Genetic and Clinical Investigations in the Familial Cardiomyopathies

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Dedication

My mother, Lisa, and her mother, Marion Westcott, share each of my ambitions, successes and difficulties. The qualities of these ladies are ever present.

I have profited from the skills of many teachers, and wish I had known many more. David Sperling at Nairobi’s Strathmore College, Sam Donaldson at Edinburgh’s James Gillespie’s High School, Hugh Miller and Andrew Flapan at the Royal Infirmary in Edinburgh, Lameh Fananapazir, Doug Rosing and Dotti Tripodi at the National Institutes of Health, and Peter Mills and Tom Crake in London are the teachers and mentors whose values I call on still. I have benefited from them more than they can know.

To Dr. Peter Mills I owe a further debt of gratitude. This thesis would never have been completed without his counsel and ever present encouragement.
Declaration

I, Dr Saidi A Mohiddin, the candidate presenting for the degree of Doctorate (Medicine) have written this thesis.

Collaborative effort has been important in much of the research presented here. When major contributions have been made by other individuals or institutions, this is acknowledged in the appropriate section of the thesis. None of the work here has been submitted for any other professional qualification. Where material has been published, this has been approved by my supervisor. All publications are identified in the appropriate sections of this thesis.

Dr Saidi A Mohiddin
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2 INTRODUCTION

2.1 HISTORICAL PERSPECTIVES AND EPIDEMIOLOGY

Hypertrophic cardiomyopathy (HCM) is the single most common cause of sudden death in otherwise healthy young people, found in approximately half of such cases.\textsuperscript{1-4} It is a genetic disease occurring in approximately 1 in 500-1,000 of the general population.\textsuperscript{5,6} The myopathy is often undiagnosed and asymptomatic cases are often unrecognised. Usually, HCM develops with progressive asymmetric LV hypertrophy (LVH) during the period of rapid body growth of adolescence, but it may be present in childhood or even before birth. Progressive hypertrophy after age 20 is uncommon, but initial diagnosis even in old age is not. The hypertrophy predominantly involves the LV, and is often more marked than in any other cardiac disease. It represents hypertrophy and hyperplasia of several cell types, including cardiac myocytes, fibroblasts, and smooth muscle cells, along with excessive collagen and matrix deposition.\textsuperscript{7,8} The normal parallel arrangement of myocytes is often disturbed (fibre disarray).

The first descriptions of Hypertrophic Cardiomyopathy (HCM) are often attributed to Teare in the late 1950s.\textsuperscript{9,10} In the half century following, what was considered an unusual or rare single clinical entity associated with a dire prognosis is increasingly understood as a group of myocardial diseases with surprisingly heterogeneous genetic causes and phenotypic expressions with a benign outcome as the more common clinical course.

A PubMed search for ‘hypertrophic AND cardiomyopathy’ yields nearly 16,000 hits, with 3181 in the last 5 years (June 2009); to date, the most active research interests have included

1. The development of therapies for the relief of outflow obstruction.
2. The beginning of an understanding of the genetic basis of hereditary left ventricular hypertrophy.
3. The development of cardiac imaging techniques to serve diagnostic and prognostic needs.
4. The development of strategies identifying and addressing risks of sudden death.

Further advances in therapy are likely to require both further development in these areas, but will also include:
(1). A more thorough understanding of the pathophysiology of the symptomatic patient with unobstructed HCM. This includes an improvement in detecting and managing 'diastolic failure', myocardial ischaemia and myocardial fibrosis.

(2). Unravelling of the cellular processes leading from mutation to disease and of the powerful factors that modify disease expression. Novel pharmacological therapies may follow.

(3). Randomised clinical trials to evaluate existing and future clinical practice. Few randomised trials interrogate the value of contemporary clinical practice.

2.2 DEFINITIONS OF HYPERTROPHIC CARDIOMYOPATHY

HCM is currently diagnosed on the basis of morphologic abnormalities, requiring the demonstration of a criterion magnitude of left ventricular hypertrophy (LVH) unexplained by an identified hemodynamic, metabolic, infiltrative or endocrine cause of LVH. Limitations to the utility of these definitions includes the many patients with LVH (who may have a variant of HCM), scenarios where LVH accompanies cardiovascular conditions also associated with LVH (hypertension and athletic training in particular), and when a genetic abnormality associated with familial HCM is identified in individuals without LVH. Some sarcomeric mutations associated with symptoms and/or risks of sudden death are associated with minimal or no LVH in some but not others from the same families.

2.2.1 CLASSIFICATIONS OF HYPERTROPHIC CARDIOMYOPATHY

Heterogeneity is a striking feature of HCM, and is evident from morphologic, clinical, prognostic and molecular perspectives. For example, any sarcomeric mutation associated with HCM identified thus far can result in a variety of disease phenotypes or no detectable disease at all. Similarly, exhaustive mutation detection in, say, patients with obstructive HCM will detect several different and often novel mutations, or will fail to find any mutations at all. Clinical, translational and basic research, as well as clinical practice needs valid and precise definitions of disease; this heterogeneity defies a single disease classification scheme. Morphologic, physiologic, prognostic, mutation-specific, gene specific and molecular-functional classification systems have been suggested; their existence describes attempts to provide a single unified classification scheme that satisfies clinical needs (in developing symptomatic, prognostic and screening strategies) and the need to understand mechanism of disease. Notably, despite these
efforts, clinical phenotypes remain disconnected from the causes of HCM; powerful and unidentified factors modify the end result of mutant gene expression.14,15

2.2.1.1 Morphological Classification

Early investigators determined that LVH varied in magnitude and its distribution in the LV.16,17 Asymmetric septal hypertrophy (ASH) describes the most commonly observed distribution of hypertrophy in the LV septum. Approximately one quarter of ASH distribution HCM patients develop a dynamic LV outflow tract obstruction as a consequence of systolic anterior movement (SAM) of the mitral valve leaflets towards the basal septum to cause obstructive HCM.18 More unusual morphologic variants include mid-cavity obstructive HCM, apical (or Japanese) HCM and dilated-phase or ‘burnt-out’ HCM (Figure 1 and Figure 2). Finally, cardiac magnetic resonance imaging’s ‘killer application’ promises to be tissue characterisation. The detection of myocardial scarring by delayed enhancement following gadolinium enhancement may prove both diagnostically and prognostically powerful.

Figure 1: Morphological Variants in Hypertrophic Cardiomyopathy.

A: Idealized figures illustrating distributions of LVH seen in HCM. In addition to ‘classical’ variants (1-4), imaging may demonstrate normal LV wall thickness, and sarcomeric mutations may also present as a dilated cardiomyopathy, either with an intervening hypertrophic stage19, or as end stage ‘burnt-out’ hypertrophic disease.20-22
Morphology and patterns of hyperenhancement (delayed gadolinium enhancement, DGE) demonstrated by cardiac magnetic resonance imaging. A, B, C: Asymmetric septal hypertrophy (ASH) with 4-chamber views demonstrating cardiac morphology at end diastole (A), end systole (B) and delayed enhancement (C). SAM with septal contact (arrow) results in LVOTO and mitral regurgitation. Delayed enhancement is seen in the basal septum (double arrows). A unilateral pleural effusion and collapse of the left lung is also seen. D, E, F: Reversed ASH in 4-chamber (D) and basal short axis (E) views. There is extensive patchy DGE in the basal anterolateral segment. G, H, I: The uncommon apical variant of HCM. 4-Chamber views demonstrate distal LVH and small end-diastolic cavitory volumes (D) and the systolic obliteration of the distal two-thirds of the LV cavity. DGE is extensive, and was demonstrated in all LV segments (I). Images courtesy of the London Chest CMR unit (M. Westwood, C. Davies, A Mathur)

2.2.1.2 Pathophysiological Classification

The physiologic abnormalities and symptoms associated with HCM show a surprising degree of infidelity to the morphology. Arrhythmia risk, abnormal myocardial relaxation/compliance, and ischaemia are demonstrated in patients with severe/mild LVH, unobstructed/plasticse, high/low risk and in symptomatic/asymptomatic patients. These features do not have a simple relationship with any morphologic or genetic feature of HCM. Furthermore, physiologic disturbances appear to contribute to the evolution of the clinical expression of disease more so, and often in the absence of, demonstrable changes in LV morphology.
For example, an individual who is asymptomatic in his 20's may develop exertional limitations in his 40's, despite minimal or no changes in cardiac morphology. Ischaemic, obstructive, restrictive, arrhythmic, systolic and chronotropic abnormalities are not mutually exclusive, and may progress and interact in the absence of obvious changes in LVH. Difficulties in taking objective measurements of the parameters other than gross morphology and flow velocities, and the lack of rational therapies render physiologic classification of largely theoretical interest. Nonetheless, we increasingly speak of restrictive or diastolic failure HCM, dilated phase HCM, ischaemic HCM, HCM with associated atrial myopathy, and are keenly pursuing ways to more accurately identify pro-arrhythmic HCM. The potential for therapy tailored to abnormal physiology such as using renin-angiotensin-aldosterone system (RAAS) modulation for restrictive HCM, or defibrillator therapy for pro-arrhythmic HCM provide powerful motivation for the further development of a disease understanding and classification on a physiological basis.

2.2.1.3 Genetic Classification

The genomics revolution has failed to make substantial contributions to the clinical management of HCM. Despite the tremendous advances in the identification of mutant genetic causes of HCM, genetic classifications have thus far proven to be of quite limited practical significance. Our ability to associate natural history with a defined mutation has at least three major limitations: (1) A genetic cause for HCM is currently not found in approximately 40% of proband patients; (2) the large number and rarity of most individual mutations; and (3) the high degree of infidelity between genotype and phenotype, even within a single pedigree, a feature of the disease that attests to the power of undefined modifying influences. Nonetheless, although a single HCM causing mutation does not dictate the cardiac phenotype, or even cause the disease in many individuals, it does appear to influence clinical phenotype.

For a few of the many mutations thus far detected that have been extensively studied in many affected individuals from different families, several clinical features are more prominent than expected. This includes sudden death risk, atrial fibrillation, mid cavity disease and LVH with accessory pathways and conduction disease.
2.2.1.4 Molecular classifications

Classifications based on the gene affected rather than on the precise mutation, or on the gene products likely role (for example considering all actin mutations together, or all actin, troponin or tropomyosin mutations as thin filament mutations, or all sarcomeric mutations versus non sarcomeric HCM) has also not proven useful. Thus beta myosin or 'thick-filament' HCM does not appear to be appreciably different from 'thin-filament' HCM. Over the next few years, we are likely to see more non-sarcomeric genes implicated in HCM, and comparisons between sarcomeric and non-sarcomeric HCM will be made.

2.2.1.5 Molecular or Motor Function Classification:

The development of a classification scheme that is derived from a mechanistic understanding of the molecular dysfunction that results from mutant protein expression remains an important and attractive goal of cardiomyopathy research. It stands to reason that as the functional consequences of a mutation are necessary for the development of HCM, some dimension of the phenotypic severity will correspond to the severity of the molecular disability.

Mutations in sarcomeric genes are the commonest causes of HCM, accounting for 50-60% of cases in tertiary referral populations. For the many dozen mutations in these genes that are known to cause HCM, there is a very limited understanding of the functional consequences of mutant protein expression and how this may lead to the HCM phenotype. The design and validation of assays of molecular/contractile function (for example Vactin, ATPase activity and pCa50) may yet contribute towards a more thorough understanding and classification system. At present, no studies convincingly associate bench-top assays of mutant protein function with any 'assay' of clinical outcome. Some researchers have been tempted to talk in terms of 'gain of function' mutations causing LVH and DCM resulting from 'loss of function' mutations, however parameters of in-vitro acto-myosin interaction bear no relation to the resulting phenotype.

An alternative perspective is that the development of LVH in HCM is the output value of an equation where sarcomeric dysfunction is a necessary, but not sufficient, input variable. Quite apart from its property as a necessary variable, a mutation also has an unrelated further property influencing, but not dictating, other phenotypic characteristics. These characteristics
include LVH distribution \textsuperscript{33,42}, sudden death risk\textsuperscript{12,31,43,44} and atrial myopathy. \textsuperscript{29} The non-sarcomeric input variables that are responsible for variable penetrance and phenotypic heterogeneity are not defined but must be reasonably (genetically) polymorphic to account for the dramatic disease heterogeneity encountered in individuals with identical mutations. A better understanding of the cardiomyocyte’s hypertrophic circuitry and of the genetic polymorphisms in the components of this circuitry is important for a more complete mechanistic understanding of HCM’s aetiology. The hypertrophic circuit may also provide insights into other myopathic processes such as due to hypertensive heart disease, LV remodelling following myocardial infarction and heart failure (Figure 3).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{hypertrophic_circuit.png}
\caption{The Hypertrophic Circuit of a Cardiomyocyte.}
\end{figure}

The concept of a cellular circuit effecting altered transcription leading to cardiac hypertrophy may help in the understanding of why similar phenotypes may result from different hypertrophic stimuli, and why the same stimulus can lead to different phenotypes. The final phenotype depends on the nature(s) of the hypertrophic stimulus, as well as the component circuitry – which will have genetically determined differences between individuals.

2.2.1.6 Clinical Classification

A pragmatic clinical approach, based purely on the indications for available interventional strategies, is concerned with the division of HCM into symptomatic and asymptomatic disease, into obstructive and non-obstructive HCM, and into categories of estimated risk of sudden death from arrhythmia or stroke. In these schemes, morphologic features (for example the LVH magnitude, left atrial involvement, LV dilatation), clinical variables (prior clinical events, family history and results of routine cardiac testing such as
exercise testing), and occasionally the precise genetic cause (for example the detection of a 'malignant' mutation) are helpful in predicting clinical outcome. The approach is based on the few clinical interventions currently available (Figure 4). The scheme is critically dependent on the accuracy of the HCM diagnosis; does a hypertensive patient with asymmetric or symmetric LVH require a defibrillator if he/she has risk factors?

![Figure 4: Management of Symptoms and Risks of Sudden Death in HCM.](image)

This decision making therapeutic cascade considers prognostic and symptomatic indications for intervention. At present, the presence of LVOTO is only considered important in symptomatic HCM. *LVOTO is an independent risk factor for poor prognosis, and future indications for either ICD therapy or ASA might consider one or both of these interventions in asymptomatic individuals with obstruction.

ABPR = Abnormal Blood Pressure Response to exercise; LVH = LV Hypertrophy; NSVT = Non Sustained Ventricular Tachycardia; LVOTO = LV outflow tract obstruction. Adapted from Mohiddin and Knight. Alcohol Septal Ablation.
2.3 Genetics

2.3.1 Genetic Basis of the Familial Hypertrophic Cardiomyopathies

Since the earliest description of HCM, its genetic basis has been appreciated. In common with many other hereditary diseases, research in the last 2 decades has brought significant advances in the understanding of its molecular basis, and has revealed an unexpected degree of heterogeneity.

Most cases of HCM are inherited as an autosomal dominant disease. Hence, half of the parents, siblings, and children are at risk of inheriting the disease mutation. However, fewer than half actually develop HCM, as the disease penetrance (number of subjects with HCM / number of subjects with disease mutation x 100) is often much lower than 100%. The ability of HCM to 'skip' one or more generations in some families complicates most stepwise strategies when screening for familial disease. Truly sporadic HCM, proven by the demonstration of the absence of an identified disease mutation in both parents, is uncommon.

Most of the molecular defects identified to date are missense mutations (single base-pair substitution) changing the identity of an evolutionarily-conserved amino acid in one of several genes that encode contractile proteins of the sarcomere (Table 1). In as many as 10% of probands, HCM is associated with double (homozygous or double heterozygous) disease causing mutations. To date, studies comprehensively sequencing all sarcomeric genes in HCM cohorts detect mutations in only about half. It is likely that non-sarcomeric genes account for a considerable fraction of all HCM cohorts; these are now being detected. LVH is also a feature of several multi-system hereditary conditions diseases (Table 1).
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<td>&gt;15</td>
<td>Expanded trinucleotide repeat and missense mutations</td>
</tr>
<tr>
<td>Leopard Syndrome (PTPN11)</td>
<td>2</td>
<td>Biventricular outflow obstruction</td>
</tr>
<tr>
<td>Mitochondrial DNA deletions</td>
<td>see table 2</td>
<td>LVH, LV systolic dysfunction,</td>
</tr>
<tr>
<td>Alpha-B Crystallin myopathy (CRYAB)</td>
<td>1</td>
<td>Skeletal myopathy, lens opacities</td>
</tr>
</tbody>
</table>

Table 1. Genetic Heterogeneity In HCM.

Adapted from Fananapazir and Mohiddin in Hypertrophic Cardiomyopathy, Conn’s Current Therapy, 2005, LVH, LV hypertrophy; A-V, atrio-ventricular; WPW, Wolff-Parkinson-White syndrome

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2.3.1.1 Sarcomeric Hypertrophic Cardiomyopathy

Up to half HCM cases result from the dominant-negative effects of a mutant protein component of the sarcomere, the highly organized contractile unit of cardiac and skeletal muscle. At least nine different genes are implicated, including those encoding beta myosin heavy chain, cardiac actin, myosin binding protein C, alpha tropomyosin, the troponins, titin and the light chains of myosin. Most are missense mutations, permitting the synthesis of a full-size sarcomeric protein with a substitute amino acid at one or another position.

These were the first detected causes, and HCM has been described as a disease of the sarcomere. One conventional hypothesis is that LVH develops to compensate for the abnormal sarcomeric contractility that results from the incorporation of ‘poison peptides’ into the contractile assembly. Thus, increasing contractile unit number, and reducing cavity sizes and wall tension accommodate impaired contractility (Figure 5). At present, more than 400 mutations in these nine genes have been causally associated with HCM. (http://genetics.med.harvard.edu/seidman/cg3/index.html)

Figure 5. Compensatory Theory of Hypertrophy.

More weak rowers needed; myocytes incorporating mutant gene product may need to be larger with more numerous ‘weaker’ sarcomeres to compensate for their relative contractile impairment. (Larson, Farside)
A myocyte is composed of sarcomeres arranged in series and in parallel. In turn, each sarcomere, the basic force-generating element of muscle, consists of a thick filament arranged in parallel to a thin filament. The thin filament is anchored to a dense transverse band known as the Z-line. Interaction between the filaments results in their relative motion, shortening the sarcomere and creating a contractile force that acts at the Z-line to cause contraction of the entire muscle cell. In molecular terms, the thick filament is composed principally of the polymerised tails of numerous beta-myosin heavy chains, which are intertwined in pairs, while the thin filament consists chiefly of polymerised actin, which forms from actin monomers in a fashion resembling two stacks of coins gradually twisting around each other.

Interaction between myosin and actin is the molecular basis of contraction. Each beta-myosin has a head hinged to its long tail; in pairs, the heads extend away from the heavy chain, oriented toward the thin filament, and are therefore referred to as cross-bridges. Each head can bind actin and the intracellular fuel ATP; the head also comprises two other proteins, the essential and regulatory light chains of myosin that are thought to provide a modulating influence on head function. The myosin-binding site on actin is in the groove between the “stacks of coins.” In the resting state, the site is occupied by alpha-tropomyosin, which inhibits actin-myosin interaction. Three other proteins, troponins C, I, and T, are also components of the thin filament. The muscle cell's electrical depolarisation results in calcium influx. Binding of calcium to troponin C then induces a conformational change in alpha-tropomyosin, exposing the myosin-binding site on filamentous actin.

The beta-myosin head can now bind to actin; the head also hydrolyses its bound ATP, which dissociates, causing a conformational change in the head—a change that exerts directional force on the thin filament. On binding the next ATP, the head dissociates and beta-myosin returns to its former conformation. If ATP and calcium remain available, the process can continue, with progressive translocation of the thin filament relative to the thick filament.

It seems likely that in one way or other, all sarcomeric gene mutations associated with HCM impair the function of the sarcomere as a molecular motor. This view is supported by several in vitro assays of mutant protein or myocyte function. For example, in the translocation test, myosin is bound by its tail to a glass slide, leaving the head free to interact with
filamentous actin. For several different sarcomeric mutations, translocation velocity is altered. In some cases, such as the alpha-tropomyosin mutation Val95Ala, the velocity is depressed, but for two essential-light-chain mutations, the velocity is increased. This type of evidence, now obtained for several mutations in several genes, provides evidence that different mutations have distinctive functional consequences. The infidelity between precise molecular defect and final phenotype becomes all the more perplexing.

Several cardiac sarcomeric genes—beta-myosin heavy chain, the essential and regulatory light chains, and alpha-tropomyosin—are also expressed in skeletal muscle. In some beta-myosin mutations, a skeletal myopathy develops that resembles central core disease, in which mitochondria are absent from the core of many slow muscle fibres. Light-chain mutations have been associated with a skeletal myopathy involving ragged red fibres similar to those seen in mitochondrial myopathies.

2.3.1.2 Non-Sarcomeric Hypertrophic Cardiomyopathy

As many as half the HCM patients do not have a sarcomeric mutation despite most having a cardiac phenotype indistinguishable from that in patients with a sarcomeric mutation. Similarly indistinguishable from HCM can be the LVH in certain multisystem hereditary disorders; for example in Leopard syndrome, Friedreich's ataxia and in Neuroacanthocytosis. Transgenic studies also indicate that non-sarcomeric molecular lesions can cause cardiac hypertrophy.

Mutations affecting energy production in the myocyte are an increasingly recognized aetiology of HCM. Mutations in PRKAG2, a gene that encodes a regulatory subunit of cyclic AMP dependent kinase (AMPK), are associated with a cardiomyopathy syndrome characterized by HCM that progresses to LV dysfunction, atrio-ventricular bypass tracts, atrial fibrillation, and bradycardia from heart block and sinus node disease. AMPK is activated by increases in AMP relative to ATP and is thought to act as an energy charge sensor. Activated AMPK increases ATP production, inhibits non-essential cellular processes, and alters gene expression.

Mutations in nuclear and mitochondrial genes encoding mitochondrial proteins have also been associated with HCM (table 1). Expanded trinucleotide repeats and missense (mainly null) mutations in the FRDA gene encoding fraxatin, a nuclear encoded mitochondrial protein,
results in Friedreich’s ataxia. Up to 75% of patients have LVH similar to that in HCM. The absence of frataxin may lead to abnormal iron metabolism and oxidative damage to mitochondria. Mutations affecting nuclear and mitochondrion-encoded components of the electron transport system and beta-oxidation have been associated with a variety of cardiomyopathic states (Table 2).

Myocellular Ca2+ concentrations are tightly regulated. Increases in intracellular Ca2+ initiate sarcomeric contraction, and its rapid removal leads to relaxation. Ca2+ dependent signalling in myocytes also mediates the hypertrophic gene response to a variety of hypertrophic stimuli. To date, no abnormalities in Ca2+ regulatory genes have been reported as certain causes of human HCM. However, a multitude of transgenic models have shown that abnormalities in several of these genes are sufficient to produce hypertrophic cardiomyopathy.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene Affected/Gene Product</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friedreich’s ataxia</td>
<td>FRDA/frataxin-nuclear encoded mitochondrial protein</td>
<td>LVH, LVD, HB</td>
</tr>
<tr>
<td>Kearns-Sayre</td>
<td>mtDNA deletions/mitochondrial proteins</td>
<td>LVH, LVD, HB</td>
</tr>
<tr>
<td>MELAS</td>
<td>mtDNA deletions/mitochondrial proteins</td>
<td>LVH, LVD, HB</td>
</tr>
<tr>
<td>MERFF</td>
<td>mtDNA deletions/mitochondrial proteins</td>
<td>LVH, LVD, HB</td>
</tr>
<tr>
<td>Myotonic dystrophy-1</td>
<td>DMPK/Myotonic dystrophy protein kinase</td>
<td>HB, VT</td>
</tr>
<tr>
<td>Myotonic dystrophy-2</td>
<td>ZNF9/Zinc finger protein</td>
<td>HB, VT</td>
</tr>
<tr>
<td>Duchenne muscular dystrophy</td>
<td>DMD/dystrophin – cytoskeletal protein</td>
<td>LVD</td>
</tr>
<tr>
<td>Becker muscular dystrophy</td>
<td>DMD/dystrophin (allelic with Duchenne)</td>
<td>LVD*</td>
</tr>
<tr>
<td>Limb-girdle muscular dystrophy**</td>
<td>Several – at least 15 different AD and AR variants</td>
<td>HB, LVD</td>
</tr>
<tr>
<td>Emery Dreifuss - X linked</td>
<td>STA/Emerin- nuclear membrane protein</td>
<td>HB, LVD</td>
</tr>
<tr>
<td>Emery Dreifuss - AD &amp; AR</td>
<td>LMNA***/Lamins A and C- nuclear membrane proteins</td>
<td>HB, LVD</td>
</tr>
<tr>
<td>Desmin related myopathies</td>
<td>Several include desmin, alpha-B crystallin</td>
<td>LVH, LVD, HB, RCM</td>
</tr>
</tbody>
</table>

Table 2: Cardiac Involvement in Selected Hereditary Neurological Syndromes

* less severe than Duchenne muscular dystrophy; ** Some variants, such as those associated with mutations in the sarcoglycan, are associated with LV dilatation and dysfunction; *** LNMA mutations also associated with limb-girdle muscular dystrophy type 1B, Charcot Marie Tooth disease type 2B-1, and can also result in a dilated cardiomyopathy, often with conduction abnormalities in the absence of skeletal muscle weakness. MtDNA – mitochondrial DNA; LVH- LV hypertrophy; LVD-LV dilatation and systolic dysfunction; HB-heart block; RCM-restrictive cardiomyopathy; AD-autosomal dominant; AR-autosomal recessive.

Adapted from Mohiddin and Fananapazir, in Neuroacanthocytosis Syndromes.
2.3.2 Genetic Basis of the Dilated Cardiomyopathies

Dilated cardiomyopathy (DCM) is characterized by left ventricular dilatation and systolic dysfunction, and patients are at risk of death from congestive cardiac failure, cardiac arrhythmias and thromboembolic complications. Pathologic changes include myocyte atrophy and death and myocardial fibrosis.

In most cases, the cause of DCM is never determined. It has been estimated that DCM is familial (FDC) in approximately one-third of cases and transmission may be autosomal dominant or recessive, or X-linked.\textsuperscript{73-75} DCM may be an important and life threatening complication of several neuromuscular (and metabolic) diseases such as the muscular dystrophies, mitochondrial syndromes and myotonic dystrophy (table 2).\textsuperscript{67,75}

In addition to the relatively low likelihood of a case of DCM being genetically determined, screening strategies are also complicated by a more variable age-related penetrance than is the case for HCM, and because inheritance patterns other than autosomal dominant are more likely. However, it is important to consider FDC in all patients with DCM unless there is strong evidence that an alternative cause is responsible. Early, preferably pre-clinical diagnosis of family members at risk may allow prognostically beneficial therapy proven to prevent progressive systolic impairment and death from heart failure.

Products of genes associated with FDC include those localized to the cytoskeletal, nuclear envelope, sarcomere and mitochondrion; still others are implicated by transgenic studies. It has been suggested that FDC results from abnormalities in propagation of contractile force generated by the sarcomere to the extracellular matrix.\textsuperscript{41} Non-familial DCM, such as resulting from ischaemic heart disease, viral myocarditis, alcohol abuse, vitamin deficiency, chemotherapy and haemacromatosis, are characterized by diffuse myocardial damage and cell death.\textsuperscript{77-80} In FDC, it is likely that cell damage and death result from a variety of insults such as from sarcomeric contraction improperly constrained by the cytoskeleton and cellular ‘ischaemia’ resulting from energy deficit.

2.4 Clinical Features of Hypertrophic Cardiomyopathy

HCM is occasionally detected \textit{in-utero} or in infancy. However, the disease usually develops during the period of rapid body growth in puberty. Significant increases in LV wall thickness
after the age of 18 years are unusual. Occasionally, HCM is diagnosed for the first time in the elderly, although in most cases it is thought that the LV hypertrophy has been present for decades. Late-onset HCM, proven with serial cardiac imaging is unusual; late diagnosis or late-onset of symptoms are not synonymous with true late-onset cardiac hypertrophy. Age-related penetrance defines most stepwise or cascade family screening strategies.

2.4.1 CLINICAL ASSESSMENT

History: Some patients with severe LV hypertrophy may be asymptomatic, but complaints of chest discomfort, dyspnoea, palpitations, fatigue, presyncope, and syncope are common. Symptoms are frequently induced by postural changes or by exertion, dehydration, arrhythmia, after large meals and vasodilating drugs. The construction of a family pedigree and identification of any family members with cardiovascular histories (including sudden death and stroke) is useful.

Clinical Examination: Examination may be normal, and must not be solely relied upon when screening at-risk family members. Patients with obstructive HCM may have a bifid arterial pulse and there is often a prominent LV apical impulse. Auscultation may reveal an added third or fourth heart sound, and in patients with obstruction, a harsh systolic murmur at mid-sternum radiates to the axilla, increases in intensity on assuming an upright posture and becomes fainter with squatting. In a few, there may be signs right heart failure. Electrocardiography (ECG) and echocardiography (echo) are the principal diagnostic tools.

Electrocardiography: The 12-lead electrocardiogram is highly variable and may be normal. The most common finding is voltage criteria for LV hypertrophy with ‘strain’ pattern and changes frequently precede echocardiographically detectable LV hypertrophy.

Cardiac Imaging: Two-dimensional echo is the primary method for diagnosing HCM and the usual diagnostic criteria require the demonstration of LV wall thickness of greater than 13 mm (usually asymmetric hypertrophy of the septum) in the absence of another cause for cardiac hypertrophy. In athletes or patients with hypertension, wall thickness should exceed 15 mm. Echocardiography also; establishes the severity and distribution of left and right ventricular hypertrophy; demonstrates the presence or absence of LV obstruction and its site and severity (LV outflow tract or intra-LV cavity), and the presence or absence of mitral valve regurgitation
and aberrant papillary muscles; provides estimates of LV systolic function, pulmonary arterial pressure, and the severity of left atrial enlargement.

In addition to providing a more accurate definition of regional cardiac wall thickness, LV mass and systolic function, cardiac magnetic resonance imaging (CMR) allows the detection of regional myocardial scarring through imaging of delayed enhancement (DGE) following intravenous infusion of the contrast agent gadolinium. DGE may prove an important prognostic feature of HCM.

**Exercise Testing:** With adequate precautions treadmill exercise testing is safe and objectively assesses exercise tolerance. Exercise-induced arrhythmia and a hypotensive blood pressure response may identify patients at increased risk for sudden death. The use of metabolic exercise testing is largely confined to research indications and will often demonstrate markedly impaired exercise capacity even in individuals that report few or no symptoms.

**Ambulatory electrocardiographic monitoring:** Holter monitoring detects non-sustained ventricular tachycardia in approximately one quarter of HCM patients or even more in some cohorts. NSVT is recognized as a principal risk factor for sudden death; in common with all other risk factors used in primary prevention of sudden death algorithms, NSVT has poor positive predictive accuracy. Holter monitoring may also detect other important arrhythmias, notably atrial fibrillation (AF). Most expert opinions on HCM will consider warfarin anticoagulation after a single episode of paroxysmal AF is detected.

**Myocardial perfusion imaging:** Stress induced myocardial perfusion defects, typically with myocardial scintigraphy or cardiac MRI are present in up to two thirds of patients. Assays of coronary sinus lactate concentrations during rapid pacing have demonstrated that perfusion defects truly represent myocardial ischaemia. Perfusion defects may be subendocardial and circumferential, resulting in an apparent LV cavity dilatation during stress myocardial scintigraphy. Fixed defects at scintigraphy probably represent large areas of myocardial scarring. CMR first-pass perfusion imaging (gadolinium) combined with high spatial resolution cardiac imaging and tissue characterization is preferred when available. The clinical and research utility of CMR perfusion imaging in HCM has yet to be fully evaluated.
Cardiac catheterisation: Right and left heart catheterisation provides measures of cardiac output, ventricular filling pressures and LV pressure gradients under basal conditions and following provocation. These hemodynamic indices are valuable for guiding symptomatic therapy and for assessing the efficacy of therapeutic interventions and research outcomes. Coronary artery angiography excludes congenital and acquired coronary disease and assesses the suitability of septal arteries for alcohol septal ablation (see management).

Electrophysiological studies: Standard electrophysiology (EP) protocols often provide evidence of sinus node, atrioventricular node, and His-Purkinje disease, and may induce atrial or ventricular arrhythmias. EP abnormalities are more frequently demonstrated in sudden death (SD) survivors, but the validity of EP in SD primary prevention in adult patients is unproven; few centres now perform EP studies routinely. EP provoked ventricular arrhythmias may play a small role in assessing children at high risk of SD.

Genetic testing: An early hope was that mutation-specific natural histories would provide an ability to predict clinical events. This has proven to be the case for only a handful of specific mutations, with most capable of causing any of the HCM phenotypes. Additionally, genetic testing remains time and resource expensive, and will identify mutations in only 50-60% of patients. Nonetheless, the clinical utility of genetic diagnoses lies in the ability to make a preclinical diagnosis. Identifying children at risk of developing disease – and those not – allows lifestyle and professional choices to be made early, may assist SD prevention and focuses clinical resource

2.4.2 RISKS OF SUDDEN DEATH, STROKE AND HEART FAILURE

Several studies have identified that HCM is the commonest cause of SD in the young, usually defined as death occurring within 24 hours of the onset of symptoms in individuals under the age of 45 years. Estimated risks of SD have varied widely, a phenomenon at least partly due to the provenance of the cohort of patients studied. The annual mortality in tertiary centre cohorts is greater than that in studies of ‘community based’ patients. An annual risk for death in relatively unselected HCM patients is likely to be 0.5-1% per year, rising to 2% or even higher in certain groups, such as children with HCM. Few studies adequately differentiate cause of sudden death – these include SD assumed secondary to ventricular
arrhythmia, heart failure related death (with arrhythmia as a frequent terminal event), and stroke. In a retrospective analysis of data from a cohort of 368 HCM patients, a summation of risk factors identified patients at higher risks of SD. In this study the presence of one risk factor was associated with an annual death risk of 1.2%, two with 3%, and three with 10.7%.98

This and other studies do not specifically address stroke and congestive heart failure related causes of death. Similarly, there are no trials examining efficacy of intervention intended to improve prognosis (implantable cardioverter-defibrillators (ICD), anticoagulation or heart failure medication). In current risk assessment strategies, the risk factors are added; however, the importance of each risk factor cannot be equal. Certainly, each on its own has a low positive predictive value. Their relative importance, non-binary status and any important synergistic interactions remain unexamined. For example a patient with recurrent syncope and a family history of multiple sudden deaths would receive the same risk estimate as a patient with an abnormal exercise blood pressure response and a single episode of syncope. The sizeable fraction of patients in the retrospective ICD cohort studies (discussed below) with only one risk factor yet who receive appropriate shocks attests to the importance of the clinical experience. Additionally, more recently identified risk factors (Table 3) will need to be incorporated into prognostic assessments. Several difficulties are evident; (i) their independence from and interaction with other RFs are not known, (ii) their relative contributions are to arrhythmia, stroke or heart failure death risk are unknown, (iii) the risk reduction benefit from specific interventions, for example treatment of LV outflow obstruction or avoidance of physical exertion, is also unknown.

Finally, the patient’s age has considerable bearing on risk assessment. Sudden death is commonest in the second and third decade of life, and uncommon prior to age 14 years. Children and young adults with HCM are thought to be at the highest risks of SD, but the risk factors that predict SD in the young are likely to be different than those that predict death in older adults.92 Younger adults with risk factors have more cumulative risk than older adults with the same risk profile; additionally older adults have survived despite the estimated risk. Unpublished data from children aged <20 suggests that many of the risk factors used for adults will not identify high risk children.93 Instead, of a number of candidate risk factors, only septal wall thickness and electrophysiological studies were independent predictors of SD in
multivariate analysis. Children with inducible VT and ST ≥ 20 mm had a five-year SD rate of 25%; without inducible VT and ST ≥ 20 mm the five-year event rate was 8%. (Table 4).92

<table>
<thead>
<tr>
<th>Established Risk Factors</th>
<th>Candidate Risk Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac arrest †</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>Sustained VT †</td>
<td>Left atrial enlargement</td>
</tr>
<tr>
<td>Non-sustained VT *</td>
<td>Myocardial ischaemia</td>
</tr>
<tr>
<td>Family history of premature SD*</td>
<td>DGE imaging</td>
</tr>
<tr>
<td>Unexplained syncope*</td>
<td>'Malignant' mutation</td>
</tr>
<tr>
<td>LV wall thickness ≥30mm*</td>
<td>Environmental**</td>
</tr>
<tr>
<td>Abnormal exercise BP*</td>
<td></td>
</tr>
<tr>
<td>LVOTO</td>
<td></td>
</tr>
<tr>
<td>LV dilation</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Conventional and Candidate Risk Factors for Predicting Premature Death in HCM.

† ICD indicated for secondary prevention; * Risk factors used in summation to risk stratify for the purposes of primary prevention ICD implantation.96 Environmental refers to common situations that may increase risk, for example hypertension, competitive sports, recreational drug taking. VT, ventricular tachycardia; DGE, delayed gadolinium enhancement; LVOTO, LV outflow obstruction. Modified from Maron et al.1

<table>
<thead>
<tr>
<th></th>
<th>Septal thickness &lt; 20mm</th>
<th>Septal thickness ≥ 20mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS negative</td>
<td>0% (0%, 0%), N=45</td>
<td>8% (0%, 17%), N=50</td>
</tr>
<tr>
<td>EPS positive</td>
<td>0% (0%, 0%), N=7</td>
<td>25% (5%, 41%), N=26</td>
</tr>
</tbody>
</table>

Table 4: Predicting SD events in HCM patients less than 20 years old.
The table shows 5 year probabilities (95% confidence interval)[N= number of patients in each stratum].92

2.4.2.1 Ventricular Arrhythmia

A consensus document prepared for the AHA, ACC and ESC1 unequivocally recommends ICD implantation for secondary prevention of arrhythmic SD – i.e. after failed SD, syncope or haemodynamic compromise with documented spontaneous ventricular arrhythmia. ICD implantation is considered a class 2a indication (level of evidence C) in HCM patients with one or more ‘major’ risk factor for SD. In other words, the guidance states that while many experts would favour ICD implantation at this level of perceived risk, some would not (see table for interpretation of guidance). In large part, this is because there are no prospective studies examining either accuracy of risk prediction or success of intervention. A single risk factor
probably confers approximately 1% annual risk of death (inclusive of arrhythmic, stroke, heart failure). The low event rate, ICD implantation costs/morbidity and the absence of trial evidence have led most centres to consider a threshold of 2 risk-factors more reasonable.

Strategies for the prevention of arrhythmic SD have not been tested prospectively. Retrospective studies describe a high intervention rate in patients receiving ICDs for primary prevention.\textsuperscript{103-105} These studies also detail a relatively high rate of ICD complications, in part reflecting the challenges of ICD therapy in active young individuals (about one quarter receive inappropriate shocks). Additionally, the risk factor profiles of those patients receiving appropriate ICD discharges were no different from those not receiving shocks. Furthermore, in one study, one third of the patients shocked appropriately had only a single risk factor.\textsuperscript{103} Our ability to risk stratify remains severely limited.

2.4.2.2 Thrombo-Embolic Events
Stroke risks in HCM increase with advancing age.\textsuperscript{106} In large part, this is thought to reflect an increased frequency of atrial fibrillation occurring as part of a progressive left atrial myopathy (increased LA size, decreased LA systolic function). Approximately 2% of HCM patients per year develop AF, and they have sizeable stroke risks (OR of 17.7 compared with HCM without AF).\textsuperscript{107}

Significant left atrial enlargement (>50 mm) developing without evidence of AF is considered a sufficient indication for anticoagulation in some centres. The rationale for this is that the atrial myopathy often includes severe impairment of LA contractile and capacitance function which may predispose to stroke, as well as the high likelihood that undetected PAF is present (or will shortly develop) in such patients.

2.4.2.3 Systolic Left Ventricular Failure
While symptoms associated with congestive heart failure are common and multifactorial in HCM, the development of progressive LV systolic impairment and LV dilatation is rather less common. Estimates are that approximately 10% of patients enter a well defined burnt-out phase of the disease, sometime termed end-stage (ES) disease.\textsuperscript{20-22} Many more patients develop more subtle degrees of systolic impairment; after all, the basic molecular fault in most cases is an abnormal component of the contractile apparatus.
Outpatient review with regular cardiac imaging is indicated; patients with ES disease have increase risks of adverse events, including mortality/transplant rates of approximately 10% per annum.\textsuperscript{21,22} No reliable method of predicting those patients at greatest risk of developing ES disease has been developed. It has been suggested that left atrial dilatation may be an early sign of the development of end-stage (ES) disease.\textsuperscript{108} Patients with ES disease invariably have myocardial scarring detectable at DGE imaging. Even though DGE imaging is positive in two thirds of all HCM patients, serial CMR imaging with detailed assessment of LV volumes and quantification of DGE has a potential role in the detection of early ES disease.\textsuperscript{109,110}

Patients with dilating phase HCM are usually considered suitable for consideration of all the prognostic pharmacological and device modalities indicated for systolic heart failure. The efficacy of any of these interventions in this group of patients has not been demonstrated, but is assumed to be similar to that in the cohorts examined in the large heart failure trials.

2.4.3 Obstruction and Mitral Regurgitation

In one quarter of HCM patients, almost exclusively where the LVH has an ASH distribution, mitral-septal contact in systole can result in a dynamic LV outflow tract obstruction (LVOTO). This systolic anterior motion of the mitral valve (SAM) also interrupts leaflet coaptation producing a variable degree of mitral regurgitation (MR). Echocardiography with Doppler best demonstrates and quantifies all these abnormalities. Obstructive HCM is often associated with symptoms, particularly on effort. LVOTO is also associated with a poorer prognosis.\textsuperscript{111-113}

In symptomatic individuals, pharmacological treatment with beta blockade, verapamil or disopyramide (alone or in combination) may reduce measured outflow gradients. Invasive reduction of LVOTO is indicated if symptoms in patients with LVOTO resulting from SAM persist despite maximum pharmacological treatment (see figure 4 in section 1.2). The premise of all LVOTO therapies is that outflow obstruction and MR contribute to symptoms; the relatively poor correlation between both LVOTO magnitude and MR with symptom status probably reflects the additional and variable importance of myocardial ischaemia, impaired LV filling, small stroke volumes and arrhythmias.
The determinants of SAM are uncertain; is there a 'pull' or 'push' force on the mitral apparatus? The conventional understanding is that the anterior leaflet is pulled towards the LVOT as the result of the Venturi effect produced as systolic flow accelerates across the convexity of the ventricular surface of the anterior mitral leaflet. A negative pressure pulls the anterior leaflet away from the posterior leaflet and towards the septum. Alternatively, as the result of distorted LV geometry, the systolic effort pushes the mitral leaflets towards the LVOT. The uncertainty regarding mechanisms resulting in SAM contributes to the vigorous debate concerning the preferred invasive treatment modality. Following the initial description of alcohol septal ablation (ASA) in 1995, it has gained rapid acceptance and is now the most commonly chosen treatment option for LVOTO reduction. More ASA procedures than operations have been performed despite the passage of more than 40 years since surgical LV septal myectomy (LVM) was developed.

The debate concerning the relative merits of ASA and LVM is not informed by any randomised studies. The only randomised trials addressing drug-refractory symptomatic LVOTO treatment options examine dual chamber (DDD) pacing. DDD, ASA and LVMM, have all been subject to the contrary opinions of enthusiasts and detractors. A fourth option, mitral valve replacement, is considered only in the presence of other indications for mitral valve replacement.

2.4.3.1 Surgical Myectomy

In the 1960's, a surgical approach to resect the sub-aortic ventricular septum through the retracted aortic valve at aortotomy was developed. The Morrow procedure or LVM is still considered the 'Gold Standard' treatment by several influential opinions. Modern surgical results, as reported by the few centres with significant experience, are impressive, with low operative mortality, substantial symptom improvement, increased exercise tolerance, and perhaps an improved prognosis. While few doubt the potential benefits of LVM, its designation as 'Gold Standard' is not supported by randomised trial data or contemporary real-world practice. Additionally, published results are from the few centres with considerable experience where complication rates are low. These include aortic incompetence, heart block, ventricular septal defects, and all the complications of open-heart surgery. Surgical techniques are undoubtedly superior when more complex anatomic problems are to be addressed, for
example if the subvalvar apparatus is abnormal or there is co-existing mid-cavity obstruction.\textsuperscript{125,126}

2.4.3.2 Pacemaker Therapy

Pacing therapy (DDD) with right ventricular pre-excitation reduces LVOTO acutely and chronically.\textsuperscript{127,128} This effect is likely to be the result of (paradoxical) septal movement away from the direction of SAM, but reductions in cardiac output may also contribute. Apical RV lead placement and carefully chosen paced A-V intervals are important for successful therapy. Uniquely in HCM research, pacing has been examined in prospective placebo controlled randomised trials. M-PATHY was a cross-over study where 3 months of active pacing were compared with placebo pacing.\textsuperscript{129} No benefits of active pacing were demonstrated over an apparently large placebo effect.

The hemodynamic and symptomatic benefits of pacing are reported to be progressive throughout the first 6 months following implantation and persist after cessation of pacing.\textsuperscript{130} Potential bias from crossover treatment effects and type 2 error due to the abbreviated duration of therapy may have contributed to a failure to demonstrate treatment benefit and the large placebo effects. Significant hemodynamic and symptomatic improvement after DDD pacing were demonstrated in the larger randomised double-blinded PIC study.\textsuperscript{131}

Potential adverse effects on LV systolic and diastolic function of chronic RV pacing\textsuperscript{132,133}, equivocal trial data and the development of ASA have resulted in the relegation of pacing to a poor third choice. Nonetheless, pacemaker has low complication rates compared with ASA and LVMM. The rate of ICD implantation in HCM patients is increasing and almost all are dual chamber systems to minimize inappropriate shocks. A therapeutic trial of pacing therapy in ICD patients with symptomatic LVOTO may prove reasonable before ASA or LVM. Furthermore, pacing-ICD therapy will increase the safety of both ASA and LVM.

2.4.3.3 Non-surgical Septal Reduction

ASA's rapid acceptance has undoubtedly been influenced by the convictions of proponents and a greater willingness of patients for less invasive procedures. In the non-randomised and uncontrolled studies now available, ASA substantially reduces LVOT gradient, improves
symptoms, increases exercise tolerance and leads to desirable changes in cardiac anatomy and physiology. These include reduced LV filling pressure, abolition or attenuation of MR, smaller LA size, and reduction in LVH. Early concerns regarding arrhythmogenicity of the ‘infarct’ scar are not supported by data, but the potential for long-term complications cannot be dismissed. The major complication in contemporary ASA cohorts remains complete heart block, and permanent pacing is required in approximately 10% of treated patients. However, it should be noted that despite ASA’s comparative youth, the number of patient treatment years must now exceed that of LVM. ASA has evolved such that its procedural mortality and efficacy are similar to that of LVM.

2.4.3.4 Additional Indications for Invasive Therapy

Proven LVOTO: LVOTO worsens if end diastolic LV volumes are reduced (postural change, dehydration, vasodilatation, post-prandially etc) or if inotropy is increased (after ventricular extrasystoles, exertion). A systolic murmur apparent on standing from a squat is strongly suggestive of provoked LVOTO, as are symptoms of postural presyncope and an intolerance of extrasystoles. Labile LVOTO may be missed if echocardiographic or invasive studies do not include techniques to provoke SAM. Most studies (LVM, DDD and ASA) include patients with provokable LVOTO, and symptomatic benefits are demonstrated.

The Valsalva manoeuvre and exercise can be used to demonstrate provokable LVOTO, though Valsalva detects fewer patients with provoked LVOTO than exercise will. Pharmacological agents include amyl nitrate, sublingual glycerin trinitrate and intravenous isoproterenol. Dobutamine is not recommended as it can provoke LVOTO in normal hearts. The Brokenborough effect following ventricular extrasystoles can demonstrate provokable LVOTO in the catheter laboratory, either through the irritant effects of manipulating a catheter in the ventricle, or by paced extrasystoles. This latter technique is particularly useful during ASA where temporary pacing can introduce identically timed extrasystoles every few cardiac cycles.

Mitral Valve Abnormalities and Mid-Cavity Obstruction: Benign structural abnormalities of the mitral valve are common in HCM. More important abnormalities include anomalous insertions of the papillary muscles/chordae onto the mitral leaflets or septum, and displacement of the papillary muscles. Surgery is strongly favoured if significant structural
mitral abnormalities co-exist with SAM, but the MR of uncomplicated SAM should be abolished or improve following successful ASA.

Pronounced papillary muscle hypertrophy may result in the uncommon and poorly understood mid-cavity (mid-ventricular) variant.\textsuperscript{140,141} Mid-cavity obstruction can co-exist with LVOTO, or can become apparent after successful LVOTO treatment.\textsuperscript{125} Potential adverse consequences include reduced contribution of the distal LV segment reduction to cardiac output, effects of elevated afterload on the distal LV segment (including metabolic consequences, reduction of myocardial perfusion pressures, aneurysm formation) and any (poorly defined) consequences of muscle contracting onto muscle.

Sustained monomorphic VT is uncommon in HCM, but when present is frequently associated with a distal LV aneurysm resulting from mid-cavity obstruction. (personal observation) The detection of monomorphic VT should prompt a search for apical abnormalities that may not be apparent on standard echo views (Figure 6).

\textbf{Figure 6. Echocardiographic and Angiographic Findings in Mid-Cavity Obstructive HCM.}

This patient, was referred for alcohol septal ablation. Doppler studies were interpreted as demonstrating an outflow gradient of approximately 70 mmHg (A). A more careful examination of Doppler signals reveals that the high velocity signal is in early diastole (arrow), and that the systolic outflow velocity is normal (double arrow). The location and timing in the cardiac cycle of this high velocity Doppler signal (arrow) are demonstrated in a 4-Chamber view (B) and
in colour M-Mode (C). The presence of an apical aneurysm, undetected by echocardiography, is demonstrated on the LV angiograms (D-G). In systolic frames (D and F), it can be seen that the apical and the proximal LV are disconnected by mid-ventricular obliteration. In early diastole (E and G) and with relief of the mid-ventricular obstruction, the high pressure apical cavity empties into the relaxing proximal LV generating the high Doppler velocities. From Mohiddin and Knight, Alcohol Septal Ablation in the Oxford Textbook of Interventional Cardiology.45

Several groups report that pacing142, ASA143 and surgery 140 can reduce mid-cavity gradients. However, although mid-cavity disease is often associated with drug-refractory symptoms, it is not understood why symptoms develop or if gradient reduction improves these.

Asymptomatic LVOTO: A fundamental understanding in general cardiology is that mitral regurgitation and aortic stenosis are associated with increased risks of heart failure and sudden death, even in asymptomatic patients. Are LVOTO and MR in obstructive HCM independently associated with worse prognosis? Retrospective studies report that LVOTO is independently associated with greater risks of death.111-113 These reports include important differences concerning mode of death (heart-failure or arrhythmic), the importance of LVOTO as a binary or continuous risk factor, and the significance of symptom status.

Maron et al.111 analysed records from 1101 patients followed for a mean of 6.3 years. Patients with LVOTO (resting gradient>30 mmHg) had a relative risk (RR) of death of 1.6, but risk did not increase with increments of LVOTO above 30 mmHg. Death was most often related to heart failure or stroke rather than from arrhythmic SD. In half as many patients followed for 4.5 years, Autore et al report that LVOTO predicted cardiovascular death best in asymptomatic patients (RR 2.4). In patients with functional class III or IV symptoms, but not LVOTO were powerful predictor (RR 7.9).112 After a median follow up of 5.1 years, Elliott et al. also report reduced survival in patients with LVOTO.113 They detect an incremental risk, with an RR for all cause death and transplantation of 1.24 per 20 mmHg of LVOTO. A very low SD risk in the least symptomatic leads the authors to conclude that septal reduction therapy in the asymptomatic is not indicated for the prevention of SD.

At present, the desire to relieve symptoms is the only indication for invasive LVOTO reduction. Long-term consequences on cardiac structure and function of chronic LVOTO and MR may include progressive atrial enlargement and atrial fibrillation, pulmonary hypertension and LV systolic impairment. Furthermore, LVOTO reduction leads to modest LVH regression
remote from the ablated myocardium, suggesting that some of the LVH develops in response to afterload, in addition to the ‘primary’ hypertrophy. Future approaches to LVOTO may include prognostic indications and treatment protocols may resemble those for the management of asymptomatic and symptomatic structural valve disease.

Finally, it must be remembered that HCM is a complex syndrome where several mechanisms are responsible for symptoms. A patient with successful LVOTO reduction still has HCM, and remains subject to other contributors to symptom limitation, disease progression and SD risk.

2.4.4 Diastolic Function - Atrio-Ventricular Coupling

In HCM, clinical practice is largely based on a relatively limited understanding of the physiology of the disease process; in an almost complete absence of randomised trial data, dynamic outflow obstruction is often managed invasively, defibrillators are used when sudden death risk is deemed high, and symptoms of heart failure are treated with negative inotropes and diuretics. While there is an accumulation of clinical evidence that they are effective treatments, mechanisms underlying heart failure-related symptomatology in HCM are less well understood and symptomatic patients are difficult to treat.

Abnormalities of LV filling are thought to be a major cause of exercise intolerance in many patients, particularly those without LVOTO. Abnormalities affecting LV relaxation, LV compliance and atrial function all contribute to sub-optimal diastolic performance, and elevated LV filling pressures or limited preload reserve may result in exercise limitation. LV filling can be referred to as atrio-ventricular (A-V) coupling to emphasize the importance of both atrial and ventricular properties. Limitations in methods available for assessing these properties inhibit our ability to assess patients and to develop intervention strategies.

2.4.4.1 Symptomatic Non-Obstructive Hypertrophic Cardiomyopathy

The management approach to the symptomatic HCM patient with LVOTO assumes that obstruction makes a significant contribution to the development of symptoms. LVOTO reduction is often very successful in symptom improvement, but successful LVOTO treatment may still be associated with significant residual symptoms. For the symptomatic HCM patient without LVOTO, therapeutic options are few and often unsatisfactory.
Clinical investigations in the symptomatic non-obstructive patient often detect evidence of ischaemia, abnormal diastolic indices, systolic abnormalities, atrial enlargement, myocardial scarring at MRI and atrial/ventricular arrhythmias. No individual or profile of abnormality reliably distinguishes symptomatic from asymptomatic patients, or correlates sufficiently well with measured exercise limitation. However, abnormal LV filling is thought to make major contributions to heart failure symptoms and exercise intolerance in such patients. In the study of 'Diastolic Failure', HCM can be regarded as a 'super' model. LV filling is a complex and interactive process determined by preload, afterload, systolic events, and the passive and active properties of the cardiac chambers and vascular structures. Though it is easy to demonstrate abnormal LV filling in HCM cohorts, the specific determinants of the diastolic abnormalities and their clinical importance have been difficult to characterize. AS LV filling depends on several interacting cardiac properties, a comprehensive description of diastole, 'diastolic profiling', will include several parameters.

Definitions of Diastole: The definition and temporal limits of diastole can be described in cellular or hemodynamic terms. Following peak LV force generation during early systole, energy-dependent sequestration of calcium into the sarcoplasmic reticulum leads to rapid dissociation of actin-myosin fibril cross-links and the beginning of 'cellular' diastole. As a result, rapid LV pressure decline results in aortic valve closure; 'haemodynamic' diastole begins shortly after 'cellular' diastole. A period of isovolumic relaxation follows until atrial pressure exceeds LV pressure and the mitral valve opens. Early rapid filling occurs until atrio-ventricular pressure equilibration again develops (diastasis) and there is little or no flow across the valve. The passive capacitance and intra-cavitary pressure in the left atrium and pulmonary veins and as well as LV relaxation velocity, LV restoring forces (a suction effect as the LV 'springs' from end systolic toward equilibrium volume) and compliance all influence early LV filling.

If sinus rhythm is present, late diastolic atrial contraction causes a second LV filling phase, providing the final stretch to myocardial fibres and thereby increasing LV stroke volume via the Frank-Starling mechanism. This phase of LV filling is dependent on the interaction of atrial systolic force (itself dependent on atrial preload and atrial inotropy) and the LV's passive pressure-volume characteristics (compliance). Cellular diastole is terminated by energy dependent actin-myosin cross-linking and cycling after intracellular calcium is again increased
effecting excitation-contraction coupling. The mitral valve closes as LV pressure rises to exceed LA pressure and diastole, as defined haemodynamically, comes to an end.

**Preload Reserve:** Preload reserve refers to the heart’s capacity to increase cardiac output through increasing cardiac filling (Starling relationship). In the resting HCM heart, LV volumes are typically small, frequently with near obliteration of end systolic cavity volume suggesting further decreases in end-systolic volume can make only minimal contributions to an increased stroke volume. As such, increasing ventricular end diastolic volume and reliance on preload reserve is probably more important for increasing cardiac output than in a normal heart. Apparently ‘normal’ age-related decreases in LV compliance and atrial elastance are described in normal hearts\textsuperscript{152-159} and may have much greater consequences in an ageing HCM heart dependent for increased cardiac output on preload reserve. Several cellular, histological, and anatomic/chamber abnormalities have been well described and can, theoretically, impair atrio-ventricular coupling (Table 5).

<table>
<thead>
<tr>
<th>Functional Abnormality</th>
<th>Potential consequence for LV Filling</th>
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<tr>
<td>Myocellular</td>
<td>Sarcomeric impairment</td>
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<tr>
<td></td>
<td>Delayed calcium</td>
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<td></td>
<td>sequestration\textsuperscript{58,60-64}</td>
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<tr>
<td>Myocardial</td>
<td>Fibrosis\textsuperscript{65-67}</td>
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<td></td>
<td>Disarray\textsuperscript{168}</td>
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<td></td>
<td>Ischaemia\textsuperscript{69}</td>
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<tr>
<td>LV Architecture</td>
<td>Small volumes</td>
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<tr>
<td></td>
<td>Increased wall thickness\textsuperscript{69-70}</td>
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<tr>
<td>Left Atrial</td>
<td>Systolic impairment</td>
</tr>
<tr>
<td></td>
<td>Reduced compliance\textsuperscript{70-74}</td>
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<tr>
<td>Electrical</td>
<td>Conduction delay</td>
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<td></td>
<td>Sinus tachycardia</td>
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<td></td>
<td>Atrial Fibrillation</td>
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<td></td>
<td>LV and LA systolic impairment</td>
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<td></td>
<td>Prolonged myocyte relaxation</td>
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<td>Reduced compliance</td>
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<td>Dysynchronous contraction and relaxation</td>
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<td>Impaired contraction and relaxation</td>
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<td>Reduced end systolic size, reduced compliance</td>
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<td></td>
<td>reduced compliance</td>
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<td></td>
<td>smaller atrial kick'</td>
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<td></td>
<td>passive LV filling impaired, Elevated pulmonary venous pressures</td>
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<tr>
<td></td>
<td>Dysynchronous contraction and relaxation</td>
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<td></td>
<td>Abbreviated LV filling</td>
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<tr>
<td></td>
<td>Loss of atrial kick, abbreviated LV filling</td>
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</tbody>
</table>

Table 5: Contributions to Impaired Atrio-Ventricular Coupling and LV filling in HCM.

Adapted from Mohiddin and McKenna. In Diastolic Heart Failure.\textsuperscript{23}

**2.4.4.2 Clinical Significance of Diastolic Abnormalities in HCM**
Small LV volumes, abnormal relaxation kinetics, reduced chamber compliance, atrial systolic abnormalities and Myocellular dysfunction are features of HCM that may restrict preload reserve, resulting in elevated pulmonary venous pressures and/or inadequate cardiac output. Tolerance of exertion, particularly as diastole is abbreviated by increased heart rates, may be severely compromised by restrictions in LV filling.

In HCM patients, resting abnormalities in LV diastolic filling parameters were documented decades ago.166,175-192 Much of the earlier work, while documenting perturbations in LV relaxation and compensatory augmentation of atrial emptying failed to either analyse or to demonstrate a strong relationship (or any relationship) between selected parameters of LV filling and functional capacity or exercise performance. Most studies utilize echocardiographic, nuclear or catheter data to assess diastole. Several limitations are evident in these studies; (1) there is no Gold Standard measure that assesses all major components of LV filling in diastole; (2) the demonstration of elevated LV filling pressures does not necessarily identify the failing component(s) of diastole; (3) it is unclear which pressure measurement best represents LV filling (e.g. mean PAWP, mLAP, post-a/pre-a LVEDP); (4) many measurements are load (pre- and after-) load dependent (5) resting measurements of LV filling cannot assess non-constant (e.g. inotrope/load dependent LV relaxation and atrial contractility) and non linear (e.g. LV compliance) variables; (6) inclusion of obstructed and non-obstructed patients may confound the results as symptoms/exercise limitation in these patients may have different causes, and the presence of obstruction/mitral regurgitation may invalidate the diastolic assay under investigation; (7) in some of the studies, inclusion of a high percentage of asymptomatic patients limits the power of a study to associate the measured diastolic parameter with functional impairment.

2.4.4.2.1 Invasive Hemodynamic Studies

Chan et al compared PAWP, cardiac index (CI) and blood pressure (BP) after diuresis and infusion of fluid volume in 13 HCM patients.193 In the HCM patients and normal controls, all three indices were higher in the resting upright state after fluid infusion. At peak upright exercise testing, PAWP was also significantly higher in patients when fluid replete compared to the same patients after diuresis, but CI and BP were similar. Thus, as a higher PAWP in the volume replete was not associated with greater exercising CI, the authors concluded that
stroke volume was insensitive to preload. In this study, the mean PAWP at peak exercise was 25 mmHg, similar to that seen in exercising heart failure patients and compared to only approximately 10 mmHg in normal volunteers. LV volumes were not measured.

Frenneaux et al. and Lele et al. reported results of invasive (Swann-Ganz) exercise testing. Subjects were relatively young (mean 34 years) and mildly symptomatic or asymptomatic as most (87%) were in New York Heart Association (NYHA) functional class I or II, and several had LVOTO. Cardiac index increased by 340%, but did not correlate with PCWP. Patients did, however, demonstrate large changes in mean PCWP; 5±6 mmHg at rest increasing to 24±11 mmHg at peak exercise. An analysis of their data shows a poor relationship between exercise induced augmentation of stroke volume and increases in PAWP ($R^2 = 0.05$, my own calculations, from published data tables). Thus, PAWP increases to levels associated with pulmonary oedema and there is no simple relationship between PAWP and cardiac output. Similar findings are reported by Kitzman et al. in a mixed group of patients with LVH; stroke volume did not increase despite significant increases in preload – again, as measured by mean PAWP.

LVEDP is the appropriate measure on the pressure-volume plot that describes the Starling relationship. The apparent paradox whereby changes in stroke volume and filling pressures appear unrelated may be explained by poor validity of mean PAWP as surrogate measure for LVEDP; the correlation between mean PAWP and LVEDP is poorest in patients with LVH. Mean PAWP may better reflect pulmonary venous hydrostatic pressures and therefore assess exercise limitation by dyspnoea rather than by limited cardiac output. Simultaneous LV volume/pressure measurements and those of LA function would more completely assess LV filling than any single pressure measurement. As yet, no studies compare pressure/volume characteristics with symptom status or functional parameters.

2.4.4.2.2 Echocardiographic Studies

Echocardiography can estimate chamber volumes, relaxation properties, and more recently Doppler derived measurements are being used to estimate LV filling pressures.

Echocardiographically derived diastolic abnormalities are consistently demonstrated in HCM, but an association of these with symptoms or exercise performance has been less easily demonstrated. Using conventional resting transmural Doppler measurements (E/A
ratio), Maron et al. detected abnormal diastolic indices in most HCM patients, and noted that the severity of abnormality were increased in patients without LV obstruction. Nihoyannopoulos et al. found similar patterns of abnormality in resting transmitral flow characteristics, and showed a weak correlation with peak oxygen consumption (VO₂) during exercise testing. However, there were no differences in E/A Doppler ratios between symptomatic and asymptomatic patients. In this study, and in a further study from the same group that employed radionuclide angiography, evidence for LA systolic failure was also detected. In a study comparing exercise performance to transmitral Doppler measurements as well as pulmonary vein flow and LA fractional shortening, Briguori et al. found no evidence for a relationship between resting transmitral flow indices and VO₂. A reasonable correlation between LA fractional shortening and VO₂ was detected, suggesting atrial systolic function may be an important determinant of exercise capacity.

Difficulties in demonstrating robust associations with symptoms or exercise performance are not surprising given the now well-described phenomena of pseudo-normalization and load-dependence of the E/A ratio. These parameters perform particularly poorly at estimating LV filling in HCM. More recently developed echo measurements including pulmonary vein Doppler, mitral flow propagation velocity, tissue Doppler and a variety of derived ratios and intervals are more reliable at predicting LV filling pressures. A brief description of these newer measurements may help in the interpretation of the clinical studies that use them.

In HCM patients, Nagueh et al. compared a variety of Doppler parameters (including; E/A, the ratio transmitral E to early mitral annular velocity Ea (E:Ea); early mitral propagation flow velocity; pulmonary vein inflow characteristics, E and A acceleration/deceleration times) to a variety of LV diastolic pressure measurements (LV minimal pressure, pre-A LVEDP and post-A LVEDP). E:Ea and propagation velocity correlated well with pre-A LVEDP (r=0.76 and r=0.67 respectively). In a larger group of patients without HCM, they report that E:Ea and a different measure of LV filling pressure, the mean diastolic LV pressure (M-LVDP), are strongly correlated. However, the E:Ea ratios were in an indeterminate range in more than half the patients in whom predicted M-LVDP could not be assigned as normal or elevated. Correlations between E:Ea and LVEDP have subsequently been shown by others, but may be significantly better in patients with poor LV systolic function. In summary, while E:Ea may contribute to
diastolic assessment, the available studies use quite different measures of LV filling pressure (pre-A LVEDP, M-LVDP, LVEDP). The accuracy of filling pressures predicted by E:Ea may be altered by changes in preload and may be different in different patient populations.

In a study of 85 HCM patients, Matsumura et al. found a reasonable correlation between resting E:Ea ratio and VO₂, and that E:Ea values were highest in symptomatic patients. These findings have recently been validated in a group of patients with apical HCM. However, although the r-values were relatively modest (r=-0.42 and r=-0.47), the studies' findings are all the more surprising when considered in comparison to those earlier studies where filling pressures were measured invasively, but where no association was found with exercise performance.

2.4.4.3 Atrial Function

To date, little has been written about the role of atrial function on preload reserve and exercise tolerance. Briguori et al. reported an association between resting LA fractional shortening and exercise capacity. They suggest that LA fractional shortening reflects LVEDP and that elevated LVEDP determines exercise capacity. An alternative view is that LA systolic function contributes to LVEDP, preload reserve and exercise capacity. While Sachdev et al. found that that LA size predicted exercise capacity in patients with non-obstructive HCM, the same group were unable to demonstrate that LA active emptying fraction, ejection force, and kinetic energy (all measurements obtained at rest) predicted exercise capacity.

LA pressure-volume studies and assessments of LA function during stress are largely lacking, though the few studies available in HCM suggest decreased inotropic reserve and decreased atrial compliance accompany LA enlargement. The role that primary abnormalities of atrial inotropic/reservoir function may have in abnormal LV filling remain largely theoretical. The potential for enhanced atrial function to compensate for abnormal LV relaxation/compliance, and the characteristics of a secondary atrial myopathy that may develop in response to chronic and progressive LV disease also deserve further evaluation. As has been mentioned above, dilation may predict the onset of cardiac failure from ES disease. The contribution of LA dysfunction to the syndrome that is HCM is a neglected topic.

2.4.4.4 Measurement Tools and Prospective Intervention Strategies
Therapeutic options for the symptomatic HCM patient without LVOTO are limited. Renin-angiotensin-aldosterone blockers, calcium antagonists, and statins have been suggested as potential treatments. Each of these proposed therapies aims at a particular pathophysiological abnormality, for example at LV fibrosis/stiffness. The identification of appropriate patient groups and monitoring of therapy will require an ability to assess the components of LV filling. No readily available diagnostic techniques allow this, but echocardiographic approaches are likely to form the basis of any practical tool. The ideal tools will measure parameters that are load dependent in a linear or predictable way, and will incorporate a mechanism to vary load. Non-invasive and easily obtained parameters or clinical surrogates would greatly assist in the monitoring of patients and treatments.
3 GENETIC STUDIES

1. GENERAL METHODS

3.1.1.1 Collection of Proband DNA and Mutation Detection

Patients referred to the Inherited Heart Diseases section of the NHLBI were evaluated and recruited for protocols approved by the institute (98-H-0100, 99-H-0065 and 87-H-0057).

3.1.1.2 Methods

Patients: All HCM patients were considered for recruitment for genetic studies. Consecutive, unrelated proband patients with HCM diagnosed using standard clinical criteria received genetic counselling prior to consenting for this study. A 12-lead ECG and echo were recorded in all cases and a pedigree tree was obtained. Each Proband was assigned a unique identification comprising a composite of a unique family number and a number defining their position in the pedigree. All first degree relatives were invited for clinical screening. Following the identification of any novel genetics results, other family members might also be invited; alternatively, a field-trip to the family’s locale would be organised.

Field Trips: Patients evaluated at NHLBI were referred from all US states and from other countries. Field trips were confined to the continental US states and allowed the clinical evaluation of and DNA collection from family members at their relative convenience. A field trip usually comprised two physicians, two research nurses, a portable 12 lead electrocardiograph and echocardiogram, and equipment for collection of whole blood.

DNA Extraction: Genomic DNA extracted from whole blood as according to the manufacturer’s instructions (Gentra). After the determination of the concentration of extracted and hydrated DND (generally > 200 ng/microlitre), this was stored at 4 degrees centigrade.

Polymerase Chain Reaction (PCR): PCR formed the basis of all genetics experiments. Generally, 1 microlitre of DNA in a concentration of DNA of 50 ng/microlitre was used for all PCR reactions. PCR reactions were completed using genomic DNA, primers (in pairs or in separate forward or reverse reactions) and a premixed formulation incorporating nucleotides and Taq polymerase (ReadyToGo Beads, Amersham). PCR conditions were determined from the
manufacturer's guidelines, from predicted PCR conditions (Primer Design, ABI Prism) and by preliminary tests of PCR conditions for each primer pair. For PCR indications requiring radioactive labelling, 33P labelled nucleotide was added to the PCR mix.

**Primer Design:** Whenever available, published primer pairs were used. For novel genes, primer design was undertaken. Genomic gene structures were predicted using the basic local alignment sequence tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) a tool that aligns cDNA gene sequences against searchable genomic DNA sequences.

As much of this work was undertaken before the completion of the Human Genome Project, libraries of recently sequenced clones were the major source of genomic DNA matches to cDNA sequences. The genomic structure of a candidate gene was thus identified and ,primer pairs designed complimentary to intronic sequences flanking a given exon (PrimerDesign, DNASTar). Primer pairs were manufactured (commercial vendors), and tested for optimal PCR conditions.

**Single Stranded Conformational Polymorphisms (SSCP):** Electrophoretic separation of 33P-labelled PCR products (amplified genomic DNA incorporating exon coding sequences) identified anomalous conformers. SSCP was used to screen for exonic coding variants that would thereafter be sequenced.

**DNA Sequencing:** Cycle sequencing (ABI Prism 310, Applied Biosystems) according to the manufacturer's instructions (BigDye Terminator, Applied Biosystems) in forward and reverse directions provided sequence pherograms. Pherograms were analysed (SeqMan, DNASTar) for heterogeneity and compared with wild-type sequences for the detection of mutations (missense or insertion/deletion). Repeat sequencing from stored DNA and restriction digest were used to confirm the presence of any mutation.

**Linkage Analysis:** The resources required for Linkage analysis were not available in our genetics laboratory. Collaborative research with larger laboratories was sought following the detection of suitably large pedigrees with autosomal dominant unusual disease phenotype. Phenotyping and the collection/purification of DNA were completed by our group.

3.2 **Sarcomeric Gene Mutations**
Mutant sarcomeric genes are the commonest causes of HCM yet; (1) their diversity; (2) the clinical utility of a genetic diagnosis and; (3) the mechanisms of pathogenicity are poorly understood. This section describes studies to improve understanding in these areas. Additionally, identifying HCM patients that do not have sarcomeric mutations facilitates other research: the search for non-sarcomeric causes of HCM. Some of the results of these efforts have been presented in abstract form and peer reviewed journals (Table 6).

<table>
<thead>
<tr>
<th>Scientific Sessions</th>
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<th>Peer Reviewed Articles</th>
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Table 6: Published Research and Presentations of Findings: Sarcomeric Mutations.
3.2.1 Detection of Mutations in Beta Myosin Heavy Chain

3.2.1.1 Abstract

Aims: Mutation-specific natural histories promise to improve risk stratification, pre-clinical diagnosis and genetic counselling in HCM. Detection of genetic causes of HCM is currently not routinely available, and the ideal detection strategy is unknown. We investigated the utility of genetic screening in HCM by performing mutational analysis of β-myosin, one of the commonest causes of the disease.

Methods and Results: Genomic DNA from 100 consecutive unrelated patients with HCM and from 200 normal unrelated subjects was screened with SSCP for mutations in the first 23 exons of MYH7 encoding the motor region of β-myosin. Anomalous conformers were sequenced and mutations confirmed by restriction fragment length polymorphism. The clinical characteristics of patients with MYH7 mutations were compared with those in whom MYH7 mutations were not detected.

Seventeen missense mutations were identified in 19 patients. Notably, 13 or 76% were novel. Mutations were detected in both alleles in 2 patients: one was homozygous for Lys207Gln and another heterozygous for Pro211Leu and Arg663His. No missense mutations were detected in control subjects. Left ventricular (LV) wall thickness was similar in patients with MYH7 mutations and in those where MYH7 mutations were absent. HCM patients with MYH7 mutations had more marked left atrial enlargement and presented more frequently with syncope than other HCM patients.

Conclusions: These β-myosin mutations detected in 19% of patients most likely account for only a small fraction of all MYH7 associated HCM; detection methods should therefore allow identification of previously undescribed mutations. The genetic diversity and rarity of most of the mutations renders phenotype-genotype correlation difficult. Co-existence of more than one sarcomeric mutation in the same patient may be more common than is appreciated, could confound linkage studies and may contribute to the marked phenotypic diversity characteristic of HCM. Further studies are necessary to ascertain the clinical consequences of novel and compound gene abnormalities, and to determine whether correlating functional domain to
phenotype provides more useful information about clinical significance of the molecular defects.

3.2.1.2 Introduction

HCM demonstrates non-allelic and allelic heterogeneity, and at the time this research was undertaken, mutations in any of 9 genes encoding sarcomeric components were identified as causes of HCM. The most extensively studied gene is MYH7, coding for β-myosin heavy chain.31,32,38,39,221-225 The familial hypertrophic cardiomyopathy database listed 72 mutations in this gene that by September 2001 when this study's findings29 were published were associated with HCM (June 2009: there are now 194).226 Almost invariably, these are missense mutations affecting the motor or head-lever region of the molecule.

Determination of the molecular causes of HCM has enabled description of mutation-specific natural histories. For example, some β-myosin mutations are associated with high disease penetrance and a high incidence of sudden death; others with low penetrance and relatively benign prognosis.32,43 Importantly, some sarcomeric mutations are associated with mild LV hypertrophy but a poor prognosis.12,13 Molecular diagnosis also allows pre-clinical diagnosis in children and establishes the diagnosis in adults with mild disease. Hence, increasingly, a genetic diagnosis is considered potentially important in the management of HCM. However, despite recent advances, the utility of genetic screening in individual patients is unclear.

It is also uncertain which screening method should be utilized in clinical practice. Mutations may be detected by, (1) specifically testing for each of more than one hundred identified mutations, for example by restriction fragment length polymorphism (RFLP); (2) sequencing the several hundred exons of these 9 genes, with or without preliminary screening such as with SSCP; or (3) by studying suitably large kindreds using genetic linkage analysis. Following detection of novel mutations, it is then desirable to prove causality (even in genes known to cause HCM) and to determine the natural history of the associated HCM.

We sought to determine the prevalence and diversity of MYH7 mutations in a consecutive cohort of unrelated HCM patients and in normal controls to: (1) evaluate a sequencing based strategy for routine mutation detection in unselected patients, (2) determine the degree of
heterogeneity in normal individuals, and (3) evaluate feasibility of describing the natural histories of the associated HCM.

3.2.1.3 Methods

Patients: A hundred consecutive unrelated proband patients with HCM referred to the National Institutes of Health were studied. Informed consent was obtained under protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (Protocol 99-H-0065).

Clinical Studies: Studies included 2-D echocardiography, 12-lead electrocardiography, and in selected cases, treadmill exercise testing. HCM was defined as LV wall thickness >13 mm in the absence of another cause for cardiac hypertrophy. A family history of HCM was defined as present if obtained at the initial clinic interview and does not include results of subsequent family screening. A family history of sudden death (SD) was defined as the sudden death of 1 or more first degree family members under the age of 45 years. In selected cases, family studies were pursued when mutational analysis had detected novel findings.

Genetic Analysis: Genetic analysis was performed on genomic DNA extracted from whole blood (Genta). Using previously described primer pairs and methods, each of the first 23 exons of MYH7 was amplified by the polymerase chain reaction. SSCP was performed for each exon for every patient to identify anomalous conformers. Anomalous conformers were sequenced (BigDye Terminator, ABI Prism 310), to detect sequence abnormalities (SeqMan, DNASTar). RFLPs were predicted from mutant and wild type sequences (MapDraw, DNASTar) and mutations confirmed by digestion when appropriate endonucleases were available.

Genomic DNA from 200 unrelated control individuals was used as controls; for each novel mutation detected, 100 control DNAs were screened by RFLP for that mutant. Uniquely in this area of research, further 100 unrelated controls were screened by SSCP for each exon in which a mutation had been detected in HCM patients in order to determine if MYH7 heterogeneity was found in a normal population. This was felt to be important as prior studies had indicated that HCM causing mutations are likely to be rare in any cohort of genetically unrelated individuals, including cohorts with HCM.
Statistics: Data are expressed as mean ± SD. Continuous variables were analysed using Student's t-test for unpaired data. Non-continuous variables were analysed using Fisher's exact test. A p-value of <0.05 was considered significant.

3.2.1.4 Results
Prevalence of Known and Novel β-Myosin Mutations in the HCM Population, and Genotype-Phenotype Correlations: Results of genetic analysis in the 100 patients with HCM and their clinical findings are presented in Table 7 and Table 8 respectively.

The mutations affect each of the functional domains of the motor unit of myosin (Figure 7). Seventeen different missense mutations were detected in 19 patients in 11 of the first 23 exons of MYH7 (encoding the motor domain of beta myosin). Of these, 13 mutations, or 79%, were novel. The total number of MYH7 mutants associated with HCM increased to 85 from 72, an increase in 18%, as a result of this study. Of the 4 previously described mutations, one (Arg403Gln) is associated with a poor prognosis, one (Leu908Val) is associated with a relatively benign prognosis, and two (Ser782Asn and Arg663His) are associated with atrial fibrillation. Mutant alleles with the Leu908Val and Arg663His substitutions were found most commonly, present in 2 and 3 patients respectively. RFLP confirmed the presence of all novel mutations except for Asn479Ser for which no available endonuclease discriminates.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Exon</th>
<th>Charge Change</th>
<th>Morphology</th>
<th>Clinical Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arg41&gt;Gly</td>
<td>5</td>
<td>positive-neutral</td>
<td>ASH</td>
<td>No FH</td>
</tr>
<tr>
<td>2</td>
<td>Ser54&gt;Glu</td>
<td>5</td>
<td>No</td>
<td>ASH</td>
<td>FH</td>
</tr>
<tr>
<td>3</td>
<td>Lys56&gt;Glu</td>
<td>12</td>
<td>positive-negative</td>
<td>mid-cavity</td>
<td>No FH</td>
</tr>
<tr>
<td>4</td>
<td>Arg65&gt;Gln</td>
<td>13</td>
<td>positive-neutral</td>
<td>ASH</td>
<td>SD, DCM in family</td>
</tr>
<tr>
<td>5</td>
<td>Asn67&gt;Ser</td>
<td>15</td>
<td>No</td>
<td>ASH</td>
<td>FH, No FH</td>
</tr>
<tr>
<td>6</td>
<td>Glu70&gt;Ala</td>
<td>15</td>
<td>negative-neutral</td>
<td>ASH</td>
<td>FH with much SD</td>
</tr>
<tr>
<td>7</td>
<td>Gly65&gt;Arg</td>
<td>16</td>
<td>neutral-positive</td>
<td>ASH</td>
<td>AF</td>
</tr>
<tr>
<td>8</td>
<td>Arg76&gt;His</td>
<td>18</td>
<td>positive-neutral</td>
<td>mid-cavity</td>
<td>no FH, 'arrhythmia' in family</td>
</tr>
<tr>
<td>9</td>
<td>Arg76&gt;His</td>
<td>18</td>
<td>positive-neutral</td>
<td>mid-cavity</td>
<td>PAF, SD and HCM in family</td>
</tr>
<tr>
<td>10</td>
<td>Arg16&gt;Pro</td>
<td>18</td>
<td>positive-neutral</td>
<td>ASH</td>
<td>PAF, no FH</td>
</tr>
<tr>
<td>11</td>
<td>Ile70&gt;Thr</td>
<td>20</td>
<td>No</td>
<td>ASH</td>
<td>FH</td>
</tr>
<tr>
<td>12</td>
<td>Ser71&gt;Asn</td>
<td>21</td>
<td>No</td>
<td>mid-cavity</td>
<td>PAF, FH</td>
</tr>
<tr>
<td>13</td>
<td>Val75&gt;Gly</td>
<td>21</td>
<td>No</td>
<td>ASH</td>
<td>DCM in family</td>
</tr>
<tr>
<td>14</td>
<td>Met74&gt;Leu</td>
<td>22</td>
<td>No</td>
<td>PAF, no FH</td>
<td>FH</td>
</tr>
<tr>
<td>15</td>
<td>Gly81&gt;Glu</td>
<td>22</td>
<td>No</td>
<td>ASH</td>
<td>PAF, no FH</td>
</tr>
<tr>
<td>16, 17</td>
<td>Leu76&gt;Val</td>
<td>23</td>
<td>No</td>
<td>mid-cavity</td>
<td>PAF, no FH</td>
</tr>
<tr>
<td>18</td>
<td>Pro77&gt;Leu*Arg80&gt;His</td>
<td>7 &amp; 18</td>
<td>*no</td>
<td>ASH</td>
<td>AF, AF in family</td>
</tr>
<tr>
<td>19</td>
<td>Lys91&gt;Gln</td>
<td>20</td>
<td>positive-neutral</td>
<td>apical</td>
<td>AF, heterozygotes bradycardic</td>
</tr>
</tbody>
</table>

Table 7. Beta myosin (MYH7) mutations identified in 100 unrelated patients with HCM.

ASH, asymmetrical septal hypertrophy; SD, sudden cardiac death; AF, chronic atrial fibrillation; PAF, paroxysmal AF; FH, family history of HCM; DCM, dilated cardiomyopathy.

<table>
<thead>
<tr>
<th>All Patients</th>
<th>MYH7 Mutations n=100</th>
<th>Non-MYH7 HCM n=81</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age at diagnosis (years)</td>
<td>35±18</td>
<td>36±18</td>
<td>ns</td>
</tr>
<tr>
<td>asymptomatic</td>
<td>8 (5%)</td>
<td>7 (9%)</td>
<td>ns</td>
</tr>
<tr>
<td>cardiac arrest</td>
<td>3</td>
<td>3</td>
<td>ns</td>
</tr>
<tr>
<td>presyncope</td>
<td>66</td>
<td>51 (79%)</td>
<td>22</td>
</tr>
<tr>
<td>syncope</td>
<td>33</td>
<td>22 (58%)</td>
<td>11</td>
</tr>
<tr>
<td>chest pain</td>
<td>72</td>
<td>58 (72%)</td>
<td>14</td>
</tr>
<tr>
<td>dyspnoea</td>
<td>86</td>
<td>70 (86%)</td>
<td>16</td>
</tr>
<tr>
<td>palpitations</td>
<td>68</td>
<td>52 (64%)</td>
<td>16</td>
</tr>
<tr>
<td>CHF</td>
<td>13</td>
<td>8 (10%)</td>
<td>5</td>
</tr>
<tr>
<td>ICD</td>
<td>8</td>
<td>5 (6%)</td>
<td>3</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>32</td>
<td>23 (28%)</td>
<td>9</td>
</tr>
<tr>
<td>septum (mm)</td>
<td>21±7</td>
<td>21±7</td>
<td>21±7</td>
</tr>
<tr>
<td>posterior wall (mm)</td>
<td>11±2</td>
<td>11±2</td>
<td>11±2</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>45±6</td>
<td>44±7</td>
<td>46±6</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>26±6</td>
<td>24±5</td>
<td>27±6</td>
</tr>
<tr>
<td>FS (%)</td>
<td>42±7</td>
<td>41±7</td>
<td>41±7</td>
</tr>
<tr>
<td>left atrium (mm)</td>
<td>44±10</td>
<td>52±11</td>
<td>43±9</td>
</tr>
<tr>
<td>ASH (obstruction)</td>
<td>72</td>
<td>69 (85%)</td>
<td>13</td>
</tr>
<tr>
<td>Apical LVH</td>
<td>5</td>
<td>4 (5%)</td>
<td>1</td>
</tr>
<tr>
<td>mid-ventricular</td>
<td>13</td>
<td>8 (10%)</td>
<td>5</td>
</tr>
<tr>
<td>Exercise duration (sec)</td>
<td>450±199</td>
<td>438±236</td>
<td>453±194</td>
</tr>
<tr>
<td>ABPR</td>
<td>23</td>
<td>5/11 (45%)</td>
<td>18/58 (31%)</td>
</tr>
</tbody>
</table>

Table 8: Clinical Characteristics of HCM Patients With and Without Mutant vs. Beta Myosin.

(), per cent; ns, not significant; LVIDd and LVIDs, LV internal dimension in diastole and systole; FS, Fractional shortening; and ABPR, abnormal blood pressure response on exercise (systolic BP rise<20 mmHg at peak exercise).
The domain affected by each individual mutation was predicted from the crystalline structure of the highly homologous chicken fast skeletal myosin (PDB id: 2MYS). This structure encompasses the motor region of myosin which recent studies suggest comprises five major subdomains; the N-terminal subdomain (black); the upper 50-kDa subdomain (blue); the lower 50-kDa subdomain (grey) and the converter subdomain (green). Following the converter, a long helical segment of the heavy chain (blue) binds the essential light chain (ELC) and the regulatory light chain (RLC) together forming the lever arm (Houdusse et al., 2000). The domains are connected by single-stranded joints such as the relay (yellow) which allow movement of the subdomains relative to each other. The motor region is shown with the positions of residues affected by each mutant marked; novel mutations are labelled in red. Approximate positions of residues Lys^{207}, Pro^{211} and Gly^{571} are shown; residues Lys^{207} and Pro^{211} are contained in loop 1 overlying the nucleotide pocket which is not resolved on the crystal structure. The region around Gly^{571} is also missing, and is in a part of the lower 50K-subdomain thought to make contact with actin. Finally Gin^{862} and Leu^{868} are distal within the lever arm and are not seen on this model.

**Double heterozygosity and homozygosity:** Unexpectedly, both MYH7 alleles were abnormal in two patients with end-stage HCM. This was one of the first demonstrations of double
heterozygosity/homozygosity in HCM, a finding subsequently made also by others.\textsuperscript{26,27,37,38,51}

Patient 18 demonstrated double heterozygosity for the Arg\textsuperscript{663}His and novel mutation Pro\textsuperscript{211}Leu (Figure 8a). He presented at 56 years of age with fatigue. An echocardiogram at 63 years demonstrated a markedly enlarged left atrium (61 mm), cardiac hypertrophy (septum 20 mm, posterior LV wall 10 mm), normal internal LV dimensions and systolic function (end-diastolic dimension, 36 mm; end-systolic dimension, 22 mm; fractional shortening, 39%), and LV outflow obstruction (estimated gradient, 55 mm Hg). An echocardiogram at 76 years showed LV wall thinning (septum 16 mm), impaired LV systolic function, no outflow obstruction, and associated with chronic atrial fibrillation and symptoms of congestive cardiac failure. Of 3 siblings, one also inherited both mutations; one the Arg\textsuperscript{663}His mutation only and the third neither (Figure 8a).

New diagnoses of HCM complicated by atrial fibrillation in the siblings with mutant MYH7 were made following family screening; the sibling with wild-type MYH7 had normal clinical findings.

Patient 19 is homozygous for the novel Lys\textsuperscript{207}Gln mutation (Figure 8b). Asymptomatic HCM was diagnosed at the age of 47 years following routine electrocardiography. At the age of 64 years an echocardiogram showed a markedly dilated left atrium (62 mm), apical HCM with a basal septal dimension of 15 mm increasing to 21 mm towards the apex, posterior LV wall 10 mm, LV end-diastolic dimension 52 mm, LV end-systolic dimension 30 mm and no LV outflow gradient.

The most recent echocardiogram, at the age of 74 years shows increased left atrial (66 mm) and LV (56 mm in diastole) dimensions. He was treated with an implantable cardioverter-defibrillator following several episodes of syncope, and has received several appropriate ICD discharges interventions. He has had admissions with pulmonary oedema and was in NYHA functional class III and chronic atrial fibrillation. One surviving sibling, all 4 children, and three grandchildren are heterozygous for Lys\textsuperscript{207}Gln (Figure 8b). This sibling, aged 80 years was subsequently diagnosed with mid-ventricular obstructive HCM and an interventricular septal wall thickness of 35 mm. The echocardiograms of a son (43 years), two daughters (46 and 40 years) and three grand children (9, 3 and 15 years) with the Lys\textsuperscript{207}Gln allele were normal. Several of the family members including all three children heterozygous for Lys\textsuperscript{207}Gln, but without cardiac hypertrophy, had a marked resting sinus bradycardia.
Figure 8: Patients with Compound MYH7 Mutants: DNA Sequences and Pedigrees.
A. Sequencing pherograms for patient 18 demonstrate double heterozygosity (Arg^{663}His and Pro^{211}Leu). In the pedigree, ages are in parentheses. One sibling has both mutations, another has Arg^{663}His only, and a third neither. The siblings with mutations were diagnosed with HCM and atrial fibrillation. The sibling with normal MYH7 alleles has a normal ECG and echocardiogram. B. Pherograms demonstrate homozygosity for Lys^{207}Gln in patient 19. A sibling heterozygous for Lys^{207}Gln was diagnosed with mid-cavity HCM, but all other family members with a single mutant allele were normal.
Comparison of clinical findings of MYH7 and non-MYH7-associated HCM: Two groups of HCM patients are identified; those with, and those without, MYH7 mutations. The groups of HCM patients were similar with regards age, gender, family history of HCM or sudden death, exercise performance, and need for pharmacological and/or other therapies. However, patients with MYH7 associated-HCM were more likely to present with syncope, and had greater left atrial dimensions despite a similar severity of LV hypertrophy and higher LV fractional shortening, Table 2. These differences remain significant after exclusion of the two patients with double mutations. Patients with MYH7-associated HCM also had a greater tendency to develop atrial fibrillation, but this did not reach statistical significance.

Genetic Analysis of Normal Controls: All novel mutations (except when no endonuclease was available) were absent from the DNA of 100 unrelated normal subjects following RFLP (200 alleles). SSCP and direct sequencing of all anomalous conformers for MYH7 exons 5,7,12,13,15,16,18,20,21,22 and 23 in 100 other unrelated normal individuals (200 alleles) identified several neutral single nucleotide polymorphisms. (SSCP for exon 15 was performed on DNA of all 200 subjects as RFLP was unavailable for Asn479Ser). Thus, in 200 control alleles no MYH7 missense mutations were present in the 11 exons affected and each novel mutation was absent from 400 control alleles.

3.2.1.5Discussion

Yield and Utility of Genetic Screening Methods: Our study demonstrates MYH7 mutations are detected in about twenty per cent of patients with HCM. Most of these have not been described, indicating that mutations identified to date account for a fraction of all MYH7 associated HCM. Only 7 of 19 patients (4 of 17 mutations) had mutations that had been reported previously. Three of these (Arg405Gln, Arg663His and Leu908Val) are amongst a relatively few mutations with well-described natural histories that were similar to those of the unrelated patients in the present study.29,31,39

This magnitude of MYH7 heterogeneity in HCM patients indicates that genetic screening must be able to identify novel mutations. As most mutations are rare, and may even be unique to certain families, genetic screening strategies relying on identification of previously determined mutations (such as RFLP) will have limited yields; clinically applicable techniques will most likely
rely on sequencing based strategies. Until automated high throughput technologies, such as those based on chip hybridisation arrays become reliable, considerable time and expense is required for reliable mutation detection in HCM.

The validity of ascribing causative roles to MYH7 mutations detected in individual HCM patients can be questioned. Pedigree expansion with linkage analysis, mutant protein functional assays and transgenic models contribute to greater proof of causal relationships. In practice, definitive proof of causality will not be available for most detected mutations. The absence of a mutation from a number of control DNAs, identified by RFLP, is often cited as proof of cause.

This study has shown that MYH7 heterogeneity in HCM patients is such that most mutations will also be absent in a large population of unrelated HCM patients, just as in normal controls. While it has been assumed that MYH7 sequence abnormalities are rare in the normal population, there has been little proof of this. We did not detect any missense sequence abnormalities by SSCP in 100 normal subjects in each of the exons identified as having mutations in this series of HCM patients, and did not find the specific abnormalities by RFLP in 100 other controls. Thus, MYH7 is extremely conserved and coding abnormalities are rare in the absence of cardiomyopathy. For future novel mutations found in these exons of this gene, it should not now be necessary to demonstrate their absence from control DNA as a routine element of the genetic diagnosis. For other genes, particularly myosin binding protein C, this determination is yet to be made. Thus, these novel mutations are likely to cause HCM as (1) they involve highly-conserved amino acids; (2) the mutations were absent in 400 alleles from normal subjects; (3) MYH7 shows no sequence variation in 200 normal alleles and; (4) HCM was present only in family members with mutation.

A further complicating factor is that several sarcomeric gene mutations may be present in the same patient. Homozygosity and double heterozygosity for sarcomeric gene mutations have been described previously but are assumed to be rare.50,51,227,228 In this series of consecutive unrelated patients, abnormalities in both alleles of MYH7 were noted in 2% of the patients or in 10% of those with MYH7 mutations. If sufficiently frequent, occurrence of multiple mutations would confound linkage studies. It would also diminish our ability to attribute the disease to a particular mutation and may, in part, explain the phenotypic diversity
characteristic of HCM. The likelihood of heterozygosity should also be considered in genetic counselling of at-risk family members.

Screening for molecular causes of HCM is only valuable for management of patients if the natural histories of the identified mutations are known and shared by families in which HCM is caused by the identical genetic defect. Unfortunately, the genetic diversity, the rarity of most mutations, and limited number of affected subjects, render phenotype-genotype correlations difficult. Therefore, this aspect of management for improving risk stratification in HCM is still very much in the clinical research domain.

**Clinical Differences between HCM Caused by MYH7 and Non-MYH7 Associated HCM:** Our findings suggest that MYH7 associated HCM differs from non-MYH7 associated HCM in certain important clinical respects. Despite better systolic function and similar LV wall hypertrophy and frequency of LV outflow obstruction, patients with MYH7 associated HCM had greater left atrial enlargement. This implies that MYH7-associated HCM is complicated by a greater LV stiffness; alternatively MYH7 mutations contribute directly to a primary atrial myopathy. Affected patients were also more likely to present with syncope, perhaps related to an increased incidence of arrhythmias such as atrial fibrillation.

**Implications for Mutation-Phenotype Correlations:** These findings indicate that most HCM patients have an uncharacterised mutation whose rarity may disallow adequate description of the associated natural history in unrelated kindreds. Furthermore, once obtained, application of this information may be limited to few patients. The large number of MYH7 mutations now described will permit testing of the hypothesis that site and nature of mutations within the distinct functional domains of β-myosin determine clinical outcomes. A recent study linked a Phe764Leu mutation in MYH7 to dilated cardiomyopathy. In our study, a patient with obstructive HCM (septum of 32 mm) had a mutation in the preceding residue (Val763Gly). However, a sibling with this mutation presented with cardiac failure and dilated cardiomyopathy as a teenager, suggesting mutations in this region may predispose to DCM rather than HCM. In the two individuals with double mutant alleles, one or both mutations occurred in a loop (loop 1) overlying the nucleotide-binding pocket. Thus, mutations in this region may, when homozygote, result in only mild and/or poorly penetrant disease.
3.2.1.6 Conclusions

MYH7 mutations are found in one fifth of HCM patients and are rare in normal subjects. Most HCM patients have mutations with undescribed natural histories. Mutation detection methods will have to rely on sequence-based methods. Compound-heterozygosity occurs more frequently than has been appreciated and may be responsible for some of the phenotypic diversity and incomplete disease penetrance and for sporadic cases of HCM. Further work on genotype-functional consequence-phenotype correlation is needed and may be facilitated by grouping mutant proteins by functional domain affected.

A major advantage of a candidate gene based screening method of consecutive unselected patients, such as in this study, is that patient selection is not biased towards those with a well-defined family history where the causative mutation is likelier to have a more penetrant and severe phenotype.

3.2.1.7 Study Limitations

Results of mutational analysis clearly depend on the patient population and sensitivity of SSCP (estimated to be 85%). For simplicity we analysed only one gene. Analysis of other sarcomeric genes was beyond the scope of this investigation, but may demonstrate that multiple heterozygosity may be even more frequent and further complicate the phenotypic expression of HCM.
3.2.2 CLINICAL CHARACTERISTICS OF BETA MYOSIN HYPERTROPHIC CARDIOMYOPATHY

3.2.2.1 Abstract

Background: Mutations affecting MYH7, encoding beta myosin, are the most commonly detected causes of HCM. We hypothesized that the severity of the HCM resulting from any particular sarcomeric mutation is influenced by the nature and severity of the resulting molecular functional defect. Additionally, routine mutational analysis frequently detects novel mutations that may be ‘private’ to the affected family, and for which little or no clinical information is available. We therefore sought to determine if (1) β-myosin mutations are associated with an HCM phenotype distinguishable from HCM not caused by β-myosin mutations, (2) mutations affecting the same functional sub-domains of β-myosin (mutant sub-domains) share phenotypic features.

Methods and Results: We compared findings in 90 unrelated patients with HCM caused by one of 47 mutations in the motor unit of β-myosin and 125 unrelated HCM patients without β-myosin mutations. β-myosin HCM was diagnosed earlier and was more frequently associated with syncope, heart failure and family history of sudden death. Despite their younger age and a similar magnitude of left ventricular hypertrophy and outflow obstruction, β-myosin patients had larger left atria and more frequently developed chronic atrial fibrillation, syncope and heart failure admissions. These complications and systolic impairment progressed more rapidly in β-myosin patients. Non-significant trends were apparent when comparing mutant sub-domains; a family history of sudden death was commonest for upper 50kDa mutations, heart failure was more frequent in upper and lower 50kDa HCM, atrial fibrillation was commonest for lower 50kDa, and left atrial enlargement greatest in upper and lower 50kDa HCM.

Conclusions: HCM phenotypes are difficult to distinguish on the basis of the causative genetic defect. However, HCM resulting from β-myosin mutations presents earlier, and disease complications occur more frequently. The greater atrial dimensions and frequency of heart failure symptoms suggest that atrial myopathy and diastolic dysfunction are particularly prominent in β-myosin HCM. Mutations affecting the upper or lower 50kDa sub-domains were associated with more severe and complicated natural histories; this may facilitate prognostic predictions following the detection of a novel or ‘private’ causative mutation.
3.2.2.2 Introduction

Of the 8 genes encoding the principal components of the human sarcomere, the most extensively studied gene is MYH7, coding β-myosin heavy chain. MYH7 mutations are also the most commonly detected causes of HCM and most are missense mutations affecting the motor or head-lever domain of β-myosin heavy chain. The Harvard Medical Genetics database lists more than 190 MYH7 mutations associated with HCM, (http://genetics.med.harvard.edu/seidman/cg3/index.html) and investigations continue to detect novel mutations. Genetic diagnoses have the potential to contribute to the management of HCM. For example, some MYH7 mutations have been associated with high disease penetrance and a high incidence of sudden death; others with low penetrance and more benign prognosis. However, our ability to associate natural history with a given sarcomeric mutation has at least two major limitations: [1] the large number and rarity of most individual mutations and, [2] the high degree of variability between genotype and phenotype.

The varied phenotypes evident within single families are a characteristic of HCM, and genotype-phenotype descriptions need to encompass such variability. In such a scheme, a mutation can be viewed as conferring a probability that HCM will develop, and probabilities that complications will occur. Indeed, it is the likelihood of any particular outcome associated with a genotype that is most likely to be useful for making clinical decisions. The evaluation of several individuals with each of the genotypes is required for the estimation of outcome probabilities. Such studies would be restricted to the more commonly detected mutations, whereas the majority of mutants are rare or even apparently unique. Families are often small, with few members available for study.

It is extremely unlikely that the large number of disease causing mutations will ever be adequately studied individually. If mutants can be clustered into groups based on certain criteria, phenotype studies may become more feasible. These criteria may include gene affected, functional domain affected, amino acid charge change or results of in-vitro functional assays. This clearly represents compromise, and the relative and absolute merits of mutational groups based on such assumptions are poorly tested. However, the findings presented in the
previous section suggested that despite similar a severity of LVH, β-myosin HCM had clinical features distinct from HCM resulting from other causes. We therefore sought to evaluate whether these findings were confirmed in a larger group of patients with β-myosin HCM.

Furthermore, we assessed a strategy for intra-gene grouping phenotype studies. As recent studies suggest myosin’s motor domain comprises five major sub-domains, the disruption of a subdomain’s function by any mutation of its constituent residues may each result in similar consequences. Motility assays that assess the unloaded velocity of actin translocation by mutant β-myosin correlate poorly with disease severity and penetrance, suggesting that the cardiomyopathy results from more subtle disturbances of sarcomeric function. Sarcomeric disturbances resulting from different mutations may include abnormalities of actin binding and release, nucleotide binding, hydrolysis and release, conformational changes in the motor domain, transmission of the power stroke, and interactions with the myosin light chains. Recent studies suggest myosin comprises five major sub-domains; these relatively rigid subdomains articulate about flexible ‘joints’ during myosin’s power stroke. We hypothesized that as the disruption of a subdomain by a mutation in any of its constituent residues might result in similar molecular consequences, phenotypic characteristics may be shared by mutants affecting the same sub-domain.

We therefore sought to determine whether HCM resulting from mutant β-myosin has a natural history distinct from non β-myosin HCM and if MYH7 mutations affecting the same sub-domain of the β-myosin motor region are associated with similar phenotypes.

3.2.2.3 Methods

Subjects: Subjects studied were all unrelated proband HCM patients attending the Inherited Cardiac Diseases clinic and studied under approved protocols described above. A proband patent was defined as the first member of a family evaluated at NHLBI, and a family history of HCM was considered positive if a suspicious family history was elicited at the first clinic interview. All proband patients with MYH7 motor domain mutations detected at the NIH comprised the β-myosin HCM group, and were compared to a series of control HCM patients in whom MYH7 motor domain mutations had not been detected following mutation detection (non β-myosin HCM). The MYH7 mutation group comprised those identified in the study above,
and others in whom prior or subsequent mutational analysis had been completed. HCM patients in whom more than one MYH7 mutation had been detected were excluded.

**Genetic Screening and Myosin Functional Domain affected:** Mutational analysis of the first 23 exons of MYH7 was performed on genomic DNA extracted from whole blood as described above. The functional domain of β-myosin affected by each mutation was predicted from the crystalline structure of the motor region of the highly homologous chicken fast skeletal myosin (PDB Id: 2MYS): the N-terminal, the upper 50-kDa, the lower 50-kDa, the converter and a long helical segment comprising the lever arm (Figure 9). Mutations were also divided into two groups (conserved and non-conserved substitutions) defined by the presence or absence of a charge changing amino acid substitution.

![Functional Domain of Beta Myosin Affected by Each Mutant](image)

**Figure 9.** Functional Domain of Beta Myosin Affected by Each Mutant. This was predicted from the crystalline structure of the highly homologous chicken fast skeletal myosin (PDB Id: 2MYS). This structure encompasses the motor region of myosin, which recent studies suggest comprises five major subdomains. The motor region of beta myosin is shown from 4 views: the N-terminal subdomain (black); the upper 50-kDa subdomain (blue); the lower 50-kDa subdomain (grey) and the converter subdomain (green). Following the converter, a long helical segment of the heavy chain (light blue) binds the essential light chain (ELC) and the regulatory light chain (RLC) together forming the lever arm. Sub-domains are connected by single-stranded joints such as the relay (yellow) that allow movement of the subdomains relative to each other.

**Clinical History:** Patient histories were reviewed for clinical data including age of diagnosis, family history of sudden death, and history of complications including congestive heart failure.
(CHF), atrial fibrillation (AF), and cardiac arrest. A family history of sudden death was defined as the death of 1 or more first degree family members under the age of 45 years.

**Echocardiography:** Transthoracic 2-dimensional echocardiograms were obtained in all subjects. The following were recorded from standard views: left atrial (LA) size, LV internal dimensions in diastole and end-systole, septal thickness and Doppler LV outflow tract velocity.

**Statistics:** Continuous variables were analysed using Student's t-test and categorical variables with Fisher's exact test. To further compare features between β-myosin and non β-myosin HCM patients taking age and length of follow-up into account, the data were analysed using the general linear model. If there was significant interaction between age (or length of follow up) and β-myosin status, the features of correspondent age (or length of follow up) groups were compared. A p-value of <0.05 was considered significant. Data are expressed as mean ± SD.

**3.2.2.4 Results**

**Subjects:** 90 β-myosin and 125 non β-myosin HCM patients were studied. The 90 β-myosin patients had 47 MYH7 missense mutations of 45 residues: 7 mutations were in the N-terminal subdomain (9 unrelated patients), 11 were in the upper 50-kDa (15), 9 were in the lower 50-kDa (23), 10 in the converter (22 patients) and 10 in the lever arm (21 patients) (Table 1). The mutation was charge-changing in 47 of the 91 β-myosin patients, and in 29/47 mutations.

**Comparisons of β-myosin with Non β-myosin HCM:** Patients with MYH7 mutations were over 10 years younger than non β-myosin patients when diagnosed with HCM and also had a slightly longer duration of follow-up (Table 10). Gender ratios were significantly different between the groups; the proportion of male and female patients were equal for β-myosin HCM and there were significantly more men in non β-myosin HCM. β-myosin patients also more frequently described family histories of sudden death. Despite their younger age and similar severity of septal hypertrophy and LV outflow tract obstruction, β-myosin patients more frequently had a history of syncope and were more likely to have had episodes of CHF. There was a greater tendency towards the development of AF in β-myosin patients, who also developed significantly greater LA enlargement.
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Table 9: Allocation of MYH7 Mutants to Domain of Beta Myosin Affected.

Domain assignments and the number of unrelated patients with each mutation are tabulated. All domains of the motor region are represented, with similar numbers of mutants in each.
Clinical and echocardiographic features of β-myosin and non β-myosin HCM patients were compared for six different age groups: <20 years, 20-29 years, 30-39 years, 40-49 years, 50-59 years, and ≥60 years (Figure 10). Symptoms of dyspnoea, syncope, chest pain (not shown), palpitations (not shown) and the development of AF and CHF episodes were more prevalent with increasing age in both HCM groups. Syncope increased in prevalence between successive age groups for β-myosin patients but remained relatively unchanged in non β-myosin HCM. Complications of CHF (P=0.002) and syncope (P=0.003) developed earlier and were more prevalent in β-myosin than in non β-myosin patients.
Clinical features and echocardiographic dimensions in HCM patients of different ages with or without β-myosin mutations. *; P<0.05 comparison of non β-myosin with β-myosin HCM of the same age-group.

There was a tendency towards the development of chronic AF at a younger age in β-myosin patients. LA enlargement was greater in β-myosin patients than in non β-myosin patients (P<0.001). The LA enlarged with age, and was similar in the oldest age groups. The magnitude of septal hypertrophy was similar in all age groups except in the <20 years group where septal thickness was greater in β-myosin patients (P=0.03). There was a significant reduction in septal thickness between age groups when all patients are considered together (P<0.001). LV systolic function measured as the fractional shortening (FS) appeared to decline steadily between age groups in β-myosin patients, and was significantly depressed compared to non β-myosin HCM in the oldest age groups (P<0.05). FS in the oldest β-myosin age group was significantly lower
than in the youngest β-myosin age group (45±9% and 34±11%; P=0.03) but similar in oldest and youngest non β-myosin age groups (46±8% and 41±9%; P=ns).

As decline in functional status and the development of complications in HCM is frequently measured against the length of time from initial diagnosis to the most recent evaluation, or follow-up, β-myosin and non β-myosin patients were compared for similar follow-up periods: <4-years, 5-9 years, 10-14 years, 15-19 years and >20 years (Figure 11). For each follow up duration, β-myosin patients were approximately 10 years younger than non β-myosin patients, reflecting their younger age at diagnosis (P<0.001). Taking length of follow up into account, syncope was significantly more prevalent in β-myosin than in non β-myosin patients (P=0.04), but the prevalence of dyspnoea (P=ns) and chronic AF (P=ns) were not significantly different. β-myosin patients, but not non β-myosin patients, showed a steady increase in the frequency of CHF between successive follow up durations, and CHF was significantly more common in β-myosin patients with the longest duration of follow up (P=0.009). The duration of follow up had an independent effect over above the effect of β-myosin status on the development of dyspnoea (P=0.026) and chronic AF (P=0.009). LA sizes in β-myosin HCM were significantly greater than in non β-myosin HCM (P<0.001). The duration of follow up had an independent effect to that of β-myosin status on increasing LA size (P<0.001) and on declining FS (P=0.008). Septal wall thickness was significantly greater in β-myosin than non β-myosin patients in the <4-year follow up group (P=0.01).
Figure 11. The Natural History of Beta Myosin HCM: The Effect of Time from Diagnosis.

1; P=0.01 comparison between non β-myosin and β-myosin HCM for the same follow up duration.

β-myosin Functional Domain Analysis: Echocardiographic measurements of septal wall thickness and LV outflow gradients were similar for all sub-domain groups and non β-myosin HCM. (Figure 12) Despite these similarities and the relatively small number of patients in each domain (Table 9), several significant differences are apparent. A family history of SD was far commoner in patients with upper 50 kDa mutations, present in 53% (8/15) of cases compared with 24% (18/75) in all other β-myosin HCM (P=0.03) and only 14% (17/125) non- β-myosin HCM (P=0.001) Lower 50 kDa mutations were was frequently associated with chest pain (P=0.04), Dyspnoea (P=0.03) and presyncope (P=0.04) than upper 50 kDa HCM. Paroxysmal AF was detected in 14% (3/22) of converter patients in comparison with 44% (30/68) of all other β-myosin HCM (P=0.01), 57% (13/23) of lower 50 kDa HCM (P=0.005) and 47% (7/15) of upper 50 kDa HCM (P=0.06). Similarly chronic AF developed in only 5% (1/22) of converter HCM in
comparison to 25% (17/68) of all other β-myosin HCM (P=0.06) and 35% (8/23) of lower 50kDa HCM (P=0.02). In converter HCM, episodes of CHF were reported by only 14% (3/22) in contrast to 35% (24/68) of all other β-myosin HCM (P=0.06) and by 52% (12/23) of lower 50kDa HCM (P=0.01).

LA dimension was greatest in the upper and the lower 50 kDa patient groups. LA size in the upper 50 kDa was significantly greater than in converter (P=0.006), lever arm HCM (P=0.04) and non β-myosin HCM (P=0.0002). LA size in lower 50 kDa HCM was also greater than in converter HCM (P=0.01) and non β-myosin HCM (P=0.001). Differences in FS between domains were not significant. The fractional shortening (FS) in converter domain patients was significantly lower than non β-myosin HCM (P=0.009), but similar between non β-myosin HCM and all other domains. The comparatively low rates of CHF in the converter group is in contrast to the lower FS; however, abnormal LV filling in diastole, rather than LV systolic impairment is held to be the important determinant of such symptoms.

3.2.2.5 Discussion

A strategy to correlate phenotype with the affected functional domain is fundamentally limited by the well described infidelity between mutation and phenotype or penetrance within even a single pedigree.32,233,234

This study reports clinical findings associated with the largest single series of MYH7 mutations to date. We have shown that β-myosin HCM has features distinct from HCM resulting from other causes despite the phenotypic heterogeneity characteristic of HCM. In addition, our data indicate that mutations affecting the same part of the β-myosin molecule may result in similar clinical outcomes, and that the charge change effect of mutant substitutions has no or limited effects on phenotypic expression,

HCM patients with β-myosin mutations were diagnosed at a younger age, were more likely to have a family history of sudden death, and to develop complications at a younger age than those without β-myosin mutations. Several adverse symptoms and echocardiographic changes are also apparent sooner after diagnosis in β-myosin HCM. Importantly, these differences were apparent despite the younger ages of β-myosin patients and similar magnitudes of LV hypertrophy and LV obstruction.
A more severe atrial pathology was a particular feature of β-myosin HCM in our series, and may account for the greater tendency of AF. LA remodelling has been proposed as a surrogate marker of an elevated LV diastolic burden and our findings may reflect greater LV diastolic dysfunction resulting from β-myosin HCM. Alternatively the more severe degrees of LA enlargement may represent a more severe primary atrial myopathy. Alpha myosin heavy chain is the predominant isoform in normal human atrial muscle. However, there is a switch in expression to the β-myosin isoform in abnormally loaded atria, presumably as part of an adaptation to elevated atrial afterload. We suggest that the atrial response to LV diastolic abnormalities is especially maladaptive in β-myosin HCM, as normal α isoform is replaced by mutant β-myosin.

A history of sudden death was more frequent in upper 50 kDa HCM, a sub-domain that includes the malignant Arg403His mutant. In support of our findings, a study comparing only 11 MYH7 mutations reports that actin binding region mutations (included in the upper 50 kDa) were associated with poorer prognosis. In our study, AF, CHF and LA enlargement were also more severe in HCM associated with upper (and lower) 50kDa sub-domain mutations.

3.2.2.6 Conclusions

Despite similar magnitudes of LV hypertrophy and LV outflow tract obstruction, there are differences between HCM associated with β-myosin mutations and non β-myosin causes. Several characteristic features of HCM are more severe and progress more rapidly in β-myosin HCM. There are some phenotypic similarities in HCM resulting from different mutations in the same β-myosin sub-domain. Mutations in the upper and lower 50 kDa subdomains may result in the most severe forms of HCM. The finding that each β-myosin sub-domain may have similar features could facilitate prognostic predictions when the natural history of the causative MYH7 mutation is as yet undefined.
3.2.2.7 Limitations

The limitations of this study include; (1) The relative frequency of some mutants may bias the sub-domain description toward that of the mutant. In particular, the Arg663His mutation is over represented; (2) The non β-myosin group is used as a control to contrast the behaviours of β myosin HCM. Phenotypic distinctions between defined non β-myosin or of sarcomeric and non-sarcomeric causes of HCM are also possible and are not addressed in this study.
3.2.3 Molecular and Phenotypic Effects of Heterozygous, Homozygous and Compound Heterozygote Beta Myosin Heavy Chain Mutations

This work was undertaken in collaboration with the University of Vermont, Department of Molecular Physiology and Biophysics, Burlington, Vermont, USA, and Dakota Cardiovascular, P.C. Rapid City, SD, USA.

All clinical assessment, family screening and mutation detection/genotyping was completed at or by NHLBI. β-myosin functional assays were performed by the Vermont group on mutant and wild-type protein obtained at NHLBI from biceps muscle biopsy. The cardiologists at Dakota Cardiovascular greatly facilitated the large-scale screening, in South Dakota, of the larger of the two families (Figure 14) studied here.

3.2.3.1 Abstract

HCM has variable penetrance and phenotype. Heterozygous mutations in MYH7, encoding β-myosin heavy chain, are the commonest cause of HCM, and it has been proposed that 'enhanced' mutant actin-myosin function is the causative molecular abnormality. We studied individuals from families where members have two, one or no mutant MYH7 alleles to examine for dose effects. In one family, a member homozygous for Lys207Gln had cardiomyopathy complicated by left ventricular dilatation, systolic impairment, atrial fibrillation and defibrillator interventions. Only one of five heterozygous relatives had HCM. Leu908Val and Asp906Gly mutations were detected in a second family in which penetrance for Leu908Val heterozygotes was 46% (21/46) and 25% (3/12) for Asp906Gly. Despite the low penetrance, hypertrophy was severe in several heterozygotes. Two individuals with both mutations developed severe HCM. The velocities of actin translocation ($V_{\text{actin}}$) by mutant and wild-type (WT) myosins were compared in the in-vitro motility assay. Compared to WT/WT, $V_{\text{actin}}$ was 34% faster for WT/D906G and 21% for WT/L908V. Surprisingly $V_{\text{actin}}$ for L908V/D906G and K207Q/K207Q mutants were similar to WT. However, the apparent enhancement of mechanical performance with mutant/WT myosin was not observed for mutant/mutant myosin. This suggests that Vactin may be a poor predictor of disease penetrance or severity and that assays of power production may be more appropriate, or that the limited availability of muscle samples from
patients prohibits any definitive conclusions. Finally, severe HCM in heterozygous individuals occurs despite very low penetrance suggesting these mutations alone are insufficient to cause HCM and that uncharacterised modifying mechanisms exert powerful influences.

3.2.3.2 Introduction

HCM has been causally linked with gene mutations encoding various components of the sarcomere, including β-myosin heavy chain (β-MHC). Although expression of mutant proteins initiates a cascade of events ultimately leading to pathological left ventricular (LV) hypertrophy, a true mechanistic understanding of this cascade and its initiation are poorly understood. Simplistically, the extent to which a mutant protein’s function has been altered should correlate with the severity of the HCM phenotype and, therefore, characterizing a mutant protein’s functional capacity may aid in developing a mechanistic view of this hypertrophic response. However, variations in penetrance that occur for a given mutant protein, even within a given family, suggest that other factors make powerful contributions to the development of this disease.

As many as 20% of HCM patients possess one more missense mutation of the gene encoding β-MHC. Myosin is a hexameric protein consisting of two heavy chains and two associated light chains per heavy chain. Each heavy chain has a globular catalytic head domain that has actin binding, ATP hydrolytic and motor activities. Beyond the globular head, two heavy chains associate in an α-helical coiled-coil rod allowing the myosin molecule to polymerise into thick filaments within the sarcomere. Thus, each myosin molecule is a double-headed structure, where both heads are necessary for myosin to exhibit its maximal force and motion generation. In most cases, the point mutations are associated with only one β-MHC allele, leading to a patient that is heterozygous for the mutation such that 50% of the expressed myosin will be of the mutant form.

Although earlier reports suggest that diminished mutant myosin function was the primary cause for HCM, recent reports from our laboratory suggest that the same mutant myosins have enhanced function. These and other data led to numerous hypotheses regarding the aetiology of HCM. For example, LV hypertrophy may develop as a compensatory response to a mutant’s diminished function. Alternately, enhanced function could lead to the
hypercontractile state characteristic of HCM\textsuperscript{19,41} and may result in heterogeneous force profiles within the muscle along with depletion of sarcomeric energy reserve \textsuperscript{30,247} leading to myocyte injury, fibrosis and hypertrophy.\textsuperscript{248}

HCM patients with two mutant sarcomeric alleles have been identified by us and others.\textsuperscript{27,50,51,228,229,249} Such patients provide an opportunity to compare clinical phenotypes and mechanical capacity of mutant $\beta$-MHC associated with individuals having one or two mutant alleles. With two mutant alleles, expression of mutant myosin in which both heavy chains carry a mutation will exist, which may help define the impact of the mutation on myosin molecular function. In addition, the penetrance and severity of the disease may be gene dose dependent which would result in patients with a more severe clinical phenotype. Here we have identified several patients with point mutations in both $\beta$-MHC alleles. These patients were uniformly of a severe HCM phenotype. Mutant myosin prepared from biopsy of the upper portion of the biceps muscle obtained from these patients and family members were characterized for the effect of the mutation on myosin’s ability to move actin in a motility assay.

3.2.3.3 Methods

Patients and Genetic Analysis: Patients with HCM and their family members in pedigrees where two mutant MYH7 alleles had been identified were studied.\textsuperscript{27} Informed consent was obtained under protocols approved by the Institutional Review Boards of the NHLBI, (99-H-0065 and 98-H-0100) and the University of Vermont (IRB00000485).

Clinical studies and genetic analysis were completed as described in the section on General Methods. Disease penetrance (%) was defined as: (phenotype and genotype positive individuals/ genotype positive individuals)*100.

Biopsy and Myosin Preparation: Skeletal muscle myosin was isolated from samples of human biceps tissue (~10 mg) obtained through a small incision over the upper portion of the biceps muscle in the non-dominant arm under local anaesthesia. The biceps muscle is made up of about 40-60% slow fibres,\textsuperscript{250} which express the cardiac $\beta$-MHC isoform. Therefore, the isolated myosin will be a mixture of both normal and mutant heavy chains as well as fast and slow skeletal myosin isoforms. This concern was mitigated by comparison of motility results for myosins isolated from the biceps versus cardiac and coleus tissues (see Results). Myosin
purification from small tissue samples for use in the in vitro motility assay has been previously described in detail.39,224,251

The In-Vitro Motility Assay: Standard methods were used for the in vitro motility assay with special care taken to remove all non-functional myosin.39,252 This last step is critical so that the internal load contributed by non-functional myosin is eliminated. Actin prepared from chicken skeletal muscle was fluorescently labelled by incubation in tetramethylrhodamine isothiocyanate-phalloidin overnight.252,253 Assays were carried out at 30°C using a 30-μl chamber to which the following solutions were added and removed39: 1) 100 μg/ml myosin; 2) bovine serum albumin; 3) 1 μM unlabeled actin in actin buffer (25 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl2, 10 mM DTT, oxygen scavengers, pH 7.4); 4) actin buffer with 1 mM MgATP; 5) 6 x 30 μl washes with actin buffer; 6) 10 nM labeled actin; 7) 1 mM MgATP in actin buffer with 0.375 % methylcellulose. Step 3 and a comparable step in the initial myosin isolation procedure removes denatured, rigor-like, non-functional myosin that might act as a load to the free movement of actin filaments in the motility assay.39,254 Actin movement was visualized, recorded, and digitally analysed to determine actin filament velocity, \( V_{\text{actin}} \).

3.2.3.4 Results

Genetic Testing and Clinical Correlates of the Molecular Defects: Two HCM pedigrees with two mutant MYH7 alleles were studied. The mutant alleles were K207Q/K207Q in family A (figure 7) and L908V/D906G in family B (figure 8). The D906G is a novel mutant reported here for the first time. Mutants K207Q and L908V had been reported previously.27,31

In family A (Figure 13), the proband was homozygous for K207Q, a non-conservative mutation in a loop spanning the ATP-binding pocket. He had been diagnosed with HCM at the age of 47. Both parents are assumed to be heterozygous. His father died in his mid 40’s with a history strongly suggestive of HCM, but his mother died at the age of 92 years. The proband has survived to his 70’s, but his HCM had been complicated by chronic AF, multiple episodes of syncope and therapeutic interventions of a defibrillator-cardioverter device. Serial echocardiograms demonstrate progressive LV dilatation and systolic impairment and reduction in septal thickness, severe LA enlargement and the development of pulmonary hypertension. No other family member was homozygous and 9 were heterozygous, including a sister who was
subsequently diagnosed with HCM at the age of 80 years. She has a history of frequent presyncope and syncope and a septal wall thickness of 38 mm with mid ventricular obstruction. There was no history of hypertension. Eight other family members heterozygous for K207Q had not developed LV hypertrophy at the ages of 46, 43, 40, 18, 15, 14, 13, and 9 years (20% penetrance over the age of 18 years). Five of these individuals have a resting sinus bradycardia suggesting an alternative phenotypic expression (Table 1).

![Pedigree of Family with MYH7 Mutant K207Q.](image)

Figure 13. Pedigree of Family with MYH7 Mutant K207Q.
The proband is marked with an arrow. The numbering of individuals corresponds to that in the table below (Table 1).

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<td>IV-3</td>
<td>15</td>
<td>54</td>
<td>Normal</td>
</tr>
<tr>
<td>IV-4</td>
<td>13</td>
<td>44</td>
<td>Normal</td>
</tr>
<tr>
<td>IV-5</td>
<td>9</td>
<td>48</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Table 11. Cardiac Findings in individuals heterozygous for Beta Myosin Mutant K207Q.
For the second family (Figure 14), a four-generation pedigree was founded after two brothers from a family with HCM married two sisters from another family also with HCM. We established that each family had a different MYH7 mutation affecting the rod region of βMHC, a conservative substitution L908V and D906G, a charge changing substitution. Two descendants have both L908V and D906G and several dozen others have either L908V or D906G. Both L908V/D906G compound heterozygotes developed HCM, but the penetrance for L908V/WT was 46% (21/46), and 25% (3/13) for D906G/WT (P=ns). The two double heterozygotes both had prominent mid-LV cavity hypertrophy, moderate to severe LA enlargement and paroxysmal AF. LV phenotype in penetrant heterozygotes (both for the D906G and the L908V) varied from mild LV hypertrophy to severe LV hypertrophy with LV outflow obstruction. Sudden death and defibrillator discharges were also reported for several individuals with proven or obligate heterozygous genotypes.

![Figure 14. Pedigree with MYH7 Mutants D906G and L908V.](image)

Only family members with a mutant allele are shown; genotype negative individuals are excluded for clarity and to promote anonymity.

**The in vitro motility assay**: The effects of the MYH7 mutations on myosin mechanical function were assessed by characterizing skeletal muscle β-MHC from biceps muscles. This approach was similar to that previously used by ourselves and other investigators.39,232 The biceps
express β-MHC as well as fast skeletal myosin isoforms. To control for any differences in actin filament velocities resulting from differential expression of the fast skeletal, normal, and mutant β-MHC rather than an effect due to the mutant myosin itself, we compared the results from our previous studies of the L908V mutation isolated from both heart and soleus muscle biopsies to data for the L908V myosin from the biceps (Figure 15).39,232

![Figure 15. A Comparison of the In-Vitro motility of Mutant and Wild-Type Beta Myosin.](image)

The Motility Assay compares the velocity of actin translocation ($V_{\text{actin}}$) produced by mutant (L908V) and wild-type beta-myosin extracted from biceps, cardiac and soleus muscle biopsies are compared. Biceps and cardiac muscle myosins produce similar $V_{\text{actin}}$, suggesting biceps is a suitable source for β-myosin heavy chain. The cardiac and soleus data were published previously by the Vermont group.256.

Normal biceps β-MHC (WT/WT) from control samples moved actin with a $V_{\text{actin}}$ of 1.4±0.1 μm/s, whereas L908V/WT moved actin 21% faster (p<0.05, figures 3 and 4). The increased $V_{\text{actin}}$ for the L908V agrees qualitatively with our previous observation of faster $V_{\text{actin}}$ for the L908V when isolated from either heart (where no skeletal myosin is expressed) or soleus tissue samples (Figure 15).39,232 Thus, any confounding effects due to the known mixture of fast and slow
myosin isoforms that are normally expressed in the biceps\textsuperscript{250} are minimal, allowing the biceps to serve as a valid tissue preparation for assessing mutant myosin mechanical performance.

Enhanced $V_{\text{actin}}$ was also observed for D906G/WT $\beta$-MHC where $V_{\text{actin}}$ was 1.9 ± 0.1 μm/s or 34% faster than control (P<0.005, Figure 15). In contrast, L908V/D906G $\beta$-MHC had $V_{\text{actin}}$ similar to controls (1.5 ± 0.1μm/s). Thus the effect on $V_{\text{actin}}$ of the individual mutations do not appear to be additive and in fact may even cancel each other. A similar result was observed for the K207Q/K207Q mutation with $V_{\text{actin}}$ of 1.5 μm/s being similar to normal values. However, as myosin from a K207Q heterozygote was not available for comparison it is not possible to compare this double mutant with a K207Q/WT. It is important to note that due to the limited number of patients (L908V/D906G, n=2; K207Q/K207Q, n=1) it may not be possible to draw any statistical conclusions.

3.2.3.5 Discussion

The homozygote and double heterozygote patients in this and other reports have a clinical course characterized by either severe LV hypertrophy\textsuperscript{51,249} and/or progressive LV systolic dysfunction, severe LA enlargement and AF,\textsuperscript{36,27,50} suggesting a gene dose effect on the severity of phenotypic expression. The phenotypic progression to LV dilatation and systolic impairment is relatively uncommon, described in only about 10% of HCM patients.\textsuperscript{257,258} Animal models exist that demonstrate that the cardiac pathologies of the heterozygote and homozygote 'malignant' sarcomeric protein mutations result in significantly different cardiomyopathies.\textsuperscript{259,260} To our knowledge, no non-penetrant homozygote or double heterozygotes have been reported in humans or homozygous transgenic animal models, consistent with a view that gene dosage also affects penetrance.\textsuperscript{259,260}

Assaying myosin's molecular mechanics allows us to test the hypothesis that the extent of alterations to mutant myosin function may be related to the severity of the HCM cardiac phenotype. If so, then the clinical prognosis for HCM patients may be predicted based on readily measurable parameter of myosin molecular motor function. Therefore, we isolated myosin from the biceps muscle of patients with the following mutations: L908V/WT, D906G/WT, L908V/D906G and K207Q/K207Q and assessed their ability to propel actin filaments in the \textit{in vitro} motility assay. This assay serves as a molecular model system to
characterize the actomyosin interaction that relates to unloaded shortening in muscle. Of note, the L908V/WT and the D906G/WT mutations enhanced rather than compromised myosin’s unloaded velocity generating capacities. The L908V and D906G are located in the S2-segment of the myosin rod, far from the motor domain where ATP hydrolysis and actin binding occur. This raises the important question of how the mutations in the S2 segment can affect the mechanical performance of the motor domain. The potential for long distance communication between the catalytic site and domains either proximal or distal to this site are possible through structural elements within the myosin motor domain (for examples see review 261). Another explanation is that muscle myosin needs both heads to generate maximum force and motion245 and that coordination of the heads requires the region of the coiled-coil rod near the S2-segment to unwind or breathe.256 It is possible that the L908V and the D906G mutations exert their effect through alterations to head-head interactions by virtue of their effect on S2-segment flexibility.

With patients heterozygous for either the L908V or the D906G mutations, the predominant mutant myosin specie is a heterodimeric myosin with one normal and one mutant head, assuming random association of the β-MHC. Therefore, the 20-35% enhanced $V_{\text{actin}}$ for these mutations may not be the maximum possible effect. Indeed, myosin from a transgenic mouse homozygous for the R403Q HCM mutation had a 60% enhancement in $V_{\text{actin}}$ compared to a 16% increase for the heterozygote.224 On the basis of these findings and on the observed severe clinical phenotype, myosin from patients expressing both L908V and D906G in separate heavy chains was expected to alter $V_{\text{actin}}$ to a greater extent than myosin heterozygous for either mutation. However, $V_{\text{actin}}$ was similar to normal controls (Figure 16). A trivial explanation for the lack of an effect is due to the limited number of patients (n=2), which prevents any statistically definitive statements from being made. This, too, was the case for the single patient where both MYH7 alleles code for K207Q, a mutation affecting a surface loop that spans the entrance to the nucleotide-binding pocket. Therefore, the true effect of having a double mutation on myosin function cannot be determined at this time, although the severity of the clinical phenotype for these patients strongly suggests that a mutational effect does exist for these myosins. The paucity of data on double mutants is due to the apparent rarity of such
patients, and the probability that the same double mutations are present in other HCM pedigrees must be exceedingly low.

![Graph](image)

Figure 16. Comparisons of $V_{\text{actin}}$ for Wild-Type and Mutant Myosin.

* t-test significance at $p<0.05$.

In the present study, $V_{\text{actin}}$ was the only measured index of contractile function. However, the normal functioning of the heart is critically dependent on its ability to generate power (the product of force and velocity). Therefore, changes in $V_{\text{actin}}$ alone may not represent the fundamental molecular abnormality that ultimately results in the HCM phenotype. Power producing capacities for mutant myosins have yet to be determined in previous studies and it may be possible that a mutant myosin exhibits increased $V_{\text{actin}}$ yet generates either reduced or greater power than normal myosin. Finally, a more precise description of myosin power production may uncover how the primary insult in myosin function leads to the development of the HCM phenotype, which is more severe in patients with double β-MHC mutations.
3.3 **Non-Sarcomeric Causes of Hypertrophic Cardiomyopathy: Candidate Gene Approach for the Detection of Novel Mutations.**

Methods for the identification of new genetic causes of HCM include the Candidate Gene approach and Linkage Analysis. The candidate gene approach identifies genes that are likely, on the basis of known or presumed function of their protein product, to be involved in some aspect of the hypertrophic response. Thus, following the detection of a β-myosin mutation through linkage analysis, a candidate gene approach rapidly led to the detection of mutations in all other ‘candidate’ sarcomeric genes. Genes that regulate intracellular calcium concentrations, as well as those that are implicated in calcium-sensitive hypertrophic signalling are identified as candidate causes of HCM. The role of the products of these genes in mediating cardiac hypertrophy has been shown by a variety of in-vitro and transgenic animal studies. Publications and presentations derived from this work are tabulated below (Table 12).

<table>
<thead>
<tr>
<th>Scientific Sessions</th>
</tr>
</thead>
</table>

*Table 12. Published Research and Presentations of Findings: Non-Sarcomeric Mutations.*
3.3.1 IDENTIFICATION OF CANDIDATE GENES AND MUTATION DETECTION

3.3.1.1 Introduction

Intracellular calcium concentrations are tightly regulated. Release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) is an important signal transduction mechanism that plays a critical role in numerous cellular functions. In cardiac myocytes, excitation-contraction (E-C) coupling is a Ca\(^{2+}\)-dependent process; depolarisation opens voltage-dependent Ca\(^{2+}\)-channels (L-type or dihydropyridine receptors, DHPR), allowing a small influx of extracellular Ca\(^{2+}\) (\(I_{\text{Ca}}\)). \(I_{\text{Ca}}\) is insufficient to evoke full contraction, but triggers a larger release of Ca\(^{2+}\) from the SR through the ryanodine receptor (RyR), a process termed Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (CICR).\(^{262,263}\) CICR elevates [Ca\(^{2+}\)], to fully contracting levels, and the magnitude and duration of cardiac contraction depends to a great extent on CICR and the Ca\(^{2+}\) extrusion mechanisms that restore Ca\(^{2+}\) to resting levels. As Ca\(^{2+}\) is both the trigger and the output of RyRs, CICR is expected to be an all-or-none event. In intact cells, however, CICR is finely graded by \(I_{\text{Ca}}\), implying the existence of mechanisms that terminate CICR. Several agents, including FKBP12.6, sorcin and phospholamban modulate RyRs and CICR.\(^{70,262-264}\)

In addition to initiating cardiac contraction, Ca\(^{2+}\) mediates many intracellular signals including those responsible for cellular proliferation and cell death, which, in turn, lead to cardiac hypertrophy and heart failure.\(^{70,265-273}\) Experimental perturbation of several components involved in the control of cytoplasmic [Ca\(^{2+}\)] both in vitro and in vivo leads to cardiomyopathic states, characterized by LV hypertrophy, altered myocardial contractility, diastolic dysfunction, and arrhythmia.\(^{70,271}\) Cytosolic [Ca\(^{2+}\)] is reportedly elevated in certain models of cardiomyopathy, and in others the inhibition of Ca\(^{2+}\) mediated signals prevents cardiomyopathy.\(^{70,265-270,273}\)

3.3.1.2 Methods

Identification of Suitable Genes: A literature review identified several candidate genes, coding proteins implicated in myocellular calcium regulation or in calcium dependent hypertrophic signalling. The final selection from the large number of candidate genes thus identified was made according to (1) the prior description of cardiac hypertrophy in transgenic animal models, (2) the cardiac specificity of the gene expression, (3) the importance of a gene product's role in
calcium dependent signal transduction or calcium regulation, as inferred from in-vitro studies, and (4) on the basis of information available regarding the evolutionary conservation of the protein sequence.

Following the identification of candidate genes, the genomic DNA structure was established using BLAST and primer design, SSCP and sequencing was completed as described previously. Clinical investigations, including expanded family studies are also described above.

3.3.1.3 Results

Candidate Genes Studied: The genes identified for mutational analysis are listed in Table 13. Genomic DNA from either 100 or 200 unrelated HCM probands were screened for mutations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Cellular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RyR2</td>
<td>Cardiac or ryanodine type 2</td>
<td>Calcium channel, mediating CICR from the SR</td>
</tr>
<tr>
<td>SRI</td>
<td>Sorcin</td>
<td>Inhibitor of RyR and CICR</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
<td>Inhibitor of SERCA re-uptake of calcium into the SR</td>
</tr>
<tr>
<td>CABIN1</td>
<td>CABIN1</td>
<td>Inhibits calcineurin-mediated signal transduction</td>
</tr>
<tr>
<td>FKBP 12.6</td>
<td>FK506 binding protein 1B</td>
<td>Inhibitor of RyR and CICR</td>
</tr>
</tbody>
</table>

Table 13. Genes identified as candidate causes of HCM.

Included in this table are those involved in calcium signalling that have been identified with roles important in hypertrophic signalling. The concept of the hypertrophic circuit of the cardiomyocyte was introduced earlier in this thesis (Figure 3).

Mutation Detection: Coding changes affecting the amino acid sequence were detected in RyR2, PLN and SORCIN. RyR2 and PLN coding changes were subsequently identified as being present in alleles from control subjects (polymorphisms) or did not co-segregate with the disease in family members. A heterozygous SRI allele (coding sorcin F112L) was identified in two unrelated HCM patients, and was absent in the DNA from 198 other unrelated HCM patients and 200 unrelated control subjects.

Conclusion: Mutations in genes encoding this class of protein are unlikely to make important contributions as causes of HCM. Rarely, mutations in these genes may, however cause HCM,
and we have identified one such mutation for further study. As this class of proteins is implicated in hypertrophic cellular pathways, any polymorphisms identified are worthy of examination for their potential to modify disease expression.
3.3.2 **Sorcin Mutant (F112L)**
This work was undertaken in collaboration with Hector Valdivia’s laboratory in the Department of Physiology, University of Wisconsin.

The F112L sorcin mutation was detected at NHLBI. All subsequent clinical assessment, family screening, mutation detection/genotyping, smooth muscle cell culture and confocal microscopy were completed at or by NHLBI. Cloning, expression and mutagenesis of sorcin and functional assessment were performed by collaborators at the University of Wisconsin. Results from this collaboration were presented at the American Heart Association’s annual scientific Sessions (2002) and were awarded the Samuel Levine Young Investigator award (S A Mohiddin).

The publication of this sorcin variant has subsequently led to basic research efforts by other groups, including the development of a mutant animal model and the description of mutant effects in cardiac myocytes.274,275

### 3.3.2.1 Abstract

**Background:** HCM is considered a disease of the sarcomere. However, in many cases a sarcomeric mutation is not found, indicating diverse aetiologies. Transgenic studies identify important roles for Ca\(^{2+}\) signalling in hypertrophy. We determined whether F112L sorcin, a mutation affecting a protein that regulates Ca\(^{2+}\) release by the ryanodine receptor (RyR), was associated with HCM.

**Methods:** Family members of proband patients with mutations were clinically evaluated. We determined sorcin’s distribution in human tissue, and assessed mutant protein function.

**Results:** A missense mutation in sorcin resulting in substitution of a highly conserved amino acid (F112L) was identified in two unrelated subjects. F112L-Sorcin was significantly associated with an apical form of HCM (logarithm of odds (LOD) score 3.57) and hypertension (LOD score 3.68) in the two families. F112L-Sorcin was absent in unaffected family members and 200 control subjects. Sorcin was localized to cardiac myocytes, vascular smooth muscle cells, endothelial cells, and glomerular podocytes. The recombinant mutant F112L-sorcin displayed markedly attenuated conformational changes in response to Ca\(^{2+}\), a property that allows wild-type sorcin to translocate from the cytosol to membrane-bound targets (RyR). Wild type (WT)
Sorcin abolished RyR activity, whereas F112L-Sorcin had no effect. Finally, WT decreased the amplitude of Ca\(^{2+}\) sparks in ventricular myocytes, but F112L-sorcin had limited effects.

**Conclusion:** We describe for the first time the association of a sorcin mutation with HCM and hypertension. The F112L mutant lacks the inhibitory action of sorcin on RyR-mediated Ca\(^{2+}\) release. Our findings suggest that the F112L mutation alters myoplasmic [Ca\(^{2+}\)] by impairing modulation of RyRs by sorcin. In turn, this may account for some HCM and hypertension cases through several potential mechanisms. Sorcin may represent a novel target for pharmacotherapy of hypertension and heart failure.

### 3.3.2.2 Introduction

HCM demonstrates both non-allelic and allelic genetic heterogeneity, and results from one of more than a hundred mutations in genes encoding sarcomeric proteins.\(^4\)\(^1\) Identified molecular defects account for only half of the cases, and it is likely that non-sarcomeric genes may also be responsible. Non-sarcomeric causes of HCM are largely uncharacterised, and may be associated with distinct or compound phenotypes.

We identified a heterozygous SRI allele (coding sorcin F112L) in two unrelated HCM patients (see previous Chapter), and did not detect this allele in in the DNA of 198 other unrelated HCM patients and in 200 unrelated control subjects. We therefore investigated whether mutations in sorcin (chromosome 7q21.1), encoding a 22 kDa protein (198 amino acids) that regulates intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) are associated with human HCM.\(^16\)-\(^24\)

### 3.3.2.3 Methods

**Subjects.** Informed consent was obtained in accordance with a study protocol approved by the IRB of NHLBI. Following the initial detection of the F112L sorcin allele in two unrelated families, we sought to evaluate all family members for HCM and hypertension and all were genotyped. Clinical investigations included history, physical examination, 12-lead electrocardiography, echocardiography and, in some, CMR imaging. Individuals were either invited to the NIH Clinical Center in Bethesda, MD, or were evaluated during a field-trip. HCM was defined as a maximal LV wall thickness of 13 mm or greater in the absence of another explanation for the
increased wall thickness (>15 mm in patients with hypertension), presence of asymmetrical LV hypertrophy, and/or LV outflow obstruction. Hypertension was defined as systolic and diastolic blood pressures of greater than 160 mm Hg and 90 mm Hg, respectively.

**Genetic analysis:** The genomic structure of *sorcin* was determined using Basic Local Alignment Search Tool (BLAST) searches of genomic sequence databases, and PCR primers designed as described above. Amplifications of exon 5 (encoding the 112 phenylalanine amino acid residue) was performed using primers; 5'-ATCCTTAGAAATATCGAAATACAG-3' and 5'-ACTTCTACCAATGAATGGAGAGTTCTA-3'. PCR conditions were 35 cycles at 94 °C, 65 °C and 72 °C for 30 seconds each. Control DNA was screened using SSCP, and any anomalous conformers were sequenced (ABI Prism). The computer package for determining the association between phenotype and the F112I sorcin mutation was LINKAGE (Version 5.1).276

**Tissue preparation and Immunofluorescence:** Samples of control human renal artery and kidney were flash-frozen in dry ice and isopentane immediately upon surgical removal. Samples of the LV were obtained at autopsy from an adult subject without heart disease. All tissues were stored at −80 °C until use. Tissues were embedded in Tissue-Tek OCT compound (Ted Pella, Redding, CA), snap frozen in liquid nitrogen and serially sectioned in a cryostat at a thickness of 5-10 µm, placed on slides coated with 3-aminopropyltriethoxysilane and stored at −80 until processed.

Cultured human smooth muscle cells were also studied (37°C fetal bovine serum). Culture preparations were treated with thapsigargin following which cells were fixed at various time points. Thapsigargin increases cytosolic Ca by inhibiting the reuptake of Ca into the sarcoplasmic reticulum (SR).277

For immunolabelling the human tissue samples we used the following primary antibodies singly or in combination; rabbit polyclonal anti-sorcin278; monoclonal anti-sorcin (Zymed, CA); monoclonal anti-Von Willebrand factor-VIII (Dako, CA); monoclonal anti-smooth muscle alpha-actin (Dako, CA); and monoclonal anti-synaptopodin (Research Diagnostics, NJ). Sections were incubated with primary antibodies overnight at 4 °C, and sections were then incubated for 1
hour at room temperature with the appropriate FITC- and/or rhodamine conjugated anti-rabbit and mouse secondary antibodies (Vector, CA). For negative controls, the primary antibody was replaced by normal IgG. Sections were mounted using Vectashield mounting medium with nuclear counter stain (Vector laboratories) and examined with a laser scanning confocal fluorescence microscope (Leica TCS-4D DMIRBE, Germany).

Cultured smooth muscle cell preparations were labelled with similar primary antibodies, nuclear stains, and with ruthenium red (Sigma) to label the SR.

**Structural modelling of sorcin:** The structural model of the Ca$^{2+}$-binding domain of sorcin (residues 33-198) is based on the homology of sorcin with other members of the penta-EF hand family of proteins. Mutant and wild-type models were generated (Insight II-Biopolymer software, Biosym Technologies). A Richardson style rendering of the protein secondary structure was produced (WebLab viewer, Biosym Technologies).

**Planar bilayer technique:** Recording of single RyRs in lipid bilayers was performed as described previously. Briefly, a phospholipid bilayer of PE:PS was formed across an aperture of ~200 μm diameter in a delrin cup. The cis chamber (900 μl) was the voltage control side connected to the head stage of a 200A Axopatch amplifier, while the trans chamber (800 μl) was held at virtual ground. Both chambers were initially filled with 50 mM cesium methanesulfonate and 10 mM Tris/Hepes pH 7.2. After bilayer formation, cesium methanesulfonate was raised to 300 mM in the cis side and 100 to 200 μg of SR vesicles were added. After detection of channel openings, Cs’ in the trans chamber was raised to 300 mM to collapse the chemical gradient. Single channel data were collected at steady voltages (-30 mV) for 2-5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10 kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5-2 kHz. Data acquisition and analysis were done with Axon Instruments software and hardware (pClamp v8.0, Digidata 200 AD/DA interface).

**Cloning, expression and mutagenesis of sorcin:** The human sorcin gene was obtained from ATCC (New York, NY) and amplified by PCR from the clone YH18DO8.RI to introduce Xhol sites. The PCR product was then subcloned into pGEX-5x-3 (Pharmacia) using standard procedures. The mutant F112L-sorcin was constructed according to the manufacturer’s instructions
Wild-type sorcin and F112L-sorcin were expressed in *E. coli* BL21 (Promega) and then purified in a Glutathione Sepharose column, also according to manufacturer's instructions. The recombinant proteins were cleaved from the column with Factor X activated (Novagen) at room temperature overnight.

**Isolation of cardiac cells and $\text{Ca}^{2+}$ imaging:** Ventricular cells were isolated by collagenase digestion of perfused mice hearts. Cells were suspended in Tyrode medium and kept at room temperature until used, usually within 6 hours. The cardiac myocytes were permeabilized with saponin (0.01 % for 45-60 s) in an internal solution containing (mM): 120 potassium aspartate, 3 MgATP (free [Mg$^{2+}$] = 1 mM), 10 phosphocreatine, 5 U ml$^{-1}$ creatine phosphokinase, and 8% dextran, pH 7.2. For measurements of intracellular [Ca$^{2+}$], myocytes were perfused with the internal solution described above plus 30 μM Fluo-4 salt and EGTA/CaCl$_2$ ratios set to yield 100 nM to 300 nM free [Ca$^{2+}$]. Free [Ca$^{2+}$] was calculated according to equilibrium constants given by Fabiato using a computer program (MaxC, Stanford University, CA) and verified with a Ca$^{2+}$ electrode. Saponin and sorcin were applied onto the cells with a perfusion system that uses a fast step three-channel micropipette (Warner Instruments). Confocal images (BioRad MR-1) were recorded with a plan apofluor 60X oil immersion objective. The scan line was oriented along the long axis of the cell. Fluo-4 was excited at 488 nm, with emitted fluorescence measured at 515 nm. Ca$^{2+}$ transients were reconstructed by stacking 512 consecutive line scans and performing a time-intensity plot using IDL 5.4 software. [Ca$^{2+}$], was calculated using a pseudo-ratio method assuming a $K_a$ and resting [Ca$^{2+}$] of 1.1 μM and 100 nM, respectively. Ca$^{2+}$ spark frequency, amplitude and intensity were measured with a computer program running in IDL software. All experiments were performed at room temperature (21-23 °C).

**Ca$^{2+}$ titration of sorcin:** As other members of the penta-EF hand family of proteins, sorcin undergoes structural changes with [Ca$^{2+}$]. The structural changes were monitored by measuring the intrinsic fluorescence of the protein. Direct fluorescence titrations of sorcin were carried out in PBS buffer, 1 mM EGTA and different CaCl$_2$ concentrations to yield the free [Ca$^{2+}$] specified in Fig 4. The emission spectrum of sorcin (1-3 μM) in the range of 303 to 400 nm was monitored with a CPS-220 spectrofluorometer (Photon Technology International) at room temperature.
3.3.2.4 Results

Genetic Analysis: The missense mutation in exon 5 of sorcin (Figure 17a, b), resulting in substitution of leucine for phenylalanine at amino acid residue 112 (F112L-Sorcin), was not detected in 200 unrelated control subjects. F112 is an invariant residue in a completely conserved region of sorcin that forms a helix-loop-helix motif known as an EF hand (Figure 17c). No other missense or nonsense coding abnormalities were detected.

Members of the two affected families were screened for HCM and F112L-Sorcin, and are designated family A, of European and Native American origins, and family B, which is African-American (Figure 18). Clinical data was available in 9 of 10 family members with F112L-Sorcin. Eight of the 9 subjects were noted to have HCM, several with an apical distribution of LV hypertrophy (Figure 19). The electrocardiogram was abnormal in 6 of the 9 family members with F112L-Sorcin, including a junctional tachycardia in one subject. All of the 8 patients with cardiomyopathy had a history of hypertension. The one family member with the F112L-Sorcin but without LV hypertrophy was hypertensive. Two members of the family, without this mutation, aged greater than 50 years, also had hypertension, but neither had LV hypertrophy by echocardiography. The mutation was not present in any family member without hypertension, and was present in all family members with LV hypertrophy.
Figure 17. The Detection of a Novel Mutation in Sorcin, a Candidate cause for HCM.

Panel A. PCR products of exon 5 in an affected family member have anomalous electrophoretic mobility (arrow).
Panel B. The corresponding pherogram illustrates the T to C transition at codon 112 in exon 5 (arrow) resulting in substitution of leucine for phenylalanine. Forward (F) and reverse (R) sequencing is shown in the top 2 rows, and WT sequence is shown in the lowest row. Panel C. Human and hamster sorcin are 95% similar. Substitution F112L is at an invariant residue in a completely conserved sequence forming the helix-loop-helix motif of the third of sorcin’s five EF hands.
Figure 18. Genotype and Phenotype of two Families with Mutant Sorcin (F112L).

Panels A, B and C. Short axis and four-chamber two-dimensional echocardiographic views, respectively, illustrating apical hypertrophic cardiomyopathy in the patient III-1 (kindred B). Panels D, E, and F are cardiac MRI images from patient III-6 (kindred A); D is a long axis view in end-diastole, and E and F are short axis end-diastolic and end-systolic images demonstrating severe hypertrophy with almost complete obliteration of the LV cavity.
**F112L-Sorcin is associated with cardiomyopathy and hypertension:** HCM has a prevalence of about 0.1 - 0.2% in the general population and the apical variant is even less common. Assuming a phenocopy rate of 1% and a disease penetrance of 90%, (spouses coded unknown), the logarithm of odds (LOD, see later section) score for the association of the F112L-Sorcin and cardiomyopathy was 3.48 for family A (p<0.0001), and 3.57 for both families combined (p<0.00005). To examine the association of hypertension with F112L-Sorcin, variable phenocopy rates were used; 1%, 3%, 5%, 7%, and 14% for the age groups of 29 years or less, 30-49 years, 50-59 years, 60-69 years, and 70-79 years, respectively. Assuming a disease penetrance of 90% (spouses coded unknown) the association between the F112L-Sorcin and hypertension in family A was 3.65, and 3.68 for both families combined (p = 0.00002).

**Sorcin is present in human cardiac myocyte, smooth muscle cells of arteries, podocytes of renal glomeruli, and cardiac myocytes:** Using immunoblot analysis with polyclonal and monoclonal antibodies, we determined that sorcin was present in human heart, renal artery and kidney tissues (data not shown). Immunocytochemistry indicated that sorcin is confined to a peripheral, sarcolemmal location of cardiac myocytes where it may co-localize with both RyR and DHPR (Figure 20). In contrast, sorcin was evenly distributed throughout the cytoplasm in smooth muscle cells, endothelial cells, and podocytes of the glomerulus (Figure 20). Sorcin was much more abundant in the smooth muscle cells of arterioles than in larger conduit arteries, suggesting its greater importance in resistance-sized vessels. Endothelial cells of the glomerulus did not contain sorcin, in contrast to endothelial cells of the arterioles. In cultured smooth muscle cells, a dynamic and dramatic alteration in the intracellular distribution of sorcin was demonstrated following treatment with thapsigargin (Figure 21).
Figure 20. Distribution of Sorcin in Human Heart and kidney.

A: Human left ventricle. (1) Sorcin (labelled green) is confined to a sarcolemmal, or at least peripheral, location in cardiomyocytes (2). Endothelial cells (arrow) from an intracardiac capillary contain both factor VIII (green) and sorcin (red). (3) A small intracardiac artery is shown in cross section. Sorcin (red) is present in media smooth muscle cells and endothelial cells (labelled green for factor VIII).

B: Human renal artery. (1) Endothelial cells labelled with factor VIII (red) of the medium sized renal artery are weakly positive for sorcin (green). Nuclei are stained blue. The media smooth muscle cells are also weakly positive for sorcin. (2) In contrast, smooth muscle and endothelial cells of a resistance-vessel sized adventitial arteriole (arrow) in the same renal artery are strongly positive for sorcin (green, with nuclei stained red). (3) An adventitial artery at higher magnification.

C: Human renal glomeruli. (1) Sorcin (red) is confined to podocytes identified by dual labelling with anti-synaptopodin antibody (green); (2) In contrast to the endothelial cells in panels A and B, glomerular endothelial cells labelled for factor VIII (green) do not express sorcin (red). (3) The vascular pole of a glomerulus demonstrates the distribution of sorcin (red) in the afferent/effenter arterioles and in the podocytes.
Figure 21. Intracellular Localization of Sorcin in Cultured Vascular Smooth Muscle Cells.

Sorcin (labelled green) is localized in cultured coronary smooth muscle cells before and at various time points following treatment with thapsigargin (TG). At baseline, sorcin is confined to the cytosolic compartment where it appears to be evenly distributed. Eighty seconds following treatment with TG, cytosolic sorcin appears in spark-like concentrations, with this appearance persisting for at least three minutes. At 300 seconds, cytosolic sorcin is prominently arranged in a striated arrangement, thought to represent co-localization with the endoplasmic reticulum (ryanodine staining not shown). Sorcin is now also detected in the nuclear compartment, suggesting a role for sorcin in nuclear signalling. By 10 minutes, Sorcin appears to have largely returned to a baseline distribution, thought some cells still demonstrate sorcin content.

Predicted changes in the 3-D structure of sorcin induced by the F112L mutation: Sorcin shares remarkable similarity with other members of the penta EF-hand family of proteins. The canonical EF-hand is a helix-loop-helix motif that coordinates Ca$^{2+}$ with high affinity and is typically recognized in analyses of the protein primary structure. In sorcin, the five EF-hand motifs encompass the majority of the protein (amino acids 33-190) and are proposed to adopt a three-dimensional arrangement that is similar to that of calpain, another member of the penta EF-hand family. We used the crystallographic coordinates of the structurally conserved regions of calpain to model the spatial arrangement of the five EF-hand motifs of sorcin. Crystallographic data have now confirmed the validity of the model. Sorcin's F112 is located in the third EF-hand, at the end of the first helix and before the beginning of the Ca$^{2+}$-binding loop (Figure 17 and Figure 22). The net effect of the F112L mutation is the replacement of the 7-carbon alkyl group of phenylalanine that includes a planar phenyl ring by the 4-carbon...
alkyl group of leucine. In the model, F112 stabilizes the third EF-hand by linking the first with the second helices through hydrophobic interactions. In energy minimization runs, the nonpolar core of the phenyl ring cannot be replaced by the shorter alkyl group of leucine, leading first to destabilization of the third EF-hand, followed by repositioning of the adjacent EF-hands. This cascade of effects finally results in a thermodynamically stable F112L-sorcin (Figure 22) that is radically different from WT-sorcin. Thus, the amino acid F112 appears to contribute importantly to the tertiary structure of sorcin.

![Figure 22. Three-Dimensional Structure of Wild-Type and F112L Mutant Sorcin.](image)

All images show a Richardson style rendering of secondary structure, with rods corresponding to α-helices, arrows to β-sheets, and ribbons to random coil. (A) Minimal structure of WT-sorcin. The phenylalanine residue at position 112 is represented as a CPK structure. (B) F112L-sorcin before energy minimization. Note the gap between α-helices D and E of the third EF-hand resulting from the absence of the phenyl group of phenylalanine. (C) F112L-sorcin after energy minimization. Note the severe destabilization of the protein's original conformation.

*Effect of the F112L sorcin mutation on Ca^{2+}-induced conformational changes:* Sorcin undergoes conformational changes upon binding Ca^{2+}. In the absence of Ca^{2+}, sorcin is largely soluble and likely to be located in cytosolic compartments. Ca^{2+} binding causes structural rearrangements that expose large hydrophobic surfaces, allowing sorcin to interact...
with membrane-bound target proteins. These Ca\(^{2+}\)-dependent transitions are proposed to constitute a signalling mechanism that prompts sorcin to exert its cellular effects: \textit{in vitro}, these transitions are detected as reductions of the protein's intrinsic fluorescence.\textsuperscript{284,285} Figure 23 (A) shows that increasing [Ca\(^{2+}\)] gradually quenches the intrinsic fluorescence of WT-sorcin. Similar to hamster sorcin recombinant human WT-sorcin also undergoes structural reorganization that exposes tryptophan residues to solvent, decreasing its intrinsic fluorescence.\textsuperscript{279} A plot of fluorescence maximum (330 nm) as a function of [Ca\(^{2+}\)] yields a sigmoid relationship (Figure 23 B, inset) with ED\(_{50}\)(Ca) (the [Ca\(^{2+}\)] that causes half-maximal fluorescence quenching, = 2.3 \(\mu\)M).

In sharp contrast, F112L-sorcin has an intrinsically higher fluorescence intensity/nmol protein (Figure 7B), displays smaller changes in absolute fluorescence, and a lower [Ca\(^{2+}\)] is needed to transition the protein (ED\(_{50}\)(Ca) = 0.12 \(\mu\)M). Thus, the F112L mutation appears to reduce sorcin's plasticity, an effect likely to hinder its response to intracellular Ca\(^{2+}\) signals.

**Figure 23.** Fluorescence Emission Spectra of Wild-Type and F112L-sorcin.

(A) Quenching of the intrinsic fluorescence of WT-sorcin (A) and F112L-sorcin (B) by increasing concentrations of Ca\(^{2+}\). (C) Normalized fluorescence emission at 330 nm for WT-sorcin and F112L-sorcin. Data points were fitted with the equation \(E = E_{\text{max}} / [1 + ([\text{Ca}^{2+}]/IC_{50})]\), where \(E\) is fluorescence emission at 330 nm and IC\(_{50}\) is the [Ca\(^{2+}\)] necessary to produce half-maximal fluorescence quenching (WT-sorcin = 2.8 \(\mu\)M; F112L-sorcin = 200 nM). Results are representative of 6 experiments with less than 10% error.

**Effect of F112L-sorcin on RyR:** We tested the ability of F112L-sorcin to modulate cardiac RyRs. Figure 24A and B show traces from continuous recordings of cardiac RyRs reconstituted in planar lipid bilayers (at +40 mV and activated by ~5 \(\mu\)M cytosolic [Ca\(^{2+}\)]). In the absence of
sorcin, the channel switches between periods of reduced and high activity, which is reflected in the diaries of channel activity (Figure 24 E and F) as sweeps of high $P_o$ alternating with low $P_o$. Cis (cytosolic) addition of 1 μM and 2 μM WT-sorcin, severely reduced and completely abolished, respectively, bursts of high $P_o$, producing marked inhibition of channel activity (Figure 24 C and E), as reported previously. By contrast, similar and higher concentrations of F112L-sorcin failed to suppress the bursts of channel activity (Figure 24D), producing little effect on overall channel $P_o$ (Figure 24 F). Thus, F112L-sorcin is incapable of reproducing the effect of its parent molecule. The structural disarray of F112L-sorcin has a functional correlate.

Figure 24. Effect of Wild-Type and F112L Sorcin on Cardiac Ryanodine Channel Openings.
Ryanodine Channels are reconstituted in lipid bilayers. Single-channel openings are shown as upward deflections in all panels. Cs$^+$ is the charge carrier and flows from the trans (luminal) to the cis (cytosolic) side of the channel. Holding potential: -40 mV. Scale bar is 200 ms (x-axis) and 30 pA (y-axis). (A) and (B) RyR activity under a constant [Ca$^{2+}$] of ~5 μM before (labelled “control”) and ~1 min after the addition of the specified concentration of WT-sorcin (C) or F112L-sorcin (D). Traces in each panel are from a single experiment, which is representative of 6 (WT-sorcin) and 4 (F112L-sorcin) experiments. (E) and (F) are bar representations of the open probability ($P_o$) integrated every 1000 milliseconds. Average $P_o$ under each condition is given in the text.
**Cellular effects of WT-sorcin and F112L-sorcin:** Intracellular Ca\(^{2+}\) waves and Ca\(^{2+}\) sparks, the elementary Ca\(^{2+}\) release events are manifestations of RyR activity in whole cells.\(^{263,291}\) WT-sorcin and F112L-sorcin were perfused onto permeabilized cardiac ventricular myocytes to compare their ability to modulate Ca\(^{2+}\) waves and Ca\(^{2+}\) sparks. To increase the frequency and duration of spontaneous Ca\(^{2+}\) release events, cells were loaded with Ca\(^{2+}\) prior to permeabilization by pacing them at 1 Hz for 30 s. Permeabilized cells were also perfused with an internal solution containing 150 nM free Ca\(^{2+}\) instead of the normal 80-100 nM Ca\(^{2+}\). (4) Increasing Ca\(^{2+}\) load sensitises RyRs to cytosolic Ca\(^{2+}\),\(^{292}\) and the elevated \([\text{Ca}^{2+}]_{\text{c}}\) in the internal solution drives RyRs closer to threshold. Under these conditions, mouse ventricular myocytes display several foci of Ca\(^{2+}\) release readily detected by line-scan confocal microscopy (Figure 25 A, control). These "hot spots" produce Ca\(^{2+}\) release events that propagate along the cell's longitudinal axis without colliding with Ca\(^{2+}\) release events from neighbouring sites. These propagating "Ca\(^{2+}\) wavelets" have a greater amplitude and duration than normal Ca\(^{2+}\) sparks but lower than overt Ca\(^{2+}\) waves.

Perfusion of ventricular myocytes with 1 μM WT-sorcin blunts Ca\(^{2+}\) wavelets and turns them into discontinuous Ca\(^{2+}\) release events (Figure 25 A, WT-sorcin). The modified release events resemble isolated Ca\(^{2+}\) sparks indicating that WT-sorcin restrains RyR activity. These results are in line with the inhibitory effect of WT-sorcin observed on reconstituted RyRs. By contrast, cells perfused with an identical amount of F112L-sorcin exhibit discrete Ca\(^{2+}\) wavelets, although their intensity and periodicity are lower than control (Figure 25 B). While WT-sorcin decreases the average intensity of Ca\(^{2+}\) wavelets from 2.17 to 1.43 F/Fo, F112L-sorcin decreases it from 2.08 to 1.82 F/Fo. Thus, F112L-sorcin may partially block Ca\(^{2+}\) release events in whole cells, in contrast with its complete lack of effect on RyR activity in bilayer experiments.
Figure 25. Effect of Wild-Type and F112L Sorcin on Ca\textsuperscript{2+} Wavelets and Ca\textsuperscript{2+} Sparks.

Left panels are line-scan images of permeabilized cardiac ventricular myocytes before (labelled “control”) and after perfusion with 1 \textmu M WT-sorcin (A) or 1 \textmu M F112L-sorcin (B). Bar scale: x ms (x-axes) and x \textmu m (y-axes). Right panels are surface plots of Ca\textsuperscript{2+} levels of the corresponding figure.

3.3.2.5 Discussion

We have undertaken a candidate-gene approach to determine whether mutations affecting a protein that regulates Ca\textsuperscript{2+} release from intracellular stores are associated with HCM. Numerous transgenic studies have established the importance of similar proteins in cardiac hypertrophy\textsuperscript{41,70,271} This study is the first to determine that abnormalities in this class of genes are associated with HCM, and the first to establish a role for sorcin in human disease.

We report that a sorcin missense mutation (F112L) is associated with an unusual (apical) form of HCM and systemic hypertension in two families. We also demonstrate that sorcin is present in endothelial cells, vascular smooth muscle cells, cardiac myocytes and podocytes of the renal glomerulus. Ca\textsuperscript{2+} dependent signalling has been shown to mediate several central behaviours of these cell types, and sorcin may therefore play a significant role in the modulation of Ca\textsuperscript{2+}
signalling in critical components of the cardiovascular system. The mutant (F112L-Sorcin) also has a reduced capacity to inhibit RyRs; we hypothesize that F112L-Sorcin precipitates a chain of events that result in the cardiovascular alterations observed in these individuals.

**Association of F112L sorcin mutation with HCM and hypertension:** The causality of F112L-sorcin for the HCM phenotype is supported by the following: (a) highly significant co-segregation of the phenotype with the mutation; (b) presence in affected members of two unrelated families from different genetic backgrounds, (c) absence in all unaffected members of both families, (d) absence in 400 chromosomes from control subjects, and (e) the fact that F112 is highly conserved across several species, reflecting its functional significance. Hypertension, which also co-segregated with F112L, could also account for cardiac hypertrophy. Hypertension is typically associated with symmetrical LV hypertrophy, whilst the affected patients in our study mostly had asymmetric septal hypertrophy or the uncommon apical HCM variant. While hypertension has been associated with asymmetric LV hypertrophy, apical LV hypertrophy is not described in large studies of hypertensive patients.293,294 Additionally, fewer than half of hypertensive patients develop LV hypertrophy.293,295 It is therefore likely that the severity of LV hypertrophy and its distribution are due, at least in part, to the cardiac expression of F112L. As with most molecular defects that have been linked with HCM, phenotypic penetrance associated with sorcin is incomplete suggesting that other environmental and genetic factors may also be important in determining disease expression.

**F112L-sorcin, RyR and DHPR:** Sorcin is a Ca\(^{2+}\)-binding protein (K\(_{D, Cao}\) ~ 1 \(\mu\)M) member of the penta EF-hand group of proteins.279 In cardiac myocytes, sorcin localizes to the sarcolemma and the dyadic junctions, where DHPRs are in close proximity to RyRs.278 In *in-vitro* experiments, sorcin is able to bind to RyRs and to reduce its \(P_o\) with a \(K_d\) ≤ 1.0 \(\mu\)M.(19) Under conditions of low Ca\(^{2+}\), sorcin dimerizes and remains soluble in the cytosol. In high [Ca\(^{2+}\)] sorcin’s EF hands bind Ca\(^{2+}\) and conformational changes expose hydrophobic residues following which sorcin translocates from soluble (cytosol) to membrane-bound target proteins, presumably RyRs.284,285,287 It is hypothesized that sorcin dissociates from RyRs when [Ca\(^{2+}\)] is low, re-associating after CICR when [Ca\(^{2+}\)] is high. In such a scenario, sorcin’s [Ca\(^{2+}\)]-dependent inhibition of RyRs provides a restraint for CICR. Additionally, sorcin has been found to bind to the pore-forming subunit of the cardiac DHPR, albeit with unknown functional
consequences. Given the proximity of DHPRs and RyRs in the dyadic junction, it is tempting to speculate that Ca\(^{2+}\)-dependent translocation of sorcin mediates inter-channel communication, modulating DHPR voltage sensitivity according to Ca\(^{2+}\) release from intracellular stores.

We propose that the F112L-Sorcin mutation fails to modulate RyRs by disrupting sorcin’s third EF hand and impairing Ca\(^{2+}\) responsiveness. In support of this hypothesis we have shown that activity of isolated RyR are unaffected by F112L-Sorcin and that the formation of Ca\(^{2+}\) waves in cardiac myocytes is minimally affected by F112L-Sorcin. Sorcin’s dose-dependent effect on RyR activity (Figure 23 and Figure 24) suggests the pathologic mechanism is haploinsufficiency. Alternatively, mutant sorcin may have a dominant negative effect; for example by competing with WT for Ca\(^{2+}\) or by dimerizing with WT and inhibiting its response to increases in [Ca\(^{2+}\)].

Further studies are necessary to determine interactions of WT-sorcin with F112L-sorcin and effects of sorcin on DHPRs. Sorcin’s role in cardiac E-C coupling, endothelial or smooth muscle cell vasomotor control, glomerular function, and in other aspects of Ca\(^{2+}\) signalling are unknown, but its distribution and the mutant’s known functional consequences suggest potential mechanisms by which F112L sorcin results in cardiomyopathy and hypertension.

**F112L-sorcin and cardiomyopathy:** Alterations of E-C coupling and Ca\(^{2+}\) handling are common findings in animal models of cardiac hypertrophy and in hypertrophic and failing human hearts.\(^70,265-270\) This study suggests that F112L-sorcin, by improperly regulating CICR, may be directly responsible for cardiac hypertrophy. We confirm that sorcin is present in cardiac myocytes, and demonstrate that F112L-Sorcin is associated with apical or asymmetric HCM and is incapable of modulating the activity of isolated RyRs. Net alteration in Ca\(^{2+}\) transients may account, at least in part, for the LV hypertrophy, as the frequency and amplitude modulation of local Ca\(^{2+}\) release events play a fundamental role in controlling several cell functions which include hypertrophy, hyperplasia, and cell death.\(^267,270\) Additionally, hypertension may contribute to the cardiac phenotype.

**F112L-sorcin and hypertension:** The demonstration that sorcin is present in the smooth muscle cells of smaller arterioles, endothelium and glomerulus, suggests several roles in blood pressure regulation, through effects on vasomotor tone and/or intravascular volume.
a) **Vascular smooth muscle:** Local and global increases in smooth muscle cell \([\text{Ca}^{2+}]\) control myogenic tone\(^{297,298}\), determining systemic vascular resistance. Abnormal regulation of DHPR or RyR by F112L-sorcin may impair \([\text{Ca}^{2+}]\) homeostasis in arterial smooth muscle cells, resulting in hypertension. Incomplete inhibition of RyRs by F112L-sorcin may prevent quenching of CICR resulting in global \([\text{Ca}^{2+}]\) elevation and vasoconstriction.

(b) **Modulation of endothelial function:** Endothelial cells are actively involved in controlling arterial vascular tone through production of several vasoactive substances including endothelin and nitric oxide\(^{298,299}\). The presence of sorcin in endothelial cells is a novel and intriguing finding of this study and suggests that sorcin may, through \([\text{Ca}^{2+}]\)-dependent mechanisms, play a role in endothelial cell-mediated vasomotor control.

(c) **Renal control of blood pressure.** Sorcin is markedly expressed in podocytes and in the afferent and efferent glomerular arterioles (Figure 20). Podocytes contribute to the integrity and regulation of the filtration barrier of the glomerulus\(^{300}\). Their foot processes contain a contractile apparatus that responds to vasoactive substances like angiotensin II and endothelin. These lead to an increase in intracellular \([\text{Ca}^{2+}]\) by opening intracellular \([\text{Ca}^{2+}]\) stores\(^{300,301}\). Abnormalities of podocyte function or in afferent/efferent arteriolar tone may result in hypertension.

### 3.3.2.6 Summary

A missense mutation of a conserved residue of sorcin (F112) reduces its ability to modulate RyRs. F112L-sorcin mutation is associated with apical HCM and hypertension. HCM may result from the expression of F112L-sorcin in ventricular myocytes, while hypertension may result from dysfunctional sorcin activity in vascular and glomerular cells. Our findings establish sorcin as a cause of non-sarcomeric HCM, propose functions for this protein in cardiovascular and other organs, and may lead to novel therapeutic targets for the therapy of hypertension, cardiac failure and LV hypertrophy. The association of HCM with sorcin suggests mutations in similar genes may also cause human HCM and our findings may represent a template for a novel and distinct form of hypertension with cardiomyopathy.
3.4 **Linkage Analysis for the Detection of Novel Genetic Causes of Hypertrophic and Dilated Cardiomyopathy.**

Large pedigrees with a hereditary disease where the clinical features are unusual or unique may be suitable for genetic approaches to identify the genetic locus linked with the disease. Linkage analysis associates any given genetic locus (each locus is usually a polymorphic region within the genome, such as a microsatellite marker or a single nucleotide polymorphism) a hereditary trait. Meiotic crossover creates allele recombinants; the probability that a known locus on a chromosome is inherited along with an unknown disease causing gene decreases the further apart these two locations are on a chromosome, and with an increasing number of meioses. Gene sequences that are close together on the same chromosome are linked; a map comprising of a series of markers across the genome in each member of a pedigree can be analysed for the probability that any given marker is linked with the disease. A logarithm (power of 10) of odds score (LOD) describes this relationship. A LOD score of 3 indicates the chances of a marker not identifying the disease causing locus as less than 1 in 1000. Each locus includes several genes, and following the identification of a disease associated locus, these genes are examined for mutations. The power of Linkage analysis may be compromised by incomplete penetrance and by multiple heterozygosity, two phenomena now known to complicate the development of familial HCM. Similarly, phenocopy (similar disease caused by a different aetiology) also challenges Linkage analysis. The prevalence of hypertension and heart failure in unselected populations remain hazards for cardiac evaluation and subsequent linkage of cardiac phenotypes with LVH or LV dilation with genetic maps. Estimated phenocopy rates are thus incorporated into most Linkage analyses.

The resource demands required for genome-wide scanning for Linkage analysis were unavailable in our laboratory. Collaborations with larger laboratories were developed when suitable pedigrees were identified, expanded and characterised by our section. Publications and presentations derived from this and related research is presented in the table below (Table 14).
Scientific Sessions


Gene Sequences

  - Homo Sapiens nebulin-related anchoring protein isoform S (NRAP) mRNA, complete cds. Accession AY177620.
  - Homo Sapiens nebulin-related anchoring protein isoform C (NRAP) mRNA, complete cds. Accession AY177621.
  - Mus Musculus nebulin-related anchoring protein isoform S (NRAP) mRNA, complete cds. Accession AY177622.
  - Mus Musculus nebulin-related anchoring protein isoform C (NRAP) mRNA, complete cds. Accession AY177623

Peer Reviewed Articles


Table 14: Published Research and Presentations of Findings: Linkage Analysis.
3.4.1 Association of Hypertrophic Cardiomyopathy, Sensorineural Deafness, and a Mutation in Unconventional Myosin VI (MYO6)

This work involved collaboration with the Section on Human Genetics, Laboratory of Molecular Genetics, and the Hearing Section, Neuro-Otology Branch and Section on Gene Structure and Function, Laboratory of Molecular Genetics of the National Institute on Deafness and Other Communication Disorders (NIDCD), NIH.

Clinical assessment family screening and DNA collection included a field trip to study family members. Cardiac evaluation was completed by our section. Audiometric assessment and linkage analysis was performed by NIDCD.

3.4.1.1 Abstract

Introduction: Familial hypertrophic cardiomyopathy (HCM) is typically confined to a cardiac phenotype and is caused by mutations in genes encoding sarcomeric proteins. Occasionally HCM may be one component of a hereditary multi-system disorder. Sensorineural hearing loss (SNHL) is similarly genetically heterogeneous. Mutations in the MYO6 gene, encoding unconventional myosin VI, have been found to cause nonsyndromic SNHL, i.e. SNHL in the absence of any other related clinical features.

Methods and Results: Thirty-six members of a kindred in which autosomal dominant SNHL co-segregates with HCM were evaluated by history, physical exam, electrocardiography, echocardiography and audiometry. Ten had SNHL, four additionally had HCM. Six had SNHL without echocardiographic evidence for left ventricular hypertrophy (LVH). Four of these 6 patients had abnormalities on 12 lead electrocardiogram (ECG), three of the six had a prolongation of the QT interval. Cardiac symptoms were mild or absent in most affected family members. Genetic analyses suggested linkage of a cardio-auditory phenotype to a region at 6q13 encompassing the MYO6 locus. A novel missense mutation (H246R) of MYO6 affecting the highly conserved motor domain was detected in all affected members.

Conclusions: We describe a possible cardio-auditory syndrome associated with a dominant missense mutation in a gene encoding an unconventional myosin. The cardiac manifestations may escape detection in pedigrees with MYO6-associated SNHL. Careful cardiologic
characterization of these families may clarify the etiologic relationships among HCM, SNHL, and myosin VI.

3.4.1.2 Introduction

Hereditary SNHL shows a great degree of non-allelic and allelic genetic heterogeneity, and can be dominant, recessive, X-linked or mitochondrial.\textsuperscript{309-311} Hereditary SNHL is classified according to mode of inheritance and the presence of clinically detectable extra-auditory manifestations (syndromic deafness) or their absence (nonsyndromic). The distributions of mutant gene expression are not necessarily restricted to clinically affected organ systems, and mutant genes associated with "nonsyndromic" deafness may therefore have subtle extra-auditory manifestations.

Genetic syndromes restricted to a cardio-auditory phenotype include the long QT syndrome (LQTS) caused by mutations in KvLQT1 (KCNO1) or in KCNE1 where QT prolongation has autosomal dominant expression (Romano Ward syndrome), but congenital SNHL with LQTS is autosomal recessive (Jervell and Lange-Nielsen syndrome).\textsuperscript{74} In the LQTS, cardiac structure is normal. Schonberger et al recently described a syndrome of dilated cardiomyopathy (DCM) and SNHL which was linked to 6q23-24 in two families.\textsuperscript{74} DCM and SNHL penetrance were both age-related, though SNHL onset was at a much younger age and appears to have been more penetrant. Non-sarcomeric causes of HCM are largely uncharacterised, and may be associated with distinct or compound phenotypes.

Here a pedigree co-segregating progressive, late-onset, autosomal dominant SNHL, QT interval prolongation and cardiac hypertrophy is described. Linkage analysis indicates the causative gene is located on chromosome 6q12. We have identified a mutation in MYO6, a gene encoding a non-muscle or unconventional myosin, in all affected members of the pedigree.

3.4.1.3 Methods

A proband patient diagnosed with hypertrophic cardiomyopathy at the age of 35 years following presentation with chest pain and dyspnoea was referred for evaluation at the Inherited Heart Diseases section of the NHLBI. The initial evaluation was notable for a strong family history of bilateral hearing loss, a family history of poorly characterised cardiac problems and the recent diagnosis of electrocardiographic QT prolongation in two of his three children.
The hereditary pattern of hearing loss excluded a mitochondrial inheritance and instead was strongly suggestive of autosomal dominant inheritance. The apparent association of hereditary hearing loss, LV hypertrophy and QT prolongation suggested a possible novel hereditary disease; we therefore evaluated all consenting family members.

**Subjects**: Subjects were studied according to NHLBI (98-H-0100 and 99-H-0065) and NIDCD (97-DC-0180) protocols approved by the respective Institutional Review Boards. Written consent was obtained from all family members who participated in the study. Family members were evaluated by interview, clinical examination, 12-lead ECG, transthoracic echocardiography, pure-tone and speech audiometry, tympanometry, and acoustic reflex testing.

**Clinical Evaluation**: Hypertrophic cardiomyopathy was diagnosed when echocardiographic measurements of left ventricular (LV) wall dimensions were greater than 13 mm in the absence of other causes of LVH. The QT interval was determined manually from the 12-lead ECG by an experienced electrophysiologist blinded to patient identity and results of aetiological evaluations, and the corrected QT (QTc) was calculated using the Bazett formula. The QTc was considered prolonged if \( \geq 470 \) msec in males and \( \geq 480 \) msec in females.\(^{312}\) Pure tone audiometry was performed at 0.25, 0.5, 1, 2, 4, 6, and 8 kHz for air conduction and at 0.5, 1, 2, and 4 kHz for bone conduction. Pure tone averages (PTA) were calculated for air conduction thresholds at 0.5, 1, 2 and 4 kHz. Individuals were considered affected if they showed bilateral PTAs greater than the 95th percentile when compared to age/sex-adjusted normative data.\(^{313}\)

Genetic analysis: Genomic DNA was obtained from peripheral blood as described above. PCR of polymorphic short tandem repeat (STR) markers was performed with fluorescent dye-labeled primers, and STR amplicons were analysed (ABI 377 DNA Sequencer, Genescan and Genotyper software, Applied Biosystems). After excluding linkage to several known loci for SNHL and LQTS, a genome-wide scan was performed using the Weber v.9a marker set (10 cM resolution, Marshfield Medical Research Foundation). Two LOD scores were calculated using the FASTLINK version of the LINKAGE software package.\(^{314}\) Individuals were assigned to one of three liability classes based on the SNHL phenotype: class 1 (age > 50) 95% penetrance and 1/100 phenocopy rate; class 2 (age 25 to 50) 90% penetrance and 1/1000 phenocopy rate; class 3 (age < 25) 30% penetrance and 1/10,000 phenocopy rate. Simulated LOD scores were
generated using SLINK\textsuperscript{315} and calculated under the assumptions described above. Additional simulations were based on the cardiac phenotype alone and a single, highly penetrant, syndromic trait. MYO6 was evaluated for mutations using previously described primer pairs and direct sequencing (Big-Dye Terminators, Applied Biosystems).\textsuperscript{304}

3.4.1.4 Results

Clinical Findings: The proband (III-3) was diagnosed with hypertrophic cardiomyopathy at the age of 35 years following evaluation for exertional dyspnoea and chest pain (Figure 26). Several episodes of presyncope and syncope had occurred since age 20. One episode of self-limiting atrial fibrillation was associated with severe presyncope. A 12-lead ECG showed sinus rhythm (SR), left atrial hypertrophy (LAH), left axis deviation (LAD), a q-wave in lead AVf, biphasic septal T-waves and a corrected QT interval (QTc) of 584 msec (Figure 27). Transthoracic echocardiography shows a non-dilated and hyperdynamic LV, systolic anterior motion of the mitral valve and normal LV outflow velocities. Magnetic resonance imaging of the heart measured the anterior septum to be 21 mm, posterior wall 12 mm (normal septum and posterior wall dimensions 6-11mm), ejection fraction 65% and LV mass 270 gm (mean in normal adults 87±12 gm). (Figure 28) There was no family history of syncope or sudden death at a young age.

The proband had bilateral, symmetric, mild-to-severe SNHL affecting high frequencies to the greatest degree, resulting in a down-sloping audiometric configuration. The onset was in the early postlingual period of the first decade of life, followed by steady and gradual progression. Speech audiometry and acoustic reflex testing results were consistent with cochlear hearing loss. A history of progressive hearing loss affecting several generations within the family was evident, suggesting autosomal dominant inheritance. The onset and natural history of SNHL was similar in other affected family members (age range: 5-54 years). The two youngest affected subjects had SNHL affecting only middle and low frequencies (at five and ten years of age). In contrast, affected adults had hearing loss affecting all frequencies to varying degrees, with a variety of audiometric configurations. The highest observed pure-tone average (0.5 / 1 / 2 / 4 kHz) in the better-hearing ear was 55 dB-HL in subject III-2, who was 43 years old (Figure 29).
Genotype and phenotype are summarized on the pedigree. Several descendants of II-3 and II-4 were studied; none had cardiomyopathy or SNHL and have been omitted from the pedigree to promote anonymity. An "R" below the pedigree symbol indicates a carrier of the mutant Arg246 allele of MYO6; an "H" indicates homozygosity for the normal His246 allele. Also shown are sequencing pherograms illustrating mutant MYO6 (H246R). PQT= prolonged QT interval.

Leads V1-V3 are shown for all; lead II is also shown for II-1, II-2, III-2 and III-5. All ECGs recorded at 10 mm/mV at rate 25 mm/sec. II-1, III-2 and III-3 all have LVH on echocardiography. II-2, III-5 and IV-6 had normal echocardiograms. II-2 has a very prolonged QT interval and III-5 has borderline first-degree heart block. The ECG in IV-6 was considered
normal for age (5 years). A stylised recording is shown at bottom to identify the component waves of the ECG (P,Q,R,S and T) and the QT interval.

Figure 28. Cardiac Magnetic Resonance Imaging in Mutant MYO6.

Magnetic resonance cardiac imaging in proband III-3. The panel on the left is a short-axis view of the patient’s mid-left ventricle in cross-section and a normal left-ventricle is shown in the panel on the right for comparison. Both images are to the same scale. There is marked hypertrophy of the proband’s LV walls, particularly of the intraventricular septum. RV, right ventricle; LV, left ventricle; IVS, intraventricular septum.

Figure 29. Composite Puretone Audiograms of Individuals with Mutant MYO6.
The rates of progression and audiogram configurations are highly variable, although all affected subjects were consistent in that the hearing loss was bilateral and sensorineural.

Clinical evaluation of family members subsequently determined that other family members who have SNHL also have ECG and echocardiographic abnormalities (Table 15). The most typical phenotype includes bilateral low-frequency SNHL progressive in severity from the end of the first decade of life, apical LVH and a prolonged QT interval. In the youngest family member (IV-6, age 5 years) with early signs of SNHL, no ECG or echocardiographic abnormalities were detected, and in another with early SNHL, (IV-3, age 10 years) the ECG showed right axis deviation (RAD), LAH and increased voltages despite a normal echocardiogram. Family member II-3 was not evaluated in our audiological clinic (age 74 years) and is reported to have a late-onset hearing loss, several ECG abnormalities and has inferior wall hypokinesia, mitral annular calcification, and mitral and aortic valve regurgitation on echocardiography. She has no descendants with SNHL or cardiomyopathy. It was thought that this individual was both a cardiac and auditory phenocopy. There were no instances of ECG abnormalities detected in individuals with normal hearing. We concluded that a possible cardio-auditory syndrome was segregating in this family, with SNHL as the most penetrant feature.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Age (Years)</th>
<th>MY06</th>
<th>SNHL</th>
<th>ECG</th>
<th>Other ECG abnormalities</th>
<th>ECHO</th>
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<tr>
<td>II-1</td>
<td>75</td>
<td>mutant</td>
<td>Yes</td>
<td>581</td>
<td>q wave in lead AVL, LVH, LAH, LBBB</td>
<td>Apical LVH, SAM, hypodynamic non-dilated</td>
</tr>
<tr>
<td>II-2</td>
<td>89</td>
<td>mutant</td>
<td>Yes</td>
<td>559</td>
<td>Borderline 1st HB</td>
<td>Normal for age</td>
</tr>
<tr>
<td>II-3</td>
<td>74</td>
<td>wild-type</td>
<td>No</td>
<td>572</td>
<td>q in lead AVL, LVH, LAH, wide QRS complex, 1st HB</td>
<td>Mild 1st, mild MR, inferior hypokinesia</td>
</tr>
<tr>
<td>III-1</td>
<td>39</td>
<td>mutant</td>
<td>Yes</td>
<td>468</td>
<td>q in leads II, III, AVF</td>
<td>Apical LVH</td>
</tr>
<tr>
<td>III-2</td>
<td>45</td>
<td>mutant</td>
<td>Yes</td>
<td>505</td>
<td>q in leads II, III, AVF, Posthemiblock, biphasic t-waves</td>
<td>Apical LVH, trivial SAM</td>
</tr>
<tr>
<td>III-3</td>
<td>36</td>
<td>mutant</td>
<td>Yes</td>
<td>584</td>
<td>q in lead AVL, LAD, LAH, biphasic t-waves</td>
<td>Septal LVH</td>
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<tr>
<td>III-4</td>
<td>50</td>
<td>mutant</td>
<td>Yes</td>
<td>435</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>III-5</td>
<td>47</td>
<td>mutant</td>
<td>Yes</td>
<td>459</td>
<td>Borderline 1st HB, LAH</td>
<td>Normal</td>
</tr>
<tr>
<td>III-6</td>
<td>50</td>
<td>mutant</td>
<td>Yes</td>
<td>510</td>
<td>None</td>
<td>Not available</td>
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<tr>
<td>IV-1</td>
<td>42</td>
<td>mutant</td>
<td>No</td>
<td>509</td>
<td>Borderline QT deviation</td>
<td>Normal</td>
</tr>
<tr>
<td>IV-2</td>
<td>12</td>
<td>mutant</td>
<td>No</td>
<td>447</td>
<td>LAH, RAD possibly normal for age</td>
<td>Normal</td>
</tr>
<tr>
<td>IV-3</td>
<td>50</td>
<td>mutant</td>
<td>Yes</td>
<td>425</td>
<td>None</td>
<td>Normal</td>
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<tr>
<td>IV-4</td>
<td>28</td>
<td>mutant</td>
<td>No</td>
<td>425</td>
<td>None</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*II-3 was considered a double phenocopy with late-onset hearing loss and cardiac abnormalities consistent with valvular and ischemic disease

Prolonged QTc durations are in bold type.

LVH-left ventricular hypertrophy
LAH-left atrial hypertrophy
LBBB-left bundle branch block
RBBB-right bundle branch block
RAD-right axis deviation
SAM-systolic anterior motion of the mitral valve
MR-mitral valve regurgitation
AF-atrial fibrillation
QRS-Q-wave, T-wave, QT interval and QRS complex on the ECG (see Figure 2A)

Table 15. Cardiac Abnormalities and Hearing Loss Associated with Mutant MY06.

Clinical details on selected members of the pedigree. Individuals can be identified from Figure 26.
**Genetic Analysis:** The pedigree structure and size of family 641 (Figure 26) allows a simulated maximum LOD score of 4.4, assuming a dominant, syndromic trait with greater than 90% penetrance in all generations. Consideration of the cardiac phenotype resulted in maximum simulated LOD scores of only 1.8 (assuming 50% penetrance for LV hypertrophy). After excluding loci associated with familial SNHL (at that time), we performed a 10 cM resolution genome wide scan. Affection status for the purposes of linkage analysis was determined by the presence of SNHL. Individuals were assigned to one of three liability classes, based on the SNHL phenotype: class 1 (age > 50) 95% penetrance and 1/100 phenocopy rate; class 2 (age 25 to 50) 90% penetrance and 1/1000 phenocopy rate; class 3 (age < 25) 30% penetrance and 1/10,000 phenocopy rate. No markers showed perfect co-segregation with the SNHL phenotype. The maximum LOD score obtained was 2.98 with marker D6S1681 at 6q13. Analyses with additional markers at 6q13 revealed that all affected individuals had the same haplotype spanning 30 cM (D6S1017 to D6S1031). Two individuals, IV-1 and IV-10, were normal for audiological examination at ages 12 and 28 years respectively, also inherited this haplotype - raising the possibility of incomplete penetrance.

Genes localized to chromosome 6q13 include KCNQ5 and MYO6 encoding non-muscle myosin VI; the latter was recently associated with dominant (DFNA22) and recessive (DFNB37) nonsyndromic sensorineural deafness. Given that mutations of KCNQ4 caused nonsyndromic deafness (DFNA2) and KCNQ1 cause the cardio-auditory phenotype Jervell and Lange-Nielson syndrome, we first sequenced the coding exons of the paralogous gene, KCNQ5. No mutations in KCNQ5 were found among affected members. Sequence analysis of the coding exons of MYO6 revealed a novel transition mutation, 737A>G, in all affected members of family 641. This is predicted to lead to the substitution of a conserved histidine residue for arginine in the motor region of the myosin VI protein (H246R, Figure 30). This mutation was not detected in the genomic DNA from 351 individuals of a wide variety of ethnic backgrounds (702 chromosomes). PLN (encoding phospholamban), a gene recently associated with dilated cardiomyopathy localized to 6q22.1, was also sequenced to test the hypothesis that the SNHL and HCM phenotypes were etiologically independent but associated due to the physical linkage of the causative genes on chromosome 6. No mutations in PLN were identified following sequencing of several affected individuals.
3.4.1.5 Discussion

We describe the novel association of progressive, postlingual autosomal dominant SNHL and hypertrophic cardiomyopathy. There is a previous report of profound, prelingual autosomal dominant hearing loss associated with HCM; the same group also report a greater frequency of hearing loss in HCM patients than in other groups with LVH. In our pedigree, linkage and mutation analysis implicate a missense allele (H246R) of the myosin VI gene (MY06). Another missense allele of MY06 (C442Y) was recently identified as the cause of dominantly inherited and progressive SNHL (DFNA22); no other clinical features were reported in affected individuals. No cardiac abnormalities were detected in affected individuals with recessive missense mutations in MY06 (DFNB37). In the mouse, mutations of Myo6 cause hearing loss and vestibular dysfunction, with no other phenotype reported. However, MY06 expression in the human heart was demonstrated by Northern blot. The cardiac phenotype reported here may suggest that only certain MY06 mutations are associated with cardiomyopathy. In our study, the cardiac findings are relatively benign and mild in all except the proband. Unless echocardiograms and ECGs are part of the clinical evaluation, a mild or incompletely penetrant cardiac phenotype may be overlooked. Lastly, we cannot exclude the possibility that the cardiac and SNHL phenotypes in family 641 are unrelated.

The histidine to arginine mutation affects the highly conserved motor region of myosin VI. Myosin VI is a non-muscle (unconventional) myosin with a property unique from most other...
myosins; most myosins have motor activity towards the ‘plus-end’ of actin filaments, dimers of myosin VI are directed towards minus end.\textsuperscript{324-326} Several non-muscle myosins have been associated with human hearing:\textsuperscript{325-328}

HCM typically results from mutations affecting genes encoding components of the myocyte’s contractile apparatus.\textsuperscript{41} More recently, non-sarcomeric causes have been identified, but none of these genes encodes a non-muscle myosin. Transgenic knockouts of non-muscle myosin II-B develop severe structural cardiac defects similar to human congenital heart disease, suggesting that at least some unconventional myosins are required for cardiac development.\textsuperscript{329} There is no evidence for congenital cardiac abnormalities in the family we report here. Rather, like the hearing abnormality, penetrance of the cardiomyopathy is age-related suggesting it develops as a consequence of prolonged abnormal myosin VI function. Myosin VI dimerizes and the mutant protein may have dominant negative effects, impairing the dimer’s motor function.

Myosin VI is expressed in the human heart, although its intracellular localization and role(s) are not described.\textsuperscript{321} In the cochlea the function of myosin VI is unknown. Myosin VI is found in the cuticular plate of hair cells where it may be important developmentally and provide protection by restraining mechanical forces.\textsuperscript{321-323} Myosin VI is also associated with the Golgi complex and clathrin coated vesicles in several cell types, and may provide motor power for intracellular transport, exocytosis and endocytosis.\textsuperscript{325,330-332} Cells lacking myosin VI function have smaller Golgi complexes and protein secretion is significantly reduced.\textsuperscript{332} Myosin VI is found in the leading edges of growth factor-stimulated fibroblasts, and it may participate in cell migration and shape changes.\textsuperscript{333,334} Finally, cell division and morphogenesis in Drosophila are dependent on myosin VI function.\textsuperscript{334,335} Thus, myosin VI contributes to a broad range of cytoskeletal and cellular motility functions. In cardiomyocytes, myosin VI is likely to participate in similar functions, and the development of cardiac hypertrophy (and SNHL) may follow from a persistent deficiency in one or more of these functions.

### 3.4.1.6 Conclusions

Progressive autosomal dominant SNHL and mild cardiac hypertrophy may result from the expression of mutant non-muscle myosin VI. Subtle cardiac manifestations may escape detection or lead to misdiagnosis in other individuals with SNHL and MY06 mutations.
3.5 AUTOSOMAL DOMINANT DILATED CARDIOMYOPATHY: IDENTIFICATION OF A NOVEL DISEASE LOCUS ON CHROMOSOME 10q26

Clinical assessments of the members of the family described here, including phenotyping were accomplished during a field trip. DNA was also obtained, as described in the section on general methods. Linkage analysis was performed the University of Utah and at Harvard Medical School (Mark Keating’s Laboratory). Following the identification of the disease locus, candidate gene sequencing was performed in our laboratory.

3.5.1.1 Abstract:
Background: Dilated cardiomyopathy (DCM) is a prevalent disorder characterized by cardiac chamber dilatation and left ventricular (LV) systolic dysfunction. Previous genetic linkage and candidate gene analyses with DCM families and sporadic patients have revealed heterogeneous associated loci and genetic causes.

Methods and Results: A family with fifteen individuals affected with DCM was identified. Blood was collected from all consenting family members for genotypic analysis. After excluding linkage to established DCM loci, a genome-wide 10 Centimorgan linkage scan using microsatellite markers was performed and linkage to chromosome 10q26 was identified. The peak two-point LOD score of 4.45 was obtained with D10S1651, with a multipoint LOD score of 5.69.

Conclusions: A new locus for autosomal dominant DCM on chromosome 10q26 is identified.

3.5.1.2 Introduction
Dilated cardiomyopathy (DCM) is characterized by progressive cardiac chamber dilatation and systolic dysfunction. Morbidity and mortality occur as a result of end-organ hypo-perfusion, thromboembolic events, arrhythmias and sudden cardiac death. DCM is aetiologylogically heterogeneous and a strong genetic component is evident. In some pedigrees, highly penetrant autosomal inheritance is clearly seen. In most cases, familial disease is not readily apparent. Prospective detailed evaluations of relatives of DCM patients detect familial disease, defined as
one or more relatives with DCM, in 20-25% of the cases.\textsuperscript{336,337} Autosomal dominant transmission patterns are most readily detected, but X-linked, recessive, and mitochondrial transmission also occurs. We sought to determine the cause of DCM in a five generation family with multiple members referred for management of congestive heart failure due to DCM. A simplified pedigree is shown below (Figure 31).
Figure 31. A Family with Autosomal Dominant Inherited Dilated Cardiomyopathy.

Conventions for gender (females denoted by circle) and affection status (symbols filled in if the DCM phenotype is diagnosed) are used.
3.5.1.3 Methods and Results

Phenotypic Characterization Studies were performed in under protocols approved by the Institute Review Boards of the National Institutes of Health, and the University of Utah. Family members were evaluated by clinical history, physical examination, 12-lead electrocardiography, Doppler and 2-dimensional echocardiography. The diagnostic criteria for DCM were determined from echocardiographic analysis, and consist of an LV ejection fraction of < 50%, or a fractional shortening <27% on M-mode, or both. The presence of an LV end diastolic dimension of > 5.5 cm was used as criteria for ventricular dilatation.338-341 Evidence of secondary causes of cardiac dysfunction, such as coronary artery disease, myocarditis, hypertension and excessive alcohol consumption were elicited from the medical history, physical exam and additional testing, if clinically indicated. Clinical evaluation and the assignation as affected or unaffected phenotype was performed by the NHLBI. Individuals <35 years of age with normal echocardiographic measurements were classified with uncertain disease status due to age-related penetrance of DCM.

Initial evaluation of family history revealed 10 previously identified members with history of CHF. We have phenotypically characterized and collected blood from 61 family members. As a result of exhaustive screening of all living relatives, we identified a total of 16 individuals affected with left ventricular dysfunction. In addition to DNA from all living family members, we also obtained banked tissue samples for genotyping four individuals who died as a result of DCM.

DNA collection Genomic DNA was extracted from whole blood according to standard protocols. A gel extraction protocol (Qiaquick, Qiagen) modified for tissue extraction was used to obtain genomic DNA from formalin fixed, paraffin imbedded autopsy specimens.342

Genotypic analysis genome-wide scan was performed with 400 highly polymorphic (average heterozygosity >70%) short tandem repeat (STR) Genethon markers spaced at 10 Centimorgan intervals.343 The fluorescently tagged primers (Perkin Elmer ABI PRIZM linkage mapping set version 2.0) permitted PCR product detection on a Megabase automated capillary sequencer, followed by allele calling using the Genetic Profiler program (Amersham/Pharmacia). PCR based manual genotyping, using 32P-labelled STR primers was performed using established
protocols.\textsuperscript{343} PCR products were resolved by electrophoresis on 6% denaturing polyacrylamide gels (Sequagel) and visualized by exposure to autoradiograph film (XAR, Kodak) at $-70^\circ$C for 1 to 24 hours, prior to development. All genotyping was performed blinded to each patient's clinical status.

**Linkage analysis** Linkage analysis was performed using the LINKAGE 5.1 software package running on a DEC Alpha 3000 computer administered by the genetics department at Harvard Medical School.\textsuperscript{276,344} Initial evidence for linkage was obtained with marker D10S1651 on the long arm of chromosome 10. Following the genome-wide scan, genotyping in the region around chromosome 10q26, showing evidence for linkage, was performed using published STR markers, in order to confirm linkage and refine the map location of the DCM trait. Multipoint linkage analysis was performed using the VITESSE software package.\textsuperscript{345} LOD score calculations for the four non-recombinant markers provided in table 1 were calculated using allele frequencies determined from a panel of unrelated individuals.

**Identification and mutation screening of candidate genes** Positional candidate genes were screened for mutations by direct sequencing. When the defined genomic structure was available, primers were designed within the flanking intron to permit amplification of the exonic and splicing sequences as described above. Three genes expressed in ventricular myocardium were selected for sequencing; NRAP (gene encoding nebulin related anchor protein), PICOT and ADAM12. PICOT is a putative inhibitor of protein kinase C and may play a role in inhibition of hypertrophic signals in cardiomyocytes and in the regulation of contractility.\textsuperscript{346} ADAM12 is a disintegrin metalloprotinase, a class of proteins that may contribute to cardiac remodelling.\textsuperscript{218,347} NRAP colocalizes with nebulin in cardiomyocytes where it is thought to play a role in anchoring myocyte contractility and in myofibril organisation. PCR products from two affected individuals were directly sequenced for mutations in PICOT and ADAM12 (by the Harvard group). The human NRAP sequence had not been determined and we undertook to describe the genomic structure of this gene (see next chapter) before mutation detection could be completed. No mutations in ADAM12, PICOT or NRAP were detected following exhaustive screening of these genes. The identity of the mutant gene responsible for some cases of DCM at the 10q26 locus remains unknown.
3.5.2 GENOMIC ORGANIZATION, ALTERNATIVE SPLICING, AND EXPRESSION OF HUMAN AND MOUSE N-RAP, A NEBULIN-RELATED LIM PROTEIN OF STRIATED MUSCLE

The work described in this section was begun to enable a search for N-RAP mutations in a pedigree where a DCM phenotype was linked to a 10q26 locus. N-RAP had been described in the mouse, and the human analog localised to 10q24-26. To determine the human cDNA sequence and the genomic structure of N-RAP, we first used BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and other bioinformatics resources to examine human sequences (genomic DNA libraries and expressed sequence tags – ESTs) for homology with the murine sequence. It became apparent that the published murine sequence was erroneous, and that the true gene product significantly differed in length and sequence. In consequence, we sought to determine the correct mouse sequences, and to determine the human sequence for the first time. We also established the tissue distribution of NRAP and described splice variants.

All the work described here was completed at NIH. Collaboration was formed with the laboratory of Robert Horowits at the National Institute of Arthritis and Musculoskeletal and Skin diseases. Dr Horowits’ group published the first murine NRAP sequences and had developed a research programme dedicated to understanding NRAP’s cellular role. The Horowits’ laboratory repeated some of their published experimental work using the corrected NRAP sequence. All other work was performed in our laboratory.

3.5.2.1 Abstract

Introduction: Linkage analysis identifies 10q24-26 as a disease locus for dilated cardiomyopathy (DCM), a region including the N-RAP gene. N-RAP is a nebulin-like LIM protein that may mediate force transmission and myofibril assembly in cardiomyocytes. We describe the sequence, genomic structure and expression of human N-RAP, as well as an initial screen to determine whether N-RAP mutations cause cardiomyopathy.

Methods and Results: Human expressed sequence tag databases were searched with the published 3528 base-pair (bp) mouse N-RAP open reading frame (ORF). Putative cDNA sequences were interrogated by direct sequencing from cardiac and skeletal muscle RNA. We identified two human N-RAP isoforms with ORFs of 5085 bp (isoform C) and 5190 bp (isoform...
S), encoding products of 193-197 kDa. Genomic database searches localize N-RAP to human chromosome 10q25.3 and match isoforms C and S to 41 and 42 exons. Only isoform C is detected in human cardiac RNA; in skeletal muscle, approximately 10% is isoform C and approximately 90% is isoform S. We investigated apparent differences between human N-RAP cDNA and mouse sequences. Two mouse N-RAP isoforms with ORFs of 5079 and 5184 bp were identified with ~ 85% similarity to human isoforms; published mouse sequences were inaccurate and included cloning artifacts truncating the ORF. Murine and human isoforms have similar gene structure, tissue specificity, and size. N-RAP is especially conserved within its nebulin-like and LIM domains. We expressed both N-RAP isoforms and the previously described truncated N-RAP in embryonic chick cardiomyocytes. All constructs targeted to myofibril precursors and the cell periphery, and inhibited myofibril assembly. Several human N-RAP polymorphisms were detected, but none were unique to cardiomyopathy patients.

Conclusions: N-RAP is highly conserved and exclusively expressed in cardiac and skeletal muscle. Genetic abnormalities remain excellent candidate causes for cardiac and skeletal myopathies.

3.5.2.2 Introduction
Dilated cardiomyopathy (DCM) is characterized by left ventricular (LV) systolic dysfunction and dilatation. DCM is inherited in about one third of cases and transmission may be autosomal dominant or recessive, or X-linked. Several genetic causes of DCM have been described and mutations in several other genes have been implicated in transgenic animal studies. Many genes associated with DCM in human and transgenic studies are involved in propagation of contractile force generated by the sarcomere to the extracellular matrix, leading to the suggestion that compromised force transmission is one primary cause of DCM. The DCM-causing genes at many other loci remain unidentified.

Linkage analysis in a DCM family identifies a locus at 10q24-10q26 as a primary cause of DCM. The N-RAP gene has been mapped to this region of the genome, and is a prime candidate for involvement in DCM by virtue of its putative role in transmitting contractile force. N-RAP up-regulation has been found to precede the development of DCM in mouse models of this disease. In addition, N-RAP co-purifies with cardiac intercalated disks, where it is thought to
link myofibrils to intracellular membrane-associated complexes for effective force transmission.\textsuperscript{353} An analogous role has been postulated for N-RAP at the myotendinous junctions of skeletal muscles. \textsuperscript{348}

Initial analysis of the mouse \textit{N-RAP} cDNA sequence indicated that it encodes a protein containing an N-terminal LIM domain and more than two nebulin-related super repeats.\textsuperscript{348} In vitro studies with recombinant \textit{N-RAP} fragments have shown that the \textit{N-RAP} LIM domain can bind talin\textsuperscript{354} and  \(\alpha\)-actinin,\textsuperscript{353} while the super repeats bind actin and the tail region of vinculin.\textsuperscript{354} Evidence that errors in sequence identity had been made were available; although the cDNA sequence predicted that mouse \textit{N-RAP} is 133 kDa, immunoblot analysis identified \(N\)-\textit{RAP} migrating at 190 kDa on denaturing gels.\textsuperscript{348}

In addition to its postulated role in linking the ends of myofibrils to membrane-associated complexes, studies in cultured chick cardiomyocytes implicated \textit{N-RAP} in the process of myofibril assembly. As these cells spread in culture, immature fibrils containing punctate \(\alpha\)-actinin Z-bodies, \(\alpha\)-actin, and muscle tropomyosin are formed.\textsuperscript{355-359} Time-lapse studies showed that the closely spaced \(\alpha\)-actinin beads in these structures aggregate laterally to form nascent myofibrils.\textsuperscript{359} These structures incorporate titin and muscle myosin, the latter of which may exist as preformed bipolar thick filaments that are oriented and incorporated into the nascent myofibrils by their interactions with the titin filaments.\textsuperscript{357,359,360} Like non-muscle myosin IIb, \textit{N-RAP} is present in all of the myofibril precursors, but is not found in the mature sarcomeres.\textsuperscript{361} In mature myocytes, \textit{N-RAP} contains distinct regions that target to the cell periphery, the actin filaments, and the Z-lines when expressed as GFP-fusion proteins, and over-expression of any of these regions of \textit{N-RAP} can inhibit myofibril assembly in the cultured cardiomyocytes.\textsuperscript{362} This functional data led to the proposal that \textit{N-RAP} is an organizing centre during the first steps of myofibril assembly, controlling the integration of \(\alpha\)-actinin and actin to form the first premyofibril complex at the membrane.\textsuperscript{362}

Here we report the cDNA sequence, genomic structure, and subcellular localization of human \textit{N-RAP}. We also compare these data to newly determined mouse \textit{N-RAP} cDNA sequence as well as mouse genomic data, and show that the \textit{N-RAP} gene and gene product are highly conserved.
between species. Finally, we describe polymorphisms in human N-RAP and a screen for N-RAP mutations in a family with DCM linked to chromosome 10q24-26.

3.5.2.3 Materials and Methods

Subjects: All subjects were studied following their informed consent obtained under protocol 98-H-0100 of the National Heart, Lung and Blood Institute of the National Institutes of Health.

Identification of Human N-RAP cDNA Sequence and Genomic Structure: Complementary database searches and direct sequencing identified human cDNA and genomic sequences. Searches of human expressed sequence tag (EST) databases were performed using the Basic Local Alignment Tool (BLAST) of the National Center for Biotechnology Information (NCBI, at http://www.ncbi.nlm.nih.gov/BLAST/) with serial segments of the 3528 bp mouse N-RAP cDNA open reading frame (ORF) as query. Human EST sequences allowed the design of complementary primers (PrimerSelect, DNastar, WI) for the polymerase chain reaction (PCR) and the amplification and direct cycle sequencing (BigDye terminator, ABI Prism 310, Applied Biosystems, CA) of sections of N-RAP cDNA. Total RNA was extracted from adult human heart and skeletal muscle (Quiagen, CA), and 1st strand cDNA synthesized with either random hexamer or oligo-dT primers (RT-PCR beads, Amersham Biosciences Corp., Piscataway, NJ). Rapid amplification of cDNA ends (RACE) identified the terminal 5' and 3' cDNA sequences in adult human skeletal and cardiac muscle cDNA as per manufacturers instructions (Marathon cDNA, Clontech, CA). Briefly, gene specific primers and adaptor primers complimentary to incorporated oligonucleotide sequences at the 5' and 3' ends of full-length cDNA allow PCR amplification and sequencing of cDNA ends. Overlapping cDNA sequences were aligned into a single contig (Seqman, DNastar, WI) that was extended and confirmed by additional rounds of direct sequencing.

Completed N-RAP cDNA sequences were entered as the query in BLAST and BLAST Like Alignment Tool (BLAT, at http://genome.cse.ucsc.edu/index.html) against non-redundant (NR) and high throughput genomic sequence databases to identify intron/exon boundaries. Genomic DNA structure was required to conform to the constraints of initiation, and termination, splicing and polyadenylation. PCR primer pairs were designed complementary to the flanking intronic regions of each exon to allow confirmation of genomic sequence by direct
sequencing. For genomic DNA Sequencing, human genomic DNA was extracted from whole blood (Puregene, Gentra, MN).

Identification of Mouse N-RAP cDNA Sequence and Genomic Structure: Using PCR amplification, RACE and direct sequencing, the full-length mouse N-RAP was re-sequenced from adult mouse cDNA (Marathon cDNA, Clontech, CA) using methods similar to that used in human N-RAP sequencing. In addition, the full length open reading frame of adult mouse N-RAP was amplified by RT-PCR, cloned and sequenced. mRNA was isolated from adult mouse skeletal or cardiac muscle (Poly(A)Pure, Ambion Inc., TX) and cDNA was synthesized (GIBCO BRL SuperScript Preamplification System, Life Technologies, MD) with random hexamers as primers. The full-length N-RAP open reading frame was amplified using a forward (5'-ATGAATGTGCAGGCCTGCTCT-3') and reverse (5'-TCACATCAGCAGAGTCTTCTT-3') primer (Advantage2 PCR Enzyme system, Clontech, Palo Alto, CA) using an amplification protocol of 1 cycle at 95 °C for 1 minute; 35 cycles at 95 °C for 30 seconds, then 68 °C for 3 minutes; and then 1 cycle at 68 °C for 3 minutes. PCR products were gel purified and cloned into the pcDNA3.1/NT-GFP-TOPO plasmid vector (Invitrogen Corp., Carlsbad, CA). Plasmids were propagated in One Shot TOP10 E. coli cells (Invitrogen, Carlsbad CA) and purified using the Quantum Prep Plasmid Miniprep Kit (BioRad Laboratories, Inc., Hercules, CA). The inserts were completely sequenced as described below.

In addition to amplification of the full-length open reading frame as described above, defined regions of the adult murine N-RAP transcript were amplified using primer pairs flanking exon 12 (forward: 5'-GACCGATGTGGCCAGGCTTACTCAGAAG-3'; reverse: 5'-CAGGGGAACCAGCTCAGGTTTTG-3') and exons 37-40 (forward: 5'-TGATGGGCATGAAAGGGACAGGAT-3'; reverse: 5'-ATCCCCGGGCCCCCTCTGTT-3'). The amplification protocol used was 1 cycle at 95 °C for 1 minute; 35 cycles at 95 °C for 30 seconds, then 68 °C for 1 minute; and then 1 cycle at 68 °C for 1 minute.

RNA Electrophoresis: PCR amplification of human skeletal cDNA using primer pairs flanking exon 12 of N-RAP (forward: 5'-GTTACCCGGGACTATGAGGAGCAG-3'; reverse: 5'-TCAGCGGTAATTCAGACTCAGC-3') produced two electrophoretically sized products of approximately 550 and 650 bp. Each band was excised from the gel and the DNA extracted.
Following sequencing to confirm the products as N-RAP sequence, P$^{32}$ labelled probes complementary to each product were generated by PCR. An additional probe complementary to an 113 bp sequence towards the 3' end of N-RAP was also generated by PCR (forward: 5'-CCAGTGATTTCCGGTACAAAGAGG; reverse: 5'-GAGCCTGCAAACGTAGGAAATG). Human multiple-tissue northern blots (MTN, Clontech, CA) were probed according to the manufacturers instructions, to confirm the size of full-length N-RAP mRNA.

**Immunofluorescence and Immunoblot:** Human biopsy samples and adult mouse tissues were dissected and stored at -80 °C until use. Samples were prepared for immunoblot analysis as previously described (Luo et al., 1997b). Briefly, samples were homogenized in 12 volumes of homogenisation buffer (20 mM Tris, 2 mM EDTA, 20 µg/ml leupeptin and 100 µg/ml PMSF, pH 8.0) and mixed 1:1 with 2x SDS sample buffer. Equal amounts of protein were electrophoresed on Novex 4-20% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and transblotted onto polyvinylidenedifluoride (PVDF) paper. Filters were exposed to a specific polyclonal anti-N-RAP antibody diluted 1:1000, horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody diluted 1:5,000 (Amersham), and ECL detection reagents as previously described.

For sectioning, tissues were embedded in Tissue-Tek OCT compound (Ted Pella, Redding, CA), snap frozen in isopentane cooled with dry ice, and serially sectioned in a cryostat at a thickness of 10 µm. The sections were transferred to slides coated with 3-aminopropyltriethoxysilane and stored at -80 °C. Sections were washed with PBS (phosphate buffered saline, pH 7.4), overlaid with blocking solution (5% goat serum in PBS) for 30 minutes, and then exposed to blocking solution containing a 1:1000 dilution of primary anti-N-RAP antibody (Luo et al., 1997b) for 1 hour. The sections were washed 3 times with an excess of PBS, and then overlaid with blocking solution containing a 1:100 dilution of fluorescein linked antibody from donkey against rabbit immunoglobulins (Amersham Biosciences Corp., Piscataway, NJ). After incubation for 1 hour, the sections were again washed 3 times with an excess of PBS, and then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Blocking and antibody incubations were at 37 °C.

Primary cultures of chick cardiomyocytes were prepared from 7-10 day chick embryos as previously described. After 1 day in culture, the cells were transfected with the full-length
mouse N-RAP isoform S or C open reading frames newly cloned into pcDNA3.1/NT-GFP-TOPO as described above, as well as with the vector encoding unfused GFP. Cardiomyocytes were also transfected with a previously described GFP-N-RAP construct (Carroll et al., 2001). The transfection mixtures contained 0.5 μg of plasmid DNA and 6 μl of FuGene (Boehringer Mannheim Corp., Indianapolis, IN), a non-liposomal transfection reagent, in normal growth media. Samples were fixed 3 days after transfection and stained with monoclonal antibody against sarcomeric α-actinin (Sigma, St Louis, MO) diluted 1:20; bound primary antibody was detected using a 1:100 dilution of rhodamine-linked secondary antibody (rabbit anti-mouse whole antibody, Sigma) as previously described.  

Stained sections and cultured cells were observed with a Zeiss Axiovert 135 microscope equipped for incident-light fluorescence and phase contrast microscopy using a 63x oil immersion objective with a numerical aperture of 1.25. Images were collected using a Photometrics CoolSnap fx CCD camera (Roper Scientific, Inc., Tucson, AZ ) interfaced with a Power Macintosh computer. Quantitative image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The percentage of the area of each cardiomyocyte that contained mature myofibrils as visualized by α-actinin staining was taken as a measure of myofibril content, as previously described.

**Mutation Detection:** Genomic DNA was screened for single-strand conformation polymorphisms (SSCP – as previously described in section on general methods) for every exon in 50 unrelated patients with either DCM or hypertrophic cardiomyopathy that had progressed to a dilated phase. Anomalous conformers were sequenced in both directions. Every exon of N-RAP was sequenced from the genomic DNA of two affected members of the family with 10q24-26 linked DCM.

### 3.5.2.4 Results

**cDNA Sequence and Gene Organization of Human and Murine N-RAP:** Two human N-RAP cDNA isoforms were identified with ORFs of 5085 bp (isoform C; GenBank accession AY177621) and 5190 bp (isoform S; GenBank accession AY177620), encoding products of 193 and 197 kDa, respectively. These open reading frames are ~50% longer than predicted by the previously
published mouse N-RAP sequence. To address this discrepancy, we re-sequenced murine N-RAP cDNA and found that it was highly similar to the newly sequenced human cDNA. We identified mouse N-RAP isoforms C and S (Genbank accession numbers AY177623 and AY177622, respectively), with open reading frames of 5079 and 5184 bp, respectively.

Genomic database searches localize human N-RAP to chromosome 10q25.3 and match isoforms C and S to 41 and 42 exons respectively (BLAST search, UCSC genome build freeze July 2002, Genbank accession AL390197); genomic database searches localize murine N-RAP to mouse chromosome 19 (NCBI document ID 20890983), and indicate a gene organization similar to human N-RAP. As illustrated below (Figure 32 A), human and mouse N-RAP genes are each distributed over approximately 75 kb of genomic DNA. For human N-RAP, exonic and flanking intronic sequences were confirmed by sequencing from genomic DNA. For mouse N-RAP, the full-length ORF was amplified from skeletal and cardiac mRNA, cloned into plasmids, and sequenced. In both humans and mice, the presence of exon 12 in isoform S and its absence in isoform C is the only difference between the two isoforms.

The exon boundaries in the ORF are completely conserved between mouse and human N-RAP. An N-terminal LIM domain (residues 6-57) is encoded by the first two exons plus the first codon of exon 3; the rest of exon 3 encodes a short linker region. Exons 4-41 encode 46 copies of 31-38 residue repeats that are homologous to nebulin modules (Figure 32 B,C). For the most part, each exon encodes a single module; the exceptions are exons 19, 24, 30, 35, and 40, which each encode two or three modules (Figure 32 B).

The first 11 modules, encoded by exons 4-14, are single repeats that share 40% similarity with single repeats near the N-terminus of nebulin. Modules 12-46, encoded by exons 15-41, are organized into five super repeats each containing 7 modules (Figure 32 D); the N-RAP super repeats are highly homologous to each other and to nebulin super repeats, sharing 41% identity and 64% similarity to super repeats near the centre of human nebulin.

The highly conserved organization of exons between human and mouse N-RAP is reflected in the primary sequence (Figure 33). Human and mouse N-RAP are 84% identical and 90% similar. The 52-residue LIM domain is most highly conserved, differing by only one residue between human and mouse N-RAP. The linker region is 75% similar between human and mouse, while
the simple repeat and super repeat regions are 87% and 93% similar, respectively. The similarity decreases to 73% in the C-terminal residues that follow the super repeats.

(A) Schematic representation of exons found in the human and mouse N-RAP genes. The N-RAP gene spans more than 75,000 base pairs in each species, and the exon organization is conserved. (B) N-RAP domain organization and corresponding exons. Exons 1 and 2 together encode a LIM domain; exons 4-14 encode single repeating modules;
and exons 15-41 encode 5 super repeats, each of which contains 7 single modules. Exon boundaries correspond to module boundaries, except where dashed vertical lines indicate multiple modules encoded by a single exon. Exon 12 is alternatively spliced (filled box). The alignment of human N-RAP modules (C) and super repeats (D) is shown. Similar residues are boxed.

The alignment also includes the original mouse N-RAP sequence. The main difference between the present results and the previously determined translated sequence is a frame shift within module 32 resulting from a single nucleotide insertion relative to the new sequence; this change put a premature stop codon in frame in module 33, resulting in a truncated open reading frame that did not include the last two super repeats (Figure 33). In addition, the previously determined N-RAP sequence did not contain module 9, which is encoded by the alternatively spliced exon 12. Three additional short regions of sequence mismatch between the original and the new mouse N-RAP sequences are evident in the simple repeat region; these are the result of frame shifts from single nucleotide insertions or deletions. Finally, the previously published cDNA does not include exons 37-40.

Alternatively Spliced N-RAP Transcripts in Skeletal and Cardiac Muscles: A 12-tissue human tissue northern blot incubated with a probe complementary to 113 bp near the 3' end of N-RAP identifies a single transcript in heart and skeletal muscle of a size equivalent to that predicted by our sequencing data (Figure 34 A). As expected, N-RAP isoforms differing by only 105 bases were not resolved by this technique.

In order to detect and compare the relative levels of each isoform expressed, we PCR amplified a region that included exon 12 and several flanking exons from human and mouse skeletal and cardiac muscle cDNA. Two bands were detected in skeletal muscle with sizes corresponding to those expected for products including or excluding exon 12 (Figure 34 B); the results show that in both mouse and humans, more than 90% of N-RAP transcripts in skeletal muscle include exon 12. In contrast, only the smaller PCR product was detected in cardiac muscle, showing that cardiac N-RAP does not contain exon 12. These differences were confirmed by direct sequencing of both products in human cDNA.

Since exons 37-40 are not found in the originally sequenced mouse N-RAP cDNA, we searched for alternative splicing in this region by PCR amplifying exons 36-41 from mRNA. A single prominent band was detected in mouse skeletal and cardiac muscle with a size corresponding
to that expected from the new N-RAP sequence (Figure 34). In contrast, no PCR product was detected at 205 bp, which would correspond to a transcript that does not include exons 37-40.
Figure 33. Alignment of Human and Murine N-RAP Protein Sequences.

Included for comparison is the previously published mouse N-RAP sequence. The domain boundaries are indicated above the sequence.

A. Northern Blot

B. RT-PCR Analysis

Figure 34. Differential Expression of N-RAP Splice Variants in Cardiac and Skeletal Muscle.

(A) Northern blot detection of N-RAP in 12 human tissues. A 5-6000 base pair sized product is identified only in skeletal and cardiac muscle. (B) PCR amplification of NRAP cDNA from skeletal muscle (S) and heart (H) between exons 10-16 in humans and exons 9-14 and 36-41 in mouse. The expected sizes using the human exon 10-16 primers are 650 bp and 550 bp for isoforms S and C, respectively, and the expected sizes using the mouse exon 9-14 primers are 549 bp and 444 bp for isoforms S and C, respectively.

N-RAP Protein in Skeletal and Cardiac Muscles: Immunoblotting identifies a single ~190 kDa band as N-RAP in adult mouse skeletal and cardiac muscles (Figure 35A), as previously reported and in agreement with the sequences presented above. A band with the same mobility is detected in blots of adult human skeletal muscle and heart, along with a prominent band migrating at ~65 kDa. Immunofluorescence with this antibody demonstrates that N-RAP is specifically concentrated at the intercalated disks in human hearts (Figure 35 B,C), a localization
pattern that is identical to that previously observed in mouse hearts.\textsuperscript{353} The results are consistent with the smaller band detected in human striated muscles being a proteolytic fragment of N-RAP, but do not rule out the possibility that this is an unrelated intercalated disk protein that cross-reacts with the N-RAP anti-serum.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure35.png}
\caption{N-RAP is Localized to the Intercalated Discs in Skeletal and Cardiac Myocytes.}
\end{figure}

(A) Total proteins from mouse and human striated muscles separated by SDS polyacrylamide gel electrophoresis and stained with coomassie blue (left panel), and corresponding blot probed with anti-N-RAP antibody (right panel). Lanes 1 and 2 were loaded with
adult mouse skeletal muscle and heart homogenates, respectively, and lanes 3 and 4 were loaded with human skeletal muscle and heart homogenates, respectively. Immunoblot detection reveals a major band at ~190 kDa in all samples, corresponding to the molecular weight predicted from the full-length cDNA sequences. An additional band is detected at ~65 kDa in the human samples. The positions of molecular weight markers and predominant muscle proteins (myosin heavy chain and actin) are indicated. (B,C) Frozen section of human cardiac muscle stained with N-RAP antibody and imaged with phase contrast (B) or fluorescence (C) optics. N-RAP is concentrated at the intercalated disks (arrowheads). Autofluorescent, phase dense lipofuscin granules are also visible (arrows).365

We used primary cultures of embryonic chick cardiomyocytes as a model system in which to observe the targeting and phenotypic effects of N-RAP isoforms. Our previous work showed that myofibrillar components are diffusely localized after 1-2 days in culture, but after 3-4 days are organized into mature striated myofibrils.361,362 We found that this time-course of myofibril accumulation could be reproducibly quantified by measuring the percentage of the cardiomyocyte area containing mature myofibrils, as defined by broad periodic bands of α-actinin staining.362 Our previous studies showed that GFP-tagged N-RAP domains could inhibit myofibril accumulation in this system.362

We expressed full-length mouse N-RAP isoforms S and C as GFP-fusion proteins in primary cultures of embryonic chick cardiomyocytes; for comparison, we also transfected cells with the truncated mouse GFP-N-RAP construct previously thought to be the full-length cDNA.362 This construct is identical to N-RAP isoform C except that it does not include the last two super repeats. To visualize myofibrillar structures, cells were fixed and stained with antibodies against sarcomeric α-actinin. Cells were transfected at day one and fixed at day four, since our previous study showed that this protocol yields maximal effects on myofibril assembly.362

We found that the truncated N-RAP was associated with myofibril precursors and the cell periphery, but did not remain associated with mature sarcomeres (Figure 36 A-D). Many examples were found in which the truncated N-RAP appeared to inhibit myofibril accumulation (Figure 36 C,D), as previously reported.362 In contrast, GFP alone was diffusely distributed in transfected cells, and did not affect myofibril accumulation (Figure 36 E,F).

Like the truncated N-RAP, both full-length N-RAP isoforms were associated with myofibril precursors and the cell periphery, but did not remain associated with mature sarcomeres (Figure 37 A-B, E-F). In addition to incorporating into myofibril precursors, we observed many cardiomyocytes in which over expression of either isoform apparently inhibited myofibril
assembly (Figure 37 C-D, G-H). Quantification of the extent of myofibril accumulation shows that both of the full-length N-RAP isoforms as well as the truncated isoform C significantly inhibit myofibril assembly when compared to myocytes expressing GFP alone (Figure 38); in addition, the N-RAP isoforms and the truncated N-RAP were equally effective in inhibiting myofibril assembly.

Figure 36. Expression of N-RAP Inhibits Myofibril Accumulation.

Cultured embryonic chick cardiomyocytes transfected with GFP-tagged NRAP constructs (A-D) or GFP alone (E,F). Double exposures show α-actinin staining (left panels) and GFP fluorescence (right panels). Truncated N-RAP fusion
protein is found in myofibril precursors characterized by closely spaced or continuous α-actinin staining (arrows) and at the cell periphery, but not in mature sarcomeres identified by periodic wide bands of α-actinin (A-B). In addition, many cells were observed in which truncated N-RAP appeared to have inhibited myofibril accumulation when compared to neighbouring untransfected cells (C,D). In contrast, cells transfected with GFP alone exhibited GFP fluorescence diffusely distributed throughout the cell (F), with no effect on α-actinin organization (E).

Figure 37. Chick Cardiomyocytes Transfected with N-RAP Splice Variants.
Constructs encode GFP-tagged mouse *N-RAP* isoform C (A-D) or isoform S (E-H). The GFP-tagged proteins are visualized in green (right panels), and alpha-actinin staining is visualized in red (left panels). Each *N-RAP* fusion protein is found in myofibril precursors characterized by closely spaced or continuous alpha-actinin staining (arrows) and at the cell periphery, but not in mature sarcomeres (A-B, E-F). In addition, many cells were observed in which each construct appeared to have inhibited myofibril accumulation (C-D, G-H).

![Bar chart](image)

**Figure 38. Myofibril Content in Cardiomyocytes expressing N-RAP constructs.**

Each full-length *N-RAP* isoform, as well as the truncated isoform C that lacks the C-terminal two super repeats, inhibits myofibril accumulation to the same extent when compared to cardiomyocytes expressing GFP alone. Each bar represents the mean ± sem from 24-27 cells. Values for the *N-RAP* constructs are significantly different from GFP alone (p < .01).

**Genomic Sequencing and Cardiomyopathy:** Several polymorphisms causing single amino-acid sequence changes were detected in 50 unrelated DCM or HCM patients and normal controls (table 1). We found no coding sequence or splice donor/acceptor abnormalities that were unique to members of the family with DCM linked to chromosome 10q24-26.

**3.5.2.5 Discussion**

The genomic and cDNA sequences for human and mouse *N-RAP* have been identified. *N-RAP* is expressed as at least two splice variants in cardiac and skeletal muscles, where there is a degree of isoform specificity. Sequence analysis indicates that *N-RAP* has a highly conserved N-
terminal LIM domain followed by either 10 or 11 simple repeats and then by 5 highly conserved C-terminal super repeats, each of which contains 7 single repeats. The N-RAP super repeats are most similar to those found in the central region of nebulin, a large actin-binding protein of skeletal muscle composed of a C-terminal SH3 domain, 22 central super repeats, and simple repeats linking the super repeats to the non-repetitive C-terminal and N-terminal regions.\textsuperscript{363,364}
The N-RAP simple repeats are most similar to the simple repeats near the N-terminus of nebulin.

N-RAP is a much larger protein than previously reported, containing an additional two super repeats at the C-terminal end (Figure 33). Cloning and sequencing artefacts are likely to have been responsible for errors in the published sequence. The sizes of the \textit{N-RAP} mRNA detected by northern blot and the N-RAP protein detected by immunoblot, both previously\textsuperscript{348} and in this report, are consistent with the predictions of the sequence presented here. In addition, we did not detect transcripts missing exons 37-40 by RT-PCR (Figure 34), as predicted from the previously published mouse \textit{N-RAP} sequence. Interestingly, human N-RAP cDNAs with large numbers of exons missing are represented in the Genbank database (e.g. accession numbers AK056605, AK096875). However, to date there is no evidence that these smaller forms represent actual functional transcripts.

The two \textit{N-RAP} splice variants identified differ by a single 105 bp exon which encodes a single 4.1 kDa nebulin-like repeat. These size variants, and perhaps other isoforms, have to date not been resolved by RNA or protein electrophoresis. The repetitiveness and conservation of domain coding within individual exons suggests that yet further splice variations may alter the number, specificity or separation of the nebulin-like repeats. The inclusion of exon 12 in the predominant skeletal isoform adds a single nebulin-like repeat, and may be fundamental to differences in the role of N-RAP in skeletal and cardiac muscle; in adult cardiac muscle N-RAP is concentrated at the intercalated disc, whereas in skeletal muscle N-RAP is localized at the myotendinous junctions.\textsuperscript{348}

In cultured embryonic chick cardiomyocytes, N-RAP is found in the earliest myofibril precursors found at the cell periphery.\textsuperscript{361} We found that both full-length N-RAP isoforms targeted to the cell periphery and to myofibril precursors when expressed as GFP-fusion proteins (Figure 37),
and that they were equally effective in inhibiting myofibril assembly (Figure 38). Previously we showed that the N-RAP LIM domain targets to the cell periphery, while the simple repeat region targets to Z-lines and the super repeats target to actin filaments.\textsuperscript{362} Over-expression of each N-RAP region interfered with myofibril assembly, leading to a model in which N-RAP acts an organizing centre during the first steps of myofibrillogenesis, controlling the assembly of $\alpha$-actinin and actin into Z-bodies with associated thin filaments.\textsuperscript{362} Inhibition of this process by over expressing full-length N-RAP suggests that the stoichiometry of the molecular components found in pre-myofibrils is critical for proper assembly to take place. Alternatively, murine and chick N-RAP may have significant differences, or yet other N-RAP isoforms may be expressed in the developing myocyte. The truncated N-RAP previously described\textsuperscript{362} contains the LIM domain, the simple repeats, and three of the five super repeats, and exhibits localization and inhibition of myofibril assembly that is indistinguishable from the new full length N-RAP constructs.

Unfortunately, our expression studies in chick cardiomyocytes have not revealed any functional differences between the N-RAP isoforms. This may be because the single module encoded by the mouse skeletal muscle-specific exon has a binding partner that is only expressed in skeletal muscle; a second possibility is that the isoform difference is a mammalian adaptation that does not occur in avian muscles. Further work will be needed to test these hypotheses.

The early upregulation of N-RAP in mouse models of DCM\textsuperscript{352}, along with its likely involvement in force-transmission and cardiomyocyte development\textsuperscript{348, 354, 362}, indicate that genetic abnormalities affecting N-RAP are excellent candidate causes of familial cardiomyopathy. A screening and sequencing strategy using genomic DNA has not identified mutations likely to cause DCM in a family with DCM linked to a locus that includes N-RAP, or in 50 unrelated cardiomyopathy patients. However, several polymorphisms have been detected (Table 1) and these may contribute to a genetic milieu that modifies the expression of several cardiac and skeletal muscle diseases.
<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>CODON</th>
<th>SUBSTITUTION</th>
<th>Δ CHARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>207</td>
<td>GTG-GGG</td>
<td>VAL-GLY</td>
<td>0-&gt;0</td>
</tr>
<tr>
<td>208</td>
<td>GTG-GCG</td>
<td>VAL-ALA</td>
<td>0-&gt;0</td>
</tr>
<tr>
<td>249</td>
<td>TAT-TGT</td>
<td>TYR-CYS</td>
<td>0-&gt;0</td>
</tr>
<tr>
<td>282</td>
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<td>ALA-THR</td>
<td>0-&gt;0</td>
</tr>
<tr>
<td>344</td>
<td>GCT-ACT</td>
<td>ALA-THR</td>
<td>0-&gt;0</td>
</tr>
<tr>
<td>360</td>
<td>CAG-CGG</td>
<td>GLN-ARG</td>
<td>0-&gt;P</td>
</tr>
<tr>
<td>484</td>
<td>GAC-AAC</td>
<td>ASP-ASN</td>
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</tr>
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<td>490</td>
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<td>0-&gt;0</td>
</tr>
<tr>
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<td>ASN-ILE</td>
<td>0-&gt;0</td>
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<td>674</td>
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<td>ALA-VAL</td>
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<td>884</td>
<td>CGC-TGC</td>
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<tr>
<td>1566</td>
<td>CGC-TGC</td>
<td>ARG-CYS</td>
<td>0-&gt;P</td>
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<tr>
<td>1681</td>
<td>GCT-GTT</td>
<td>ALA-VAL</td>
<td>0-&gt;0</td>
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</tbody>
</table>

Table 16. Coding changes detected in human N-RAP.

Residues are numbered according to the longer skeletal isoform (N-RAP isoform S). Residue charges are indicated as neutral (0), negative (N), or positive (P).
4 CLINICAL STUDIES

4.1 COHORT CLINICAL STUDIES

Prospective and retrospective observational studies of cohorts of HCM patients comprise the majority of clinical HCM research. Indeed, the viability of large research HCM units has depended on their ability to maintain data on large cohorts of patients referred for tertiary opinion. From these databases specific data sets are examined for relationships with outcomes usually of premature death or success following intervention for symptomatic LVOTO. In the clinic, the majority of our working knowledge comes from these studies. The risk of bias in these studies is very real. Publication bias, bias inherent in studies of tertiary centre cohort studies (or any with implicit or explicit inclusion criteria) and those resulting from double-unblinded, non-randomised studies completed by enthusiastic stake-holder principal investigators dependant on profile for future funding.

This section describes a retrospective study that examines claims, published in the New England Journal of Medicine\textsuperscript{366}, that the presence of systolic compression of epicardial arteries (commonly referred to as myocardial bridging) in children with HCM is powerfully and independently associated with SD risk. The authors also advocated cardiac surgery or coronary stenting in treatment.

Other retrospective studies that were completed at NIH are not presented here; Table 17 includes references to that work.
Scientific Sessions:


Peer Reviewed and Commissioned Articles

- Moak JF, Leifer ES, Tripodi D, Mohiddin SA, Fananapazir L. MD Long Term Follow Up of Children and Adolescents Diagnosed with Hypertrophic Cardiomyopathy: The Importance of Septal Thickness and Inducible Ventricular Tachycardia in Predicting Outcome. (JACC, In Review)

Table 17. Published Research and Presentations of Findings: Clinical Studies.
4.1.1 Myocardial Bridging in Children with Hypertrophic Cardiomyopathy

Quantification of sudden death (SD) risk in HCM remains somewhat of a holy grail. Children and adolescents (aged 18 years or less) diagnosed with HCM are thought to have particularly high sudden death risks (Figure 39). In addition, lifestyle changes (such as avoidance of exercise) are more subject to non-compliance and therapeutic strategies (such as implantable defibrillators) are subject to more complications than for adult patients.

In 1998, Yetman et al published a retrospective study in the New England Journal of Medicine with an astonishing claim; almost all SD in children with HCM was likely to be caused by myocardial ischaemia resulting from myocardial compression of epicardial coronary arteries (coronary 'bridging'). The authors suggested that, in affected individuals, surgical division of the muscular bridge overlying the coronary arteries may lead to an improved prognosis. Furthermore, in their study, myocardial bridging developed independently of the magnitude of hypertrophy, and other important clinical features of the disease.

The claim that a single risk factor could accomplish risk stratification with nearly perfect predictive power (Figure 39) stood in contrast to the marked heterogeneity that is a prominent feature of HCM. The clinical implications of the study, particularly with the status afforded by the NEJM, included routine cardiac catheterisation in all children with HCM. A large minority would subsequently be considered for with major cardiac surgery.

Children with HCM referred to the Inherited Cardiac Diseases section at the NHLBI were routinely considered for inclusion in research protocols, many of which included cardiac catheterisation and myocardial perfusion scintigraphy. We therefore examined the cohort of children that had undergone selective coronary angiography at the NIH.

4.1.1.1 Abstract

Background: It has recently been suggested that systolic compression of epicardial coronary arteries is an important cause of myocardial ischaemia and sudden death (SD) in children with hypertrophic cardiomyopathy (HCM). We examined the associations between systolic compression of the coronary vessels and their intramural (septal) branches with myocardial perfusion abnormalities and clinical outcome in children with HCM.
Results. Angiograms from 57 children with HCM were reviewed for the presence of coronary and septal artery compression. Coronary compression was present in 40% of the children. The left anterior descending artery was most commonly affected and multiple sites found in 4 children. Myocardial perfusion abnormalities were more frequently present in children with coronary compression than in those without, (94% versus 47%, p=0.002). Coronary compression was also associated with more severe septal hypertrophy and higher left ventricular (LV) outflow gradient. Septal branch compression was present in 65% of the children and was significantly associated with coronary compression, severity of LV hypertrophy and outflow obstruction. Multivariate analysis demonstrated that LV septal thickness and septal branch compression, and not coronary compression, were independent predictors of myocardial perfusion abnormalities. Coronary compression was not associated with symptom severity, ventricular tachycardia on Holter or induced at electrophysiologic study or a worse prognosis.

Conclusions. Compression of coronary arteries and their septal branches are common in HCM children and are related to the magnitude of LV hypertrophy. Several mechanisms contribute to the development of myocardial ischaemia in HCM, and our findings suggest that coronary compression does not make an important contribution. Rather, LV hypertrophy and compression of intramural arteries may significantly contribute to the development of myocardial ischaemia in HCM.

4.1.1.2 Introduction
HCM is often associated with disabling symptoms, arrhythmias and sudden cardiac death. Several mechanisms, including myocardial ischaemia, are thought important in the aetiology of sudden death (SD).\textsuperscript{84,89,90,98,372-378}

HCM patients often have regional myocardial perfusion abnormalities as demonstrated by exercise thallium scintigraphy and positron emission tomography (PET) and by net production of myocardial lactate.\textsuperscript{89,379-384} SD is most common in paediatric populations (Figure 39), and stress induced myocardial perfusion abnormalities are associated with increased risk.\textsuperscript{384-387} There are several mechanisms potentially responsible for ischaemia in HCM including the obstruction, by systolic compression, of epicardial and intramyocardial arteries.\textsuperscript{89,366,388}
Yetman et al. suggested that systolic compression of epicardial arteries in children with HCM persists into diastole resulting in coronary insufficiency and myocardial ischaemia.\textsuperscript{366} In their study, compression was associated with reduced survival, ventricular tachycardia, chest pain and impaired exercise performance. The magnitude of LV hypertrophy and severity of obstruction were not associated with compression. In contrast, prognosis in children without coronary compression was excellent (Figure 39). It is suggested that surgical division of the bridge may improve prognosis.

![Figure 39. Survival Estimates of Children Diagnosed with HCM.](image)

Based on these studies,\textsuperscript{366,385-387,389} the annual mortality from cardiac deaths in children is estimated at approximately 4% per year. It is reported that in the absence of coronary bridging, survival is significantly improved.

To validate their findings, we investigated relations between SD, systolic coronary compression of intra- and epicardial arteries, myocardial perfusion abnormalities and severity of hypertrophy in a larger group of HCM children who had been followed for a longer period and whose management represented more current practice.

**4.1.1.3 Methods**

Children with HCM who underwent selective coronary angiography at the NIH between January 1989 and April 1999 were studied. HCM was defined echocardiographically as a hypertrophied,
non-dilated LV in the absence of another cause for the increased cardiac mass. Cardiac events were sudden death or resuscitated cardiac arrest. Survival was determined according to the age of the children, not years from diagnosis, as the majority of cardiac events occur in the second and third decades of life.\textsuperscript{390}

Right and left heart hemodynamic measurements and selective coronary angiograms were obtained at catheterisation. All coronary segments showing evidence of compression were assessed quantitatively to determine the severity of compression. Coronary compression was defined as a maximum systolic compression \( >50\% \).\textsuperscript{366} Identification of exact diastolic/systolic boundaries from selective coronary angiograms is not accurate; angiographic determination of diastolic persistence of compression is not reliable and was not measured. Septal perforator branches of the LAD were considered compressed when they were angiographically obliterated in systole. Treadmill exercise tests were performed using the Bruce protocol. Exercise thallium scintigraphy was available for most subjects. A region of reduced thallium uptake was determined to be reversible or fixed, and the presence or absence of apparent cavity dilatation was also determined.

Holter recordings were analysed for the presence or absence of ventricular tachycardia (VT). The QT, QTc intervals and QTc dispersion were measured from the 12-lead ECG. Electrophysiology (EP) studies were performed in a subset of the children using previously described methods.\textsuperscript{90}

Two-sample data were compared by Student’s t-test. Cardiac survival rates for patients with and without coronary compression were determined by Kaplan-Meier estimates and compared with the log rank test. Multivariate logistic regression analysis was used to determine the independent contributions of LV wall thickness, LV outflow obstruction, septal compression and bridging to thallium perfusion abnormalities. A \( p<0.05 \) indicated significant differences.

4.1.1.4 Results

Characteristics of the Study Population: 57 children with HCM admitted to the National Institutes of Health had selective coronary angiograms. These children had a high prevalence of risk factors associated with sudden death (Table 18).
Table 18. Prevalence of Risk Factors for Sudden Death in the Study Population.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Prevalence</th>
</tr>
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<tbody>
<tr>
<td>Cardiorespiratory arrest, syncope and/or presyncope</td>
<td>35 (61%)</td>
</tr>
<tr>
<td>Family history of &gt;2 premature sudden death</td>
<td>12(21%)</td>
</tr>
<tr>
<td>Myocardial ischaemia</td>
<td>31 (54%)</td>
</tr>
<tr>
<td>VT during ambulatory Holter monitoring</td>
<td>14 (25%)</td>
</tr>
<tr>
<td>Genetic abnormality associated with a poor prognosis</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>Severe LV hypertrophy (LV wall thickness &gt;30 mm)</td>
<td>12 (21%)</td>
</tr>
<tr>
<td>Flat* or hypotensive BP response to baseline exercise</td>
<td>26 (46%)</td>
</tr>
<tr>
<td>More than one of the features listed above</td>
<td>53 (93%)</td>
</tr>
</tbody>
</table>

Angiographic Findings: We detected coronary compression more frequently than previously reported and at sites other than the mid LAD. Coronary compression (Figure 40) affected 28 coronary segments in 23 (40%) children. Multiple coronary sites were identified in 4 (7%) children: 2 coronary segments in 3, and 3 segments in 1 child. Compression was of the mid-LAD in 13 (46%), proximal LAD in 1 (4%), distal LAD in 2 (7%), a diagonal branch in 5 (18%), an obtuse marginal branch in 4 (14%), and the posterior descending artery in 3 (11%). The mean length of the compressed segment was 15±7 mm, range 6-39 mm, with a mean narrowing of 76±18%. The maximum compression was between 75-100% in 13 (46%) and complete systolic occlusion was seen in 8 (29%). Coronary compression less severe than used in the current definition was detected in additional children. Septal artery compression was detected in 37/57 children (65%). It was detected in all 23 children with coronary compression, but only 14/34 without coronary compression, p<0.001.

Clinical Features: The ages of diagnosis and cardiac catheterisation and the symptom status were similar in children with and without compression (Table 19). Cardiac events (2 sudden deaths, 4 cardiac arrests) occurred in 2 children with a compressed coronary and 4 without. The cumulative survival rate at 20 years of age in children with coronary compression was 85±10% and without was 82±8%, p=0.9. In addition, LV systolic dysfunction occurred in 3 children without coronary compression, in 1 child following transmural myocardial infarction (none in children with coronary compression). One child in each group underwent cardiac transplantation for severe symptoms and exercise limitation.

Echocardiography: Mean septal thickness in children with coronary compression was 28±8 mm Vs. 19±8 mm in those without coronary compression, p<0.001, and 26±9 mm compared to
17±5 mm in children with or without septal compression, \( p<0.0005 \). Children with coronary or septal compression had higher LV outflow gradients than children without any compression (Table 19 and Table 20).

**Hemodynamic Variables:** Mean resting LV outflow gradient was greater in patients with either coronary or septal compression than without (Table 19 and Table 20)

---

**Figure 40. Coronary and Septal Perforator Compression in Children with HCM.**

Diastolic frames are on the left. (A). 6-year old boy with compression of the mid-LAD and its septal branches. (B). 13-year old girl with compression of the posterior descending artery and its septal branches. (C). Compression of septal branches of the LAD in the absence of LAD compression. Large arrows indicate sites of bridging of epicardial arteries and small arrows indicate compression of septal branches.
<table>
<thead>
<tr>
<th>Coronary Compression</th>
<th>All children</th>
<th>No Compression</th>
<th>Compression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (range)</td>
<td>10±6</td>
<td>11±6 (.03-18)</td>
<td>10±6 (0.1-17)</td>
<td>0.23</td>
</tr>
<tr>
<td>Age at angiography (range)</td>
<td>15±4</td>
<td>15±4 (.6-20)</td>
<td>14±4 (.4-20)</td>
<td>0.35</td>
</tr>
<tr>
<td>Follow up (years): from diagnosis</td>
<td>8±6</td>
<td>9±6</td>
<td>8±6</td>
<td>0.7</td>
</tr>
<tr>
<td>from catheterisation</td>
<td>4±4</td>
<td>5±4</td>
<td>3±3</td>
<td>0.08</td>
</tr>
<tr>
<td>Male</td>
<td>43 (75%)</td>
<td>28 (82%)</td>
<td>15 (65%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Family history of SD</td>
<td>12 (21%)</td>
<td>8 (24%)</td>
<td>4 (17%)</td>
<td>0.74</td>
</tr>
<tr>
<td>Clinical presentation asymptomatic</td>
<td>10 (18%)</td>
<td>6 (18%)</td>
<td>4 (17%)</td>
<td>0.99</td>
</tr>
<tr>
<td>syncope</td>
<td>23 (40%)</td>
<td>15 (44%)</td>
<td>8 (35%)</td>
<td>0.67</td>
</tr>
<tr>
<td>cardiac arrest</td>
<td>4 (7%)</td>
<td>3 (9%)</td>
<td>1 (4%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Echocardiographic indices interventricular septum (mm)</td>
<td>23±9</td>
<td>19±8</td>
<td>28±8</td>
<td>0.0004</td>
</tr>
<tr>
<td>posterior LV wall (mm)</td>
<td>11±3</td>
<td>12±4</td>
<td>11±3</td>
<td>0.43</td>
</tr>
<tr>
<td>septum to posterior LV wall ratio</td>
<td>2.2±1.1</td>
<td>1.8±0.9</td>
<td>2.7±1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>LV outflow gradient (mm Hg)</td>
<td>28±35</td>
<td>16±28</td>
<td>45±37</td>
<td>0.002</td>
</tr>
<tr>
<td>Cardiac catheterisation systolic LV (mm Hg)</td>
<td>123±28</td>
<td>116±21</td>
<td>133±34</td>
<td>0.03</td>
</tr>
<tr>
<td>LV outflow gradient (mm Hg)</td>
<td>23±31</td>
<td>14±24</td>
<td>37±34</td>
<td>0.005</td>
</tr>
<tr>
<td>septal compression (%)</td>
<td>37 (65%)</td>
<td>14 (41%)</td>
<td>23 (100%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Exercise thallium scintigraphy abnormal study</td>
<td>(n=48)</td>
<td>(n=30)</td>
<td>(n=22)</td>
<td></td>
</tr>
<tr>
<td>number of abnormal segments</td>
<td>86/288 (30%)</td>
<td>34/180 (19%)</td>
<td>52/108 (48%)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 19. Characteristics of Children with Systolic Coronary Compression. P values refer to comparisons of 'No Compression' with 'Compression'.

<table>
<thead>
<tr>
<th>Septal Perforator Compression</th>
<th>Absent (n=20)</th>
<th>present (n=37)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echocardiographic indices interventricular septum (mm)</td>
<td>17±5</td>
<td>26±9</td>
<td>0.0004</td>
</tr>
<tr>
<td>posterior LV wall (mm)</td>
<td>12±4</td>
<td>11±3</td>
<td>0.30</td>
</tr>
<tr>
<td>septum to posterior LV wall ratio</td>
<td>1.5±0.5</td>
<td>2.5±1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>LV outflow gradient (mm Hg)</td>
<td>13±29</td>
<td>35±36</td>
<td>0.02</td>
</tr>
<tr>
<td>Cardiac catheterisation systolic LV (mm Hg)</td>
<td>117±16</td>
<td>127±31</td>
<td>0.2</td>
</tr>
<tr>
<td>mean aortic pressure (mm Hg)</td>
<td>78±13</td>
<td>69±7</td>
<td>0.01</td>
</tr>
<tr>
<td>LV outflow gradient (mm Hg)</td>
<td>10±15</td>
<td>30±31</td>
<td>0.01</td>
</tr>
<tr>
<td>coronary bridging (%)</td>
<td>0/20</td>
<td>23/37 (76%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Exercise thallium scintigraphy abnormal study</td>
<td>(n=17)</td>
<td>(n=31)</td>
<td></td>
</tr>
<tr>
<td>number of abnormal segments</td>
<td>4 (24%)</td>
<td>27 (87%)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 20. Characteristics of Children with Systolic Septal Perforator Compression. P values refer to comparisons of 'No Compression' with 'Compression'.
Exercise thallium scintigraphy: Reversible myocardial abnormalities were present in 31 of 48 (65%) children (Figure 41). Abnormalities in myocardial thallium uptake were much commoner in children with coronary and septal compression (Table 19 and Table 20). LV hypertrophy was greater in patients with reversible thallium abnormalities (septum, 26±9 mm versus 16±3 mm, p=0.0001) and LV outflow gradients at cardiac catheterisation were higher (26±33 mm Hg versus 7±12 mm Hg, p<0.05).

![Stress Images](image1)

![Rest Images](image2)

Figure 41. Myocardial Perfusion Scintigraphy.
Images obtained during exercise, in the upper row, and following rest and re-injection below. Reversible apical, subendocardial, posterior and septal defects are seen in this child with mid LAD compression. These defects cannot all be due to LAD compression.

The distribution of thallium abnormalities were often unrelated to the coronary artery compressed, and were similar in the children with LAD compression to those in children with compression of another coronary. Stress-induced apparent LV cavity dilatation thought to represent subendocardial ischaemia and not easily attributed to occlusion of a single coronary, was present in over a third of the children. Almost half of the children without coronary compression had reversible abnormalities. Multiple regression analysis identifies LV septal thickness and septal perforator compression, and not LV obstruction or coronary compression, as independent predictors of reversible myocardial thallium uptake abnormalities (Table 21).
Thus, factors other than coronary compression are important in the aetiology of perfusion abnormalities. In contrast, compression of septal perforators may contribute to myocardial perfusion abnormalities.

<table>
<thead>
<tr>
<th>Univariate analyses</th>
<th>Odds Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septal wall thickness (per Sd of 9 mm)</td>
<td>9.90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV outflow obstruction (per mm Hg)</td>
<td>1.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bridging (absent or present)</td>
<td>3.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Septal perforator compression (absent or present)</td>
<td>7.30</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multivariate model with all 4 variables</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septal wall thickness (per Sd of 9 mm)</td>
<td>9.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV outflow obstruction (per mm Hg)</td>
<td>1.01</td>
<td>0.2</td>
</tr>
<tr>
<td>Bridging (absent or present)</td>
<td>1.61</td>
<td>0.14</td>
</tr>
<tr>
<td>Septal perforator compression (absent or present)</td>
<td>3.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 21. Relation of Myocardial Perfusion Abnormalities to Selected Clinical Parameters.

**Exercise and Electrophysiology**: There were no significant differences in mean exercise duration, resting and peak systolic blood pressure in children with and without coronary or septal compression. The mean QT and QTc intervals, and QTc dispersion were not significantly different in children with and without. Non-sustained VT was recorded in 17% of the children during ambulatory ECG monitoring. EP was performed in 36 (63%) of the children; sustained VT was induced in 28% of studies. Differences in VT on Holter and VT induced at EP study in children with or without coronary compression were not statistically significant.

**4.1.1.5 Discussion**

**Myocardial ischaemia in HCM**: Evidence for myocardial 'ischaemia' in HCM is provided by stress-induced; (1) chest pain and electrocardiographic changes; (2) reversible myocardial perfusion defects at thallium scintigraphy (3) limitations in coronary flow reserve (CFR); and (4) anaerobic metabolism in patients with HCM who have a history of chest pain.\(^{89,379-384,391}\)

Myocardial ischaemia results from true hypoxia (mismatch of flow supply to myocardial
demand), or from abnormalities in production and utilization of high-energy phosphates.\textsuperscript{30,392,393}

CFR limitation in HCM, like thallium perfusion defects, is often most striking in the subendocardium and does not typically show single coronary distribution.\textsuperscript{89} CFR is lowest in HCM patients with microvascular abnormalities detected following biopsy.\textsuperscript{381,383,384,388,394}

Myocardial perfusion may also be limited by elevated LV diastolic pressure, abbreviated diastolic intervals and systolic arterial compression as illustrated in Figure 42.\textsuperscript{395}

\textbf{Figure 42. Impaired Coronary Perfusion Pressure in a Patient with Non-Obstructive HCM.}

Simultaneous recordings of arterial pressure and LV pressure in a young patient with severe LV hypertrophy, no outflow obstruction, septal artery compression but no coronary compression. (A) The gradient between arterial and LV pressure in diastole, as indicated by the shaded area, drives myocardial perfusion. (B) Following infusion of isoproterenol, LV diastolic pressures have risen dramatically, aortic diastolic pressure has fallen and the diastolic period is abbreviated. Myocardial perfusion pressure and duration are severely diminished, as shown by the dramatic reduction in the shaded area.

\textbf{Definitions and Prevalence of Coronary Compression:} Coronary angiography demonstrates systolic compression of epicardial coronary arteries in a proportion of otherwise normal hearts. A length of a normally epicardial artery is crossed by a myocardial band or ‘bridge’ that contracts and compresses the underlying artery in systole.\textsuperscript{396-398} In this article, bridging refers to the muscle bridge and the angiographic findings are termed coronary compression.
In adult populations without cardiomyopathy, systolic coronary compression is seen at angiography in 0.5% to 12%, most often affecting the mid left anterior descending artery (LAD). Muscle bridges are found much more frequently at autopsy or aorto-coronary bypass (5-86%). These also most frequently cross the mid LAD but have been described over most epicardial arteries. Therefore, anatomic muscular bridges not always lead to coronary compression visible at angiography. An intra myocardial segment of an epicardial artery, most often of the mid LAD, should possibly be considered a (frequent) coronary variant rather than a coronary anomaly. Pharmacological agents like nitroglycerin and isoproterenol increase the angiographic severity and incidence of compression, with detection rates increasing to 40% in patients undergoing angiography.

In hypertrophic hearts coronary compression is more common; myocardial hyperdynamic and hypertrophic adaptations may extend to previously ‘silent’ bridges that become compressive. Coronary compression is found in 30 to 80% of adults with HCM or with aortic stenosis. Studies in adults with HCM have described no relationship to ischaemia. Coronary Compression and Myocardial Ischaemia: It has been previously suggested that coronary compression: (1) results in coronary insufficiency and myocardial ischaemia and; (2) may provide protection against the development of coronary atheroma. The latter, reviewed elsewhere, is not considered here.

Coronary flow occurs predominantly in diastole, with only 15% of myocardial flow occurring in systole. Systolic compression may result in ischaemia, if the contribution of systolic flow becomes critical, or if a sufficient severity of compression persists into diastole. Compression may result in elliptical narrowing of the coronary and not concentric narrowing typical of coronary disease. Relations between the minimum diameter and luminal diameter are different from a circle; a concentric decrease in arterial diameter of 50% decreases luminal area by 75%, the area will only be reduced by approximately 30% if the artery is distorted into an ellipse. To reduce flow significantly, diastolic coronary compression must remain at maximum severity (Figure 43).
Multiple case reports implicate compression as the cause for ischaemic syndromes in patients with otherwise normal coronary arteries. Surgery to de-roof or bypass the bridge and coronary stenting have been associated with symptomatic improvement in case reports and small uncontrolled series. Noble et al. demonstrated increased myocardial lactate production following atrial tachy-pacing in symptomatic patients with coronary compression, although approximately 25% of their patients may have had myocardial hypertrophy, itself associated with myocardial lactate production and symptoms. Quantitative angiography and coronary Doppler flow studies detect some diastolic persistence of compression, but its hemodynamic significance is not established. No controlled study demonstrates increases in morbidity or mortality associated with coronary compression, and the 5-year survival is similar to that of an unselected population. In several of the case reports describing ischaemic syndromes (chest pain or myocardial infarction sudden death or ventricular tachycardia) patients also had myocardial hypertrophy, possibly representing HCM. The literature remains equivocal.

Lastly, in HCM and other hypertrophic conditions, angiographic obliteration in systole of the septal perforator branches of the LAD and PDA (often termed septal blanching), occurs.
commonly. It is present in approximately 70% of patients with aortic stenosis or HCM, and has previously been associated with the degree of LV hypertrophy and thallium perfusion abnormalities.\textsuperscript{413-415} Doppler studies demonstrate reversal of coronary flow in systole, an abnormality correlating with severity of septal compression.\textsuperscript{415,416} Therefore, in the presence of systolic septal compression, epicardial compression may not be of additional significance in limiting coronary flow and surgical release of the compressed coronary will not improve myocardial perfusion.

4.1.1.6 Conclusions
There are several mechanisms for SD in HCM other than ischaemia, and ischaemia is multifactorial in aetiology. This study finds no evidence that epicardial compression is an important cause of myocardial perfusion abnormalities in HCM children. Previously described associations may have been confounded by associated features, such as the magnitude of myocardial hypertrophy. Rather, our data support the hypothesis that LV hypertrophy and septal artery compression are important determinants of the severity of perfusion abnormalities. It remains possible that in a few patients, a severe degree of coronary compression contributes to ischaemia. In the children studied here, there was no evidence that this contribution was significant even in those with the most severe compression. Currently, there is little evidence to suggest relief of coronary compression in children with HCM will improve symptoms or prognosis.
4.2 **PROSPECTIVE RANDOMISED STUDIES**

Prospective randomised controlled trials (RCT) are largely absent from HCM research, and despite the relatively extensive published HCM related research. In the development of therapies for HCM, the three central themes have been: (1) Prevention of sudden death (SD); (2) Relief of symptoms associated with LVOTO; and (3) Attempts in prevention/regression of LV hypertrophy. NO RCT examine SD prevention.

Two RCT compared the effects of dual chamber (DDD) pacing on LVOT gradients and symptoms in obstructive HCM, with results that are generally interpreted as showing no benefit for pacing, but are best viewed as equivocal.\(^{129,131,417,418}\) In consequence, DDD pacing for this indication has largely been abandoned in favour of alcohol septal ablation (ASA) and LV myectomy (LVM) despite these latter modalities never being subjected to similar scrutiny by randomised trial.

Finally, two small randomised studies examining effects on LV mass of pharmacological agents followed from studies in animals of statin therapy and renin-angiotensin-aldosterone system (RAAS) antagonism (refs).\(^{419,420}\) A small study randomised 19 HCM patients to losartan or placebo and reported a small reduction in LV mass following a year of losartan therapy compared with placebo.\(^{419}\) Routine clinical practice has not followed the results of this study. A pilot study of statin therapy in humans did not corroborate the animal data and found no effects on LV mass of atorvastatin (80mg) compared with placebo for 9 months in 22 randomised patients.\(^{420}\)

Thus clinical practice in HCM is based on little or no evidence of the quality expected in modern cardiology. Even the effect of the DDD trials has been to favour more invasive but less completely evaluated alternative treatments. Given the considerable research interest in HCM evident in any literature searches, it is surprising that RCTs have not been designed and completed. Reasons for this may include:

1. **Clinical heterogeneity and cohort size**: Cohort sizes offering sufficient statistical power to demonstrate treatment effect is the most evident obstacle. For example, with the primary outcome as mortality, it has been estimated that as many as 34,000 HCM patients would be needed for the screening phase of a trial of ASA against DDD that
eventually enrolled 600 patients in each treatment arm.\textsuperscript{421} The heterogeneity in disease and the high prevalence of asymptomatic patients determines the low screening to trial-eligible ratio. As this claim is made by the principal investigators of M\_PATHY, a trial of DDD pacing for the same indication that has proved influential in it's abandonment\textsuperscript{129}, the conclusion that recruitment difficulties are 'virtually insurmountable' is curious. Mortality rates are low, but other outcome measures are also of interest. Symptom status currently determines indications for invasive treatment for LVOTO. The influential DDD trials report symptom status and exercise testing as the principal outcome measure where a large placebo effect is reported in at least one of these studies. Objective measures of well-being and exercise capacity rather than mortality are surely the relevant outcome measures for a treatment indicated on symptomatic grounds. Similarly, magnitudes of LVH, LV scarring at delayed gadolinium enhancement, LVOTO, systolic function, left atrial size, pulmonary pressures and other measurements are useful surrogate markers of disease severity.

2. **Established Practice**: Nonetheless, only two or three Centres world-wide have had the cohort size required for single-centre trials; multi-site collaboration, an essential model in the coronary disease and heart failure trial successes is necessary. The large centres may be inhibited from pursuing single site trials by virtue of mutually-exclusive established stakes in a particular treatment. Thus, a tertiary centre promoting ASA may not have a similar expertise/experience in LVM necessary for a meaningful comparative trial, and visa-versa.

3. **Collaborative Studies**: The two pacing studies depended on multi-site collaborations in the USA and Europe. These efforts have not been replicated for ASA or LVM. One of the reasons for this may be the limited number of centres that offer both treatments, and the understanding that either therapy has the best outcomes in high volume centres; clearly this complicates randomisation. Pacing enthusiasts argue that the same is true of DDD therapy in that despite the availability of pacemaker implanting services, the management of pacing parameters for this indication is specialized. Anecdotally, in patients receiving implants elsewhere, we have seen inappropriate pacing set-ups including high septal lead placements, excessively short programmed A-V delays and AAI pacing. Additionally, as rates of procedural complications in these trials were
higher than expected in routine pacing practice, it is likely that operators relatively inexperienced with this pacing indication predominated.

However, problems with equal access to alternative invasive techniques should not be impediments to trials of pharmacological therapy or device therapy in the assessment of strategies to prevent death from arrhythmia, stroke and heart failure, or of treatments aimed at reversing left ventricular hypertrophy and fibrosis. It remains difficult to account for the reluctance of the HCM research community to perform such studies; an environment of antagonistic competitiveness between rival investigators and an entrenched approach to management developed over decades of clinical practice may be important contributing factors.

At NIH, we completed two single-site randomised trials. A double-blind placebo controlled trial investigating RAAS antagonism began in 1994 and was completed in 2003. The results were presented at the Scientific Sessions of the American Heart Association in 2003 (Table 22). Results of a randomised study comparing ASA with DDD pacing was presented at the Scientific Sessions of the American Heart Association in 2004 (Table 22).

<table>
<thead>
<tr>
<th>Scientific Sessions</th>
</tr>
</thead>
</table>

Table 22. Published Research and Presentations of Findings: Randomised Trials.
4.2.1 Regression of Left Ventricular Hypertrophy in Hypertrophic Cardiomyopathy: Randomised Placebo Controlled Trial of Inhibitors of the Renin-Angiotensin System

The NIH group led by Dr Fananapazir began this trial in 1994 before I joined in 1998; recruitment was completed in 2003. The primary outcome was change in LV mass following six months treatment with a angiotensin converting enzyme inhibition (ACE-I), angiotensin receptor blockade (ARB), ACE plus ARB or placebo. CMR measures of LV mass were obtained in collaboration with Dr Andrew Arai of the department of Cardiac Energetics of the NHLBI. Patient assessment and recruitment, cardiac catheterisation, exercise testing and safety monitoring were performed by our group. Key to safety considerations were study criteria excluding patients with LVOTO at rest or on provocation (isoprenaline infusion and catheter measured gradients) and patients with symptoms worse than NYHA II. A consequence of the latter is that the study was not designed to detect improvement in symptom status following treatment, but was better powered to detect deterioration in well-being.

4.2.1.1 Abstract

Background: We postulated that angiotensin converting enzyme (ACE) inhibition and/or angiotensin-II receptor (AT1) blockade causes regression of left ventricular mass in hypertrophic cardiomyopathy (HCM).

Methods and Results: Eighty-nine patients with non-obstructive HCM with preserved left ventricular systolic function, aged 20-55 years, participated in a double-blind study and were randomised to receive placebo, losartan (50 mg/day), enalapril (10 mg/day) or losartan (50 mg/day) plus enalapril (10 mg/day) for six months. Enalapril and the combination therapy significantly reduced left ventricular mass, but losartan alone did not. Left ventricular mass regression was independent of variations in arterial pressure. The drug regimens did not adversely affect left ventricular volumes, or treadmill heart rate and blood pressures. Cough, hypotension, and/or light-headedness occurred in six patients or 26% on combination therapy, three patients or (13%) on enalapril, and two patients (10%) on losartan. No patient died during a mean follow-up period of 4.0±1.5 years. The group treated with combination therapy, but not other treatment groups, reported a significant improvement in NYHA symptom status.
Conclusions: Enalapril is well tolerated in non-obstructive HCM, and reduces left ventricular mass. ACE inhibitors may be considered in HCM patients with symptoms of heart failure and preserved or impaired LV systolic function. Further studies are necessary to confirm this finding and to evaluate its potential beneficial effects on long-term outcome.

4.2.1.2 Introduction
Left ventricular (LV) hypertrophy is a major independent risk factor for morbidity and mortality from cardiovascular disease. The severity of myocardial hypertrophy in HCM is also associated with adverse outcomes. In addition to adverse outcomes that may include end stage heart failure and increased risk of sudden death, it is also associated with LV diastolic dysfunction, myocardial fibrosis, myocardial perfusion abnormalities, and disabling symptoms.

The renin-angiotensin-aldosterone system (RAS or RAAS) comprises a series of enzymatic steps that generate or modulate several biologically active species including angiotensin-II (Ag-II) and aldosterone. In addition to the classical or endocrine RAAS, a local or paracrine/autocrine RAAS may respond to localized stimuli and tissue requirements. Mechanical stretch and autonomic activity activate cardiac RAS cascades, and many or all RAS components, including aldosterone, are produced in situ.

Several adverse cardiovascular effects of RAAS activity are attributed to Ag-II activity at the angiotensin-1 receptor (AT₁) receptor. Activation of AT₁ receptors on fibroblasts, cardiomyocytes, smooth muscle cells, and endothelial cells promotes hypertrophy, mitogenesis, fibrosis, vasoconstriction, and arrhythmogenesis. In contrast, Ag-II activity at AT₂ receptors is reported to oppose some or all the effects of AT₁ activity. AT₁ receptor antagonists do not inhibit AT₂ receptors, and may actually augment their activity by increasing Ag-II availability. This, along with findings suggesting that local RAS production of Agll may occur independently of renin and ACE activity (thus ‘escaping’ systemic AEE inhibition), suggests that the optimal inhibition of local cardiac RAS requires combination therapy.

Animal models and clinical studies of hypertensive cardiomyopathy have demonstrated that ACE, AT₁ and aldosterone inhibition ameliorate myocardial fibrosis, myocyte hypertrophy, and perfusion abnormalities, and may reduce the incidence of atrial fibrillation and heart
failure. Observations that these effects did not correlate with the activity of circulating ARAS components or with the magnitude of blood pressure reduction suggested a role for local RAAS in the development of the cardiomyopathy.

The LV hypertrophy in HCM develops principally as a consequence of mutant gene expression (often encoding sarcomeric proteins) rather than to pressure overload. HCM is characterized by myocyte hypertrophy, hyperplasia and disarray, and hyperplasia of several other cell types including fibroblasts endothelial cells and smooth muscle cells. Myocardial fibrosis is a common pathologic feature and is often more pronounced than in hypertensive cardiac hypertrophy. Evidence that RAAS activity also contributes to the severity of this form of cardiomyopathy was suggested by the observation that in comparison to the ACE insertion (I) polymorphism, the deletion (D) polymorphism is associated with increased plasma ACE concentrations and occurs at an increased frequency in HCM patients than in unaffected family members or in unrelated controls, and may also influence the severity of LV hypertrophy. Common polymorphisms in both AT1 and AT2 receptors may also influence the magnitude of hypertrophy. Finally, in a mouse model of human sarcomeric gene HCM, AgII blockade reduces the degree of myocardial fibrosis.

The utility of RAS manipulation in HCM remains clinically unproven. The primary hypothesis of this study is that RAAS activity is involved in the genesis and maintenance of LV hypertrophy in HCM, and ACE inhibitors and/or AT1 receptor antagonism will cause regression of the increased LV mass, leading to improved symptoms and cardiac functional indices.

### 4.2.1.3 Methods

**Patients and Study Design:** This double blind, placebo controlled study was performed under informed patient consent according to a protocol approved by the Intramural Research Board of National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH). The primary goal of this study was to determine whether six months of ACE inhibition and/or AT1 receptor blockade therapy reduces LV hypertrophy in patients with HCM.

Between 1994 and 2003 all patients evaluated in the HCM clinic of NHLBI were evaluated non-invasively (clinical history and echocardiography) for entry criteria. Consent for invasive evaluation was obtained in one hundred and thirty-one consecutive patients. Of these 42
subjects did not meet criteria (Table 23), and 89 patients were randomised to receive daily placebo, an ACE-inhibitor (enalapril 10 mg/day), an AT1 receptor blocker (losartan 50 mg/day), or enalapril (10 mg) plus losartan (50 mg). Randomisation by the pharmacy department was performed separately for men and women. Patients in the four arms of the study were well matched with regards to clinical characteristics (Table 24).

Safety considerations: As vasodilators may aggravate LV outflow obstruction, only patients with non-obstructive HCM were included in the study. Following randomisation, heart rate and blood pressure were monitored for 3 days on drug therapy. In the absence of an adverse event, the patient received study drug for six months. Haemoglobin, platelets, electrolytes, renal and hepatic function were checked at baseline and at 1, 3, and 6-month follow-up. Urinary pregnancy test was performed monthly in women of child-bearing age. Only patients with mild or no symptoms (NYHA I or II) were recruited because of concerns regarding potential adverse effects of RAAS inhibition on symptoms in patients with unobstructed HCM.

Evaluations: Studies at baseline and at completion of the 6 months of the protocol included: (1) echocardiography; (2) symptom limited treadmill and bicycle exercise tests; (3) exercise radionuclide angiography; (4) cardiac magnetic resonance imaging (CMR); (5) cardiac catheterisation and angiography; and (6) measurements of plasma ACE and Ang-II concentrations, and of renin activity (PRA). ACE genotype was also determined.

CMR: End expiratory cine CMR was performed on a 1.5T cardiac MRI scanner using a four-element cardiac phased array coil. Computer assisted planimetry was used to quantify myocardial dimensions, volumes, ejection fraction and mass. In 10 water-filled phantoms, measured volumes correlated with actual volumes: actual volume=1.0187 x measured volume - 0.0262, R²=0.9942.

Radionuclide angiography: Parameters of LV filling measured peak filling rate (PFR), time to peak filling rate (TPFR), and atrial contribution to LV stroke volume by methods described previously. LV systolic function was determined by estimating rest and exercise LV ejection fractions.
RAS: ACE genotype was determined by previously described methods. Blood samples for PRA, ACE, and Ang-II concentrations were collected after >15 minutes lying supine following an overnight fast.

**Statistical Analysis:** Data are expressed as mean ± 1 SD. Data were compared using Student's t test. Pearson's test was used to examine the correlation between variables. A two-sided p<0.05 was considered significant. Statistical significance was tested following Bonferroni correction for multiple comparisons.

---

<table>
<thead>
<tr>
<th><strong>Inclusion:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>age, 20-55 years</td>
</tr>
<tr>
<td>LV wall thickness &gt;16 mm* by MRI</td>
</tr>
<tr>
<td>LV outflow gradient of 30 mm Hg gradient at rest, and 55 mm Hg following isoproterenol infusion to a heart rate of about 120 beats per minute at cardiac catheterisation</td>
</tr>
<tr>
<td>NYHA functional class I-II.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Exclusion:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>significant other cardiovascular disease</td>
</tr>
<tr>
<td>chronic atrial fibrillation</td>
</tr>
<tr>
<td>bleeding disorder or anaemia</td>
</tr>
<tr>
<td>renal impairment (blood urea nitrogen &gt; 22 mg/dl and serum creatinine &gt;1.4 mg/dl);</td>
</tr>
<tr>
<td>K+ &lt;3.3 mmol/l or &gt;5.1 mmol/l</td>
</tr>
<tr>
<td>hypertension: basal systolic and diastolic pressures of &gt;160 mm Hg or &gt;95 mm Hg, respectively, on two occasions separated by one hour of rest</td>
</tr>
<tr>
<td>hypotension: sitting systolic arterial pressure &lt;100 mm Hg confirmed 30 minutes later</td>
</tr>
<tr>
<td>pregnancy and lactation</td>
</tr>
<tr>
<td>impaired LV systolic function: LV ejection fraction &lt;50% (radionuclide angiography)</td>
</tr>
<tr>
<td>dependence on diuretics, verapamil, β-blockers, or antiarrhythmic drugs</td>
</tr>
<tr>
<td>sensitivity to ACE inhibitor e.g. angioedema</td>
</tr>
<tr>
<td>ACE inhibitor or losartan treatment &lt;6 months prior to the study</td>
</tr>
</tbody>
</table>

Table 23. **Study Criteria for the Randomised ACE/ARB blocker Study in HCM.**

*, 16 mm selected instead of the usual 13 mm to facilitate detection of remodelling.
### Placebo

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Enalapril</th>
<th>Losartan</th>
<th>Enalapril plus Losartan</th>
</tr>
</thead>
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<tr>
<td>Gender (males)</td>
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<td>16 [70]</td>
<td>14 [67]</td>
<td>14 [61]</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>38±8</td>
<td>34±9</td>
<td>37±11</td>
</tr>
<tr>
<td>NHYA</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>palpitation</td>
<td>14 [64]</td>
<td>12 [52]</td>
<td>8 [38]</td>
<td>12 [52]</td>
</tr>
<tr>
<td>Echocardiogram</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV wall thickness (mm)</td>
<td>27±7</td>
<td>24±6</td>
<td>25±6</td>
<td>23±6</td>
</tr>
<tr>
<td>septum (mm)</td>
<td>17±4</td>
<td>17±5</td>
<td>17±5</td>
<td>17±5</td>
</tr>
<tr>
<td>posterior wall (mm)</td>
<td>10±4</td>
<td>9±2</td>
<td>9±1</td>
<td>9±1</td>
</tr>
<tr>
<td>LV IDd (mm)</td>
<td>44±5</td>
<td>48±4</td>
<td>45±5</td>
<td>44±6</td>
</tr>
<tr>
<td>LV IDs (mm)</td>
<td>25±4</td>
<td>27±3</td>
<td>26±5</td>
<td>25±4</td>
</tr>
<tr>
<td>left atrium (mm)</td>
<td>37±6</td>
<td>44±6</td>
<td>44±6</td>
<td>41±7</td>
</tr>
</tbody>
</table>

Table 24. Randomised ACE and ARB Inhibition: Baseline Clinical Characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Enalapril</th>
<th>Losartan</th>
<th>Enalapril plus Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>22</td>
<td>23</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Gender (males)</td>
<td>14 [64]</td>
<td>16 [70]</td>
<td>14 [67]</td>
<td>14 [61]</td>
</tr>
<tr>
<td>Age (years)</td>
<td>34±9</td>
<td>38±8</td>
<td>34±9</td>
<td>37±11</td>
</tr>
<tr>
<td>NHYA</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>palpitation</td>
<td>14 [64]</td>
<td>12 [52]</td>
<td>8 [38]</td>
<td>12 [52]</td>
</tr>
<tr>
<td>Echocardiogram</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV wall thickness (mm)</td>
<td>27±7</td>
<td>24±6</td>
<td>25±6</td>
<td>23±6</td>
</tr>
<tr>
<td>septum (mm)</td>
<td>17±4</td>
<td>17±5</td>
<td>17±5</td>
<td>17±5</td>
</tr>
<tr>
<td>posterior wall (mm)</td>
<td>10±4</td>
<td>9±2</td>
<td>9±1</td>
<td>9±1</td>
</tr>
<tr>
<td>LV IDd (mm)</td>
<td>44±5</td>
<td>48±4</td>
<td>45±5</td>
<td>44±6</td>
</tr>
<tr>
<td>LV IDs (mm)</td>
<td>25±4</td>
<td>27±3</td>
<td>26±5</td>
<td>25±4</td>
</tr>
<tr>
<td>left atrium (mm)</td>
<td>37±6</td>
<td>44±6</td>
<td>44±6</td>
<td>41±7</td>
</tr>
</tbody>
</table>

Table 24. Randomised ACE and ARB Inhibition: Baseline Clinical Characteristics.

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<tr>
<th></th>
<th>Placebo</th>
<th>Enalapril</th>
<th>Losartan</th>
<th>Enalapril plus Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>22</td>
<td>23</td>
<td>21</td>
<td>23</td>
</tr>
</tbody>
</table>

**4.2.1.4 Results**

**ACE Genotype, Circulating RAS hormones, and Cardiac Hypertrophy in HCM:** ACE levels varied markedly in the study patients - from 2 to 85 U/L (laboratory normal range, 6.1 to 21.1 U/L). Prior to therapy, the DD genotype was associated with higher serum ACE levels than either ID or II genotypes (PRA, plasma Ang-I, and Ang-II levels were similar, Figure 44). However, neither LV mass nor maximum LV wall thickness was different between ACE genotypes (Figure 45). In addition, circulating hormone concentrations (ACE, PRA, and Ang-II) showed no discernable relationship with either LV mass or maximum LV wall thickness (Figure 46).

**Changes Following Inhibition of RAS:** Several parameters were measured prior to, and following treatment.

**Hormone activity:** Enalapril, alone or in combination with losartan, reduced plasma ACE and increased plasma PRA concentrations. No significant changes were observed with losartan or placebo (Figure 47).
Angiotensin converting enzyme (ACE) I/D gene polymorphisms are associated with different serum concentrations of the components of the renin-angiotensin system.

LV mass was not significantly greater in the patients with the DD genotype compared to that in patients with the ID and the II genotypes.
Figure 46. Effect of RAAS Inhibition on Component Species.

Significant reductions in plasma angiotensin converting enzyme (ACE) and increases in plasma renin activity (PRA) in the enalapril plus losartan and enalapril study arms.

Figure 47. Absence of Correlation Between LV Mass and Plasma ACE Concentration.
Regression of LV mass: LV mass decreased significantly in the enalapril (p<0.0005), and enalapril plus losartan arms (p<0.005) arms, compared with no change in the placebo arm. LV regression in the enalapril and combination therapy groups was significant compared to placebo (Table 25). LV diastolic, systolic and stroke volumes were unaffected in any of the treatment groups.

<table>
<thead>
<tr>
<th>MRI</th>
<th>Placebo</th>
<th>Enalapril</th>
<th>Losartan</th>
<th>Enalapril plus Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV end-diastolic volume (ml)</td>
<td>-5.3±3.5</td>
<td>6.4±12.9</td>
<td>2.5±24.3</td>
<td>-2.0±13.2</td>
</tr>
<tr>
<td>LV end-systolic volume (ml)</td>
<td>-0.6±9.3</td>
<td>-0.8±18.2</td>
<td>0.5±18.4</td>
<td>-0.7±11.1</td>
</tr>
<tr>
<td>LV stroke volume (ml)</td>
<td>-4.7±12.8</td>
<td>-5.6±15.8</td>
<td>2.3±13.7</td>
<td>-1.3±13.5</td>
</tr>
<tr>
<td>LV mass (gm)</td>
<td>-1.3±1.8</td>
<td>-16.5±17.5*</td>
<td>-6.7±19.9</td>
<td>-18.2±26.0*</td>
</tr>
</tbody>
</table>

Exercise test
- maximum heart rate (bpm) | 0±11 | 7±41 | 12±37 | 7±41 |
- maximum systolic blood pressure | -2±30 | 13±45 | 10±52 | 7±44 |
- maximum diastolic blood pressure | 5±25 | 4±19 | 10±26 | 6±27 |
- duration (seconds) | -21±165 | 15±224 | 40±178 | -9±416 |

Radionuclide angiography
- basal ejection fraction (%) | -1.2±7.6 | 1.5±9.1 | -0.7±7.6 | -1.0±10.0 |
- exercise ejection fraction (%) | -6.5±5.4 | 3.7±8.2 | 15±24 | 1.7±8.7 |
- TPFR | 3±142.4 | 4±49.3 | 15±38 | 3±10.5 |
- atrial contribution to stroke volume | 7.8±3.1 | -0.5±9.6 | 0.1±7.0 | -0.6±8.2 |

Cardiac catheterization
- heart rate (bpm) | 1±12 | 1±12 | 1±14 | 2±11 |
- right atrial pressure | 0±3 | 0±3 | 0±3 | 0±3 |
- right ventricular diastolic pressure | 0±4 | 0±4 | 0±3 | 0±4 |
- pulmonary artery systolic pressure | 14±7 | 0±4 | -2±5 | 4±5 |
- pulmonary artery mean pressure | 0±5 | 0±4 | -2±5 | 4±5 |
- PCWP | 0±5 | 0±4 | -2±5 | 4±5 |
- cardiac index (l/min/m²) | -0.12±0.4 | 0.12±0.5 | -0.3±0.4 | 0.2±0.5 |
- aortic systolic pressure | 2±13 | 11±4 | 15±12** | 17±20** |
- aortic diastolic pressure | -1±10 | 8±11 | 4±10 | 8±18** |
- aortic mean pressure | -1±11 | 4±13 | 4±10* | 14±16*** |
- LV systolic pressure | 1±16 | 10±9* | 9±14 | 19±16*** |
- LV end-diastolic pressure | 1±8 | 1±8 | 0±7 | 5±6 |
- basal LV outflow gradient | -2±10 | 1±8 | -3±7 | 3±9 |

Table 25. Therapeutic Effects: Changes in Cardiovascular Indices.

Data represent mean ± 1 SD changes in the indices; baseline minus follow-up values (positive values: indicate reduction and negative values an increase); pressures in mm Hg, volumes in ml and mass in gm; TPFR, time to peak filling rate; PCWP, pulmonary capillary artery wedge pressure *, p<0.05; **, p<0.01; ***p<0.001; and ****p<0.0001 compared to placebo.
Symptoms: The NYHA functional class improved significantly only in the enalapril plus losartan arm of the study (1.8 ± 0.4 to 1.5 ± 0.5, p=0.015). Individual symptoms were unchanged.

Heart rate and blood pressure: No significant changes between baseline and study exit in heart rate and blood pressure responses to exercise and exercise duration or metabolic parameters were noted in any of the study-arms (Table 25). Similarly, were not altered by any therapy.

Radionuclide Parameters of LV function: There were no changes in radionuclide indices of LV systolic and diastolic function at rest or exercise in any of the study arms.

Hemodynamic changes: Enalapril reduced resting LV systolic pressure by about 10 mmHg. The haemodynamic effects of losartan alone, and enalapril plus losartan were also modest. ACE inhibition did not increase LV outflow tract gradients. There was no correlation between changes in arterial pressures and LV mass regression. Cardiac output and LV filling pressures (LV end-diastolic pressure and pulmonary arterial capillary wedge pressure) were unchanged in the placebo group over the six month period, and did not change significantly in the three treatment groups compared to placebo.

4.2.1.5 Complications

The study was terminated due to light-headedness or hypotension during the first three trial days of therapy in 9 subjects. The drugs were terminated after 1.3 to 4.2 months of therapy in a further 7 patients due to side effects (Table 26).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>All Patients</th>
<th>Placebo</th>
<th>Enalapril</th>
<th>Losartan</th>
<th>Enalapril plus Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotension</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
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<tr>
<td>Light-headedness</td>
<td>5</td>
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<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Fatigue</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cough</td>
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<td>0</td>
<td>1</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Hearing loss</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16</strong></td>
<td><strong>1</strong></td>
<td><strong>4</strong></td>
<td><strong>2</strong></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>

Table 26. Adverse Events in the Treatment and Placebo Arms of the Study.
4.2.1.6 Discussion

This study of a randomised placebo-controlled study of the effects of RAAS inhibition in human HCM reports regression of LV mass in patients treated with ACE inhibition (with enalapril) alone, or with ACE inhibition plus ARB (with losartan). A non-significant trend towards LV regression was found in patients receiving losartan alone. Enalapril is safe and well-tolerated in HCM patients without resting or provoked LVOTO, and therapy for 6 months results in LV mass regression (about 5%). Combination enalapril plus losartan therapy resulted in a similar reduction (about 11%), but was associated with a higher incidence of hypotensive side effects. The effect of 50 mg of losartan alone on LV mass was statistically insignificant, but a larger study may have detected significance in the trend towards a smaller effect on mass regression.

Role of RAS in the Cardiac Hypertrophy of HCM: This study's results strongly support the hypothesis that RAS activity contributes to the cardiac hypertrophy of HCM. Many studies examining the role of RAS inhibition in patients with LV hypertrophy resulting from essential hypertension have demonstrated the effectiveness of ACE-inhibitors, AT1 receptor blockers and aldosterone inhibitors at reducing LV mass independently of blood pressure reduction. Similarly, our study suggests that the LV hypertrophy of HCM is subject to modulating influences by RAS activity that are independent of the primary cause (gene mutations) of the hypertrophy.

As reported by others, we observed an increase in plasma ACE hormone levels associated with the ACE gene deletion (D) allele. This study does not, however, support previous reports that commonly occurring ACE gene polymorphisms influence the magnitude of LV hypertrophy in HCM. Neither the ACE genotype nor indices of plasma RAS activity correlated with LV mass. This difference in findings may be attributed to the greater 'noise' resulting from the varied genetic causes of HCM in subjects in our study compared to studies examining for ACE polymorphism effects in single large pedigrees. Nonetheless, our findings suggest that any affect of ACE polymorphisms and circulating RAS activity on the magnitude of LV hypertrophy is small and that alterations in tissue RAS activity are central to the treatment effect.
As AT1 receptors are believed to mediate most of the adverse effects of RAS and receptor blockade has reduces LV mass in hypertensive LV hypertrophy and in various animal models, the absence of an effect the AT1 receptor blocker losartan in contrast to the efficacy of ACE inhibition was an unexpected finding. Several explanations could account for this. An effect on LV hypertrophy smaller than that of enalapril may be detectable by a larger study. Similarly, the use of larger doses of losartan may have had efficacy. However, the losartan dose used here is comparable to those used in similar studies of hypertensive LV hypertrophy, and the hypotensive effects of losartan were similar to those of enalapril in this study. The differences in LV mass regression may therefore represent a divergence of the anti-hypertensive and anti-hypertrophic effects of the two drugs. As ACE inhibition interrupts the RAS cascade more proximally, the effects of enalapril on downstream RAS targets other than the AT1 receptor may be responsible for these differences.

The proportional reduction in cardiac mass is modest, representing a 7-8% reduction in total cardiac weight. Significant cardiac hypertrophy remains; optimising RAAS antagonism with prolonged therapy and/or combinations with aldosterone antagonists may improve mass reduction, but it is likely that trophic signals other than those generated through RAS are also important.

Role of RAS in the Cardiac Fibrosis of HCM: Although conditions causing pathologic LVH also result in fibrosis, the development and regression of the two pathologic entities demonstrates temporal divergence and appear to be mechanistically distinct. In doses too small to cause regression of LVH, the angiotensin converting enzyme (ACE) inhibitor lisinopril reduces fibrosis and normalizes myocardial stiffness in rats with spontaneous hypertension. Sub-antihypertensive doses of the aldosterone antagonist spironolactone prevent LV fibrosis but not LVH in rats with hyperaldosteronism. Similarly, in hypertensive patients, angiotensin (AT) receptor blockade with losartan reduces myocardial fibrosis and chamber stiffness as measured by the Doppler transmural deceleration time. In contrast, reduction in both blood pressure and LVH with the beta-blocker labetalol is not accompanied by reduction in collagen content or myocardial stiffness. Finally, there is temporal discordance in the regression of LVH and myocardial fibrosis following successful surgical treatment of aortic stenosis and regurgitation. These findings all suggest that reverse remodeling of the collagen
matrix may be possible by interventions aimed directly at the fibrotic process such as modulation of the renin-angiotensin-aldosterone system (RAAS).

The effects of RAAS inhibition in animal models of HCM includes a regression in interstitial fibrosis.\textsuperscript{452} The extent of collagen accumulation in human HCM hearts has been correlated with NYHA class, exercise limitation in HCM patients and with pulmonary arterial wedge pressure during exercise.\textsuperscript{106,446,460} Notably, there was a significant improvement in well-being as assessed by NYHA status in the enalapril plus losartan arm of the study.

Serum markers of myocardial collagen metabolism\textsuperscript{461,462} and CMR imaging with delayed gadolinium enhancement\textsuperscript{463,464} were not included in this study as the study began before these techniques were described. Future studies of RAAS inhibition will need to include such assessments with the hypothesis that microscopic (interstitial) and macroscopic fibrosis are important therapeutic targets and that reduction in fibrosis is associated with symptom improvement.

**Clinical Implications for the Management of HCM:** Therapy reducing LV mass is only of benefit if also associated with an improvement in symptoms and/or prognostic benefit. In this respect, several clinically important issues are not resolved by our study. Considerations for patient safety included the selection of relatively asymptomatic study subjects in whom a treatment effect on functional status will be minimal. The study reports a reduced NYHA score in one of the three treatment arms, but we must remain cautious about interpreting these results.

Similarly, the short duration of therapy and the low event rate do not allow assessment of prognostic effect. Our results also cannot be extended to obstructive HCM as patients with resting or provokable LV outflow obstruction were excluded because the vasodilator effects of enalapril and losartan may worsen obstruction and associated symptoms. Additionally, in obstructive HCM as in hypertension and aortic stenosis, the elevated LV systolic pressure may itself contribute to cardiac hypertrophy\textsuperscript{104}, and effects of reduced blood pressure on LV mass cannot be discounted. Finally, it is not determined whether reductions in mass are sustained after cessation of therapy, or even with continuation of therapy beyond six months.
HCM patients often develop symptoms and signs of heart failure due to either LV diastolic or systolic dysfunction. Cardiac hypertrophy is an independent predictor of a poor prognosis in HCM and in other cardiac diseases where regression is associated with an improved outcome. As ACE inhibition is safe and well tolerated in non-obstructive HCM, improves LV diastolic dysfunction and ameliorates heart failure in several other cardiac disease states, it is reasonable to treat heart failure symptoms associated with impaired or preserved LV systolic function in patients with non-obstructive HCM with ACE inhibitors.

Further studies are necessary to confirm our findings and to determine the long-term consequences of LV mass regression.
4.2.2 Relief of Drug-Refractory Symptoms Associated with Dynamic Left Ventricular Outflow Tract Obstruction: Randomised Comparison of Pacemaker Therapy and Alcohol Septal Ablation.

The three principal invasive modalities for the therapy of refractory symptoms associated with LVOTO have vocal detractors and enthusiasts. For example, LVM is held by some as the ‘Gold Standard’ treatment by virtue of its status as the first, and therefore most enduring, apparently efficacious treatment. If numbers of procedures or patient years following intervention are the parameters used to select a gold standard, then LVM would be replaced by ASA as the preferred treatment. Relative benefits or each modality cannot be compared without a trial randomising patients to the best practitioners of each treatment.

A fundamental problem with LVM is patient reluctance to undergo cardiac surgery; patient preference has promoted the less invasive therapies of ASA and DDD. Furthermore, the modern trend within cardiology away from physician referrers (to cardiac surgeons) towards cardiologists completing both assessment and treatment has not been confined to treatment of coronary disease; proponents of ASA have been invasive cardiologists with an additional interest in HCM. Similarly, the most ardent supporters of DDD pacing have been electrophysiologists.

The relationship between advocate, stake-holder, HCM physicians referring patients to themselves for their preferred treatment clearly represents a conflict of interest. However, these advocates probably deliver the best expressions of the particular therapy. The DDD trials included lead investigators with little or no pacing expertise, some of whom were clearly supporters of other treatments before the trials were completed.

The DDD trials, at best, identified pacing as a therapy with a neutral outcome. At worst, they deny patients a minimally invasive and efficacious treatment with that does not preclude an escalation to other therapies. On the contrary, DDD pacing neutralises risks from iatrogenic AV block following ASA or LVM, and the combination of DDD with ICD function negates concerns regarding the pro-arrhythmic potential of ASA. Finally, the evolution of safer pacemaker-defibrillator (ICD) therapy introduces the prospect of an a priori treatment with pacers-ICDs of symptomatic HCM patients with obstruction that have additional indications for ICD implantation.
Despite the fact that LVM was developed at NIH by Andrew Morrow in the 1960's, HCM physicians at NIH have been proponents of pacing. We compared DDD pacing with ASA in a randomised trial that assessed changes in LV outflow tract gradients as the primary outcome; cohort data had indicated ASA was more effective at reducing LVOT gradients. The initial and final assessments of patients including clinic reviews, echocardiography, CMR and cardiac catheterisation were completed at NHLBI. Patients randomised to pacing received device implantation and pacer management at NHLBI, and ASA was performed by interventional cardiologists at nearby but independent institutions (Fayaz Shawl at the Washington Adventist Hospital and Kenneth Kent at Georgetown University Hospital. CMR was performed on all ASA patients at baseline and 6 months following treatment (Andrew Arai, NHLBI).

4.2.2.1 Abstract

Background: ASA and DDD pacing are alternative therapies to cardiac surgery in symptomatic obstructive HCM.

Methods and Results: We performed a prospective randomised comparison of ASA and DDD pacing in 70 consecutive obstructive HCM patients with drug-refractory symptoms. The study arms were matched well for age, gender and disease severity. LV outflow gradient was reduced from 99 ± 40 mm Hg to 32 ± 33 mm Hg (68% reduction, P<0.0001) in the ASA arm of the study, and from 91 ± 46 mm Hg to 43 ± 39 mm Hg (48% reduction, P<0.0001) in the DDD arm of the study. The 19 mm Hg greater reduction in LV outflow gradient following ASA was significantly greater than after DDD (P=0.038). After ASA, 3 patients (9%) required DDD pacing for heart block and 1 died three weeks following ASA. Both treatments resulted in similar symptomatic improvement, health-related quality of life, and exercise performance. As assessed by CMR, LV mass decreased from 278 ± 85 gm to 261 ± 88 gm, (17 gm or 6%; P<0.001) following ASA. ASA was not associated with changes in LV ejection fraction, LV volumes or occurrence of spontaneous arrhythmia.

Conclusions: In patients with symptomatic obstructive HCM, both ASA and DDD pacing reduce LV outflow gradients. ASA is more effective at reducing gradients, but both therapies were associated with similar improvements in symptoms. Because of risk of complications, a reasonable approach may be to consider DDD (implantable defibrillator) first. ASA or cardiac...
surgery may then be reserved for patients with persistent symptoms and residual LV outflow obstruction.

4.2.2.2 Introduction
Pharmacological therapy often fails to control symptoms adequately in obstructive HCM, or is associated with intolerable side effects. LVM was adopted (and subsequently refined) as the first effective treatment for the relief of such symptoms; DDD pacing and ASA are performed as alternative less invasive therapeutic options. None of these therapies have been compared in prospective trials.

The primary aim of this prospective randomised study was to determine whether ASA results in greater reduction in LV outflow gradients than DDD therapy in obstructive HCM associated with severe drug-refractory symptoms. The secondary aims of the study were to characterize the effects of the two therapies on (1) symptoms, patient satisfaction, and exercise tolerance; (2) LV remodelling; (3) LV function; and (4) arrhythmia propensity.

4.2.2.3 Methods
Study Design: The study was performed after informed consent according to a protocol approved by the Intramural Research Board of National Heart, Lung, and Blood Institute, National Institutes of Health. Obstructive HCM was defined as LV hypertrophy in the absence of another cause for the increased cardiac mass, and basal LV outflow gradient >30 mm Hg or >50 mm Hg following isoproterenol infusion at cardiac catheterisation. Inclusion criteria included; septal thickness of ≥16 mm (comparable to patients undergoing surgical LV myectomy or LVMM); patients age ≥18 years; and New York Heart Association functional class III/IV despite conventional drug therapy. Exclusion criteria included significant coronary artery disease and chronic atrial fibrillation.

Eighty-seven consecutive patients with obstructive HCM were screened between September 1999 and July 2001. All patients were offered LVMM as an alternative to participating in the trial; 8 declined participation in the study (1 underwent LVMM), and 9 did not meet entry criteria. Seventy patients were assigned to the two arms of the study using the method of randomly permuted blocks. The arms of the study matched well with regards to age, gender, symptoms, LV outflow obstruction and cardiac indices.
In-patient evaluations at baseline and 6-months after therapy were completed after the withdrawal of all cardiovascular drugs: (1) assessment of symptoms; (2) echocardiography; (3) treadmill and bicycle exercise tests; (4) radionuclide angiography (RNA); (5) cardiac catheterisation and angiography; (7) 24-48-hour ambulatory electrocardiography (ECG) monitoring; and (8) electrophysiology (EP) studies. Patients were also evaluated (symptoms and echocardiography) at a 3-month out-patient visit. A Health Related Quality of Life (HRQL) questionnaire was administered prior to randomisation, and at 3 months and 6-months. HRQL was assessed using physical and mental component summary scores derived from the Short Form 36 Health Survey (SF-36) Version 1.0.

<table>
<thead>
<tr>
<th></th>
<th>ASA (n=35)</th>
<th>DDD (n=35)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male)</td>
<td>15 [43]</td>
<td>14 [40]</td>
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</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>49 ±14</td>
<td>47 ±17</td>
<td>ns</td>
</tr>
<tr>
<td>range</td>
<td>20 - 82</td>
<td>20 - 76</td>
<td></td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>3.4 ± 0.5</td>
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<td>ns</td>
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<tr>
<td>Symptoms</td>
<td></td>
<td></td>
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<tr>
<td>angina</td>
<td>29 [83]</td>
<td>29 [83]</td>
<td>ns</td>
</tr>
<tr>
<td>dyspnoea</td>
<td>33 [94]</td>
<td>34 [97]</td>
<td>ns</td>
</tr>
<tr>
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<td>21 [60]</td>
<td>ns</td>
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<td>ns</td>
</tr>
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<td>28 [80]</td>
<td>ns</td>
</tr>
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<td>30 [86]</td>
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<td>beta blocker</td>
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<td>verapamil</td>
<td>31 [89]</td>
<td>27 [77]</td>
<td>ns</td>
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<td>diuretic</td>
<td>13 [37]</td>
<td>4 [11]</td>
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<td>disopyramide</td>
<td>9 [26]</td>
<td>9 [26]</td>
<td>ns</td>
</tr>
<tr>
<td>amiodarone</td>
<td>2 [6]</td>
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<tr>
<td>LVMM</td>
<td>1 [3]</td>
<td>0 [0]</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 27. Comparison of the Patients’ Baseline Clinical Characteristics in the DDD Vs. ASA Study.
Magnetic resonance imaging (MRI) studies were performed at baseline and repeated within a week and 6-months following ASA. End expiratory cine MRI was performed on a 1.5T cardiac MRI scanner using a four-element cardiac phased array coil. Computer assisted planimetry was used to quantify myocardial dimensions, volumes, ejection fraction and mass. In water-filled
phantoms, measured volumes correlated with known volumes (range, 7.5 to 465 ml, MRI = 1.00 x Volume, R2=0.9998, P<0.0001).

ASA: A septal branch was occluded proximally with a 1-2 mm angioplasty 'over-the-wire' balloon catheter (Figure 48). Angiographic dye verified absence of dye-reflux into the LAD, and excluded abnormal arterial connections. Echo contrast agent (Optison) was injected beyond the inflated balloon catheter to ensure the opacified myocardium was adjacent to the area of maximum LV outflow acceleration. Alcohol was injected over 5-10 minutes. Balloon inflation was maintained for 10 minutes to allow diffusion of alcohol into the myocardium and to prevent reflux into the main artery. If >30 mm Hg basal LV gradient continued to be present another septal artery was ablated. LV outflow gradient was monitored using a pigtail catheter in the LV and the femoral sheath. Coronary angiography verified occlusion of the target vessel. Patients were discharged 3-5 days later. A second ASA was recommended if patients had residual significant symptoms associated with significant LV outflow obstruction within the first 3-months following the initial procedure.

DDD: Device implantation and programming has been described previously. Patients with significant symptoms and residual obstruction at 3-months were offered ASA or cardiac surgery.

Statistics: Data are presented as mean (± SD). Differences from mean values were compared by Student's t-test. Pearson's correlation coefficient was used to evaluate the correlation between variables. A two-tailed P value of <0.05 following Bonferroni correction was considered significant.
Figure 48. Illustration Describing the ASA Procedure.

Left upper panel. Coronary angiogram illustrating targeted septal artery (shown by arrows). Right upper panel. Occlusion of septal artery after ASA. Left lower panel. Diagram of obstructive hypertrophic cardiomyopathy; Right lower panel. Diagram of myectomy following ASA with relief of left ventricular outflow obstruction.

4.2.2.4 Results

Procedure Details and Adverse Outcomes

ASA: ASA was not technically feasible in 1 patient, 2 patients declined the procedure, and 1 patient died during follow-up. ASA was repeated in 5 patients. Only 1 artery was ablated in 24 patients, 2 arteries in 5 patients, and 3 arteries in 3 patients. Basal LV outflow gradient was reduced to <20 mm Hg in all patients (provoked LV gradient of <60 mm Hg was used as a guide.
to success of the procedure in 3 patients without rest gradient). An average of 3.0 ± 1.7 ml of alcohol, range, 0.9 to 7.0 ml, was injected per procedure. Cardiac enzymes increased in all patients (creatine kinase, 1170 ± 555 U/l, range, 384-2454 U/l (normal, 38-252 U/l); creatine kinase-MB, 154 ± 69 ng/ml, range, 23-355 ng/ml (normal, 0-5.0 ng/ml); and troponin I, 400 ± 297 ng/ml, range, 52-1055 ng/ml (normal, 0-2.0 ng/ml).

Heart block occurred in 6 of the 32 patients who underwent successful ASA: 3 were temporary, but 3 required permanent DDD pacing (one of whom died one month after ASA). Three patients with initially negative EP study received ICD-DDD devices for sustained VT induced at the 6-month follow up study.

**DDD pacemaker therapy:** Satisfactory DDD pacing was achieved in all 35 patients in the pacemaker arm of the study. DDD therapy was complicated by 3 lead displacements and 1 pneumothorax. One patient received an upgrade to a DDD-ICD device for sustained VT induced at the end of study.

**Hemodynamic Changes:** LV outflow gradients were reduced by 68% and 48% following ASA and DDD respectively, an average of 19 mm Hg (P=0.038) greater reduction in the ASA arm (Table 28). Aortic pressures increased significantly following ASA but were unchanged in the DDD arm. Cardiac filling pressures were unchanged in both arms of the study. There was a small but significant decrease in resting ejection fraction in the DDD arm but no change was observed in the ASA arm.

**Symptoms, Functional Class, and HRQL:** Both therapies significantly improved symptoms, notably, angina and light-headedness, (Table 28). Changes in symptoms were not significantly different between the two study arms. Similarly, both therapies were associated with similar improvement in functional class status, (Figure 49). There was no further symptomatic improvement in the ASA study arm between the 3-months and 6-months evaluations. In contrast, functional class improved in the second half of the study period in the DDD arm (Figure 49).
For the patients with complete HRQL data (ASA, n=29; DDD, n=32) changes in LV outflow gradient changes were comparable to the overall cohort. There was no relation between reduction in LV gradients and changes in HRQL in patients undergoing either ASA or DDD. Both ASA and DDD were associated with equivalent HRQL benefits at 3-months that persisted to 6-months (Table 28).

**Exercise Performance:** Treadmill exercise duration increased in both arms of the study: ASA (n=26), from 275 ± 203 sec. to 384 ± 212 sec., an increase of 98 ± 150 sec., or 36%; P=0.001; and DDD (n=28) from 297 ± 184 sec. to 357 ± 154 sec., an increase of 60 ± 133 sec., or 20%; P=0.02. The difference in exercise duration between the two study arms was non-significant.

Bicycle exercise duration increased from 295 ± 206 sec. to 342 ± 176 sec., a change of 47 ± 136 sec., or 16%, in the ASA arm (n=27), P=0.10; and from 328 ± 152 sec. to 348 ± 161 sec., a change of 20 ± 99 sec., or 6%, in the DDD arm (n=33) of the study. P=0.24. The difference in exercise duration between the two study arms was not significant.
Maximum exercise [Oxygen] consumption did not improve significantly with ASA (14 ± 5 l/min at baseline versus 15 ± 5 l/min at 6-month) or with DDD pacing (14.0 ± 5 l/min at baseline versus 14.0 ± 6 l/min at 6-months).

**LV Function:** Neither therapy was associated with further elevation in LV end diastolic or pulmonary wedge pressures (Table 28) indicating ASA and DDD do not contribute to deterioration of LV diastolic dysfunction. Rest and exercise RNA-derived LV ejection fractions were unchanged following ASA (Table 28). DDD pacing, however, was associated with a small but significant decrease in LV ejection fraction.

**Cardiac Remodelling Following ASA:** LV outflow tract obstruction was markedly improved in patients who underwent MRI studies (n=26). LV masses measured before and within one week of ASA were similar (Table 29). However, LV mass decreased significantly by an average of 17 gm or 8% at 6-months. LV diastolic and systolic volumes were unchanged. However, LV stroke volume decreased at one week, but recovered by 6-months.

<table>
<thead>
<tr>
<th></th>
<th>DDD</th>
<th>Baseline</th>
<th>ASA 1-Week</th>
<th>6-Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass (gm)</td>
<td>264 ± 66</td>
<td>278 ± 85</td>
<td>280 ± 88</td>
<td>261 ± 88**†††</td>
</tr>
<tr>
<td>LVEDV (ml)</td>
<td>123 ± 29</td>
<td>119 ± 23</td>
<td>112 ± 23</td>
<td>115 ± 22</td>
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<tr>
<td>LVESV (ml)</td>
<td>33 ± 12</td>
<td>29 ± 10</td>
<td>29 ± 8</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>LV SV (ml)</td>
<td>91 ± 22</td>
<td>90 ± 21</td>
<td>83 ± 20*</td>
<td>85 ± 19</td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>74 ± 6</td>
<td>76 (7)</td>
<td>74 ± 7</td>
<td>73 ± 7</td>
</tr>
</tbody>
</table>

Table 29. LV remodelling following ASA: MRI findings.

* = p<0.05; ** = p<0.01; **** = p<0.00001 versus baseline; and ††† = p<0.001 compared to 1 week.

**ECG Changes and Arrhythmia Following ASA:** The ECG was unchanged from baseline in 15 patients (45%) at 6 months following ASA. Right bundle branch (RBBB) developed acutely in 16 patients or 48%, and persisted in 10 patients or 30%. RBBB was associated with left posterior hemiblock in 3 patients, anterior hemiblock in 2 patients, and first degree AV block in 1 patient. Left bundle branch block developed acutely in 3 patients but resolved by 6-months.
There were no significant differences in the prevalence of either spontaneous atrial or ventricular arrhythmias (data not shown). At the 6-month EP study, sustained polymorphic VT was induced 2 ASA and 1 DDD patient in whom baseline studies were negative. All three patients received implantable pacemaker-defibrillators.

4.2.2.5 Discussion

This is the first prospective randomised comparison of two therapies for symptomatic obstructive HCM. Both ASA and DDD therapies significantly reduced LV outflow gradients. ASA resulted in a slightly greater reduction (19 mm Hg) in LV outflow gradient. However, the improvement in symptoms, functional class, HRQL indices, and exercise performance were similar in the two study arms.

Although the results of LVMM have improved recently, few centres have experience with this procedure and it may be associated with significant short-term and long-term adverse outcomes.472-475 Although some authorities consider LVMM as the 'gold standard' in management of drug refractory obstructive HCM, this derives mainly from the length of experience (now more than 40 years) with the operation. The strong incentive to identify less invasive and safer alternative therapies has lead to the development of ASA and DDD pacing30,117,118,127,130,469. Both therapies have merits and potential problems.

Several studies127,128,130,131,417,418,469,476-479 have demonstrated DDD pacing significantly reduces LV outflow obstruction and improves drug-refractory symptoms. However, some investigators have reported that DDD pacing may be associated with LV diastolic dysfunction.133 Others have cautioned that the associated symptomatic improvement may in part be due to a placebo-effect23,129,480. It is important to recognize that this may also apply to the other two therapeutic options. Importantly, the design of the studies that report a placebo effect do not account for the fact that the chronic effects of DDD pacing are greater than its acute effects477,478, and there may also have been a carry-over effect of DDD pacing in the placebo arm. Assessment of subjective improvement in one of these studies was very likely influenced by unusually high complication rates of pacemaker implantation.120 The best results have been reported when attention has been paid to lead placement, and pacemakers are programmed appropriately to achieve maximum ventricular pre-excitation without interference with left
atrial emptying. The present study demonstrates that DDD pacing is safe in obstructive HCM, and confirms that it significantly reduces LV outflow gradients as measured by cardiac catheterisation, and improves symptoms as assessed by several means.

Several studies have also reported on the ability of ASA to relieve LV outflow obstruction and symptoms in obstructive HCM. As ASA is associated with heart block necessitating permanent DDD pacing in 10-20% of patients, it is not clear how much of any treatment effect is attributable to ASA and how much to DDD therapy.

We achieved reduction of LV outflow gradient and symptomatic improvement with only about 10% of patients requiring DDD devices. In our study, ASA was associated with a 17 gm reduction in LV mass. As this is similar to the amount of myocardium removed at LVMM it may reflect the direct effects of alcohol on the myocardium rather than regression of hypertrophy following reduction in LV systolic pressure. ASA did not significantly impair LV function. Spontaneous arrhythmia and late conduction abnormalities were not significantly increased.

**Therapeutic Recommendations:** A reasonable therapeutic algorithm in obstructive HCM complicated by severe drug-refractory symptoms is to consider DDD pacemaker therapy (± ICD) first, followed by ASA after about six months of pacing if significant symptoms and significant LV outflow obstruction remain. LVMM may then be considered if ASA is not feasible or also fails to relieve symptoms and LV outflow obstruction. This algorithm is based on the following observations: (i) The current study shows similar symptomatic benefit from the minimally invasive DDD and ASA; (ii) DDD therapy is less invasive and safer than either ASA or LVMM; (iii) DDD/ASA therapies are effective in many patients and do not preclude LVMM; (iv) Pacemaker-defibrillator devices are being increasingly used for HCM and the algorithm offers an opportunity to address risks of sudden death as well as symptom improvement; (v) As frequent adverse effect of both ASA and LVMM is heart block, prior DDD therapy may increase the safety of the other procedures. This algorithm advocates a conservative initial approach, with an emphasis on risk as well as symptom reduction.

**Limitations of the Study:** Our patients had more LV outflow obstruction than those included in many reports. The LV outflow gradients were however, of similar magnitude to those reported
by some authors who have reported satisfactory results with DDD pacing. Longer follow up studies may increase our understanding of the relative efficacies of the two therapies. However, previous experience indicates that six months is adequate to examine the results of therapy in obstructive HCM, and there were few additional changes noted in the present study between the 3-month and 6-month evaluations in the ASA arm of the study.
5 CONCLUDING REMARKS

5.1.1 APPRAISAL OF CLINICAL RELEVANCE
My approach to each patient in the clinic is to (1) address the certainty of the clinical diagnosis of cardiomyopathy, (2) assess symptoms and indications for invasive or pharmacological therapy, (3) perform estimates of risk from SD, heart failure and stroke, and (4) undertake genetic counselling including initiating genetic testing and family screening. The research presented in this thesis was designed to improve our ability to manage patients. This has been through clinical studies; examining new and established therapies, evaluating new prognostic measures, developing methods to assess diastole and using basic science; hoping to provide insights into mechanisms of disease.

A prosaic, but objective, assessment of impact can be made by counting citations, by other articles, of my published work. From searches on Google Scholar (http://scholar.google.co.uk), there have been more than 600 citations to articles I have co-authored, of which 110 citations are to first author papers.

Citation counting may assess a research profile; an objective assessment of clinical impact is more difficult to make. My clinical practice in heart muscle disease is derived from and encourages my research activities. I have had the learning opportunities afforded by working in two of the world’s largest centres for cardiomyopathy research and patient management (NIH and at the Heart hospital, London). It is in this context that we have established (along with Dr PG Mills) the Heart Muscle Disease (HMD) clinic at the London Chest hospital. This service is designed to provide state-of-the-art cardiomyopathy services and to develop a research infrastructure. Access to CMR, with a dedicated scanner, is an important feature. The current research interests of the HMD clinic include:

(1) Ethnic differences in HCM phenotypes. We have described striking differences in the prevalence of apical HCM between Caucasian and South East Asian populations. We are currently seeking to determine if single-gene mutations account for the differences in disease type, or if other hereditary or environmental factors are accountable.

(2) Symptomatology in apical HCM. This form of HCM is often associated with refractory symptoms. Symptoms, which include frequent presentations with acute chest pain, are not well managed or understood.
(3) Expanding and examining the clinical utility of CMR in heart muscle diseases. The contributions made to prognosis by magnitude of LVH and by impaired systolic function are increasingly understood. The diagnostic, symptomatic and prognostic importance of myocardial scarring (tissue characterisation with late gadolinium enhancement) and perfusion defects are not established.

5.1.2 FUTURE DIRECTIONS
Some of the major HCM research frontiers are described below. It is also a belief that future HCM research can now begin to make important contributions to the understanding of more common cardiac diseases.

(1) Molecular mechanisms leading from mutant to LVH. As discussed in the introduction section of this thesis, the molecular basis for the manifold relationships between hypertrophic stimuli and cardiac phenotype is poorly understood (Figure 3). Determining the causes of phenotypic variability (these ‘modifying factors’ may include environmental states as well as common genetic polymorphisms) resulting from identical or similar sarcomeric mutations may identify novel modifying treatments. Equally importantly, this may provide an understanding of why much more common causes of cardiac injury also manifest variably - for example from hypertension, valve disease, myocardial infarction.

(2) Importance of LV diastole and atrial function. Better methods for assessing atrio-ventricular coupling during LV diastole are needed. The basis for many symptoms and exertional limitation in HCM are attributed to diastolic failure, but these are currently poorly assessed or understood. The complexity of LV filling, dependant as it is on both variable and constant properties of the LV, the atria and loading conditions means that our concepts of diastole need considerable development. A single parameter (for example E/E'200,482) will not suffice, and we need to develop tools assessing LV relaxation, LV compliance, atrial capacitance, atrial elastance and so on. In its role as a ‘supermodel’ of diastolic failure, HCM has facilitated the development of some of these tools, and will continue to do so. Clearly, success in this area of HCM research will be relevant to the ‘epidemic’ of diastolic heart failure in the general population.483

(3) Improved prognostic stratification. The indications and efficacy of ICD therapy to prevent arrhythmic death, of warfarin to prevent stroke and heart failure medication to improve symptoms and longevity will need to be established in HCM. Sudden death is predicted poorly103 using current schemes98, which also do not reliably differentiate
between SD from arrhythmia, stroke or heart failure. Future prognostic stratification is likely to use a combination of current and new risk factors and will aim to independently consider risks of arrhythmia, stroke and heart failure.

(4) Outflow Tract Obstruction. The HCM community have not completed randomised trials of septal ablation (ASA) and myectomy (LVM) to the detriment of patient care. A resurgent interest DDD pacing for outflow obstruction is supported by the increasing use of ICDs and the incidence of heart block complicating ASA and LVM. An example of a prospective trial is shown in Figure 50.

Figure 50. Trial Evaluating ASA, LVM and DDD therapy in Obstructive HCM.

The primary objective is to compare the efficacy of LVM and ASA on symptom status. Evaluating a strategy of prior pacer-ICD in those with indications for ICD for the prevention of sudden death (SD) is a secondary aim.
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