phantom stability

As T1 mapping is utilised over long periods in clinical research projects, its long-term stability is important. Weekly imaging of the phantoms was then continued with an identical protocol over a 52 week period to investigate long-term phantom stability and precision.

Phantoms: One-year weekly stability results and discussion

There was reassuring consistency in derived ECVs and T1 measurements (*Table 5.3* and *Figure 5.3*), with no evidence of drift in shorter T1 relaxation times with time. Native myocardial T1 values remained robust over the study period (with native myocardial T1 = $-0.01 \times \text{number of weeks} + 972$). Larger CoVs were seen with longer T1 relaxation times, typically in pre-Gd blood measurements. There was progressive drift in native blood T1 measurements, which increased on average by 169ms over the year in accordance with the equation: Native blood T1 = $3.25 \times \text{number of weeks} + 1492$.⁴⁰⁷

	T1						T2		
	<u>High resolution protocol</u>			<u>Low resolution protocol</u>			<u>Spin-echo protocol</u>		
	Mean	SD	CoV (%)	Mean	SD	CoV (%)	Mean	SD	CoV (%)
'Native Myocardium' (A), ms	972	12.6	1.3	972	13.6	1.4	56	1.3	2.2
'Native Blood' (B), ms	1579	56.4	4.6	1550	61.9	4.0	234	8.0	3.4
'Native Myocardium' (C), ms	498	5.1	1.0	497	13.5	1.4	48	0.9	1.9
'Native Blood' (D), ms	372	6.4	1.7	372	6.5	1.8	149	3.0	2.0
ECV, %	27.4	0.9	3.2	27.6	0.9	3.1			

Table 5.3: Variability over 52 week period for T1, T2 values and phantom ECV measurements (calculated with haematocrit of 0.43).

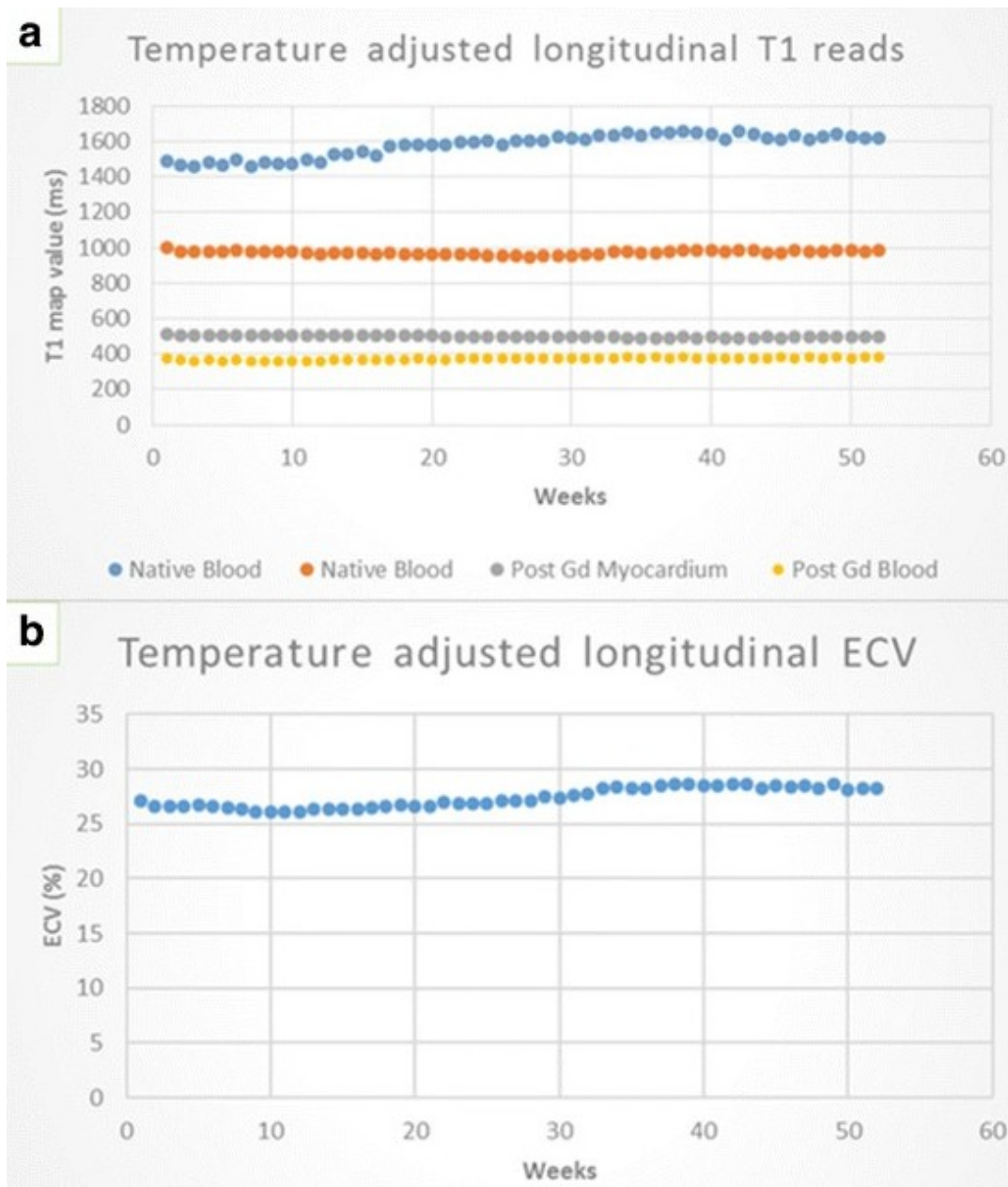


Figure 5.3: Longitudinal trend in T1 and ECV values over a one year timeframe, illustrating gradual positive drift in native blood T1 values whilst native myocardial T1, post- contrast T1 values and ECV remained more stable in comparison. Reproduced from Vassiliou *et al*, J Cardiovasc Magn Reson 2016.³⁹⁶

These findings reinforce the rationale for regular and routine phantom T1 measurements in centres at which T1 mapping is applied. A gradual downward drift in T1 is predictable based on likely loss of water from the gels with time, although and upward drift might occur due to T2 changes which affect the MOLLI distorted values of T1. Acquiring a full slow T1 and T2 measurement at regular intervals was impractical due to scanner time availability, as each slow T1 measurement would involve several hours of scan time or organisation of regular repeated overnight scans.

Fundamentally, whilst phantoms show significant gradual drifts over time, they are nevertheless still useful in safeguarding patient data quality, as they would enable correction against sudden changes in the values output by scanner software relating to numerous hidden control parameters which could be inadvertently altered (for example, emergency reinstallation of software after a machine failure). For the medico-legal aspects, these methods remain strictly “experimental”, with no guarantee, warranty or quality assessment done by the manufacturer. A “sudden-jump” correction would not be feasible without a record of frequent scanner-specific phantom measurements before unexpected scanner events.

5.1.4 RV T1 mapping technical development

Within the technical work, several different approaches were attempted before settling on the main work. The results and discussion for the volunteer normal-subject tests of various ideas and reasons for their failure are included within this *Methods* section as they form part of the experience governing development of the eventual method finally adopted for the main patient and normal subject study.

Myocardial T1 mapping has almost exclusively been applied to LV pathology thus far, with inadequate spatial resolution in its current forms to image the thinner, sub-pulmonary RV. There are numerous considerations and technical challenges posed when trying to successfully quantify the ECV in the RV, due to its complex geometry and thin mobile walls with adjacent strong signals from blood and epicardial fat. Working closely together with in-house physicists, I have sought to address each of these issues during our sequence development process (summarised in Table 5.4).

Aspects of CMR Sequence	Technical Approach Taken
Image Quality and Resolution	
Increase SNR	• Thinner slices • Choice of surface coil (loop coils) • Prone imaging
Increase spatial resolution	• Navigator-based and multi-breathhold techniques • Systolic imaging • Segmentation of images
Accounting for cardiac and respiratory motion	• Motion correction (MoCo) • Practical steps: respiratory belt, good breath-hold commands • Careful timed acquisition in cardiac diastasis
Addressing Fat Suppression	• Optimisation of fat suppression with frequency adjust • Fat-water separated imaging
Off-Resonance – impact on accuracy and precision	• Control of scanner parameters (flip angle etc.)
Field Maps	• To account for field inhomogeneities rather than attributing directly to regional ECV variations
Partial Volume Effects	• Reduced by contrast administration to shorten T1 values of interest • Consideration of 4-parameter fit model
Data Analysis	
ROI contouring and selection	• Meticulous contouring of epi/endocardial borders • Independent observers for inter-observer reproducibility
T1* maps	• Improve accuracy of blood T1 measurements

Table 5.4: Technical factors relevant to RV T1 mapping.

In the initial phase, we sought to apply and address the pertinent issues relating to MOLLI, adapting and targeting LV MOLLI parameters to improve image quality.

5.1.4.1 Modifications to the MOLLI sequence

The RV is positioned anteriorly in the chest just behind the sternum, and can be better visualised with CMR due to its proximity to the anterior receiver coil delivering increased signal-to-noise ratio (SNR), which also enables finer spatial resolution. Adaptations to 5(3)3 MOLLI (WIP 448B version) for pre-Gd imaging and 4(1)3(1)2 MOLLI for post-Gd imaging^{189, 201, 202} were carried out to select thinner slices and the narrowest possible field of view (FoV). Modified MOLLI sequences were applied to maximise visualisation of RV myocardium during diastole of the cardiac cycle in normal subjects, systemic RV and patients with rTOF who provided written consent. Subjects were scanned on a 1.5T Siemens Avanto platform in the supine position with a thoracic phased-array 12-channel surface coil.

The feasibility of this modified MOLLI approach was tested with a pilot study in systemic RV patients who had previously undergone atrial redirection surgery (Mustard or Senning procedures) with significantly hypertrophied RVs. Five systemic RV patients were recruited for MOLLI RV T1 mapping, during which data was acquired twice each in a mid-SAX slice before contrast and approximately 15 minutes after the bolus administration of 0.15mmol/kg

Gd-DTPA. The hypertrophied systemic RV (*Figure 5.4*) in these patients yielded mean RV free wall ECVs of $29.2 \pm 1.5\%$ (*Table 5.5*). As protocol amendments were made during our pilot study and we delineated ROIs differently from published work in a similar patient population, our results cannot be directly compared. Careful contouring of ROIs on T1 maps enabled ECV calculation in the free wall, although further work was necessary (as explained next) to establish corresponding ECV in normal controls.

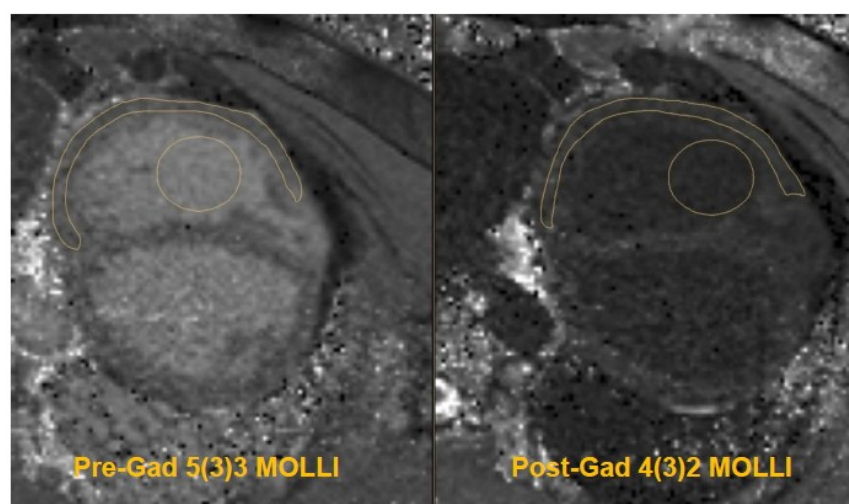


Figure 5.4: T1 maps derived from MOLLI images acquired of systemic RV patient for pilot study

	Pilot study	Plymen et al
	n=5	n=14
Age, years	33 ± 6	34 ± 7
Male/female	4/1	8/6
NYHA class I/II	4/1	12/2
<u>CMR parameters</u>		
Systemic RVEDVi, ml/m²	109 ± 23	79 ± 13
Systemic RVESVi, ml/m²	48 ± 13	35 ± 4
Systemic RVEF, %	57 ± 4	59 ± 5
RV ECV, %	RV free wall	Septal
	29.2 ± 1.5	25.4 ± 3.6

Table 5.5: Pilot study patient demographics and ECV calculations. Pilot study patients were of similar age with larger systemic RVs in comparison to published data by Plymen et al.²²¹ The derived RV ECV cannot be directly compared due to differences in sequence methodology and ROI delineation.

Discussion: Modifying MOLLI imaging

Although this approach provided reasonable image quality for ECV quantification in thickened systemic RVs (as used by other groups), it is well known that this approach is not able to generate sufficient image resolution for the thinner RV in normals and patients with rTOF (*Figure 5.5*).

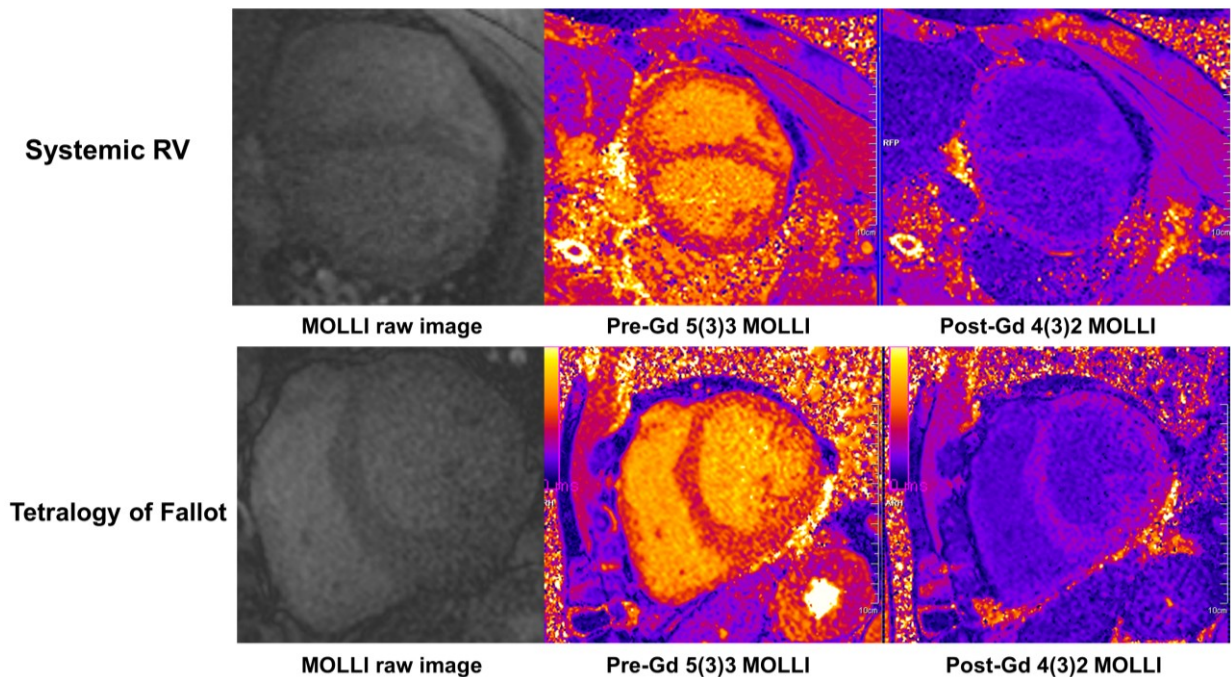


Figure 5.5: Representative MOLLI in SAX slice in systemic RV patient in top row with clearly visible RV myocardium in contrast to second row in patient with rTOF in whom the thinner subpulmonary RV myocardium cannot be clearly delineated without corruption by adjoining blood and fat.

In order to further improve SNR, additional healthy volunteers and rTOF subjects were imaged prone with loop coils during systolic phases of the cardiac cycle. Although systolic imaging has the strong benefit of compressing the RV wall into a thicker state, closing some trabeculations and expelling trapped blood, the end-systolic pause duration was found to be even shorter than at diastole, giving insufficient time for a single-shot image acquisition without being degraded by wall motion.

There was no demonstrable improvement with prone imaging (*Figure 5.6*) and although it might conceivably reduce anterior-posterior respiratory motion (leaving only the internal cranio-caudal cardiac motion with respiration more correlated with the typical diaphragm navigator approach of MRI), it was also ruled out due to its discomfort within the narrow MRI scanner bore, especially for post-operative patients.

In addition to image resolution issues, T1 maps generated in narrow myocardial walls adjoining bright tissues such as fat and blood are susceptible to artefacts such as Gibbs ringing, which would potentially introduce much larger errors than that for the thicker LV. MOLLI measurements also result in systematic under-estimation of myocardial native T1 values, relating to T2 decay during the inversion pulse and imperfect inversion.^{201, 202, 204, 211,}

⁴⁰⁸ The layer of epicardial fat adjacent to RV myocardium is not addressed with this technique, which further contributed to measurement errors simply by partial-volume impact where part of an RV myocardial pixel overlaps the epicardial fat which strongly affects the measured T1.²²⁸ High-resolution MOLLI were also carried out at 3T field strength, but the nominally greater SNR of 3T is a complex and moot point for T1-weighted cardiac protocols and did not support enough of an increase in image resolution to aid in RV visualisation (unlike some other applications where enhanced signal of the stronger 3T magnetic field is more straightforward to apply). Recalling that each single-shot MOLLI image duration must be completed within a diastolic pause time, improved resolution can only be delivered by increased application of “parallel imaging” whose direct expenditure of SNR was not evidently supported more by 3T imaging than at 1.5T. As these various modifications to the MOLLI sequence were still suboptimal, this approach was discontinued.



Figure 5.6: MOLLI acquisition in normal subject with limited visualisation of thin RV myocardium despite systolic and prone imaging.

5.1.4.2 Respiratory-navigator gated, fat-water separated segmented Saturation Recovery Single-Shot Acquisition (SASHA)

Fat-water separated imaging

The need to discriminate fat from water is of particular relevance towards optimising RV T1 quantification. Fat identification has been carried out previously with methods that include

chemical shift fat suppression,^{409, 410} T1-weighted imaging in which fat is observed with bright signal intensity, and multi-echo Dixon methods for water and fat separated image reconstruction.⁴¹¹⁻⁴¹⁷

The multi-echo Dixon fat and water separation method is a sensitive technique when combined with field map estimation to improve robustness of the separation of fat and water, in the difficult regions near the heart where main-field B0 distortion often contains local distortions^{418, 419} that are too “sharp” for any feasible external shimming hardware to address. It is based on the difference in resonance frequencies between water and fat, which is exploited by acquiring several (at least three) images at different echo times when water and fat are in-phase and out-of-phase (where the underlying assumption of a homogenous B0 field is corrected using a B0-field map calculated from the multiple-echo-time images). Its in vivo application has been demonstrated in patients with coronary artery disease, ARVC and cardiac masses (lipomas) with superior CNR to TSE imaging.⁴¹¹

The drawback of fat-water separation is that multiple echo-time images must be acquired registered with each other, namely during the same breath-hold or at least interleaved with each other so finely in time that respiratory (and cardiac) motion has exactly equal impact on all of them. This is a severe drawback, because we are already confined to the diastolic pause and finding it difficult to obtain sufficient image resolution in that time without even having to acquire at least 3 images in support of the fat-water technique.

Navigator RV T1: The Sequence

In collaboration with the National Institutes of Health/NHLBI, Bethesda, United States (Dr Peter Kellman, Staff Scientist, Physics), we were able to obtain and modify his sequence prototype for navigator based, segmented, fat-water separated spoiled gradient echo imaging of high resolution within a short acquisition window (*Figure 5.7*). The pulse sequence will subsequently be denoted as ‘navigator RV T1’, and was implemented on a 3T scanner (Skyra, Siemens Healthcare) with an 18 element anterior matrix coil and 12 elements of a matrix spine coil. A 3T system was selected to improve SNR, whilst simultaneously minimising B1 field effects by means of the parallel transmit system, which may arguably be more amenable to a saturation-recovery method, and in our case was also partly determined by scanner time and improved RF receiver coils on our newer 3T system.

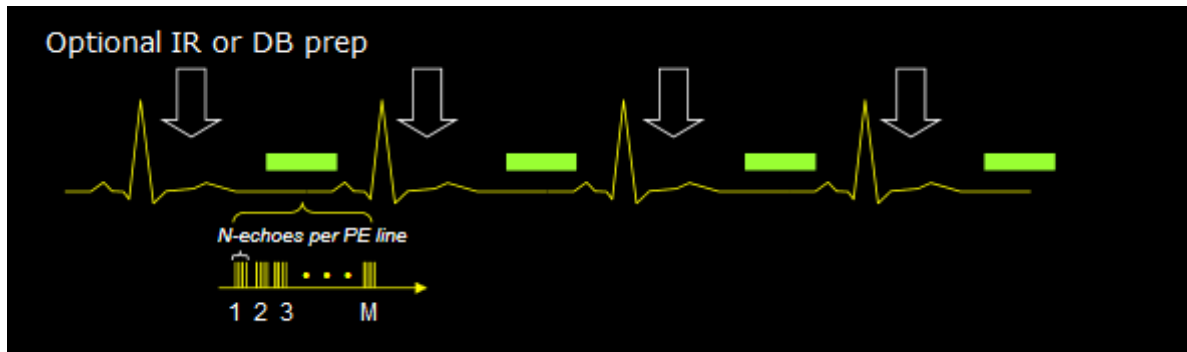


Figure 5.7: Schematic of pulse sequence in which $N=2$ echoes and $M=12$ (comprising 6 segments with 2 interleaves with each interleave collecting data for 2 echo points). Reproduced from Kellman.⁴²⁰ The IR was replaced with a saturation preparation pre-pulse, and four different echo times were acquired by two-fold interleaving of 2-echo readouts (the faster fat-water phase accumulation at 3T than 1.5T is a disadvantage).

Our aim was to quantify native RV myocardial T1. Our modified sequence acquired 6 raw-data lines (phase-encode steps) for each of the four images required for fat-water separation over two cardiac cycles. This required a total of approximately 15 accepted cardiac cycles per measured saturation-recovery delay Tsr. Free-breathing acquisitions were respiratory-gated using a diaphragm MRI navigator. A saturation-recovery protocol employed non-selective saturation at 10 different recovery times (Tsr) in incremental steps before an anchor image without magnetisation preparation was taken (*Figure 5.8*), requiring a total of 11scans to complete the data for a T1 measurement. Each series generated four spoiled gradient echo images, a water-separated, fat-separated image and frequency map. Typical imaging parameters were: FOV 300x300mm, acquired matrix 288x288 with a head-foot phase encode direction (to redirect the impact of residual respiratory ghosting errors); flip angle 24°, 100% phase resolution with no partial-Fourier, parallel-imaging at rate 3, TEs = 1.76, 3.12, 4.49, 5.85ms (dTE = 1.36, 1.67, 1.36ms), Tsr 100-900ms at equal intervals for 10 timepoints imaging every cardiac cycle. For the fully-recovered image required by SASHA T1-measurement (see *Section 1.4.2*), it was necessary to enforce >5 seconds trigger delay for complete Mz recovery between cardiac cycles of data acquisition for the anchor image. For this scan, a type of guided breathing was attempted using the patient intercom. For this work, the acquisition window for MRI image acquisition was ~100ms in diastole timed to avoid wall motion using a cine acquisition in the subject before commencing T1 mapping. The image resolution achieved was 1.04 x 1.04 x 5mm³ acquired voxel size.

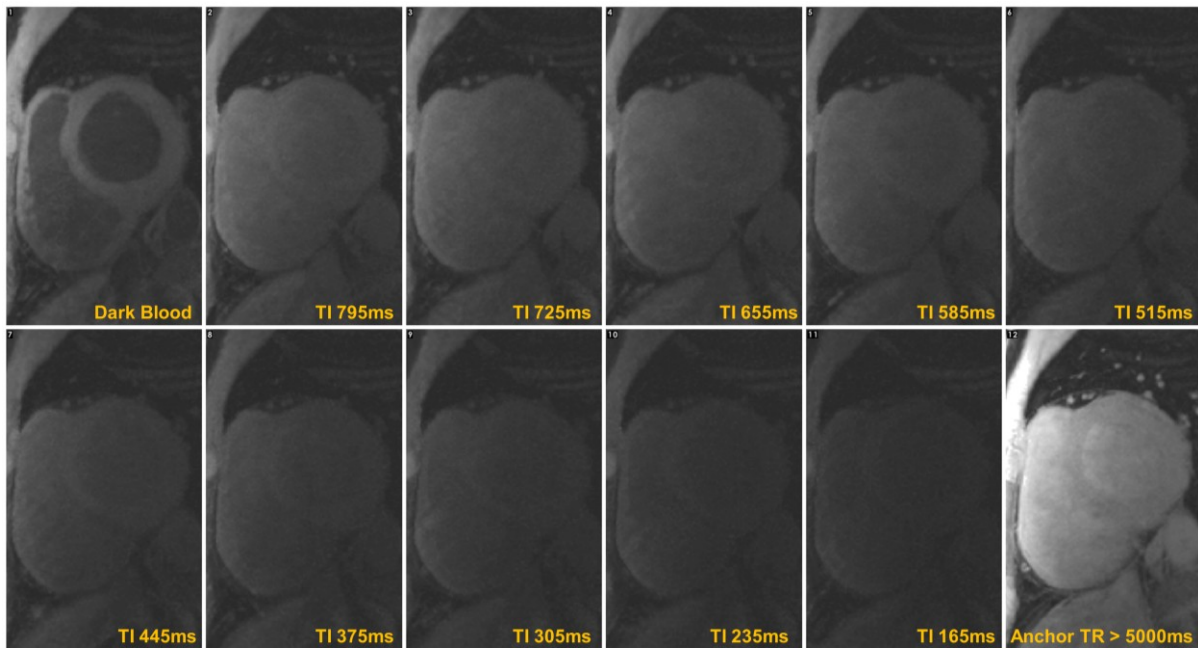


Figure 5.8: Water images of navigator RV T1 in the SAX orientation in a normal volunteer with dark blood acquisition, followed by stepwise increasing TIs and a final anchor image. The dark-blood acquisition in this study was solely in order to support ROI delineation at the final stage of image analysis onto the thin RV wall. This blood suppression was achieved by the “double-inversion-recovery” approach which is, unfortunately, fundamentally incompatible with T1 mapping. The dark-blood image was not an input to the T1 calculation.

Imaging parameters were optimised by scanning normal volunteers whose results enabled us to refine the selection for flip angle and number of segments. Time to echoes (TEs) were shortened to minimise the extent of artefacts caused by sternal wires visible in almost all patients with rTOF (*Figure 5.9*), and were subsequently reduced to a 3 TE protocol. The navigator acceptance window was set strictly to accept data only in end-expiration to minimise ghosting. It was essential to enforce a head-foot phase encode direction in order to redirect otherwise marked artefacts caused by bright chest wall ghosting (adjacent to the anterior receiver coil necessarily placed in proximity for sufficient SNR), such that these did not impact upon the RV wall. SNR assessment was also undertaken, which demonstrated ample SNR of the navigator RV T1 approach of ≈ 119 (greater than the minimum SNR of ≈ 40 proposed as needed in the non-saturated SASHA image).¹⁹⁸

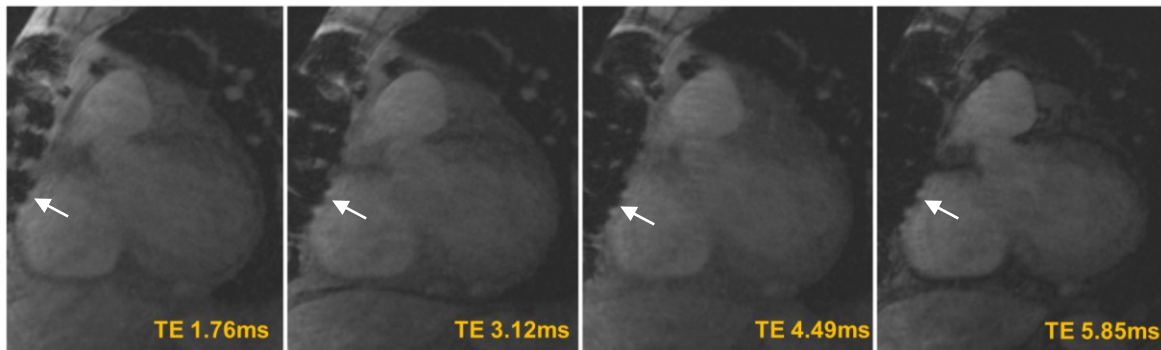


Figure 5.9: Increasing dephasing and loss of signal in the RV myocardium in patient with previous cardiac surgery caused by sternal wire artefact (arrowed) with higher TEs across images from left panel to right.

After localiser scouts and cine imaging for volumetric analysis, a SAX slice orthogonal to the RV free wall was selected. Cardiac diastasis timing was derived. Cardiac shim and frequency adjustment centred over the heart was applied to minimise off-resonance errors before the sequence was applied (although the field-map of fat-water would further account for such errors, it would break down if careful setup were not performed); all of these methods were highly operator-dependent. Acquisitions were performed free-breathing for each series between Tsr 100-900ms, whilst guided breathing instructions were given to the subject during anchor acquisitions to maximise data acquisition in end-expiration. Depending on each subject's RR interval, each T1 datapoint took approximately 45 to 90 seconds to acquire, whilst the anchor image took between 5 to 10 minutes to acquire.

Calculation of native T1 values

T1 image analysis was performed offline using CMRtools (Cardiovascular Imaging Solutions) for ROI demarcation on water-separated images, from which image signal intensities were fitted to a three-parameter exponential recovery curve⁴²¹ using MATLAB R2014a.

Navigator RV T1 feasibility pilot and reproducibility

All subjects provided written informed consent with study approval from the National Research Ethics Service. Pilot data was obtained in healthy volunteers and patients with rTOF for feasibility assessment. Each subject recruited for the pilot completed a single scan with two complete runs of navigator RV T1 acquired to investigate intra-session precision. To assess inter-scan reproducibility, further subjects were recruited and scanned on two separate dates (mean 4 ± 1 day between scans). An identical imaging protocol was followed for the second scan. Images were anonymised before analysis by two independent

observers. Inter-scan and inter-observer reproducibility were assessed as outlined in the *Methods - Statistics* section.

For the feasibility pilot, 8 subjects were recruited, which included 4 patients with rTOF and 4 age and gender-matched healthy volunteers (6 males, mean age 37 ± 6 years). All subjects completed the scan (3-echo) protocol of two runs of navigator RV T1 with native T1 quantification obtained. The results are shown in *Table 5.6*.

Parameter	All (n =8)		rTOF (n=4)		Normals (n=4)	p value
	T1, ms	CoV, %	T1, ms	T1, ms	T1, ms	
Age, years	37 $\pm$ 6		41 $\pm$ 4		33 $\pm$ 6	0.09
Gender, male/female	6/2		3/1		3/1	1.00
Native RV	Run 1	1503 $\pm$ 87	5.1	1497 $\pm$ 94	1510 $\pm$ 93	0.84
	Run 2	1574 $\pm$ 150		1583 $\pm$ 220	1565 $\pm$ 64	0.88
RV Blood	Run 1	1937 $\pm$ 114	4.5	1921 $\pm$ 112	1954 $\pm$ 131	0.71
	Run 2	1908 $\pm$ 171		1801 $\pm$ 87	2016 $\pm$ 173	0.07
Native septal	Run 1	1469 $\pm$ 24	6.3	1451 $\pm$ 19	1487 $\pm$ 12	0.02
	Run 2	1533 $\pm$ 122		1567 $\pm$ 12	1500 $\pm$ 177	0.50

Table 5.6: Results of navigator T1 pilot in patients with rTOF versus healthy controls. CoV refers to the intra-session precision between consecutive runs 1 and 2.

For inter-session reproducibility assessment, five healthy volunteers (4 males, mean age 33 ± 3 years) each completed two scans with derived RV and LV native myocardial T1 values listed in *Table 5.7*. Inter-observer reproducibility of native RV myocardial T1 was mean CoV 6.4%, RV blood T1 mean CoV 1.1% and LV (septal) myocardial T1 mean CoV 2.5%. Although acceptable inter-observer reproducibility was seen, there was much higher variability seen between scans with inter-scan CoV ranging from 6.2-13.7%. The greatest variation was seen in native RV T1 results (*Figure 5.10*). Mean native LV and blood T1 values were also noted to be higher than published values (at 3T field strength).

Parameter		Mean Inter-Observer	Inter-Observer Reproducibility				Inter-scan Reproducibility	
			Observer 1	Observer 2	Inter-observer	Inter-observer CoV (%)	Inter-scan	Inter-scan CoV (%)
Native RV T1, ms	Scan 1	1535 ± 81	1515 ± 112	1555 ± 94	-40 ± 127	5.8	Observer 1 61 ± 230	11.2
	Scan 2	1410 ± 204	1454 ± 199	1365 ± 223	89 ± 110	6.9	Observer 2 190 ± 191	13.7
RV Blood T1, ms	Scan 1	1917 ± 113	1914 ± 106	1920 ± 121	-6 ± 20	0.7	Observer 1 -70 ± 181	6.2
	Scan 2	1995 ± 159	1983 ± 146	2006 ± 172	-22 ± 38	1.4	Observer 2 -87 ± 205	7.1
Native septal T1, ms	Scan 1	1426 ± 49	1438 ± 52	1414 ± 47	24 ± 19	1.5	Observer 1 -137 ± 96	7.3
	Scan 2	1555 ± 111	1576 ± 124	1534 ± 108	42 ± 73	3.4	Observer 2 -120 ± 87	6.6

Table 5.7: Results of navigator RV T1 inter-observer and inter-scan reproducibility assessment.

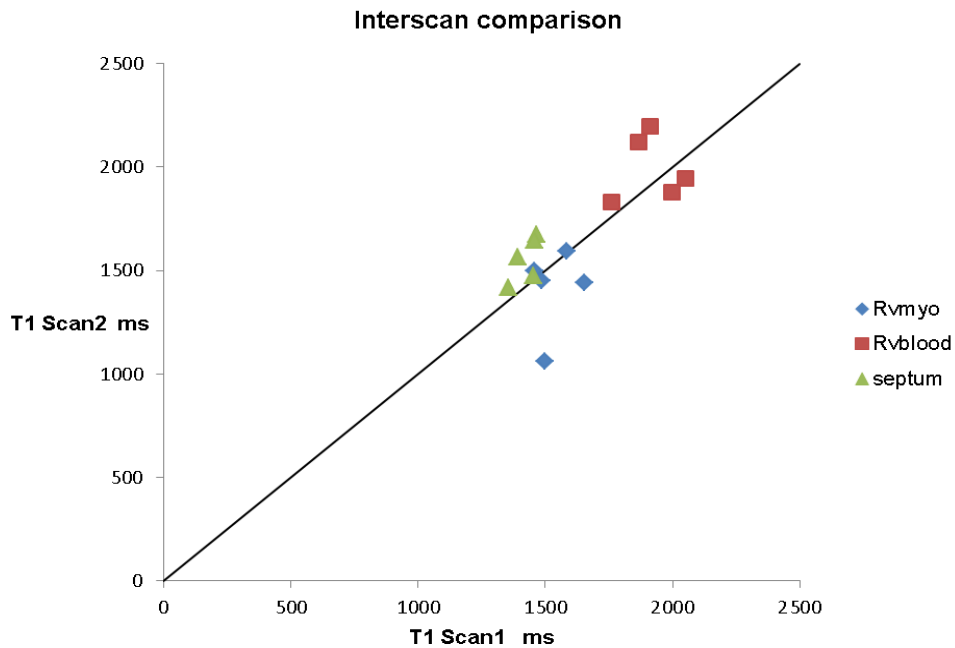


Figure 5.10: Results of inter-scan reproducibility of RV myocardial and blood, as well as LV septal T1 measurements.

Discussion: Navigator RV T1

With the navigator RV T1 method, the improvement in SNR and image resolution was unfortunately gained at the expense of increased respiratory-motion artefacts, recognised as induced by small residual errors in the assembled rawdata for each image over multiple respiratory cycles, despite the rigorously applied respiratory gating. This resulted in poor intra-session and inter-scan reproducibility, which would have masked the detection of genuine differences in native T1 values in pathology when compared to healthy subjects. The main source of error was introduced by significant respiratory ghosting that was often seen, particularly for ‘anchor’ acquisitions (*Figure 5.11*). This was most marked at the RV free wall region of interest, which obscured myocardial tissue visualisation and likely caused contamination of derived measurements.



Figure 5.11: Images from normal volunteer illustrating respiratory ghosting artefact in the ‘anchor’ image panel, in which the RV free wall myocardium cannot be discerned.

Solutions to reduce ghosting were sought, which included attempts to reduce anterior-posterior chest wall movement by scanning subjects prone, as well as applying a chest binder (Cough-Lok belt) to suppress anterior-posterior motion. Although prone imaging reduced the ghosting artefact, it did not prove to be a practical solution for patients having to undergo CMR for an hour, especially for scanning in the early post-operative period after cardiac surgery. We found that applying a chest binder triggered abdominal breathing in subjects, which then further exacerbated the ghosting artefacts seen. The inclusion of a second navigator in the anterior-posterior direction and/or incorporating chest wall movements into the image acceptance algorithm by means of respiratory bellows was also contemplated, but proved to be technically difficult and could not be readily included.

Fat-water separation took place reliably whenever the sequence was applied, effectively ‘removing’ epicardial fat from the water-separated image. However, the higher than expected myocardial and blood T1 measurements were thought to be due to noise (3-parameter curve fitting), as well as measurement errors caused by adjacent blood signal contamination compromising accuracy and precision. Elimination of adjacent blood signal was not attempted following the initial work showing difficulty with respiratory ghosting, but would have been the next stage (as in the *Results section 5.2.3*). Although T1 overestimation is recognised at low SNR values with saturation recovery imaging, relating to the Rician distribution that results in increased signal intensities at short Tsr values during magnitude image reconstruction, the SNR in this data was not so low as to cause this as measured earlier.¹⁹⁸

The option of imaging during cardiac systole was also considered briefly, but was not possible as systolic imaging did not allow the acquisition of long saturation recovery delays necessary for curve-fitting in a way that could be performed consistently for multiple cardiac

cycles for segmented raw-data acquisition (unless constant RR intervals). Sternal wire artefacts were more pronounced at 3T field strength, which required selecting RV free wall ROIs distant from artefacts. We considered modifying the image plane to long axis views (such as four chamber or LVOT) if RV myocardium could not be adequately imaged in the SAX orientation due to sternal wire artefacts. Fortunately, this was not required in any of the patients scanned. It also became apparent with the lengthy native navigator RV T1 scan that an infusion-based equilibrium-contrast approach would be required for ECV calculation to counter the effects of T1 drift after contrast was given. This would have further complicated an already extremely challenging scan proforma.

5.2 Results and Discussion

Right ventricular T1 mapping by free-breathing fat-water separated dark blood single shot saturation-recovery imaging

The respiratory-ghosting failure results of our navigator RV T1 work using high-resolution segmented imaging led us to focus on enabling single-shot imaging for subsequent experimental work. The coarser image resolution of single-shot imaging cannot separate RV myocardium from blood, and therefore demanded the optimisation of dark blood cardiac imaging (now by a different approach that is compatible with T1 mapping). During this work, a second technical difficulty occurred which demanded considerable effort to understand and improve saturation pulse efficiency. We assessed these variables together with optimisation of sampling strategies and imaging parameters.

5.2.1 Dark blood motion-sensitized preparation (MSPrep)

The navigator RV T1 sequence illustrated that the high-resolution accumulation of data over multiple respiratory cycles into one raw-data set was too vulnerable to respiratory ghosting, which did not appear to arise from the bright blood.

To eliminate these ghosts it was necessary to move to single-shot imaging, that is, where the complete raw-data for each image was obtained within one cardiac cycle to abolish the ghosting artefacts in *Section 5.1.4.2*. However, the larger pixel size of single-shot imaging would not be able to discriminate reliably the thin RV wall from RV blood akin to MOLLI methods. We therefore had to null the blood signal to proceed.

We explored dark blood imaging options to suppress blood signal adjacent to the RV myocardium of interest. CMR dark blood imaging is typically performed using double inversion recovery methods⁴²²⁻⁴²⁶ or spatial presaturation.^{427, 428} Double inversion sequences

suppress blood based on its flow and T1 properties by applying a non-selective 180° pulse followed immediately by a slice selective -180° pulse to reinvert the image slice alone, with a delay before imaging so that inflowing blood is imaged at the zero of its inversion-recovery. This has stringent limitations on the myocardial tissue being in the same place (typically, before and after systolic contraction) for the two steps required, and is incompatible with applying the series of different timings of further inversion or saturation pulses to obtain T1 mapping. The alternative approach of spatial presaturation suppresses blood through flow only by exciting and dephasing the blood magnetisation upstream of the image slice followed by a sufficient delay for it to flow into the slice. This would be incompatible with short Tsr values and tend to fail if the inflow conditions are not satisfied, as fully acknowledged by the Calgary group⁴²⁹ who attempted this method and have not taken it further.

We considered the integration of motion-sensitized preparation (MSPrep) into RV T1 mapping. This utilises motion-sensitising gradients to dephase all moving blood spins prior to imaging with a preparation sequence consisting of 90-180-90 non-selective RF pulses and a pair of identical unipolar gradients around the 180 pulse.⁴³⁰ This causes signal attenuation of stationary and moving tissues due to T2 decay, whilst accelerating the timing to $<10\text{ms}$ for the whole procedure minimising T2 decay. The method additionally induces two independent intravoxel dephasing mechanisms by a diffusion effect (phase dispersion due to incoherent microscopic motion, which would also reduce stationary myocardial signal) and velocity encoding (both incoherent and coherent larger-scale motion within flowing blood), of which the latter mechanisms was shown to dominate in the heart.⁴³⁰ Manipulation of field-of-speed (FOS) allows adjustment of the motion sensitive gradient pulse amplitudes to optimise image contrast between moving blood and myocardium in the MSPrep approach. This method is very sensitive but feasible, and our initial *in vivo* acquisitions illustrated that it requires individual specific adjustments of FOS and timing of application in the cardiac cycle to suppress blood signal without concurrently compromising myocardial signal as shown in *Figure 5.12*. The direction of motion sensitisation was perpendicular to the image slice in this work; although some exploration of other directions did not seem to be more effective. It is possible that some direction along the trabeculations would be more effective near the RV wall.

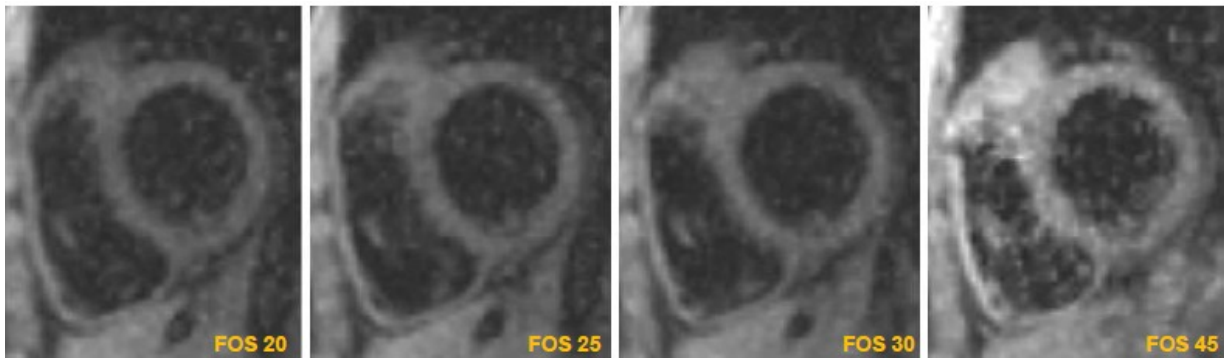


Figure 5.12: Increasing FOS values from left to right in panel showing progressively weaker blood suppression, most evident in the superior aspect of the RV in this SAX slice in a normal volunteer. The images have been acquired at identical cardiac diastasis timings, with more bright blood signal visible in the RV cavity in the FOS 45 image compared to FOS 20.

Small losses in myocardial signal due to T2 or diffusion-sensitivity losses caused by MSPrep were considered inevitable. However, these were expected to be the same multiplicative factor for every point of the repeated scans within the SASHA experiment and would therefore be expected to cancel out for the curve fit. Any small variation in MSPrep effectiveness due to changing cardiac timings between the repeated scans for SASHA was of much more concern, and the optimisation before each patient was carefully tested for $\pm 50\text{ms}$ timing having little impact on brightness. If it did, the timing was re-evaluated to a new range or MSPrep weakened to ensure no variability in myocardial signal loss with small changes in cardiac timing.

5.2.2 Optimising saturation efficiency

The previous navigator RV T1 approach had also highlighted the critical importance of achieving saturation efficiency $\eta < 0.5\%$ of the equilibrium M_0 to avoid systematic errors in SR-based T1 quantification, particularly when utilising 2-parameter fits for improved SNR as shown in *Table 5.4, Section 5.1.4*. Indeed, for this setup a 3-parameter fit was attempted and found to be intolerably noisy. We sequentially examined three saturation pulses for maximal saturation efficiency: (1) BIR4_90 saturation pulse (2) Siemens standard 3-pulse composite saturation (CompSat) and (3) 6-saturation pulse train (published for use with SASHA).⁴³¹

We were granted access to and tested a modified BIR4_90 saturation pulse via our NIH collaborator (Dr Peter Kellman, USA). The BIR4 pulse train had been designed²¹⁷ and evaluated at NIH with peak $B1 = 20.5\text{microTesla}$, achieving $\leq 0.5\%$ saturation over 70-100% of the designated $B1$ and $\pm 125\text{Hz}$ off-resonance as shown in *Figure 5.13*. The $B1$ robustness is important as the transmitted field is non-uniform *in vivo* due to absorption by

the body, in addition to the insurmountable “off-resonance” distortion of B0 by tissue in proximity to the relatively empty lungs.

Through our collaborative links, we were also subsequently provided access to a novel 6-pulse train with optimised flip angles (Dr Kelvin Chow, University of Alberta, Calgary, Canada). This pulse train had showed promising saturation efficiency at 3T with residual longitudinal magnetisation <1% in early simulation and *in vivo* imaging. In-house modifications had to be made (by Dr Peter Gatehouse, Principal Physicist and Senior Lecturer in Physics, Cardiovascular Biomedical Research Unit, Royal Brompton Hospital) to both saturation pulses for 1.5T imaging. We then conducted comparative simulation, phantom and *in vivo* scanning of healthy volunteers of all three pulses. Subjects underwent scans assessing intrasession reproducibility and image quality assessment of either a single saturation pulse with multiple runs or combination acquisitions alternating between two of the three saturation pulses. Systematic experiments showed that BIR4 and the 6-saturation pulse train were not able to attain <1% saturation needed, in contrast to the Siemens CompSat pulse. Sequential testing did not reveal the cause for the non-ideal saturation efficiency of both supposedly improved pulse trains, with substantial failure of BIR4 to saturate, leaving an unacceptable approximately 10-20% of M_0 after application. The Siemens CompSat pulse was therefore chosen for RV T1 mapping. A check of CompSat efficiency was thereafter routinely acquired *in vivo* with optimisation if necessary before the main RV T1 mapping sequence for every scan.

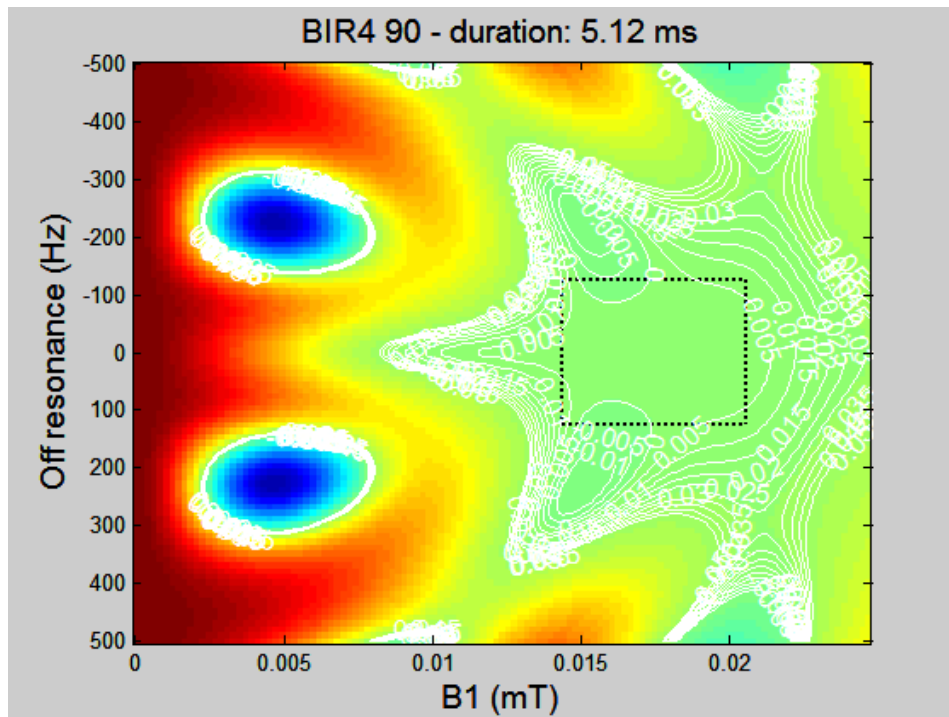


Figure 5.13: BIR4 performance for myocardial tissue ($T_1=1100\text{ms}$, $T_2=45\text{ms}$) with design range of FA and off-resonance variation denoted by dotted box. Pulse evaluation at NIH provided suppression to $<0.5\%$ of M_0 over 70-100% FA range (image courtesy of Dr Peter Kellman).

5.2.3 Optimising saturation recovery imaging

Apart from MSPrep and CompSat optimisation, other technical aspects were also studied. We chose to move from previous navigator RV T_1 work on 3T back to a 1.5T platform because the multiple-echo acquisition with 3 echoes minimally needed to support fat-water imaging could be more efficiently performed at 1.5T. This was a fundamental limitation of 3T imaging because the gradient system performance is limited by patient nerve stimulation and cannot run twice as fast at 3T, as would be needed in this situation due to the doubled off-resonance frequency of fat compared to water. On more practical aspects, our 3T system (Siemens Skyra) has some uncertainty over its limited designed peak B1 performance that we would not be able to improve. The impact of sternal wires within the echo-times available (similar on both field strengths due to gradient system performance) was also more severe at 3T than at 1.5T. We were fortunate to have the loan of a 32-channel thoracic phased-array coil, with significantly superior SNR performance over standard clinical arrays available at our centre. This coil was probably crucial to the project because it enabled shorter image acquisition times using factor 4 acceleration by receiver coil parallel imaging. The single-shot image duration for acquiring the 3 TEs needed for fat-water was thereby made short enough to run within diastasis in most hearts.

Single-shot images acquired were of low SNR, which was improved by ‘warping’ each image by complex non-rigid distortion processing so that images within the series could be averaged.^{104, 215} This process could not correct through-plane motion, but performed well enough to support free-breathing acquisitions when configured to reject frames corresponding to significant respiratory motion. The “complex” refers to operation of the MoCo method at an intermediate stage of image reconstruction not normally displayed before the magnitude values of image pixels are taken. The MoCo and fat-water technique were implemented in a team led by Dr Peter Kellman at National Institute of Health, Bethesda, USA. The support of, in particular Dr Sven Zuehlsdorff and Dr Renate Jerecic at Siemens Medical Systems for enabling this collaboration was pivotal. Access to the advanced 6-pulse composite-saturation pulses was also given by Dr Kelvin Chow (University of Alberta, Calgary, Canada).

We specifically investigated the following factors:

- Number of single-shot images acquired for complex non-rigid MoCo averaging – Data was acquired for RV native T1 measurements with a range of 10 to 20 single-shot images per series. Separate acquisitions were made with a range of MoCo image acceptance from 25%-75%. There was no detectable improvement in the measurement accuracy with collecting more images or allowing lower MoCo image acceptance values.
- Breath-hold (BH) versus free breathing (FB) – Acceptable intra-session reproducibility for RV native T1 measurements was seen for both, but there was less variation with BH acquisitions. However, the long ‘anchor’ acquisitions (where TR > 6 seconds) made BH scanning problematic and heavily reliant on patient cooperation for reproducible end-expiratory BHs in relation to the long repetition time. Although most subjects scanned were able to comply when instructed over the patient intercom, it would not be routinely practicable to enforce reproducible end-expiratory breath-holds during long anchor scans.
- Diastolic versus systolic image acquisition – systolic imaging resulted in ~ 5% under-estimate of RV native T1 compared to diastolic measurements.
- Saturation sampling strategies - Fixed Ts protocols (pre-Gd Ts~600ms, post-Gd Ts~200ms) have been shown to improve precision of saturation recovery T1 mapping up to heart rates of 120bpm.²¹⁷ We evaluated 2-parameter against 3-parameter curve fitting for RV native T1, with 3-parameter measurements showing greater variation due to increased noise.

- Heart rate variability – After saturation pulse optimisation, there was minimal impact of variation in heart rate on measured RV native T₁, in line with previous work which showed 2-parameter SASHA maintained excellent precision up to heart rates of 120bpm.²¹⁷ Sensitivity to the heart-rate during initial tests eventually alerted us to the failure of the BIR4 type saturation which is in use at some other centres. The reason for this failure remains unsolved by expert reviewers who examined the methods we used and did not identify any obvious errors.

In view of these findings, default settings for the sequence included 10 single-shot images at fixed 50% acceptance for complex non-rigid MoCo timed during cardiac diastasis. Data was acquired free-breathing as per the published fixed T_s protocol, with RV native T₁ and ECVs calculated by automated pixelwise 2-parameter fitting (following a final stage of MoCo alignment of all size of the averaged T₁-weighted and anchor images). The RV T₁ sequence is depicted in *Figure 5.14*:

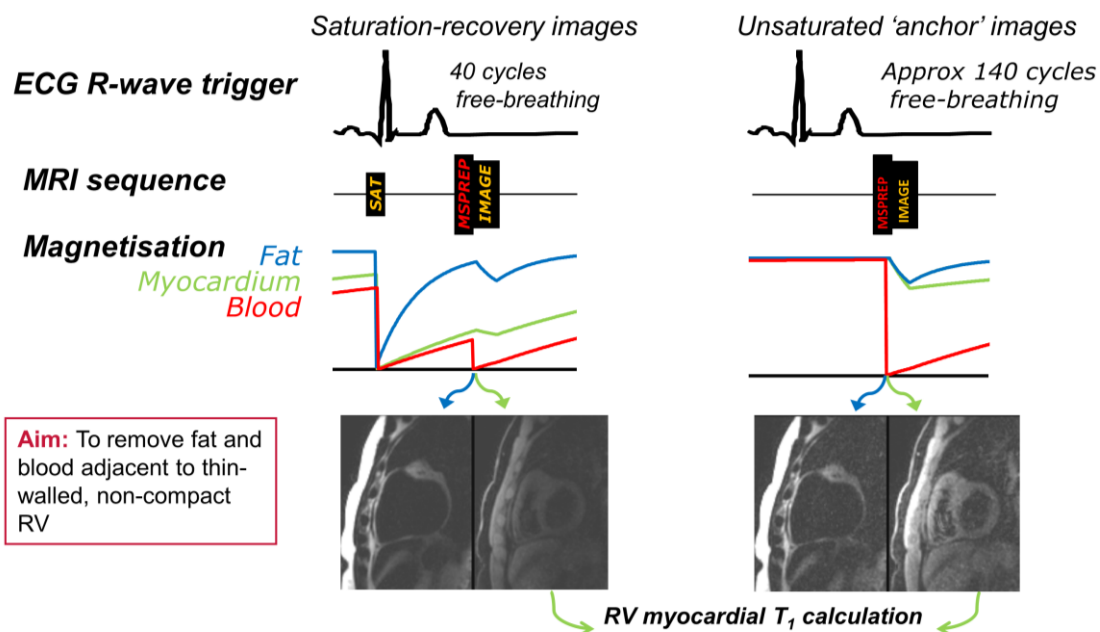


Figure 5.14: Myocardial T₁ measurement by multi-echo fat/water separated saturation-recovery single-shot acquisition (SASHA) with blood signal suppression. Image courtesy of Dr Peter Gatehouse.

5.2.4 RV T1 Mapping Scan Protocol

Having established the preferred imaging parameters, RV T1 mapping data was acquired during free-breathing (FB) on a 1.5T Siemens Avanto with a thoracic phased-array 32 channel surface coil (16 anterior, 16 posterior elements). Particular care was taken to ensure optimal anterior and posterior coil positions over the chest wall for adequate coverage of the cardiac region of interest.

Non-rigid complex MoCo averaging of fat-water separated, single-shot saturation recovery imaging was applied in accordance to the RV T1 scan protocol illustrated in *Figure 5.15*. Further to the separation of the adjacent fat, blood signal was nulled using a motion-sensitized preparation (MSPrep), the entire sequence for RV T1 measurement being identified in the following as RVT1.

Same day venepuncture was performed for haematocrit measurement via the intravenous access required for gadolinium contrast injection.

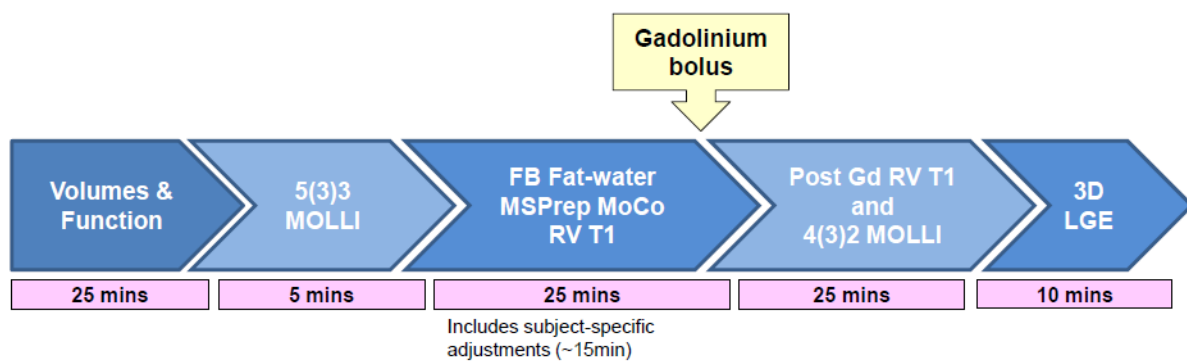


Figure 5.15: Schematic of RV T1 mapping scan protocol.

Volumetric and velocity mapping flow data was acquired as per the CMR acquisition protocol as outlined in the *Methods* section. Diastolic still time specific to each subject was determined from a high resolution cine taken in the four-chamber view. A mid short-axis cine meticulously aligned orthogonal to the RV free wall was then obtained. Importantly in this alignment, the orientation for LV measurement by the same sequence, although highly desirable for comparison purposes, had to be over-ridden in the interest of minimising partial-volume effects in the RV wall pixels.

A corresponding short-axis slice at a similar level but aligned specifically for the LV was then selected for LV T1 mapping by the much more standard Modified Look-Locker Inversion Recovery (MOLLI) technique.²⁰¹ A 5(3)3 MOLLI acquisition strategy was applied (twice, in

the same plane, simply for averaging) for native LV myocardial and blood T1 quantification in a single mid short-axis plane. Post-contrast 4(1)3(1)2 MOLLI were acquired at approximately 15 minutes after contrast administration to derive LV ECV. Typical imaging parameters for MOLLI were: MOLLI FOV 360 x 306mm, slice thickness 8mm, single shot bSSFP image duration $\approx 200\text{ms}$, flip angle 35 degrees, acceleration factor 2. The image matrix and sequence timings of the MOLLI imaging were not altered at any time during the study.

RV T1 mapping was undertaken in stepwise fashion with the RVT1 pulse sequence as follows (*Figure 5.16*):

1. **Optimisation of composite saturation** - The efficiency of the composite saturation pulse was assessed by acquiring the chosen RV SAX slice in end-expiration with saturation switched on, before repeating the RV SAX slice identically but with saturation switched off. A low-resolution image with crucially centre-out ky-ordering acquisition at very low flip-angle with very short T_{sr} ($\approx 5\text{ms}$) after saturation was set up for this purpose running at the R-wave in each cardiac cycle and averaged over 10 cycles for sufficient SNR (as image should be virtually entirely noise if near perfect saturation was achieved).

A region of interest (ROI) was then delineated within the RV cavity for both images, from which a signal intensity ratio was calculated. Optimised composite saturation efficiency η was accepted as $<1\%$ of M_0 within that placed ROI or estimated in the RV wall nearby. The presence of fat signal would sometimes be problematic in this approach due to its much shorter T_1 , so fat-water imaging was again used. The system transmitter voltage was adjusted if saturation efficiency exceeded 1% of M_0 before repeating the signal intensity ratio check. This saturation optimisation was prioritised at the relevant RV wall region only.

2. **Optimisation of MSPrep parameters** – Once composite saturation was deemed satisfactory, fat-water scouts were piloted. These consisted of 4 single-shot, fat-water separated images in the same RV SAX slice, which were acquired after frequency adjustment was carried out localised to the right-heart and adjacent chest-wall. The inherent “Tune-up” shim settings were used with no subject-specific adaptation of the B0-shimming. Multiple acquisitions were performed to obtain images that encompassed a range of field-of-speed (FOS), MSPrep run-time and image

acquisition timings during diastasis in the cardiac cycle (*Figure 5.17*). The MSPrep was always applied immediately before the start of the image, so that the T1 recovery of blood post-Gad would still be minimal. These multiple acquisitions were vital for enabling the selection of optimised MSPrep parameters.

The FOS setting was chosen by the value at which best myocardial definition with minimal blood pool contamination was seen; whilst diastolic image timing was set to minimise image quality loss by cardiac motion. Typical FOS settings applied ranged between 40-50 cm/s to avoid excessive sensitivity to motion that could otherwise lose myocardial SNR due to its small residual motion. Typical timings (for MSPrep followed immediately by image) were determined similarly as a trade-off between loss of myocardial signal and too much remaining blood. It was essential to survey typically in 50ms timing steps and acquire these at both sides (earlier and later) than the proposed timing for the main experiment. This was for robustness to mitigate against small changes in cardiac timing without losing RV myocardial signal. The slice chosen for RVT1 was towards the RV base, as blood motion in apical regions was possibly insufficient to drive the motion-sensitive dephasing reliably.

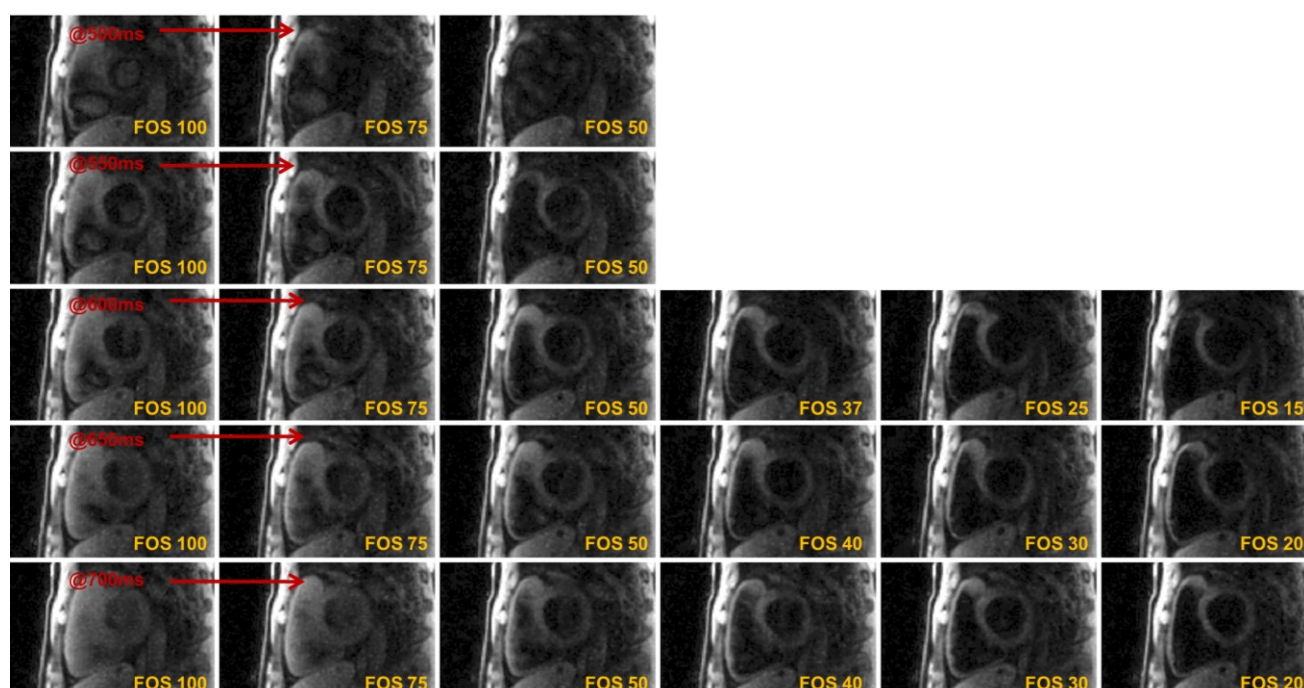


Figure 5.17: Typical variation of FOS and cardiac timing (in ms) to determine optimal MSPrep parameters in a normal volunteer. This enabled selection of imaging timing of 650ms and FOS 50cm/s as the optimal parameters for this subject.

3. **RV T1 quantification** – The free-breathing sampling scheme applied comprised four 10-cycle scans at $T_s \sim 600\text{ms}$ and two anchor scans at $T_s > 6$ seconds (in which 10 cycles were acquired with long recovery gaps). T_s values sampled were chosen in line with validated saturation recovery sampling methods to improve precision.²¹⁷ Complex MoCo averaging⁴³² was configured at fixed 50% acceptance, that is, data from the 5 shots at the most similar respiratory phase were incorporated into the averaged image. Imaging parameters were: TE = 1.0, 2.7, 4.3ms, forward-echo only, FA 10° , FOV 360x270mm, slice thickness 6mm, 6x1.9x2.1mm acquired voxels, TGRAPPA rate 4, requiring a diastolic shot duration of 175ms.

The TE values were reduced in an effort to maintain signal near sternal wires, an important limitation that rules out several other MRI methods with longer TE, and also hinders the use of 3T; as although the chemical-shift is also correspondingly faster, the limited gradient-speed enforced is similar at 1.5T and 3T, and this undesirably demands the use of interleaved as well as multi-echo imaging for fat-water at 3T. Potentially significantly, variable flip-angle imaging was not available for this work and should be noted for any future study. A centre-out ky ordering was used.

Pre-Gadolinium: Two complete runs of the sampling scheme (ie. 2xTsr 600ms and 1x anchor; all repeated twice) were acquired to enable native RV T1 quantification. Native LV T1 was not always quantified simultaneously by saturation recovery imaging as the RV SAX slice was not aligned for the LV septum, although where possible this measurement was made on images subject to some doubts over partial-volume effects due to misalignment.

Post-Gadolinium: 0.15mmol/kg of Gadovist was administered by hand as an intravenous bolus as well as a large saline flush, with concurrent activation of a timer. This allowed imaging from 3 minutes onwards post contrast administration with each post-Gd run comprising four 10-cycle scans at $T_s \sim 200\text{ms}$ and two anchor scans at $T_s > 3$ seconds. Images were acquired continuously until approximately 20 minutes post contrast administration, typically yielding 5-7 complete runs (depending on the subject's RR interval). 4(1)3(1)2 MOLLIs were acquired interleaved with each completed free-breathing run of fat-water separated, MSPrep saturation recovery imaging.

4. ***Three-dimensional late gadolinium enhancement*** – Whole-heart fibrosis and scar assessment was then carried out in a transaxial plane as described within the *Methods* section.

Two-parameter fit pixelwise T1 maps were generated for each run with semi-automated processing (in collaboration with Dr Peter Kellman, National Institutes of Health, Bethesda, MD, United States). ROIs were drawn within RV free wall myocardium, from which pre and post-contrast RV T1s were taken simply as mean values within the ROI using CMR42 (Circle Cardiovascular Imaging). Care was taken to ensure ROIs did not include areas of LGE. LV myocardial and blood T1s were derived from ROIs drawn on matched MOLLI acquisitions pre and post-contrast. ECV was calculated as defined in *Section 1.4.1*.^{159, 169}

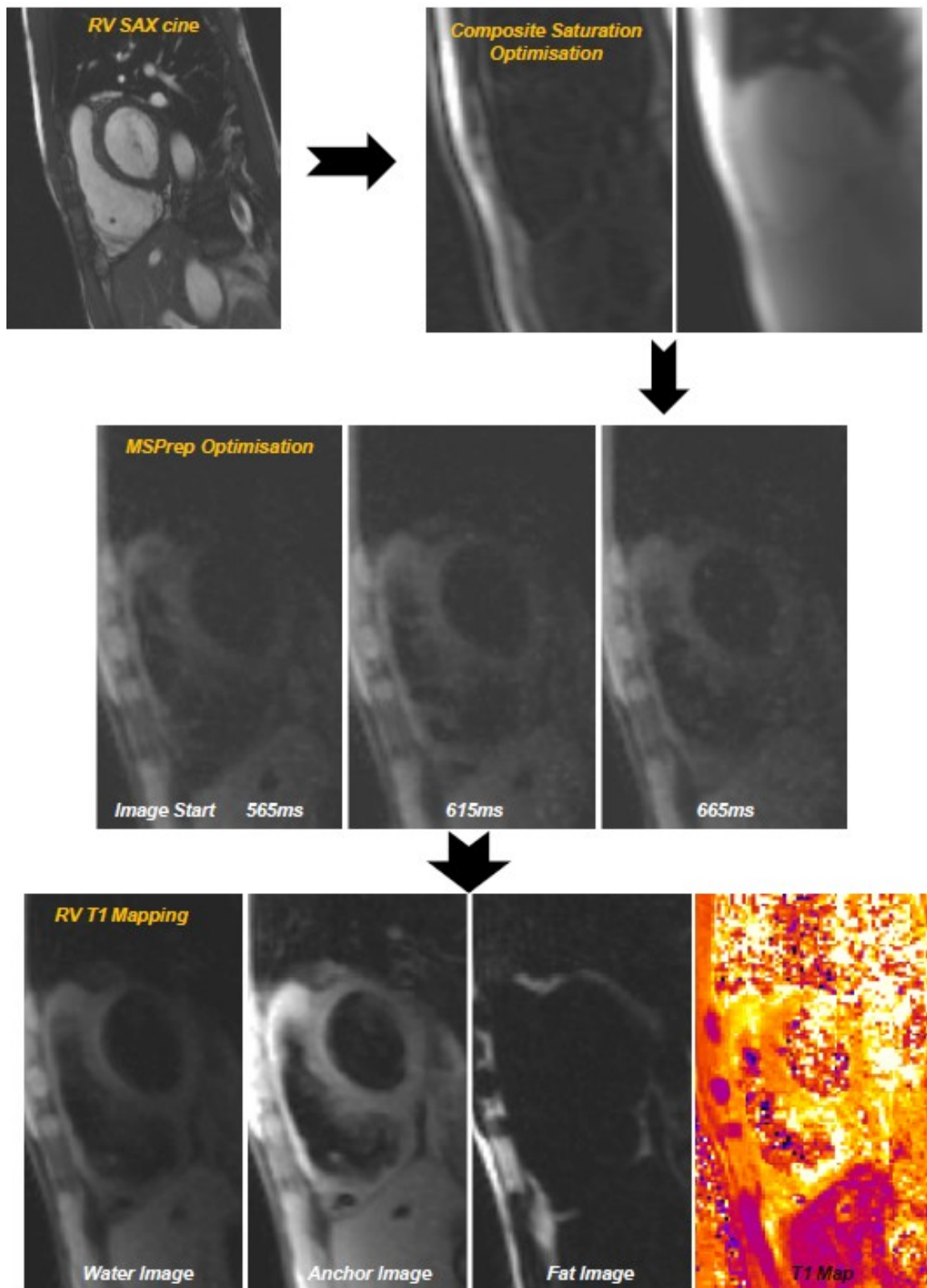


Figure 5.16: Sequential outline of RV T1 mapping in normal volunteer. The same RV SAX slice is selected throughout. MSPrep optimisation images demonstrate that an image start time of 615ms in this instance provides the best myocardial definition when compared to 565ms and 665ms (while the RV is not unusable at those times lest any drift should occur, a loss of RV signal at either of those

times would have ruled out proceeding with the use of 615ms at 40cm/s FOS and would have required further test scans). Typically 6 to 12 test scans for MSPREP must be expected.

Diastolic imaging was therefore carried out at 615ms with a FOS 40cm/s to produce representative images of free-breathing, fat-water separated saturation recovery imaging and the corresponding pixelwise T1 map in the bottom panel.

5.2.5 RV T1 Mapping Reproducibility

Healthy volunteers were recruited who had no history of cardiovascular disease, were not on any medications and lifelong non-smokers. Patients with rTOF attending for clinical CMR scans were also approached for separate attendance for RV T1 research CMR. The majority of patients with rTOF approached were asymptomatic. All subjects provided informed written consent with study approval from the National Research Ethics Service and completed the scan protocol outlined in the previous sub-section. RV myocardial T1 and ECV quantification was derived, with LV T1 and ECV measured concurrently by MOLLI. Inter-observer reproducibility was calculated by CoV = (within-subject standard deviation/mean) x100%.

Data for 22 subjects (11 patients with rTOF and 11 age and gender-matched healthy volunteers) were acquired (*Figure 5.18* and *Table 5.8*). Patients with rTOF had significantly larger RV volumes and lower, but preserved, biventricular ejection fractions when compared to normals. RV T1 maps were obtained in all subjects, with no significant difference in RV native T1 and ECV of patients with rTOF compared to controls who had thinner RV walls: mean RV T1 in rTOF 1233±52ms vs normals 1270±97ms (p=0.28) and RV ECV in rTOF 43.4±6.2% vs normals 44.5±7.0% (p=0.70). There were no significant differences in LV T1 and LV ECV differences between the two groups. Inter-observer reproducibility showed that CoV was 1.8% for native RV T1 and RV ECV CoV 6.8% (*Table 5.9*).

The similar native RV T1 and ECV seen in patients with rTOF and controls may reflect the modest sample size, or the inclusion of clinically stable patients with rTOF with minimal residual haemodynamic lesions and consequently less extreme fibrosis burdens. Our results may also reflect technical limitations. Importantly, saturation was optimised to <1% in the RV but non-uniformity of saturation was often seen across the heart, potentially skewing LV measurements by the RVT1 SASHA-based method. Higher RV ECVs may in part reflect higher fibrosis content, but may also be the result of imperfect subject-specific optimisation of MSPrep to minimise partial volume contamination by blood. This effect is likely to be more prominent in the thinner normal RV, causing falsely elevated RV ECVs in them. The known underestimation of native T1 by MOLLI compared to SASHA may also explain some of the RV-LV difference seen.

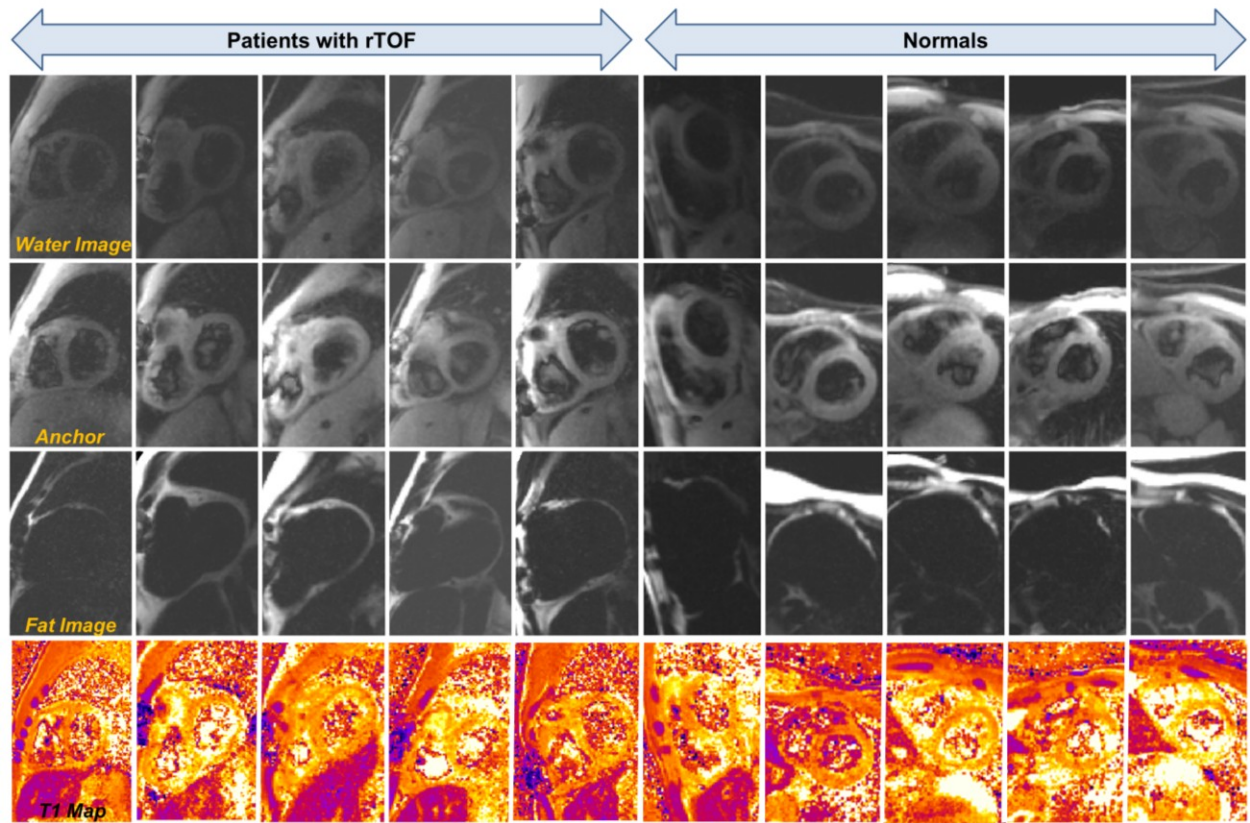


Figure 5.18: Representative short-axis images from subjects scanned by fat-water separated, MsPrep dark blood imaging for RV T1 mapping. Images for five subjects with rTOF and five healthy volunteers are shown (one subject per column). *Top row* – MoCo averaged water-only image at Ts600ms, *Second row* – MoCo averaged water-only anchor image at same window/level, *Third row* – MoCo averaged fat only image, *Bottom row* – T1 map generated from registration and 2-parameter fit of the six images per sampling scheme.

The different image orientation for most rTOF compared to normals was not considered likely to be relevant: the phase-encode direction was not changed. This orientation is simply a display convention and the angle of this plane was near to 45 degrees in some axis used by the scanner reconstruction that governed the displayed orientation.

	rTOF (n=11)	Controls (n=11)	p value
Age, years	37.5 ± 9.5	35.3 ± 8.9	0.59
Gender (Male/Female)	7/4	6/5	0.73
 RVEDVi, ml/m ²	 102.8 ± 22.4	 75.6 ± 16.3	 0.04
RVESVi, ml/m ²	49.2 ± 14.0	25.4 ± 8.7	<0.01
RVSVi, ml/m ²	53.6 ± 10.5	50.4 ± 9.7	0.47
RVEF, %	52.6 ± 5.1	66.7 ± 6.4	<0.01
RVMi, g/m ²	37.3 ± 11.6	30.3 ± 6.0	0.09
LVEDVi, ml/m ²	85.9 ± 16.4	76.4 ± 13.5	0.16
LVESVi, ml/m ²	35.8 ± 10.0	25.6 ± 6.1	0.01
LVSVi, ml/m ²	50.0 ± 8.2	50.8 ± 8.6	0.83
LVEF, %	58.8 ± 5.2	66.6 ± 3.9	<0.01
LVMi, g/m ²	58.0 ± 10.2	59.9 ± 7.4	0.62
Pulmonary regurgitant fraction, %	6.2 ± 13.6	0.7 ± 1.2	0.44
RAAi, cm ² /m ²	13.7 ± 5.4	10.6 ± 1.4	0.08
LAAi, cm ² /m ²	9.8 ± 3.7	10.7 ± 1.5	0.49
 <u>SASHA</u>			
Native RV T1, ms	1233 ± 52	1270 ± 97	0.28
Post Gd RV T1, ms	364 ± 37	341 ± 34	0.15
RV ECV, %	43.4 ± 6.2	44.5 ± 7.0	0.70
 <u>MOLLI</u>			
Pre Gd LV myocardial T1, ms	1016 ± 35	1019 ± 29	0.85
Post Gd LV myocardial T1, ms	461 ± 38	455 ± 28	0.67
Pre Gd blood T1, ms	1600 ± 60	1631 ± 82	0.32
Post Gd blood T1, ms	308 ± 28	289 ± 26	0.12
MOLLI LV ECV, %	26.3 ± 3.2	25.1 ± 2.7	0.33

Table 5.8: Study subject demographics and T1 mapping results reported as mean ± standard deviation. Note that MOLLI was used for LV values as the new method was targeted on the RV wall.

	<i>Parameter</i>	<i>Observer 1</i>	<i>Observer 2</i>	<i>Inter-Observer *</i>	<i>CoV (%)</i>
SASHA	Native RV T1, ms	1248 ± 86	1254 ± 79	-6 ± 33	1.8
	Post Gd RV T1, ms	366 ± 55	350 ± 46	17 ± 21	4.9
	RV ECV, %	41.9 ± 7.1	44.4 ± 6.9	-2.5 ± 3.3	6.8
MOLLI	Native LV T1, ms	1015 ± 33	1020 ± 32	-5 ± 19	1.3
	Post Gd LV T1, ms	457 ± 39	458 ± 29	-1 ± 18	2.6
	Pre Gd Blood T1, ms	1616 ± 72	1615 ± 72	1 ± 6	0.2
	Post Gd Blood T1, ms	299 ± 28	298 ± 28	2 ± 4	1.0
	LV ECV, %	25.8 ± 3.4	25.6 ± 2.7	0.2 ± 1.8	5.0

Table 5.9: Results of inter-observer variability analysis.

*Mean±SD of the signed differences between both observers

5.2.6 Limitations

Histological validation of the proposed new RVT1 technique has yet to be carried out. Whilst a prospective study was planned with ethics approval in place for patients with rTOF undergoing elective PVR, the preceding technical development towards a robust sequence was essential. There are outstanding technical issues that must be addressed prior to embarking upon a prospective clinical study. Subsequent to further technical modifications, intra-session precision, intra- and inter-observer reproducibility needs to be determined with larger patient and volunteer numbers. Patients with rTOF included thus far do not represent the full spectrum of haemodynamic and phenotypic variability in this cohort. Wider ranges of RV ECV may be anticipated with the inclusion of more extreme phenotypes.

The operator-dependence of this kind of CMR work cannot be overstated. CMR is sometimes described as “operator-independent” in contrast to echocardiography, and would be totally specious in this context. The free-breathing approach was adopted mainly due to the full-recovery “anchor” image required by saturation-recovery imaging.

5.2.7 Future work

The need for further technical work has already been alluded to. Specific issues continuing on from this work includes:

1. The fixed 50% MoCo acceptance was likely to be changing the final averaged output image magnitude depending on the quality of the accepted images used for averaging. The exact behaviour of the CXMOCO-averaging code was never completely characterised and requires further study. The T1-weighted images used in this SASHA application are very sensitive to alterations in the image magnitude, unlike all previous applications of the CXMOCO-averaged free-breathing mode.
2. The impact of the MsPrep upon T1 estimation although theoretically minimal as it should cancel out as a constant factor affecting all T1-weighted and anchor images, remains suspect and unproven. For example, it was unclear whether MsPrep could distort T1 if the B1-field was miscalibrated. High-quality B1-mapping was not available *in vivo* within the project timeframe within a short acquisition duration and should be targeted to tackle non-uniformity across the heart.
3. T1 measurements from the final pixelmaps could be improved upon, for example with a magnitude-weighted ROI-average for T1 by using the anchor image. Potentially, some phase-information might have been available also to discriminate remaining blood signal

from myocardium and could possibly have been used to filter blood data out of the final ROI to improve accuracy.

5.3 Conclusions

Multi-echo fat/water separated SASHA with blood signal suppression shows early promise for a clinically unmet need for RV T1 mapping in CHD. Further technical work is needed prior to clinical assessment of relationships between RV T1 and ECV in rTOF with clinical parameters and outcomes. The clinical utility of CMR for assessing lower level fibrosis in the RV remains speculative due to the challenges described. The routine use of T1 phantoms (also for other cardiac applications of ECV) is supported by our findings, and has been amalgamated into continuing practice at our CMR unit.

Chapter 6 Genomic Signatures in Repaired Tetralogy of Fallot: Myocardial Fibrosis and Implications for Cardiac Remodelling

For the genomics work presented in this chapter, an introduction and literature review were presented in *Introduction section 1.5*. The design and experimental work included within this chapter were conducted in the timeframe between October 2012 and June 2015.

Each of these Chapter 6 conclusions is reiterated in the overall setting of the thesis Conclusions (Chapter 8).

6.1 Aims and Methods

Aim: To investigate genomic signatures of the pathological RV in patients with rTOF.

We hypothesize that unbiased genome wide analysis of the RV in rTOF with RNA sequencing may predict RV fibrosis extent and subsequently restrictive physiology phenotypes. We anticipate that fibrosis pathway genes may be implicated in differential gene expression profiles in rTOF.

6.1.1 Patient Population

Patients with rTOF aged ≥ 16 years scheduled for elective pulmonary valve replacement (PVR) were approached for study inclusion. All eligible patients were under the care of the Adult Congenital Heart Disease service at the Royal Brompton Hospital, UK. Institutional ethics committee approval was in place, with informed consent obtained from all study participants. Tissue studies were compliant with UK Human Tissue Act guidelines. Patients provided specific signed permission for RV myocardial biopsies to be taken intra-operatively under direct vision during open heart surgery when judged technically feasible by the operating surgeon. Clinical data from non-invasive investigations performed as part of the surgical work-up was available, which included electrocardiography, chest radiograph, echocardiography and CMR. RV restrictive physiology was defined by laminar antegrade flow in the pulmonary artery in late diastole, present throughout the respiratory cycle on echocardiography (as per *Section 2.5.2*). RV myocardial tissue samples from 30 patients with rTOF were promptly snap-frozen in liquid nitrogen at the time of tissue sampling intra-operatively.

RV control tissue for comparison was obtained from structurally normal hearts donated for cardiac transplantation, from which RV myocardial biopsies were made available via the Cardiovascular Biomedical Research Unit Biobank of the Royal Brompton & Harefield NHS Foundation Trust. Donor RV tissue was collected and stored at the time of surgery in a similar fashion.

6.1.2 RNA Sequencing

RNA was extracted from frozen RV samples using TRIzol (Thermo Fisher Scientific) in accordance with the manufacturer's protocol and quantified by ultraviolet spectrophotometry. RNA quality was assessed on the Agilent 2100 bioanalyser. RNA integrity numbers ranged from 6.3 to 9.1 (mean 8.2 ± 0.6) and 1 μ g of total RNA was used. Library preparation was then completed with Illumina TruSeq RNA sample preparation kits (Illumina, San Diego, USA) utilizing the poly-A enriched for mRNA protocol yielding samples for 27 patients with rTOF and 11 RV controls. Support for RNA extraction and library preparation was provided by Dr Leanne E Felkin, Postdoctoral Research Associate, Molecular Biology Group, Harefield Heart Science Centre, National Heart & Lung Institute, Imperial College London.

Samples were pooled (4-5 samples/pool, 2 lanes per pool) to avoid batch effects. Paired-end 100 base-pair sequencing was then carried out on the Illumina Hi-Seq platform at the MRC Clinical Sciences Centre, Hammersmith Hospital, Imperial College London.

6.1.3 Data Processing

Data processing was carried out with bioinformatics support from Miao Kui (Bioinformatician, Duke-NUS Graduate Medical School, Singapore), Aida Morena-Moral (Bioinformatics and Systems Biology, Integrative Genomics and Medicine Group, Imperial College London) and Dr Francesco Pesce (Postgraduate researcher, Department of Clinical Cardiology & Physiology, National Heart & Lung Institute, Imperial College London).

Reads from RNA sequencing were aligned to the human genome to generate gene counts, which were then processed for data normalisation, filtering and adjustment in the following sequence:

1. **Gene counts:** Gene counts were computed with HTSeq (TopHat v2.0.12, Bowtie2 version 2.2.3, Samtools version 0.1.18 and HTSeq 0.6.1). From the HTSeq output, only protein coding genes with status known in Ensemble version GRCh37 were selected (18,964 genes).

2. **Filtering of genes with low expression:** Fragments per kilobase of transcript per million mapped reads (FPKM) were computed with *DESeq2R* package (version *DESeq2_1.6.3*)⁴³³ using CDS gene lengths retrieved from Ensembl Biomart (GRCh37 version). A FPKM-based filtering criterion was applied by keeping only those genes with a value of FPKM=1 in at least 5% of the samples (in this case 2 samples). Following this, the number of genes reduced from 18,964 genes to 13,936 genes (*Figure 6.1*). The final distribution of the filtered expression data supports two overlapping major abundance classes as previously described.⁴³⁴

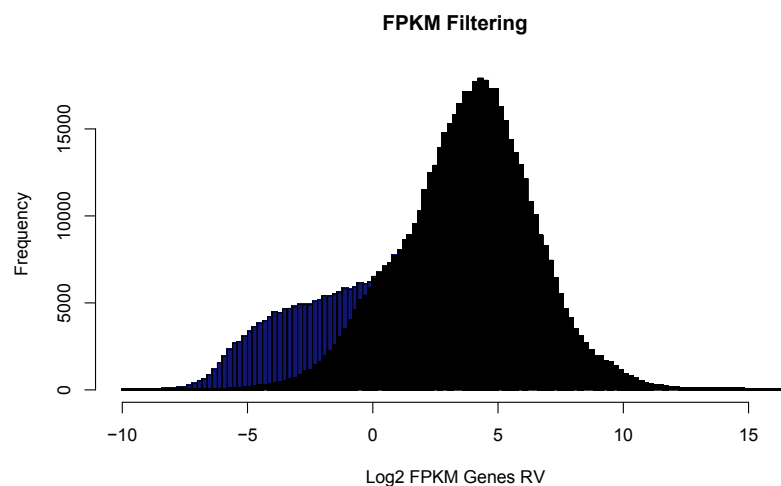


Figure 6.1: Log2 FPKM values of the genes in the RV cohort, before (light purple) and after (dark purple) filtering out genes expressed at low levels. The filtering criterion applied was FPKM=1 in at least 5% of the cohort samples (i.e. in at least two samples).

3. **Data transformation:** Variance stabilising transformation (VST) and size factors normalisation was applied to the raw gene counts by using *DESeq2R* package (version *DESeq2_1.6.3*)⁴³³ (*Figure 6.2*). The global gene expression for each RV sample after VST transformation and filtering was then plotted as in *Figure 6.3*.

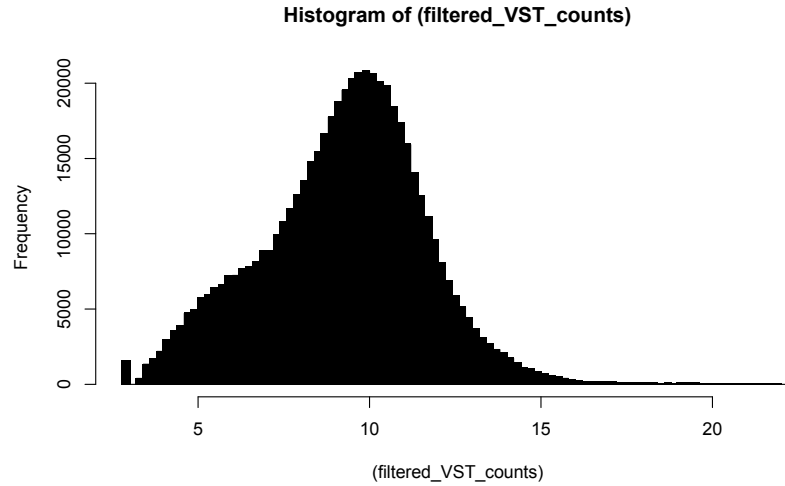


Figure 6.2: Distribution of the filtered VST gene counts in the RV data cohort.

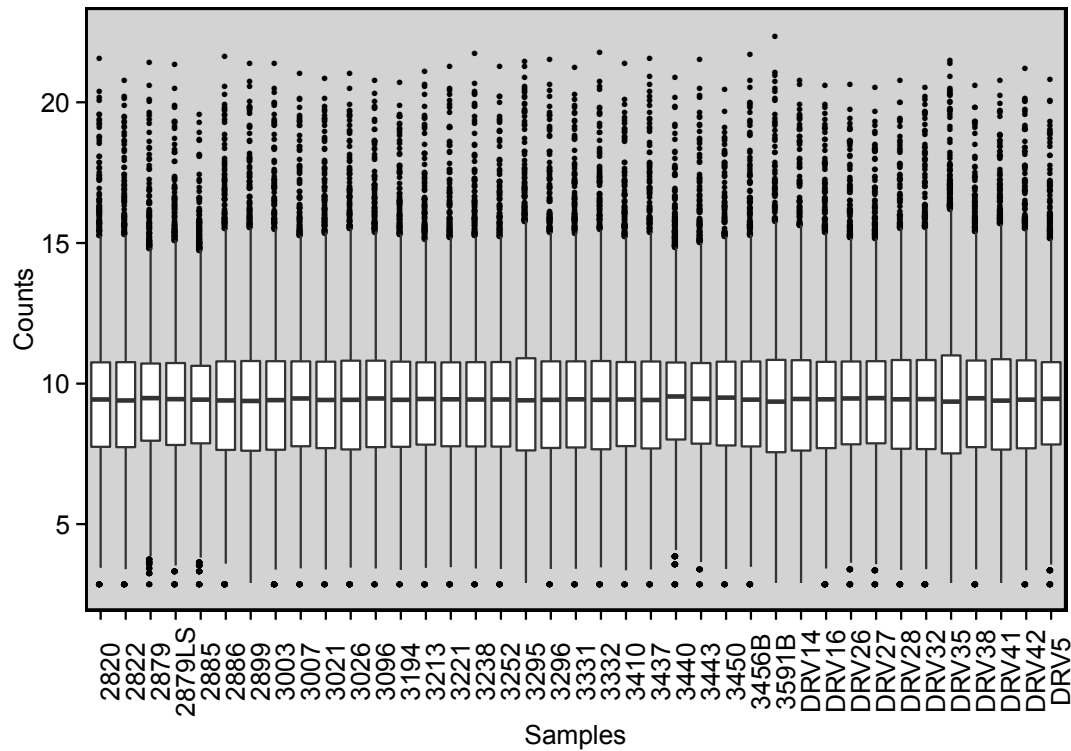


Figure 6.3: Boxplot of global gene expression after VST transformation and filtering, in which each boxplot represents each RV sample (rTOF patients denoted by case numbers, whilst controls denoted by 'DRV' prefix).

4. **Adjusting for covariates:** After filtering, the data was split into two groups comprising patients with rTOF and controls. For subsequent analyses, patients with rTOF were included only if gene expression data and histological fibrosis quantification results (see *Section 2.4*) were available. The final cohort sample size therefore consisted of 27 patients with rTOF and 11 controls.

Gene expression counts of patients with rTOF were adjusted for: 1) patient age at time of tissue collection (that is, age at PVR) and 2) gender. This adjustment was performed by taking the residuals of a multivariate linear model in which both age and gender were added as predictors.

Of the 11 control samples, age and gender data were not available for a subset (3 samples missing age and 1 sample had no gender information available). The missing gender value was imputed by clustering the expression levels of selected sex specific genes as previously applied⁴³⁵ across the control samples. This enabled the derivation of female gender for control (DRV38) as shown in *Figure 6.4*. After completing gender imputation, the gene expression counts of the control samples were adjusted for gender only by taking the residuals of a linear model in which gender was added as a predictor.

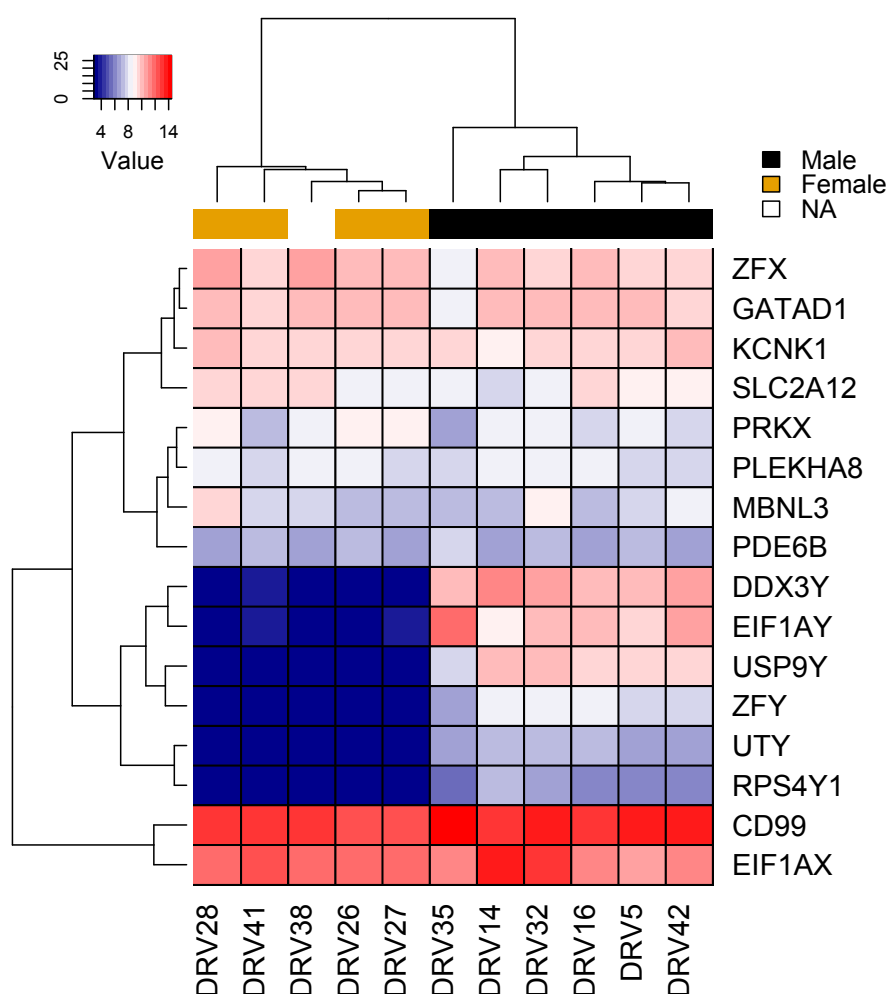


Figure 6.4: Heat map of VST transformed expression counts levels for selected sex associated genes.⁴³⁵ In the top bar, samples are colour coded by gender (*black*: male, *orange*: female and *white*: sample with missing gender).

6.1.4 RV data analyses

6.1.4.1 Differential gene expression testing

Differentially expressed genes (DEGs) between TOF patients and controls samples were computed with the R package *DESeq2* (version *DESeq2_1.6.3*) with gender as a covariate in the model. To reduce the number of missing adjusted p-values, *cooksCutoff* *DeSeq* parameter was set to False.

Functional Gene Set Enrichment Analysis (GSEA) for differential gene expression: rTOF versus controls

Ensembl gene IDs were mapped to gene symbols (Ensembl version GRCh37) and were ranked by Benjamin and Hochberg adjusted p-value of differential expression. GSEA^{436, 437} was run in classic, pre-ranked mode with 10,000 iterations for the following sets: KEGG, GO, Reactome, MiR, TF targets sets and Hallmark gene sets. These gene sets were downloaded (11th March 2015) from <http://www.broadinstitute.org/gsea/downloads.jsp>. The minimum gene set size was set to 10 whilst the maximum gene set size was set to 5,000.

6.1.4.2 Relating gene expression to fibrosis burden in rTOF

Correlations were performed between the VST transformed, filtered and adjusted gene expression counts with histological levels of fibrosis by computing the corresponding Spearman correlation and p-value with the R function *corAndPvalue* from the R package WGCNA (version *WGCNA_1.42*).^{438, 439} Applying a nominal p-value significance level of 0.05 (see section 6.1.5 regarding multiple testing), 357 genes were associated with RV fibrosis burden in rTOF (which included both positive and negative correlations).

Functional Gene Set Enrichment Analysis (GSEA) for fibrosis in rTOF

Ensembl gene IDs were mapped to gene symbols (using Ensembl version GRCh37) and were now ranked by nominal p-value of Spearman correlation with fibrosis (computed as described in 6.1.4.1). GSEA was run in classic, pre ranked mode with 10,000 iterations for KEGG, GO, Reactome, MiR, TF targets sets and Hallmark gene sets. These gene sets were downloaded (11th March 2015) from <http://www.broadinstitute.org/gsea/downloads.jsp>. As per the previous section, the minimum gene set size was set to 10 whilst the maximum gene set size was set to 5,000.

6.1.4.3 Genomic network analysis in rTOF

STRING 10⁴⁴⁰ was used for protein-protein association analysis. The top 25 DEGs (ranked by nominal p value) were used as input for STRING analysis for rTOFs vs controls, restrictive vs non-restrictive phenotypes and by correlation to fibrosis burden. These generated networks based on data from experimental protein-protein interactions and

curated databases. Network analysis data was produced with the support of Dr Catherine Francis (Clinical Research Fellow, National Heart & Lung Institute, Imperial College London).

6.1.5 Statistical Analysis

Clinical data normality was assessed by Kolmogorov-Smirnov analysis. Results are reported as mean $\pm$ standard deviation (SD) or median (IQR) according to data distribution. Groups were assessed by paired Student t tests or Wilcoxon test as appropriate. Categorical variables were compared by Fisher's exact test. Correlations were assessed by Pearson's and Spearman correlation tests.

Statistical analysis of genome data was carried out with R software (v.3.1.3) and SPSS (IBM Statistics V.22). Student's t test and Benjamin and Hochberg multiple testing corrections for false discovery rate (FDR) were applied. The FDR-adjusted p value was defined as <0.05 .

6.2 Results

6.2.1 Baseline Characteristics

A total of 27 adult patients with rTOF consented and had intra-operative RV myocardial biopsies during surgical PVR (mean age 32.0 ± 10.6 years, 78% male) with clinical characteristics as outlined in *Table 6.1*. The predominant haemodynamic lesion in this cohort prior to surgery was pulmonary regurgitation (PR), with CMR volumetric and functional data illustrating significant RV dilatation and overall preserved biventricular systolic function (*Table 6.1*).

Of the 11 RV donor control tissues analysed, the mean age (n=8) was 34 ± 13 years, with gender male/female/unknown = 6/4/1 prior to imputation. The causes of death for controls included road traffic accidents (n=3), intracranial bleed or subarachnoid haemorrhage (n=2), pulmonary fibrosis (n=1) and bacterial meningitis (n=2). The cause of death for 2 donors could not be identified as local documentation had been destroyed. Nevertheless, the donor hearts were examined as normal for transplant use. The two groups were comparable in terms of age and gender.

Patient Population (n=27)	
Age, years	32.0 ± 10.6
Gender (Male/Female)	21/6
rTOF with associated lesions *	2
Presence of symptoms, n (%)	17 (63)
Predominant haemodynamic lesion - PR/PS/Both	25/1/1
Heart rate, bpm	72 ± 10
Systolic blood pressure, mmHg	117 ± 11
Diastolic blood pressure, mmHg	71 ± 9
QRS duration on ECG, ms	152 ± 23
Cardiothoracic Ratio	0.54 ± 0.05
Previous palliative procedures, n (%)	12 patients with 14 procedures
Blalock-Taussig shunt	8
Waterston shunt	4
Brock procedure	1
Open pulmonary valvotomy	1
Characteristics of initial repair	
Age, years	4.2 ± 3.4
Transannular patch (%)	14 (52)
RVOT patch (%)	9 (33)
RV to PA conduit (%)	3 (11)
Surgical details not known (%)	1 (4)
Restrictive RV physiology at echo, n (%)	6 (22)
CMR at study recruitment	
RVEDVi, ml/m ²	146.8 ± 36.6
RVESVi, ml/m ²	73.2 ± 26.3
RVSVi, ml/m ²	73.5 ± 20.3
RVEF, %	50.9 ± 9.8
cRVEF, %	28.7 ± 9.1
RVMi, g/m ²	62.6 ± 14.2
Pulmonary regurgitant fraction, %	38.2 ± 12.4
RAAi, cm ² /m ²	14.8 ± 3.3
LVEDVi, ml/m ²	78.9 ± 17.7
LVESVi, ml/m ²	31.6 ± 12.7
LVSVi, ml/m ²	47.2 ± 8.7
LVEF, %	60.7 ± 7.6
LVMi, g/m ²	65.5 ± 14.4
LAAi, cm ² /m ²	9.4 ± 2.1
RV LGE score [†]	4.9 ± 2.1

Table 6.1: Demographics of study subjects with rTOF

* 1 pulmonary atresia, 1 absent pulmonary valve. [†] RV/LV insertion points not included

6.2.2 Differential Gene Expression in rTOF

Computation of raw gene counts revealed 18,964 genes that were differentially expressed in rTOF compared to normal controls. After filtering and normalization to account for co-variates of age and gender, a total of 4,662 genes were significantly differentially expressed with Benjamin and Hochberg adjusted p-value <0.05.

There was upregulation of 2187 genes and downregulation of 2475 genes. The dominant functional themes of upregulated genes included extracellular matrix proteins (collagens, SMAD family, bone morphogenic proteins), angiotensin pathway and transcription factors. The downregulated genes encompassed TGF β , matrix metalloproteinases, serine-threonine kinases and interleukins (IL10, IL12A, IL18, IL24) as listed in *Tables 6.2 (a) to (f)*.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
<u>Collagens</u>						
Collagen, type XXII, alpha 1	COL22A1	ENSG00000169436	30.54	1.777	0.00000398	8
Collagen, type XI, alpha 1	COL11A1	ENSG00000060718	4.52	1.513	0.00115491	1
Collagen, type XI, alpha 2	COL11A2	ENSG00000204248	323.78	1.416	0.00000056	6
Collagen, type IX, alpha 1	COL9A1	ENSG00000112280	70.63	1.412	0.00002453	6
Collagen, type IX, alpha 2	COL9A2	ENSG00000049089	45.16	1.304	0.00000440	1
Collagen, type XX, alpha 1	COL20A1	ENSG00000101203	1.96	1.294	0.00509359	20
Collagen, type X, alpha 1	COL10A1	ENSG00000123500	14.76	1.181	0.00364939	6
Collagen, type XIV, alpha 1	COL14A1	ENSG00000187955	2066.41	1.156	0.00236392	8
Collagen beta(1-O)galactosyltransferase 2	COLGALT2	ENSG00000198756	616.30	0.944	0.00006537	1
Collagen, type XVI, alpha 1	COL16A1	ENSG00000084636	1311.09	0.922	0.00001661	1
Collagen, type VIII, alpha 2	COL8A2	ENSG00000171812	201.87	0.867	0.02071743	1
Collagen, type IV, alpha 5	COL4A5	ENSG00000188153	456.53	0.783	0.00007206	X
Collagen triple helix repeat containing 1	CTHRC1	ENSG00000164932	208.76	0.734	0.00184119	8
Collagen, type IV, alpha 6	COL4A6	ENSG00000197565	582.93	0.667	0.00483240	X
Collagen, type VII, alpha 1	COL7A1	ENSG00000114270	887.46	0.614	0.04074082	3
Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	COL4A3BP	ENSG00000113163	1330.30	0.318	0.00329035	5
Collagen beta(1-O)galactosyltransferase 1	COLGALT1	ENSG00000130309	2056.97	-0.369	0.01447415	19
Collagen, type VI, alpha 3	COL6A3	ENSG00000163359	8477.09	-0.523	0.01938363	2
Ficolin (collagen/fibrinogen domain containing) 1	FCN1	ENSG00000085265	217.73	-1.508	0.00018965	9

Table 6.2(a): Collagen network genes with differential gene expression in rTOF.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
<u>Matrix Metalloproteinases and TIMPs</u>						
Matrix metalloproteinase 17	MMP17	ENSG00000198598	74.36	0.723	0.04204718	12
Matrix metalloproteinase 25	MMP25	ENSG00000008516	63.86	-0.734	0.04302554	16
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	ENSG00000196611	3.24	-1.800	0.00005448	11
TIMP metalloproteinase inhibitor 2	TIMP2	ENSG00000035862	8729.25	0.766	0.00098263	17
TIMP metalloproteinase inhibitor 1	TIMP1	ENSG00000102265	5384.59	-1.044	0.00839530	X
<u>Extracellular Matrix components</u>						
Fibronectin type III domain containing 1	FNDC1	ENSG00000164694	632.83	1.537	0.00000019	6
Extracellular leucine-rich repeat & fibronectin type III domain containing 2	ELFN2	ENSG00000166897	9.44	1.283	0.00026708	22
Fibronectin leucine rich transmembrane protein 2	FLRT2	ENSG00000185070	395.62	1.015	0.00784635	14
Leucine rich repeat and fibronectin type III domain containing 1	LRFN1	ENSG00000128011	22.25	0.778	0.00223264	19
Fibronectin type III and ankyrin repeat domains 1	FANK1	ENSG00000203780	20.94	0.581	0.03620723	10
Integrin, beta 1	ITGB1	ENSG00000150093	17709.50	-0.689	0.00630626	10
Fibronectin type III domain containing 4	FNDC4	ENSG00000115226	117.97	-0.705	0.04613300	2

Table 6.2(b): Extracellular matrix genes differentially expressed in rTOF.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
<u>Transforming Growth Factor</u>						
TGFB1-induced anti-apoptotic factor 1	TIAF1	ENSG00000221995	235.86	0.672	0.00355378	17
TGF-beta activated kinase 1/MAP3K7 binding protein 2	TAB2	ENSG00000055208	2995.24	-0.424	0.00252481	6
Transforming growth factor, beta 2	TGFB2	ENSG00000092969	496.35	-1.031	0.00708477	1
SMAD family member 9	SMAD9	ENSG00000120693	347.30	0.817	0.00005404	13
SMAD family member 7	SMAD7	ENSG00000101665	991.64	0.513	0.00127677	18
SMAD family member 6	SMAD6	ENSG00000137834	899.63	0.461	0.01698642	15
Bone morphogenetic protein 6	BMP6	ENSG00000153162	126.00	1.144	0.00004319	6
Bone morphogenetic protein 4	BMP4	ENSG00000125378	546.07	0.842	0.00000795	14
Bone morphogenetic protein 2	BMP2	ENSG00000125845	189.12	0.676	0.00139239	20
Bone morphogenetic protein 8b	BMP8B	ENSG00000116985	75.74	-0.814	0.01653561	1
Bone morphogenetic protein 7	BMP7	ENSG00000101144	449.97	-1.103	0.00171874	20
<u>Tumour Necrosis Factor</u>						
C1q and tumor necrosis factor related protein 3	C1QTNF3	ENSG00000082196	92.06	1.506	0.00000398	5
C1q and tumor necrosis factor related protein 7	C1QTNF7	ENSG00000163145	211.47	1.298	0.00000048	4
Tumor necrosis factor receptor superfamily, member 25	TNFRSF25	ENSG00000215788	704.26	0.915	0.00000131	1
TNF receptor-associated factor 1	TRAF1	ENSG00000056558	312.70	0.580	0.00047799	9
TNF receptor-associated factor 7, E3 ubiquitin protein ligase	TRAF7	ENSG00000131653	907.47	-0.456	0.00287157	16
Lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR	ENSG00000111321	1438.77	-0.465	0.01321206	12
Tumor necrosis factor, alpha-induced protein 1 (endothelial)	TNFAIP1	ENSG00000109079	2372.49	-0.506	0.00026790	17
Tumor necrosis factor receptor superfamily, member 19	TNFRSF19	ENSG00000127863	1719.69	-0.610	0.00764822	13
Tumor necrosis factor receptor superfamily, member 10c	TNFRSF10C	ENSG00000173535	37.06	-0.691	0.01826353	8
Tumor necrosis factor receptor superfamily, member 11a	TNFRSF11A	ENSG00000141655	74.11	-0.855	0.01302223	18
Tumor necrosis factor, alpha-induced protein 8-like 3	TNFAIP8L3	ENSG00000183578	349.22	-0.964	0.00827119	15
Tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	ENSG00000181634	10.16	-1.003	0.01829831	9
Tumor necrosis factor receptor superfamily, member 11b	TNFRSF11B	ENSG00000164761	695.21	-1.047	0.02575774	8
Tumor necrosis factor receptor superfamily, member 12A	TNFRSF12A	ENSG00000006327	2120.06	-1.073	0.00613420	16
Tumor necrosis factor (ligand) superfamily, member 8	TNFSF8	ENSG00000106952	17.87	-1.081	0.00284071	9
Tumor necrosis factor, alpha-induced protein 6	TNFAIP6	ENSG00000123610	212.86	-1.109	0.00119116	2
Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	ENSG00000125735	60.97	-1.251	0.00226041	19
C1q and tumor necrosis factor related protein 1	C1QTNF1	ENSG00000173918	5383.77	-1.329	0.00000006	17
Tumor necrosis factor, alpha-induced protein 8-like 2	TNFAIP8L2	ENSG00000163154	51.76	-1.534	0.00000002	1

Table 6.2(c): TGF and TNF pathway genes differentially expressed in rTOF.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
Interleukins						
Interleukin 16	IL16	ENSG00000172349	267.32	0.693	0.00046746	15
Interleukin enhancer binding factor 3, 90kDa	ILF3	ENSG00000129351	6164.89	-0.207	0.01755845	19
Interleukin 17 receptor A	IL17RA	ENSG00000177663	640.94	-0.428	0.00517796	22
Interleukin 13 receptor, alpha 1	IL13RA1	ENSG00000131724	1376.96	-0.534	0.01574806	X
Interleukin enhancer binding factor 2	ILF2	ENSG00000143621	3186.56	-0.555	0.00001255	1
Interleukin 18 receptor 1	IL18R1	ENSG00000115604	108.05	-0.706	0.00653832	2
Interleukin-1 receptor-associated kinase 1	IRAK1	ENSG00000184216	1613.54	-0.742	0.00007917	X
Interleukin 24	IL24	ENSG00000162892	26.57	-0.763	0.02591955	1
Interleukin 12A	IL12A	ENSG00000168811	39.15	-0.780	0.00018513	3
Interleukin 17 receptor B	IL17RB	ENSG00000056736	124.45	-0.902	0.00003767	3
Interleukin 18	IL18	ENSG00000150782	61.41	-0.904	0.00053635	11
Interleukin 20 receptor, alpha	IL20RA	ENSG00000016402	281.69	-0.916	0.01409140	6
Interleukin 18 binding protein	IL18BP	ENSG00000137496	326.52	-0.916	0.00102858	11
Interleukin 10	IL10	ENSG00000136634	16.80	-0.946	0.03426991	1
Interleukin 18 receptor accessory protein	IL18RAP	ENSG00000115607	32.83	-1.039	0.00427462	2
Interleukin 15 receptor, alpha	IL15RA	ENSG00000134470	359.58	-1.006	0.00000091	10
Interleukin 1 receptor-like 1	IL1RL1	ENSG00000115602	108.58	-1.124	0.01658266	2
Interleukin 12 receptor, beta 1	IL12RB1	ENSG00000096996	23.77	-1.132	0.00007863	19
Interleukin 4 induced 1	IL4I1	ENSG00000104951	25.23	-1.282	0.00199747	19
Interleukin 1 receptor, type II	IL1R2	ENSG00000115590	21.27	-1.286	0.00014856	2
Interleukin 2 receptor, alpha	IL2RA	ENSG00000134460	42.21	-1.585	0.00000009	10

Table 6.2(d): Interleukins differentially expressed in rTOF.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
Natriuretic peptide receptor 3	NPR3	ENSG00000113389	2234.81	1.579	0.00000004	5
Natriuretic peptide C	NPPC	ENSG00000163273	119.32	-2.123	0.00000000	2
Endothelin receptor type A	EDNRA	ENSG00000151617	1075.03	0.837	0.00001554	4
Endothelin receptor type B	EDNRB	ENSG00000136160	1261.57	-0.657	0.00192958	13
Endothelin converting enzyme 2	ECE2	ENSG00000145194	76.68	-1.033	0.00039317	3
Endothelin 2	EDN2	ENSG00000127129	15.93	-2.088	0.00000016	1

Table 6.2(e): Neurohormones differentially expressed in rTOF.

Gene Name	Gene Symbol	Ensembl.Gene.ID	baseMean	log2FoldChange	FDR p val	chromosome
<u>Angiotensin</u>						
Angiotensin II receptor, type 2	AGTR2	ENSG00000180772	50.80	1.362	0.00030641	X
Angiotensin I converting enzyme	ACE	ENSG00000159640	915.56	0.929	0.00038029	17
<u>Hypoxia Inducible Factor</u>						
Hypoxia inducible factor 3, alpha subunit	HIF3A	ENSG00000124440	3179.92	0.624	0.03660569	19
Hypoxia inducible factor 1, alpha subunit	HIF1A	ENSG00000100644	2564.44	-0.735	0.00105012	14
<u>T-box transcription factor</u>						
T-box 1	TBX1	ENSG00000184058	62.70	1.163	0.00044253	22
T-box 15	TBX15	ENSG00000092607	44.66	0.771	0.04670507	1
T-box 19	TBX19	ENSG00000143178	48.65	0.588	0.02892979	1
T-box 2	TBX2	ENSG00000121068	2058.64	0.551	0.00538794	17
<u>Platelet Derived Growth Factor</u>						
Platelet derived growth factor C	PDGFC	ENSG00000145431	489.29	0.294	0.00761500	4
Platelet-derived growth factor receptor-like	PDGFRL	ENSG00000104213	491.86	0.857	0.00601296	8
<u>Serine/Threonine Kinases</u>						
Serine threonine kinase 39	STK39	ENSG00000198648	1165.99	-0.478	0.01573548	2
Serine/threonine kinase 3	STK3	ENSG00000104375	585.06	-0.580	0.00000751	8
Serine/threonine kinase 17a	STK17A	ENSG00000164543	1280.84	-0.632	0.03799311	7
Serine/threonine kinase 40	STK40	ENSG00000196182	3119.08	-0.718	0.00004202	1
<u>Calmodulins</u>						
Calcium/calmodulin-dependent protein kinase IG	CAMK1G	ENSG00000008118	7.67	1.334	0.00175338	1
Calmodulin-like 6	CALML6	ENSG00000169885	9.91	0.708	0.01756129	1
Calmodulin 3 (phosphorylase kinase, delta)	CALM3	ENSG00000160014	9801.69	-0.363	0.03287020	19
Calmodulin 2 (phosphorylase kinase, delta)	CALM2	ENSG00000143933	14863.53	-0.382	0.00352491	2
Calmodulin-lysine N-methyltransferase	CAMKMT	ENSG00000143919	160.61	-0.462	0.00002263	2
Calmodulin regulated spectrin-associated protein family, member 3	CAMSAP3	ENSG00000076826	18.07	-0.970	0.02029419	19
<u>Smooth muscle related</u>						
Parvin, alpha	PARVA	ENSG00000197702	2904.07	-0.504	0.00011724	11
Calponin 1, basic, smooth muscle	CNN1	ENSG00000130176	7738.17	-1.589	0.00001119	19
<u>Miscellaneous</u>						
Lumican	LUM	ENSG00000139329	10460.23	0.958	0.00211219	12
Cardiotrophin 1	CTF1	ENSG00000150281	891.13	0.638	0.00407785	16
Lamin A/C	LMNA	ENSG00000160789	13630.84	-0.596	0.01125345	1

Table 6.2(f): Differential gene expression in rTOF.

Gene Set Name	Size	ES	NES	FDR p-val	RANK AT MAX	LEADING EDGE
HALLMARK_KRAS_SIGNALING_DN	188	0.1709	2.8056	0.000	9437	tags=69%, list=52%, signal=141%
KEGG_CALCIIUM_SIGNALING_PATHWAY	176	0.1452	2.2021	0.014	10027	tags=69%, list=55%, signal=152%
GO (Cellular Component)						
EXTRACELLULAR_MATRIX	99	0.2026	2.3393	0.011	7384	tags=61%, list=40%, signal=101%
PROTEINACEOUS_EXTRACELLULAR_MATRIX	98	0.2088	2.3855	0.017	7384	tags=61%, list=40%, signal=102%
EXTRACELLULAR_MATRIX_PART	57	0.2466	2.2094	0.019	7361	tags=65%, list=40%, signal=108%

Table 6.3(a): Upregulated gene expression sets in rTOF.

Gene Set Name	Size	ES	NES	FDR p-val	RANK AT MAX	LEADING EDGE
Hallmark						
HALLMARK_MTORC1_SIGNALING	197	-0.3972	-6.4425	0.000	6357	tags=74%, list=35%, signal=112%
HALLMARK_INFLAMMATORY_RESPONSE	197	-0.2502	-4.1457	0.000	6787	tags=62%, list=37%, signal=98%
HALLMARK_IL6_JAK_STAT3_SIGNALING	87	-0.3372	-3.6784	0.000	2688	tags=48%, list=15%, signal=56%
HALLMARK_OXIDATIVE_PHOSPHORYLATION	199	-0.2218	-3.6123	0.000	9388	tags=73%, list=51%, signal=149%
HALLMARK_PI3K_AKT_MTOR_SIGNALING	104	-0.2858	-3.3882	0.000	5520	tags=59%, list=30%, signal=84%
HALLMARK_TNFA_SIGNALING_VIA_NFKB	196	-0.1610	-2.5741	0.000	5290	tags=45%, list=29%, signal=63%
HALLMARK_INTERFERON_ALPHA_RESPONSE	93	-0.2218	-2.5085	0.001	8731	tags=70%, list=48%, signal=133%
Kegg						
KEGG_OXIDATIVE_PHOSPHORYLATION	114	-0.2673	-3.2817	0.000	8763	tags=75%, list=48%, signal=143%
KEGG_MAPK_SIGNALING_PATHWAY	260	-0.1012	-1.8810	0.024	8851	tags=58%, list=48%, signal=112%
Reactome						
REACTOME_RAF_MAP_KINASE_CASCADE	10	-0.6236	-2.4450	0.001	6878	tags=100%, list=38%, signal=160%
REACTOME_MAP_KINASE_ACTIVATION_IN_TLR_CASCADE	48	-0.2734	-2.1993	0.005	8333	tags=73%, list=46%, signal=134%
GO (BP)						
INFLAMMATORY_RESPONSE	121	-0.1600	-2.0800	0.019	8342	tags=62%, list=46%, signal=113%
JAK_STAT_CASCADE	30	-0.3100	-2.0600	0.021	2801	tags=47%, list=15%, signal=55%
REGULATION_OF_JAK_STAT_CASCADE	11	-0.5000	-2.0500	0.022	2529	tags=64%, list=14%, signal=74%

Table 6.3(b): Downregulated gene expression sets in rTOF.

GSEA functional analysis showed altered expression of MTORC1 signalling pathway (FDR p value <0.01), IL-6 JAK signaling pathway (FDR p value = 0.00) and NOTCH signaling (FDR p value = 0.04). There was upregulation of extracellular matrix pathways (*Table 6.3a*) and downregulation of MTORC1, inflammatory response, IL-6 JAK and MAPK signaling as well as oxidative phosphorylation pathways (*Table 6.3b*).

6.2.3 Genomic signatures of fibrosis in rTOF

There was widely variable extent of fibrosis present on histological analysis in RV myocardial biopsy samples of patients with rTOF (as illustrated in *Figure 6.5* with analysis method described in *Section 2.4*). The median collagen volume fraction was 14.1% (range 4.8-60.2%) derived from the analysis of 20 fields per sample at 250x magnification. When gene expression and fibrosis extent were analysed in combination, there was differential expression of 16 genes (with FDR p value<0.05). TGF β receptor associated protein 1, angiopoietin-like 4, interleukin 10 and 32 negatively correlated with fibrosis, whilst R-spondin 4, myosin light chain 12B and checkpoint kinase 1 positively correlated with fibrosis (*Table 6.4*).

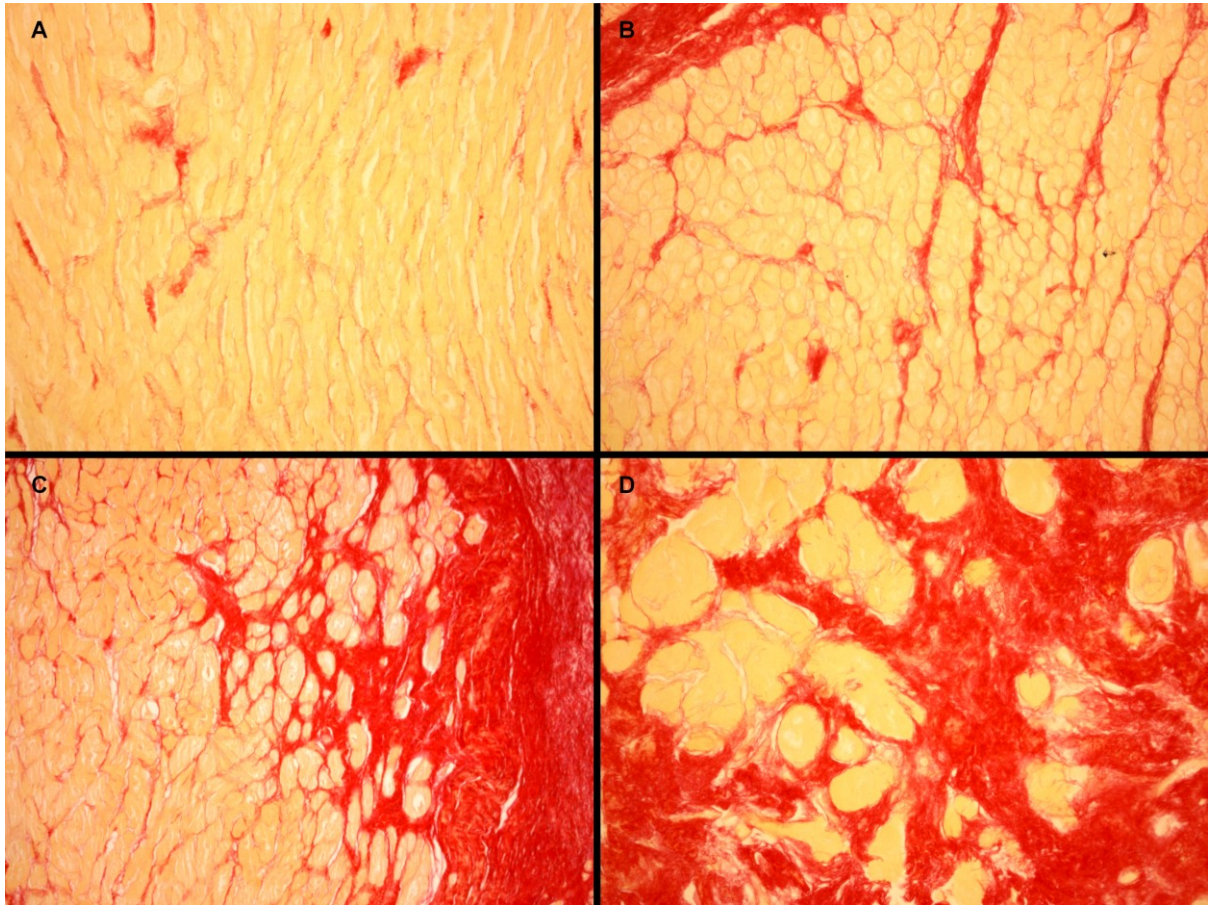


Figure 6.5: Variable extent of RV myocardial fibrosis seen with picosirius red stain, ranging from predominantly normal cardiomyocyte structure with pale yellow appearance in panel (A) to increasing extents of fibrosis from panels (B) to (D) with progressive intense red stain. Destruction of normal myocyte architecture with extensive fibrosis is seen in panel (D).

GSEA functional analysis showed upregulation of oxidative phosphorylation gene sets when more fibrosis was present (*Table 6.5a*); with downregulation of TGF β , MAPK signaling, collagen formation, extracellular matrix and transcription factor activity (*Table 6.5b*). When comparing gene expression profiles of higher fibrosis burden ($\geq 14\%$) rTOF patients with lower fibrosis burden ($\leq 14\%$) patients, there was upregulation of oxidative phosphorylation pathways with downregulation of TGF β , MAPK and MTORC1 signaling (data not shown).

Gene Name	Gene Symbol	Ensembl_ID	Spearman correlation (R)	Nominal p-value
Interleukin 10	IL10	ENSG00000136634	-0.535	0.004
Angiopoietin-like 4	ANGPTL4	ENSG00000167772	-0.487	0.010
Transforming growth factor, beta receptor associated protein 1	TGFBRAP1	ENSG00000135966	-0.470	0.013
Discoidin domain receptor tyrosine kinase 1	DDR1	ENSG00000204580	-0.439	0.022
Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2RY6	ENSG00000171631	-0.436	0.023
C1q and tumor necrosis factor related protein 1	C1QTNF1	ENSG00000173918	-0.421	0.029
Snf2-related CREBBP activator protein	SRCAP	ENSG00000080603	-0.405	0.036
Dickkopf WNT signalling pathway inhibitor 3	DKK3	ENSG00000050165	-0.397	0.040
TNF receptor-associated factor 6, E3 ubiquitin protein ligase	TRAF6	ENSG00000175104	-0.393	0.043
Interleukin 32	IL32	ENSG00000008517	-0.383	0.049
C1q and tumor necrosis factor related protein 9	C1QTNF9	ENSG00000240654	0.381	0.050
Checkpoint kinase 1	CHEK1	ENSG00000149554	0.389	0.045
Tumor necrosis factor, alpha-induced protein 8	TNFAIP8	ENSG00000145779	0.391	0.044
Plasminogen receptor, C-terminal lysine transmembrane protein	PLGRKT	ENSG00000107020	0.398	0.040
Myosin, light chain 12B, regulatory	MYL12B	ENSG00000118680	0.474	0.013
R-spondin 4	RSPO4	ENSG00000101282	0.493	0.009

Table 6.4: Differentially expressed genes correlated to fibrosis burden in rTOF.

Gene Set Name	SIZE	ES	NES	FDR p-val	RANK AT MAX	LEADING EDGE
Hallmark						
HALLMARK_OXIDATIVE_PHOSPHORYLATION	198	0.3961	6.5054	0	5255	tags=77%, list=38%, signal=121%
HALLMARK_MYC_TARGETS_V1	197	0.2951	4.7767	0	6484	tags=76%, list=47%, signal=139%
Kegg						
KEGG_OXIDATIVE_PHOSPHORYLATION	108	0.4688	5.5489	0	5129	tags=83%, list=37%, signal=131%
KEGG_CARDIAC_MUSCLE_CONTRACTION	58	0.3051	2.7564	0.0001	6336	tags=76%, list=45%, signal=139%

Table 6.5(a): GSEA gene sets upregulated with fibrosis extent in rTOF.

Gene Set Name	SIZE	ES	NES	FDR p-val	RANK AT MAX	LEADING EDGE
Hallmark						
HALLMARK_TNFA_SIGNALING_VIA_NFKB	189	-0.4017	-6.4216	0.0000	4212	tags=70%, list=30%, signal=99%
HALLMARK_INFLAMMATORY_RESPONSE	182	-0.2443	-3.9021	0.0000	6059	tags=68%, list=43%, signal=118%
HALLMARK_IL6_JAK_STAT3_SIGNALING	80	-0.3007	-3.1537	0.0000	5763	tags=71%, list=41%, signal=121%
HALLMARK_TGF_BETA_SIGNALING	52	-0.2884	-2.4552	0.0004	2429	tags=46%, list=17%, signal=56%
HALLMARK_HYPOXIA	186	-0.1460	-2.2697	0.0021	2039	tags=29%, list=15%, signal=34%
Kegg						
KEGG_JAK_STAT_SIGNALING_PATHWAY	101	-0.2670	-3.1162	0.0000	5551	tags=66%, list=40%, signal=109%
KEGG_TGF_BETA_SIGNALING_PATHWAY	73	-0.3082	-3.1047	0.0000	3746	tags=58%, list=27%, signal=78%
KEGG_ECM_RECEPTOR_INTERACTION	71	-0.2868	-2.7685	0.0000	6035	tags=72%, list=43%, signal=126%
KEGG_MAPK_SIGNALING_PATHWAY	215	-0.1542	-2.6454	0.0001	6765	tags=64%, list=49%, signal=122%
Reactome						
REACTOME_NFKB_AND_MAP_KINASES_ACTIVATION_MEDIATED_BY_TLR4_SIGNALING	68	-0.2642	-2.5332	0.0023	4125	tags=56%, list=30%, signal=79%
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	64	-0.2569	-2.4326	0.0041	6453	tags=72%, list=46%, signal=133%
REACTOME_COLLAGEN_FORMATION	48	-0.2880	-2.3797	0.0057	6453	tags=75%, list=46%, signal=139%
REACTOME_MAP_KINASE_ACTIVATION_IN_TLR_CASCADE	48	-0.2884	-2.3560	0.0060	4125	tags=58%, list=30%, signal=83%
REACTOME_SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX	58	-0.2296	-2.1085	0.0215	3542	tags=48%, list=25%, signal=64%
REACTOME_INTERFERON_SIGNALING	136	-0.1464	-2.0067	0.0318	9457	tags=82%, list=68%, signal=254%
REACTOME_TRANSCRIPTIONAL_ACTIVITY_SMAD2 to 4_HETEROTRIMER	36	-0.2760	-1.9383	0.0408	3907	tags=56%, list=28%, signal=77%
GO (Biological Process)						
INFLAMMATORY_RESPONSE	93	-0.2100	-2.3800	0.0060	6499	tags=68%, list=47%, signal=126%
TRANSCRIPTION	652	-0.0700	-2.1700	0.0180	8329	tags=67%, list=60%, signal=158%
REGULATION_OF_MAP_KINASE_ACTIVITY	57	-0.2400	-2.0900	0.0270	3067	tags=46%, list=22%, signal=58%
GO (Cellular Component)						
EXTRACELLULAR_MATRIX	81	-0.2100	-2.2400	0.0090	6683	tags=69%, list=48%, signal=132%
GO (Molecular Function)						
TRANSCRIPTION_FACTOR_ACTIVITY	260	-0.1311	-2.4251	0.0109	2014	tags=27%, list=14%, signal=31%
CYTOKINE_ACTIVITY	66	-0.2385	-2.2366	0.0276	2394	tags=41%, list=17%, signal=49%

Table 6.5(b): GSEA gene sets downregulated with fibrosis in rTOF.

6.2.4 Genomic signatures of the restrictive rTOF phenotype

Patients with RV restrictive physiology (n=6) trended towards a greater extent of fibrosis seen histologically with percentage collagen fraction $21.2 \pm 16.3\%$ vs $17.5 \pm 14.7\%$ in non-restrictive patients with rTOF (p=0.64).

There were 31 DEGs in patients with rTOF and restrictive phenotypes when compared to non-restrictive patients. Specifically, there was upregulation of extracellular matrix protein genes and B natriuretic peptide (*Table 6.6*).

Gene Name	Gene Symbol	Ensembl ID	baseMean	log2FoldChange	FDR p value
Natriuretic peptide B	NPPB	ENSG00000120937	4368.65	1.377	0.00089
Interleukin 1 receptor-like 1	IL1RL1	ENSG00000115602	61.81	1.318	0.02115
Epiregulin	EREG	ENSG00000124882	17.19	1.170	0.02184
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	ENSG00000196611	15.16	0.908	0.02957
Lysyl oxidase-like 4	LOXL4	ENSG00000138131	300.27	1.164	0.04002

Table 6.6: Genes upregulated in restrictive rTOF phenotype.

GSEA functional analysis showed altered expression of the hypoxia-induced transcription factor 1 (HIF-1) signaling and focal adhesion pathways. Gene ontology (GO) hierarchical analysis once again highlighted the importance of the extracellular matrix in the restrictive rTOF phenotype (*Figure 6.6*). When examining gene networks in the restrictive phenotype by STRING v10 analysis, interactions of fibrosis genes including TGF pathway (TGF β , SMADs), MMPs, TIMPs with fibrinolysis pathway genes were evident as illustrated in *Figure 6.7*.

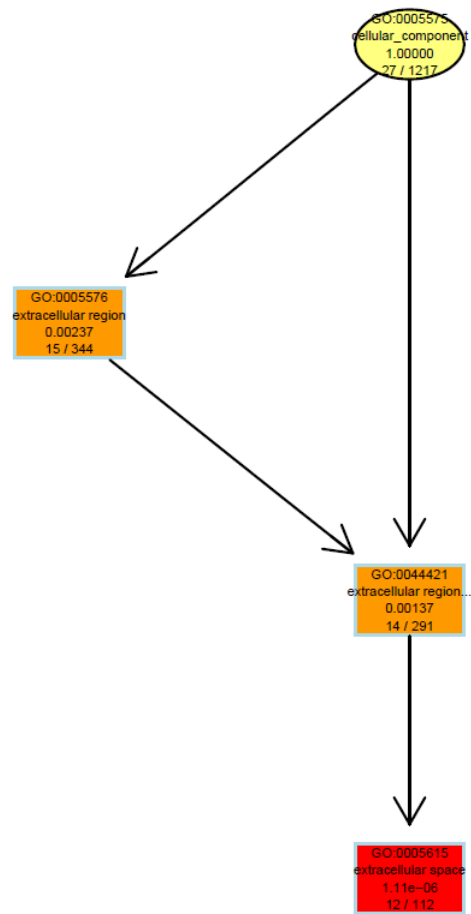


Figure 6.6: GO hierarchical analysis of cellular components gene expression in restrictive rTOF phenotype, illustrating the dominant role of the extracellular matrix in this phenotype.

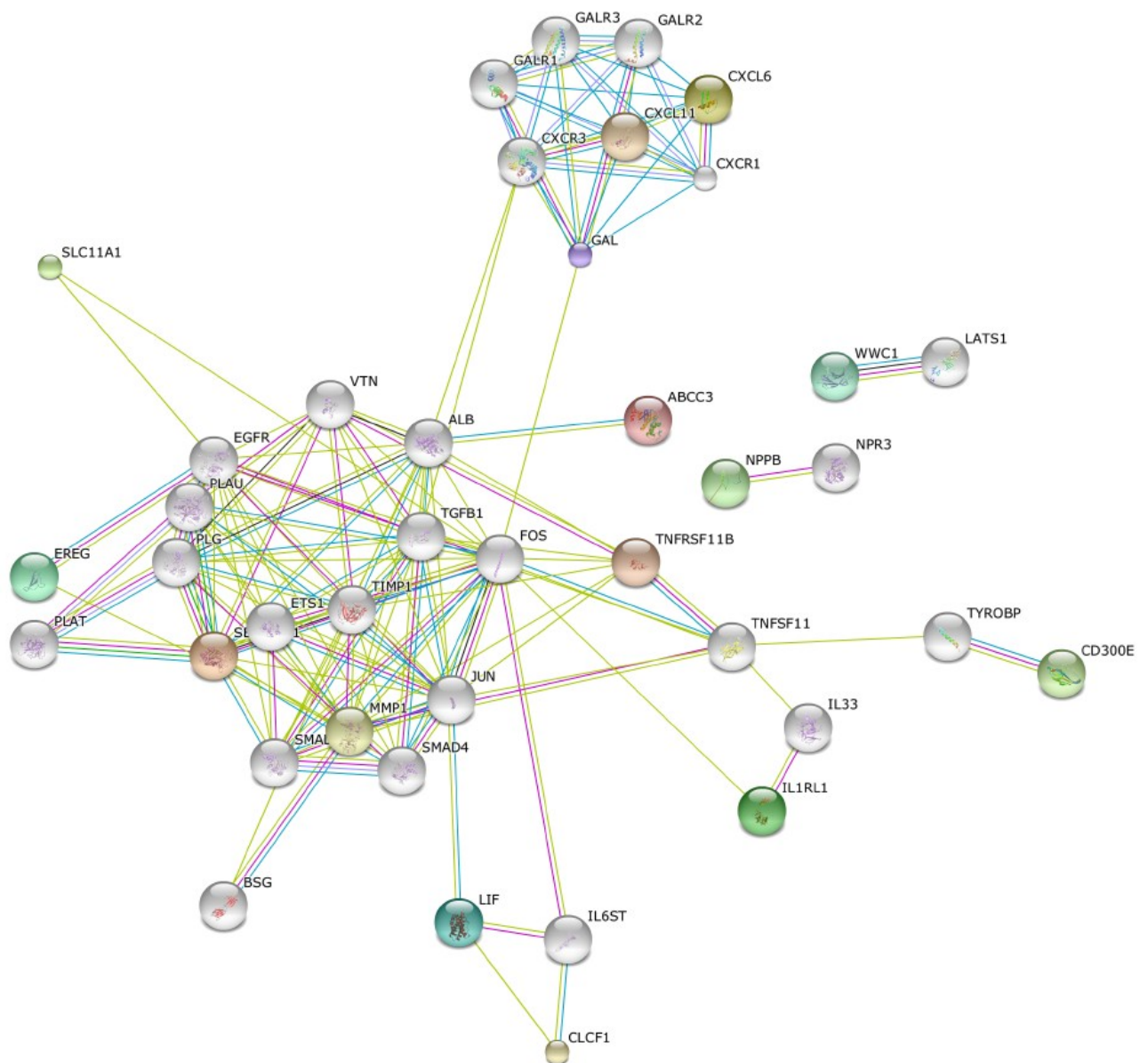


Figure 6.7: Network analysis of rTOF restrictive phenotype (seeded from top 25 genes differentially expressed in restrictive rTOFs, ranked according to the nominal p value). Protein-protein interactions are clustered around fibrosis-related genes that include TGF β , TIMP1, EGFR, MMP1 and SMADs.

6.2.5 Ubiquitin C (UBC) as a gene network hub in rTOF

The data suggests that ubiquitin C (UBC) may feature as a centralised ‘network hub’ gene in the genomic signature of the pathological RV in rTOF. Gene network analysis (STRING v10) was carried out as described in *Section 6.1.4.3* with protein-protein functional relationships illustrated in *Figures 6.8 and 6.9*. This demonstrated the striking, focal prominence of UBC in gene interactions. Evidence of UBC involvement in rTOF could be validated by Western blotting (which was outside the scope of the current study). Interactions between UBC and

differentially expressed, fibrosis-related genes, in particular TRAF6, CHEK1 and MYL12B were also evident (*Figure 6.10*). Its functional significance merits further investigation.

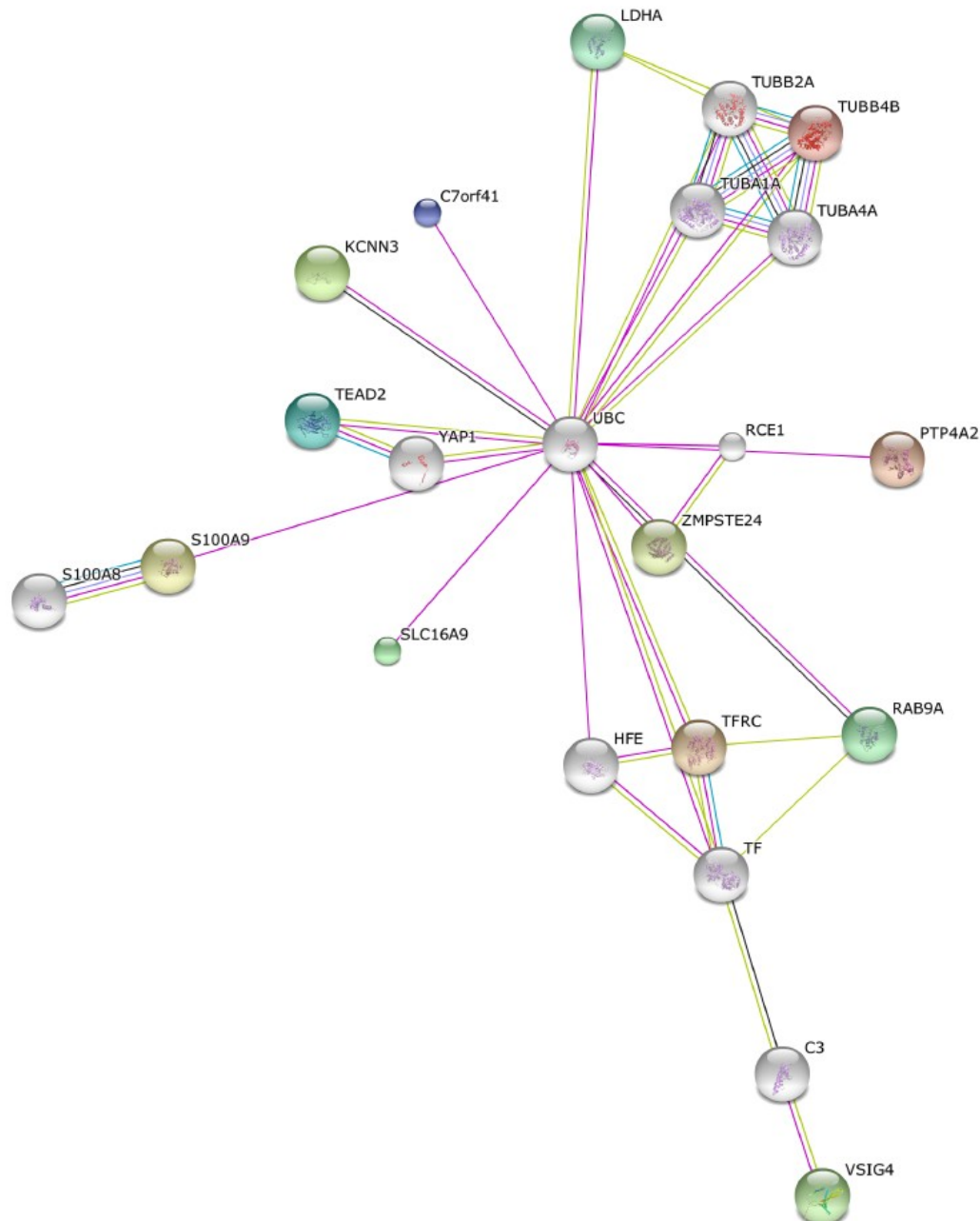


Figure 6.8: Protein-protein interactions of differentially expressed genes in rTOF with UBC as centralised seed.

6.3 Discussion

These RNA genomic findings should be interpreted in the context of this being an exploratory, hypothesis-generating observational study of a small cohort of well-phenotyped patients with rTOF and cannot and should not be over-extrapolated. The main genomic signal of our study suggests that Ubiquitin C may have pertinent molecular interactions and functional implications as a 'network hub' gene in rTOF. Differential gene expression of the RV was evident in rTOF, with emergent functional themes of changes to extracellular matrix, transcription factor expression and neurohormonal activation. There was altered expression of MTORC1, NOTCH, MAPK signalling and oxidative phosphorylation pathways. The RV fibrosis burden in patients with rTOF may influence gene expression, and appeared to modulate extracellular matrix and TGF β expression, as well as the activity of oxidative phosphorylation pathways. Patients with restrictive RV physiology had differential HIF-1 signalling, as well as fibrosis-related protein-protein interactions.

6.3.1 *Altered expression of the extracellular matrix and collagens*

Collagen is the most abundant protein in the body, constituting a vital component of the ECM. Procollagen types I and III are the dominant subtypes in the myocardium, with turnover every 80-120 days delicately regulated by fibroblasts.⁴⁴¹ Fibroblast activity is influenced by cytokines (TNF α , IL-1, IL-6), autocrine and paracrine factors that include angiotensin II, TGF β and circulatory hormones such as aldosterone. Collagen synthesis is achieved via the synthesis and cleavage of procollagen types I and III, whilst collagen degradation is mediated by MMP and TIMPs. Interstitial collagenase (MMP-1) initiates the breakdown of collagens by peptide bond hydrolysis, together with MMP-8 and MMP-13 to generate carboxyl-terminal telopeptide of collagen type I (CITP).⁴⁴²

There is increased expression of collagens in the myocardium of patients with rTOF,⁴⁴³ with significant upregulation of collagen types I and III reported in native TOF patients. Moderate upregulation of collagen type 9 and 15, together with higher fibronectin expression was also reported in a cohort of native (n=12) and rTOF (n=8) patients. Within this reported study, the levels of MMP-13, MMP-17, TIMP-1 and TIMP-4 expression correspondingly declined.²⁹¹

As described in *Section 6.2.2*, our dataset of DEGs in rTOF included an upregulation of expression of collagens, fibronectin, SMAD family 6, 7 and 9 as well as bone morphogenic proteins (BMPs). There was also evidence of altered expression of MMPs and TIMPs. Specifically, our data showed upregulation of collagen types 4, 9, 10, 11, 14, 20 and 22. The clinical significance of the increased expression of each of these collagen subtypes is

difficult to interpret due to our small sample size, as well as the constant and dynamic turnover of collagen elsewhere in the body. The dysregulation of collagen synthesis and degradation observed was in part anticipated, in view of myocardial fibrosis being the pathological hallmark of disease in TOF.

Downregulation of MMP-1 and TIMP-1 gene expression with an upregulation of MMP-17 and TIMP-2 were evident in our data. Our findings are partially discrepant compared to the reported findings of Sharma *et al*, who conversely reported downregulation of MMP-17.²⁹¹ The role of MMPs in the pathological RV and CHD is undefined to date. MMPs are associated with diastolic dysfunction and heart failure in acquired LV disease, and may contribute towards similar outcomes in rTOF. Altered MMP-1 expression has been reported in hypertension,¹⁵⁰ cardiac fibrosis (in aortic stenosis),⁴⁴⁴ dilated cardiomyopathy and ischaemic heart disease.⁴⁴⁵⁻⁴⁴⁸ Initial forays into the relevance of MMP expression in the serum of ACHD patients reported that MMP-2 levels were associated with echocardiographic parameters of biventricular and diastolic dysfunction in patients with rTOF.⁴⁴⁹ However, the interaction between MMP-2 and other biological substrates is highly complex, and serum MMP levels may not accurately reflect myocardial gene expression. Further study is required to elucidate the relevance of these biomarkers in rTOF.

6.3.2 Oxidative phosphorylation in rTOF

Disruption to oxidative phosphorylation and mitochondrial respiratory chain activities have been shown in native TOF at the time of surgical repair. Mitochondrial oxidative phosphorylation adapts to physiological changes in oxygen tension by adjusting the rate of cellular respiration. Increased mitochondria numbers, mitochondrial abnormalities (of vacuolar degeneration and damage to mitochondrial cristae)^{450, 451} with reduced mitochondrial enzyme activity^{452, 453} are present in unrepaired TOF. This reflects impaired electron transportation in the heart due to decreased capacity of mitochondria to synthesize adenosine triphosphate (ATP) in hypoxic conditions.

Our data similarly demonstrated a downregulation of oxidative phosphorylation processes, but in patients with rTOF years after initial surgical repair when cyanosis is no longer the predominant feature. This downregulation of mitochondrial pathways mirrors the findings of a murine model of RV volume overload in which dysfunctional mitochondrial bioenergetics was observed.⁴⁵⁴ Interestingly, there was then upregulation of oxidative phosphorylation pathway activity when more extensive myocardial fibrosis was present. It could be postulated that this association between oxidative phosphorylation and fibrosis may be mediated by increased generation of reactive oxygen species, with contributory effects of angiotensin II and

TGF β .^{455, 456} The drivers for persistence of decreased oxidative phosphorylation in adult rTOF and its increase in fibrosis are not known, and this observation merits more research.

6.3.3 Neurohormonal gene expression in TOF

There is growing evidence for neurohormonal activation in rTOF,³³⁴ with BNP levels predicting adverse outcomes of mortality and sustained arrhythmia in this potentially vulnerable patient cohort.⁷¹ Our data shows altered expression of natriuretic peptides and endothelins. An increase in natriuretic peptide receptor 3 (NPR3) expression was evident, together with a decrease in NPPC expression. NPR3 is a receptor for natriuretic peptide hormones, with similar binding affinities for atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide (NPPC/CNP). It regulates local concentrations for NPPA, NPPB and NPPC through functioning as a clearance receptor. In addition to its interactions with natriuretic peptides, protein-protein interactions between NPR3 and UBC are recognised (see Figures 6.11 and 6.12).^{457, 458}

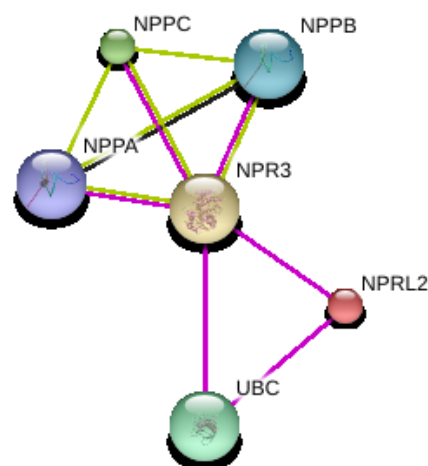


Figure 6.11: Protein-protein interactions of NPR3. Of note, there are important connections with natriuretic peptides (A to C), but also Ubiquitin C (UBC).

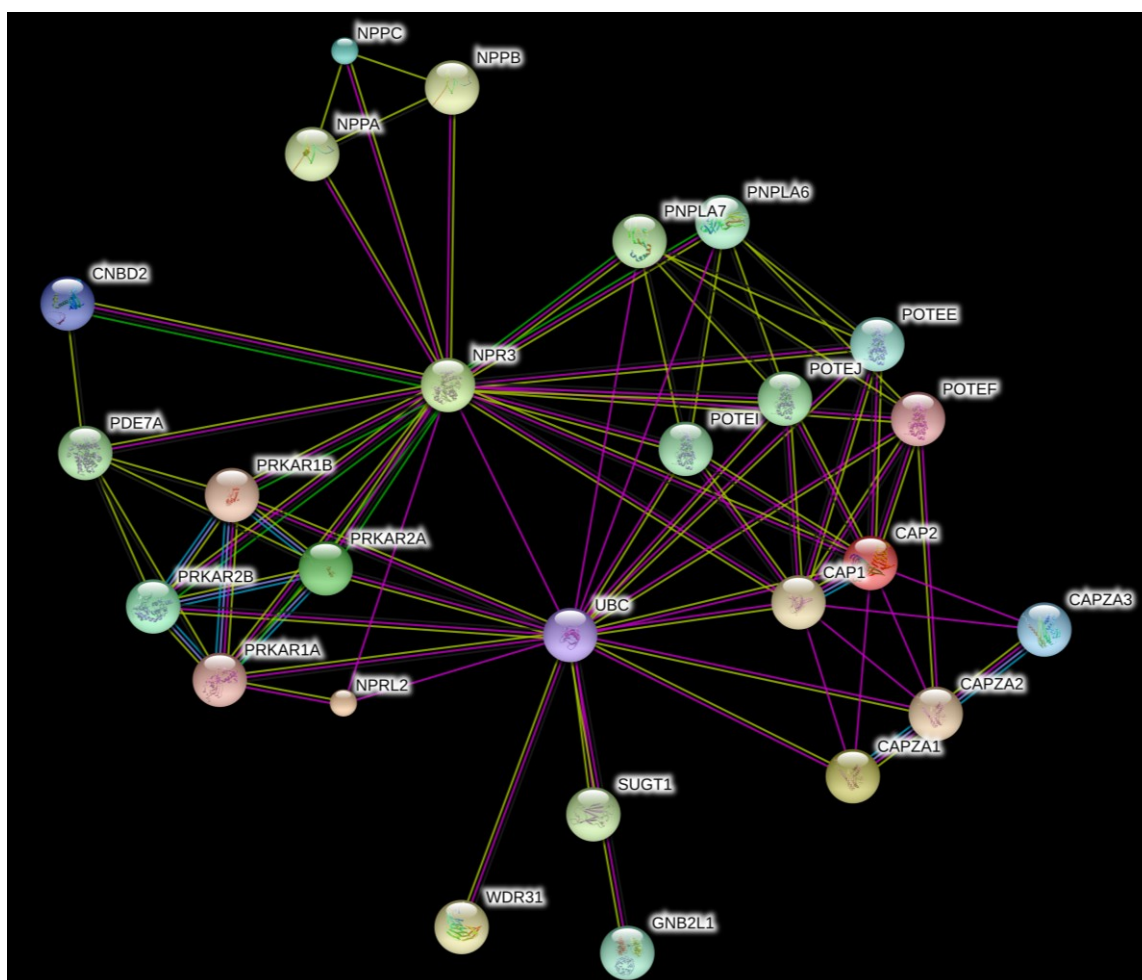


Figure 6.12: Expanded 25 protein interactants of NPR3 with centralized role of NPR3 and UBC.

Turning from the altered expression of natriuretic peptides, we next discuss altered expression of endothelins. There was downregulation of endothelin-2 (EDN2), endothelin converting enzyme 2 (ECE2) and endothelin receptor type B (EDNRB) expression, with increased expression of endothelin receptor type A (EDNRA). Endothelin-1 is the most abundant, widely expressed endothelin isoform in the heart,⁴⁵⁹ and has been shown by our group to be dysregulated in rTOF with elevated circulating levels.⁷¹ Although Endothelin-2 (ET-2) has been less studied than ET-1, it is expressed in the human heart⁴⁶⁰ and exerts effects that include positive inotropy, influencing cardiac conduction and arrhythmias, as well as modulating vascular tone and cardiac remodelling.^{461, 462} The downregulation of ET-2 and ECE2 in our study mirrors the findings of a murine *in vivo* model of heart failure in which ET-2 mRNA decreased, with reciprocal increase in ET-1 mRNA simultaneously. *In vitro* experiments of acute hypoxia in the same study stimulated a marked increase in ET-2 levels,⁴⁶³ whilst a separate murine model of ET-2 over-expression aggravated myocardial interstitial fibrosis.⁴⁶⁴

6.3.4 TGF β in rTOF

Our data demonstrates upregulation of SMAD 6 and 7 activities, together with downregulation of TGF β 2 signaling in rTOF. Perturbations to TGF β expression have been reported in both animal models and human studies of TOF. The inhibitory effects of SMAD 6 and 7 on TGF β are recognized in murine models.⁴⁶⁵⁻⁴⁶⁷ Both up and downregulation of TGF β have been reported in small series of patients with rTOF.^{291, 292} TGF β 2 expression was specifically examined in the RVOT myocardial tissue of 12 children with TOF versus 3 normal controls, as quantified by immunohistochemistry,⁴⁶⁸ where no statistically significant differences in TGF β 2 expression were detected in a modest sample cohort, in contrast to our data.

Myocardial TGF β synthesis has consistently and significantly been upregulated in animal models of heart failure, with evidence of increased levels of TGF β -activators, mRNA and protein induction in the remodeling myocardium.^{469, 470} However, direct evidence of increased TGF β activity is deficient, and the evidence *in vivo* is less consistent. The role of each TGF β isoform in modulating cardiac remodeling remains unknown. Whilst excessive TGF β promotes collagen deposition resulting in myocardial stiffening and diastolic dysfunction, unopposed loss of TGF β signaling (as suggested in our data) may result in matrix degradation, cardiac dilatation and systolic dysfunction. This could be a plausible mechanism in our study cohort patients with significant pulmonary regurgitation and volume overload.

Other signaling pathways such as those involving BMP and Notch are synergistic with TGF β signaling, thereby mediating endothelial-mesenchymal transition during cardiogenesis.⁴⁷¹ The TGF β superfamily has been implicated in the pathogenesis of conotruncal cardiac defects. Dysregulation of TGF β expression in native and rTOF is therefore not entirely surprising, as septation of the conotruncus into the ventricular outflow tract during embryogenesis is critically dependent on TGF β and BMP activity.⁴⁷²

6.3.5 The restrictive RV phenotype and fibrosis

As outlined in *Section 1.1.6*, a potential link between RV fibrosis and restrictive physiology has been reported previously.^{61, 62} Patients with restrictive physiology had gene interactions that centered on fibrosis-related pathways. Whilst we noted a trend towards higher collagen volume fraction in restrictive patients with rTOF, this was not statistically significant in our cohort. We found negative correlations between TGF β superfamily genes (namely IL10, TGFBRAP1 and TRAF6) and the extent of myocardial fibrosis, with corresponding downregulation of TGF β and MAPK signalling. It remains to be seen whether TGF β

expression in this setting is a manifestation of a compensatory response to, or the actual driver for, myocardial fibrosis. A thematic overlap of TGF β superfamily and extracellular matrix genes appeared to be differentially expressed by both fibrotic and restrictive phenotypes.

The restrictive subgroup exhibited increased gene expression of BNP (= NPPB gene symbol). Plasma BNP levels have not previously been discriminative when comparing restrictive to non-restrictive rTOF RVs in adolescents.⁶⁰ In contrast, a prospective study of 80 infants and children with TOF had plasma BNP levels measured days after surgical repair and reported that restrictive RV patients had higher BNP levels than non-restrictive patients.⁴⁷³ Contemporaneous plasma BNP levels were not available in our study patients to contextualize the BNP gene expression findings.

Altered HIF-1 signalling was evident in the restrictive phenotype in our study. Jeewa *et al*⁸⁰⁰ have previously demonstrated a cardioprotective HIF1A genotype in patients with TOF, in which a higher number of functioning HIF1A alleles corresponded to increased fibrosis at the time of surgical repair and subsequently less RV dilation, better preservation of RV function and hence greater freedom from re-intervention. It could therefore be hypothesized that an association between HIF1A allelic status and RV restriction may be possible. This, however, was beyond the scope of this study.

6.3.6 Ubiquitin in rTOF

Our data suggests that ubiquitin C may be an important central protein mediating interactions with other proteins in rTOF. Ubiquitin C (UBC) codes for polyubiquitin and can be induced in response to cellular stress challenges, and is involved in the maintenance of ubiquitin homeostasis.⁴⁷⁴⁻⁴⁷⁶ It is one of four precursor genes (UBB, UBC, UBA52 and UBA80) that combine to form ubiquitin.⁴⁷⁷⁻⁴⁸⁰ Current characterisation of UBC expression and its molecular mechanisms in humans is deficient, with early studies suggesting that the 5'-UTR intron is crucial in regulating UBC gene expression.^{481, 482} Targeted UBC disruption has demonstrated that its function cannot be compensated for by other ubiquitin genes.⁴⁷⁴

Ubiquitin is a small, widely expressed protein covalently attached to proteins in linear chains via a specific enzyme reaction. The ubiquitin-proteasome system (UPS) plays a vital role in the degradation of toxic misfolded, oxidized or damaged proteins, with $\leq 30\%$ of newly synthesized proteins being degraded by the proteasome within minutes of synthesis (see *Figure 6.13*).^{483, 484} It remains controversial as to whether cardiac UPS regulation confers cardiotoxic or cardioprotective effects, with contradictory studies providing evidence to

support both tenets.⁴⁸⁵⁻⁴⁹⁰ UPS is implicated in the pathophysiology of various heart failure syndromes in which protein folding and degradation defects contribute to cardiac dysfunction in disease states. In the LV, these include cardiac amyloid, hypertrophic, dilated and desmin-related cardiomyopathies.^{491, 492} Elevated levels of ubiquitinated proteins and ubiquitin-positive aggregates in failing DCM hearts have been demonstrated.^{493, 494} Aberrant protein degradation with increased protein turnover has been shown to contribute to both cardiac hypertrophy and, paradoxically, atrophy.^{495, 496} Amongst its myriad functions, UPS regulates Notch and VEGF signaling pathways, in addition to HIF degradation within the cardiovascular system, all of which are of recognized to be implicated in TOF.^{497, 498}

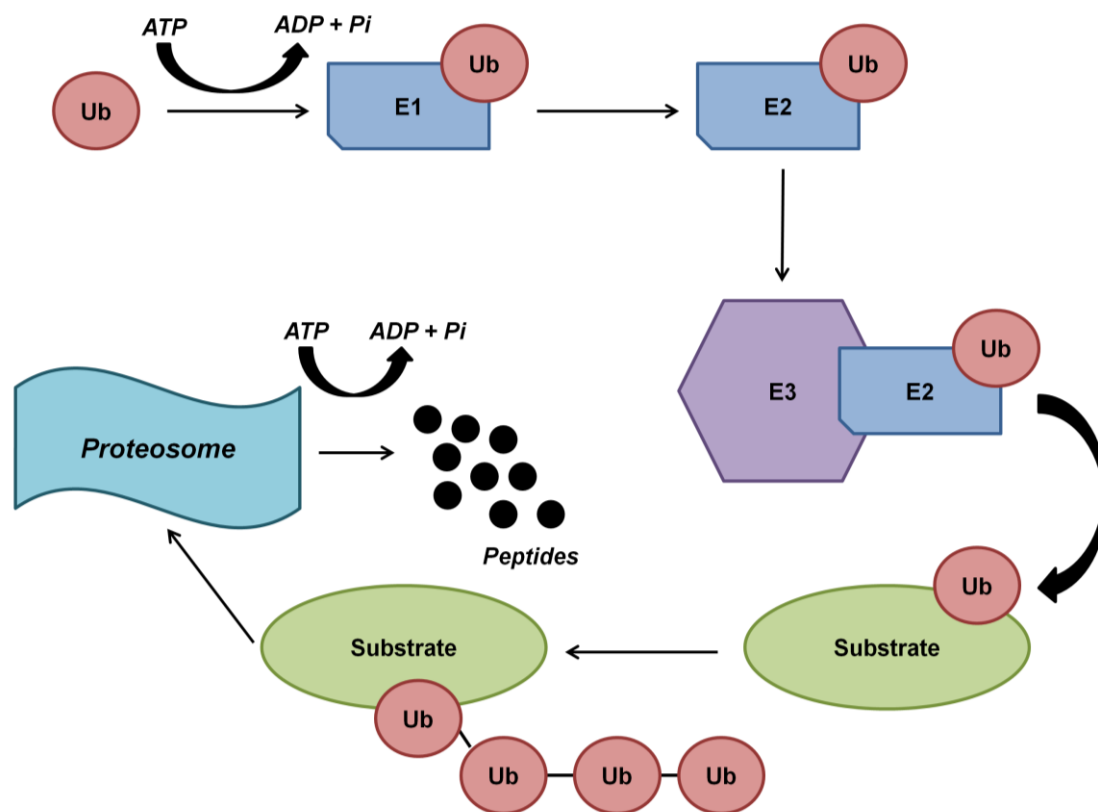


Figure 6.13: Protein degradation by UPS takes place through protein conjugation to ubiquitin (*Ub*) by ubiquitin activating enzyme (*E1*) through an ATP-dependent activation process. Activated ubiquitin is then transferred to ubiquitin conjugating enzyme (*E2*) with linkage to the lysine residue in proteins. This reaction is catalyzed by ubiquitin ligases (*E3*) repeatedly to form an ubiquitin chain. The ubiquitin-conjugated proteins will then be recognized by the 19S subunit of the proteasome and degraded into peptides by the 20S core proteasome, before being released and degraded to amino acids by peptidases in the cytoplasm.^{499, 500}

In spite of its importance, research into RV function and failure has continually lagged behind the study of the LV. The relevance of UPS in RV hypertrophy and failure has only been examined in proteomic murine models to date.^{501, 502} The induction of RV hypertrophy and failure in both these studies have elegantly illustrated that proteasome activity is impaired, with a decrease of transcript and protein levels together with an accumulation of polyubiquitinated proteins and free ubiquitin. This *in vivo* model of UPS in RV failure suggests it may be a plausible molecular mechanism applicable to the late-onset RV failure seen in patients with rTOF.

Furthermore, the involvement of UPS in rTOF may have interesting future clinical applications, as studies indicate that the UPS can selectively degrade contractile and signaling proteins in the heart to alter its morphology and physiology.^{487, 503, 504} Ubiquitination is not terminal and can be reversed by de-ubiquitinases and proteasome inhibitors,⁵⁰⁵ thereby providing a potential therapeutic target. Bortezomib (a proteasome inhibitor) has shown early promise in suppressing right ventricular hypertrophy and vascular remodeling in a murine model.⁵⁰⁶

6.3.7 Limitations

This descriptive, exploratory observational study has identified differential gene expression and dysregulation of genetic pathways and networks which facilitates further hypothesis generation. The study is, however, limited by the very small sample size of our rTOF cohort. Further biological experiments are therefore required to validate transcriptomic results. Causal relationships cannot be established, and should not be extrapolated. Larger, multicentre collaborative studies may advance our initial findings. The RV myocardium sampled in patients with rTOF were selected away from areas of macroscopic focal scarring under direct vision by the operating surgeon (that is, away from previous ventriculotomy incisions which cause localised focal fibrosis), but may nevertheless still not be representative of the rest of the RV myocardium. This sampling error introduced may therefore not be reflective of the true fibrosis burden.

RNA sequencing has intrinsic technical limitations that include finite bioinformatics software and algorithms for data processing and analysis. There remains a lack of standardisation for software choice across the field, which creates variations in the efficiency and accuracy of data analysis.⁵⁰⁷⁻⁵⁰⁹ Different tools and parameter settings influence the results of highly complex RNA sequencing datasets, which should be borne in mind when interpreting the findings and making comparisons between studies.

6.3.8 Future Work

Our observations suggest a more comprehensive and targeted genomic analysis should be undertaken to identify candidate disturbances modifying pathway integrity in rTOF. Our study cohort consists of predominantly volume loaded RVs in contrast to previously published data which featured mainly pressure-loaded RVs. There would be scientific merit in comparing the genomic profiles of patients with significant/severe pulmonary regurgitation against pulmonary stenosis, in addition to the current comparison performed with normal RVs.

The hypotheses generated would support the application of proteomics and proteogenomics, particularly with regard to investigating the TGF β pathway and ubiquitination in rTOF. Prospective validation of UBC in rTOF should be undertaken with Western blotting. The possible role of HIF1A signalling in restrictive RV phenotypes could be specifically examined.

6.4 Conclusions

To our knowledge, this study is one of the earliest (if not first) reports of genome-wide RNA sequencing in a well-characterised, albeit small cohort of patients with rTOF, incorporating age and gender-matched normal controls. The exploratory, hypothesis-generating and observational nature of the study must be emphasized, so as not to over-interpret the potential implications of the study findings, through which causation cannot be derived.

In summary, we have demonstrated differential gene expression of 4662 genes in rTOF patients with significant PR. TGF β , extracellular matrix and oxidative phosphorylation pathways were dysfunctional. Ubiquitin C appears to be a potential promising central network regulatory protein in the pathological rTOF RV. This study also showed that there may be distinct molecular signatures of rTOF fibrotic and restrictive phenotypes.

Chapter 7 Neurohormonal activation and its relation to outcomes late after repair of Tetralogy of Fallot

This chapter examines neurohormonal activation in patients with rTOF via a prospective observational study design, with our main findings published in *Heart*, 2015.⁷¹ Much of this chapter is based on our published data.

A detailed review is presented in *Section 1.6* in which the relevance of neurohormones in CHD is discussed. Cross-sectional studies have demonstrated neurohormonal activation including elevated BNP levels in adults with rTOF.^{334, 335} However, the use of BNP and its longitudinal predictive value of adverse outcomes in rTOF are unknown.

Each of these Chapter 7 conclusions is reiterated in the overall setting of the thesis Conclusions (Chapter 8).

7.1 Aims and Methods

This study aimed to investigate the prognostic value of BNP in patients with rTOF in a prospective observational longitudinal study.

My exact role in this long-term follow-up project was to collate and organise the study data acquired at time of recruitment in addition to the follow-up data. I then performed data analysis, followed by manuscript drafting and revision for publication from October 2012 to June 2013. Study participant recruitment and data collection had been completed by Dr Aidan Bolger (Clinical Research Fellow at National Heart and Lung Institute, Royal Brompton during the period 2000-2004) as part of a wider project examining neurohormonal activation and chronic heart failure in adults with CHD. The neurohormone results for a subset of 20 patients with rTOF included within this study were published in *Circulation* 2002, in which neurohormone levels in a mixed cohort of patients with adult CHD were found to relate to symptom severity and ventricular dysfunction.³³⁴

7.1.1 Patient Population and Study Design

Serial patients aged ≥16years attending tertiary adult CHD outpatient care at the Royal Brompton Hospital between September 2000 and March 2004 were invited to participate at

the time of referral for CMR. A detailed clinical and neurohormonal assessment was performed and patients were followed up for clinical events. All participants gave study specific written informed consent and the study was approved by the local ethics committee. The study protocol excluded patients with clinically decompensated cardiac disease, active infection or evidence of malignancy.

In accordance with the study protocol, all patients underwent same day 12-lead electrocardiograms (Hewlett Packard PageWriteXLi) and chest radiography (Siemens Digiscan). The cardiothoracic ratio was calculated from the posteroanterior chest radiograph. Clinical data at baseline including heart rate and blood pressure were recorded. A subset of patients also underwent cardiopulmonary exercise testing using the modified Bruce protocol, with the data included in the analysis when the respiratory quotient value (RER) was ≥ 1 and performed within six months of the study recruitment date.

CMR Assessment

Comprehensive CMR imaging was performed as described in Methods (*Chapter 2.2.3*). In brief, single-shot fast spin echo imaging with dark-blood preparation images were taken in multiple slices in transverse, coronal and sagittal planes for anatomic assessment. Four chamber and 2-chamber bSSFP cine images were acquired and used to plan a short axis stack through both ventricles. Contiguous short axis slices were used to quantify ventricular volumes, mass and function indexed to body surface area. Flow velocity mapping was performed in a plane transverse to the right ventricular outflow tract to calculate the pulmonary regurgitant fraction. Atrial areas were measured from a four chamber cine frame selected just prior to opening of the atrioventricular valves, then indexed to body surface area. All CMR derived measurements were analysed blinded to the neurohormonal data.

Neurohormone Assessment

Neurohormonal quantification was performed on peripheral blood samples as described in *Section 2.4.1*. Venepuncture was performed to obtain morning samples as previously published.⁹ Atrial natriuretic peptide (ANP), BNP and active renin were quantified with immunoradiometric assays (BNP 1pmol/l = 3.472pg/ml).⁵¹⁰ Serum aldosterone was measured by radioimmunoassay (EuroDPC) and endothelin-1 (ET-1) was assessed by enzyme-linked immunosorbent assay (Bachem). Routine clinical laboratory assays were used for full blood count, renal function and liver function.

Age- and gender-matched healthy controls were recruited during the 2000-2004 study period. The control subjects underwent height, weight, heart rate, and blood pressure

measurements as well as peripheral venous blood sampling following the same protocol as the patients with rTOF.

Study Endpoints

The primary endpoint was all cause mortality. The secondary endpoint was documented sustained arrhythmia, atrial and/or ventricular. I collated follow-up data that was obtained from hospital electronic and paper medical records. Survival status was assessed using the National Health Service electronic system linked to the national database of mortality data at the Office for National Statistics (United Kingdom).

7.1.2 Statistical Analysis

Statistical analysis was performed with SPSS (IBM Statistics Version 22) and R version 2.12.1. Variables were tested for normality with the Kolmogorov Smirnov method, and log transformation performed for non-parametric data prior to statistical analysis. Results are reported as mean±SD for parametric or median (interquartile range) for non-parametric data. Comparison between groups was assessed using unpaired Student's t test or Wilcoxon test as appropriate. Correlation was assessed with Pearson's and Spearman correlation tests. Univariate Cox proportional hazard analysis was used to ascertain relationships between demographic, clinical and neurohormonal variables. Kaplan Meier curves were plotted to present the data graphically. Multi-variate analysis was not performed due to the low event rate. Hazard ratios and two-sided 95% confidence intervals (CIs) are presented. Receiver operating curve (ROC) analyses were performed to assess the prognostic value of the natriuretic peptide axis. Cut off values were identified on a survival-ROC method implemented in the survival-ROC R-package using the Kaplan-Meier estimate and the median follow-up time as the timepoint of interest. A two-sided p value <0.05 was considered significant.

7.2 Results

7.2.1 Baseline characteristics

Ninety patients with rTOF (mean age 32.7±11.3 years, 58 male) and fifteen age and gender-matched healthy controls were prospectively recruited (*Table 7.1*). Forty-two patients had prior palliative shunts, with median age at repair of 6 (0.25-48.0) years. Forty-five (50%) of patients had transannular patches and 12 patients (13%) had previous surgical pulmonary valve replacement (PVR). Only a small proportion of rTOF patients were on cardiac medications.

Patients with rTOF had significantly higher baseline heart rates compared to controls (rTOF 84 ± 14 bpm vs 70 ± 8 bpm, $p < 0.01$). There were no other significant differences in baseline characteristics.

Study patients were typically normotensive, and 64 (71%) of study participants were asymptomatic in NYHA functional class I at the time of study inclusion. The majority had broad QRS complexes with right bundle branch block morphology (mean QRS duration 147 ± 28 ms) and cardiomegaly (mean cardiothoracic ratio 0.55 ± 0.06). Sixty-five (72%) participants completed cardiopulmonary exercise testing with RER ≥ 1 within six months of recruitment with relatively preserved exercise capacity (peak VO_2 27.0 ± 8.4 ml/kg/min, predicted peak VO_2 $79.4 \pm 18.1\%$). The mean pulmonary regurgitation fraction was $29.9 \pm 14.8\%$.

	rTOF** (n=90)	Controls (n=15)	P
Age (years)	32.7 ± 11.27	30.3 ± 4.99	0.82
Gender (Male/Female)	58/32	7/8	
Body mass index (kg/m ²)	24.4 ± 4.94	24.4 ± 2.76	0.92
Heart rate (bpm)	84 ± 15	70 ± 8	<0.01
Systolic blood pressure (mmHg)	116.6 ± 15.2	113.2 ± 14.5	0.45
Diastolic blood pressure (mmHg)	72.5 ± 11.4	73.5 ± 8.5	0.76
ANP (pmol/L)	6.9 (4.0 - 9.9)	3.3 (1.0 - 4.0)	<0.01
BNP (pmol/L)	8.9 (5.9 - 14.6)	5.4 (2.2 - 7.5)	<0.01
Endothelin-1 (pmol/L)	1.14 (0.94-1.48)	0.75 (0.44 - 0.93)	<0.01
Renin (pmol/L)	55.0 (45.5 - 66.5)	18.6 (12.0 - 22.7)	<0.01
Aldosterone (nmol/L)	357 (295 - 450)	320 (250 - 402)	0.10
Haemoglobin (g/dl)	14.8 ± 1.64	14.2 ± 1.14	0.21
Sodium (mmol/l)	138.3 ± 2.16	137.4 ± 1.84	0.15
Creatinine (micromol/l)	78.8 ± 19.4	76.8 ± 9.6	0.69
CRP (mg/l)	7.51 ± 3.57	6.13 ± 1.96	0.16
QRS duration (ms)	147 ± 28.3		
Cardiothoracic ratio (%)	55 ± 6		
Cardiopulmonary Exercise Testing (n=65 with RER >1)			
Peak VO₂ (ml/kg/min)	27.0 ± 8.4		
Predicted peak VO₂ (%)	79.4 ± 18.1		
VE/VCO₂ slope	29.5 ± 7.4		
Anaerobic threshold (ml/kg/min)	16.9 ± 5.2		
Symptoms, n (%)			
NYHA Class I	64 (71)		
NYHA Class II	20 (22)		
NYHA Class III & IV	6 (7)		
Age at primary repair, years	6.0 (IQR 0.25-48.0)		
Surgical History, n (%)			
Previous palliative procedure	42 (47)		
◦ Blalock-Taussig shunt	23 (26)		
◦ Waterston shunt	13 (14)		
◦ Brock Procedure	6 (7)		
Previous pulmonary valve replacement	12 (13)		
Nature of repair, n (%)			
◦ Transannular patch	45 (50)		
◦ RVOT patch	23 (26)		
◦ Conduit insertion	11 (12)		
◦ Surgical history not available	11 (12)		
Medications, n (%)			
Angiotensin-converting enzyme inhibitors/ angiotensin receptor blockers	5 (6)		
β blockers	6 (7)		
Digoxin	3 (4)		
Diuretics	9 (11)		
Anti-arrhythmics (Amiodarone, Sotalol)	12 (14)		

Table 7.1: Baseline demographics of rTOF patients vs controls (mean± SD or median (IQR) as appropriate) **Nine patients (10%) had pulmonary atresia subtype, 1 patient had absent pulmonary valve and 1 double outlet right ventricle.

7.2.2 Neurohormonal dysregulation in rTOF

Neurohormonal activation was evident in patients with rTOF compared to controls (*Table 7.1*) with elevated levels of ANP, BNP, ET-1 and renin found.

Seventy-one rTOF patients (79%) had abnormal BNP concentrations (≥ 5.3 pmol/l or 18.3 pg/ml).⁵¹⁰ Seventy percent of asymptomatic patients (NYHA functional class I) had abnormal BNP levels. Symptomatic patients (NYHA functional class $\geq$ II) had higher ANP, BNP, renin and aldosterone levels (*Table 7.2*). The presence of symptoms corresponded to recognised adverse clinical markers such as age, sodium, impaired peak oxygen consumption on cardiopulmonary exercise testing, higher cardiothoracic ratio and lower biventricular ejection fraction on CMR (with $p < 0.05$). Paradoxically, symptomatic patients appeared to have lower pulmonary regurgitant fraction and marginally QRS durations. (This may have been confounded by the inclusion of patients that had undergone delayed PVR (13%) based predominantly on symptom onset based on contemporary clinical practice. These patients will have had relief of pulmonary regurgitation and reduction in QRS duration but potentially incomplete symptom relief). BNP levels were higher in women than men in line with recognised gender differences (median BNP in women 10.5 (7.6-22.8) vs 8.1 (5.2-12.2) pmol/l in men), however, this was not statistically significant ($p = 0.42$).

Significantly higher levels of ANP, BNP and ET-1 were seen in patients with previous palliation prior to repair, compared to patients who underwent primary repair ($p < 0.05$). Elevated neurohormones were associated with older age at repair (notably ANP); Ln ANP-age at surgery $r = 0.55$, $p < 0.01$, Ln BNP-age at surgery $r = 0.25$, $p = 0.02$, Ln ET-1-age at surgery $r = 0.33$, $p = 0.01$.

Significant correlations between the natriuretic peptides ($r = 0.65$) and renin/aldosterone ($r = 0.53$) were evident (*Table 7.3*). There were also significant correlations between ET-1 and natriuretic peptides (Ln BNP/Ln ET-1, $r = 0.83$; Ln ANP/Ln ET-1, $r = 0.39$) as shown in *Figure 7.1*; in addition to between ANP and aldosterone ($r = 0.29$), albeit weak. The natriuretic peptide axis (ANP/BNP) correlated with the cardiothoracic ratio and indexed RA area (*Figure 7.1*). There were no anticipated associations between BNP with RVEDV and RVEF; however, symptomatic patients had higher LVEDV and lower LVEF compared to asymptomatic patients ($p = 0.02$ and 0.03 respectively). There were no other significant associations.

	Total (n=90)	Asymptomatic (n=64)	Symptomatic (n=26)	P
Age at study, years	32.7 ± 11.3	31.0 ± 10.6	36.8 ± 11.9	0.03
Neurohormones				
ANP (pmol/L)	6.9 (4.0 - 9.9)	5.3 (3.3 - 8.5)	8.5 (6.1 - 21.0)	<0.01
BNP (pmol/L)	8.9 (5.9 - 14.6)	8.9 (5.8 - 12.7)	11.4 (6.2 - 33.7)	0.04
Endothelin-1 (pmol/L)	1.14 (0.94-1.48)	1.10 (0.92 - 1.37)	1.24 (1.00 - 2.10)	0.16
Renin (pmol/L)	55.0 (45.5 - 66.5)	54.5 (45.0 - 64.8)	56.0 (50.0 - 78.5)	0.02
Aldosterone (nmol/L)	357 (295 - 450)	346 (295 - 400)	485 (355 - 787)	<0.01
Biochemical parameters				
Haemoglobin (g/dl)	14.8 ± 1.64	14.9 ± 1.33	14.6 ± 2.25	0.38
Sodium (mmol/l)	138.3 ± 2.16	138.6 ± 1.98	137.4 ± 2.33	0.01
Creatinine (micromol/l)	78.8 ± 19.4	77.5 ± 14.6	82.0 ± 28.1	0.32
Urate (micromol/l)	325.6 ± 79.0	330.9 ± 77.0	313.0 ± 83.6	0.34
CRP (mg/l)	7.51 ± 3.57	7.39 ± 2.39	7.74 ± 5.19	0.78
Cardiopulmonary Exercise Testing (RER >1)	(n= 65)	(n= 52)	(n=13)	
Peak VO₂ (ml/kg/min)	27.0 ± 8.4	28.1 ± 8.7	22.3 ± 5.1	<0.01
Predicted peak VO₂ (%)	79.4 ± 18.1	82.7 ± 17.6	65.7 ± 13.5	<0.01
VE/VCO₂ slope	29.5 ± 7.4	29.0 ± 7.4	31.2 ± 7.1	0.34
Anaerobic threshold (ml/kg/min)	16.9 ± 5.2	17.5 ± 7.3	14.6 ± 4.5	0.12
QRS duration (ms)	147 ± 28.3	145 ± 26.3	131 ± 21.5	0.04
Cardiothoracic Ratio	0.55 ± 0.06	0.54 ± 0.05	0.60 ± 0.07	<0.01
CMR parameters				
RVEDV indexed (ml/m ²)	130.1 ± 44.7	125.1 ± 34.9	143.6 ± 63.1	0.40
RVESV indexed (ml/m ²)	64.3 ± 36.7	58.3 ± 24.5	80.5 ± 55.7	0.13
RVSV indexed (ml/m ²)	65.6 ± 18.6	66.5 ± 18.9	63.2 ± 17.9	0.48
RVEF (%)	52.3 ± 11.3	53.9 ± 10.5	48.1 ± 17.9	0.03
RV indexed mass (g/m ²)	57.3 ± 17.4	56.5 ± 14.0	59.6 ± 24.7	0.58
LVEDV indexed (ml/m ²)	72.5 ± 18.8	69.4 ± 15.4	80.7 ± 24.4	0.02
LVESV indexed (ml/m ²)	27.1 ± 10.4	24.7 ± 8.2	33.3 ± 13.2	<0.01
LVSV indexed (ml/m ²)	45.5 ± 11.7	44.7 ± 10.4	47.9 ± 14.7	0.34
LVEF (%)	63.4 ± 7.9	64.6 ± 7.7	60.3 ± 8.0	0.03
LV indexed mass (g/m ²)	63.7 ± 18.7	63.0 ± 13.7	65.5 ± 28.4	0.69
≥ Moderate pulmonary stenosis, n	8	4	4	0.17
≥ Moderate pulmonary regurgitation, n	78	56	22	0.72
≥ Moderate pulmonary regurgitation or stenosis, n	80	58	22	0.41
Pulmonary regurgitant fraction (%)	29.9 ± 14.8	33.0 ± 14.2	21.6 ± 13.3	<0.01

Table 7.2: Neurohormonal activation and symptoms in patients with TOF.

*Moderate pulmonary stenosis = peak velocity ≥3m/s

VO₂= peak oxygen uptake, VE/VCO₂ = ventilatory efficiency, EDV = End-diastolic volume, ESV = End-systolic volume, SV = stroke volume, EF = ejection fraction

	Ln ANP	Ln BNP	Ln ET-1	Ln Renin	Ln Aldosterone
Ln ANP		$r = 0.65$, $p < 0.01$	$r = 0.39$, $p < 0.01$	$r = 0.04$, $p = 0.72$	$r = 0.29$, $p = 0.01$
Ln BNP			$r = 0.83$, $p < 0.01$	$r = -0.03$, $p = 0.81$	$r = 0.18$, $p = 0.11$
Ln ET-1				$r = 0.14$, $p = 0.33$	$r = 0.23$, $p = 0.10$
Ln Renin					$r = 0.53$, $p < 0.01$
Ln Aldosterone					

Table 7.3: Correlation coefficients of neurohormones in rTOF

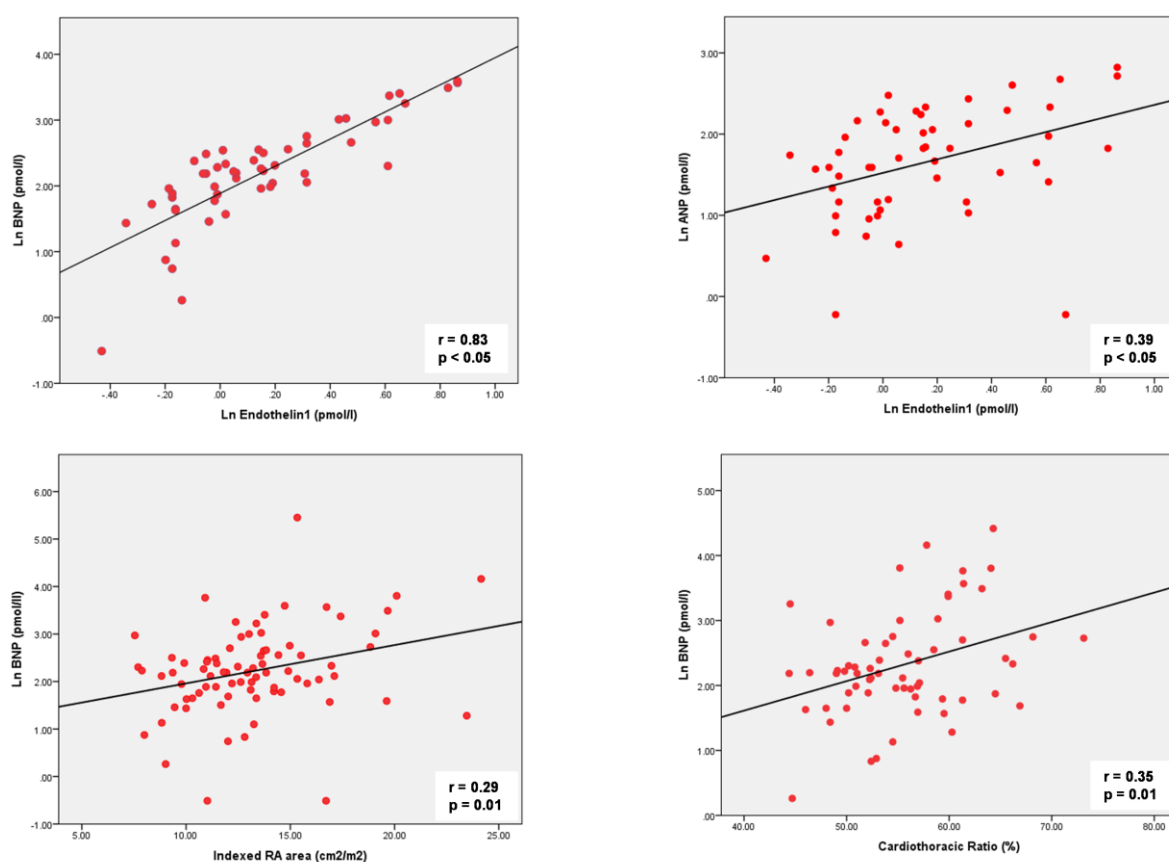


Figure 7.1: Neurohormonal and clinical variable interactions in rTOF. Reproduced from Heng *et al*, Heart 101(6):447-54, 2015⁷¹ with permission from BMJ Publishing Group Ltd.

7.2.3 Outcomes

Comprehensive follow-up data was available for 83 (92%) of patients. Median follow-up was 10 years (IQR 0.71-12.4 years) with 7 participants lost to follow-up, due to emigration (n =3) and repeated defaulting from clinical attendance (n=4).

Primary endpoint: Mortality

Eight deaths occurred during the study follow-up, with median age at death of 39.3 years (range 29.9-65.8). Causes of death were sudden cardiac death (n=4), peri-operative complications at the time of PVR with acute RV failure (n=2), and respiratory tract infection with sepsis (n=1). The cause of death for a single patient (aged 30 at death) could not be established as local documentation was no longer available. Of the 4 patients who died of sudden cardiac death, one had concomitant aortic stenosis, 2 patients experienced a presumed fatal arrhythmia within an hour of symptom onset, and one experienced recurrent and eventually fatal arrhythmia whilst in a persistent vegetative state after preceding aborted sudden cardiac death with ventricular fibrillation during pregnancy.

There was a trend towards higher BNP in deceased patients (15.5 (6.3-38.8) pmol/l) compared to survivors (8.9 (5.9-14.1) pmol/l). However, this was not significant (p=0.53). Deceased patients had significantly larger cardiothoracic ratios, higher RV volumes and bi-atrial dilatation compared to the surviving study cohort (*Table 7.4*). All deceased patients had residual haemodynamic lesions at baseline with 7 patients having at least moderate pulmonary regurgitation and one patient having aortic stenosis.

Baseline BNP predicted all cause mortality on univariate Cox proportional hazard analysis (HR 1.16 per 10pmol/L, 95% CI 1.05-1.29, p<0.01); as illustrated in *Table 7.5A* and *Figure 7.2A*. Other univariate predictors of mortality include recognised adverse markers of clinical outcome of cardiothoracic ratio, creatinine and indexed right atrial area. Time-dependent ROC characteristics analysis supported the predictive value of BNP at 5 years of follow-up with BNP≥15pmol/L related to increased mortality (HR 5.40, 95% CI 1.29-22.60, p<0.01, *Figure 7.2B*). The absolute mortality at 5 year follow-up was 19% (95% CI 0.4-34.2) in patients with BNP≥15pmol/L vs 3% (95% CI 0.0-7.5) in patients with BNP≤15pmol/L.

There was no association between mortality and the volumetric or functional parameters measured by CMR, nor with the indices of cardiopulmonary exercise capacity.

	Total (n=83)	Alive (n=75)	Deceased (n=8)	P
Age at study, years	32.7 ± 11.6	32.2 ± 11.3	37.5 ± 13.4	0.22
Neurohormones				
ANP (pmol/L)	6.9 (3.8 - 10.3)	6.9 (3.5 - 9.9)	7.4 (4.7 - 51.0)	0.30
BNP (pmol/L)	9.0 (5.9 - 15.3)	8.9 (5.9 - 14.1)	15.5 (6.3 - 38.8)	0.53
Endothelin-1 (pmol/L)	1.06 (0.93 - 1.37)	1.10 (0.95 - 1.37)	0.78 (0.65 - 0.78)	0.20
Renin (pmol/L)	55.0 (45.0 - 67.0)	55.0 (45.0 - 68.0)	52.0 (45.3 - 64.0)	0.40
Aldosterone (nmol/L)	357.0 (295.0 - 451.3)	355.0 (295.0 - 415.3)	576.0 (276.3 - 788.0)	0.18
Biochemical parameters				
Haemoglobin (g/dl)	14.7 ± 1.66	14.8 ± 1.63	14.4 ± 2.05	0.38
Sodium (mmol/l)	138.3 ± 2.11	138.3 ± 2.15	138.5 ± 1.77	0.77
Creatinine (micromol/l)	77.8 ± 19.4	76.1 ± 14.5	94.4 ± 43.5	0.28
Urate (micromol/l)	325.6 ± 79.3	326.6 ± 81.5	316.8 ± 58.0	0.74
CRP (mg/l)	5.06 ± 4.33	7.84 ± 3.72	6.50 ± 2.81	0.40
Cardiopulmonary Exercise Testing (RER >1)				
		(n= 58)	(n=7)	
Peak VO₂ (ml/kg/min)	26.3 ± 8.1	26.6 ± 8.1	23.8 ± 8.3	0.35
Predicted peak VO₂ (%)	77.1 ± 19.2	77.8 ± 19.2	71.0 ± 18.8	0.38
VE/VCO₂ slope	30.6 ± 8.0	30.4 ± 7.9	31.8 ± 9.6	0.67
Anaerobic threshold (ml/kg/min)	15.9 ± 4.8	16.1 ± 4.9	14.2 ± 3.3	0.36
QRS duration (ms)	145 ± 28	144 ± 28	148 ± 31	0.70
Cardiothoracic Ratio	0.56 ± 0.06	0.55 ± 0.06	0.65 ± 0.07	<0.01
CMR parameters				
RVEDV indexed (ml/m²)	128.1 ± 43.5	124.5 ± 37.6	165.6 ± 78.0	0.22
RVESV indexed (ml/m²)	62.3 ± 34.7	59.1 ± 27.9	95.6 ± 70.8	0.03
RVSV indexed (ml/m²)	65.6 ± 19.2	65.1 ± 19.3	70.0 ± 18.9	0.52
RVEF (%)	52.7 ± 10.7	53.4 ± 10.5	46.1 ± 11.6	0.09
RV indexed mass (g/m²)	56.7 ± 17.2	55.8 ± 16.6	65.3 ± 22.4	0.17
LVEDV indexed (ml/m²)	72.5 ± 19.4	71.4 ± 17.9	84.0 ± 30.2	0.43
LVESV indexed (ml/m²)	26.9 ± 10.6	45.6 ± 11.5	48.0 ± 16.2	0.17
LVSV indexed (ml/m²)	45.8 ± 11.9	44.7 ± 10.4	47.9 ± 14.7	0.60
LVEF (%)	63.5 ± 8.0	63.3 ± 19.0	70.7 ± 21.6	0.33
LV indexed mass (g/m²)	63.9 ± 19.2	63.0 ± 13.7	65.5 ± 28.4	0.69
Indexed RA area (cm²/m²)	13.2 ± 3.4	12.9 ± 3.2	16.9 ± 3.3	<0.01
Indexed LA area (cm²/m²)	9.4 ± 2.4	9.3 ± 2.3	12.0 ± 2.8	0.03
Pulmonary regurgitant fraction (%)	29.9 ± 14.8	33.5 ± 11.4	32.8 ± 7.6	0.90

Table 7.4: Characteristics of follow-up: survival and deceased rTOF patients.

(A)

Variable	Hazard Ratio (95% CI)	p
BNP, per 10pmol/l	1.16 (1.05-1.29)	0.01
Cardiothoracic ratio, per 1%	1.40 (1.07-1.83)	0.02
Creatinine, per 10micmol/l	1.39 (1.12-1.73)	<0.01
RA indexed area, cm ² /m ²	1.23 (1.01-1.49)	0.04

(B)

Variable	Hazard Ratio (95% CI)	p
Age at study, years	1.04 (1.00-1.07)	0.03
Age at repair, years	1.04 (1.00-1.08)	0.04
QRS duration on ECG, per 10ms	1.20 (1.02-1.42)	0.03
Peak VO ₂ , ml/kg/min	0.93 (0.86-1.00)	0.04
Cardiothoracic ratio, per 1% change	1.24 (1.12-1.37)	<0.01
ANP, pmol/l	1.77 (1.21-2.59)	<0.01
BNP, per 10pmol/l	2.06 (1.32-3.21)	<0.01
Endothelin-1, pmol/l	8.18 (1.29-51.82)	0.03
RVESVi, ml/m ²	2.46 (1.14-5.32)	0.02
RA indexed area, cm ² /m ²	1.13 (1.03-1.24)	0.01

Table 7.5: Univariate predictors of outcome **(A)** Mortality **(B)** Sustained arrhythmia.

Parameters not listed did not reach statistical significance.

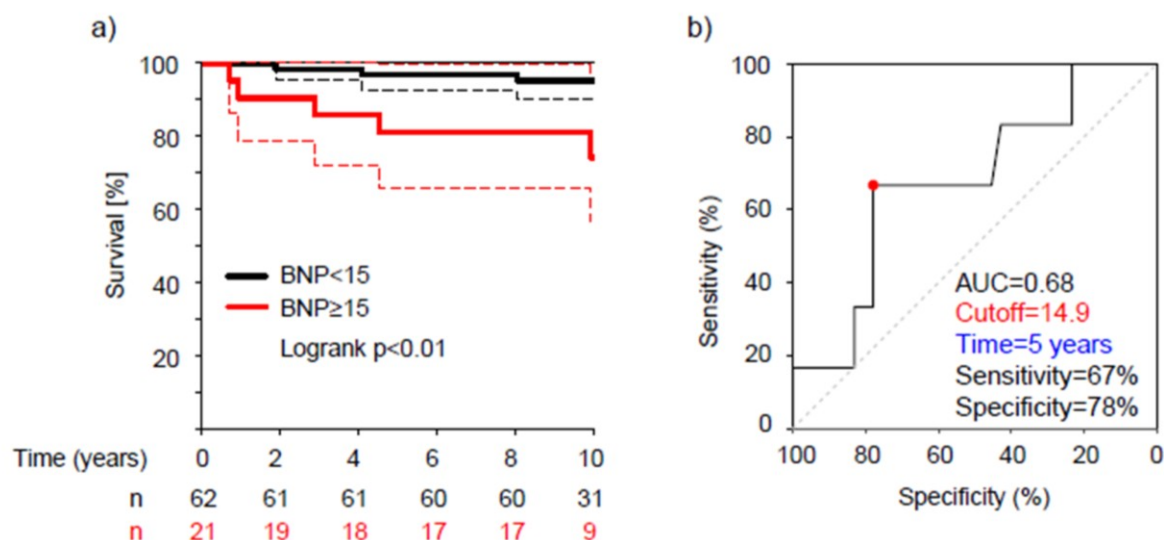


Figure 7.2: **(A)** Kaplan-Meier survival curve (dotted lines represent 95% confidence intervals) at BNP threshold of 15pmol/litre **(B)** ROC survival analysis for BNP cutoff at 14.9pmol/litre (AUC = Area under curve). Reproduced from Heng *et al*, Heart 101(6):447-54, 2015⁷¹ with permission from BMJ Publishing Group Ltd.

Secondary endpoint: Sustained atrial or ventricular tachy-arrhythmia

There were 28 events for the secondary outcome of documented sustained arrhythmia (atrial and/or ventricular) during follow-up. Nineteen patients had twenty atrial arrhythmias (atrial flutter n=11, atrial fibrillation n=6, atrioventricular nodal re-entry tachycardia/AVNRT n=3), and 7 patients had eight documented ventricular arrhythmias (ventricular tachycardia n=6, ventricular fibrillation n=2). Ten patients underwent electrical DC cardioversion for restoration of sinus rhythm.

Univariate neurohormonal predictors of sustained arrhythmia on Cox proportional hazards analysis included ANP, BNP and ET-1; together with other recognised adverse markers including patient age, QRS duration, exercise capacity, cardiothoracic ratio and indexed RA area (*Table 7.5B*).

7.3 Discussion

We have demonstrated that elevated circulating levels of natriuretic peptides, ET-1 and renin in rTOF patients predicted adverse outcomes. Specifically, BNP was predictive of mortality. Raised BNP levels were seen even in asymptomatic patients, and the extent of neurohormonal activation corresponded to functional class. There was interaction of ET-1 with the natriuretic peptide axis, which may provide new mechanistic insight.

7.3.1 The prognostic value of BNP

We and others have reported cross-sectional data demonstrating neurohormonal activation in rTOF. This study suggests a novel prognostic role of BNP in patients with rTOF. In our dataset, a BNP ≥ 15 pmol/l predicted a five-fold increase in mortality. CHD by definition constitutes structural cardiac abnormality, impaired exercise capacity and neurohormonal activation despite previous reparative surgery; CHD therefore has been likened to chronic heart failure syndrome.⁵¹¹ As BNP is secreted by cardiomyocytes due to increased myocardial wall stress,⁵¹² it is consequently released in CHD in response to pressure or volume overload states.^{513, 514}

BNP is a sensitive marker of LV ventricular dysfunction and is often elevated prior to symptom onset. BNP rises commensurate with the degree of LV dysfunction,^{67, 515} and predicts sudden death in chronic heart failure.⁵¹⁶ The attributes of BNP in the LV chronic heart failure setting may consequently extend to chronic RV pathology such as in rTOF, where ventricular myocyte stretch is recognised in rTOF due to chronic volume loading from pulmonary regurgitation. Sudden cardiac death in rTOF remains a poorly mitigated

outcome, with a long-term estimated risk between 3.7 and 6%.^{6, 9} Identifying patients at increased risk is challenging, and BNP measurements may have a role in both clinical risk assessment and decision making process in primary prevention.

There is limited data on the prognostic value of BNP in patients with rTOF. Our institution had previously shown both ANP and BNP to be strong predictors of mortality in a prospectively studied mixed cohort of 49 symptomatic complex ACHD patients, including 18 patients with rTOF. Fifty-seven percent of that study cohort had systemic ventricular dysfunction.³³⁶ By comparison, we have now demonstrated that BNP remains prognostically significant within a mostly asymptomatic cohort of patients with rTOF with generally preserved biventricular function. Although BNP elevation observed in adult patients with rTOF was modest compared to chronic heart failure patients, it did, however, provide prognostic information. BNP may therefore have clinical utility in lifelong clinical management.

7.3.2 Dysregulation of ET-1 in rTOF

Endothelin-1 (ET-1) was isolated in 1988 as a potent vasoconstrictor and stimulant of myocardial fibroblast proliferation. It has therefore been implicated in cardiac remodelling, stimulating cardiac hypertrophy through fibrosis.⁵¹⁷ Increased ET-1 levels seen in chronic heart failure may be due to pulmonary congestion upregulating pulmonary endothelin converting enzyme-1, which results in higher pulmonary production and secretion of ET-1 in a porcine model.⁵¹⁸

In our study, ET-1 was independently associated with the natriuretic peptide axes (Ln BNP/Ln ET-1, $r = 0.83$; Ln ANP/Ln ET-1, $r = 0.39$; $p < 0.05$), and was also elevated in patients with palliative shunts prior to surgical repair.

Raised ET-1 levels have been reported in children with CHD. This is postulated to relate to combinations of increased pulmonary blood flow or pressure. The mechanism of elevated ET-1 in rTOF may relate to the exposure of pulmonary vascular endothelium to increased shear forces with pulmonary regurgitation and large RV stroke volume, or residual pulmonary stenosis.⁵¹⁹ This is supported by the finding of higher ET-1 levels in patients with preceding palliative shunts to increase pulmonary blood flow with chronic LV volume loading in our study cohort. ET-1 is increased in chronic heart failure patients with a similar relationship between ET-1 and the natriuretic peptide axis seen, suggesting that neurohormonal activation may have pathophysiological links to circulating ET-1 levels.⁵²⁰ Raised ET-1 is known to be prognostic in chronic heart failure,⁵²¹ and pathophysiologically,

it would be a potentially useful peripheral biomarker in rTOF, since it is likely to increase with progressive pulmonary regurgitation, cardiac remodelling and myocardial fibrosis.

7.3.3 Applicability of neurohormone measurement to clinical practice

We illustrated (*Figure 7.1*) that BNP was associated with cardiothoracic ratio and indexed RA area ($p=0.01$), supporting mechanistic theories of right atrial size and myocyte stretch stimulating ANP and BNP release.^{334, 522} The cardiothoracic ratio is dependent on the transverse diameter of the cardiac silhouette bordered by the right atrium and LV in atrioventricular concordant hearts, and therefore the interdependence of cardiothoracic ratio and the natriuretic peptide axis is not surprising, but does add to the mechanistic understanding of neurohormonal activation in rTOF.

Previous studies have shown variable relationships between plasma BNP and NT-proBNP to exercise capacity, RV volumes and function. Small series have demonstrated relationships between BNP and RV volumes, dysfunction or pulmonary regurgitation.³³⁵ However, other work and our study have shown modest or no significant correlations of BNP to pulmonary regurgitation, exercise capacity or CMR derived measures.³³⁸ These discrepancies may be due to differences in the selection, age group and sample sizes of patients studied. In addition, some patients with rTOF in contemporary practice undergo delayed PVR, which will restore pulmonary valve competency but with residual persistent RV dilatation that may elevate BNP values. Our data reflects 90 clinically stable adult patients, most of whom did not meet current criteria based on CMR RV volumes for surgery, with preserved systolic function and only mildly impaired peak VO_2 .

Routine BNP measurements are performed in the UK to guide clinical management of patients with rTOF patients in numerous adult CHD centres. Its sensitivity and interpretation in the context of decision making for PVR and risk stratification remains incompletely characterised. Neurohormonal modulation remains an important potential therapeutic target in view of early neurohormonal activation in this patient group, although the evidence base for improvements in prognosis or clinical outcome is very limited.⁵²³ Future randomised controlled studies with larger patient numbers, longer periods of observation and serial neurohormonal assays are required. At present, decision for timing of catheter or surgical intervention is guided by tackling haemodynamic targets in a timely fashion rather than neurohormonal antagonism.

7.3.4 Limitations

This study originated in 2000-2004 with recruitment of patients referred for CMR at a tertiary adult CHD centre. This study design may have introduced selection bias of patients with haemodynamically significant lesions, as CMR may not have been routinely performed in all patients with rTOF under follow-up in this early era. Furthermore, patients with rTOF with implanted devices (pacemakers, cardiac resynchronisation devices or implantable cardioverter defibrillators) had contraindications for CMR which may have added further bias. Nevertheless, this follow-up study reflects a large cohort of patients, many of them asymptomatic and stable, representing the population with greatest clinical challenge for risk assessment. The neurohormones included within this study have variable specificity for cardiac tissue (with renin and aldosterone in particular predominantly originating from the kidneys and adrenal glands), and should therefore be interpreted in the clinical context.

Since patients with rTOF had a low event rate, we were unable to assess for other known adverse markers of clinical outcome and confounding factors. As such, our findings may therefore be difficult to generalise. Investigations in other large contemporary adult rTOF cohorts are hence justified. Further, prospective work is necessary to determine the relative, incremental value of serial BNP measurements in rTOF and examine its potential response to therapy.

7.4 Conclusion

In this long-term follow-up of a single centre prospective study, neurohormonal activation was present in patients with rTOF and correlated to symptoms. We observed for the first time potentially pertinent mechanistic interactions between the natriuretic peptide axis and ET-1 in rTOF. The early pathological neurohormone profile remains a potential therapeutic target that merits further exploration. BNP may predict mortality and sustained arrhythmia late after rTOF. Elevated BNP levels in asymptomatic patients with rTOF may represent an early clinical marker, preceding the often late onset of symptoms. The relationship between BNP, mortality and arrhythmias could be exploited as a potential therapeutic target for symptomatic and prognostic benefit. Serial assessment and larger studies are required to define the clinical utility of BNP for risk stratification, which could then be integrated into the armamentarium of clinical biomarkers during lifelong clinical care.

Chapter 8 Conclusions

Outcome prediction in rTOF remains an imprecise science, often governed by expert clinical consensus rather than evidence-based medicine due to limited trial evidence based on modest patient numbers and low adverse event rates, combined with changing surgical practice over the decades. The aim of this thesis was to provide a detailed investigation of potential biomarkers to improve outcome prediction in rTOF. I have therefore examined the roles of CMR, next generation gene sequencing and peripheral blood biomarkers in predicting clinical outcomes in patients with rTOF. The various included studies examined inter-related domains in rTOF, that is, those of cardiac remodelling and myocardial fibrosis, linked by CMR as the primary imaging modality for each study.

This thesis has described how structural and biological cardiac remodelling occurs after pulmonary valve replacement in rTOF. Novel CMR based T1 mapping techniques were developed and their feasibility was tested for measuring diffuse fibrosis of the RV in rTOF. Gene profiling with next generation sequencing of the markedly abnormal RVs in rTOF identified differential gene expression compared to normal subjects, with altered gene expression of fibrotic and restrictive phenotypes. I reported on Ubiquitin C as a potential gene network hub in rTOF. Lastly, neurohormonal dysregulation is evident in rTOF, with BNP predictive of mortality and clinical arrhythmias.

8.1 Summary of Findings

As CMR plays such a pivotal role in the studies of this thesis, I first assessed the reproducibility of CMR derived cardiac volumetric and functional data. In **Chapter 3**, I demonstrated robust volumetric, mass and functional assessment of both ventricles in rTOF with CMR. There were good levels of agreement in intra- and inter-observer measurements, confirming the precise and reliable nature of serial CMR measures. An appreciation of the strengths and potential pitfalls of CMR derived measures is pertinent, as it allows one to distinguish technical and operator-related limitations from clinically relevant disease progression.

Cardiac remodelling has been generally regarded as a gradual process following surgical PVR. The optimal timing for surgical PVR as well as its clinical outcomes in a prospectively recruited adult cohort of patients with rTOF was probed in **Chapter 4**. Our data demonstrates for the first time that the major improvement in RV volumes hitherto seen at midterm or late follow-up have already occurred almost immediately within days after surgery. In addition, we observed a continuing reduction in RVESVi, together with midterm

recovery in RVEF, suggesting a more gradual biological remodelling of the load-independent RV function following surgical intervention.

We found that preoperative RV volumes predicted normalisation of post-PVR RV volumes, with preoperative RVESVi $\leq 82 \text{ ml/m}^2$ being equally sensitive and more specific for RV normalisation than the preoperative RVEDVi threshold of $\leq 158 \text{ mL/m}^2$. We therefore propose that RVESVi may be a more appropriate marker that better reflects the early and sustained benefit of PVR on RV contractility and optimal reverse remodelling. Improved outcomes following PVR may be achieved by incorporating RVESVi into the individualised decision making process when considering the optimal timing for intervening.

As diffuse myocardial fibrosis is implicated in the pathophysiology of TOF and is not detectable by LGE CMR, we attempted to develop a RV targeted, novel T1 mapping based technique for quantifying diffuse fibrosis and ECV in the RVs of patients with rTOF in **Chapter 5**. Numerous approaches were systemically evaluated; each designed to tackle factors that would have otherwise introduced bias such as partial volume effects, fat contamination, cardiac and respiratory motion, in addition to suboptimal image resolution and the proximity of sternal wires. Multi-echo imaging to separate fat from myocardium, combined with blood signal suppression showed early promise as a feasible method in saturation-recovery T1 mapping, although further technical work is required prior to clinical assessment of relationships between RV T1 and ECV in rTOF. We also designed, manufactured and tested T1 phantoms as a quality assurance measure for single and multi-centre T1 and ECV mapping work. Our findings support its routine use in the clinical workflow of a CMR unit, and indeed globally.⁵²⁴

In **Chapter 6**, I carried out genome-wide RNA sequencing on RV biopsy samples in a well-characterised, albeit small, cohort of adult patients with rTOF, incorporating age and gender-matched normal controls for comparison. The RNA genomic findings should be interpreted in the context of the study being of a descriptive, exploratory observational design; reporting on preliminary associations that require further biological validation experimental work. Differential gene expression of the RV was evident in patients with rTOF, with emergent functional themes of changes to the extracellular matrix, transcription factor expression and neurohormonal activation. The RV fibrosis burden in patients with rTOF further influenced gene expression, and appeared to modulate extracellular matrix and TGF β expression, as well as the activity of oxidative phosphorylation pathways. Patients with restrictive RV physiology had differential HIF-1 signalling, as well as fibrosis-related protein-protein

interactions. Interestingly, there was a 'genetic signal' that Ubiquitin C may have important molecular interactions and functional implications as a 'network hub' gene in rTOF.

Lastly, the long-term clinical implications of neurohormonal activation in rTOF were investigated in **Chapter 7**. We confirmed elevated circulating levels of natriuretic peptides, ET-1 and renin in patients with rTOF, some of which were predictive of adverse outcomes. Mechanistic interactions of ET-1 with the natriuretic peptide axis were seen. Serum BNP was elevated even in asymptomatic patients, and predicted mortality and sustained clinical arrhythmia. The prognostic contribution of BNP may aid cogent risk assessment and consequent personalised clinical care for patients with rTOF at risk of premature SCD.

8.2 Future Work

The work contained in this thesis has identified further research avenues which could build upon the findings described as follows:

8.2.1 *Longitudinal predictive value of focal fibrosis in rTOF*

As alluded to previously, myocardial fibrosis is the underpinning theme of this thesis. Although LGE CMR can identify focal ventricular fibrosis in rTOF and enable its semi-quantitative assessment, its longitudinal predictive value for risk stratification and clinical outcomes is not defined. We therefore hypothesize that focal fibrosis as measured by LGE CMR predicts outcomes in rTOF, with a study in progress.

Consecutive patients with rTOF aged ≥ 16 years of age attending for clinically indicated CMRs and/or dedicated ACHD outpatient clinics at the Royal Brompton Hospital have been invited to participate in the study to reach a projected sample size of 300, thereby aiming to accrue ~2000 patient years of follow-up (as of December 2016) for clinical outcome analysis. Baseline demographics, clinical events and non-invasive investigation results will be collated into a customised electronic database. Patients will also be invited and consented for same day “biobanking”, during which venous blood sampling will be carried out to allow genotype-phenotypic correlations in a subset of the study. The required gene sequencing infrastructure is in place as part of the National Institute for Health Research Cardiovascular Biomedical Research Unit (cBRU) at the Royal Brompton Hospital. The primary endpoint will be defined as clinically documented sustained ventricular arrhythmia or all-cause mortality.

Prior patient recruitment of ~150 patients with rTOF into the above study had already taken place. I designed the study database, and have completed prospective CMR data acquisition of a further 190 adult patients with rTOF from 2012-2015 according to our acquisition protocol (*Section 2.2.3*). As outlined in the same *Section 2.2.3*, technical improvements supporting reliable high-resolution 3D LGE have enabled me to obtain such datasets in over 100 patients of the study cohort. Venous blood has been sampled and “biobanked” in approximately 80 patients over the data collection period, stored in preparation for genomic analysis. CMR volumetric data analysis and semi-quantitative LGE scoring has been completed concurrently, blinded to patient demographics. Clinical outcomes data collection is in progress with cBRU support, with projected reporting of study findings in 2017.

8.2.2 Technical development for RV T1 mapping by MRI

Intensive, rigorous MRI sequence development and technical work has been conducted towards a T1 mapping method optimised for the thin and mobile RV myocardium adjacent to sternal wires as well as fat and blood signals. Whilst we investigated and demonstrated some potential methods in *Chapter 5*, further technical ideas are still required before RV myocardial T1/ECV mapping can become reliable for clinical work. Unresolved technical issues have been described in *Section 5.2.7*, which have to be addressed by both simulation and volunteer experiments to enhance sequence precision and reliability.

Future RV T1 developmental work is heavily contingent on the continuing support and invaluable collaboration with Dr Peter Kellman, Staff Scientist, Physics, National Institutes of Health/NHLBI, Bethesda, United States. The cardiac MRI physics group at the Royal Brompton Hospital directed by Professor David Firmin also has a research agreement with Siemens Medical Systems, Erlangen, Germany for access to sequence source code, and has ethical permission for MRI sequence tests in normal healthy and patient volunteers. Our in-house work on RV T1/ECV development continues to be led by Dr Peter Gatehouse (Principal Physicist and Senior Lecturer in Physics, Cardiovascular Biomedical Research Unit, Royal Brompton Hospital), who recently secured funding from the British Heart Foundation for a PhD technical development studentship to improve RV assessment by CMR.

8.2.3 Clinical validation study of diffuse fibrosis in rTOF

Assuming there is satisfactory completion of the technical CMR sequence development, so that the eventual method is reliable for patient use, the individual threads of this thesis could be unified via a prospective cohort observational clinical study with the primary objective of validating CMR assessment of RV diffuse fibrosis (whether by T1, ECV or other parameters) against histological analysis. A secondary objective of establishing the clinical value of non-invasive CMR fibrosis measurement and its role in cardiac remodelling could be simultaneously studied.

Study specific ethics approval has been obtained (REC 14/LO/0263) for the following proposal:

Fifty patients with rTOF (aged ≥ 16 years with no contraindications to CMR and contrast administration) undergoing pulmonary valve surgery would be prospectively studied with pre-operative CMR with LGE and RV T1/ECV mapping as outlined in *Figure 8.1*, provided a reliable method is found. Non-invasive measurement of diffuse fibrosis would then be

validated against RV histology sampled at the time of surgery for “gold standard” validation. Arrangements would be made for study participants to be clinically reviewed at 6 months following PVR to evaluate post-operative changes with a repeat CMR (with the same CMR protocol). Patients will be specifically consented for permission to examine their clinical records annually for up to 10 years to establish whether the T1/ECV measurements predict clinical events.

Remodelling after Pulmonary Valve Replacement for repaired Tetralogy of Fallot – From Histology to Heart Form & Function

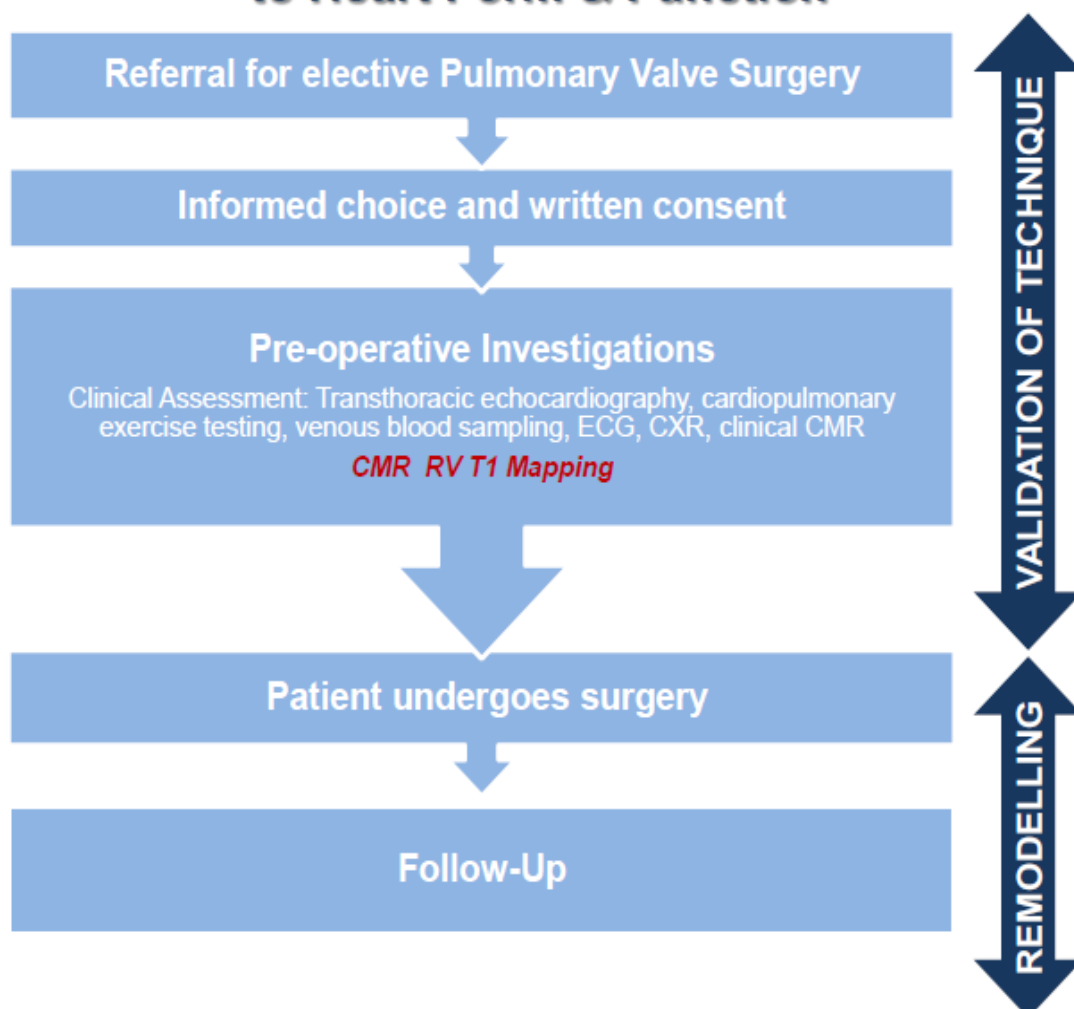


Figure 8.1: Proposed study protocol schematic for Remodelling after pulmonary valve replacement for rTOF, a clinical validation study for the proposed novel CMR RV diffuse fibrosis T1 (ECV) mapping technique.

8.2.4 Advancing genomics in patients with rTOF

The genomic findings described in *Chapter 6* are mainly exploratory and descriptive, and consequently generating further hypotheses and lines of research enquiry. As specifically listed in *Section 6.3.8*, Western blotting of ubiquitin (in particular ubiquitin C) expression levels should be carried out as the next experimental step to validate our theory of UBC as a potential network hub in rTOF. Other genomic results of interest could be validated in turn by replication with quantitative polymerase chain reaction (PCR). It could also be instructive to undertake a study focussed on applying proteogenomics or sequencing the TGF pathway in larger rTOF patient numbers, which may provide new insights into cardiac remodelling and genetic determinants of diastolic dysfunction.

Having demonstrated differential gene expression in rTOF with RNA sequencing, this technique could be applied to specific phenotypes in patients with rTOF to explore genotype-phenotype relationships. In particular, genomic profiles of patients with significant pulmonary stenosis could be compared to pulmonary regurgitation, restrictive RV to non-restrictive phenotypes, as well as preserved versus impaired ventricular function. Gene expression in patients pre versus post intervention (such as with pulmonary valve replacement) could be also be explored, although when best to time the collection of biological samples (blood or tissue if appropriate) for transcriptomics following intervention is far from clear cut.

On a broader scale, the formation of multi-centre consortia and genomic registries will transform the scale and yield from genomic research studies. Within the UK, the 100,000 Genome Project^{525, 526} is actively recruiting CHD patients. On a local level, the cardiovascular biomedical research unit biobank at the Royal Brompton and Harefield NHS Foundation Trust continues to collect and steward samples from CHD patients for ongoing and future research projects.

8.2.5 CMR detection of myocardial disarray in rTOF

A further promising recent development in CMR is cardiac Diffusion Tensor Imaging (DTI), which demonstrates the helical myocardial fibres of normal myocardium with striking angular and spatial resolution by exploiting the preferential diffusion of water along muscles.^{527, 528} This emerging technique has been successfully applied in the human heart *in vivo* by our institution,⁵²⁹ with ongoing technical improvement work.⁵³⁰⁻⁵³²

Histological myocardial disarray has been demonstrated in the RV infundibulum in rTOF in association with cardiac fibrosis,¹⁵⁵ and the mechanism postulated was that fibrosis produces cell separation, which culminates in diverse myofibril orientation. The application of

myofibre imaging in TOF to identify areas of disarray could influence surgical strategies and potentially identify substrates (isthmuses) for arrhythmia therapy. I have therefore recruited 10 patients with rTOF to participate in a feasibility pilot of CMR DTI. This pilot is a small part of an active subject of investigation led by Professors Dudley Pennell and David Firmin at our institution.

8.2.6 Diastolic dysfunction in rTOF by DENSE CMR imaging

There are proposed mechanistic links between diffuse myocardial fibrosis as imaged by CMR T1 mapping and diastolic dysfunction.^{243, 250, 259} In view of this, I investigated the options for assessing RV regional myocardial function and mechanics with CMR as an adjunct to T1 mapping in rTOF. The strengths and weaknesses of various options for MR assessment of regional myocardial mechanics were considered.⁵³³

Displacement ENcoding with Stimulated Echoes (DENSE) is a quantitative CMR technique for assessing myocardial displacement and strain.^{534, 535} In collaboration with Daniel Auger (Research Associate, Epstein Lab, Department of Biomedical Engineering, University of Virginia, USA), and Professor Frederick Epstein (Department of Biomedical Engineering, University of Virginia, USA), with post-processing support from Dr Pedro Ferreira (Image processing physicist, Cardiovascular Biomedical Research Unit, Royal Brompton and Harefield NHS Foundation Trust), I applied 2D and 3D cine DENSE sequences to the RV in 10 patients with rTOF. I analysed the data with a RV-specific processing package, and compared the results to data from five healthy volunteers.⁵³⁶ The detailed results are beyond the scope of this thesis, but we were able to measure global RV strain, and demonstrate lower peak strains of the RV outflow tract with delayed activation of the RV in rTOF when compared to healthy controls (unpublished data). Further work is required to determine if and how this technique might be extended usefully to clinical practice in CHD.

8.3 Concluding remarks

As described and demonstrated throughout this thesis, significant knowledge gaps remain in mediating adverse outcomes of arrhythmias, heart failure and sudden cardiac death in patients with rTOF. Risk factors for major outcomes remain poorly defined. This thesis has charted a preliminary attempt to investigate potential biomarkers, so as to improve outcome prediction in rTOF. The multiple hurdles to RV T1 mapping exemplify the technical complexity of imaging the RV, while the genomics RNA sequencing work has hopefully aided in directing future lines of inquiry.

Finally, this research process has reinforced the immense need for collaborative, multi-centre work in CHD (such as the CONCOR national Dutch registry of CHD and INDICATOR International TOF registry) for the evaluation of major clinical outcomes to improve longevity and decrease morbidity in patients with rTOF.

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Appendices

Abstracts and Publications List Related to Thesis

Publications:

Heng EL, Gatzoulis MA, Uebing A, Sethia B, Uemura H, Smith GC, Diller GP, McCarthy KP, Ho SY, Li W, Wright P, Spadotto V, Kilner PJ, Oldershaw P, Pennell DJ, Shore DF, Babu-Narayan SV. Immediate and Midterm Cardiac Remodelling Following Surgical Pulmonary Valve Replacement in Adults with Repaired Tetralogy of Fallot: A Prospective Cardiovascular Magnetic Resonance and Clinical Study. Revisions resubmitted to *Circulation*.

Vassiliou VS*, **Heng EL*** (joint first authors*), Gatehouse PD, Donovan J, Raphael CE, Giri S, Babu-Narayan SV, Gatzoulis MA, Pennell DJ, Prasad SK, Firmin DN. 2016. Magnetic resonance imaging phantoms for quality-control of myocardial T1 and ECV mapping: specific formulation, long-term stability and variation with heart rate and temperature. *J Cardiovasc Magn Reson* 18(1):62

Heng EL, Bolger AP, Kempny A, Davlouros P, Davidson S, Gatzoulis MA, Babu-Narayan SV. 2015. Neurohormonal activation and its relation to outcomes late after repair of tetralogy of Fallot. *Heart* 101(6): 447-54

Abstracts & Presentations:

Heng EL, Kellman P, Gatzoulis MA, Moon J, Gatehouse P, Babu-Narayan SV. 2016. Quantifying right ventricular diffuse fibrosis in tetralogy of Fallot – a novel customised approach for the challenges of the right ventricle. *J Cardiovasc Magn Reson* 18(Suppl 1):O26. Oral presentation at: SCMR 19th Annual Scientific Sessions, Los Angeles, USA.

Matthew GL, Vassiliou V, **Heng EL**, Smith GC, Sri A, Unnikrishnan N, Alpendurada F, Pennell DJ, Gatehouse P, Symmonds K, Prasad SK. 2016. Streamlining trigger delay estimation for T1 mapping. *J Cardiovasc Magn Reson* 18(Suppl 1): T11

Heng EL, Gatzoulis MA, Uebing A, Sethia B, Uemura H, Smith GC, Diller GP, McCarthy KP, Ho SY, Li W, Wright P, Spadotto V, Kilner PJ, Oldershaw P, Pennell DJ, Shore DF, Babu-Narayan SV. 2015. Early cardiac remodelling after pulmonary valve replacement in patients

with repaired tetralogy of Fallot. Oral presentation for Young Investigator Award: Cardiff International Cardiovascular Conference 2015.

Heng EL, Gatzoulis MA, Smith GC, Shore DF, Sethia B, Uemura H, Diller GP, Ho SY, Pennell DJ, Babu-Narayan SV. 2015. Early cardiac remodelling post pulmonary valve replacement in patients with repaired tetralogy of Fallot. Moderated poster session at: Challenges in repaired tetralogy of Fallot, European Society of Cardiology Congress 2015, London, UK.

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