Myocardial Fibrosis in Hypertrophic Cardiomyopathy

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I, Dr Vimal Patel, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed……

Abstract

Hypertrophic cardiomyopathy (HCM) is characterised by myocardial hypertrophy, fibrosis and abnormal vascular pathology and is usually caused by mutations in sarcomeric protein genes. Histological studies and *in vivo* imaging with cardiac magnetic resonance imaging (CMRI) have shown that myocardial fibrosis is an important entity that contributes to disease progression. However, little is known about the regulation of genes involved in collagen synthesis and metabolism, the pathways that contribute to the development of myocardial fibrosis and whether this is an early pathological process which ultimately leads to the development of the overt phenotype in genetic mutation carriers. Furthermore, the contribution of fibrosis on myocardial function has been poorly defined.

In this thesis, I identified that myocardial genetic expression of collagen is upregulated in patients with HCM and this is paralleled by elevated levels of procollagen in plasma. The genetic expression of transforming growth factor beta (TGF-β) and its downstream mediator connective tissue growth factor was also enhanced in HCM and correlated with collagen I and III RNA levels, suggesting a central role of TGF-β in mediating fibrosis. Plasma markers of collagen synthesis and metabolism were also increased in sarcomeric mutation carriers without hypertrophy, suggesting that fibrosis may be an early process that contributes to the development of the overt phenotype.

Plasma levels of procollagen I were higher in patients with non-sustained ventricular tachycardia and focal fibrosis identified by CMRI was associated with impaired systolic deformation. Diffuse fibrosis beyond that seen in healthy controls also correlated with a reduction in systolic function.

Together, the findings of this thesis support the hypothesis that myocardial fibrosis is an active process in HCM that precedes clinical phenotype. Myocardial fibrosis is at least in part mediated by the TGF- β pathway and associated with impaired systolic performance and may contribute to arrhythmic risk in HCM.

Impact statement

The findings of this thesis provide further insight into the pathways that drive myocardial fibrosis in hypertrophic cardiomyopathy and the impact of fibrosis on myocardial dysfunction. The evidence presented in this thesis also lends further support to the hypothesis that plasma markers of myocardial fibrosis are elevated in patients with HCM and that myocardial fibrosis is an early entity which may precede and subsequently contribute to the development of the overt clinical phenotype in sarcomeric mutation carriers.

Together these findings could aid in the development of therapies targeted against pathways which promote myocardial fibrosis at an early or preclinical stage of the disease. The findings of this study may also point to a future role for the measurement of plasma collagen biomarkers as a potential screening tool in family members of those affected by the disease and also in the monitoring of disease activity, progression, response to treatment and potentially risk stratification.

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Declaration of authenticity

The work submitted in this thesis to the University College London is original and has not been submitted elsewhere for any other professional qualification.

This thesis has been checked using University College London *Turnitin* plagiarism detection software within UCL Moodle. The similarity index was 19% which reflects the use of published material by the candidate as first author (appendix 3) and inclusion of a uniformly standardised cardiac MRI protocol which was being utilised for research studies at the London Heart Hospital.

1. Introduction

1.1 Definition of hypertrophic cardiomyopathy

Cardiomyopathies are defined by structural and functional abnormalities of the ventricular myocardium that are unexplained by flow-limiting coronary artery disease or abnormal loading conditions.[1] The European Society of Cardiology (ESC) Working Group on Myocardial and Pericardial Diseases proposed an updated classification of cardiomyopathies based on morphological and functional phenotypes and subcategories of familial/genetic and non-familial/non-genetic disease.[1] Hypertrophic cardiomyopathy (HCM) is defined by the presence of increased left ventricular (LV) wall thickness that is not explained solely by abnormal loading conditions. [2]

1.2 History of disease

Descriptions of a hypertrophied myocardium in association with sudden death date back to the 18th century.[3] In the 19th century physicians such as Vulpian and colleagues provided clinico-pathological reports of hypertrophic cardiomyopathy and descriptions of clinical features that would be compatible with left ventricular outflow tract obstruction (LVOTO).[4] It was not however until Donald Teare's landmark paper in 1958, that the first modern description of HCM was presented. Eight cases of ''asymmetrical hypertrophy (ASH) or muscular hamartoma'' in young adults were described with seven experiencing sudden death. The paper was relevant for both the association of left ventricular hypertrophy (LVH) and sudden death (SCD) but also because he identified a familial pattern of disease.[5]

1.3 Epidemiology and genetic basis of the disease

Hypertrophic cardiomyopathy is the commonest inherited cardiac disease with prevalence of 1 in 500 of the general population.[6] In most cases, HCM is inherited in an autosomal dominant manner and is secondary to mutations in one of 11 genes that encode proteins of the cardiac sarcomere: specifically, β-myosin heavy chain (*MYH7*, chromosome 14); myosin-binding protein C (*MYBPC3*, chromosome 11); cardiac

troponin T (*TNNT2*, chromosome 1); cardiac troponin I (*TNNI3*, chromosome 19); α-tropomyosin (*TPM1*, chromosome 15); α-cardiac actin (*ACTC*, chromosome 15); essential myosin light chain (*MYL3*, chromosome 3); regulatory myosin light chain (*MYL2*, chromosome 12); cardiac troponin C (*TNNC1*, chromosome 3); α-myosin heavy chain (*MYH6*, chromosome 14).[7] Mutations in contiguous Z -disks and calcium handling proteins account for less than 1% of cases.[7-9]

To date over 1000 mutations in genes encoding sarcomeric proteins have been reported in patients with the disease. Mutations in MYH7 and MYBPC3 account for the majority of mutation positive cases, whilst mutations in TNNT2, TNNI3, TPM1, ACTC and myosin light chains and are thought to account for 10 – 15% of cases.[7-10]. The combination of genetic diversity, heterogeneous clinical phenotypes and inconsistent study design has precluded the establishment of precise genotype–phenotype relationships.[11]

Phenocopies of HCM caused by other genetic and acquired disorders can be broadly classified into those associated with metabolic disorders, mitochondrial myopathies, neuromuscular disease and infiltrative processes.[12] These phenocopies are often associated with extra-cardiac manifestations, abnormalities in the conduction pathway and cardiac imaging may also demonstrate distinct morphological abnormalities.

1.4 Pathological findings

Hypertrophic cardiomyopathy is characterised by distinct histological and morphological abnormalities.

1.4.1 Histology

Histologically, HCM is distinguished by a triad of myocyte hypertrophy, myocyte disarray and fibrosis.[13] Another common feature is the presence of morphologically distinct intramural coronary arteries.[14]

Myocyte hypertrophy is the hallmark of HCM which is greatest in layers closest to the

left ventricular cavity.[15] Cardiomyocytes exhibit nuclear enlargement and pleomorphism with hyperchromasia with disorganized myofibrillar architecture.[13]

Myocyte disarray is the loss of normal parallel alignment of myocytes. Although, this is not specific to HCM, the presence of more than 5 – 10% by volume is considered specific to HCM.[16-20]

The observed pattern of myocardial fibrosis in HCM is complex and the literature is confusing as terminology is not consistent across pathological, imaging and clinical studies. Four distinct patterns have been described in HCM including diffuse interstitial expansion, perivascular fibrosis, replacement fibrosis following myocyte death and plexiform fibrosis associated with myocyte disarray commonly found in the septum and at insertion points.[21-25] The entity of myocardial fibrosis in HCM is explored further in subsequent sections of the introduction.

At necropsy, 83% of individuals with HCM who experienced SCD have morphologically distinct intramural coronary arteries characterised by a thickened vascular wall and decreased luminal area.[14] The vascular wall is characterised by expansion of the intimal and medial compartment secondary to proliferation of smooth muscle cells and the collagen network.[14 26] These vascular changes are not specific to HCM but are 20 times more prevalent in HCM than in normal controls and their prevalence is similar in those with and without a history of chest pain.[14] Abnormal small vessels are significantly more common in areas of extensive fibrosis and the extent of replacement fibrosis has been shown to correlate with small vessel disease.[14 27]

1.4.2 Left ventricular hypertrophy

The distribution of hypertrophy in HCM is not uniform. The most common distribution of hypertrophy is asymmetrical septal hypertrophy (ASH) but other distributions of hypertrophy include apical LVH, symmetrical concentric and eccentric hypertrophy.[28 29]

1.4.3 Myocardial architecture

Abnormalities of the myocardial architecture and mitral valve apparatus have been associated with HCM. These include the presence of myocardial crypts,[30 31] anterior mitral valve leaflet elongation,[32 33] and abnormal LV apical trabeculae.[32] These morphological abnormalities may precede the expression of LVH and predict the presence of sarcomeric mutation carriage.[34]

1.5 Pathophysiology of disease

Symptoms in HCM are explained by arrhythmias and exercise intolerance secondary to a complex interaction of LVOTO, myocardial dysfunction, and myocardial ischaemia.[35] *Figure 1.*

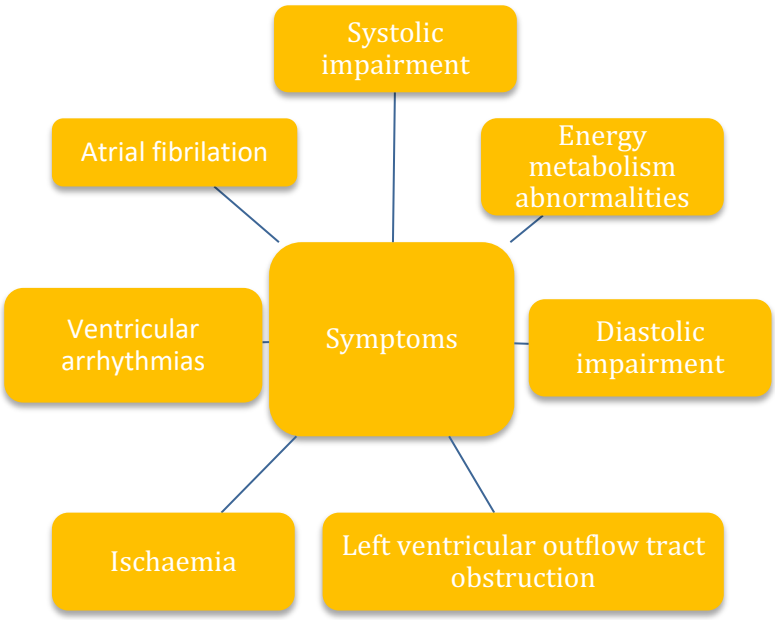

Figure 1: Mechanisms of symptoms in hypertrophic cardiomyopathy.

1.5.1 Systolic anterior motion of the mitral valve and left ventricular outflow tract obstruction

Systolic anterior motion of the mitral valve (SAM) is the anterior displacement of the mitral valve and its supporting structures into the left ventricular outflow tract. The extent of SAM is dynamic and can be enhanced by manoeuvres that increase

contractility or reduce afterload and preload. Left ventricular outflow tract obstruction is the result of complete SAM resulting in contact between the mitral valve and the inter-ventricular septum. Outflow tract obstruction is present in approximately one third of patients under resting conditions, with a further one third demonstrating latent or provocable obstruction.

Left ventricular outflow tract obstruction is defined as a pressure gradient across the left ventricular outflow tract of ≥ 30 millimetre of mercury (mmHg) at rest or ≥50mmHg with physiological provocation with Valsalva and exercise.[12] Systolic anterior motion of the mitral valve is often associated with a degree of mitral regurgitation, which is posteriorly directed and secondary to a failure of leaflet apposition.

Patients with LVOTO describe huge variations in their symptomatic status. Common symptoms include dyspnoea, chest pain and syncope, whilst and others with obstruction may not describe functional limitations.[36 37] The association between LVOTO and SCD has been widely debated. Some studies have shown no association between obstruction and SCD, whilst others have shown a positive correlation. The presence or severity of outflow tract gradient has historically not been considered an independent risk factor for SCD. However, the recent 2014 ESC guidelines for hypertrophic cardiomyopathy now includes outflow tract gradient in their SCD risk stratification model.[12 38]

1.5.2 Systolic dysfunction

LV systolic performance is often considered to be hyperdynamic as the measured ejection fraction is 'supra-normal'. However, this partly relates to small internal cavity dimensions rather than a true increase in LV contractility. A proportion of individuals develop progressive disease characterized by the development of systolic dysfunction (ejection fraction (EF) <50%). The prevalence of severe systolic impairment or 'burnt out' HCM using conventional echocardiographic criteria ranges from 2% to nearly 10% [39 40]. However, the incidence of systolic LV impairment may be much higher as clinically

significant reductions in systolic performance may occur while the measured ejection fraction remains within the normal range.[41]. Regional myocardial performance assessed using tissue Doppler and strain imaging is usually reduced and precedes the development of overt systolic failure [42 43]. End-stage HCM with progressive systolic dysfunction develops at all ages, but in the majority of patients the time from onset of heart failure symptoms to diagnosis of severe systolic impairment is about 10-15 years [39]. The development of severe systolic heart failure is associated with a poor prognosis, with rapid progression to death or transplantation and an overall mortality rate of up to 11% per year [39].

1.5.3 Diastolic dysfunction

In clinical practice, surrogates for diastolic dysfunction include left atrial (LA) dilatation, abnormal Doppler indices and raised left ventricular end diastolic (LVED) pressures upon invasive assessment.

Diastolic dysfunction is a contributory factor in exercise limitation in HCM [44-46] but conventional Doppler indices of left ventricular diastolic function do not correlate with symptoms or exercise capacity [47 48] or invasively measured diastolic parameters [49]. This may be because Doppler velocities are affected by variables independent of diastolic function, such as volume loading [50] Characteristically, patients with HCM have left ventricular diastolic impairment demonstrated by reduced early diastolic (Ea) velocities in the mitral annulus and septum and reversal of the ratio of early to late diastolic velocities (Ea/Aa) [51] The transmitral E to lateral Ea ratio has a modest correlation with New York Heart Association (NYHA) functional class and exercise capacity[51] suggesting that other factors including reduced stroke volume response to exercise, ventilation/perfusion mismatch, and abnormal peripheral oxygen utilisation may also influence exercise capacity. A recent study using magnetic resonance in 64 patients showed that LV chamber stiffness (determined as the ratio of pulmonary capillary wedge pressure (derived from the transmitral E/Ea ratio) to LVED volume) was associated with exercise capacity, and was the only significant variable on multivariable analysis [52].

1.5.4 Myocardial ischaemia

Exertional chest pain is a common symptom in individuals with HCM [53]. Individuals with HCM also have morphologically distinct intramural coronary arteries which are characterised by a thickened vascular wall secondary to proliferation of smooth muscle cells [14 26]. Several studies have shown that patients with HCM have abnormal myocardial perfusion although the exact clinical significance of this remains unclear.[54-59 60-62]

1.5.5 Energy metabolism

The myocardium depends on oxygen for high-energy phosphate production by oxidative phosphorylation. In the normal heart, adenosine triphosphate (ATP) is produced mostly by the metabolism of free fatty acids (FFA) and carbohydrates, with FFA accounting for approximately 70% of total ATP production. In health, FFA oxidation is directly related to plasma FFA concentration, whereas glucose and lactate uptake are inversely related to plasma FFA levels. When oxygen delivery to the myocardium is insufficient to meet the requirements of mitochondrial respiration, high-energy phosphate production falls and lactate, the end product of anaerobic glycolysis, accumulates. This phenomenon is familiar in ischaemic heart disease, but evidence from animal and human studies suggest that HCM is also characterised by a reduction in the concentration of high-energy phosphates in the myocardium [63]. One possible explanation is myocardial ischaemia caused by microvascular dysfunction as described earlier. Alternatively it may be the direct consequence of sarcomere protein gene mutations on myocardial contractile efficiency [64]

Skeletal muscle metabolism and uptake of oxygen are fundamental determinants of exercise capacity. Phosphocreatine, is a phosphorylated creatine molecule that can anaerobically donate a phosphate group to ADP to form ATP during the initial part of intense muscular effort. It has been shown using ^{31}P magnetic resonance that patients with HCM carrying a mutation in β myosin heavy chain had elevated phosphocreatine:ATP ratio at rest compared to controls and HCM patients with

troponin T mutations.[65] During exercise both HCM patient groups had a greater depletion of phosphocreatine. Furthermore, the recovery of phosphocreatine, a marker oxidative metabolic capacity was reduced in the β myosin heavy chain group. This data suggests that individuals with HCM have increased dependence on non-oxidative synthesis of ATP and reduced oxidative capacity [65].

1.5.6 Atrial fibrillation and thromboembolism

Atrial fibrillation (AF) is common in patients with HCM. Studies have demonstrated a prevalence of 22% with a reported annual incidence of 2 – 2.6%. The presence of AF is associated with a high burden of thromboembolic complications.[66] In a recent study of 4,821 patients, 3.6% experienced a thromboembolic complication within 10 years. This was strongly associated with the presence of AF and increasing LA dimensions. Other associations with AF include age, heart failure symptoms, maximal LV wall thickness (MWT) and vascular disease.

1.5.7 Ventricular arrhythmia and sudden cardiac death

The incidence of SCD in non-athletic individuals with HCM is very low (less than 1% per annum).[67] Despite the low incidence, the effects of SCD can be devastating with a peak incidence in adolescence and young childhood, frequently in the absence of warning signs or symptoms. [68]

The mechanisms triggering ventricular arrhythmias in HCM is poorly understood. However, intraventricular dispersion of myocardial conduction secondary to variable cardiomyocyte size, fibrosis and disarray alongside increased myofilament calcium sensitivity and abnormal calcium handling are likely to promote electrophysiological dysfunction and re-entrant pathways.[69-73] Furthermore, myocardial ischaemia secondary to abnormal intramural coronary arteries, systolic compression of epicardial arteries and an inadequate capillary density relative to muscle mass may promote ventricular arrhythmias and in part may explain the association between exercise and SCD.[74 75]

1.6 Clinical assessment

1.6.1 Electrocardiogram

Characteristic changes on the 12-lead surface electrocardiogram (ECG) include LVH, repolarization abnormalities and pathological Q waves. Patients with an apical distribution of LVH demonstrate characteristic giant T wave inversion in the mid pre-cordial leads.[76] The ECG may also assist in the identification of non-sarcomeric disease; for example, low QRS voltage and conduction abnormalities may be a marker of amyloid heart disease.[77]

During ambulatory ECG monitoring, The prevalence of NSVT has been shown to be between 20 – 31%, whilst the prevalence of paroxysmal atrial arrhythmias is 37%.[68 78]

1.6.2 Echocardiogram

Current diagnostic criteria are based upon the demonstration of LVH with a maximal end diastolic LV wall thickness of ≥15mm, which cannot be explained by abnormal loading conditions. The presence of mild LVH (≥ 13 – 14mm) may be considered diagnostic in the presence of supporting features such as a family history, abnormal ECG changes or a pathogenic mutation.[12] Right ventricular hypertrophy may be observed in up to 30% of affected individuals.

The 2D echocardiogram is used to detect SAM of the mitral valve and Doppler echocardiography is used to quantify the left ventricular outflow tract gradient (LVOTG) at rest and during provocation manoeuvres. Further assessment in the upright position or following exercise should be performed in individuals with symptoms but no evidence of LVOTG at rest.[79] Associated mitral regurgitation, which is often posteriorly directed, can be further assessed.

LA dimensions are frequently increased and are an adverse prognostic marker. Most individuals demonstrate small LV internal dimensions. The ejection fraction is a suboptimal measure of systolic performance in the presence of hypertrophy, and deformation imaging using Doppler and speckle tracking frequently demonstrate impairment in longitudinal function despite a preserved ejection fraction. Patients with progressive myocardial systolic dysfunction may exhibit myocardial thinning, LV cavity dilatation and impairment of systolic function with a spontaneous loss of LVOTO.[39 41] Conventional parameters of diastolic performance and filling pressures including assessment of the mitral inflow E:A ratio, deceleration time and transmitral E to early diastolic velocity of the lateral wall are routinely performed and often demonstrate abnormal filling patterns.

Features such as the presence of biventricular hypertrophy, myocardial speckling and thickening of valves and interatrial septum can help in identifying non sarcomeric disease such as amyloid heart disease or mitochondrial cytopathy.[12]

1.6.3 Cardiopulmonary exercise testing

Cardiopulmonary exercise testing is performed at baseline and is advocated with any change in functional status. HCM is associated with impaired exercise tolerance and reduced peak oxygen consumption independently of symptomatic status.[80 81] Up to one third of individuals may demonstrate an abnormal blood pressure response to exercise which has previously been shown to be an adverse prognostic marker.[82 83] Exercise testing may also provide a useful tool in differentiating HCM from an athletes heart with physiological hypertrophy.[84]

1.6.4 Cardiac magnetic resonance imaging

Cardiac magnetic resonance imaging allows for detailed assessment of myocardial morphology and systolic performance. Gadolinium contrast enhanced CMRI also allows *in vivo* estimation of myocardial fibrosis. Late gadolinium enhancement (LGE) imaging identifies areas of focal myocardial replacement fibrosis.[85-88] Pre-and post-

contrast T1 mapping provide a method for accurate quantification of extracellular volume (ECV). The distribution of LGE, gadolinium (Gd) kinetics and native T1 relaxation time may help to identify clinical phenocopies of sarcomeric disease including cardiac amyloidosis and Anderson-Fabry disease.[89-92] The development of interstitial fibrosis and expansion of extracellular volume (ECV) alongside subtle morphological abnormalities may precede the development of hypertrophy and may identify early expression of disease amongst gene carriers and family members. [32 34 93]

1.7 General principles of disease management

1.7.1 Medical management

In patients with symptoms caused by LVOTO, the aim of treatment is to reduce the outflow tract gradient. Options include negatively inotropic drugs (β-blockers, disopyramide and verapamil), atrio-ventricular sequential pacing, percutaneous alcohol ablation of the inter-ventricular septum and surgery. Approximately 60 to 70% of patients improve with medical therapy but high doses are frequently required and side effects are common [94]. Similarly, diastolic characteristics and myocardial ischaemia may be improved by reducing heart rate and improving filling time.

At present, pharmacological therapy of systolic heart failure in HCM is initiated only when patients develop symptoms or have a reduced ejection fraction. The drugs used (angiotensin converting enzyme (ACE) inhibitors (or angiotensin receptor blockers), β-adrenoceptor blockers, diuretics and digoxin) are identical to those employed in patients with dilated cardiomyopathy (DCM), although the effect on prognosis is unknown. In patients with paroxysmal nocturnal dyspnoea and chronically raised pulmonary pressures diuretics can be effective, but the dose and duration of therapy should be minimised particularly in patients with severe diastolic impairment or labile obstruction.

Anticoagulation

The data supporting the efficacy of anticoagulation with vitamin K antagonism in preventing stroke in those with HCM and AF is largely limited to non-randomised observational studies. Several studies have demonstrated that anticoagulation lowers the incidence of stroke in individuals with HCM and AF. [95-97] In a recent study, patients who were found to be in AF at presentation experienced a relative risk reduction of 55% with VKA treatment. The study found that at an exploratory threshold of 4% risk of a thrombo-embolic event over 5 years, the absolute risk reduction and number needed to treat for patients in AF was 13% (95% CI 2.1 –24%) and 7.7, respectively. [98] The role of novel anticoagulants in stroke prevention has yet to be evaluated.

1.7.2 Management of cardiac dysrhythmia

Implantable cardiac defibrillators (ICD) are indicated for secondary prevention and primary prevention of sudden cardiac death in high-risk individuals. Current ACCF/AHA and previous ESC guidelines used the presence of the following binary clinical parameters to define a risk algorithm: MWT >30mm, the presence of NSVT, unexplained syncope, abnormal blood pressure response to exercise (failure to augment systolic BP by at least 20mmHg) and a family history of sudden or aborted sudden cardiac death.[99 100] The presence of multiple risk factors equates with higher risk and it is accepted practice to consider the implantation of an ICD in people with 2 or more risk factors.[99-101]

This strategy has proved useful in differentiating low and high risk individuals although with limited power.[101] Additional challenges of this approach also relate to difficulties in managing individuals with a solitary risk factor (up to 40%) and inability to provide an individualized patient risk.[101] To overcome this the ESC has advocated the use of a validated risk algorithm based upon age, MWT, LA diameter, peak LVOTG, family history of SCD, unexplained syncope and NSVT to provide individualized 5 year risk.[12 38]

The guidance categorises an individual's risk as low (<4%, 5 year risk of SCD), intermediate (4-6%) and high (>6%). ICD implantation is generally not indicated in those with low risk but may be considered in the presence of intermediate risk and should be considered in those identified as high risk.[12] The insertion of an ICD requires close consideration of the life-style, socio-economic status and psychological health. Individuals should be counselled regarding potential complications as ICD related complication rates are high in HCM with one dataset demonstrating 16% of patients received inappropriate therapy and 18% experienced device related complications.[102]

Symptomatic bradycardia and AV block may be treated with anti-bradycardia pacing. However, the development of progressive conduction in HCM should prompt the consideration of non-sarcomeric disease such as Anderson Fabry disease or amyloid.[77] The role of cardiac resynchronization therapy has not been systematically evaluated in HCM but is often considered in those with a broad QRS and systolic impairment.

1.7.3 Invasive management of left ventricular outflow tract obstruction

Invasive strategies for the management of LVOTO is indicated in symptomatic individuals whose symptoms are refractory to medical therapy. There is no evidence to support invasive septal reduction therapy in asymptomatic individuals.

Right ventricular pacing

Several small trials have shown that sequential atrio-ventricular (AV) pacing with a short AV delay may be useful in treating LVOTO by promoting cardiac dyssynchrony.[103] [104] The evidence in support of this practice remains inconclusive and the use of sequential AV pacing for the management of LVOTO is generally reserved for those who are considered unsuitable for other forms of septal reduction therapy, those who have concurrent indication for device insertion or to facilitate medical optimization with beta blockers or calcium channel antagonist.[12] [105]

Surgery

Surgical septal reduction therapy to treat LVOTO involves resection of septal muscle distal to the mitral leaflet septal contact point through a retracted aortic valve. The surgical resection can be extended to the distal septum in the presence of mid-cavity obstruction and concomitant mitral valve surgery can be performed in the presence of residual outflow tract obstruction or mitral regurgitation. Long term symptomatic improvement can be achieved in above 70% of cases with low mortality and complications rates when performed in experienced centres.[106-111]

Alcohol Septal Ablation

Alcohol septal ablation (ASA) was developed in the 1990s and involves the selective injection of alcohol into an appropriate septal perforator to create a localized infarct.[112] A meta-analysis of 42 published studies demonstrated that ASA is an effective treatment for the management of LVOTO resulting in improved functional class and exercise capacity.[113] There are no randomized control trial data comparing surgical myectomy vs. ASA, but recent meta-analyses have demonstrated that ASA and surgical myectomy are comparable in improving functional class and mortality.[114 115] ASA is associated with higher incidence of high degree conduction disease requiring the insertion of a pacemaker and residual LVOTG.[114 115]

1.7.4 Cardiac transplantation

Cardiac transplantation can be considered in patients with HCM with refractory symptoms in the absence of LVOTO. In one case series HCM accounted for approximately 5% of cardiac transplants with comparable outcomes to those with alternate indications.[116] The primary indication for heart transplant is refractory heart failure symptoms and evolution to end-stage disease with only a small proportion (approx. 5%) requiring intervention for refractory ventricular arrhythmias.[116 117]

2. Myocardial fibrosis in hypertrophic cardiomyopathy

Fibrous connective tissue is formed as part of a reparative or reactive process and regardless of the organ involved, common molecular pathways are activated. Myocardial fibrosis is influenced by ischaemia, haemodynamic load and neurohumoral activation. Although fibrosis is not specific to HCM, histopathological studies consistently support a central role in the disease pathogenesis. Fibrosis may occur early in the disease and is associated with a worse prognosis but the degree of fibrosis in HCM cannot be explained by hypertrophy or disarray alone.

2.1 The extracellular matrix

The cardiac extracellular matrix (ECM) is a complex and dynamic entity composed of proteoglycans, glycoproteins, proteases, collagens, growth factors and cytokines, cardiac fibroblasts, blood vessels, lymphatic vessels and nerve endings. Modulation of these various components facilitates proper mechanical, chemical and electrical signalling between cells.[118 119] The cardiac ECM provides a structural scaffold to cardiac myocytes and vascular structures as well as maintaining normal chamber geometry and ventricular function. Beyond its structural role, the ECM facilitates mechanical, electrical and chemical signals during homeostasis and cardiac development. Cell proliferation, migration, adhesion and changes in gene expression during occur in response to physiological stress or injury.[118 120]

Cardiac myocytes are surrounded by a basement membrane which is composed of type IV collagen, alongside laminins, entactin, fibrillin and fibronectin.[120 121] The interstitial matrix is a network comprised primarily of structural fibrillar collagens I and III. The network of collagen fibres is organised in 3 distinct layers: endomysium, perimysium and epimysium. The endomysium surrounds individual muscle fibres, while the perimysium surrounds groups of muscle fibres; the epimysium encloses groups of perimysial bundles.[120] Non-collagenous glycoproteins (including fibronectin and laminin) and proteoglycans are important in cellular adhesion, cell-cell interaction and signal transduction.[120]

The ECM is linked via integrins to the cellular cytoskeleton. Integrins are transmembrane receptors, composed of α and β subunits, which serve important roles in cellular adhesions, ECM organisation, cellular signalling, proliferation and survival.[122] In mammals there are 18 α subunits and 8 β subunits, with 24 different integrin combinations.[123] In myocytes integrin subunits α_1, α_3, α_5, α_6, α_7, α_9, and α_{10} are expressed, while β_1 is the cardiac-specific sub-isoform, with some authors also detecting expression of β_3 and β_5 subunits on myocytes. Cardiac fibroblasts (CF), also express the integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$, but also uniquely express the subunits α_2 and α_v which associates with β_1, β_3 and β_5.[124 125]

Altered expression of integrins is observed in animal models of myocardial fibrosis, infarction and hypertrophy.[124-126] In patients with ischaemic cardiomyopathy, levels of integrin β_{1D} were reduced compared to controls whilst alterations in the expression of cardiac integrins have been demonstrated pre and post device therapy.[127-130] The diversity of combinations in cardiac integrins and the varied expression through development, health, ageing and disease is likely to reflect varied functional roles for integrins.[125]

Cardiac integrins may also play a role in angiotensin II (Ang II) and TGF- β mediated fibrosis. Exposure of CF to Ang II results in increased expression of integrins $\alpha_V\beta_3$ and $\alpha_8\beta_1$.[131 132] Furthermore, Ang II is a potent inducer of Osteopontin (OPN) which binds to a variety of integrins and β_1 integrins regulate the angiotensinogen gene in CFs, in response to mechanical stretch[124 133] Integrin dependent mechanical activation of TGF- β has been well described in epithelial cells which contains the $\alpha_V\beta_6$ integrin receptor.[124] Epithelial cells are not expressed in the heart, but elevated levels of the mesenchymal integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$ have been identified in animal models of fibrotic cardiac disease and have been shown to play an important role in controlling human myofibroblast differentiation via TGF- β activation.[134] Integrin $\alpha_V\beta_8$, has also been shown to activate in a proteinase dependent manner, although its role in cardiac fibrosis is yet to be established.[135]

2.2 Fibroblasts and regulation of the extracellular matrix

Cardiac fibroblasts are the major cellular component of the human myocardium. CFs are critical in maintaining homeostasis of the ECM by regulating collagen synthesis and degradation. Collagen I and III is synthesised by CF as a procollagen precursor, maturation of this precursor results in the formation of mature collagen and the release of amino- and carboxy-terminal propeptide (PINP/PIIINP and PICP/PIIICP) by-products into the interstitium and plasma. Degradation of mature collagen is regulated by matrix metalloproteinases (MMP) which are inhibited by tissue inhibitors of metalloproteinases (TIMP). The by-products of collagen degradation are carboxy-terminal telopeptides (ICTP/IIICTP), *Figure 2.*

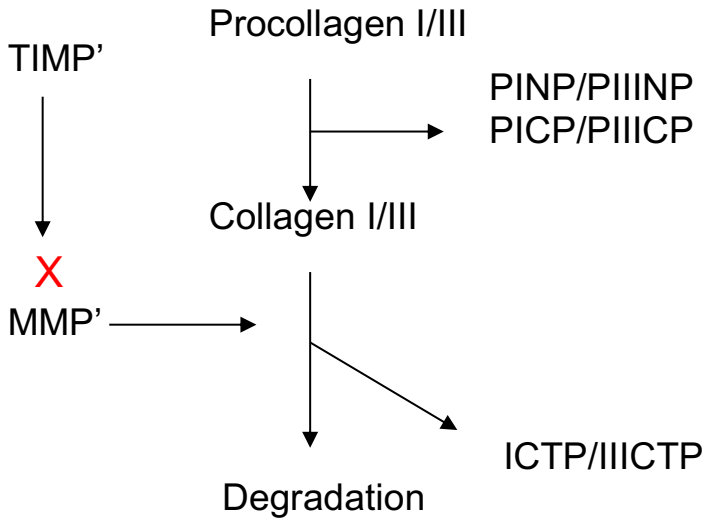

Figure 2 Collagen metabolism

Abbreviations: PINP Aminoterminal propeptide of type I procollagen, PIIINP Aminoterminal propeptide of type III procollagen, PICP Carboxyterminal propeptide of type I procollagen, PIIICP Carboxyterminal propeptide of type III procollagen, MMP Matrix metalloproteinase, TIMP Tissue inhibitors of metalloproteinases, ICTP Carboxyterminal telopeptide of type I collagen, IIICTP Carboxyterminal telopeptide of type III collagen

In response to stress and pro-fibrotic mediators, resident CF undergo transition to the active myofibroblast phenotype promoting ECM turnover and collagen synthesis.[136] In addition to resident CF, during periods of cardiac remodelling myofibroblasts can also be derived from other cell types including smooth muscle cells, epithelial and endothelial cells and circulating bone marrow derived progenitor cells, monocytes and fibrocytes.[137 138]

2.3 Driving factors of myocardial fibrosis in hypertrophic cardiomyopathy

Cardiac fibroblasts are under external influence and in response to a variety of stimuli including mechanical stretch, ischaemia, oxidative stress and inflammatory mediators can undergo transformation to express the myofibroblast phenotype and promote collagen accumulation. These stimuli are particularly relevant to the HCM disease model and are likely in some part to drive the development of myocardial fibrosis.

2.3.1 Mechanical stretch

Plasma levels of B-natriuretic peptides (BNP) are markers of myocardial stretch. Elevated levels of BNP have been demonstrated in both endomyocardial samples and serum of patients with HCM.[139-144] The causative mechanism of myocardial stretch may relate to raised left ventricular filling pressures and also outflow tract obstruction. Furthermore, mutations involving the intrinsic myocardial stretch receptor, the Z discs, have been linked to the development of the HCM phenotype.[145 146]

2.3.2 Oxidative stress

Markers of oxidative stress are increased in individuals with HCM. Right ventricular septal endomyocardial samples from individuals with HCM have shown increased expression of major lipid peroxidation product, 4-hydroxy-2-nonenal-modified protein - when compared to normal controls and levels correlate with LV dimensions and inversely with systolic function.[147] Serum levels of 8-isoprostaglandin F, a stable marker of oxidative stress, has also been shown to elevated in HCM and highest in those with LVOTO.[148]

2.3.3 Ischaemia

Myocardial ischaemia is another putative driver of myocardial fibrosis. Myocardial ischaemia is likely to be a result of increased myocardial metabolic demands and the presence of morphologically distinct and abnormal blood vessels**.** Histological examination may demonstrate features consistent with subacute and chronic

ischaemic injury including areas of coagulative necrosis, neutrophilic infiltrate, myocytolysis, granulation tissue healing and replacement fibrosis.[27 149 150].

Studies examining changes in coronary sinus pH have demonstrated that a proportion of patients produce lactate under stress.[151-153] *In vivo* perfusion imaging techniques have also demonstrated myocardial ischaemia as a prominent feature in HCM.[54-59] Using positron emission tomography patients with HCM have shown a failure to augment myocardial blood flow during coronary vasodilatation, which is most marked in the sub-endocardium.[60-62] Several studies have also demonstrated an association between replacement myocardial fibrosis, impaired myocardial blood flow and perfusion defects using CMRI. [62 154-157]

2.3.4 Inflammation

Several markers of inflammation have been shown to be elevated in individuals with HCM. The acute inflammatory cytokine interleukin-6 is elevated in patients with HCM compared with controls.[158 159] Circulating levels of pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α) are also higher in patients with HCM when compared to healthy controls, and were found to be highest (although not significantly so) in patients with end-stage HCM.[159] In a study of patients with obstructive HCM, myocardial TNF-α levels were increased at baseline and decreased following non-surgical septal reduction therapy.[160]

In a recent study, elevated plasma levels of high sensitivity CRP, interleukin 1β, interleukin 1 receptor antagonist, interleukin 6 and interleukin 10 were observed in HCM compared to controls. Histological assessment of endomyocardial biopsies demonstrated the presence of inflammatory cell infiltrate in 37% of patients and correlated with histological fibrosis. Nuclear factor kβ activity was also identified in half of the endomyocardial biopsies. Plasma levels of TNFα, high sensitivity CRP and interleukin 1 receptor antagonist were shown to correlate with levels of late enhancement.[161]

2.3.5 Energy depletion

Myocardial phosphocreatine to adenosine triphosphate (PCr:ATP) ratio is a marker of myocardial energy metabolism and can be measured by 31P magnetic resonance spectroscopy. The PCr:ATP ratio has been shown to be reduced in individuals with HCM and may precede the development of hypertrophy.[162-164] These findings support the hypothesis that energy deficiency may be an early driver of the clinical phenotype. A recent study evaluating the role of myocardial energy deficiency in HCM suggested that energy deficiency contributes to diastolic function and also exercise capacity.[165]

2.4 Mediators of myocardial fibrosis

Myocardial fibrosis is mediated by a complex interaction of hormones, growth factors and proteins, *figure 3*. Potentially mediators of myocardial fibrosis in HCM are discussed in more detail.

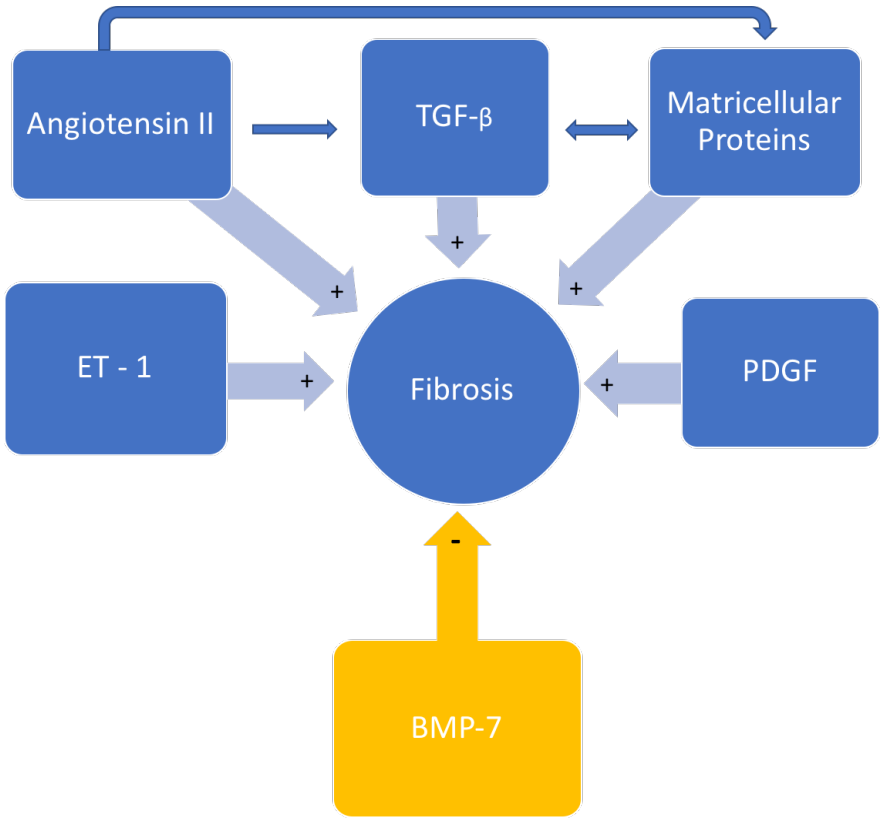

Figure 3: Putative mediators of myocardial fibrosis.

Abbreviations: TGF-β Transforming growth factor beta, ET-1 Endothelin 1, PDGF Platelet derived growth factor, BMP-7 Bone morphogenetic protein 7

2.4.1 Matricellular proteins

Matricellular proteins are a group macromolecules that are secreted into the extracellular matrix that primarily modulate cellular function without serving a structural role.[166] Although a diverse group of proteins, they share several characteristics which include: i) high levels of expression only during development and in response to injury; ii) ability to bind to a variety of cell-surface receptors, components of the extracellular matrix, growth factors, cytokines, and proteases; iii) ability to induce de-adhesion or counter-adhesion and iv) limited involvement in tissue homeostasis.[166-168] Members of the matricellular protein family include TSP-1, 2 and 4, secreted protein acidic and rich in cysteine (SPARC or Osteonectin), tenascin C (TN-C) and X (TN-X), OPN, periostin and members of the CCN family.

Osteopontin

Osteopontin is an arginine–glycine–aspartate-containing adhesive glycoprotein. It is not expressed in the healthy myocardium but is upregulated in response to hypoxia, injury and volume/pressure overload.[169] Transcription is up-regulated by Ang II and by other growth factors including TGF-β and fibroblast growth factor-basic (bFGF).[170 171] Osteopontin expression has been shown to be markedly increased in the infarcted myocardium.[172] In animal models of cardiac hypertrophy and fibrosis, OPN expression is markedly upregulated and mediates a cardiac hypertrophy and fibrosis.[173 174] Knock out animal models have shown a reduction in myocardial fibrosis, hypertrophy and accelerated systolic dysfunction in response to stress.[169 173 175] In humans, increased myocardial expression of OPN in DCM correlates with myocyte hypertrophy, LV dilatation, impairment of systolic function and increased levels of type I collagen.[176] Elevated levels of OPN have also been identified in individuals with HCM relative to controls and patients with ischaemic heart disease (IHD).[177] Plasma levels of OPN have been shown to correlate with the severity of heart failure and predict mortality.[178]

CCN Family

The CCN gene family (Cyr61/CTGF/NOV) consists of six members: *CCN-1* – 6. Of this group, the matricellular protein CCN2 has been most studied and implicated in cardiac disease. CCN2 expression is strongly driven by TGF-β partially through Smad dependent signalling.[179 180] Ang II and ET-1 are also regarded potent inducers of CCN2.[181 182] Ang II induction of CCN2 appears to be partially dependent on TGF-β and treatment of cultured cardiomyocytes and microvascular endothelial cells with anti-TGF-β neutralizing antibody inhibited Ang II driven CCN2 expression.[183] Elevated RNA and protein levels of CCN2 have been demonstrated in fibrotic disease in both animal models of cardiac disease and humans, including myocardial infarction, diabetes and heart failure.[180 184] Despite its association with myocardial fibrosis, CCN2 on its own is considered a weak promotor of fibrosis, and requires TGF-β and other fibrogenic stimuli to produce a sustained and profound fibrotic response.[180 185]

SPARC

SPARC (osteonectin/BM-40) is a highly conserved, multifunctional glycoprotein. Induction of SPARC is mediated by TGF-β and other members of its family.[186] SPARC shares a synergistic relationship with TGF-β and can promote TGF-β expression and signalling.[187] SPARC has also been shown to have an inhibitory role in PDGF, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) signalling.[188] SPARC levels in the myocardium are significantly elevated in response to infarction and in models of hypertrophy and fibrosis.[189-191] SPARC loss has been associated with disorganised granulation tissue, impaired scar maturation and with an increased incidence of cardiac rupture and heart failure.[192] SPARC null mice had reduced collagen content in models of hypertrophy and fibrosis.[191]

Periostin

Periostin is a 90-kDa secreted protein involved in cell adhesion and contains four repetitive fasciclin domains. Periostin is expressed in the neonatal heart where it has

a role in fibroblast recruitment and modulation, resulting in maturation of the collagenous system.[193 194] In the adult myocardium, increased expression of periostin in response to insult is driven by TGF- and bone morphogenetic protein (BMP).[195] In the infarcted myocardium, periostin has been shown to play an important role in early scar formation and protection against acute myocardial rupture but may contribute to long term adverse remodelling.[196-198] In animal models of hypertrophy, periostin was associated with myocardial hypertrophy with loss of function being associated with reduced hypertrophy and fibrosis.[199-201] Overexpression of perostin has been associated with myocardial hypertrophy and also progressive ventricular failure and myocardial fibrosis.[196 202 203]

Tenascin

Tenascins are a family of highly conserved oligomeric glycoproteins with 4 paralogs labelled C, R, X and Win mammals. Tenascin C is the founding member and most implicated in cardiac pathophysiology. Tenascin C synthesis can be upregulated by environmental factors including hypoxia, mechanical stretch and in response to inflammatory cytokines and growth factors including TGF-β and Ang II.[204 205] In animal models TN-C has been shown to play an important role in myofibroblast recruitment, ECM deposition and angiogenesis.[167 206-209] Increased expression has been shown in human infarcted myocardium, myocarditis and dilated cardiomyopathy.[210-213]

Thrombospondins

The thombospondin (TSP) family consist of 5 multifunctional proteins that can be divided into 2 subgroups. Subgroup A, contains TSP-1 and TSP-2 which form trimers and subgroup B containing TSP -3, -4 and - 5, which are homopentamers. Each thrombospondin has a distinct pattern of expression but TSP 1, -2 and -4 have been shown to play roles in cardiac fibrosis.[214-217] Thrombospondin-1 expression is enhanced by the pro-inflammatory cytokines and growth factors including TGF-β, PDGF, bFGF, Ang II, tumour necrosis factor (TNF) and interleukin-1 (IL-1).[188 218 219] Thrombospondin-1, is a angiostatic mediator that also plays an important role in TGF-

β activation and inhibition of MMP activation.[220 221] It is upregulated in the infarcted and pressure overloaded ventricle and is considered an important mediator of matrix preservation and preventing progressive ventricular dilatation.[215 222] Thrombospondin-2 has also been shown to play a role in matrix preservation by inhibiting MMPs whilst TSP-4 null animal models have shown accelerated fibrosis suggests an anti-fibrotic role.[217 223]

2.4.2 Angiotensin II

Angiotensin II is an oligopeptide that is elevated in fibrotic heart disease. Angiotensin II has been shown to promote fibroblast proliferation, collagen synthesis, myocardial fibrosis and TGF-β synthesis via activation of the angiotensin receptor type 1 (AT-1).[214 224-227] Transforming growth factor-β has been identified as a critical downstream mediator of Ang II which via TGF-β/Smad 3 signalling mediates collagen synthesis in response.[228] Antagonism of AT-1 inhibits the expression of TGF-β1 in models of fibrotic heart disease.[229 230] In the absence of TGF-β, Ang II does not result in myocardial fibrosis and hypertrophy.[231] Angiotensin II may further promote myocardial fibrosis by promoting the expression of Interleukin-6 (IL-6) and suppression of microRNAs 29b and 33a.[232-234]

In a Troponin T animal model of HCM, treatment with Losartan was shown to reduce the collagen volume fraction by 49% which was paralleled by a 50% reduction in the expression of collagen 1α(I) and TGF-B1.[235] In another αMHC model of HCM chronic and early treatment was shown to prevent the development of LVH and fibrosis, although in this study the use of Losartan did not reverse established pathological remodelling as seen in the previous study.[200]

The benefits for angiotensin antagonism in human studies have unfortunately been conflicting and underwhelming. In a small study involving 23 patients with HCM, the addition of valsartan was shown to reduced levels of procollagen I but with no differences seen in levels of BNP and procollagen III.[236] In another study Losartan treatment in patients with non-obstructive HCM resulted in improvement in markers

of diastolic function and also a reduction in NT-pro BNP.[237] In another study using Losartan therapy in HCM identified a signal demonstrating attenuation and even regression in some patients of both LV mass and the extent of LGE although no differences in the levels of PICP between the control and losartan group were noted.[238] Other small pilot studies have also conferred a benefit of angiotensin antagonism using a angiotensin II receptor antagonist on LV mass, systolic performance and exercise tolerance.[239 240] However these positive findings were not supported in the larger INHERIT trial which demonstrated that Losartan treatment at 1 year did not result in a difference in LV mass and the extent and de novo occurrence of LGE. This study argues against the benefit of angiotensin antagonism in those with established disease.[241] The role of targeting this pathway in sarcomeric mutation carriers with no or early phenotypic expression is in process.

2.4.3 Endothelin 1

The endothelin group of proteins are well established vasoconstrictors that play a role in cardiovascular disease including systemic and pulmonary hypertension. Endothelin-1 has also been shown to be a mediator of fibrosis which may act downstream of TGF-β and angiotensin II.[185] Endothelin-1 binds to two distinct receptors, ET-A and ET-B, which are present in both cardiac fibroblasts and myocytes respectively.[242 243] Endothelin-1 promotes fibroblast proliferation, expression of the myofibroblast phenotype and enhance collagen synthesis.[243-246] In animal models ET-1 induced myocardial fibrosis whilst antagonism of endothelin was served a protective role in myocardial fibrosis.[247-249] Levels of myocardial and circulating ET-1 are elevated in patients with chronic heart failure but long-term antagonism of the endothelin receptor does not alter the course of the disease.[250 251]

2.4.4 Platelet derived growth factor

Four platelet derived growth factors (PDGF A to D) ligands have been identified which form homodimers (PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD) and heterodimers (PDGF-AB). All forms of PDGF bind to tyrosine kinase receptors, PDGFR α and β. All known

PDGF isoforms can generate cardiac fibrosis and hypertrophy when overexpressed from cardiomyocytes. However, the degree and location of fibrosis vary between the different ligands, which are likely a result of differential activation of the two PDGF receptors.[252-254] In rodent models of myocardial infarction increased PDGF-A, PDGF-D and PDGFR levels are coincident with angiogenesis, and inflammatory and fibrogenic responses.[255] Levels of PDGF-B and PDGF-C were reduced.[255] Similarly, cardiac-specific overexpression of PDGF A, C and D, but not PDGF B have been shown to enhance TGF-β1 transcription and promote the development of cardiac fibrosis.[256] In the infarcted myocardium, perivascular cells demonstrate activation of the PDGF-B/PDGFR- β pathway and may be a potential source of myofibroblast.[257] However, in a rodent model of ischaemic reperfusion injury, recombinant human PDGF-B upregulated messenger RNA expression of anti-mesenchymal transition factor BMP-7.[258] Antagonism of PDGF receptors by Imatinib results in reduced expression of fibrogenic mediators including TGF-β, reduced collagen type I expression and scar formation.[259]

2.4.5 Transforming growth factor-beta

Transforming growth factor β is a cytokine which exerts a pleotrophic effect on all cell types involved in cardiac injury, repair and remodelling. In vitro and in vivo studies have established its central role in mediating myocardial fibrosis and hypertrophy.[260] [261] Three distinct isoforms of TGF-β (TGF- β1, 2 and 3) exist and exhibit distinct patterns of regulation.[262] TGF- β1 is the most prevalent isoform in the cardiovascular system and much of our knowledge relating to TGF- β and myocardial fibrosis relates to this isoform.

Transforming growth factor β is synthesized as a precursor molecule and is incorporated into the ECM in a latent form following association with its latency-associated pro-peptide (LAP) and latent TGF-β1-binding protein (LTBP-1).[263] Only a small proportion of TGF- β requires activation from its latent state to exert a maximal cellular effect.[264] TGF- β is activated by TSP-1, integrins, Ang II, proteases including MMP2, 9 and plasmin as well as changes in the pH of the local environment.[124 220 264-269]

Active TGF- β binds to TGF- β type II receptor (TGF- βR2) which in turn results in the recruitment and phosphorylation of TGF- β type 1 receptor (TGF- βR1).[270 271] The TGF- βR1 is a kinase, and in the case of fibroblasts, it is termed activin-linked kinase 5 (ALK5). Subsequent phosphorylation of Smad 2 and 3 by ALK5 results in the formation of heterodimers with Smad 4 which can translocate into nucleus and directly promote pro-fibrotic gene expression. This process can be inhibited by Smad 7.[262 271 272]

Independent of Smad mediated transcription, TGF-β activates additional signalling cascades including extracellular signal regulated kinase (Erk), c-Jun-N-terminal kinase (JNK), TGF-β-activated kinase 1 (TAK1), p38 Mitogen activated protein kinase (MAPK) and GTPase pathways.[271] The pro-fibrotic actions of TGF- β in part also relate to its ability to strongly induce and synergistic interaction with CCN2. Elevated RNA and protein levels of CCN2 have been demonstrated in fibrotic disease in both animal models of cardiac disease and humans, including myocardial infarction, diabetes and heart failure.[180 184]

A landmark study in 2007, demonstrated that endothelial - mesothelial transformation (End-MT) contributed to the development of myocardial fibrosis. The authors demonstrated that almost a third of myocardial fibroblasts were of endothelial origin. In cultured cells, they identified that the process of End-MT was driven by TGF- β and antagonised by BMP-7. Administration of BMP-7 was also able to reduce fibrosis via a reduction in End-MT in animal models of fibrosis.[273] Parallels have identified in renal models of fibrosis.[274]

Bone morphogenetic protein-7, is a member of the TGF-β superfamily. BMP-7 signalling is regulated by Smad1, 5 and 8. The final common step involves Smad 4 regulated translocation into the nuclear envelope.[275] Recent studies have shown that BMP-7 can attenuate and facilitate reverse remodelling in animal models of AF and pressure overload induced fibrosis by antagonising the effects of TGF-β.[276 277]

Studies in HCM have been limited, but have demonstrated that myocardial TGF-β1 messenger RNA and protein levels were 2.5 and 2.8 times higher in areas of hypertrophy relative to non-hypertrophied.[278] Individuals with HCM have also been shown to demonstrate increased gene expression of TGF-β1 relative to those with aortic stenosis, stable angina and dilated cardiomyopathy.[279] Elevated TGF-β in those with HCM have been associated with increased brain natriuretic peptide (BNP), higher symptomatic status and adverse outcomes.[280]

2.5 Histological evidence for myocardial fibrosis in hypertrophic cardiomyopathy

2.5.1 Histology

Histological analysis of the myocardium is the gold standard method for identifying and quantification of myocardial fibrosis. The observed patterns of myocardial fibrosis in HCM are described below.

Replacement fibrosis, is the reactive deposition of collagenous scar in areas of myocyte death, *figure 4.* This type of fibrosis is synonymous with myocyte death following ischaemic insult but is also a prominent histological feature in HCM.[21 22] The replacement scarring may be represented as small discrete foci, but multiple foci, confluent zones of fibrosis in the subendocardium and transmural scar also occur.[21 24] Post-mortem examination of individuals with HCM who experienced sudden death revealed that chronic post-necrotic replacement fibrosis was a common finding in the absence of significant epicardial disease.[27]

Diffuse interstitial myocardial fibrosis, is the expansion of the extracellular matrix by collagen, *figure 4.* An eight-fold increase in this form of collagen was identified in asymptomatic children and young adults with HCM who suffered sudden death compared to normal controls. Expansion of the interstitial compartment was also identified in infants and was independent of the organisation of the myocardial architecture.[23] This study demonstrated that expansion of the interstitial collagen network commences at an early stage of the disease and continues during periods of

growth. Interstitial collagen accumulation is more prevalent in the dilating phase of HCM.[281] This form of fibrosis appears to be predominantly seen in the mid wall of the myocardium although there is considerable heterogeneity.[23] [282] The histological evidence suggest that interstitial fibrosis may be an early and potentially reversible primary phenomenon that determines clinical phenotype and prognosis.

Excessive collagen accumulation may also be seen surrounding the myocardial vessels, (*perivascular fibrosis)* and also in association with myocyte disarray (*plexiform fibrosis)*.[21] [22] [25] The latter is commonly identified in the septum at the insertion points.

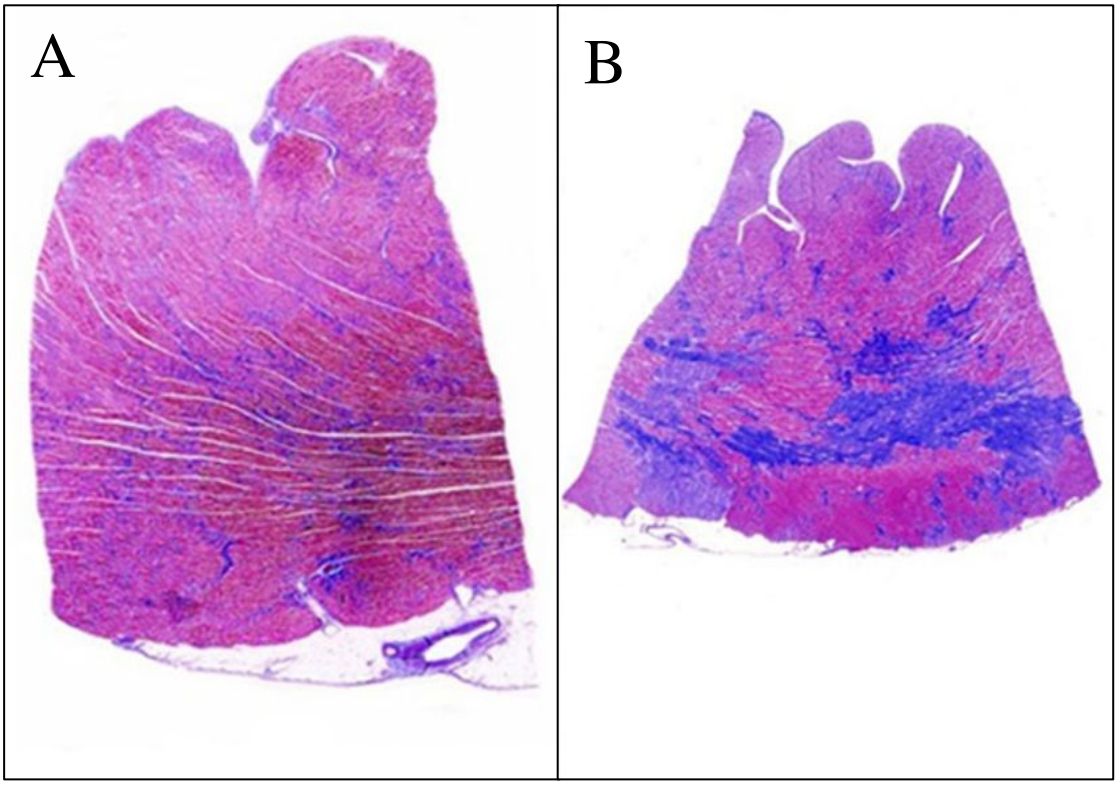

Figure 4: Myocardial tissue stained with Azan Mallory trichrome demonstrating patterns of myocardial fibrosis (blue). A. Interstital fibrosis B. Replacement fibrosis. With permission from Galati et al, 2016.[282]

2.5.2 Techniques for tissue fibrosis quantification

Dye Staining

Histological quantification of collagen can be performed by dye staining cut sections of either frozen or paraffin embedded tissue. The collagen volume fraction can be determined using automated image analysis software after selecting the region of interest. Several dyes have been established in their ability to stain collagen.

Van Gieson's stain is the most basic stain used for the identification of collagen. The combination of anionic dyes used in this technique produces differential binding of the dye to tissue components. The picric acid is able to penetrate and stain all the components of tissue whilst the larger molecules of fuschin acid is only able to enter the porous collagen network and displace the picric acid and ultimately results in the collagen network staining red and other components of tissue appearing yellow.

Masson trichrome involves utilisation of three staining dyes with differing colours and size to again produce differential staining of tissue contents based upon the porousness of each tissue component. The exact composition of the stain can very but generally red blood cells, keratin and muscle stain red, cytoplasm and adipose tissue staining pink, cell nuclei appear brown or black and collagen appearing either green or blue.

Picrosirius red binds to collagen and under bright field microscopy appears red. Picrosirius red also exaggerates the bifringency of collagen and under polarised light with collagen appearing green, red, orange or yellow on a black background. Some studies have shown that the appearance under polarised light may help to differentiate collagen type with type I collagen appearing red, orange or yellow and type III collagen appearing green.[283] Other studies have however demonstrated that polarised colours are more reflective of fibre thickness and packing with larger collagen fibres appearing bright yellow or orange whilst thinner ones are green.[284]

Hydroxyproline

Collagen contains 12.2% w/w hydroxyproline which can be accurately quantified in homogenised tissue samples using either a commercially available colorimetric assay or by performing reverse-phase high performance liquid chromatography (HPLC).[285]

To date the majority of studies in HCM which have utilised dye staining methods to quantify collagen volume fraction, however given the heterogeneity in the extent and distribution of fibrosis seen in HCM this method remains vulnerable to the influence of sampling error and may essentially influence the significance of clinical results.[286] Unlike staining methods, quantification of hydroxyproline does not provide any details relating to the pattern of collagen deposition but does provide accurate collagen quantification for an entire volume of tissue and thus eliminating sampling variability.[285]

2.6 Detection and clinical significance of myocardial fibrosis in vivo

2.6.1 Collagen biomarkers

Several small-scale studies have evaluated collagen biomarkers in HCM and have been inconsistent in their findings.[287-294] Ho et al, provided the most significant study on collagen biomarkers in HCM.[295] In this study the authors measured differences in levels of PICP, MMP-1, TIMP-1 and ICTP between individuals with pathogenic sarcomeric mutations with overt disease, subjects with proven pathogenic sarcomeric mutations without LVH and controls. Compared to controls those with overt disease demonstrated a significant elevation in PICP and its ratio with ICTP. No differences were identified in levels of MMP-1, TIMP-1 and ICTP. In those with overt disease there was no correlation between collagen biomarkers and clinical parameters including LGE. However, the most significant finding of this paper was the discovery of raised levels in PICP in gene carriers without overt LVH or LGE on CMR when compared to controls. This finding demonstrates that enhanced collagen synthesis may represent

an early clinical manifestation of sarcomeric disease and provide a useful serological marker of genetic expression.

2.6.2 Echocardiography

Myocardial scarring is characterised by increased acoustic brightness and integrated backscatter imaging was developed as a method to assess tissue reflectivity. Integrated backscatter which is calibrated relative to the pericardium and blood has been shown to correlate with histological fibrosis in those with myopathic and valvular cardiac disease.[296-299] In HCM, integrated backscatter was higher in patients compared to controls and correlated with histological findings including disarray, interstitial fibrosis and myocyte non-homogeneity.[300] Calibrated integrated backscatter has also been shown to be higher in sarcomeric mutation carriers relative to controls and an independent predictor of NSVT in those with the overt phenotype.[301 302] Cyclic variation in integrated backscatter has previously been used to determine regional myocardial function but has been superseded by strain imaging as a clinical utility.[303]

2.6.3 Contrast enhanced cardiac magnetic resonance imaging

Contrast enhanced cardiac magnetic resonance (CMRI) imaging allows in vivo evaluation of myocardial fibrosis. The contrast agents employed are Gd based bound to a chelator such as diethylenetriamine pentaacetic acid (DTPA). Following administration into the intravascular compartment, Gd-DTPA can diffuse into the interstitial compartment of both normal and abnormal regions of myocardium. Due to increased volumes of distribution[304-306] and differences in wash-in/washout time constants,[305 307] abnormal myocardium accumulates higher concentrations of Gd-DTPA and differences in image enhancement over time identify regions of fibrosis, late Gd enhancement (LGE).[307]

The distribution of late enhancement in the diseased myocardium is not uniform. The distribution and extent of myocardial may help to establish an aetiology of both the dilated and hypertrophied heart.[308] In ischaemic heart disease the presence of LGE has been shown to be an predictor of adverse outcomes.[309 310] Furthermore, the

extent and degree of transmural LGE has been shown to predict a poor response to revascularisation, medical therapy and cardiac resynchronisation.[311-314] In non-ischaemic DCM, the pattern of LGE may help to identify the presence of infiltration and cardiac involvement as a part of a multisystem disease and the presence of LGE in DCM has been shown to be an adverse prognostic marker. In patients with an LVEF <35%, the presence of LGE was shown to result in a 8 fold increased risk of experiencing a composite end point of a heart failure admission, appropriate ICD therapy or cardiac death.[315] In another study involving 472 patients, the presence of midwall fibrosis was found to be an independent predictor beyond LV function for all cause and cardiovascular mortality.[316]

In the hypertrophied heart, the distribution of LGE and Gd kinetics may help to identify clinical phenocopies of sarcomeric disease. The presence of abnormal gadolinium kinetics and circumferential sub-endocardial late enhancement is specific to cardiac amyloidosis whilst localised posterior wall LGE may be a cardiac feature of Anderson-Fabry disease.[89-92] There is strong correlation between LGE and histological *replacement* fibrosis in HCM, *figure 5.*[86-88 317] Gadolinium enhancement is not uniform and an array of patterns have been described in HCM and are likely to reflect the morphological varieties of fibrosis described above and varying extent of interstitial expansion.[318]

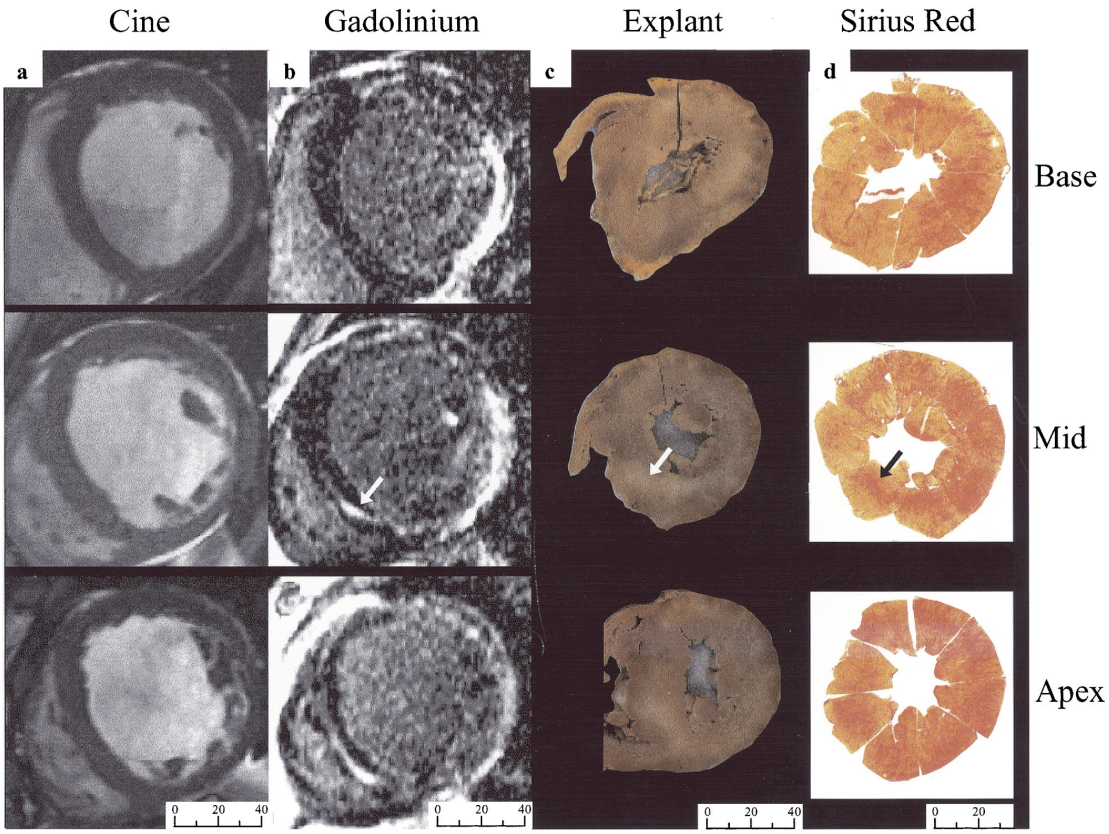

Figure 5: Comparison of ***(a)*** *in vivo diastolic cine image,* ***(b)*** *in vivo gadolinium-enhanced cardiovascular magnetic resonance,* ***(c)*** *gross specimen of sections from an explanted heart, and* ***(d)*** *histologic sections stained with sirius red. Regions of gadolinium enhancement correlate with regions of macroscopic unstained pale myocardium and regions of red-stained collagen. A representative mesocardial region, which is well defined, is marked by an arrow. Figure and caption with permission from Moon et al 2004.*[86]

Techniques for quantification of late enhancement

Studies have utilised a variety of techniques for LGE quantification. Techniques for LGE quantification including manual quantification (which requires the operator to draw regions of interest in areas of LGE) and a variety of thresholding techniques whereby the scar is defined at a specific signal intensity above the normal myocardium. The defined signal intensity can be set to 2 – 6 standard deviations above the normal myocardium or alternatively by using a technique called full width half maximum

(FWHM), whereby the threshold is set to half the maximal signal within an area of scar. Flett et al, demonstrated that in their cohort of patients with ischaemic heart disease and HCM, LGE volumes were comparable when using manual quantification, FWHM, 5 and 6 standard deviation methods. The 2-standard deviation technique produced LGE volumes which were almost double those generated by the manual, 6 standard deviation and FWHM techniques. The FWHM technique was the most reproducible, however reproducibility was the worst for the HCM group.[319] A subsequent study focusing on LGE quantification in patients with HCM concluded that although the FWHM technique was the most reproducible, it also resulted in consistent underestimation when compared to manual quantification. The authors of this study found that thresholding with 3 standard deviations was the most accurate with acceptable reproducibility.[320]

Clinical Significance of late gadolinium enhancement in hypertrophic cardiomyopathy

Several studies have evaluated the clinical significance of LGE upon clinical outcomes. In a study published by Maron et al in 2008, 202 patients underwent a CMRI with a mean follow up of 681 ± 249 days. The authors identified that the presence of LGE was more prevalent in those with HF symptoms and that the extent of LGE was an inverse and independent predictor of left ventricular EF. The annualised adverse cardiovascular event rate, which included appropriate ICD therapy, SCD and progressive heart failure symptoms was not statistically different between those with and without LGE.[321]

A later study from Rubinshtein et al, 2010 included 424 patients who underwent CMRI followed by manual LGE quantification. The authors demonstrated that during follow up (mean 43 +/- 14 months) 8 patients experienced SCD (n = 4) or appropriate ICD therapy (n = 4) and 2 patients experienced heart failure related deaths. All cardiac deaths occurred in patients with LGE. LGE remained an independent predictor of ICD therapy and SCD after adjustment of other risk factors. Although the presence of LGE

was not associated with symptomatic status, LGE was inversely correlated to EF and patients with LGE had a higher burden of ectopy and NSVT.[322]

In the same year, O'Hanlon and colleagues published a study which had enrolled 217 patients to undergo CMRI with a mean follow up of 3.1 years. The authors used the FWHM technique to quantify LGE and demonstrated that LGE was associated with a composite primary outcome of cardiovascular deaths, cardiovascular admissions, sustained ventricular arrhythmias, appropriate ICD therapy or SCD. The presence and extent of LGE was an independent predictor of heart failure related events but did not remain an independent predictor for the arrhythmic end point after adjusting for the presence of NSVT.[88] In the same journal, Bruder et al, also published a follow up study of 220 patients who underwent CMRI with a mean follow up of 1,090 days. During the follow up period, 20 patients died and 2 experienced appropriate ICD therapy. Six patients died of cancer, 9 experienced SCD and 2 died of heart failure. The presence of LGE remained a univariate predictor of all cause and cardiac mortality, whilst the extent of LGE was associated with SCD and all cause cardiac mortality. The presence of LGE remained an independent predictor for all-cause mortality after considering the presence of 1-2 conventional risk factors.[323]

Each of these published studies was limited by a combination of incomplete risk evaluation, selection and referral bias as well as differences in scanning protocols. A meta-analysis of these studies concluded that LGE was associated with all-cause mortality (OR 4.46, CI 1.53 to 13.01, p=0.006), cardiovascular death (OR 2.92, CI 1.01 to 8.42, p=0.047) and heart failure related death (OR 5.68, CI 1.04 to 31.07, p=0.045). A trend for sudden or aborted SCD was observed but statistical significance was not reached (OR 2.39, CI 0.87 to 6.58, p=0.091).[324]

Since the publication of this meta-analysis additional studies examining the influence of LGE on long term outcomes have been published. Ismail et al, examined the role of LGE in risk stratification. The study followed 711 patients who had undergone CMRI for a median follow up of 3.5 years. LGE was quantified by the FWHM technique. The authors identified that the extent, but not the presence of LGE was a strong

univariable predictor of the primary end-point of sudden or aborted SCD (event rate 3.1%). Following multivariable analysis, the association between the extent of fibrosis and the primary end-point did not remain significant after adjusting for LV-EF. The extent of LGE was also found to be a univariable predictor of all-cause mortality but again did not remain significant after the adjusting for the influence of LV-EF and NSVT.[325] A second study by Chan et al which enrolled 1,293 patients identified that the presence and extent of LGE by percent LV mass was strongly associated with sudden cardiac death (adjusted hazard ratio, 1.46 per 10% increase in LGE; *P*=0.002) and the development of LV systolic dysfunction (adjusted hazard ratio, 1.80 per 10% increase in LGE; *P*=0.03). The authors demonstrated that a LGE burden of >15% of LV mass was associated with 2-fold increase in the risk of sudden death and concluded that extensive fibrosis was an adverse prognostic marker.[326]

A more contemporary meta-analysis of 3,067 patients including the above studies concluded that LGE was associated with all cause (OR 1.80, 95% CI 1.21 to 2.67, p=0.003) and cardiovascular mortality (OR 2.78, 95% CI 1.41 to 5.47, p=0.003), sudden cardiac death (OR 2.52, 95% CI 1.44 to 4.4, p=0.001) with a strong trend being observed towards heart failure associated deaths (OR 2.47, 95% CI 0.98 to 6.24, p=0.06). Late enhancement in this meta-analysis was both associated with adverse cardiovascular outcomes which in part may relate to the inclusion of patients with small volumes of fibrosis (<10%) in 5 of the 6 studies utilized.[327]

Together, these studies suggest that fibrosis as detected by late enhancement imaging contributes the development of LV systolic dysfunction and associated symptoms and clinical outcomes. Late enhancement may also serve as a risk marker for SCD and overall adverse cardiac outcomes, however its value once other conventional clinical parameters including NSVT and EF are accounted for remains questionable.

Extracellular volume

Cardiac MRI with LGE enhancement has limitations in quantification of fibrosis as it fails to detect *diffuse* fibrosis. Techniques measuring myocardial longitudinal relaxation time constant (T1) and its spatial resolution (T1 mapping) pre- and post-

contrast may overcome this and allow for a true estimation of myocardial ECV. Several methods have been developed to overcome the confounding effect of contrast kinetics that alongside advancements in T1 mapping sequences have allowed for more robust and clinically applicable whole heart quantification of global ECV. [328-330] ECV has been shown to correlate consistently with myocardial collagen fraction assessed histologically across a range of cardiac pathologies.[286 330-334] Flett et al, evaluated the correlation between ECV and histological collagen fraction in patients with HCM undergoing surgical myomectomy.[286] Using the primed equilibrium contrast CMR (EQ-CMR) technique, the authors identified a positive correlation between ECV and histological myocardial collagen volume. The correlation however was not as strong at that observed in aortic stenosis (r^2 0.86 versus 0.62) and did not achieve statistical significance (p=0.08). The authors postulated that this reflected the heterogeneous and patchy nature of cardiac fibrosis in HCM. White et al, subsequently compared the EQ-CMR technique and the dynamic equilibration achieved by delayed post-bolus imaging upon ECV quantification derived from T1 mapping. The authors found that the correlation with histological collagen fraction and ECV using the bolus technique ($r^2 = 0.69$, $p < 0.01$) was comparable to that with EQ-CMR technique ($r^2 = 0.71$, $p < 0.01$). Furthermore, the authors found that across a spectrum of health and disease that ECV values obtained by both techniques demonstrated a high level of correlation but the bolus technique consistently gave a higher value when ECV exceeded 0.4.[331]

Subsequent evolution of T1 mapping techniques have resulted in the emergence of modified Look Locker inversion recovery (MOLLI) pulse sequences and a shortened MOLLI (ShMOLLI) pulsed sequence. MOLLI merges images from three consecutive inversion recovery experiments and allows T1 determination at the same phase of the cardiac cycle providing a more accurate measurement with less heart rate dependence. ShMOLLI confers an additional benefit of allow generating rapid and high-resolution myocardial T1-maps in a single short breath-hold over only 9 heart beats.[335] Fontana and colleagues demonstrated that single breath hold T1 mapping to quantify ECV using the ShMOLLI protocol was better tolerated amongst patients, provided a better correlation with histological collagen volume and trended towards a greater reproducibility when compared to a multibreath-hold technique.[330] A recent

meta-analysis identified 9 studies that have quantified ECV using a range of contrast protocols and TI mapping techniques. The pooled r for these nine studies describing the overall estimated correlations between collagen volume and ECV was 0.88 (95% CI:0.854, 0.914) and was not notably heterogeneous (chi-squared = 7.44, *P* = 0.489 for the Q test and $I^2 = 0.00\%$). Together, these studies demonstrate that CMR using T1 mapping provides a reliable, consistent and reproducible method for ECV quantification.[336]

Sado et al, 2012 evaluated ECV across a variety of cardiac pathologies and healthy controls. Cardiac ECV values were found to be similar between controls and patients, however the ECV in patients with HCM, DCM and aortic stenosis were comparable and significantly higher than in the control population. The amyloid and infarcted myocardium had a significantly higher ECV when compared to health and the other forms of non-ischaemic cardiomyopathies.[337] Extracellular volume has also been shown to correlate to age and in patients with heart failure and preserved EF ECV has been shown to be a predictor passive LV stiffness.[338 339] Extracellular volume is also higher in hypertensive patients although only in association with the development of LVH.[340]

In 2013, Ho et al identified that patients with HCM demonstrated a higher ECV than controls. Within the HCM cohort those with sarcomeric mutations had a higher burden of LGE and elevated ECV even in areas without late enhancement when compared to patients in whom sarcomeric mutations were not identified. The authors further demonstrated that sarcomeric mutation carriers also had a higher ECV even in the absence of LVH when compared to controls, albeit not to the extent seen in those with overt disease. Extracellular volume was also found to correlate with NT-ProBNP and global E' velocity.[93]

The significance of ECV expansion as detected by CMRI on clinical phenotype is yet to be fully established. A study involving 45 patients found that ECV was significantly associated with parameters of diastolic dysfunction, diastolic strain rate and was inversely proportion to peak oxygen consumption.[341] Another study demonstrated

that in HCM patients with preserved or mildly impaired systolic function, ECV correlated with impairment in both peak circumferential strain and early diastolic strain rate.[342] These studies suggest that expansion of the extracellular compartment influences myocardial function. ECV has also been shown to be shown a useful utility in identifying individuals at identifying patients considered to be at intermediate or high risk of SCD (ESC risk score of >4%). Using a combination of ECV in addition to the ESC risk model increased the diagnostic accuracy of identifying patients with syncope or NSVT.[343] However, in patients considered at high arrhythmic risk that carried ICD's, ECV as quantified by computed tomography did not predict the occurrence of malignant ventricular arrhythmia.[344]

2.6.4 Computed tomography and myocardial fibrosis

The development of multi-detector computed tomography (MDCT) has provided the ability to perform tissue characterisation using cardiac CT. Following the administration of iodinated contrast agent, delayed enhancement imaging with CT has been shown to accurately identify areas of established ischaemic scar on tissue histology and late enhancement on CMRI following myocardial infarction.[345] [346] Studies in HCM have demonstrated a high burden of late enhancement in those considered to be at high arrhythmic risk and correlates with the detection of ventricular arrhythmia in those carrying ICDs.[347] [348] Equilibrium contrast CT can also be used to quantify the ECV by measuring pre and post contrast attenuation. ECV quantification using this method has good correlation with CMRI based quantification of ECV and histological fibrosis across a variety of cardiac disease.[349-351] CT provides many advantages over CMRI including availability, higher spatial resolution and the presence of a linear relationship between attenuation and iodine contrast; however the exposure to the radiation and access CMRI limits its clinical utility.[352]

2.6.5 Myocardial deformation imaging as a surrogate for myocardial fibrosis.

Speckle tracking echocardiography can be used to assess regional and global myocardial deformation. Patients with HCM can demonstrate a reduction in both

regional and global longitudinal strain.[43 353] Longitudinal impairment of strain has been shown to relate to the extent of hypertrophy although several studies have also shown an association between the extent of LGE and both regional and global strain. [43 353-362] In one such study, 48 patients underwent deformation analysis using speckle tracking and CMRI with LGE quantification. `The authors found that global longitudinal strain was lower in patients whom demonstrated LGE and at a regional level segments with LGE had significantly impaired systolic strain. In multivariate analysis including LVMI, early diastolic mitral annular velocity and propagation velocity, GLS was the strongest independent predictor of the extent of LGE (standard coefficient = 0.627, p = <0.001). Furthermore, a GLS of <12.9% was associated with adverse cardiac outcomes.

Myocardial deformation can also be assessed with CMRI. Myocardial tagging imaging can be used to quantify myocardial displacement, strain and strain rates. Studies utilising this technique and have demonstrated that patients with HCM have reduced myocardial contraction in both areas of hypertrophy and non-hypertrophied areas compared to controls.[363 364] Patients with HCM have also demonstrated asynchronous contraction and a generalised reduction in diastolic strain rate indicative of slow filling and diastolic impairment.[363 364] Kim et al 2008, found that in 25 patients with HCM circumferential shortening as quantified by a myocardial tagging sequence was significantly lower in segments that displayed LGE and was more specific to areas with a focal nodular pattern of late enhancement relative to areas with poorly defined late enhancement.[365] Both ECV and LGE have been shown to associate with impairment of circumferential strain using myocardial tagging but the significance did not remain following inclusion of wall thickness.[342]

The use of myocardial tagging in daily practice has however been somewhat limited due to the complex and time-consuming acquisition and post-processing protocols. Feature-tracking CMR overcomes some of these challenges and allows the assessment of myocardial motion and strain on routine steady state in free-precession (SSFP) imaging which as a routine part of a clinical scan. Unlike echo-based strain imaging CMR does not allow tracking of the compact myocardium, however the bloods tissue contrast defines the endocardium and provides the ability to track endocardial

features through the cardiac cycle. Feature tracking is highly reproducible and has been validated against both myocardial tagging and echo strain imaging.[366-368] In patients with HCM, feature tracking shows good agreement with speckle tracking derived longitudinal strain.[369] Late enhancement and wall thickness have been shown to be independent predictors of attenuated circumferential, radial and feature tracking derived longitudinal strain in HCM.[370] In this study abnormal global longitudinal, circumferential and radial strain were associated with all-cause mortality and secondary composite end point of heart failure hospitalisation, ventricular arrhythmias and cardiovascular death. In another study both LGE and ECV were shown to be a univariable associates of impaired circumferential and radial strain derived by feature tracking, a relationship which does not hold true after inclusion of wall thickness.[342]

2.7 Aims of the thesis.

Myocardial fibrosis is a prominent feature in HCM that may precede clinical phenotype[93] [295], contributing to disease progression and clinical outcome. The regulation of genes involved in collagen metabolism and the expression of genes involved in mediating myocardial fibrosis have yet to be studied at a myocardial level. Reverse transcription polymerase chain reaction (RT-PCR) allows generation of sensitive, specific and reproducible quantitative data with a wide dynamic range of detection. The RT² Profiler™ PCR fibrosis array provides the benefits of RT-PCR and combines it with the ability of arrays to profile the expression of a panel of genes relevant to a specific pathway. I therefore choose to utilise RT² Profiler™ PCR human fibrosis array to answer the following aims:

Aim 1: To investigate myocardial expression of genes involved in collagen metabolism in HCM and association with myocardial collagen content.

Aim 2: To determine the myocardial genetic expression of pro-fibrotic mediators.

- *TGF-* β *and associated signaling cascade in HCM.*

- *Thrombospondins, Endothelin-1, Platelet derived growth factor and connective tissue growth factor.*

Markers of collagen metabolism have consistently been shown to be altered in individuals with HCM. However, correlation between markers of collagen metabolism and clinical phenotype have been inconsistent. Furthermore, to date only a solitary study has evaluated the expression of collagen biomarkers in those with sarcomeric mutations prior to the development of clinical phenotype. I therefore sought to evaluate the expression of collagen biomarkers in those with HCM and mutation carriers relative to controls. I further aimed to determine a clinical correlation between collagen biomarkers and clinical features in a well characterised cohort of patients with HCM.

Aim 3: To evaluate plasma markers of collagen metabolism in controls, asymptomatic sarcomeric gene carriers and patients with HCM.

Aim 4: To evaluate the relationship between markers of collagen metabolism to clinical phenotype in patients with HCM.

Myocardial fibrosis has been shown to be a determinant of progressive systolic dysfunction and the development of heart failure. However, the relative contribution of myocardial fibrosis in systolic performance is poorly defined. I sought to determine the association between myocardial fibrosis to systolic performance using 2D speckle tracking imaging.

Aim 5: To determine the between global and regional longitudinal myocardial deformation and left ventricular hypertrophy, replacement fibrosis and interstitial fibrosis.

3. Methods:

3.1 Summary of my role in the project

Together with Professor Perry Elliott, I developed the idea for the research project presented in this thesis. I initially obtained ethics (outlined below) to allow the collection and storage of plasma and tissue required for the studies presented in chapters 4 and 5. I was subsequently responsible for patient recruitment and sample (plasma and tissue) collection for all the patients presented in these chapters between 2010 and 2013. I established the collaborative partnerships that were required for performing the plasma and tissue experiments and took responsibility for sample preparation and delivery. Data collection, clinical profiling and offline 2D echocardiography assessment include strain analysis required for these chapters was performed by myself and a second observer where indicated. Clinical profiling and offline analysis of 2D echocardiography including strain analysis relating to the patients presented in chapter 6 was performed by myself between 2010 and 2012. Statistical analysis presented throughout the thesis was performed by myself.

3.2 Funding

The work in this thesis was undertaken during my period as a Clinical Research Fellow at the Heart Hospital, University College London Hospital. During this period, I was funded by Human Genetic Therapies, Shire Pharmaceuticals Ltd Hampshire International Business Park, Basingstoke, Hampshire, RG24 8EP, UK.

Additional funding was obtained from the Heart Hospital Charitable grants committee, 16-18 Westmoreland Street, London, W1G 8PH, UK for research consumables.

This work was undertaken at UCLH/ UCL that received a proportion of funding from the U.K. Department of Health's NIHR Biomedical Research Centre's funding scheme.

3.3 Ethics

3.3.1 Plasma and tissue

Patients

Ethical approval for the plasma and tissue work performed in this thesis was obtained from the London Central Research Ethics Committee (REC 11/LO/0913)). *Appendix 2.* Ethical approval allowed for:

- Tissue samples to be obtained from patients undergoing surgical myomectomy and retention of redundant tissue undergoing cardiac surgery and myocardial biopsy for protein and RNA expression.

- Blood and urine samples to be collected and stored from patients with HCM and gene carriers for biomarker analysis.

Controls:

Ethical approval for the collection, storage and biomarker analysis of plasma from controls was obtained from the London Central Research Ethics Committee (REC 11/LO/0913)). *Appendix 2.* Ethical approval allowed for:

Ethical approval for the experiments performed utilising control tissue was obtained from the ethical committee of clinical investigation at Hospital Puerta de Hierro-Majadahonda, Estudio de los mecanismo moleculares que regulan la insuficiencia cardiac: paper del splicing alternative; Principal Investigator: Dr Pablo Garcia Pavia.

3.3.2 Cardiac MRI:

All patients included in chapter 6 had been provided consent for their involvement in a study quantifying diffuse myocardial fibrosis using cardiac MRI. Ethical approval for

this study was provided by London Harrow research Ethics Committee (REC 07/H0715/101); Principle investigator Dr James Moon.

3.4 Study Population

3.4.1 Patient recruitment

Tissue: Myocardial tissue from the LV septum was obtained from 24 consecutive individuals fulfilling diagnostic criteria for with HCM (MWT thickness ≥ 15mm in the absence of abnormal loading conditions or ≥13mm with a first degree relative) undergoing surgical myomectomy for the management of LVOTO. There was no evidence of obstructive coronary artery disease or significant valvular heart disease. All patients were under active follow up at The Heart Hospital, University College London, United Kingdom.

Plasma: 113 patients who fulfilled current diagnostic criteria for HCM, (MWT thickness ≥ 15mm in the absence of abnormal loading conditions or ≥13mm with a first degree relative) were recruited at The Heart Hospital, UCLH, London, UK between 28TH July 2011 and 20th March 2013. Exclusion criteria were previous or co-existing cardiac disease (valvular dysfunction greater than grade 2, ischaemic heart disease and congenital heart disease), previous cardiac surgery or alcohol septal ablation, history of recent trauma (<6 months), malignancy, systemic or inflammatory disease, coagulative disorder and uncontrolled hypertension (BP >140/90).

Cardiac MRI: 56 patients had undergone a CMR with quantification of extracellular volume at The Heart Hospital, UCLH, London, UK. These patients formed the cohort for the study presented in chapter 6.

3.4.2 Healthy mutation carriers

Plasma: 20 patients with confirmed pathogenic sarcomeric mutations (*table 1*) and a family history of HCM were identified for recruitment between 1st December 2011 and 19th November 2012. All individuals had mutations in sarcomeric genes and were

identified during cascade screening. The mutations were confirmed using Sanger sequencing and have been previously published. No individual had a preceding medical diagnosis and demonstrated no electrocardiographic or echocardiographic features of HCM.

3.4.3 Controls:

Myocardial RNA: Myocardial tissue was obtained from deceased subjects. The modality of death was road traffic accident or suicide (asphyxiation or gunshot), table 2. The individuals were not known to have a personal or family history of cardiovascular disease. Samples were obtained within 24 hours of death and all subjects underwent detailed autopsy and no evidence of cardiac disease was identified. Methods undertaken for RNA extraction are described below. The samples were provided by Dr Pablo Pavia Garcia and Dr Enrique Lara, Hospital Puerta de Hierro-Majadahonda,

Table 1: Confirmed pathogenic mutations in the gene carrier group

Case	Gene	Mutation	Case	Gene	Mutation
1	MYBPC3	Trp792fs	11	MYBPC3	Glu542Gln
2	MYH7	Val606Leu	12	MYBPC3	Glu619Lys
3	TPM1	Glu192Lys	13	MYH7	Arg663His
4	TNNI3	Ala157Val	14	ACTC	Arg314Cys
5	TPM1	Ala183Val	15	MYH7	Alal797Thr
6	MYBPC3	Arg502Gln	16	MYH7	Alal797Thr
7	MYBPC3	Arg502Gln	17	MYH7	Alal797Thr
8	TNNI3	Ala157Val	18	TNNT2	Arg278Cys
9	TNNT2	Arg278Cys	19	TNNT2	Arg278Cys
10	MYBPC3	Glu619Lys	20	MYBPC3	IVS9-1G>C

MYBPC 3 = Myosin-binding protein C, *MYH7* β = Myosin heavy chain, *TPM1* = α-tropomyosin, TNNI3 = Cardiac troponin I, TNNT2 = Cardiac troponin T

Plasma: 20 healthy controls were recruited between 24th October 2012 and 27th February 2013. These were family members and healthy volunteers. Family members included for the study did not meet the diagnostic criteria for HCM and tested negative for sarcomeric mutations implicated in their family. Healthy volunteers were individuals with no active medical problems, medical treatment or family history of HCM.

Table 2: Demographics and information of control samples

Gender	Age (years)	Height (cm)	Weight (kg)	Ischaemic Time (h)	Modality of Death	Heart Weight (g)
Male	30	165	72	8	Road traffic accident	325
Female	37	151	52	20	Suicide (Hanging)	250
Male	17	181	69	17	Suicide (Gunshot)	275
Male	31	190	115	5	Suicide (Hanging)	533
Male	47	167	85	10.5	Suicide (Hanging)	361

cm = centimetres, kg = kilograms, hrs = hours, g = grams

3.5 Clinical profiling:

The following demographics were recorded in all individuals recruited in this study:

- Hospital ID
- Sex
- Date of birth
- Date of recruitment
- BP
- BMI
- Body surface area (BSA)

Participants who were recruited for the studies involving tissue and plasma underwent additional clinical profiling to include the following parameters:

- NYHA functional class

- Medical therapy
    - Beta blocker
    - Calcium channel antagonist
    - Disopyramide
    - Amiodarone
    - Spironalactone
    - Other diuretic use
    - PPAR gamma inhibitors
    - Aspirin
    - Warfarin
    - Statin

- Presence of systolic dysfunction defined as EF <55%
- History of AF
- History of LVOTO (Resting gradient >30mmHg and provocable gradient >50mmHg)
- Presence of pacemaker or ICD
- Risk factor profiling
    - MWT > 3cm
    - Presence of NSVT (three or more consecutive ventricular extrasystoles at a rate of ≥120 beats per minute (bpm))
    - Abnormal BP response to exercise (Systolic BP drop >20mmHg)
    - Family history of SCD
    - History of unexplained syncope.

3.6 Sample collection and storage

3.6.1 Tissue

Myocardial tissue was collected at time of surgical myomectomy from patient with HCM. Tissue was either snap frozen in liquid nitrogen and subsequently stored at -80 degrees or collected in RNA later™ and frozen 24 hours later at -80 degrees. Control

biopsy samples from the left ventricular free wall were obtained during autopsy. Tissue was collected in RNA later™ and frozen 24 hours later at -80 degrees.

3.6.2 Plasma

Phlebotomy was performed in an upright position. Whole blood was collected into commercially available Ethylenediaminetetraacetic acid (EDTA) and lithium heparin (LiH) treated tubes. Dietary intake was not modified prior to sampling. Centrifugation was performed immediately for 15 minutes at 2,400 x g (5702R centrifuge, Eppendorf, Germany). The resulting supernatant was equally aliquoted into 2 clean polypropylene tubes (A and B samples) using a Pasteur pipette. Samples were stored at -40°C prior to transfer on dry ice for long term storage at -80°C.

Routine laboratory blood sampling for N-terminal pro b-type natriuretic peptide (NT-Pro BNP), liver function test, full blood count, erythrocyte sedimentation rate, urea and electrolytes, creatine kinase (CK) and C-reactive protein (CRP) was performed sin patients with HCM and gene carriers.

3.7 Echocardiography

3.7.1 2-Dimensional echocardiography

All patients underwent detailed transthoracic echocardiographic examination in accordance with current BSE guidelines as a part of routine clinical assessment.[371] Pulsed wave Doppler and tissue Doppler imaging were used to measure mitral valve inflow and lateral mitral annular velocities to allow the calculation of the E/EA ratio. Left ventricular outflow tract gradient was assessed using continuous wave Doppler; LVOTO was defined as a resting LVOTG of ≥30mmHg or ≥50mmHg on physiological provocation (Valsalva or exercise). Echocardiograms were analysed offline using Echopac PC work- station (GE Medical Systems) by 2 observers (myself and Dr Joel Salazar, a cardiologist trained in echocardiography and cardiomyopathy). An average over 3 consecutive cycles was utilised for individuals with AF. The following parameters were recorded:

- Distribution of LVH
- Measurement of maximal left ventricular wall thickness (MWT)
- LA dimensions
    - Parasternal long axis diameter (mm)
    - Area (cmsq) from apical 4 chamber
- Left ventricular dimensions at end-systole and end-diastole were measured (mm)
- Indexed LV mass (g/msq)
- EF was calculated using Simpson's biplane method.
- Fractional shortening (%)
- Peak left ventricular outflow tract gradient was measured using continuous wave Doppler
- Transmitral E:A Ratio
- E/Ea Lateral wall
- Severity of valvular dysfunction.

3.7.2 Strain imaging

Two-dimensional longitudinal strain was assessed retrospectively by two observers (myself and Dr Joel Salazar, a cardiologist trained in echocardiography and cardiomyopathy) who were blinded to the CMRI data. Apical views with high frame rates (range 28-110 fps) were analysed using a speckle-tracking algorithm incorporated into an Echopac work- station (GE Medical Systems). The endocardium was manually defined in the apical 3 chamber, 2 chamber and 4 chamber acquisitions. Automated tracking was performed with manual adjustment of tracked sectors to ensure that the myocardium was appropriately defined to achieve optimal tracking through the cardiac cycle. End-systole was defined by aortic valve closure in the apical 3 chamber view. Strain profiles were obtained from the three apical views and a bull's-eye display of left ventricular myocardial deformation was generated, *figure 6*. Peak longitudinal strain was recorded for each of myocardial segments using a 17-segment model to include basal anterior, basal anteroseptum, basal inferoseptum, basal

inferior, basal inferolateral, basal lateral, mid anteroseptum, mid inferoseptum, mid inferior, mid inferolateral, mid lateral, apical anterior, apical septum, apical inferior and apical inferolateral walls and apical cap; global LV longitudinal strain was calculated from the mean strain values from each segment. Individuals with imaging which was either prohibitive to strain analysis or resulted in greater than a total of four segments that failed to track, were excluded from analysis.

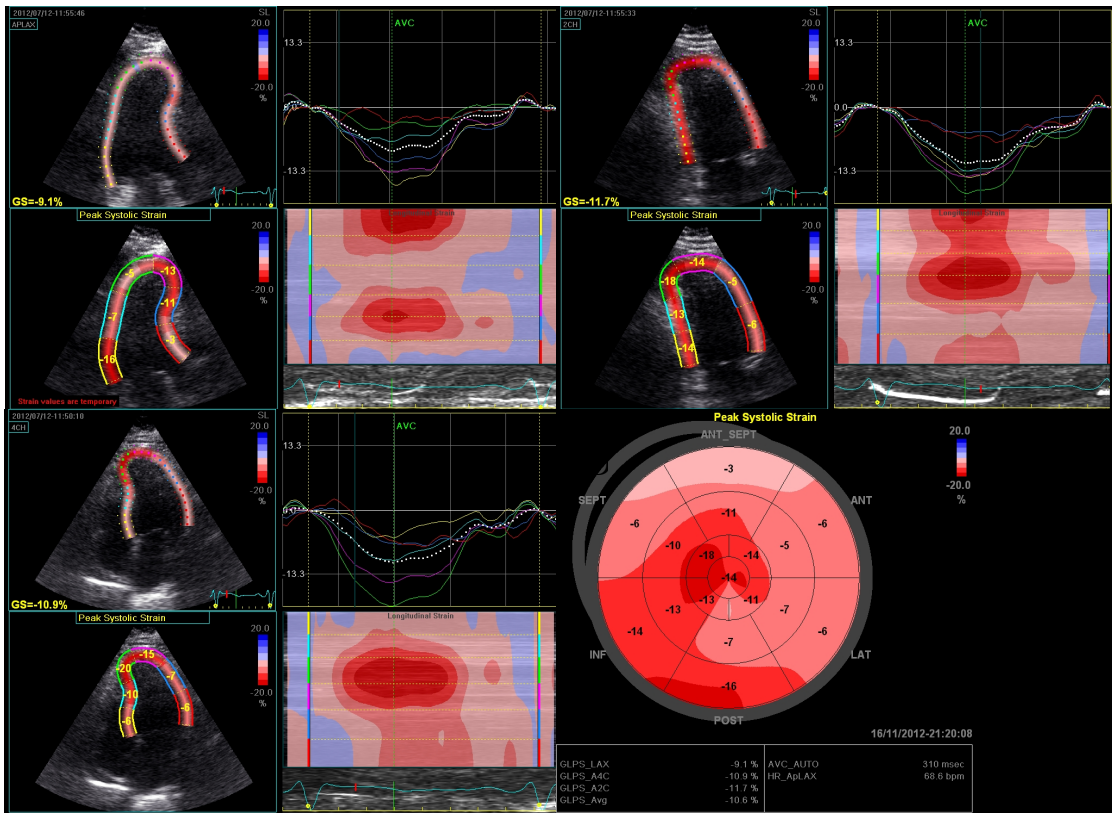

Figure 6 Strain profiles from the apical 3 chamber view (A), apical 2 chamber view (B), apical 4 chamber view (C) and a bull's-eye display of left ventricular myocardial deformation (D)

3.8 Cardiac MRI

CMRI was performed in a 1.5 Tesla scanner (Avanto, Siemens, Erlangen, Germany). Retrospective image for the data presented in chapter 5 and 6 was performed by Dr Gaby Captur (chapter 5) and Dr Daniel Sado (chapter 6), cardiologist trained in CMRI respectively.

3.8.1 Pilot

All studies started with single shot pilot images with the following settings : repeat time (TR): 3.39ms, echo time (TE): 1.7ms, slice thickness, 5mm, field of view (FOV) 360 x 360mm, read matrix 256 and flip angle 60^{o} .

3.8.2 Cine imaging

Steady state free precession (SSFP) cine imaging was then undertaken, firstly in the long axis planes with a short axis cut through the aortic valve. A standard LV short axis stack was then acquired using a slice thickness of 7mm with a gap of 3mm. Retrospective ECG gating was used with 25 phases. Typical fast imaging with steady state precession (FISP) imaging parameters were TE: 1.6ms, TR: 3.2 ms, in plane pixel size 2.3 x 1.4mm, slice thickness 7mm, flip angle 60 ° . These settings were optimised accordingly if the subject was unable to breath - hold, or had arrhythmia. Cine images were acquired and used to measure wall thickness, left ventricular mass and chamber dimensions. EF was calculated by standard thresholding using CMR tools.

3.8.3 Late gadolinium enhancement imaging

Intravenous Gadoterate meglumine (gadolinium - DOTA, marketed as Dotarem® Guerbet, S.A, France) was then administered as a 0.1mmol/kg dose via a pressure injector at a rate of 3ml/sec, with a 25ml normal saline flush. LGE assessment was then undertaken using a FLASH IR sequence with slice thickness 8 mm, TR: 9.8ms, TE: 4.6ms, α: 21 o , FOV 340 x 220 mm (transverse plane), sampled matrix size 256 x 115 - 135, 21 k – space lines acquired every other RR interval (21 segments with linear reordered phase encoding), spatial resolution 1.3 x 2.1 x 8 mm, no parallel imaging, pre - saturation bands over cerebrospinal fluid and any pleural effusions .

These parameters were optimised according to individual patient characteristics. The TI was manually set to achieve nulling of the myocardium between 300 and 440 ms. When LGE was observed, images were acquired in phase swap and cross cut to ensure artefact elimination. Where the LGE distribution appeared particularly diffuse, a TI scout was used to ascertain the specific parts of myocardium with the highest

concentration of gadolinium. If the participant was struggling with the breath - hold, FISP imaging or inversion recovery - SSFP imaging (single shot or segmented) was used as an alternative sequence.

LGE quantification was performed using a semi-quantitative method (chapter 5) and using the full width at half maximum technique (chapter 6).[319]

3.8.4 Extracellular volume

Myocardial and blood T1 was assessed at baseline and at 15 minutes after the contrast bolus in a basal, mid and apical short axis slice using the shortened modified look locker inversion recovery sequence (Sh-MOLLI).[335] For the ShMOLLI mapping, typical setting were: FOV 340 x 340mm, FOV phase 69.8%, flip angle 35^{o} , TR 1095msec, TE 1.05msec, slice thickness 8mm, 107 phase encoding steps, interpolated voxel size=0.9x0.9x8mm, base resolution 193 and phase resolution 100. Acquisition time was 206msec for a single image with a cardiac (trigger) delay time (TD) of 500ms; where necessary, to avoid misgating, the TD was decreased by 100ms for every 10 beats per minute above a resting heart rate of 80 beats per minute.

Blood haematocrit was assessed prior to the CMRI study. This information allows calculation of the ECV using the formula:

$$ECV = (1\text{-haematocrit}) \times \frac{\Delta R1 \text{ myocardium}}{\Delta R1 \text{ Blood}}$$

Where $\Delta R1 = (1/T1_{post\ contrast} - 1/T1_{pre\ contrast})$

A ROI was then drawn in the mid myocardium of each segment without reference to the LGE imaging and ECV was calculated for the basal anterior, basal anteroseptum, basal inferoseptum, basal inferior, basal inferolateral, basal lateral, mid anteroseptum, mid inferoseptum, mid inferior, mid inferolateral, mid lateral, apical anterior, apical septum, apical inferior and apical inferolateral wall using a 16-segment model.

3.9 Tissue RNA

3.9.1 Extraction and quantitative polymerase chain reaction

Performed by:
Tepnel Pharmaceutical Services , Gen-Probe Life Sciences Ltd. Oaks Business Park, Crewe Road, Wythenshawe, Manchester, M23 9HZ

RNA was extracted from 24 HCM tissue samples using the Qiagen RNeasy fibrous tissues Mini Kit. Up to 30mg of tissue was used as the input material. The tissue was disrupted and homogenised in lysis buffer using the Qiagen Tissuelyser II and stainless-steel beads. The homogenised samples were incubated for 10 minutes with Proteinase K to remove excess protein. The samples were bound to the Qiagen RNeasy columns following the standard protocol. The samples were then washed on column and genomic DNA was removed using DNase I in an on column digestion. The RNA was eluted from the columns in two 30µl aliquots of RNase Free water.

RNA from control tissue (n = 8) was extracted by Dr Enrique Lara, Hospital Puerta de Hierro-Majadahonda, Madrid, Spain. The homogenised tissue was not incubated with Proteinase K but the methods were otherwise the same. RNA samples where sent on dry ice.

All RNA elution's were quantified by optical density using the nanodrop spectrophotometer. The first elution was also run on the Agilent bioanalyzer using RNA 6000 nano chips to assess the integrity of the RNA and a RNA integrity number (RIN) value was generated where possible.

Quantitative PCR (qPCR) was performed on the extracted RNA using a RT² Profiler™ PCR Array, Qiagen, Hilden, Germany. The human fibrosis RT2 profiler™ PCR array profiles the expression of 84 key genes involved in dysregulated tissue remodelling during the repair and healing of wounds, *table 3.*

3.9.2 Analysis protocol:

32 samples were analysed using two- step RT-PCR on the Qiagen RT² Profiler PCR Array, Human, Fibrosis 384-well format. Prior to reverse transcription 1µg of RNA was treated with genomic DNA elimination solution to remove any residual DNA. The treated RNA was converted to cDNA using the Qiagen RT2 First Strand Kit. The cDNA was added to the RT² Profiler PCR Array with 2x RT2 SYBR Green Mastermix according to the manufacturers protocol and run on the 7900HT real time PCR instrument using the cycle times specified in the protocol. Four samples were processed on each array one of which being a control sample. The qPCR data was viewed in the 7900HT SDS Version 2.3 software and the cycle threshold was adjusted to the optimum point in the exponential phase of amplification. The cycle threshold values were exported into a Microsoft excel file and reformatted for input into the Qiagen web-based software RT2 Profiler PCR Array Data Analysis version 3.5.

From the panel of 84 genes available, I shortlisted the genes involved in collagen synthesis and metabolism, genes involved in TGF- β mediated signalling and genes of other established fibrotic mediators.

The following genes involved in collagen synthesis and metabolism were analysed:
COL3A1 (Collagen, type III, alpha 1), COL1A2 (Collagen, type I, alpha 2), MMP2 (Matrix metallopeptidase 2), MMP3 (Matrix metallopeptidase 3), MMP8 (Matrix metallopeptidase 8), MMP9 (Matrix metallopeptidase 9), MMP13 (Matrix metallopeptidase 13), MMP14 (Matrix metallopeptidase 14), TIMP1 (Tissue metallopeptidase inhibitor 1), TIMP2 (Tissue metallopeptidase inhibitor 2), TIMP3 (Tissue metallopeptidase inhibitor 3), TIMP4 (Tissue metallopeptidase inhibitor 4).

Table 3: The Human Fibrosis RT² Profiler™ PCR Array profiles the expression of 84 key genes involved in human fibrosis

Symbol	Description	Symbol	Description
ACTA2	Actin, alpha 2, smooth muscle, aorta	LOX	Lysyl oxidase
AGT	Angiotensinogen	LTBP1	Latent transforming growth factor beta binding protein 1
AKT1	V-akt murine thymoma viral oncogene homolog 1	MMP1	Matrix metallopeptidase 1
BCL2	B-cell CLL/lymphoma 2	MMP13	Matrix metallopeptidase 13
BMP7	Bone morphogenetic protein 7	MMP14	Matrix metallopeptidase 14
CAV1	Caveolin 1	MMP2	Matrix metallopeptidase 2
CCL11	Chemokine (C-C motif) ligand 11	MMP3	Matrix metallopeptidase 3
CCL2	Chemokine (C-C motif) ligand 2	MMP8	Matrix metallopeptidase 8
CCL3	Chemokine (C-C motif) ligand 3	MMP9	Matrix metallopeptidase 9
CCR2	Chemokine (C-C motif) receptor 2	MYC	V-myc myelocytomatosis viral oncogene homolog
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
COL1A2	Collagen, type I, alpha 2	PDGFA	Platelet-derived growth factor alpha polypeptide
COL3A1	Collagen, type III, alpha 1	PDGFB	Platelet-derived growth factor beta polypeptide

CCN2	Connective tissue growth factor	PLAT	Plasminogen activator, tissue
CXCR4	Chemokine (C-X-C motif) receptor 4	PLAU	Plasminogen activator, urokinase
DCN	Decorin	PLG	Plasminogen
ET-1	Endothelin 1	SERPINA1	Serpin peptidase inhibitor, clade A
EGF	Epidermal growth factor	SERPINE1	Serpin peptidase inhibitor, clade E
ENG	Endoglin	SERPINH1	Serpin peptidase inhibitor, clade H
FASLG	Fas ligand	Smad2	Smad family member 2
GREM1	Gremlin 1	Smad3	Smad family member 3
HGF	Hepatocyte growth factor	Smad4	Smad family member 4
IFNG	Interferon, gamma	Smad6	Smad family member 6
IL10	Interleukin 10	Smad7	Smad family member 7
IL13	Interleukin 13	SNAI1	Snail homolog 1 (Drosophila)
IL13RA2	Interleukin 13 receptor, alpha 2	SP1	Sp1 transcription factor
IL1A	Interleukin 1, alpha	STAT1	Signal transducer and activator of transcription 1, 91kDa
IL1B	Interleukin 1, beta	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced
IL4	Interleukin 4	TGF-β1	Transforming growth factor, beta 1
IL5	Interleukin 5	TGF-β2	Transforming growth factor, beta 2
ILK	Integrin-linked kinase	TGF- β3	Transforming growth factor, beta 3

INHBE	inhibin, beta E	TGF-βR1	Transforming growth factor, beta receptor 1
ITGα_1	Integrin, alpha 1 subunit	TGF-βR2	Transforming growth factor, beta receptor II
ITGα_2	Integrin, alpha 2 subunit	TGIF1	TGF-β-induced factor homeobox 1
ITGα_3	Integrin, alpha 3 subunit	TSP-1	Thrombospondin 1
ITGα_V	Integrin, alpha V subunit	TSP-2	Thrombospondin 2
ITGβ_1	Integrin, beta 1 subunit	TIMP1	TIMP metallopeptidase inhibitor 1
ITGβ_3	Integrin, beta 3 subunit	TIMP2	TIMP metallopeptidase inhibitor 2
ITGβ_5	Integrin, beta 5 subunit	TIMP3	TIMP metallopeptidase inhibitor 3
ITGβ_6	Integrin, beta 6 subunit	TIMP4	TIMP metallopeptidase inhibitor 4
ITGβ_8	Integrin, beta 8 subunit	TNFα	Tumour necrosis factor alpha
JUN	Jun proto-oncogene	VEGFA	Vascular endothelial growth factor A

The following genes involved in TGF-β mediated signalling were analysed:
TGF-β family, associated proteins and receptors: TGF-β1 (Transforming growth factor, beta 1), TGF-β2, (Transforming growth factor, beta 2), TGF-β3 (Transforming growth factor, beta 3), BMP7 (Bone morphogenetic protein 7), LTBP1 (Latent, transforming growth factor beta binding protein 1), TGF-βR1 (Transforming growth factor, beta receptor 1) and TGF-βR2 (Transforming growth factor, beta receptor 2)

Smad Signalling cascade: Smad2 (Smad family member 2), Smad3 (Smad family member 3), Smad4 (Smad family member 4), Smad6 (Smad family member 6) and Smad7 (Smad family member 7)

Cardiac integrins: ITGα_V (Integrin, alpha V), ITGβ_3 (Integrin, beta 3), ITGβ_5 (Integrin, beta 5).

The following pro-fibrotic mediators were also analysed:

- CCN2(Connective tissue growth factor)
- TSP-1 (Thrombospondin 1) and TSP-2 (Thrombospondin-2)
- PDGF-A (Platelet-derived growth factor alpha) and PDGF-B (Platelet-derived growth factor beta)
- ET-1 (Endothelin-1)

3.10 Tissue collagen quantification

In this study we elected to measure the levels of hydroxyproline as a marker of tissue collagen. Given the heterogeneous and patchy nature of fibrosis seen, this was considered the most accurate mean of determining total collagen myocardial collagen content eradicating any potential bias introduced by tissue sampling inherent to staining methods. We collaborated with Dr Chris Scotton, Principal Research Fellow based in the Centre for Inflammation and Tissue Repair, who had considerable experience and expertise in performing HPLC for hydroxyproline quantification. Tissue

preparation and HPLC was performed using an established protocol (detailed below) at University College London, Rayne Institute, 5 University Street, London WC1E 6JF.

3.10.1 Protocol

Total myocardial collagen was determined by measuring hydroxyproline content in aliquots of pulverised myocardial. Collagen contains 12.2% w/w hydroxyproline, which is detected and quantified by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxa-1,3-diazole (NBD-Cl) - derivatised acid hydrolysates. Hydroxyproline is a secondary amino acid that reacts with NBD-Cl to generate a chromophore detected at 495 nm wavelength. The specificity of the reaction is further ensured by keeping the derivatisation time to 20 minutes at 37°C, a time-point that has been confirmed experimentally to be maximal for up to 20 mmol hydroxyproline.

For each sample, approximately 20 mg of myocardial powder was weighed and hydrolysed in 2 ml 6 M hydrogen chloride (HCl) for 16 hours at 110°C in a Pyrex tube. Hydrolysates were decolourised with activated charcoal, filtered through 0.65 µm filter (Millipore Ltd., UK) and diluted 1 in 50. Aliquots (200 µl) of diluted hydrolysate were transferred to a microfuge tube and evaporated to dryness under vacuum on a Speedvac (Thermo Electron Corporation, UK). The resulting residue was reconstituted in 100 µl HPLC-grade water, buffered with 100 µl of 0.4 M potassium tetraborate (pH 9.5) and reacted with 100 µl 36 mM NBD-Cl (in methanol) to a final concentration of 12 mM NBD-Cl. The samples were then incubated in a hot block at 37°C for 20 minutes. The reaction was stopped by addition of 50 µl 1.5 M HCl. At this point 150 µl of 3.33x buffer A as described below was also added. Samples were then filtered through an HPLC low dead-volume filter (0.22 µm, Millipore Ltd., UK) into a polypropylene insert within an Amber Snap Seal vial (Laboratory Sales Ltd., UK). These vials were then loaded onto the HPLC apparatus and the samples were sequentially injected onto the HPLC column and eluted with acetonitrile gradient.

Chromatography conditions

The HPLC apparatus used for measurements was Agilent series 1100 (Agilent Technologies, USA) with a reverse-phase cartridge column (LiChroCART LiCrospher, 250mm length x 4 mm diameter, 5 µm particle size, 100 Rp-18; BDH/Merck, UK) protected by a directly coupled pre-column (LiChrosorb 4 mm x 4 mm, 5 µm particle size, 100 Rp-18; BDH/Merck, UK). The column was continuously maintained at 40°C in a heated column oven. At the beginning of each batch of samples, the HPLC system was equilibrated in running buffer A for minimum of 40 minutes. Hydroxyproline standards (50 pM, Sigma) were processed alongside the samples and inserted at equal intervals throughout the run. NBD-Cl-treated samples and standards were individually injected onto the column and eluted with an acetonitrile gradient, which was achieved by changing the relative proportions of running buffers A and B over time.

Post-column detection was achieved by monitoring absorbance at 495nm using a flow-through variable wavelength monitor. Hydroxyproline elutes as a discreet peak between five and seven minutes following sample injection on the column, between glutamine (3.5 minutes) and serine (seven to nine minutes). The column running and regeneration time for each sample was 25 minutes.

The hydroxyproline content in each sample was determined by comparing peak areas of individual sample chromatograms with the average of the standard peak areas. This value represents the hydroxyproline level in the fraction of the acid hydrolysates of the pulverised myocardial and was used to calculate total myocardial collagen based on the hydroxyproline content of collagen, dilution factors at the processing steps, the weight of myocardial powder and the total myocardial weight.

3.11 Biomarker analysis

3.11.1 Initial experiments performed in Naples

Initial efforts to quantify levels of PICP, PIIINP, ICTP, MMP1, MMP2, MMP9 and TIMP1 were performed as a part of collaborative project with Dr Giuseppe Limongelli, Monaldi Hospital, AO Colli, Second University of Naples, Naples, Italy.

In preparation for the experiments I sourced the following ELISA kits using 2B Scientific Ltd, UK:

Source: Antibodies-online
PICP: Code ABIN414990
PIIICP: Code ABIN414778

Source: Biovendor Research and Diagnostic Products, Czech Republic.
TIMP 1: Code RBMS2018R
MMP 1: Code BBT0458R
MMP 2: Code BBT0459R
MMP 9: Code RBMS2016/2R

Source: USCN Life Sciences Inc, USA
ICTP: E90665Hu

Samples were couriered on dry ice alongside the ELISA kits to Naples using World Courier, USA. The experiments were subsequently conducted by Dr. Valeria Maddaloni, Medical genetics specialist, Department of Cardiothoracic and Respiratory Sciences, Monaldi Hospital, AO Colli, Second University of Naples, Naples, Italy. Following completion of the experiments it became apparent that the samples had been incorrectly processed resulting in sample concentrations which differed by 10 – 100-fold relative to previously published results. The source of the error was not identifiable, and the results were therefore considered unreliable and decision to re-run the experiments using the stored B samples was taken.

3.11.2 Re-run of experiments with KingsPath

Once the decision to re-run the experiments was made, competitive quotes were sought from industry and a decision to run the experiments with KingsPath was made. The B samples were transported on dry ice and biomarker analysis was performed at KingsPath, King's College London, Bessemer Wing, Denmark Hill, London, SE5 9RS, UK. The following markers of collagen metabolism were measured in serum using commercially available kits:

C terminal telopeptide of type 1 collagen (ICTP): Levels were measured in EDTA plasma using a competitive radioimmunoassay (RIA) assay manufactured by Orion Diagnostica, Finland. The minimal detectable level was 0.4ug/L.

MMP1: Levels were measured in EDTA plasma by Quantakine ELISA kit distributed by R & D systems, Europe. Minimal detectable level of 0.021ug/L.

MMP3: Levels were measured in EDTA plasma by Quantakine ELISA kit distributed by R & D systems, Europe. Minimal detectable level of 0.009ug/L.

MMP9: Levels were measured in EDTA plasma by Quantakine ELISA kit distributed by R & D systems, Europe. Minimal detectable level of 0.156ug/L.

Carboxy-terminal propeptide of procollagen type I (PICP): Levels were measured in EDTA plasma by sandwich enzyme linked immunosorbent assay (ELISA) KIT manufactured by USCN Life Science Inc, Democratic Republic of China). The minimal detectable level of 26.6pg/mL.

Amino terminal peptide of type III procollagen (PIIINP): Levels were measured in EDTA plasma using a competitive radioimmunoassay (RIA) assay manufactured by Orion Diagnostica, Finaland. The minimal detectable level was 0.3ug/L.

Tissue inhibitor of metalloproteinases 1 (TIMP-1): Levels were measured in EDTA plasma by Quantakine ELISA kit distributed by R & D systems, Europe. Minimal detectable level of 0.08ug/L.

3.12 Statistics:

3.12.1 General statistics

Statistical comparisons were carried out using either SPSS Statistics, version 21.0 (IBM, USA) or Analyse-it (version 2.26, Analyse-it software Ltd, Leeds, UK). A p value <0.05 was considered significant in all analyses.

Continuous data that were normally distributed either pre- or post-logarithmic transformation are expressed with mean and s.d. Non-normally distributed data were expressed as median and interquartile range (IQR). Categorical variables are shown as frequencies and percentages. Comparisons between groups were performed using independent T test (parametric data) or Mann Whitney U test (non-parametric data). Frequencies were compared using Fisher's exact test. Correlations were quantified by simple linear regression with Pearson (parametric) or Spearman (non-parametric) correlation.

Multivariable analysis with backward elimination was used to identify predictors of echocardiogram-derived strain in chapter 6. The model included factors that were considered predictors of global strain rate, including age, LVMI (CMRI derived), global LGE % and ECV.

To assess the association between regional wall thickness and regional ECV to regional longitudinal strain in chapter 6, we utilised a mixed effect model to account for data correlation arising from related outcome measures being obtained from subjects.

3.12.2 Analysis of RNA data

The Microsoft excel file containing the cycle threshold values obtained from the qPCR analysis was uploaded to the Qiagen web-based software RT^2 Profiler PCR Array Data Analysis version 3.5. The control and disease groups were pre-selected. β2-Microglobulin was selected as the housekeeping gene. The software generates the following:

Average Ct, defined as the number of cycles required for the fluorescent signal to cross the threshold

Delta Ct : Ct for the gene of interest (GOI) – Ct for the housekeeping gene (HKG)

Delta-Delta Ct: Average Ct disease – Average Ct control

Fold change = Describes the relative change in quantity between the disease group and controls and is calculated by $2^{(-\text{Delta-Delta Ct})}$.

Fold regulation: For genes which are upregulated with a fold change of greater than 1, the fold regulation value will be numerically identical. The software transforms fold change values less than 1 (down regulated) by returning the negative inverse (i.e. -1/fold change). Thus, if gene X has a fold change value of 0.2, this is the equivalent of having a fold regulation of - 5-fold.

P value: The p values are calculated based on a Student's t-test of the replicate $2^{\wedge(-\text{Delta Ct})}$ values for each gene in the control group and treatment groups, and p values less than 0.05 are considered significant.

To allow intra-group analysis of RNA expression for each mediator in the HCM group, the relative regulation of RNA for each HCM sample was normalised to the control groups.

4. Genetic regulation of myocardial fibrosis in hypertrophic cardiomyopathy

4.1 Background:

Despite elucidation of the genetic basis of HCM, the mechanisms underlying development and progression of the clinical phenotype are poorly understood. Although numerous pathological triggers can result in myocardial dysfunction, myocardial fibrosis is likely to be one common final pathway responsible for progressive ventricular remodelling. Despite a growing body of evidence highlighting the entity of myocardial fibrosis in HCM and its role in clinical phenotype the pathways responsible for myocardial fibrosis have yet to be elucidated. The role of pro-fibrotic mediators and their association with myocardial fibrosis has yet to be evaluated in patients with HCM, A large number of mediators have been shown to role in promoting and regulating myocardial fibrosis however it is widely accepted that TGF-β mediated signalling has a central role in mediating myocardial fibrosis in cardiovascular disease. Elevated expression of TGF-β has been demonstrated in patients with HCM, however, to date the genetic regulation of TGF-β and factors which are responsible for regulating TGF-β controlling its signalling cascade in mediating myocardial fibrosis in the HCM myocardium has yet to be evaluated.[278]

In this study we sought to utilise a multiplex approach to examine the genetic regulation of pathways associated with fibrosis with a central focus on TGF-β in a well characterised cohort of patients undergoing myomectomy. We aimed to compare RNA expression of TGF-β, its associated proteins and other recognised mediators of fibrosis in the HCM myocardium relative to controls and their association with myocardial collagen content.

4.2 Aims:

1: To investigate myocardial expression of genes involved in collagen metabolism in HCM and association with myocardial collagen content.
2: To determine the myocardial genetic expression of pro-fibrotic mediators.

4.3 Results:

The final study cohort comprised 22 HCM samples and 5 controls. Prior to analysis 2 HCM samples were excluded as the patients had undergone previous ASA and 3 controls were excluded due to the presence of RNA degradation. The modality of death, ischaemic time and weight of the heart for the controls are shown in *table 2*.

4.3.1 Demographics

The HCM cohort was comprised of 12 males (55%) and 10 females (45%), whilst the control group consisted of 4 males (80%) and 1 female (20%). The HCM group was significantly older (47 $\pm$ 12 vs. 32 $\pm$ 11 y, p=0.02) than the controls group but with a comparable BMI (29 $\pm$ 5 vs. 27 $\pm$ 5 kg/m^2, p=0.37)*, table 4.* In the HCM group the mean MWT was 19.5 $\pm$ 4mm and the mean LVOTG was 113 $\pm$ 25 mmHg. The mean FS was 40 $\pm$ 8 %. LA and left ventricular cavity dimensions are shown in table 4. Results of tissue RNA regulation are shown in *table 5 and figure 7.*

Table 4 Demographics of the study cohort

	HCM						*Controls*						*p value*
n	22						5						
Male	12 (54.6%)						4 (80%)						0.3
	Mean	*s. d*	*Min*	*Max*	*Median*	*IQR*	*Mean*	*s. d*	*Min*	*Max*	*Median*	*IQR*	*p value*
*Collagen**	13.51	8.08	7.3	46.9	11.2	9.48- 15.03							
Age (yrs)	47	12	24	68	49	36 - 55	32	11	17	47	31	24 - 42	0.02
BMI (kg/msq)	29	5	21	46	28	26 - 31	27	5	21	32	26	22 - 31	0.37
MWT (mm)	19.5	4	14	29	18.5	17 - 22							
LVOTG (mmHg)	11	25	64	177	108	98 - 123							
LA (mm)	45	7	32	59	45	39 - 51							
LVEDD (mm)	45	5	34	56	45	42 - 48							
LVESD (mm)	27	5	19	38	28	22 - 30							
FS (%)	40	8	28	59	40	34 – 45							
E/A	1.49	0.81	0.63	4.29	1.26	1.05 - 1.63							

HCM = Hypertrophic cardiomyopathy, s.d = standard deviation, min = minimum, max = maximum, IQR = interquartile range; BMI = Body mass index, MWT = Maximal wall thickness, LVOTG = Left ventricular outflow tract gradient, LA = Left atrial diameter, LVEDD = Left ventricular end diastolic dimension, LVESD = Left ventricular end systolic dimension, FS = Fractional shortening, E/A = transmitral E to A ratio. *Total myocardial collagen content ug/mg of cardiac tissue, yrs = years, kg/m^2 = kilogram per metre squared, mm = millimetre, mmHg = millimetre of mercury, % = percent

Table 5 Tissue RNA regulation of mediators involved in collagen synthesis, metabolism and TGF-β related pathways, HCM vs Controls.

Gene	Fold Change	95% CI	Fold Regulation	p value
COL1A2	1.493	(1.09 - 1.89)	1.493	0.081
COL3A1	2.037	(1.51 - 2.57)	2.037	0.013
MMP1	0.786	(0.00 - 1.91)	-1.272	0.564
MMP2	0.896	(0.60 - 1.19)	-1.116	0.775
MMP3	0.672	(0.49 - 0.85)	-1.487	0.029
MMP8	0.555	(0.37 - 0.74)	-1.801	0.002
MMP9	1.433	(0.03 - 2.83)	1.433	0.588
MMP13	0.702	(0.51 - 0.90)	-1.425	0.087
MMP14	0.928	(0.77 - 1.08)	-1.077	0.617
TIMP1	1.748	(1.23 - 2.27)	1.748	0.064
TIMP2	0.987	(0.75 - 1.23)	-1.013	0.990
TIMP3	0.900	(0.67 - 1.13)	-1.111	0.583
TIMP4	1.522	(0.78 - 2.26)	1.522	0.110
TGF-β1	1.017	(0.78 - 1.25)	1.017	0.778
TGF-β2	4.761	(0.22 - 9.30)	4.761	0.008
TGF-β3	1.153	(0.88 - 1.42)	1.153	0.318
TGF-βR1	0.990	(0.75 - 1.23)	-1.010	0.961
TGF-βR2	0.944	(0.82 - 1.07)	-1.060	0.576
LTBP1	1.697	(0.90 - 2.49)	1.697	0.020
Smad2	1.137	(0.91 - 1.37)	1.137	0.287
Smad3	1.078	(0.78 - 1.38)	1.078	0.521
Smad4	1.329	(0.79 - 1.87)	1.329	0.085
Smad6	1.108	(0.70 - 1.52)	1.108	0.502
Smad7	1.028	(0.79 - 1.27)	1.028	0.714
$ITGa_V$	1.787	(1.11 - 2.47)	1.787	0.001
$ITGb_3$	1.162	(0.89 - 1.44)	1.162	0.316
$ITGb_5$	1.159	(0.83 - 1.49)	1.159	0.267
BMP7	0.265	(0.11 - 0.42)	-3.780	0.015
TSP-1	0.874	(0.00 - 1.82)	-1.145	0.123
TSP-2	1.225	(0.79 - 1.66)	1.225	0.310
CCN2	2.921	(1.15 - 4.70)	2.921	0.022

PDGF-A	0.803	(0.65 - 0.96)	-1.245	0.136
PDGF-B	0.508	(0.22 - 0.79)	-1.970	<0.001
ET-1	0.826	(0.54 - 1.11)	-1.211	0.371

CI = Confidence interval

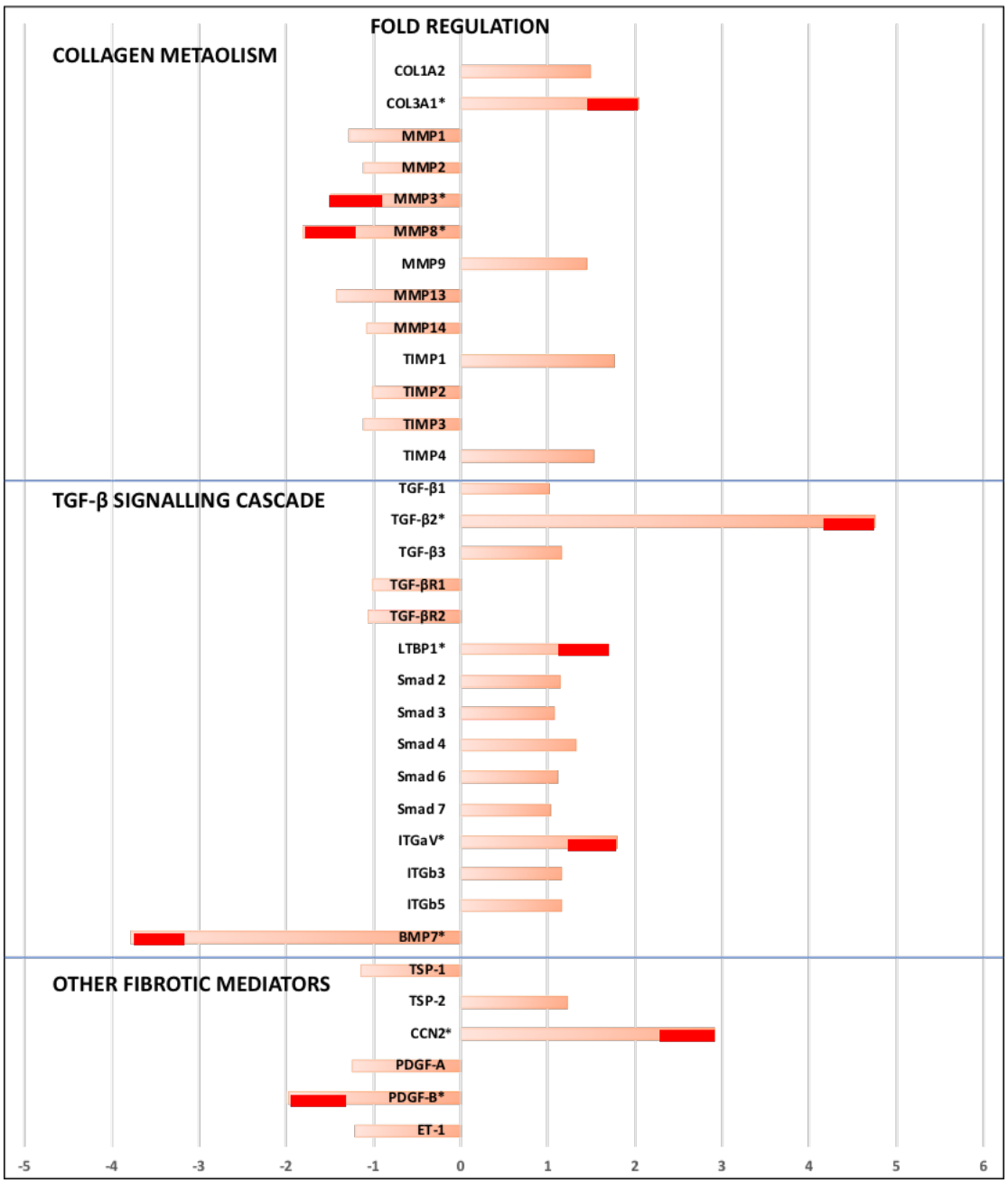

Figure 7: Tissue RNA regulation of mediators involved in collagen synthesis, metabolism and TGF-β related pathways, HCM vs controls. ***** = p <0.05***

4.3.2 Collagen content

In the HCM group the mean level of collagen was 13.51 ± 8.08 ug/mg of cardiac tissue, with a range of 7.3 – 46.9 ug/mg of cardiac tissue. When compared to controls, the RNA expression of type III procollagen was significantly up-regulated by a factor of 2.0365, p=0.013 in the HCM group. Type 1 procollagen was upregulated by a factor of 1.4928 although this failed to reach statistical significance, p=0.081.

Within the HCM group, there was a significant correlation (r = 0.494, p=0.02) between levels of myocardial collagen and the fold regulation of type III procollagen (relative to the control group). The correlation between type I procollagen and myocardial collagen did not reach significance (r = 0.343, p=0.118)

4.3.3 Matrix metalloproteinase

Expression MMP3 (-1.4873 fold, p=0.029) and MMP8 (-1.8009, p=0.002) were significantly downregulated in the HCM group. Expression of MMP 1 (-1.2723 fold, p=0.56), MMP 2 (-1.1158 fold, p=0.77) MMP 9 (-1.4334 fold, p=0.59) MMP 13 (-1.425 fold, p=0.09) and MMP 14 (-1.0772 fold, p=0.62) were also downregulated but statistical significance was not achieved.

4.3.4 Tissue inhibitors of metalloproteinase

Tissue RNA expression of TIMP 1 (1.7483 fold, p=0.064), TIMP 2 (-1.0131 fold, p =0.99) TIMP 3 (-1.1112 fold, p=0.58) and TIMP 4 (1.522 fold, p=0.11) were not significantly altered between controls and the HCM group.

4.3.5 TGF-β family, associated protein and receptors

Tissue expression of TGF-β2 showed a significant upregulation in the HCM samples when compared to the control group (4.7608 fold, p=0.008). No difference in the expression of TGF-β1 (1.0166 fold, p=0.78) and TGF-β3 (1.1529 fold, p=0.32) was observed. BMP7 was significantly downregulated in the HCM group (-3.7798,

p=0.015), LTBP1 was also significantly upregulated in HCM (1.6966 fold, p=0.02). No difference in the expression of TGF-βR1 (-1.01 fold, p=0.96) and TGF-βR2 (-1.0596, p=0.58) receptors was observed.

Within the HCM group, there was a significant correlation between fold regulation relative to the control group of TGB1 (r = 0.570, p=0.006; r = 0.528, p=0.012), TGF-β2 (r = 0.569, p=0.006; r = 0.514, p=0.014) and TGF-β3 (r = 0.738, p <0.001; r = 0.496, p=0.019) to the fold regulation of type 1 and 3 procollagens respectively. No correlation was observed for BMP7.

4.3.6 Smad signalling cascade

Expression of Smad 2 (1.1371 fold, p=0.29), Smad 3 (1.0782 fold, p=0.52), Smad 4 (1.3291 fold, p=0.09), Smad 6 (1.1075 fold, p=0.50) and Smad 7 (1.0283, p=0.71) proteins were not significantly altered in the HCM group when compared to controls.

4.3.7 Cardiac integrins

In the HCM group, the expression of ITGα_v subunit was significantly upregulated (1.7871 fold, p=0.0007) whilst no statistical difference was identified in the expression of the ITGβ_3 (1.1617 fold, p=0.32) and β_5 (1.1586 fold, p=0.27) subunits.

4.3.8 Thrombospondins, Endothelin-1, Platelet derived growth factor and Connective tissue growth factor.

When compared to controls, CCN2 was significantly upregulated (2.9207 fold, p=0.021) whilst. Levels of TSP 1 (-1.1445 fold, p=0.12), TSP-2 (1.2248 fold, p=0.31), ET-1 (-1.2105 fold, p=0.37) and PDGF-A (-1.245 fold, p=0.14) were not significantly different between the 2 groups. PDGF-B was significantly down regulated in the HCM group (-1.97 fold, p=<0.001).

Within the HCM group, there was a significant correlation between fold regulation relative to the control group of CCN2 (r = 0.841, p <0.001; r = 0.634, p=0.002), PDGF A

(r = 0.545, p=0.009; r = 0.485, p=0.022) and TSP-2 (r = 0.871, p <0.001; 0.785, p=<0.001) to the fold regulation of type 1 and 3 procollagens respectively. No correlation was observed for TSP-1, ET-1 and PDGF B.

4.4 Discussion

Collagen synthesis

In this study, we identified a significant upregulation in the RNA expression of type III collagen in the HCM myocardium relative to controls. There was also a trend towards significance for elevated RNA expression of type I collagen. The extent of collagen as quantified by hydroxyproline varied greatly, however there was a significant correlation between myocardial collagen and the extent of collagen III genetic upregulation suggesting that collagen synthesis is an active process in HCM.

Collagen metabolism

The expression of MMP 1, 2, 9, 13 and 14 in the HCM myocardium was not significantly altered relative to controls. MMP 3 and MMP 8 were significantly down-regulated. No significant differences were identified in the expression of TIMP 1, 2, 3 and 4. Degradation of mature collagen is regulated by MMPs which are inhibited by TIMPs. These findings demonstrate that the up-regulation in collagen synthesis is not counterbalanced with increased expression of genes involved in collagen metabolism at a myocardial level.

TGF-β mediated signalling and cardiac integrins

In this study, I identified a 4.8 fold up-regulation in the RNA expression of TGF-β2 isoform in the HCM myocardium. Regulation of all three TGF-β isoforms also correlated with the regulation of both procollagen I and III. RNA levels of Smad signalling cascade were not significantly altered in the HCM myocardium.

Previous studies have established the role of TGF-β in promoting myocardial fibrosis and hypertrophy.[260 261] Individuals with HCM have also been shown to demonstrate increased gene expression TGF-β1 relative to those with aortic stenosis, stable angina and dilated cardiomyopathy.[279] Elevated levels of myocardial TGF-β1 messenger RNA and protein levels have also been shown to be 2.5 and 2.8 times higher in areas of hypertrophy relative to non-hypertrophied.[278] Elevated levels TGF-β in those with HCM have been identified associate with increased BNP, higher symptomatic status and more adverse outcomes.[280]

Smad signalling is a fundamental signalling pathway involved in response to TGF-β.[262 271 272] Independent of Smad mediated transcription, TGF-β activates additional signalling cascades including Erk, JNK, TAK1, MAPK and GTPase pathways.[271] The results of this study suggest that TGF-β signalling does not directly regulate the expression of Smad proteins but its effects may be mediated through enhanced activation of pre-formed Smad or via Smad independent pathways.

TGF-β has been shown to be a stimulator of endothelial - mesothelial transformation (Endo-MT), a process which is antagonised by BMP-7.[273] BMP-7 has been shown to facilitate reverse fibrotic remodelling in cardiac disease.[276 277] I was able to demonstrate that RNA levels of BMP-7 are downregulated in the HCM myocardium.

Together, these data suggest that TGF-β is an important mediator of myocardial fibrosis and that Smad independent signalling may play an important role in TGF mediated signalling in HCM. The balance of TGF-β to BMP-7 is in favour of promoting endo-mesothelial transformation and unopposed TGF-β driven fibrosis.

I identified a significant up-regulation in the integrin a_v subunit suggesting that cardiac integrins may play a role in TGF-β activation. Integrins are transmembrane receptors that are composed of an α and β subunits and facilitate cell–ECM connections and cell–cell interactions. Integrin dependent mechanical activation of TGF-β has been well described in epithelial cells which contains the $\alpha_v\beta_6$ integrin receptor.[124] Epithelial cells are not expressed in the heart, but elevated levels of the mesenchymal integrins

$\alpha_v\beta_5$ and $\alpha_v\beta_3$ have been identified in animal models of fibrotic cardiac disease and have been shown to play an important role in controlling human myofibroblast differentiation via TGF-β activation.[134] Integrin $\alpha_v\beta_8$, has also been shown to activate TGF-β in a proteinase dependent manner, although its role in cardiac fibrosis is yet to be established.[135]

CCN family

CCN2 is pro-fibrotic mediator which acts downstream of TGF-β. I found a 2.9 fold upregulation in CCN2 and this was strongly correlated with the regulation of both procollagen I and III RNA. The CCN gene family consists of six members, of which the matricellular protein CCN2 has been most studies and implicated in cardiac disease. CCN2 expression is strongly driven and dependent on TGF-β partially through Smad dependent signalling.[179 180 183] Elevated RNA and protein levels of CCN2 have been demonstrated in fibrotic disease in both animal models of cardiac disease and humans, including myocardial infarction, diabetes and heart failure.[180 184] Despite its association with myocardial fibrosis, CCN2 on its own is considered a weak promotor of fibrosis, and requires TGF-β and other fibrogenic stimuli to produce a sustained and profound fibrotic response.[180 185] The findings in this study would therefore suggest that CCN2 in synergy with TGF-β plays a role in mediating fibrosis in HCM.

Thrombospondins

Thrombospondin regulation was not significantly altered in the HCM group, but the level of TSP-2 regulation strongly correlated with the regulation of procollagen I and III RNA suggesting a role in modulation of fibrosis. Thrombospondin-1, is a angiostatic mediator that also plays an important role in TGF-β activation and inhibition of MMP activation.[220 221] It is upregulated in the infarcted and pressure overloaded ventricle and is considered an important mediator of matrix preservation and preventing progressive ventricular dilatation.[215 222] TSP-2 has also been shown to play a role in matrix preservation by inhibiting MMPs whilst TSP-4 null animal models have shown

accelerated fibrosis suggests an anti-fibrotic role.[217 223] TSP-2 is able to bind but not activate TGF-β and thus may present as competitive inhibitor of TSP-1.[220]

Endothelin 1

In this study, no difference in the regulation of ET-1 or association with procollagen RNA was identified. Endothelin-1 has been shown to be a mediator of fibrosis which may act downstream of TGF-β and angiotensin II.[185] ET-1 has been shown to promote fibroblast proliferation, promote the expression myofibroblast phenotype and enhance collagen synthesis.[243-246] In animal models ET-1 induced myocardial fibrosis whilst antagonism of endothelin was served a protective role in myocardial fibrosis.[247-249] Elevated levels of myocardial and circulating ET-1 are elevated in patients with chronic heart failure but long-term antagonism of the endothelin receptor does not alter the course of the disease.[250 251] The findings in this study suggest that ET-1 may not play a significant role in mediating fibrosis in HCM.

Platelet derived growth factor

The regulation of PDGF A was not upregulated in the HCM group but did correlate with the RNA expression of procollagen I and III. PDGF B was significantly down regulated with no correlation with the regulation of procollagen. All four PDGF (A-D) ligands have been associated with the ability to generate myocardial fibrosis and hypertrophy different ligands, which are likely a result of differential activation of the two PDGF receptors.[252-254] PDGF A, C and D have been shown to increase expression of TGF-β and promote myocardial fibrosis.[256] PDGF-B, may also demonstrate some protective properties by promoting the expression of anti-mesenchymal transition factor BMP-7.[258] These findings suggest that PDGF may have a role in mediating fibrosis and potentially regulating mesenchymal transition.

4.5 Limitations

The HCM samples were obtained from the interventricular septum of patients with left ventricular outflow tract obstruction and may not reflect gene regulation in those without obstruction. When considering the results of this study the small sample size must be considered. Furthermore, the control group was significantly younger than the patient group and the results may in part reflect processes associated with ageing.

When aiming to compare genetic expression it was felt that the use of these samples would provide the best control relative to tissue biopsy from patients with cardiac disease undergoing surgery. The use of samples from patients with cardiac disease has its own inherent limitations and activation of common pathways such as myocardial fibrosis in cardiac disease would limit their value in such study.

There are also several considerations to consider with relation to the control samples:

(i) Samples were taken from the LV free wall and therefore may be a directly comparable to processes occurring in the intraventricular septum,

(ii) The ischaemic time for the control samples are also likely to alter the genetic regulation. However, one would anticipate that myocardial ischaemia would trigger a pro-fibrotic response in a similar to one observed in myocardial infarction and thus should not deter from the positive findings of this study.

(iii) A number of samples were taken from patients who had committed suicide. These individuals may have had neuro-hormonal activation associated with stress which may also alter cardiac genetic expression.

(iv) Tissue collection and storage also varied amongst subjects and groups. This may influence the relative preservation of RNA. However, I feel that this is unlikely to alter the validity of the results as all the samples included in the final analysis passed quality checks to ensure that the RNA was not degraded.

RNA levels may degrade overtime and the differences observed in the study may be influenced as a result and enhanced RNA expression may not necessarily translate into enhanced protein synthesis.

4.6 Conclusion:

Genetic expression of procollagen is significantly upregulated in patients with HCM relative to controls. TGF-β and CCN2 mediated signalling appear to be key mediators in promoting collagen expression.

Chapter 5 Plasma markers of collagen turnover in patients with hypertrophic cardiomyopathy and preclinical carriers of sarcomere protein gene mutations

5.1 Background

Assessment of myocardial fibrosis in clinical practice is largely dependent on patients undergoing CMRI. Although CMRI remains a valuable tool in clinical evaluation it has some limitations and may not identify activation of pro-fibrotic pathways prior to the development of established fibrosis. Furthermore, cost, access and its unsuitability for certain patient groups provide additional challenges. Plasma collagen biomarkers may serve to be a useful clinical utility that allows for direct assessment of collagen synthesis and degradation in a timely and cost-effective manner.

Studies analysing plasma markers of collagen have been small but have shown that collagen turnover is increased in HCM. However, the association between markers of collagen metabolism and clinical phenotype have been inconsistent. [287-290] Furthermore, markers of collagen synthesis have been shown to precede the development of clinical phenotype and therefore may serve as useful clinical utility in identifying mutation carriers at risk of developing the clinical phenotype and may facilitate the implementation early targeted therapy.[295]

In this study we sought to compare markers of collagen synthesis and degradation in plasma between patients with HCM, sarcomeric mutation carriers and healthy controls. Furthermore, we looked to correlate markers these markers to clinical phenotype in patients displaying the overt phenotype.

5.2 **Aims**

1: To evaluate plasma markers of collagen metabolism in controls, asymptomatic sarcomeric gene carriers and patients with HCM.

2: To evaluate the relationship between markers of collagen metabolism to clinical phenotype in patients with HCM.

Results

5.3.1 Demographics

The final study cohort comprised 102 patients with HCM, 20 gene carriers and 20 controls. 11 patients were excluded at the time of analysis due to the presence of exclusion criteria. The demographics of the study cohort are shown in *table 6*. The HCM cohort had a greater predominance of males compared to both gene carriers (p=0.02) and controls (p=0.02). The controls were younger (40 ± 11 vs. 49 ± 14 yrs, p=0.016) and had lower BMI compared to the HCM cohort (28 ± 5 vs. 25 ± 5, p=0.035). The age and BMI of the gene carrier cohort were comparable to both HCM and controls.

5.3.2 Medical therapy:

None of the controls were receiving cardioactive medications. Of the gene carriers, 1 (5%) individual was treated with a beta blocker, 1 (5%) was being treated with a calcium channel antagonist and 2 (10%) patients were taking a loop or thiazide diuretic for the management of hypertension. In the HCM group 12 (11.8%) were on disopyramide, 54 (52.9%) on beta blockers, 8 (7.8%) on amiodarone, 25 (24.5%) on calcium channel antagonists, 10 (9.9%) on angiotensin converting enzyme inhibitors or angiotensin II receptor antagonist, 21 (20.6%) on warfarin, 9 (8.8%) on spironolactone, 8 (7.8%) on loop or thiazide diuretics and 29 (28.4%) on aspirin.

5.3.3 Collagen metabolism

Levels of PICP were higher in HCM compared to controls (75.95 ± 12.23 vs 62.94 ± 17.18ug/L, p=0.002). There was no difference between the HCM group and gene carriers (74.74 ± 19.72ug/L, p=1.0), but a trend towards higher levels in gene carriers relative to the control group was observed (p=0.056), *figure 8*. PIIINP levels were

higher in HCM (5.70 ± 2.15ug/L, p =0.027) and gene carriers (5.50 ± 1.04ug/L, p=0.024) when compared to controls (4.35 ± 1.52ug/L), *figure 9*. Levels of ICTP were higher in gene carriers when compared to controls. No significant difference in levels of ICTP were observed between the HCM group and the control and gene carrier group. Furthermore, no significant differences were noted between the PICP:ICTP ratio between any of the groups. No statistical differences were observed between the groups in levels of MMP1, MMP3, MMP9 and TIMP 1. *Table 7*.

5.3.4 Control group

In the control group MMP3 levels were significantly higher in men (21.63 ± 8.56 vs. 10.01 ± 2.41ug/L, p = 0.003). Levels of PIIINP correlated with age, whilst BMI was found to have a negative correlation with MMP1 (r -0.580, p = 0.007) and positive correlation with ICTP (r 0.472, p = 0.035). No other significant gender differences or association with age and BMI were identified.

5.3.5 Hypertrophic cardiomyopathy group

In the HCM group no clinical correlation existed between levels of PICP and PIIINP with age, BMI, MWT, LVMI, LA area, EF, E/Ea ratio, LV dimensions, GLS, LGE and LVOTG. MMP 1 correlated with LA area (r = 0.22, p=0.026). MMP3 correlated with age (r = 0.236, p=0.017), LVEDd (r = 0.255, p=0.01) and E/Ea (r = -0.21, p=0.05). TIMP-1 correlated with age (r = 0.298, p=0.002), LA area (r = 0.315, p=0.001) and LVOTG (r = 0.211, p=0.036). ICTP correlated with E/Ea (r = 0.214, p=0.046). *Table 8*.

Left ventricular outflow tract obstruction

When the HCM group was dichotomised by those with and without LVOTO, the non-obstructive group contained more males (p=0.011) and a greater proportion were receiving either ACE inhibitors, mineralocorticoid antagonist or both (p=0.037). The obstructive group had a higher LVMI (139 ± 48 vs. 111 ± 4 g/msq, p=0.008) and a greater proportion were receiving beta-blocker and/or disopyramide therapy. No significant difference in age, BMI, ALT or Creatinine were observed between the

groups and comparable levels of amiodarone therapy and calcium channel antagonists were being received between the groups.

In the HCM group levels of PICP was higher in those without LVOTO (70.32 ± 14.77 vs. 77.98 ± 17.76ug/L, p=0.05). PIIINP levels were comparable between individuals with and without LVOTO. MMP1 levels were higher in individuals with LVOTO (0.82 ± 0.40 vs. 0.60 ± 0.37ug/L, p=0.006). MMP3, MMP9, ICTP, PICP:ICTP ratio and TIMP-1 were comparable between those with and without obstruction. *Table 9*.

Non-sustained ventricular tachycardia

When the HCM group was dichotomised by those with and without NSVT, gender, age, LVMI, BMI, ALT and Creatinine levels were comparable between those with and without NSVT. Amiodarone, beta-blocker and calcium channel antagonist use was comparable in those with and without NSVT. The use of disopyramide was higher in those without NSVT.

PICP levels were higher in individuals with NSVT compared to those with no evidence of NSVT (81.08 ± 16.05 vs. 71.39 ± 17.20ug/L, p=0.002). PIIINP levels were comparable between individuals with and without NSVT, *figure 10*. Levels of MMP1, MMP3, MMP9, ICTP, PICP:ICTP ratio and TIMP-1 were comparable between those with and without NSVT. *Table 9*.

Left ventricular systolic dysfunction

When the HCM group was dichotomised by those with and without an EF <55%, those with an EF <55% contained a higher proportion of patients receiving ACE inhibitors and/or a mineralocorticoid antagonist. Gender, age, BMI, Creatinine, ALT, LVMI were comparable between those with an EF < 55% and those with an EF ≥ 55%. The use of amiodarone, beta-blockers, disopyramide and calcium channel antagonists were comparable.

PICP and PIIINP levels were comparable between those with an EF < 55% and those with an EF ≥ 55%. Levels of MMP1, MMP3, MMP9, ICTP, PICP:ICTP ratio and TIMP-1 were comparable between those with an EF < 55% and those with an EF ≥ 55%. *Table 9*.

Atrial fibrillation

When the HCM group was dichotomised by those with and without AF, gender, BMI, Creatinine, ALT and LVMI were comparable. The use of amiodarone, beta-blockers, disopyramide and calcium channel antagonists were comparable between the groups. A greater proportion of patients with AF were receiving ACE inhibitors and/or mineralocorticoid antagonists. The AF group were older (59 ± 8 vs 46± 14yrs, p=0.002) and had a greater LA area (33 ± 9 vs 25 ± 5cmsq, p=<0.001). EF and E/EA were comparable.

PICP and PIIINP levels were comparable between those with and without AF. MMP 9 (51.76 ± 33.44 vs. 46.73 ± 69.33ug/L, p=0.04) and TIMP-1 (96.92 ± 23.01 vs. 84.17 ± 15.21ug/L, p=0.004) were higher in individuals with AF. No differences in ICTP, PICP:ICTP ratio and MMP3 were observed. *Table 9*.

Table 6: Demographics of the study cohort.

	HCM	Gene Carriers	HCM vs. GC	Controls	HCM vs. Control	GC vs. Control
n	102	20	p	20	p	p
Male	74	9	0.02	9	0.02	1
Age (years)	49 ± 14	43 ± 17	0.176	40 ± 11	0.016	1
BMI (kg/msq)	28 ± 5	26 ± 5	0.237	25 ± 5	0.035	1
MAP (mmHg)	91 ± 9	86 ± 10	0.022			
ALT (IU/L)	36 ± 19	27 ± 15	0.044			
Creatinine (µmol/L)	84 ± 15	74 ± 18	0.021			
Hb (g/dL)	14.8 ± 1.1	14.1 ± 1.3	0.013			
NT-ProBNP (pmol/L)	145 ± 180	13 ± 13	<0.001			
CK (IU/L)	167 ± 124	120 ± 62	0.024			
MWT (mm)	18 ± 4	9 ± 2	<0.001			
Rest LVOTG (mmHg)	6 [4.00 - 23]	4 [3 - 5]	<0.001			
LVEDD (mm)	47 ± 6	47 ± 4	0.945			

LVMI (g/msq)	119 ± 36	66 ± 17	<0.001			
LA Diameter (mm)	45 ± 8	35 ±5	<0.001			
LA area (cmsq)	27 ±	18 ± 4	<0.001			
EF (%)	66 [63 - 70.0]	66 [63 - 68]	0.335			
FS (%)	37 [33 - 42]	35 [33 - 39]	0.234			
GLS (%)	-14 ± 4	-22 ± 2	<0.001			
E:A	1.25 [0.84 - 1.60]	1.14 [0.85 - 1.75]	0.77			
E/Ea	8.66 [6.30 - 12.16]	6.10 [4.40 -7.35]	0.001			
LGE (%)	16 ± 14					

HCM = hypertrophic cardiomyopathy, GC = Gene carriers, BMI = body mass index, MAP=mean arterial pressure, ALT = alanine transaminase, Hb = haemoglobin, NT-ProBNP=N-terminal pro-brain natriuretic peptide, CK = Creatine kinase, MWT = maximal wall thickness, LVOTG = left ventricular outflow tract gradient, LVEDD = left ventricular end diastolic dimension, LVMI – Indexed left ventricular mass, LA = left atrial, EF = ejection fraction, FS = Fractional shortening, GLS = global longitudinal strain, E:A = transmitral E to A ratio, E/Ea = transmitral E to early diastolic velocity of the lateral wall, LGE = late gadolinium enhancement.

kg/msq = kilogram per metre squared, mm = millimetre, mmHg = millimetre of mercury, % = percent, IU/L = international units per litre, µmol/L = micromoles per litre, g/dL = grams per decilitre, pmol/L = picomoles per litre, g/msq = grams per metre squared, cmsq = centimetre squared.

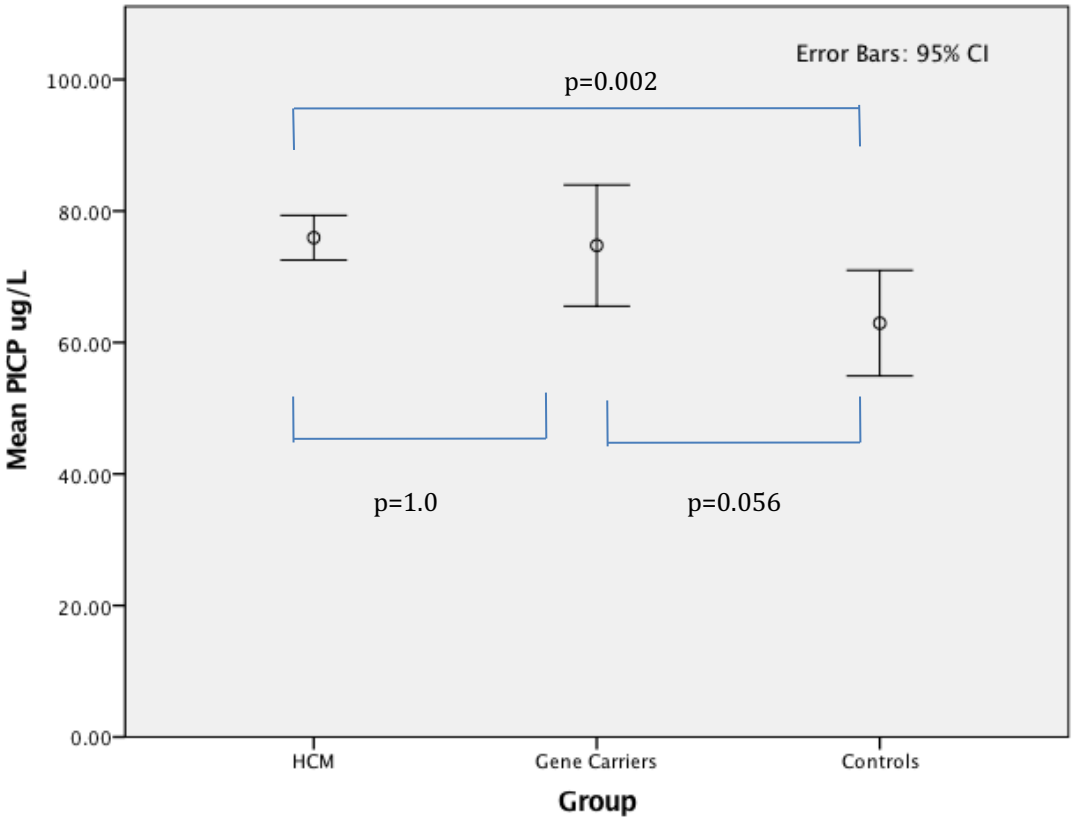

Figure 8: Levels of PICP in hypertrophic cardiomyopathy (HCM), gene carriers and controls.

PICP=Carboxyterminal propeptide of type I procollagen, ug/L = microgram per litre, CI = confidence intervals

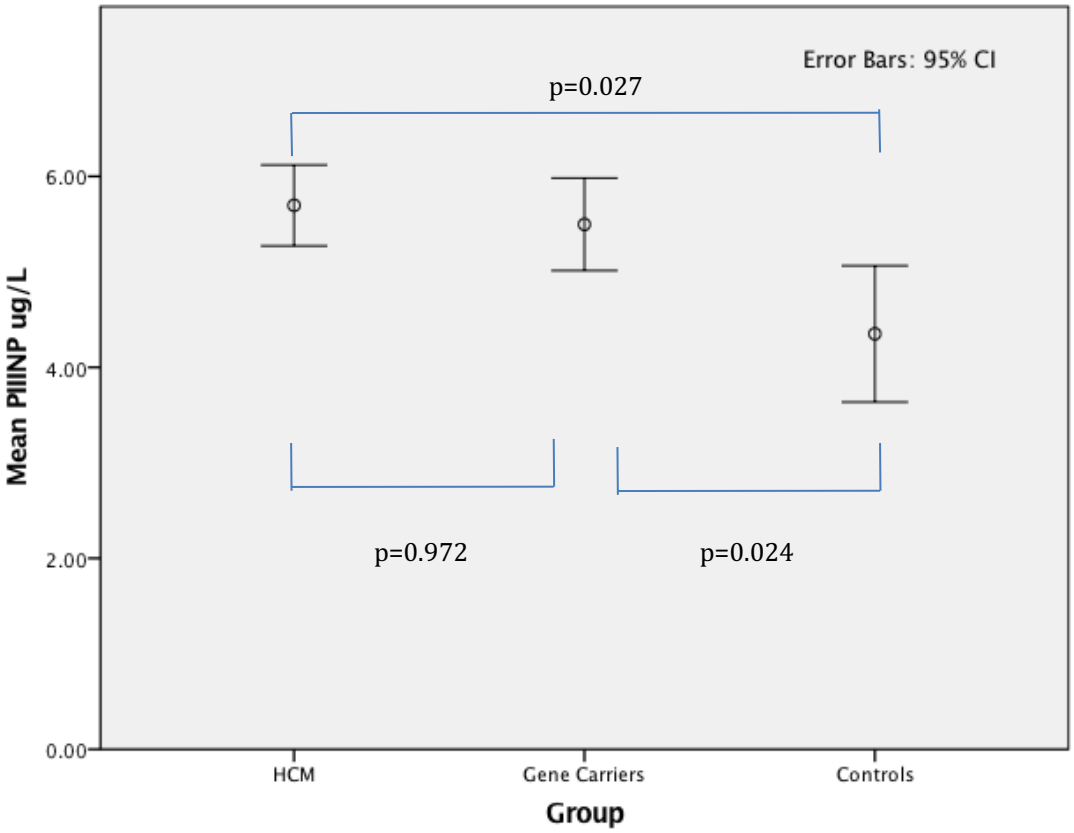

Figure 9 Levels of PIIINP in hypertrophic cardiomyopathy (HCM), gene carriers and controls.

PIIINP=Aminoterminal propeptide of type III procollagen, ug/L = microgram per litre, CI = confidence intervals

Table 7 Plasma levels of collagen biomarkers in hypertrophic cardiomyopathy (HCM), gene carriers (GC) and controls.

Biomarker	HCM	GC	Controls	HCM vs GC p value	HCM vs Controls p value	GC vs Controls p value
n	102	20	20			
PICP (ug/L)	75.95 ± 17.29	74.76 ± 19.72	62.94 ± 17.18	1	0.002	0.056
PIIINP (ug/L)	5.70 ± 2.16	5.50 ± 1.04	4.35 ± 1.52	0.972	0.027	0.024
PICP:ICTP	26.89 ± 14.90	21.90 ± 9.33	23.72 ± 6.05	0.268	1	0.999
MMP1 (ug/L)	0.66 ± 0.39	0.62 ± 0.32	0.76 ± 0.52	1	1	1
MMP3 (ug/L)	16.74 ± 7.46	14.82 ± 6.59	15.24 ± 8.31	0.887	1	1
MMP9 (ug/L)	47.91 ± 62.64	46.65 ± 74.23	47.29 ± 31.28	1	1	0.993
ICTP (ug/L)	3.32 ± 1.54	3.71 ± 0.9	2.70 ± 0.54	0.18	0.247	0.017
TIMP-1 1 (ug/L)	87.17 ± 18.07	89.78 ± 16.33	81.88 ± 10.74	1	0.83	0.49

PICP=Carboxyterminal propeptide of type I procollagen, PIIINP=Aminoterminal propeptide of type III procollagen,
MMP=Matrix metalloproteinase, TIMP=Tissue inhibitors of metalloproteinases, ICTP=Carboxyterminal telopeptide of type I collagen, ug/L = micrograms per l

Table 8 Correlation of collagen of biomarkers to clinical parameters in hypertrophic cardiomyopathy

	PICP (ug/L)		**PIIINP** (ug/L)		**PICP : ICTP**		**MMP1** (ug/L)		**MMP 9** (ug/L)		**MMP 3** (ug/L)		**ICTP** (ug/L)		**TIMP1** (ug/L)	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Age	0.146	0.143	-0.045	0.655	0.034	0.733	0.179	0.072	0.094	0.346	0.236	0.017	0.043	0.670	0.298	0.002
BMI (kg/msq)	0.131	0.190	0.061	0.539	0.001	0.989	0.013	0.897	-0.165	0.097	-0.004	0.966	0.073	0.465	0.171	0.086
MWT (mm)	-0.41	0.683	-0.015	0.881	-0.079	0.433	-0.007	0.942	-0.09	0.366	-0.11	0.272	0.07	0.484	-0.043	0.667
LVMI (g/msq)	0.065	0.513	-0.071	0.478	-0.099	0.324	0.100	0.317	-0.098	0.328	-0.037	0.710	0.155	0.120	0.115	0.248
LAA (cmsq)	0.135	0.177	0.025	0.807	0.129	0.2	0.222	0.026	0.106	0.29	0.182	0.068	-0.055	0.585	0.315	0.001
EF (%)	-0.166	0.096	-0.043	0.665	-0.119	0.232	0.03	0.767	-0.015	0.88	-0.14	0.162	0.03	0.765	-0.164	0.099
E/Ea	-0.167	0.119	0.174	0.106	-0.246	0.021	0.292	0.006	0.052	0.632	-0.21	0.05	0.214	0.046	0.194	0.07
LVEDD (mm)	0.162	0.104	0.006	0.953	0.128	0.199	-0.062	0.535	-0.078	0.438	0.255	0.01	-0.06	0.547	0.09	0.371

GLS (%)	-0.58	0.656	-0.142	0.274	0.15	0.247	0.057	0.664	-0.083	0.526	-0.025	0.847	-0.167	0.2	0.045	0.733
LGE (%)	0.153	0.345	0.123	0.451	0.117	0.474	-0.179	0.27	-0.09	0.579	0.128	0.432	-0.035	0.828	0.015	0.923
Rest LVOTG (mmHg)	-0.058	0.569	0.104	0.305	-0.16	0.114	0.12	0.238	-0.015	0.879	-0.139	0.169	0.182	0.072	0.211	0.036

PICP=Carboxyterminal propeptide of type I procollagen, PIIINP=Aminoterminal propeptide of type III procollagen, MMP=Matrix metalloproteinase, TIMP=Tissue inhibitors of metalloproteinases, ICTP=Carboxyterminal telopeptide of type I collagen

BMI = body mass index, MWT = maximal wall thickness, LVMI = Indexed left ventricular mass, LAA = left atrial area, LVOTG = left ventricular outflow tract gradient, EF = ejection fraction, E/Ea = transmitral E to early diastolic velocity of the lateral wall, LVEDD = left ventricular end diastolic dimension, GLS = global longitudinal strain, LGE = late gadolinium enhancement.

kg/msq = kilogram per metre squared, mm = millimetre, mmHg = millimetre of mercury, % = percent, g/msq = grams per metre squared, cmsq = centimetre squared, ug/L = micrograms per litre.

Table 9 Collagen biomarkers in hypertrophic cardiomyopathy dichotomised by clinical phenotype

Biomarker	**LVOTO**			**NSVT**			**EF < 55%**			**AF**		
	No	Yes	p	No	Yes	p	No	Yes	p	No	Yes	p
N	75	27		54	48		95	7		78	24	
PICP (ug/L)	77.98 ± 17.76	70.32 ± 14.77	0.049	71.39 ± 17.20	81.08 ± 16.05	0.002	76.38 ± 17.25	70.16 ± 18.12	0.320	74.76 ± 17.27	79.81 ± 17.14	0.189
PIIINP (ug/L)	5.64 ± 2.22	5.84 ± 2.01	0.59	5.62 ± 2.36	5.78 ± 1.93	0.44	5.69 ± 2.22	5.74 ± 1.05	0.65	5.61 ± 1.96	5.98 ± 2.73	0.719
PICP:ICTP	27.23 ± 11.47	25.94 ± 22.05	0.2	25.61 ± 17.22	28.33 ± 11.77	0.07	27.17 ± 15.26	23.06 ± 8.41	0.059	26.42 ± 15.11	28.43 ± 14.39	0.56
MMP1 (ug/L)	0.6 ± 0.37	0.82 ± 0.40	0.006	0.66 ± 0.42	0.65 ± 0.35	0.95	0.66 ± 0.42	0.63 ± 0.39	0.87	0.62 ± 0.31	0.76 ± 0.57	0.578
MMP3 (ug/L)	17.32 ± 7.10	15.13 ± 8.31	0.192	16.72 ± 7.80	16.77 ± 7.14	0.975	16.65 ± 7.64	17.97 ± 4.41	0.653	16.33 ± 7.66	18.08 ± 6,74	0.318
MMP9 (ug/L)	45.18 ± 50.95	55.51 ± 88.16	0.81	35.03 ± 20.92	62.41 ± 86.80	0.114	49.19 ± 64.61	30.59 ± 16.43	0.403	46.73 ± 69.33	51.76 ± 33.44	0.038

ICTP (ug/L)	3.26 ± 1.58	3.48 ± 1.44	0.682	3.45 ± 1.81	3.18 ± 1.17	0.649	3.33 ± 1.59	3.16 ± 0.65	0.939	3.28 ± 1.34	3.45 ± 2.10	0.957
TIMP1 (ug/L)	83.54 ± 18.54	91.99 ± 16.03	0.07	87.45 ± 19.16	86.85 ± 19.96	0.881	87.36 ± 18.49	84.54 ± 11.53	0.798	84.17 ± 15.21	96.92 ± 23.01	0.004

PICP=Carboxyterminal propeptide of type I procollagen, PIIINP=Aminoterminal propeptide of type III procollagen, MMP=Matrix metalloproteinase, TIMP=Tissue inhibitors of metalloproteinases, ICTP=Carboxyterminal telopeptide of type I collagen, ug/L = micrograms per litre.

LVOTO = left ventricular outflow tract obstruction, NSVT = non-sustained ventricular tachycardia, EF = ejection fraction, AF = atrial fibrillation.

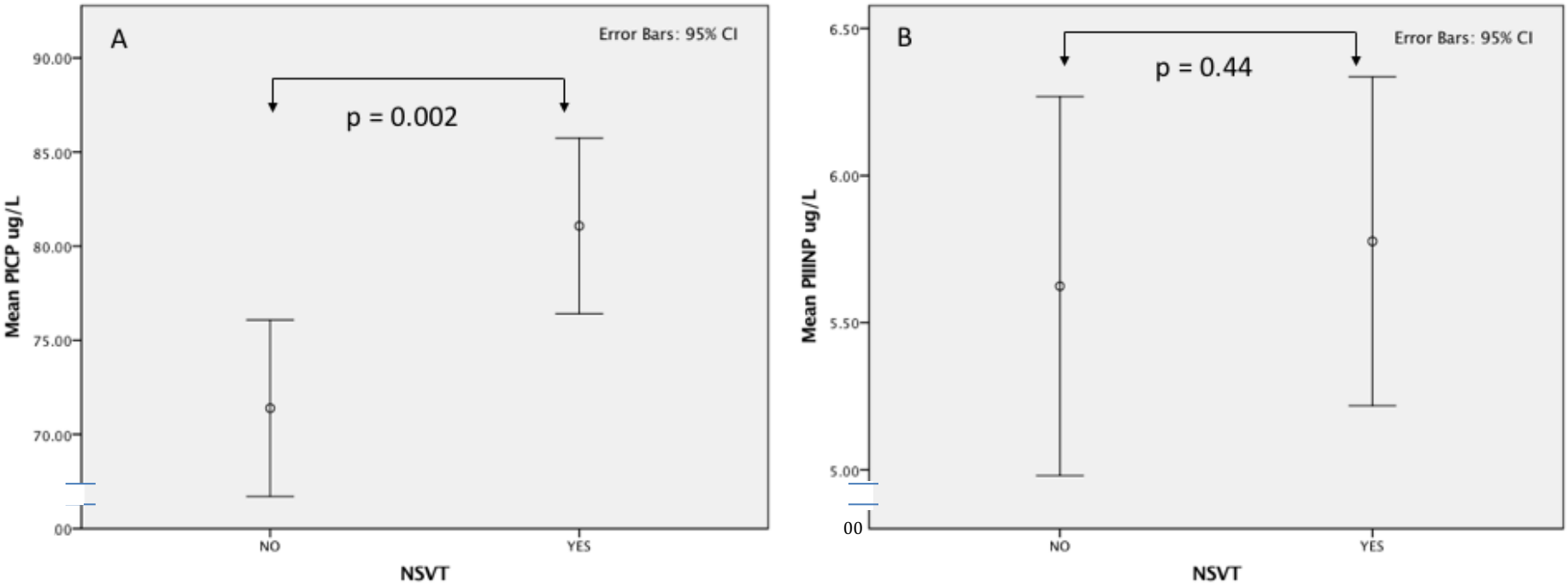

Figure 10 Levels of (A) PICP and (B) PIIINP in HCM patients with and without non-sustained ventricular tachycardia

PICP=Carboxyterminal propeptide of type I procollagen, PIIINP=Aminoterminal propeptide of type III procollagen, ug/L = micrograms per litre, NSVT = non-sustained ventricular tachycardia.

5.4 Discussion:

5.4.1 Collagen synthesis

In this study, I identified a significant elevation in plasma levels of both procollagen I and pro-collagen III in patients with HCM when compared to controls. Plasma expression of PIIINP was also significantly elevated in gene carriers when compared to controls and comparable to those seen in HCM. Levels of PICP showed a trend towards increased levels in gene carriers (p =0.056). Levels of ICTP were also elevated in gene carriers without any significant differences seen in the PICP:ICTP ratio between the groups. These findings demonstrate that enhanced collagen synthesis and turnover is an early, active process present in mutation carriers that is likely to contribute to the development and progression of the clinical phenotype.

Several investigators have identified elevated levels of markers of collagen type I (PICP and PINP) and type III (PIIINP) synthesis in HCM whilst others have been unable to demonstrate differences compared to controls.[287-290] Ho et al measured differences in levels of PICP, MMP-1, TIMP-1 and ICTP between individuals with pathogenic sarcomeric mutations with evidence overt disease, subjects with proven pathogenic mutation without LVH and controls.[295] Compared to controls, those with overt disease demonstrated a significant elevation in PICP and its ratio with ICTP. No differences were identified in levels of MMP-1, TIMP-1 and ICTP. In those with overt disease there was no correlation between collagen biomarkers and clinical parameters including LGE. However, the most significant finding of this paper was the discovery of raised levels in PICP in gene carriers without overt LVH or LGE on CMRI when compared to controls. This finding demonstrates that enhanced collagen synthesis may represent an early clinical manifestation of sarcomeric disease and provide a useful serological marker of genetic expression.

An association between markers of collagen synthesis and markers of diastolic dysfunction has been reported, whilst an elevated PICP or PINP to ICTP ratio (which represent the ratio of collagen type I synthesis to degradation) has been shown to

consistently correlate to markers of diastolic and systolic dysfunction at rest across several studies.[287-289] A correlation between markers of collagen synthesis and other clinical markers of disease has been inconsistent amongst studies.

When evaluating the association of plasma collagen markers and clinical phenotype, I identified elevated levels of PICP in patients with NSVT. These findings may point towards an association between collagen synthesis and ventricular arrhythmias. In this study, I also identified that the group with LVOTO had lower levels of PICP when compared to patients without obstruction, despite the non-obstructive group having a higher proportion of men, a lower LVMI and greater use of ACE inhibitors and/or mineralocorticoid antagonists. These findings suggest that myocardial fibrosis is an intrinsic process in HCM and not driven by increased afterload as observed in hypertension and aortic stenosis. No other clinical correlation between PICP and PIIINP were identified, including the extent of LGE. The lack of significant clinical correlations may in part relate to the relatively small sample size or may reflect the chronic nature of the process.

5.4.2 Regulation of collagen degradation

MMP2 has consistently been shown to be elevated in HCM and correlate with levels of BNP.[289 291-293] Furthermore, levels of MMP2 are highest in those with systolic dysfunction, severe heart failure symptoms and in patients with AF.[291-293] Elevated levels of MMP2 levels were also shown to be significantly associated with an adverse heart failure related outcome.[293] MMP9 has been shown in 2 studies to be elevated in HCM whilst no significant difference was identified in another.[289 291 292] Roldan et al, demonstrated that MMP9 was associated with LGE on CMRI and correlated with both exercise incapacity and LVOTG.[292] In another study MMP9 was shown to predict future LV dysfunction.[294] MMP1 has been shown to be reduced or undetectable in HCM. Other studies have not found any significant difference in levels of MMP1, MMP3 or MMP9 between controls and HCM.[287 289 291 292] Tissue inhibitors of metalloproteinase, TIMP1 and TIMP2 are also elevated in HCM with the latter being elevated in patients

with HCM with LV systolic dysfunction when compared to those without LV dysfunction and controls.[287 289 291-293]

In this study, we found no significant difference in the levels of MMP 1, MMP 3, MMP 9 and TIMP-1 between the HCM group, gene carriers and controls. Within the HCM cohort, MMP 1 was higher in the obstruction group although no correlation was identified with LVOT gradient. MMP 1 correlated with both LAA and E/Ea. MMP-9 was higher in those with AF and MMP 3 correlated positively with LV dimensions. TIMP-1 levels associated positively with LAA and rest LVOT gradient. These findings suggest that a complex interaction between collagen synthesis and degradation contributes to disease pathophysiology and cardiac remodelling.

5.5 Limitations

The study is in part limited by its power and population size. Although strict inclusion and exclusion criteria were employed, the expression of biomarkers may be influenced by non-cardiac pathophysiological processes and we are unable to conclude whether markers of collagen synthesis are proportional to the activity of pro-fibrotic pathways and collagen synthesis at a myocardial level.

Another limitation of this study was that the control samples were not matched with regards to gender, age and BMI. Although many of the markers did not correlate to these parameters in the HCM and control group the differences in the group may be a confounding factor.

5.6 Conclusions

Plasma biomarkers of collagen synthesis are increased in patients with HCM and sarcomeric mutation carriers in the absence of overt disease. Enhanced collagen synthesis is an early, active process likely to be involved in disease development and long-term outcomes.

6. Relationship between myocardial hypertrophy, fibrosis and myocardial deformation in hypertrophic cardiomyopathy

6.1 Background:

Patients with HCM often have supra-normal EF which is in part related to the small ventricular geometry. However, in most patients, HCM is characterised by slow progression to systolic and diastolic LV impairment which can result in death from heart failure or cardiac transplantation.[372] The mechanisms of this gradual decline in cardiac performance are only partly understood, but almost certainly reflect a complex and dynamic interplay between cardiomyocyte dysfunction and progressive changes in the myocardial interstitium.

Assessment of myocardial deformation allows for the assessment of global and regional systolic performance and several studies have shown that longitudinal myocardial deformation is abnormal in HCM, [43 353-356 373] The contribution of interstitial fibrosis to cardiac dysfunction is not known and a greater understanding of this interaction could direct therapy in patients with early left ventricular dysfunction.

In this study, we sought to determine the relationship between myocardial deformation, diffuse and replacement myocardial fibrosis using a combination of two-dimensional echocardiographic deformation imaging and contrast cardiac magnetic resonance imaging

6.2 Aim:

1: To determine the between global and regional longitudinal myocardial deformation and left ventricular hypertrophy, replacement fibrosis and interstitial fibrosis.

6.3 Results

6.3.1 Baseline characteristics

The study sample comprised 37 patients (79% male) with a mean age of 48 ± 14 years. 19 patients were excluded due to suboptimal echocardiographic imaging which was prohibitive to strain analysis. Twenty-five (65.8%) had asymmetrical septal hypertrophy, 1 (2.6%) had concentric LVH and 12 (31.6%) had an apical distribution of hypertrophy. Baseline characteristics of the study population are shown in *table 10*. Examples of strain maps obtained from patients with different distributions of hypertrophy are shown in *figure 11*.

6.3.2 Correlations with global strain rate

The results obtained from CMRI and echocardiography are displayed in *table 11*. The mean GLS in the cohort was -14 ± 5%. There was a significant correlation between the GLS and CMRI derived LVMI (r = 0.71, p=<0.001), EF (r = -0.36, p=0.027), mean LV wall thickness (r = 0.75, p=<0.001), MWT (r = 0.43, p=0.008) and % LGE (r = 0.43, p=0.008), *figure 12*. The correlation between GLS and global ECV failed to reach statistical significance (r = 0.25, p=0.138). There was no correlation between GLS, LVOTG and LA dimensions. There was a significant correlation observed between GLS and both E/Ea (r = 0.37, p=0.027) and NT-Pro BNP (r = 0.41, p=0.012).

In a multivariable model including predictors of global strain rate, the combined R squared of the model which factored age, LVMI, global LGE % and ECV was 0.57. LVMI (B 0.076, p=<0.001, CI 0.048 – 0.104, β = 0.65) remained an independent predictor. Global LGE % showed a weak trend towards significance (B 0.14, p=0.076, CI -0.016 – 0.295, β = 0.218).

In previous work from our department we documented the healthy volunteer range of ECV to be 0.20 – 0.34.[330] Individuals where global ECV was greater than 0.35 had impaired GLS, EF and a trend towards higher NT-Pro BNP. There was no significant difference between wall thickness, LVOTG, LA dimensions and E/Ea. *Table 12* and *Figure 13*.

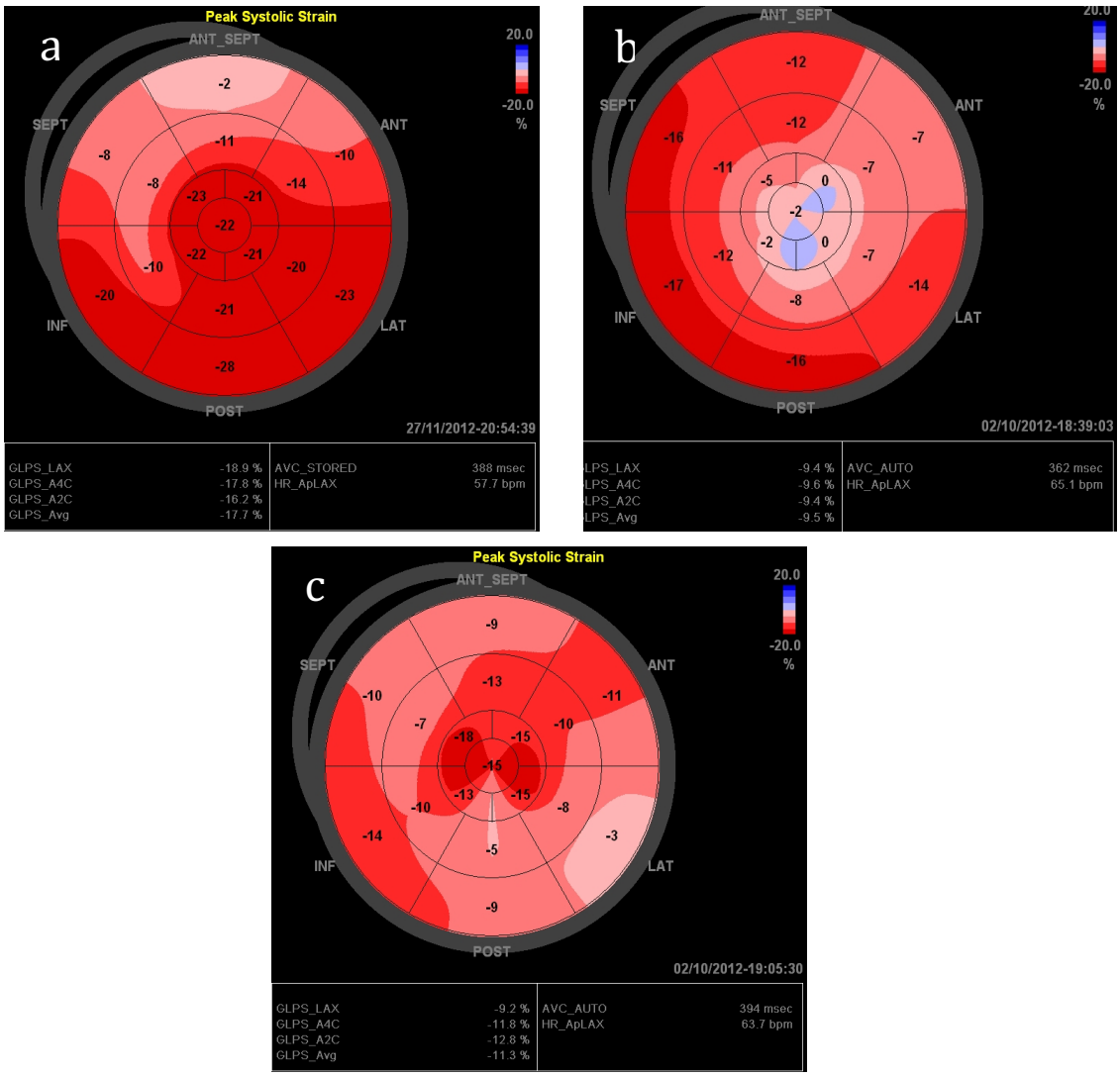

Figure 11: Longitudinal strain bullseye plot in patients with (a) asymmetrical hypertrophy, (b) apical hypertrophy and (c) concentric hypertrophy.

GLPS_LAX = mean longitudinal strain from apical 3 chamber, GLPS_A4C = mean longitudinal strain from apical 4 chamber, LGPS_A2C = mean longitudinal strain from apical 2 chamber, GLPS_Avg = mean global longitudinal strain.

Table 10: Baseline characteristic of the study population (n = 37). All results displayed as frequencies and mean ± s.d

Age (years)	48 ± 14
Male	28 (76%)
Distribution of LVH	
Asymmetrical septal hypertrophy	24 (64.9%)
Concentric	1 (2.7%)
Apical	12 (32.4%)
LVOTO	9 (24.3%)
Height (cm)	173 ± 9
Weight (kg)	84 ± 14
Body mass index (kg/msq)	28 ± 4

LVH = left ventricular hypertrophy, LVOTO = left ventricular outflow tract obstruction, cm = centimetres, kg = kilogram, kg/msq = kilogram per metre squared.

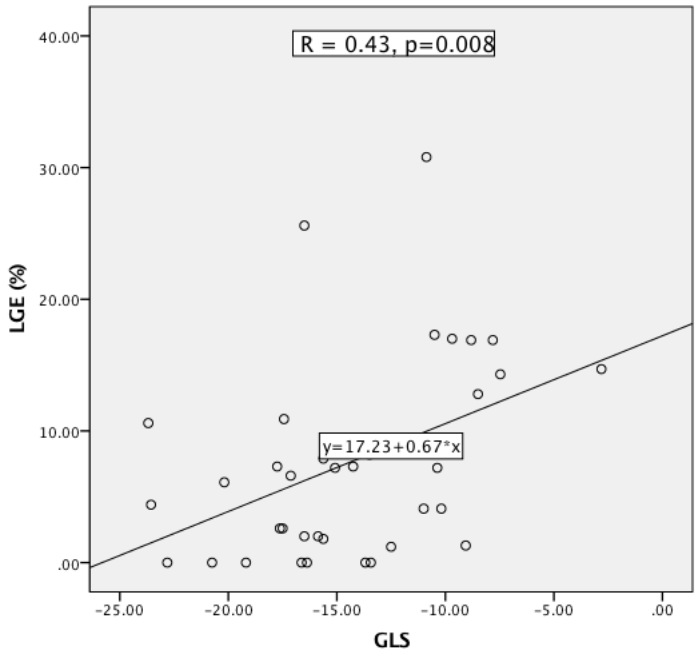

Figure 12: Association between late gadolinium enhancement (LGE) and global longitudinal strain (GLS).

Table 11: Characteristics of the study population and associated correlation coefficient with global longitudinal strain. All data displayed as mean ± standard deviation or median and interquartile range.

n = 37	mean ± s.d median [IQR]	Correlation Coefficient	p
Age (years)	48 ± 14	-0.12	0.493
NT Pro BNP (pmol/L)	64 [28 - 109]	0.41	0.012
CMRI Parameters			
LV Mass (g)	251 ± 83	0.77	<0.001
LVMI (g/msq)	114 [85 - 161]	0.71	<0.001
EF (%)	75 ± 8	-0.36	0.027
LA Area (cmsq)	31 ± 7	-0.01	0.955
LA Area Indexed (cm/msq)	15 ± 4	-0.09	0.608
Mean WT (mm)	10 ± 3	0.75	<0.001
Maximal WT (mm)	18 ± 5	0.43	0.008
Global LGE (%)	7 [1 – 12]	0.43	0.008
Global ECV	0.32 ± 0.04	0.25	0.138
Echo Parameters			
LVOTG (mmHg)	7 [4.00 – 39]	0.133	0.433
E/Ea	8.3 [6.2 - 13.7]	0.368	0.027
GLS (%)	-14 ± 5		

NT-ProBNP=N-terminal pro-brain natriuretic peptide, LVMI – Indexed left ventricular mass, EF = ejection fraction, LA = left atrial, WT = wall thickness, LGE = late gadolinium enhancement, ECV = extracellular volume, LVOTG = left ventricular outflow tract gradient, E/Ea = transmitral E to early diastolic velocity of the lateral wall, GLS = global longitudinal strain.

pmol/L = picomoles per litre, g = grams, mm = millimetre, g/msq = grams per metre squared, cmsq = centimetre squared, cm/msq = centimetre per metre squared, mmHg = millimetres of mercury.

Table 12: Group descriptive by global ECV. All data displayed as mean ± s.d unless stated.

	ECV <0.35	ECV ≥ 0.35	p
n	29	8	
Age (years)	50 ± 14	41 ± 14	0.114
NT Pro BNP (pmol/L)	56 [20 - 104]	105 [60 – 162]	0.086
CMRI Parameters			
MWT (mm)	18 ± 5	19 ± 6	0.510
EF (%)	77 ± 5	67 ± 9	0.020
LA Area (cm2)	30 ± 7	34 ± 9	0.120
LA Area Indexed (cmsq/m)	15 ± 3	17 ± 5	0.221
LVMI	113 [83 – 14]	130 [98 – 192]	0.354
Echo Parameters			
LVOTG (mmHg)	7 [4.0 – 37]	6 [5 – 65]	0.651
LA Area (cmsq)	27 ± 6	30 ±	0.268
LA Area Indexed (cmsq/m)	12 ± 3	13 ± 4	0.357
EF Simpsons (%)	69 ± 5	61 ± 7	0.001
E/Ea	8.1 [6.1 – 14.8]	9.5 [8.2 – 11.8]	0.537
GLS (%)	-15.30 ± 4.54	-11.49 ± 4.81	0.045

NT-ProBNP=N-terminal pro-brain natriuretic peptide, LVMI – Indexed left ventricular mass, EF = ejection fraction, LA = left atrial, WT = wall thickness, LGE = late gadolinium enhancement, ECV = extracellular volume, LVOTG = left ventricular outflow tract gradient, E/Ea = transmitral E to early diastolic velocity of the lateral wall, GLS = global longitudinal strain.

pmol/L = picomoles per litre, g = grams, mm = millimetre, g/msq = grams per metre squared, cmsq = centimetre squared, cm/msq = centimetre per metre squared, mmHg = millimetres of mercury.

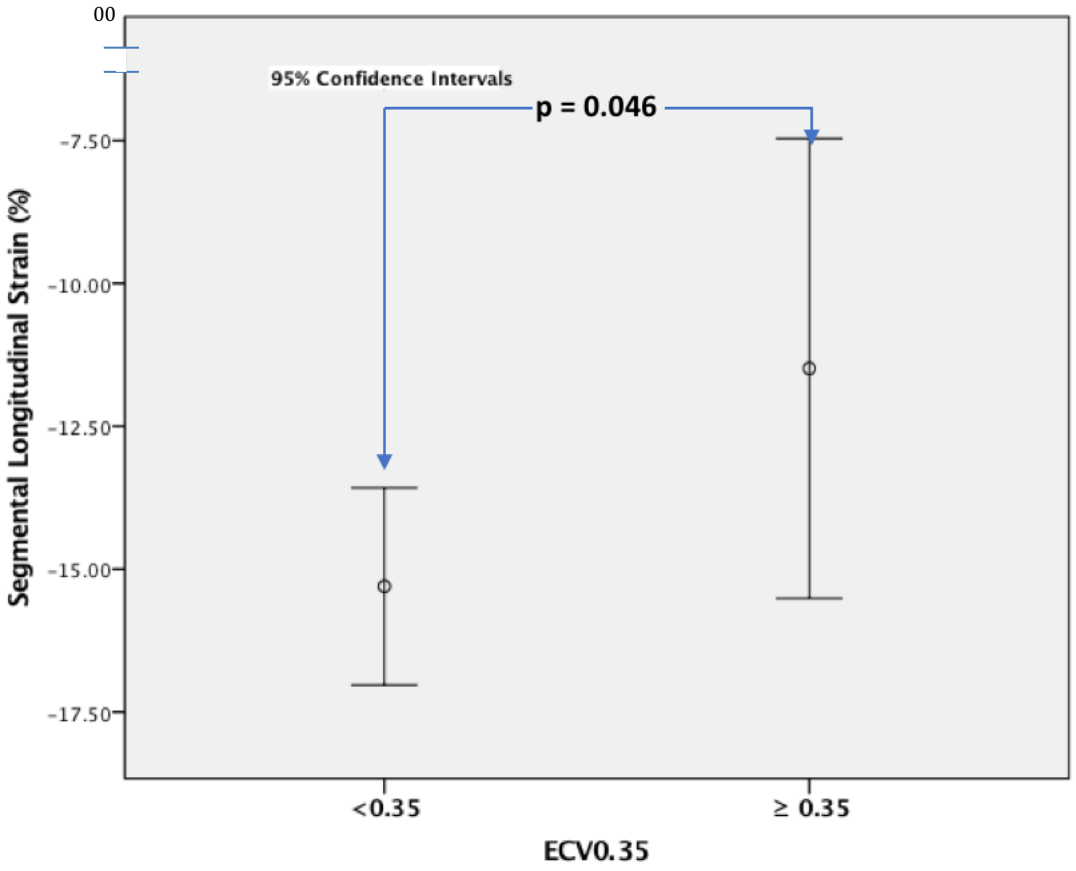

Figure 13: Segmental longitudinal strain by segmental Extracellular volume (ECV).

6.3.3 Correlation with regional strain

Segmental echocardiographic derived regional strain was correlated with regional CMRI derived wall thickness and ECV over 16 segments excluding the apical cap. Echocardiographic derived regional strain and its correlation with CMRI derived wall thickness and ECV is displayed in *table 13*.

The association of regional wall thickness and ECV on RLS was investigated using a mixed effect model. The mixed effect found the intra-cluster correlation within each subject was 24% and the random component of the mixed effect model was statistically significant ($p<0.001$), suggesting appropriate choice of this approach for analysing the data. The model demonstrated a significant relationship between both WT (estimated regression coefficient 0.48, CI 0.37- 0.58, $p <0.001$) and ECV (estimated regression coefficient 18.64, CI 9.49 – 27.78, $p <0.001$) to RLS.

Table 13: Relationship between regional wall thickness and regional ECV to regional longitudinal strain.

	Regression coefficient	**Confidence interval**	**p**
Regional wall thickness	0.48	0.37- 0.58	<0.001
ECV	18.64	9.49 – 27.78	<0.001

6.4 Discussion:

In this current study we identified that LGE was a univariate predictor of GLS with a trend being observed in a multivariable model suggesting that progressive replacement fibrosis may contribute to impaired systolic performance. The relationship between global ECV and GLS did not reach statistical significance, however, an association between ECV and longitudinal strain at a segmental level was observed. Expansion of the extracellular compartment beyond that seen in healthy controls was also associated with a significant reduction on global strain rate even after correcting for the influence of left ventricular hypertrophy.

Speckle tracking echocardiography can be used to assess regional and global myocardial deformation. HCM is characterized by a regional and global reduction in longitudinal deformation prior to the development of overt systolic dysfunction.[43] [353] An impairment in GLS has been demonstrated to be an independent predictor of adverse cardiac outcomes including heart failure related admissions and deaths. [357] [358] Consistent with these findings we found that study GLS correlated inversely with EF and directly with levels of NT-Pro BNP.

The findings of this study were also consistent with previously published studies that have demonstrated longitudinal strain to be lower in patients with HCM exhibiting LGE with GLS correlating inversely with the extent of LGE.[355] [359-362] The results of this study also suggest that progressive expansion of the extracellular volume with

interstitial fibrosis contributes towards progressive systolic impairment. The role of ECV in myocardial dysfunction has been poorly described but in accordance with findings of this study, ECV has been shown to be a univariable associate of impaired circumferential and radial strain and correlate with levels of NT-ProBNP.[93 342]

Ejection fraction is considered a poor surrogate for systolic function in the context of LVH and impairment of longitudinal deformation has been shown to be most marked in regions of hypertrophy.[43 353-356] Consistent with these studies, the current study also demonstrates that global strain is influenced primarily by the degree of hypertrophy. Impaired longitudinal deformation in the hypertrophied myocardium and may reflect a combination of direct sarcomeric dysfunction and the sub-endocardial distribution of myocyte disarray directly influencing longitudinal function.[374 375] Furthermore, myocardial ischaemia in areas of hypertrophy may also impair systolic performance.

6.5 Limitations:

The study is limited by the relatively small sample size. Although echocardiographic studies were not performed on the same day as the CMRI, the mean gap between the studies was 5 months and this believe that the results allow a fair comparison. Furthermore, the strain analysis was performed on scans acquired during routine clinical scans. Despite stringent criteria for inclusion and exclusion there was a wide range of frame rates and the images had not been optimised for strain acquisition.

6.6 Conclusion.

LV strain correlates with the extent of hypertrophy and focal fibrosis in HCM. Expansion of the ECV was associated with segmental systolic dysfunction and GLS when the ECV exceeded the range observed in the normal population. Together these findings suggest that the myocardial fibrosis contributes towards impaired myocardial systolic performance.

7. Final conclusions

Together, the findings of this thesis support the hypothesis that myocardial fibrosis is an early entity in HCM that precedes clinical phenotype. Myocardial fibrosis is associated with impaired systolic performance and may contribute to arrhythmic risk in HCM. Myocardial fibrosis is at least in part mediated by the TGF- β pathway.

7.1 Collagen synthesis and turnover in hypertrophic cardiomyopathy

In this thesis, I identified that genes involved in collagen synthesis are upregulated in myocardial tissue. There was a significant upregulation in the RNA expression of type III collagen in the HCM myocardium relative to controls. There was also a trend towards significance for elevated RNA expression of type I collagen. The relative genetic expression of type III collagen correlated to levels of myocardial collagen. This pattern is paralleled by a significant elevation in plasma levels of both procollagen I and pro-collagen III in patients with HCM relative to controls.

The enhanced synthesis of collagen is not counteracted by enhanced collagen metabolism. Tissue gene expression and plasma levels of MMP's were not significantly upregulated to counteract the increase in collagen synthesis resulting in unopposed development of myocardial fibrosis.

7.2 Collagen metabolism in gene carriage

Plasma markers of collagen synthesis are significantly altered in gene carriers and comparable to those seen in HCM. Levels of procollagen III are significantly increased when compared to controls. A strong trend for procollagen I was observed. Levels of ICTP were also increased in gene carriers relative to controls suggesting that both collagen synthesis and degradation are enhanced in gene carriers. These findings confirm that enhanced collagen synthesis and turnover is an early, active and continued process present in gene carriers that is likely to contribute to the

development and progression of the clinical phenotype. In this setting plasma markers of collagen synthesis may be a potential clinical utility for screening.

7.3 Regulation of collagen synthesis

In the HCM samples, expression of TGF-β2 and its downstream mediator CCN2 were markedly upregulated. Relative expression of all three TGF-β isoforms and CCN2 correlated with procollagen I and III gene expression. Expression PDGF B was downregulated in HCM whilst expression of PDGF A and TSP-2 correlated with expression of procollagen. These findings suggest that TGF-β mediated signalling is a key determinant of promoting myocardial collagen synthesis. Furthermore, the relative balance between TGF-β and BMP-7 is in favour of promoting endo-mesothelial transformation and unopposed TGF-β driven fibrosis

7.4 Clinical correlation

Plasma levels of PICP were higher in individuals with NSVT suggesting an association between myocardial fibrosis and the risk of sudden ventricular arrhythmias. The lack of consistent correlations between plasma markers of collagen turnover and other clinical markers of disease may be explained by the fact that myocardial fibrosis is an active and chronic process that is likely to influence the clinical phenotype over a long-term period.

Focal fibrosis identified by CMRI was associated with a reduction in systolic deformation. Furthermore, expansion of the extracellular volume by diffuse fibrosis beyond that seen in healthy controls resulted in a reduction in systolic longitudinal function.

7.5 Future directions

The work performed in my thesis has identified that increased collagen synthesis in gene carriers and HCM is an active process that is likely to contribute towards disease development and clinical markers of disease. The work carried out in this thesis could be explored to overcome some of the limitations identified and to fully understand that impact of myocardial fibrosis in HCM.

7.5.1 Genetic profiling

The majority of patients included in this study had not undergone extensive genotyping and thus I was unable to look at the role of genetic mutations upon myocardial fibrosis. The contribution of genetic mutation on myocardial fibrosis would be best studied in a large cohort of patients that had undergone extensive genetic profiling with next generation sequencing.

7.5.2 Tissue work

I chose to use a RT-PCR array to evaluate to reliably determine the regulation of genes involved in myocardial fibrosis. This process allowed me to profile a wide selection of genes that were involved in collagen synthesis and metabolism. There were however limitations which may be overcome with future work:

a. Sample size: The study cohort was small and under-representative of the diverse clinical phenotype seen in HCM. All samples were obtained from patients that has symptomatic LVOTO and samples were taken from the areas of septal hypertrophy. Future studies should:
    i. Aim to replicate the studies on larger scale in a group of genotyped patients,

ii. Utilise biopsy specimens from patients with no LVOTO, non-hypertrophied regions and study samples from patients at different stages of their disease including those with severe systolic impairment.

b. Protein expression and biological integration: Although the study highlighted the enhanced gene expression of TGF-β, we did not determine protein expression of TGF-β and its downstream mediators. Furthermore, the development and progression of myocardial fibrosis in HCM is likely to be complex interaction of cellular and molecular mediated signaling which is influenced by sarcomeric mutations, disease pathophysiology and the environment. The use and integration of microarrays and quantitative proteomics will in the future allow a greater understanding of the complex regulation of gene expression and protein synthesis that regulate myocardial fibrosis.

7.3.5 Plasma work

Future studies that would add scientific and clinical benefit would include:

a. Biomarker analysis with longitudinal follow up of a large cohort of gene carriers. This type of study would help to determine the utility of collagen biomarkers in the identification of gene carriers and prediction of disease development.
b. Longitudinal follow up of patients with HCM and correlation of clinical phenotype with collagen biomarkers. This study would provide further insight of the role of collagen turnover in disease progression and would help to determine the role of collagen biomarkers in risk prediction.

7.3.6 Targeted therapies

Enhanced understanding of disease pathophysiology and its association with genotype and clinical phenotype will ideally lead to individualised therapies for patients. Understanding the role of fibrosis in disease development and the contribution of TGF-

β activation may potentially allow for targeted antagonism of these pathways and improve patient outcome.

7.3.7 Current studies

The hypertrophic cardiomyopathy registry is a large multicenter trial that is currently underway and is designed to help establish a clearer understanding of the clinical significance of fibrosis upon clinical outcomes in HCM. The trial aims to establish a large registry of patients that have undergone genetic profiling with a view of evaluating the impact of genetic, CMR and collagen biomarkers variables on clinical outcomes in HCM. The ultimate aim is to generate a risk prediction model based upon these parameters. The design of this study should overcome many of the limitations encountered in this body of work and will not only provide further insight in the role myocardial fibrosis upon clinical outcomes but will also aim to determine the clinical utility of collagen biomarkers in clinical practice.[376]

8. References

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9. Appendix

Appendix 1: Abbreviations

ACCF American college of cardiology foundation
ACE Angiotensin converting enzyme
ACTA2 Actin, alpha 2, smooth muscle, aorta
ACTC a-cardiac actin
AF Atrial fibrillation
AGT Angiotensinogen
AHA American heart association
AKT1 V-akt murine thymoma viral oncogene homolog 1
ALK5 Activin-linked kinase 5
Ang II Angiotensin II
ASA Alcohol septal ablation
ASH Asymmetrical septal hypertrophy
AT-1 Angiotensin receptor type 1
ATP Adenosine triphosphate
AV Atrio-ventrincular
BCL2 B-cell CLL/lymphoma 2
bFGF Fibroblast growth factor basic
BMI Body mass index
BMP Bone morphogenetic protein
BNP Brain natriuretic peptide
BP Blood pressure
bpm Beats per minute
BSA Body surface area
CAV1 Caveolin 1
CCL11 Chemokine (C-C motif) ligand 11
CCL2 Chemokine (C-C motif) ligand 2
CCL3 Chemokine (C-C motif) ligand 3
CCN2 Connective tissue growth factor
CCN2 Connective tissue growth factor
CCR2 Chemokine (C-C motif) receptor 2
CEBPB CCAAT/enhancer binding protein (C/EBP), beta
CF Cardiac fibroblasts
CI Confidence intervals
CK Creatine kinase
cm Centimetre
CMRI Cardiac magnetic resonance imaging
cmsq Centimetre squared

COL1A2 Collagen, type I, alpha 2
COL3A1 Collagen, type III, alpha 1
CRP C-reactive protein
CXCR4 Chemokine (C-X-C motif) receptor 4
DCM Dilated cardiomyopathy
DCN Decorin
DTPA Diethylenetriamine pentaacetic acid
Ea Early diastolic velocity at the mitral annulus
Ea/Aa Ratio of the early to late diastolic velocities at the mitral annulus
ECG Electrocardiogram
ECM Extracellular matrix
ECV Extracellular volume
EDTA Ethylenediaminetetraacetic acid
EF Ejection fraction
EGF Epidermal growth factor
End-MT Endothelial – mesothelial transformation
ENG Endoglin
EQ-CMR Equilibrium contrast cardiac magnetic resonance imaging
Erk Extracellular signal regulated kinase
ESC European society of cardiology
ET-1 Endothelin 1
ET-A Endothelin receptor type A
ET-B Endothelin receptor type B
FASLG Fas ligand
FFA Free fatty acids
FGF Fibroblast growth factor

g/msq Grams per metre squared
Gd Gadolinium
GLS Global longitudinal strain
GREM1 Gremlin 1
HCl Hydrogen chloride
HCM Hypertrophic cardiomyopathy
HGF Hepatocyte growth factor
HPLC High performance liquid chromatography
ICD Implantable cardiac defibrillator
ICTP Carboxyterminal telopeptide of type I collagen
IFNG Interferon, gamma
IIICTP Carboxyterminal telopeptide of type III collagen
IL-1 Interleukin-1
IL-6 Interleukin-6

IL10 Interleukin 10
IL13 Interleukin 13
IL13RA2 Interleukin 13 receptor, alpha 2
IL1A Interleukin 1, alpha
IL1B Interleukin 1, beta
IL4 Interleukin 4
IL5 Interleukin 5
ILK Integrin-linked kinase
INHBE inhibin, beta E
ITGα_1 Integrin, alpha 1 subunit
ITGα_2 Integrin, alpha 2 subunit
ITGα_3 Integrin, alpha 3 subunit
ITGα_V Integrin, alpha V subunit
ITGβ_1 Integrin, beta 1 subunit
ITGβ_3 Integrin, beta 3 subunit
ITGβ_5 Integrin, beta 5 subunit
ITGβ_6 Integrin, beta 6 subunit
ITGβ_8 Integrin, beta 8 subunit
JNK c-Jun-N-terminal kinase
JUN Jun proto-oncogene
kg/msq Kilogram per metre squared
LAP Latency-associated pro-peptide
LGE Late gadolinium enhancement
LiH Lithium heparin
LOX Lysyl oxidase
LTBP1 Latent transforming growth factor beta binding protein 1
LV Left ventricle
LVED left ventricular end diastolic
LVH Left ventricular hypertrophy
LVOTG Left ventricular outflow tract gradient
LVOTO Left ventricular outflow tract obstruction
MAPK p38 Mitogen activated protein kinase (MAPK)
mm Millimetre
mmHg Millimetre of mercury
MMP Matrix metalloproteinase
MMP1 Matrix metallopeptidase 1
MMP13 Matrix metallopeptidase 13
MMP14 Matrix metallopeptidase 14
MMP2 Matrix metallopeptidase 2
MMP3 Matrix metallopeptidase 3
MMP8 Matrix metallopeptidase 8

MMP9 Matrix metallopeptidase 9
MYBPC 3 Myosin-binding protein C
MYC V-myc myelocytomatosis viral oncogene homolog
MYH6 a-myosin heavy chain
MYH7 b Myosin heavy chain
MYL2 Regulatory myosin light chain
MYL3 Essential myosin light chain
NBD-Cl 7-chloro-4-nitrobenzo-oxa-1,3-diazole
NFKB1 Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NSVT Non-sustained ventricular tachycardia
NYHA New York heart association
OPN Osteopontin
PCR Polymerase chain reaction
PCr:ATP Phosphocreatine to adenosine triphosphate
PDGF Platelet derived growth factor
PDGFA Platelet-derived growth factor alpha polypeptide
PDGFB Platelet-derived growth factor beta polypeptide
PDGFR Platelet derived growth factor receptor
PICP Carboxyterminal propeptide of type I procollagen
PIIICP Carboxyterminal propeptide of type III procollagen
PIIINP Aminoterminal propeptide of type III procollagen
PINP Aminoterminal propeptide of type I procollagen
PLAT Plasminogen activator, tissue
PLAU Plasminogen activator, urokinase
PLG Plasminogen
qPCR Quantitative PCR
RIN Ribonucleic acid integrity number
RNA Ribonucleic acid
s.d Standard deviation
SAM Systolic anterior motion of the mitral valve
SCD Sudden cardiac death
SERPINA1 Serpin peptidase inhibitor, clade A
SERPINE1 Serpin peptidase inhibitor, clade E
SERPINH1 Serpin peptidase inhibitor, clade H
Sh-MOLLI Shortened modified look locker inversion recovery sequence
Smad2 Smad family member 2
Smad3 Smad family member 3
Smad4 Smad family member 4
Smad6 Smad family member 6
Smad7 Smad family member 7
SNAI1 Snail homolog 1

SP1 Sp1 transcription factor
SPARC Secreted protein acidic and rich in cysteine
SSFP Steady state free precision
STAT1 Signal transducer and activator of transcription 1
STAT6 Signal transducer and activator of transcription 6
TAK1 Transforming growth factor-β-activated kinase 1
TD Delayed time
TGF- β3 Transforming growth factor, beta 3
TGF-ß Transforming growth factor beta
TGF-ßR1 Transforming growth factor receptor type 1
TGF-ßR2 Transforming growth factor receptor type 2
TGF-β1 Transforming growth factor, beta 1
TGF-β2 Transforming growth factor, beta 2
TGF-βR1 Transforming growth factor, beta receptor 1
TGF-βR2 Transforming growth factor, beta receptor II
TGIF1 TGF-β-induced factor homeobox 1
TIMP Tissue inhibitors of metalloproteinases
TIMP1 Tissue inhibitor of metallopeptidase 1
TIMP2 Tissue inhibitor of metallopeptidase 2
TIMP3 Tissue inhibitor of metallopeptidase 3
TIMP4 Tissue inhibitor of metallopeptidase 4
TN-C Tenascin C
TN-X Tenascin X
TNF Tumour necrosis factor
TNFα Tumour necrosis factor alpha
TNNC1 Cardiac Troponin C
TNNI3 Cardiac Troponin I
TNNT2 Cardiac Troponin T
TPM1 alpha-tropomyosin
TSP Thrombospondin
TSP-1 Thrombospondin 1
TSP-2 Thrombospondin 2
VEGF Vascular endothelial growth factor
VEGFA Vascular endothelial growth factor A

Appendix 2: Patient and control information and consent forms.

University College London Hospitals NHS
NHS Foundation Trust

INFORMATION ABOUT THE RESEARCH

Measuring Biomarkers in People with Inherited Cardiomyopathies (Adult information sheet and consent form for Blood and Urine)

Chief Investigator: Dr. Perry Elliott, Department of Cardiology, The Heart Hospital, 16-18 Westmoreland Street, London. W1G 8PH

We would like to invite you to take part in this research study. Before you decide if you wish to be enrolled in the study you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The aim of this research is to provide a greater understanding of the pathways that are involved in the development and progression of inherited cardiomyopathies. We also aim to identify markers of disease activity to help in routine clinical assessment and management.

Why have I been invited?

You have been asked to take part because you have a genetic predisposition or have been diagnosed with an inherited cardiomyopathy.

Do I have to take part?

There is no obligation on your part to enter this study. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. If you agree, we will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. Participation or non participation will not affect the standard of care you receive in any way.

What will happen to me if I take part?

IMPORTANT: THIS IS NOT A TRIAL OF TREATMENT – YOUR TREATMENT DOES NOT CHANGE.

The investigations and interventions in this research will be a part of the investigation and treatment that you would normally receive. The exception to this is that we will be required to take an additional sample of blood and urine for the purpose of further analysis and investigation.

Clinical Assessment and Tests

Your symptoms will be assessed and recorded during your routine outpatient appointment. As a part of the study we will be recording the results of tests that you would have done as a routine part of your assessment.

Blood and Urine Samples

As a part of the study we will be analysing your blood and urine. In addition to the routine blood test that you normally have done we will require 2 additional small tubes of blood and a sample of urine.

What will happen to any samples I give?

Samples will be labelled with a unique patient study number. Samples will be stored securely and analysed at facilities with in UCLH, GOSH and UCL. Access to samples will be restricted to researchers involved in taking measurements from them. Samples for the purpose of this study may be sent to external laboratories which may be outside the UK. No patient identifiers will be sent. At the end of the study the samples may be stored in accordance with the Human Tissue Act. Samples will not be used without further consent.

You may also wish to donate your blood and urine as an absolute and unconditional gift. If you do, you will retain no future rights to these samples and to any information that may be derived from them. No testing for serious communicable disease such as HIV or hepatitis B will be performed on these samples.

Will any genetic tests be done?

DNA is the hereditary material containing the genetic instructions for development and disease. Analysis of your DNA will **NOT** be performed. We will **NOT** use these samples to obtain genetic information that may have implications for you or your family.

RNA carries the genetic information and chemical “blueprint” that is essential for protein synthesis. With your consent we may measure the expression of RNA.

What are the possible disadvantages and risks of taking part?

All aspects of this study are safe.

What are the possible benefits of taking part?

To you as an individual, there is little direct benefit. However, the information we get from this study will help improve our understanding of the condition and will hopefully help us to improve the care we give our patients in the future.

What if there is a problem?

As a matter of routine, researchers have to inform all participants in all studies about their rights in the unlikely event that harm should occur. This project has been approved by an independent research committee who believe the risk to participants is minimal. Compensation for any injury caused by taking part in

this study is covered under the NHS indemnity scheme. Your right in law to claim compensation for injury where you can prove negligence is not affected. The ethics committee advises (as a matter of routine) that people with private health-care cover should inform their insurers about participation in research studies.

What if I wish to make a complaint?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Contact Dr. Perry Elliott on 02034567898 ext 64801). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be identified.

What will happen if I don't want to carry on with the study?

We will not perform any further testing on the samples you have provided and no further information will be acquired for the research. Information that has already been collected may still be used.

Will your GP be informed of your participation?

We would routinely inform your GP unless there are any objections.

What will happen to the results of the research study?

We intend to publish the results of this study in a medical journal and present them at conferences.

Your individual test results from investigation will be stored and analysed. A report of the investigations and the results will be recorded in your clinical notes. In the event that we identify a problem not already known about, your clinical care provider will be informed.

Who is organising and funding the research?

This study is being organised by Dr. Perry Elliott, Reader in inherited cardiac disease - The Heart Hospital, UCLH.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by London – Central UCLH Research Ethics Committee.

Where can I obtain independent advice about whether to enter the study?

You can ask the UCLH research and development office or consult your GP for general advice.

University College London Hospitals NHS
NHS Foundation Trust

Patient Identification Number:

Study Number: 11/LO/0913

CONSENT FORM

Title of Project: Measuring Biomarkers in People with Inherited Cardiomyopathies (Adult information sheet and consent form for Blood and Urine)

Name of Researchers: Dr. Perry Elliott.

	Please Initial
I confirm that I have read and understood the information sheet dated 21/06/2012, version 4 for the above study. I have had the opportunity to consider the information, ask questions and have any queries answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
I give permission for my blood and urine to be stored indefinitely and for further research to be carried out using them **with/without** (delete appropriate) the need to contact me again. I understand that no research will be performed unless ethics committee approval has been obtained. (If you do not agree, your sample will be destroyed at the end of the study)	☐
I give permission for the measurement and analysis of RNA	☐
I agree to take part in the above study.	☐
I agree that my GP may be informed of my involvement in the study.	☐

_____ _____ _____

Name of Patient Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

University College London Hospitals NHS
NHS Foundation Trust

INFORMATION ABOUT THE RESEARCH

Measuring Biomarkers in People with Inherited Cardiomyopathies (Adult information sheet and consent form for Myocardial Tissue - Controls)

Chief Investigator: Dr. Perry Elliott, Department of Cardiology, The Heart Hospital, 16-18 Westmoreland Street, London. W1G 8PH

We would like to invite you to take part in this research study. Before you decide if you wish to be enrolled in the study you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The aim of this research is to provide a greater understanding of the pathways that are involved in the development and progression of inherited cardiomyopathies. We also aim to identify markers of disease activity to help in routine clinical assessment and management.

Why have I been invited?

You have been asked to take part have because you DO NOT have a genetic predisposition or have NOT been diagnosed with an inherited cardiomyopathy and have undergone or are undergoing a cardiac biopsy or surgical procedure as a part of your routine investigation or treatment.

Do I have to take part?

There is no obligation on your part to enter this study. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. If you agree, we will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. Participation or non participation will not affect the standard of care you receive in any way.

What will happen to me if I take part?

IMPORTANT: THIS IS NOT A TRIAL OF TREATMENT – YOUR TREATMENT DOES NOT CHANGE.

The investigations and interventions in this research will be a part of the investigation and treatment that you would normally receive. The exception to this is that we will store and further analyse the heart tissue obtained at the time of biopsy or surgery.

Clinical Assessment and Tests

Your symptoms will be assessed and recorded during your routine outpatient appointment. As a part of the study we will be recording the results of tests that you would have done as a routine part of your assessment.

Cardiac Tissue Samples

We would like to analyse samples of heart muscle that have been obtained during cardiac biopsy or cardiac surgery as a part of your ongoing care. We will not obtain extra tissue for the purpose of research.

What will happen to any samples I give?

Samples will be labelled with a unique patient study number. Samples will be stored securely and analysed at facilities with in UCLH, GOSH and UCL. Access to samples will be restricted to researchers involved in taking measurements from them. Samples for the purpose of this study may be sent to external laboratories which may be outside the UK. No patient identifiers will be sent. At the end of the study the samples may be stored in accordance with the Human Tissue Act. Samples will not be used without further consent.

You may also wish to donate your tissue as an absolute and unconditional gift. If you do, you will retain no future rights to your tissue and to any information that may be derived from them. No testing for serious communicable disease such as HIV or hepatitis B will be performed on these samples.

Will any genetic tests be done?

DNA is the hereditary material containing the genetic instructions for development and disease. Analysis of your DNA will **NOT** be performed. We will **NOT** use these samples to obtain genetic information that may have implications for you or your family.

RNA carries the genetic information and chemical “blueprint” that is essential for protein synthesis. With your consent we may measure the expression of RNA.

What are the possible disadvantages and risks of taking part?

There are no additional risks to taking part in the research.

What are the possible benefits of taking part?

To you as an individual, there is little direct benefit. However, the information we get from this study will help improve our understanding of the condition and will hopefully help us to improve the care we give our patients in the future.

What if there is a problem?

As a matter of routine, researchers have to inform all participants in all studies about their rights in the unlikely event that harm should occur. This project has been approved by an independent research committee who believe the risk to participants is minimal. Compensation for any injury caused by taking part in this study is covered under the NHS indemnity scheme. Your right in law to claim compensation for injury where you can prove negligence is not affected.

The ethics committee advises (as a matter of routine) that people with private health-care cover should inform their insurers about participation in research studies.

What if I wish to make a complaint?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Contact Dr. Perry Elliott on 02034567898 ext 64801). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be identified.

What will happen if I don't want to carry on with the study?

We will not perform any further testing on the samples you have provided and no further information will be acquired for the research. Information that has already been collected may still be used.

Will your GP be informed of your participation?

We would routinely inform your GP unless there are any objections.

What will happen to the results of the research study?

We intend to publish the results of this study in a medical journal and present them at conferences.

Your individual test results from investigation will be stored and analysed. A report of the investigations and the results will be recorded in your clinical notes. In the event that we identify a problem not already known about, your clinical care provider will be informed.

Who is organising and funding the research?

This study is being organised by Dr. Perry Elliott, Reader in inherited cardiac disease - The Heart Hospital.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by London - Central Research Ethics Committee.

Where can I obtain independent advice about whether to enter the study?

You can ask the UCLH research and development office or consult your GP for general advice.

University College London Hospitals NHS
NHS Foundation Trust

Patient Identification Number:

Study Number: 11/LO/0913

CONSENT FORM

Title of Project: Measuring Biomarkers in People with Inherited Cardiomyopathies
(Adult information sheet and consent form for Myocardial Tissue - Controls)

Name of Researchers: Dr Perry Elliott.

	Please Initial
I confirm that I have read and understood the information sheet dated 21/06/2012, version 1 for the above study. I have had the opportunity to consider the information, ask questions and have any queries answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
I give permission for my cardiac tissue to be stored indefinitely and for further research to be carried out using them **with/without** (delete appropriate) the need to contact me again. I understand that no research will be performed unless ethics committee approval has been obtained. (If you do not agree, your sample will be destroyed at the end of the study)	☐
I give permission for the measurement and analysis of RNA	☐
I agree to take part in the above study.	☐
I agree that my GP may be informed of my involvement in the study.	☐

Name of Patient	Date	Signature

Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in

University College London Hospitals
NHS Foundation Trust
Department of Cardiology
The Heart Hospital
16-18 Westmoreland Street
London. W1G 8PH

INFORMATION ABOUT THE RESEARCH

Measuring Fibrosis (Scarring) in the Heart with MRI.

Investigators: Dr. James Moon, Dr. Andrew Flett, Dr Dan Sado

We would like to invite you to take part in a research study. Before you decide whether you wish to be enrolled in the study you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Heart muscle scarring, or fibrosis makes people breathless. It is difficult to measure, especially when it is diffuse. We want to develop a new technique of heart scanning (magnetic resonance scan) which will enable us to measure the amount of fibrosis (or scar tissue) in the heart.

Why have I been invited?

You have been chosen to take part because you have a condition that is known to cause fibrosis of the heart muscle (even though the amount of fibrosis may be very small).

Do I have to take part?

There is no obligation on your part to enter this study. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. Participation or non participation will not affect the standard of care you receive in any way.

What will happen to me if I take part?

IMPORTANT: THIS IS NOT A TRIAL OF TREATMENT – YOUR TREATMENT DOES NOT CHANGE.

We are developing a technique to measure fibrosis or scarring in the heart. To do this we need to ask you to have a heart scan and during this, some blood tests.

1. 'Heart' scans.

One of these is an echocardiogram (you may have had this already), a safe test using an ultrasound probe on your chest. The other scan is a cardiovascular magnetic resonance (CMR) scan. CMR uses a magnet and radio waves to form pictures of your heart; there are no known harmful effects (there are no x-rays). The scans are highly specialised, so they have to be done at The Heart hospital or Great Ormond Street hospital in London.

What will happen when I come for the scan?

We ask patients to arrive approximately 20 minutes before their actual CMR scan appointment time to do the paper work and get changed into a gown (there is a private changing area for this). It is important not to bring metal or jewellery into the scanner room (such as wiring or clasps in your bra, or zips, or buckles in other clothes). This is because metal interferes with the pictures. We will measure your height, and weight. A small cannula (drip) is inserted in to a vein in the arm by an experienced doctor/technician. This allows a blood sample to be taken and later a small amount of a dye to be given as part of the scan itself. This dye is called gadolinium; it is very widely used and usually has no side effects at all; very occasionally (less than 1/100 times) it causes a temporary mild headache or nausea. The scan itself takes just over an hour. It involves lying on your back inside a large open-ended tube (which is the magnet). An ECG (heart) monitor (3 sticky pads) is put on the chest and we will ask you to hold your breath (for between 2 and 15 seconds) when we are actually taking the pictures.

Are there any side effects from the scan and will it hurt?

The scanner is noisy but not at all painful. Some people experience feelings of claustrophobia during the scan, but there is constant contact via an intercom system so you can request the investigation be paused at any time. We can play the radio or music (bring your own CD if you wish).

2. Blood and tissue samples

We take extra blood when the cannula ('drip') is inserted for the dye with the heart scan. We need 2-3 small tubes (each about the same as a teaspoon). If you have had or are having a heart biopsy already, we would like to be able to analyse the histology slides. If this test is not required by your doctor then no biopsy will be performed. Participating in the study means you agree to donate your blood and tissue as an absolute and unconditional gift to UCL and that you will retain no future rights to your blood or to any information that may be derived from your blood.

3. Other Tests

If you have not had it done previously, we will perform an electrocardiogram (ECG). This test involves applying 12 sticky electrodes to your body and takes a few minutes to do. It is a perfectly safe test that allows us to assess the electrical function of your heart.

If you have not previously had one, we will also ask you to perform a cardiopulmonary exercise test if possible. During this test, you will be asked to peddle on an exercise bicycle while breathing into a mask. This allows us to

assess the function of your heart and lungs. Again, this is a very safe test that is routinely performed on patients everyday in the NHS.

What are the possible disadvantages and risks of taking part?

The MRI scan is very safe. Patients where there are potential or real problems (eg patients with a pacemaker, or severe kidney disease) will not be invited to participate in this project.

What are the possible benefits of taking part?

To you as an individual, there is little direct benefit except that it will give you an idea of how well your heart is functioning. However, the information we get from this study will help improve our understanding of heart disease and will hopefully help us to improve the care we give our patients in the future.

What if there is a problem?

As a matter of routine, researchers have to inform all participants in all studies about their rights in the unlikely event that harm should occur. This project has been approved by an independent research committee who believe the risk to participants is minimal. Compensation for any injury caused by taking part in this study is covered under University College London's Indemnity Scheme; under this arrangement it is not necessary to prove fault/negligence. You are also covered against injury caused by negligence by the NHS indemnity scheme. Your right in law to claim compensation for injury where you can prove negligence is not affected. The ethics committee advises (as a matter of routine) that people with private health-care cover should inform their insurers about participation in research studies.

What if I wish to make a complaint?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Contact Andrew Flett / Daniel Sado on 02075738888 ext 4910) If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

What will happen if I don't want to carry on with the study?

Information collected may still be used. Any stored blood that can still be identified as yours will be destroyed if you wish.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the

hospital/surgery will have your name and address removed so that you cannot be recognised.

What will happen to any samples I give?

We will need to collect blood samples during your MRI to measure the amount of contrast. Blood tests will be taken by an experienced researcher qualified to do so. Samples will be stored within UCLH. Access to samples will be restricted to researchers involved in taking measurements from them. Samples will remain in the UK at all times.

Will any genetic tests be done?

No

What will happen to the results of the research study?

We intend to publish the overall results of this study in a medical journal and present them at conferences.

Your individual test results from the blood and heart scan will be stored and analysed. A report of the MRI will be generated and the results will be communicated to the doctor who referred you. In the event that the scan identifies a problem not already known about, your GP may be informed. Such conditions would include scarring from a previous heart attack.

Who is organising and funding the research?

This study is being organised by Dr. James Moon, Senior lecturer and Honorary Consultant in cardiology – The Heart Hospital. It is being funded by the British Heart Foundation charity.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by UCLH Research Ethics Committee.

Where can I obtain independent advice about whether to enter the study?

You can ask the UCLH research and development office or consult your GP for general advice.

University College London Hospitals NHS Foundation Trust

Department of Cardiology
The Heart Hospital
16-18 Westmoreland Street
London. W1G 8PH

Patient Identification Number:

Study Number:

CONSENT FORM

Title of Project: Measuring fibrosis (scarring) in the heart with MRI scanning

Name of Researchers: Dr. Andrew Flett, Dr Dan Sado, Dr. James Moon.

Please initial box

1. I confirm that I have read and understand the information sheet dated 8/10/2010, version 5 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by members of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I give permission for my blood and tissue to be stored indefinitely and for further research to be carried out using them without the need to contact me again. I understand that no research will be performed unless ethics committee approval has been obtained. (If you do not agree, your sample will be destroyed at the end of the study) ☐

5. I agree to take part in the above study. ☐

6. I agree that my GP may be informed of my involvement in the study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 3: First author publications quoted in this thesis

Patel V, Critoph CH, Elliott PM. Mechanisms and medical management of exercise intolerance in hypertrophic cardiomyopathy. Current pharmaceutical design. 2015;21(4):466-72. [published Online First: 2014/12/09]

Patel V and Elliott P. Hypertrophic cardiomyopathy. J.C Kaski et al, Chest pain with normal coronary arteries, Springer-verlag, London 2013.

Appendix 4: Acknowledgements

I am forever grateful to the following people for their help during the preparation of this thesis:

Professor Perry Elliott, he initially gave me the opportunity to work in the inherited cardiac disease department at a very early stage of my career. I have learnt so much from him which will stay with me through my working career and beyond.

Dr Christopher Chritoph, a friend and colleague who has supported me throughout this process.

Dr Petros Syrris, for his continued support in co-ordinating the experiments.

Dr Joel Salazar, for his help as the second observer in analysis of echo studies.

Dr Dan Sado and Dr Gaby Captur, for their assistance in CMRI analysis.

Finally, my wife Krupa and my children Riayn and Amara for their support and sacrifice during this period.