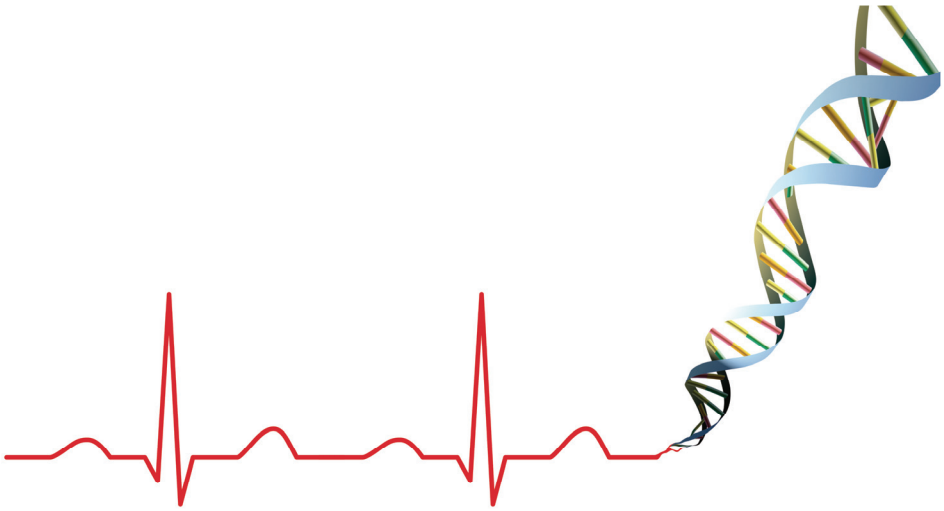
Genetic aspects and family studies of noncompaction and hypertrophic cardiomyopathy



Yvonne Maria Hoedemaekers

ISBN 978 90 9025438 8

Page layout: Eddy Drost
Cover design: Tom de Vries Lentsch and Yvonne Hoedemaekers
Printed by Ipskamp Drukkers B.V., Enschede

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.
Part of the research described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-2006T014).

Genetic Aspects and Family Studies of Noncompaction and Hypertrophic Cardiomyopathy

Genetische aspecten en familie onderzoek van noncompaction en hypertrofische cardiomyopathie

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
vrijdag 25 juni 2010 om 11:30 uur

Yvonne Maria Hoedemaekers
geboren te Venray

ERASMUS UNIVERSITEIT ROTTERDAM

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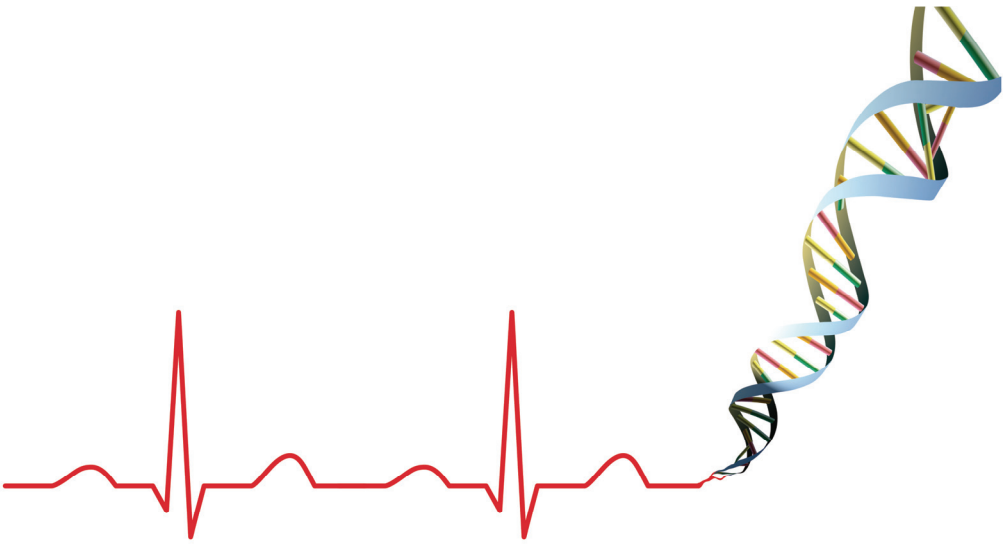
Knowing is not enough, we must apply; willing is not enough, we must do
Goethe

Voor Eddy,
Pap en mam,
Lilian en Stefan,
Miriam en Don,
Sara, Eva en Nynke

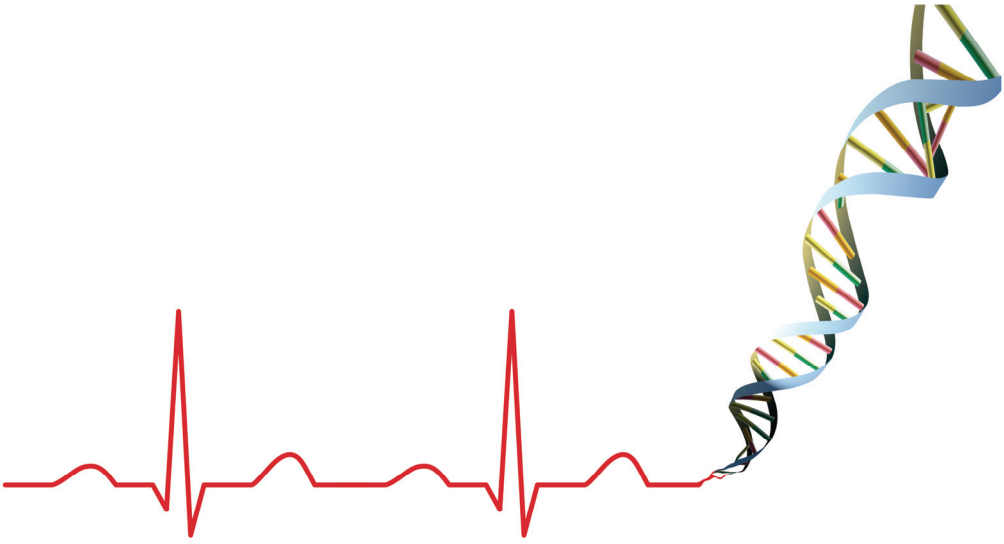
Contents



Chapter 1

Introduction

1.1 General introduction and goal of the study
1.2 Noncompaction cardiomyopathy
Adapted from:
Noncompaction cardiomyopathy
Yvonne M. Hoedemaekers, Kadir Caliskan, Danielle F. Majoor-Krakauer.
Textbook CLINICAL CARDIOGENETICS From basic science to clinical medicine. Springer 2010.
1.3 Hypertrophic cardiomyopathy



1.1 Introduction

The first reports of familial cardiac disorders appeared over 60 years ago.[1-3] Since then, knowledge on cardiogenetic disorders has increased tremendously. And now cardiogenetics is a rapidly expanding field, including the familial cardiomyopathies, arrhythmias, congenital heart diseases and cardiovascular disorders. Most of these disorders have an autosomal dominant inheritance pattern. In the last decade many genes involved in these disorders have been identified and more discoveries are sure to follow.

The growing insight in the genetic conditions of the cardiovascular system has led to a substantial increase in the number of patients referred to clinical genetic departments for genetic counselling and DNA diagnostics. Close collaboration of departments of cardiology and genetics resulted in the initiation of multidisciplinary cardiogenetic outpatient clinics to provide the optimum care and facilitate family studies of hereditary cardiologic conditions. This way family studies of more and more adult patients could be initiated which led to increasing numbers of at risk relatives visiting the cardiogenetic units. Similarly, the cardiogenetic units were involved in counselling and family studies of the families of paediatric patients. Over the years the total number of cardiogenetic counselling sessions rose from 53 in 2000 to 482 in 2008 at the department of clinical genetics of the Erasmus Medical Centre in Rotterdam (Figure 1). In 2007 that number made up 12.6% of the total number of counselling sessions in Rotterdam.

This is not only a local trend, it is observed nationwide. According to the numbers of the Dutch Society of Clinical Genetics (VKGN) the number of cardiogenetic counselling sessions rose from 538 in 2000, representing 4.5% of the total amount of counselling sessions, to 3216 in 2008, rising to 11.8% of all counselling sessions (Figure 2).

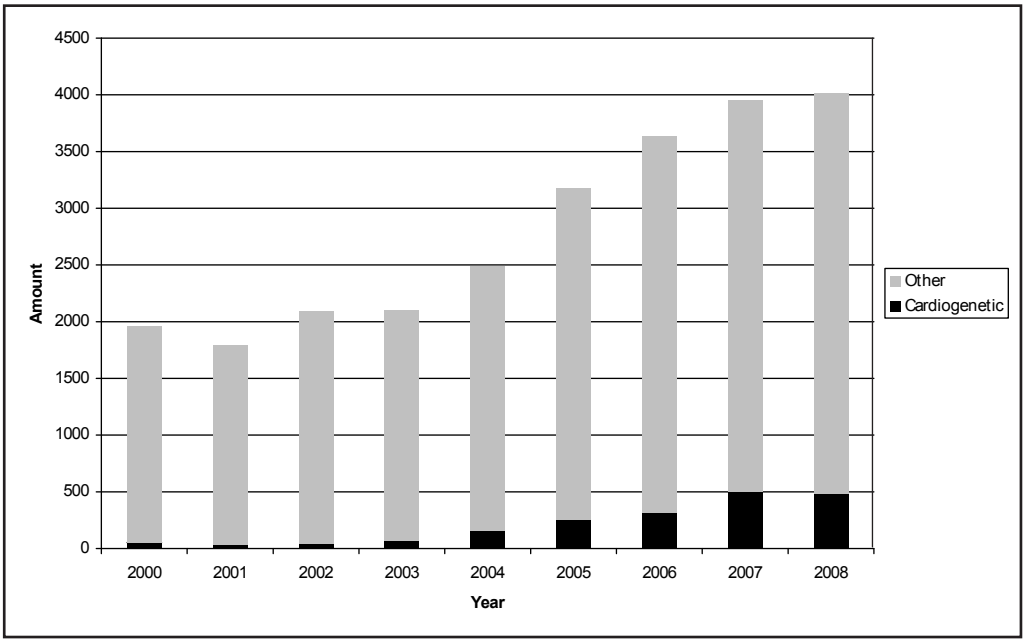


Figure 1. Amount of cardiogenetic and other counselling sessions at the department of clinical genetics of the Erasmus Medical Centre in Rotterdam during the period 2000 – 2008.

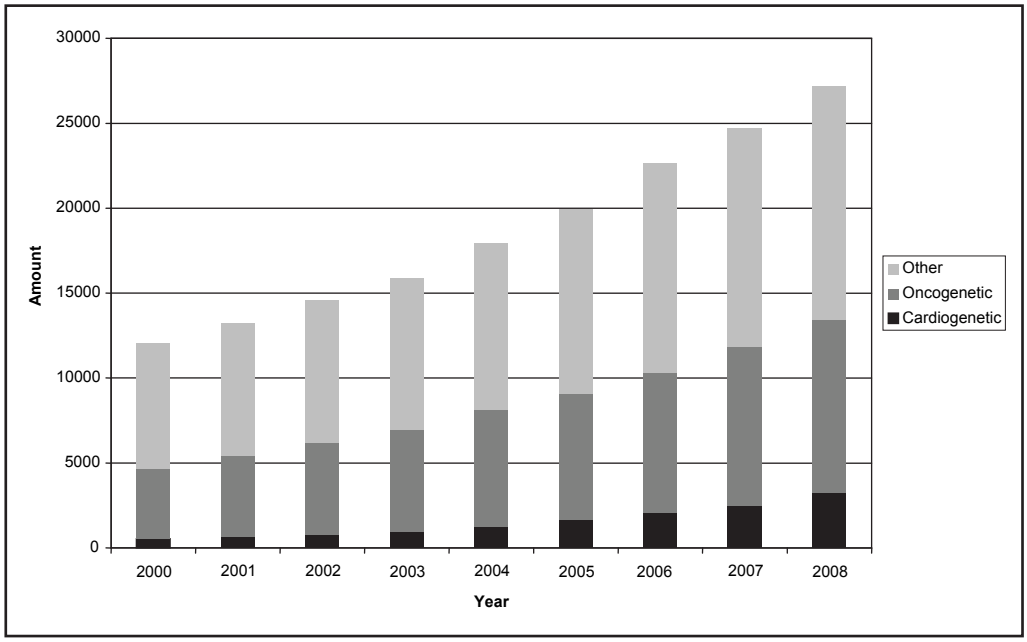


Figure 2. Genetic counselling according to subject in the Netherlands during the period 2000 – 2008. (Source: VKGN)

Since 2005 the most frequently counselled disorders at the cardiogenetic clinic are the cardiomyopathies, making up 71% of the cardiogenetic counselling sessions in Rotterdam in 2007 with hypertrophic cardiomyopathy at a firm first place (HCM; 50%) and noncompaction cardiomyopathy in second position (NCCM, 14%) (Figure 3). Approximately 27 % of the counselling sessions concern congenital structural heart defects in adults or children. The proportion of counselling sessions for familial arrhythmias, i.e. long QT syndrome (LQTS), Brugada syndrome, catecholaminergic induced polymorphic ventricular tachycardia (CPVT), Wolf-Parkinson-White syndrome (WPW), short QT syndrome (SQTS), idiopathic ventricular fibrillation, familial atrial fibrillation, atrial stand-still syndrome, familial conduction disease and sick-sinus syndrome, is smaller than the cardiomyopathies and structural heart defects, reflecting partly the lower prevalence of these disorders and perhaps also because there is less awareness of the genetic contribution to these disorders among the referring physicians. Genetic counselling and diagnostics of other cardiovascular diseases, like familial hypercholesterolaemia and familial thoracic and abdominal aneurysms complete the spectrum of disorders counselled at the cardiogenetic clinic.

This dissertation focuses on genetic family studies of noncompaction and hypertrophic cardiomyopathy.

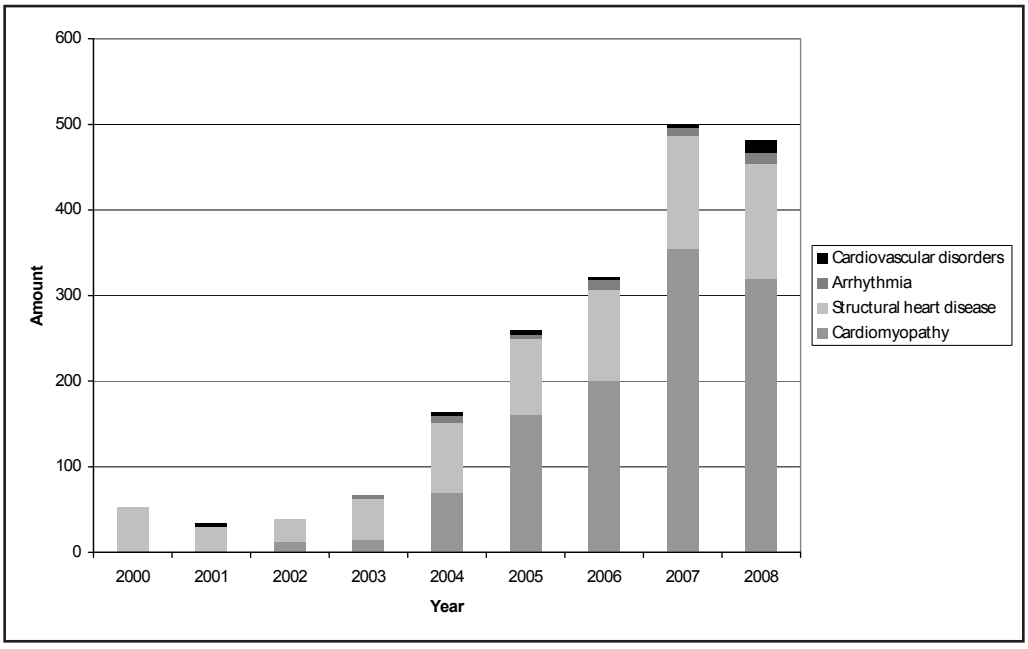


Figure 3. Different cardiogenetic subjects counselled in the period 2000-2008 at the Erasmus Medical Centre.

1.1.1 Goal of the study

Noncompaction cardiomyopathy is a relatively newly discovered cardiomyopathy. Although several genes associated with NCCM have been identified and therefore the heterogeneity of NCCM, the exact genetic origin and heredity of NCCM are not as well established as those of hypertrophic cardiomyopathy. Since both diseases can co-occur within the same family, a common pathway might be expected.

Identifying NCCM as a genetic disorder and finding the genes involved in the development of the specific features of NCCM is important because it enables accurate identification of relatives at risk of developing a cardiomyopathy, who may benefit from early monitoring and treatment to reduce morbidity and mortality. It is equally important that DNA diagnostics allow reliably excluding the familial predisposition in at risk relatives. This way relatives who are not at risk can be reassured and discharged from periodic cardiologic follow-up.

Hypertrophic cardiomyopathy is genetically heterogeneous. Depending on the amount and choice of genes examined and the examined population, DNA diagnostic strategies may identify the genetic cause in over 60 - 70% of HCM cases, indicating that in approximately 30 - 40% the genetic defect for HCM remains unidentified, and as a result presymptomatic DNA analysis of relatives is not possible.

The genetic heterogeneity of both HCM and NCCM and the elaborate intrafamilial variability warrant genotype-phenotype relationship studies. This thesis aims to identify familial NCCM and its genetic causes, to find additional genes involved in hypertrophic cardiomyopathy and to establish genotype-phenotype relationships.

1.1.2 Outline

Part 1 of this thesis focuses on the genetic aspects of NCCM. **Chapter 2** presents the first report of the identification of cardiac-myosin heavy chain (*MYH7*) defects in two families with noncompaction cardiomyopathy, linking non-compaction to hypertrophic, restrictive, and dilated cardiomyopathies. **Chapter 3** describes the results of cardiologic family studies in a large cohort of NCCM patients, consisting of 49 adults and 9 children. The cardiologic family studies were performed according to the guidelines for family screening in HCM, as described in chapter 8. **Chapter 4** presents the broad genetic spectrum as well as the mutation spectrum of NCCM yielded by investigating a large number of genes. Here the diagnostic implications of DNA analysis in NCCM are discussed as well. Focus on rare occasions of prenatal sonographic detection of NCCM is presented in **chapter 5** of this thesis, reporting the first two prenatal NCCM cases with a known genetic defect and presenting a review of previously described sonographic prenatal diagnosis of NCCM. **Chapter 6** describes the results of linkage analysis in a large NCCM family indentified by an aborted sudden cardiac death in the proband participating in the 2007 Rotterdam marathon. Next, **chapter 7** deals with the subjects of compound heterozygousity for truncating mutations in the myosin binding protein C gene (*MYBPC3*) leading to NCCM.

In part 2 the focus shifts to the genetic aspects of hypertrophic cardiomyopathy. **Chapter 8** discusses the screening and genetic counselling as performed in the cardiogenetic outpatient clinic of the Erasmus Medical Centre in Rotterdam. In **chapter 9**, evidence is given for a more severe phenotype caused by *MYBPC3* mutations than previously reported. **Chapter 10** discusses four-and-a-half-LIM domain (*FHL1*) gene mutations as a rare cause of familial hypertrophic cardiomyopathy. The *FHL1* gene is known to be associated with myopathies.
In literature a more severe phenotype is sometimes attributed to the co-occurrence of several genetic defects. In **chapter 11** the results of screening for a second or third genetic sarcomere defect in a cohort of severely affected and in a cohort of mildly affected HCM patients are presented. **Chapter 12** investigates the role of cardiac aldosteron as a modifying factor in HCM patients. **Chapter 13** recapitulates the results described in this thesis and comments on their significance and implications for daily clinical practice.

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1.2 Noncompaction Cardiomyopathy

Yvonne M. Hoedemaekers[1], Kadir Caliskan[2], Danielle F. Majoor-Krakauer[1].
[1] *Department of Clinical Genetics, Erasmus University Medical Centre, Rotterdam, the Netherlands.*
[2] *Department of Cardiology, Erasmus University Medical Centre, Rotterdam, the Netherlands.*

1.2.1 Introduction

Noncompaction of the left ventricle or noncompaction cardiomyopathy (NCCM) is a relatively new clinicopathologic entity, first described by Feldt et al in 1969.[1] NCCM is characterised by a prominent trabecular meshwork and deep intertrabecular recesses communicating with the left ventricular (LV) cavity, morphologically reminiscent of early cardiac development, and is therefore thought to be caused by an arrest of normal embryogenesis of the myocardium.[2, 3] Initial presentation includes congestive heart failure, thrombo-embolic events and (potentially lethal) arrhythmias, including sudden cardiac death. NCCM may be part of a more generalized cardiomyopathy, involving both the morphologically normal and the predominantly apical, abnormal LV segments. The cardiologic features of NCCM range from asymptomatic in adults to severe congenital forms.[4-7] Recently, NCCM was classified by the American Heart Association (AHA) as a separate primary, genetic cardiomyopathy, based on the predominant myocardial involvement and genetic aetiology.[8] The European Society of Cardiology (ESC) considers NCCM as unclassified, due to the lack of consensus whether NCCM is a separate individual cardiomyopathy or a non-specific morphological trait that can be found solitary or in combination with other forms of cardiomyopathy like hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) or with congenital heart disease.[9] The majority of NCCM diagnosed in adults is isolated. Non-isolated forms of NCCM are more frequent in childhood and may co-occur with congenital heart malformations, or may be part of a malformation or chromosomal syndrome.[7] The combination of NCCM and neuromuscular disorders is observed in adults as well as in children.
The majority of NCCM, isolated and non-isolated, is hereditary and NCCM appears to be genetically heterogenous.[10, 11] An important proportion of isolated NCCM in children and adults has been associated with mutations in the same sarcomere genes, that are involved in HCM, DCM and restrictive cardiomyopathy (RCM).[10] Not finding a genetic defect does not preclude a genetic cause of NCCM. In approximately half of familial NCCM the genetic defect remains unkown.[11] Shared sarcomere defects and the occurrence of HCM and DCM in families of NCCM patients indicate that at least some forms of NCCM are part of a broader cardiomyopathy spectrum.

The literature differentially refers to this form of cardiomyopathy as left ventricular noncompaction (LVNC), noncompaction cardiomyopathy (NCCM), noncompaction of the left

ventricular myocardium (NCLVM), left ventricular hypertrabeculation (LVHT), spongiform cardiomyopathy, embryonic myocardium, honeycombed myocardium, persisting myocardial sinusoids, myocardial dysgenesis, ventricular dysplasia or spongy myocardium. In analogy with the nomenclature of hypertrophic (HCM) and dilated cardiomyopathy (DCM), the term noncompaction cardiomyopathy is preferable. Therefore noncompaction cardiomyopathy, abbreviated as NCCM, will be used in this thesis to denote this entity.

Definition

NCCM is defined by prominent trabeculations on the luminal surface of the left ventricular apex, the lateral wall and rarely the septum in association with deep recesses that extend into the ventricular wall but which do not communicate with the coronary circulation. It is associated with a clinical triad of heart failure, arrhythmias and / or thrombo-embolic events.[12, 13] Diagnosis of NCCM relies on two-dimensional transthoracic echocardiography and / or cardiac magnetic resonance imaging (MRI) (Table 1). Improvements in cardiac imaging techniques have led to increased recognition and diagnosis of NCCM. Figure 4 displays echocardiographic and cardiac MRI images of two NCCM patients, showing the abnormal segmental trabeculations as the hallmark of this new entity.

Features of noncompaction observed in cardiologic patients and normal controls illustrate the necessity of defining criteria in order to differentiate accurately normal physiological trabecularisation from NCCM.[14]

Table 1. Echocardiographic diagnostic criteria for NCCM.

I. Chin et al.[2]
Focussing on trabeculae localised at the LV apex on the parasternal short axis and apical views and and on LV free-wall thickness at end-diastole NCCM is defined by a ration of X/Y ≤ 0.5 with: X = distance from the epicardial surface to the trough of the trabecular recess Y = distance from the epicardial surface to the peak of the trabeculation
II. Jenni et al.[12]
1. An excessively thickened left ventricular myocardial wall with a two-layered structure consisting of a compact epicardial layer (C) and a noncompacted endocardial layer (NC) of prominent trabeculations and deep intertrabecular recesses 2. A maximal end-systolic NC/C ratio > 2, measured at the parasternal short axis 3. Colour-Doppler evidence of deep perfused intertrabecular recesses 4. Absence of coexisting cardiac anomalies
III. Stollberger et al.[15]
1. More than three trabeculations protruding from the left ventricular wall, apical to the papillary muscles and visible in a single image 2. Perfusion of the intertrabecular spaces from the ventricular cavity visualised on colour-Doppler imaging

In 1990 the first diagnostic criteria for NCCM by Chin et al. were derived from the observations made in eight NCCM patients.[2] These diagnostic criteria defined NCCM by the ratio of the distance from the epicardial surface to the trough of the trabecular recess (X) to the distance from the epicardial surface to the peak of the trabeculations (Y), with ratio X/Y ≤ 0.5.

More than a decade later Jenni et al. proposed new diagnostic criteria for isolated NCCM, consisting of four echocardiographic features: 1) an excessively thickened left ventricular

myocardial wall with a two-layered structure consisting of a compact epicardial layer (C) and a noncompacted endocardial layer (NC) of prominent trabeculations and deep intertrabecular recesses; 2) a maximal end-systolic NC/C ratio > 2, measured at the parasternal short axis; 3) colour-Doppler evidence of deeply perfused intertrabecular recesses; 4) absence of coexisting cardiac anomalies.[12]

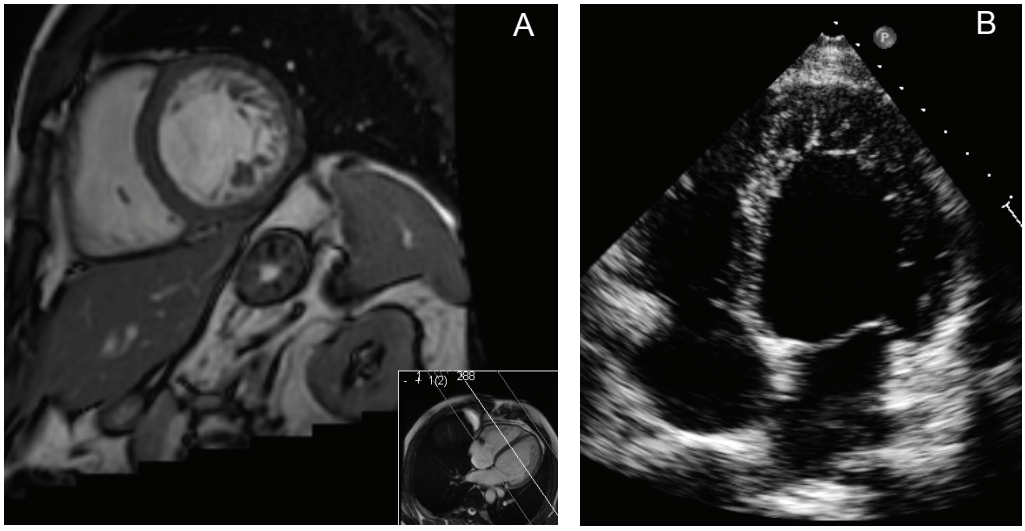


Figure 4. Cardiac MRI (A) and echocardiography (B) of a 43-year-old patient illustrating a two-layered myocardium with prominent intertrabecular recesses.

In 2002 Stollberger et al. proposed other diagnostic criteria for NCCM, wherein the diagnosis was a function of the number of trabeculations (> 3) protruding from the left ventricular wall, apically to the papillary muscles and visible in a single image plane with obligatory perfusion of the intertrabecular spaces from the ventricular cavity visualised on colour-Doppler imaging.[15]

More recently MRI criteria for NCCM introduced by Petersen et al. indicated that a noncompacted / compacted ratio (NC/C) of >2.3, measured in end-diastole, can differentiate with sufficient sensitivity between the normal variation of noncompaction of the LV in the population, noncompaction in other cardiovascular disorders and NCCM.[16]

The most recent classification system of NCCM as proposed by Belanger et al. (2008) included dividing noncompaction into four categories (none, mild, moderate and severe) according to noncompaction to compaction ratio and the size of the noncompaction area.[13] This new classification scheme used the following criteria: 1) absence of congenital heart disease, hypertrophic or infiltrative cardiomyopathy and coronary artery disease; 2) evidence of prominent trabeculations in the apex in any view (noncompacted to compacted ratio does not require to be >2); 3) concentration of the noncompacted area in the apex; 4) blood flow through the area of noncompaction.

The Jenni criteria have been the most convenient to work with in daily clinical practice and have been most widely applied in studies. However, further efforts to reach universal consensus with

respect to the diagnosis of NCCM are clearly needed. A disparity in diagnosis has been observed when comparing the application of three different sets of NCCM criteria (Chin, Jenni and Stollberger) in a cohort of 199 heart failure patients; 79% fulfilled the Chin criteria, 64% fulfilled the Jenni criteria and 53 % the criteria proposed by Stollberger. In only 30% of patients there was consensus among the three criteria on the diagnosis. Moreover, 8.3% of normal controls fulfilled one or more criteria with a higher prevalence in black controls.[14]

For now, it is disputable whether any of these diagnostic criteria are sufficiently sensitive to diagnose patients with mild noncompaction, and identify patients who may benefit from careful surveillance. For instance, in NCCM family studies a substantial proportion of (mostly asymptomatic) relatives showed mild to moderate features of NCCM.[11] Longitudinal studies of mild forms of NCCM will be needed to determine whether the current diagnostic criteria are suitable for diagnosis of family members in familial NCCM, or should be adapted in analogy to the criteria proposed for diagnosis of attenuated forms of familial HCM in relatives.

Pathology

Macroscopy

The noncompacted endocardial layer of the myocardium is comprised of excessively numerous and prominent trabeculations with deep intertrabecular recesses that extend into the compacted myocardial layer. The apical and midventricular segments of the left ventricular inferior and lateral wall are predominantly affected.[17, 18]

In a pathoanatomical study of NCCM, Burke et al. described the morphology and microscopy of 14 paediatric NCCM cases.[18] The macroscopic appearance varied from anastomosing trabeculae to a relatively smooth endocardial surface, with narrow openings of the recesses to the ventricular cavity. Three types of recess patterns were distinguished: 1) anastomosing broad trabeculae; 2) coarse trabeculae resembling multiple papillary muscles; 3) interlacing smaller muscle bundles or relatively smooth endocardial surface with compressed invaginations, identified primarily microscopically (Figure 5). In this study no morphological differences were found between isolated and non-isolated NCCM.[18]

Jenni et al described pathology of seven adult NCCM cases.[12] The pathoanatomical localisation of the noncompacted myocardium corresponded to the echocardiographic findings. Two patients also showed involvement of the right ventricular apex.[12]

In a review of published pathology of NCCM Stollberger et al. distincted three particular morphologic features of NCCM in adults and children: 1) Extensive spongiform transformation of the LV. 2) Prominent coarse trabeculations and deep recesses, covered with endocardial tissue and not communicating with coronary arteries. 3) Dysplastic thinned myocardium with excessive trabeculations.[19] The first morphology was frequently associated with other cardiac malformations, compared to the second and third.

In 1987, in an autopsy study of 474 normal hearts of all ages, it was found that prominent trabeculations may be observed in as many as 68% of the hearts, although more than three trabeculations were only identified in 3.4%.[20]

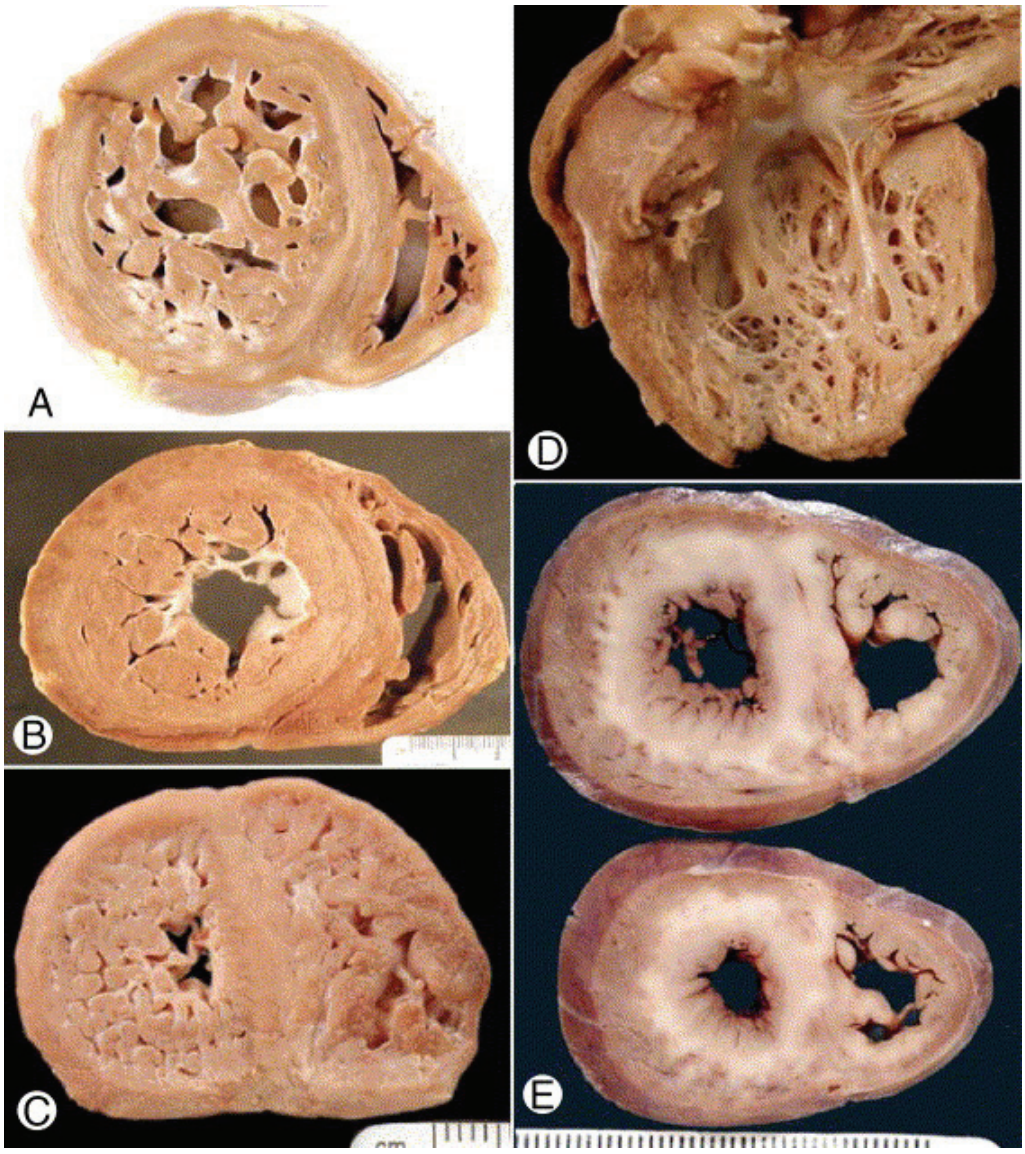


Figure 5. NCCM gross pathology with a variety of NCCM patterns:

A. Anastomosing broad trabeculae
B. Coarse trabeculae resembling multiple papillary muscles
C. Interlacing smaller muscle bundles resembling a sponge
D. Trabeculae viewed en face
E. Subtle NCCM on gross section, requires histological confirmation

Reproduced with permission from “Left ventricular noncompaction: a pathological study of 14 cases; Burke et al; *Human Pathology* 2005;36;403-411”

Microscopy

Two patterns of myocardial structure in the superficial noncompacted layer in NCCM have been described by Burke et al.:1) anastomosing muscle bundles forming irregularly branching endocardial recesses with a staghorn-like appearance; 2) multiple small papillary muscles, resulting in an irregular surface appearance (Figure 6).[18] In most patients, these patterns overlapped. Endocardial fibrosis with prominent elastin deposition was found in all 14 cases and subendocardial replacement fibrosis, consistent with microscopic ischemic infarcts, was present in 10.[18] Right ventricular involvement was identified in 6 cases.[18]

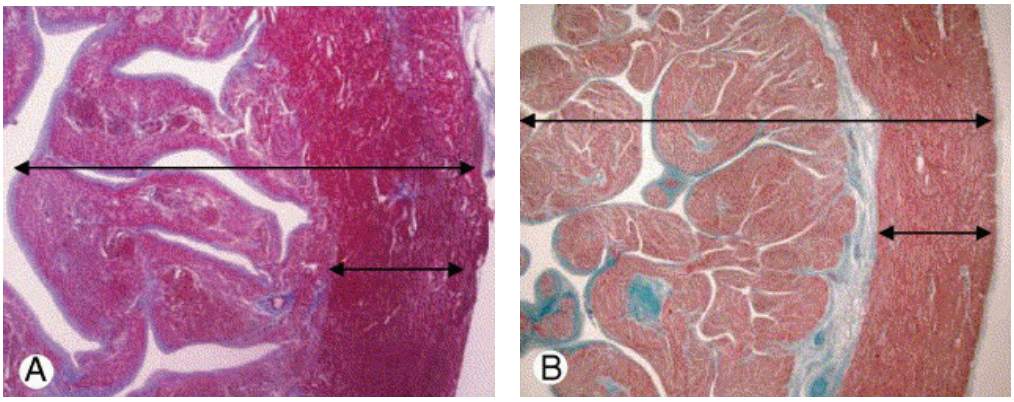


Figure 6. Histological features in NCCM
The ratio of noncompact versus compact myocardium is larger than two.
A. Relatively smooth endocardial surface (left) with anastomosing broad trabeculae.
B. Polypoid pattern of trabeculae; prominent fibrous band separating the noncompact from the compact myocardium.

Reproduced with permission from: "Left ventricular noncompaction: a pathological study of 14 cases; Burke et al; *Human Pathology* 2005;36;403-411"

Histological examination in another study showed that ventricular endocardium covered the recesses in continuity with the LV cavity and identified ischaemic lesions in the thickened endocardium and the prominent trabeculae.[12] Interstitial fibrosis ranged from absent to severe. No fibre disarray was identified in any of these cases. Signs of chronic inflammation and abnormalities of intramyocardial blood vessels were present in some patients.[12]

In one adult case report abundant extracellular matrix and myocardial fibre disarray were reported.[21]

Freedom et al. proposed two criteria for the pathological diagnosis of NCCM: 1) absence of well-formed LV papillary muscles and 2) histological verification of more than 50% penetration of invaginated endocardial recesses toward the epicardial surface. The endothelium that covers the recesses extends close to the surface of the compact layer. The recesses neither communicate nor connect with the coronary circulation.[22]

1.2.2 Epidemiology

Estimates of prevalence of NCCM were derived from large retrospective studies of patients referred for echocardiography. Population studies for NCCM have not been performed. In 1997 Ritter et al identified NCCM in 17 of 37555 (0.045%) patients who had an echocardiographic exam.[23] Similarly, in 2006 Aras et al reported a prevalence of 0.14% in over 42000 patients and in 2008 Sandhu identified definite or possible NCCM in 13 / 4929 (0.26%) patients referred for echocardiography.[24, 25] Prevalence was much higher (3.7%) in patients selected for a LV ejection fraction ≤ 45%.[25] Depending on the diagnostic criteria applied, even higher prevalence of NCCM (15.8% by Belanger; 23.6% by Kohli) were reported recently, indicating that NCCM may be more prevalent than previously indicated.[13, 14] In line with this are the observations that a substantial proportion of patients are asymptomatic, suggesting that true prevalence of NCCM may be higher, because asymptomatic patients may go unnoticed in the studies of cardiologic patients.[11, 13] In a large study on childhood cardiomyopathies, NCCM was the most frequent cardiomyopathy after DCM and HCM, with an estimated prevalence of 9% in paediatric cardiomyopathies.[26]

1.2.3 Aetiology and molecular genetics

The aetiology of NCCM is rapidly being unravelled as more and more genetic defects in different genes are found, indicating that NCCM is genetically heterogeneous. Causes for acquired NCCM are scarce. One report suggested that candida sepsis was associated with cardiologic features mimicking NCCM.[27] Currently, genetic defects are identified in 41% of NCCM patients (35% of adults and 75% of children).[10] Most genetic defects are inherited as autosomal dominant traits (Table 2), with exception of rare genetic causes of syndromal NCCM, predominantly diagnosed in children. A small proportion of patients has a de novo mutation.

However, absence of a genetic defect does not exclude a genetic aetiology. By performing systematic cardiologic family studies it was shown that no genetic defect could be found in approximately half of the familial forms of NCCM, indicating that further studies are needed to find additional genetic causes for NCCM.[11]

There is evidence that some forms of NCCM are part of a spectrum of cardiomyopathies, including hypertrophic, dilated and restricted cardiomyopathy. A shared genetic aetiology consisting of genetic defects in the same sarcomere genes, sometimes even with identical mutations, has been found in these types of cardiomyopathy. Co-occurrence of NCCM, HCM and DCM within families endorses a shared genetic susceptibility to these different forms of cardiomyopathy.[10, 11] The phenotypic variability of cardiomyopathies within families, including variability in age at onset and severity of clinical features, might be explained by additional modifying factors, additional genetic variants or defects, or may depend on yet unidentified exogenous or systemic factors.

Table 2. Genes associates with noncompaction cardiomyopathy (NCCM).

Gene	Locus	Protein	Other associated disorders	Reference
ACTC1	15q14	α-Cardiac Actin	Hypertrophic and dilated cardiomyopathy Congenital myopathy with fiber-type dysproportion	10, 11, 28, 50
CASQ2	1p13.3-p11	Calsequestrin	Catacholaminergic polymorphic ventricular tachycardia Hypertrophic cardiomyopathy	10, 11
DTNA	18q12.1-q12.2	α-Dystrobrevin		35, 134
KCNH2	7q35-q36	Potassium voltage-gated channel, subfamily H, member 2	Long QT syndrome 2 Short QT syndrome	135
*LDB3**	10q22.2-q23.3	LIM-Domain Binding protein	Dilated cardiomyopathy Late onset distal myopathy Myofibrillar myopathy	10, 11, 36, 134, 136
LMNA	1q21.2	Lamin A/C	Dilated cardiomyopathy Emery-Dreifuss muscular dystrophy Lipodystrophy Restrictive dermopathy Werner syndrome Hutchinson-Gilford Progeria Limb girdle muscular dystrophy 1B Charcot-Marie-Tooth 2B1	10, 11, 61, 62
MYBPC3	11p11.2	Cardiac Myosin-Binding Protein C	Hypertrophic and dilated cardiomyopathy	10, 11
MYH7	14q12	β-Myosin Heavy Chain	Hypertrophic, dilated and restrictive cardiomyopathy Myosin storage myopathy Distal myopathy Scapuloperoneal myopathy	10, 11, 28, 29
PLN	6q22.1	Phospholamban	Hypertrophic and dilated cardiomyopathy	10, 11
SCN5A	3p21	Sodium Channel Type 5 α-Subunit	Long QT syndrome 3 Brugada syndrome Sick sinus syndrome Familial heart block Paroxysmal ventricular fibrillation Cardiac conduction defect Dilated cardiomyopathy	137
*TAZ***	Xq28	Taffazin	Barth syndrome Dilated cardiomyopathy	10, 11, 35, 134, 136, 138-145
TNNI3	19p13.4	Cardiac Troponin I	Hypertrophic, dilated and restrictive cardiomyopathy	10, 11
TNNT2	1q32	Cardiac Troponin T	Hypertrophic, dilated and restrictive cardiomyopathy	10, 11, 28
TPM1	15q22.1	A-Tropomyosin	Hypertrophic and dilated cardiomyopathy	10, 11

*Legend. Except TAZ related disorders, all are autosomal dominantly inherited *Cypher/ZASP **G4.5*

Molecular defects in NCCM

Isolated NCCM has been associated with mutations in 14 different genes (Table 2). Defects in sarcomere genes have been identified to be the most prevalent genetic cause occurring in 32% of all patients with isolated NCCM.[10] In two DNA studies in cohorts of approximately 60 isolated NCCM patients mutations were identified in 17 to 41% of the patients depending on the number and choice of analysed genes.[10, 28] In the study by Dooijes et al of 56 patients the yield was slightly higher 41% in all and 50% in case of confirmed familial disease.[11] In children with isolated NCCM the yield of testing for sarcomere genes was as high as 75%.[10, 11]

Over 40 different mutations in sarcomere genes encoding thick (*MYH7*), intermediate (*MYBPC3*) and thin filaments (*TNNT2*, *TNNI3*, *TPM1*, *ACTC*) have been described. In particular in *MYH7,* the most frequent NCCM-associated gene, accounting for up to 20% of isolated NCCM (19% in adults and 25% in children).[10, 28] Fifty percent of the *MYH7* mutations currently associated with NCCM cluster in the ATP-ase active site of the head-region in the N-terminal part of MYH7.[10] This is an evolutionary well-conserved region of MYH7. As the ATP-ase active site is required for normal force production, impaired force generation might play a role in the aetiology of NCCM. Mutations in this region have been associated with NCCM with or without Ebstein anomaly.[28, 29] Other *MYH7* mutations (30%) were found in the C-terminal rod-region of the MYH7 protein that plays an important role in the formation of the core of the thick filament. Mutations in this region of the gene are more commonly associated with skeletal myopathies. Relatively few cardiomyopathy mutations are situated in this region.

Sarcomere mutations were common causes for NCCM in adults as well as in children.[10, 11] Multiple or compound / double heterozygous mutations were identified in 25% of the children and in 10% of the adult NCCM patients.[10] HCM complex genotypes have been described in 7%.[30] In HCM, double heterozygousity for truncating sarcomere mutations have been previously associated with severe congenital forms mostly inherited in an autosomal recessive mode.[31-33] In NCCM double mutations were associated with severe disease in two children and were also observed in adults.[10] Non-sarcomere genetic causes for isolated NCCM include mutations in the calcium-handling genes calsequestrin (*CASQ2*) and phospholamban (*PLN*), in taffazin (*TAZ*), α-dystrobrevin (*DTNA*), lamin A/C (*LMNA*) and LIM domain binding 3 (*LDB3*), potassium voltage-gated channel (*KCNH2*) and sodium channel type 5 (*SCN5A*) genes.[34-36] However, mutations in these genes were only rare causes of NCCM in single families.[37]

The absence of a mutation in approximately half of familial NCCM could be explained by phenotype assignment errors, the involvement of other yet unidentified genes, the presence of mutations in non-analysed gene sequences and incomplete sensitivity of the methods used.[10]

1.2.4 Pathogenesis

Mutations in different genes associated with NCCM affect different mechanisms in the cardiomyocyte leading to changes that may individually cause NCCM or lead to a common cellular disturbance resulting in NCCM.

Mutations in sarcomere genes may have their effect through defective force generation (either by a dominant negative mechanisms where the mutant protein acts as a “poison polypeptide” or by haploinsufficiency); mutated cytoskeletal proteins may lead to a defective force transmission; myocardial energy deficits may be the result of mutations in ATP-regulatory genes and a fourth possible mechanism is abnormal calcium homeostasis either due to changes in calcium availability or myofibrillar sensitivity for calcium.[38]

The development of NCCM features might be a compensatory response to dysfunction in one of these mechanisms.

The variable phenotypic expression of (sarcomere) gene mutations leading to different types of cardiomyopathy has not been explained. The localisation of the mutations may partly explain phenotypic diversity. Another theory is “dose-effect”; the extent of the defective mechanism may determine which phenotype develops. Thirdly, there might be independent pathways leading to the different cardiomyopathies. Finding identical mutations in different phenotypes suggests a role for additional factors, either environmental or molecular.

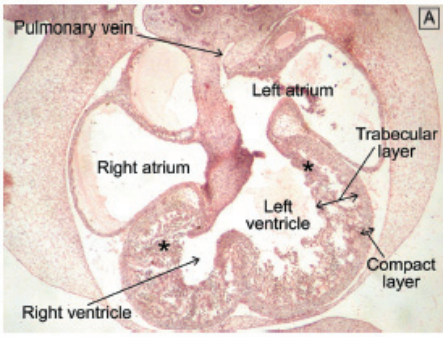
Isolated NCCM

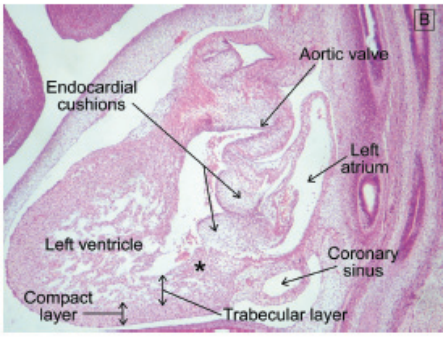
The first hypothesis on the pathogenesis of NCCM stemmed from observations that the morphology of NCCM was reminiscent of the embryonic stages of cardiac development. Consequently it was postulated that NCCM could be the result from an arrest of compaction of myocardial fibers.[39] Figure 7 illustrates the striking resemblance between NCCM and the physiological embryonic noncompaction in the 8^{th} – 10^{th} embryonic week. However, the possible mechanisms causing the arrest remain unclear. Epicardium derived cells are thought to play an important role in myocardial architecture and in the development of noncompaction.[40, 41] Mutations in genes involved in myocardial genesis like peroxisome proliferator activator receptor binding protein (*PBP*), jumonji (*JMJ*), FK506 binding protein (*FKBP12*), transcription factor specificity protein (*Sp3*), homeobox factor *NKX2.5*, bone morphogenetic protein 10 (*BMP10*), lead to congenital NCCM in knock out mice.[42-46] However, in human NCCM no mutations in these genes have been described.

Until now, there is very little insight in factors that influence the variability in age at onset and severity of symptoms of NCCM, or any other familial form of cardiomyopathy.

In the majority of patients NCCM is diagnosed in adulthood, similar to HCM and DCM, which are rarely congenital.[47, 48] Of course it could be that in NCCM the lesions detected in adult patients were present from birth on, but remained unnoticed until symptoms developed and high-resolution cardiac imaging techniques were applied. However, the detection of sarcomere defects in NCCM patients may suggest otherwise, since mutations in sarcomere genes are known to cause late onset HCM and DCM. Similarly, sarcomere mutations might lead to late onset NCCM. Longitudinal cardiologic studies of unaffected carriers of pathogenic mutations are necessary to provide insight whether noncompaction may develop later in life. The pathogenetic mechanism(s) of sarcomere defects in cardiomyopathies are not fully understood. It is possible that the pathological myocardial changes in the adult onset sarcomere related

cardiomyopathies are caused by a compensatory response to impaired myocyte function resulting from mutations in the sarcomere genes.[38, 49]





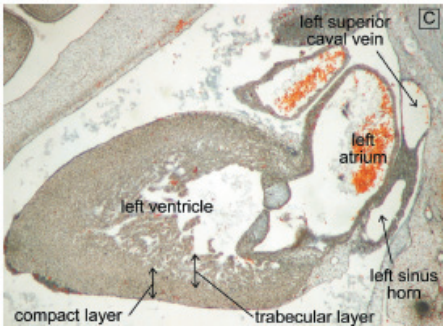


Figure 7. Human embryos at Carnegie stage 16 (A), stage 18 (B) and after closing of the embryonic interventricular foramen (C).
During development there is an extensive trabecular layer forming the greater part of the ventricular wall thickness compared to the extent of the compact layer.
The trabecular layer becomes compacted and forms the papillary muscles of the atrioventricular valves (asterisks).

Reproduced with permission from "The morphological spectrum of ventricular noncompaction; Freedom et al; *Cardiology in the Young* 2005;15:345-364"

Non-isolated NCCM

NCCM has been observed in a number of neuromuscular disorders, metabolic and mitochondrial disease, congenital malformations and chromosomal syndromes.
Some of these disorders may share pathogenetic mechanisms with NCCM. Alternatively NCCM might be secondary to other cardiac malformations or other malformations or even vice versa. Another possibility is that the co-occurrence is coincidental. Congenital heart malformations for instance are relatively frequent (birth prevalence 0,008) and may therefore occasionaly coincide with NCCM without a mutual aetiology.

Congenital heart disease

The co-occurrence of congenital heart disease and noncompaction is predominantly observed in children. Tsai et al. showed that 78% of 46 children with NCCM had a congenital heart defect.[7] The large number of structural heart malformations reported in association with noncompaction are presented in Table 3, indicating that septal defects, patent ductus arteriosus and Ebstein's anomaly are the most prevalent congenital heart defects in NCCM.
Increasingly, congenital cardiac malformations (septal defects, Ebstein anomaly, patent ductus arteriosus, Fallot's tetralogy, aortic coarctation and aortic aneurysms) are being reported in familial cardiomyopathies (HCM, DCM and NCCM) linked to sarcomere mutations, suggesting that these specific sarcomere defects may have been involved in cardiac morphogenesis.[11, 29, 50-54] But since there is rarely more than one patient with a congenital heart defect, even in families with multiple cardiomyopathy patients, the association of sarcomere defects and heart defects still demands further exploration.

Neuromuscular disease

Similar to HCM and DCM, NCCM has been associated with neuromuscular disorders. Stollberger and Finsterer identified NCCM-like morphological features in Duchenne and Becker muscular dystrophy and in myotonic dystrophy.[55-58] The gene mutated in Duchenne and Becker muscular dystrophy, is part of the dystrophine complex, a complex of muscle membrane associated proteins, connecting the cytoskeleton to the surrounding extracellular matrix and may also play a role in cell signalling. The dystrophine gene is expressed in skeletal and cardiac myocytes. A large proportion of patients and also female carriers have cardiac symptoms, including DCM.[59, 60] Other genes previously associated with neuromuscular disorders, like adult onset myofibrillar myopathy (*LDB3* or *Cypher/ZASP*), limb girdle muscular dystrophy (LGMD) (*LMNA*), scapuloperoneal myopathy (*MYH7*), myosin storage distal myopathy (*MYH7*) and Barth syndrome (*TAZ*), have recently been associated with isolated NCCM (Table 1 and 4). ZASP, lamin A and C, β-myosin heavy chain and taffazin are all expressed in cardiac and in skeletal muscle tissue. ZASP has a function in cytoskeletal assembly. Mutations in ZASP can lead to DCM and to skeletal myopathy. Lamin A and C, proteins situated in the nuclear membrane, play an important role in maintaining nuclear architecture. *LMNA* mutations have been described in three NCCM patients.[11, 61, 62] In one of

Table 3. Congenital heart disease associated with NCCM.

Congenital heart disease in NCCM	Proportion of CHD		References
	In NCCM studies*	Case reports	
Aberrant origin of right / left subclavian artery	1 / 12 (8%)	1	146, 147
Absent aortic valve		1	148
Anomalous pulmonary venous return	2 / 26 (8%)		18, 146
Aortic coarctation	6 / 204 (3%)		7, 11, 113, 146, 149
Aortico-left ventricular tunnel		1	150
Aortic stenosis	2 / 46 (4%)	2	7, 22, 151
Aortopulmonary window	1 / 21 (5%)		113
Atrial septal defect	22 / 135 (16%)	3	7, 11, 29, 113, 152, 153
Atrio-ventricular diverticulum		1	154
Bicuspid aortic valves	3 / 64 (5%)	3	7, 113, 119, 155
Bicuspid pulmonary valve	1 / 14 (7%)		18
Cardiac aneurysms		4	81, 156-158
Coronary ostial stenosis	1 / 14 (7%)		18
Cor triatriatum	1 / 46 (2%)		7
Dextrocardia	2 /58 (3%)	1	1, 7, 146
Dextro malposed great arteries	1 / 12 (8%)		146
Dextroversion		1	159
Double inlet left ventricle	1 / 46 (2%)		7
Double orifice mitral valve		4	160-162
Double outlet right ventricle	1 / 54 (2%)		149
Ebstein's anomaly	6 / 117 (5%)	10	7, 11, 153, 163-168
Fallot's tetralogy	1 / 71 (1%)	1	11, 147
Hypoplastic left heart syndrome	3 / 54 (6%)		149
Hypoplastic right ventricle	3 / 58 (5%)		7, 146
Isomerism of the left atrial appendage	4 / 66 (6%)	8	22, 146, 149, 169
Left sided superior vena cava	1 / 46 (2%)		7
Mitral valve atresia		1	148
Mitral valve cleft	2 / 54 (4%)	1	149, 158
Mitral valve dysplasia	2 / 14 (14%)		18
Mitral valve prolaps	1 / 46 (2%)		7
Patent ductus arteriosus	16 / 182 (9%)	1	7, 11, 149, 153
Persistent left superior vena cava	1 / 14 (7%)	1	18, 157
Pulmonary atresia	6 / 125 (5%)	1	11, 149, 153
Pulmonary stenosis	4 / 97 (4%)	1	11, 18, 146, 153
Pulmonary valve dysplasia	2 / 14 (14%)		18
Single ventricle	1 / 12 (8%)	1	146, 170
Subaortic membrane	2 / 55 (4%)		149
Transposition of the great arteries	1 / 46 (2%)	1	7, 171
Tricuspid atresia	2 / 54 (4%)		149
Tricuspid valve dysplasia	1 / 14 (7%)		18
Ventricular septal defect	23 / 218 (11 %)	3	1, 7, 11, 18, 113, 146, 149, 151, 157

*Legend. *Cumulative number of NCCM patients with congenital heart defect (CHD) described in one or more NCCM studies*

them there was familial LGMD as well as DCM.[11] Over 200 mutations have been described in *LMNA*, causing over 20 different phenotypes, including isolated DCM, limb girdle muscular dystrophy (LGMD), Emery–Dreifuss muscular dystrophy, Hutchinson–Gilford progeria, partial lipodystrophy and peripheral neuropathy. For many of the phenotypes there is no clear genotype-phenotype correlation, phenotypes may overlap and different phenotypes are associated with single mutations.[62] Up to 25% of patients with a LMNA mutation may remain cardiologically asymptomatic.[63] The β-myosin heavy chain is part of type II myosin that generates the mechanical force needed for muscle contraction. Tafazzins have no known similarities to other proteins. Two regions of the protein may be functionally significant, one serving as a membrane anchor and soluble cytoplasmic protein and the other may serve as an exposed loop, interacting with other proteins.

Table 4 presents a list of neuromuscular disorders in which NCCM has been identified. In addition one case of noncompaction in a patient with Friedreich ataxia has been reported.[64] Friedreich ataxia is associated with symmetric, concentric, hypertrophic cardiomyopathy.

Table 4. Neuromuscular disorders associated with NCCM / hypertrabeculation.

Neuromuscular disorders	Gene	Inheritance	Features	Ref.
Adenosine Monophosphate Deaminase 1 (MADA deficiency)	*AMPD1*	AD	Exercise-induced myopathy, muscle weakness, cramps; prolonged fatigue after exertion; benign congenital hypotonia	172
Becker and Duchenne muscular dystrophy	*DMD*	XR	Muscle weakness and wasting; hypotonia; waddling gait; pseudohypertrophy; cognitive impairment; cardiomyopathy; respiratory failure	55-57, 132, 173, 174
Charcot-Marie-Tooth 1A (HMSN 1A)	*PMP22*	AD	Distal limb muscle weakness and atrophy; distal sensory impairment	175
Myotonic dystrophy I	*DMPK*	AD	Myotonia; weakness; muscle wasting; adult cognitive deterioration; cataract; arrhythmia	58, 176, 177
Myotonic Dystrophy II	*ZNF9*	AD	Muscle pain; myotonia; weakness (proximal / deep finger / neck flexor); cataract; cardiac conduction abnormalities; palpitations; tachycardia; hypogonadism; frontal balding	178
Infantile epilepsy-encephalopathy syndrome (Ohtahara syndrome)	*ARX*	XR	Age-dependent epileptic encephalopathy with “burst-suppression” on EEG; physical and mental retardation	179
Limb girdle muscular dystrophy 1B	*LMNA*	AD	Muscle weakness and wasting restricted to the limb musculature, proximal greater than distal	11, 132
Succinate dehydrogenase deficiency		AR	Encephalomyopathy; cardiomyopathy; generalised muscle weakness; cerebellar ataxia; optic atrophy; tumour formation in adulthood	180

Legend. AD: autosomal dominant; XR: X-linked recessive; AR: autosomal recessive.

Syndromes

NCCM can occur as part of a syndrome in combination with dysmorphic features and other congenital malformations. When there are other congenital defects or when there are dysmorphic features in a patient, one of the chromosomal defects listed in Table 5 or one of the syndromes in Table 6 could be considered in the differential diagnosis.

Mitochondrial

Mitochondrial disorders often lead to multi-organ disease, including central and peripheral nervous system, eyes, heart, kidney and endocrine organs. One of the cardiac features observed in mitochondrial disease is noncompaction cardiomyopathy. Cardiac features may be the first or only feature in patients suffering from a mitochondrial disorder. In a study of 113 paediatric patients with mitochondrial disease NCCM was identified in 13%.[65] Pignatelli et al showed that five of the 36 paediatric NCCM patients who underwent a skeletal muscular biopsy, had morphologic and biochemical evidence for a mitochondrial defect, including a partial deficiency of complex I-III of the mitochondrial respiratory chain.[66] Mutations in mitochondrial DNA (mtDNA) and in nuclear DNA have been identified in the mitochondrial disorders associated with NCCM.[67-69] MtDNA mutations are maternally transmitted, whereas nuclear DNA genes involved in oxidative phosphorylation are transmitted as Mendelian traits with an autosomal dominant or recessive inheritance.

Miscellaneous

NCCM has been described in patients with heterotaxy with polysplenia, polycystic kidney disease, congenital adrenal hyperplasia, nefropathic cystinosis and myelofibrosis.[11, 66, 70-74] Whether these co-occurrences are coincidental or represent shared aetiologies with NCCM is unknown.

Among the possibly acquired forms of NCCM there are reports about an infectious cause.[27] Recently, an aetiologic role for macro- and microvascular abnormalities was suggested.[75-84] NCCM has also been described in patients with coronary heart disease.[85-87] Since coronary artery disease is a frequent disorder, this association may well be coincidental. Aortic elasticity was significantly altered in a group of 20 NCCM patients (aortic stiffness index of 8.3 ± 5.2).[78] Microvascular abnormalities in NCCM including decreased coronary flow reserve with wall motion abnormalities in more extended regions of the myocardium than the noncompacted area have been observed.[77] In addition, several case studies reported hypoperfusion of the non-compacted region in NCCM patients using myocardial perfusion SPECT, positron emission tomography, Thallium myocardial imaging or MRI.[75, 76, 79, 80, 82, 83] It is thought that fibrosis, thrombus formation, hypokinesis and necrosis may be the underlying mechanisms of hypoperfusion.[76, 79, 80]

Other pathogenic hypotheses for NCCM include adaptation to changes in the cardiovascular and / or haemodynamic climate; myocardial dissection or tearing of the inner layer of the cardiac muscle due to dilatation.[19, 22]

Table 5. Chromosomal defects associated with noncompaction cardiomyopathy (NCCM).

Chromosomal defects	Features	Ref.
Deletion		
1p36	Microcephaly; sensorineural hearing loss; deep-set eyes; flat nose; cleft lip / palate; cardiomyopathy; septal defects; patent ductus arteriosus; dilated aortic root; feeding problems; gastro-oesophageal reflux; short fifth finger and clinodactyly; mental retardation (severe); seizures; hypotonia	191-194
1q43-q43	Microcephaly; upslanting palpebral fissures; epicanthus, broad nasal bridge, micrognathia; low set ears; bow-shaped upper lip; widely spaced teeth; short webbed neck; congenital heart defects; mental retardation (severe); speech impairment; seizures; corpus callosum agenesis	195
5q35.1q35.3	Facial hirsutism; synophrys; downslanting palpebral fissures; atrial septal defect and patent ductus arteriosus; NCCM with sick sinus syndrome and second degree heart block; feeding problems; gastro-oesophageal reflux; joint hypermobility	196
22q11.2	Velo-cardio-facial syndrome: short stature; microcephaly; retrognathia; narrow palpebral fissures; square nasal root; prominent tubular nose; cleft palate; velopharyngeal insufficiency; congenital heart defect (85%): ventricular septal defect; Fallot's tetralogy; inguinal / umbilical hernia; slender hands and digits; learning disability; mental retardation; schizophrenia; bipolar disorder	66
Numeric		
4q trisomy / 1q monosomy	Senile-like appearance; narrow palpebral fissures; telecanthus; epicanthus; broad nasal bridge; low-set ears; long philtrum; dimple below lower lip; anteriorly displaced anus; rocker-bottom feet; mental retardation; hypotonia, hypoplastic corpus callosum	197
Trisomy 13	Microcephaly; hypotelorism; cleft lip / palate; coloboma; low-set ears; septal defects; patent ductus arteriosus Polydactyly; overlapping fingers; mental retardation (severe); hypotonia; seizures	198
Trisomy 21	Short stature; bachycephaly; flat facial profile; conductive hearing loss; epicanthal folds; upslant; iris brushfield spots; protruding tongue; congenital heart malformation; duodenal atresia; Hirschsprung disease; joint laxicity; single transverse palmar crease; excess nuchal skin; mental retardation; hypothyroidism; leukemia	11, 149
Mosaic trisomy 22	Microcephaly; hypertelorism; preauricular pits / tags; low-set ears; micrognathia, long philtrum; septal defects; double aortic arch; clinodactyly; hypoplastic nails; hemiatrophy; mental retardation	199
45,X0	Turner syndrome: short stature; short webbed neck; low hair line; broad nasal bridge; low-set ears; congenital heart defects: aortic coarctation; bicuspid aortic valves; aortic dilatation; lymph-oedema of hands and feet; renal abnormalities: single horseshoe kidney; renal vascular abnormalities; delayed puberty; amenorrhea; infertility; hypothyroidism	200, 201
Loci		
6p24.3-21.1	NCCM; bradycardia; pulmonary valve stenosis; atrial septal defect; left bronchial isomerism; azygous continuation of the inferior vena cava; polysplenia; intestinal malrotation (heterotaxia)	153
11p15	NCCM; mild pulmonary stenosis; mild mitral valve prolapse; atrial septal defect	202

Table 6. Syndromes associated with NCCM / hypertrabeculation.

Syndrome	Gene	Inheritance	Features	Reference
Barth syndrome / 3-Methylglutaconic aciduria	*TAZ*	XR	Growth retardation, dilated cardiomyopathy, skeletal myopathy, intermittent lactic acidemia, granulocytopenia, recurrent infections	35, 37, 134, 136, 138-145
Branchio-oto-renal syndrome I / Melnick Fraser syndrome	*EYA1*	AD	Long narrow face; hearing loss (sensory / conductive / mixed); preauricular pits; microtia; cup-shaped ears; lacrimal duct stenosis; cleft palate; bifid uvula; branchial cleft fistulas / cysts; renal dysplasia / aplasia; polycystic kidneys; vesico-ureteric reflux	181
Congenital adrenal hypoplasia	*NR0B1*	XR	Failure to thrive; hypogonadotropic hypogonadism; crytorchidism; hyperpigmentation; primary adrenocortical failure; adrenal insufficiency; gluco-mineralocorticoid insufficiency; salt-wasting; delayed puberty	66
Contractural arachnodactyly / Beals syndrome	*FBN2*	AD	Marfanoid habitus; micrognathia; frontal bossing; crumpled ear helices; ectopia lentis; high-arched palate; septal defects; bicuspid aortic valve; mitral valve prolapse; patent ductus arteriosus; aortic root dilatation; pectus carinatum; kypkoscoliosis; hip / knee / elbow contractures; arachnodactyly; ulnar deviation of fingers; talipes equinovarus; hypoplastic calf muscles; motor development delay	182
Cornelia de Lange Syndrome I	*NIPBL*	AD	Short stature; microcephaly; long philtrum; micrognathia; low-set ears; sensorineural hearing loss; synophrys; myopia; long curly eyelashes; ptosis; anteverted nostrils; depressed nasal bridge; cleft lip / palate; thin upper lip; widely-spaced teeth; congenital heart defect; pyloric stenosis; hypoplastic male genitalia; structural renal anomalies; phocomelia; oligodactyly; syndactyly of 2nd and 3^{d} toes; single transverse palmar crease; cutis marmorata; hirsutism; low posterior hair line; mental retardation; language delay; auto-mutilation	113
Leopard syndrome	*PTPN11* *RAF1*	AD	Short stature; triangular face; low-set ears; sensorineural hearing loss; hypertelorism; ptosis; epicanthal folds; broad flat nose; cleft palate; short neck; pulmonic stenosis; HCM; subaortic stenosis; complete heart block; bundle branch block; winged scapulae; hypospadia; absent / hypoplastic ovary; unilateral renal agenesis; spina bifida occulta; dark lentigines (mostly neck and trunk); café-au-lait spots	183

Continued on next page

Melnick Needles osteodysplasty	*FLNA*	XD	Short stature; micrognathia; large ears; hypertelorism; exophtalmos; cleft palate; misaligned teeth; long neck; mitral / tricuspid valve prolapse; NCCM; pulmonary hypertension; pectus excavatum; omphalocele; hydronephrosis; tall vertebrae; bowing of humerus / radius / ulna / tibia; short distal phalanges of the fingers; pes planus; coarse hair; delayed motor development; hoarse voice	[184]
Nail Patella Syndrome	*LMX1B*	AD	Short stature; sensorineural hearing loss; ptosis; cataract; cleft lip / palate; malformed sternum; hypoplasia of first ribs; glomerulanephritis; renal failure; scoliosis; elbow deformities; hypoplastic or absent patella; clinodactyly; talipes equinovarus; longitudinal ridging nails; slow nail growth; koilonychias; anonychia; aplasia pectaralis minor / biceps / triceps / quadriceps	[185, 186]
Noonan syndrome	*PTPN11* *KRAS* *SOS1* *RAF1*	AD	Short stature; triangular face; low-set ears; hypertelorism; downslanting palpebral fissures; epicanthal folds; myopia; micrognathia; high arched palate; low posterior hairline; webbed neck; septal defects; pulmonic stenosis; patent ductus arteriosus; pectus carinatum superiorly / pectus excavatum inferiorly; cryptorchidism; clinodactyly; woolly hair; mental retardation (mild); bleeding tendency; malignant schwannoma	[187]
Roifman syndrome		XR	Short-trunk dwarfism; long philtrum; strabismus; narrow and downslanting palpebral fissures; long eyelashes; retinal dystrophy; narrow upturned nose; NCCM; hepato-splenomegaly; spondylo-epiphyseal dysplasia; eczema; hyperconvex nails; hypotonia; (mild) mental retardation; hypogonadotropic hypogonadism; recurrent infections; antibody deficiency	[188]
Syndromic microphtalmia / MIDAS syndrome (MIcrophtalmia, Dermal Aplasia, Sclerocornea)	*HCCS*	XD	Short stature; microcephaly; hearing loss; microphtalmia; sclerocornea; cataract; iris coloboma; retinopathy; septal defects; cardiac conduction defects; cardiomyopathy; overriding aorta; anteriorly placed anus; hypospadia; linear skin defects; corpus callosum agenesis; hydrocephalus; mental retardation; seizures	[189, 190]

Legend. AD, autosomal dominant; XD, X-linked dominant; XR, X-linked recessive

1.2.5 Clinical aspects

Heart failure is among the most frequent presentations of NCCM, followed by supraventricular and ventricular arrhythmias, including sudden cardiac death, and thrombo-embolic events. However, as in other cardiomyopathies, there is a great variability in presentation, even within families, ranging from a fully asymptomatic course to severe heart failure necessitating cardiac transplantation. The age of presentation is also highly variable varying from prenatal and neonatal diagnosis to diagnosis at the age of 94 years.[6,11, 88-93] Prenatal diagnostic imaging detects more often bilateral ventricular hypertrophy / hypertrabeculations than the typical left ventricular morphologic changes observed postnatally and in adults (own observation). The fourth to fifth decade of life is the median age for diagnosis in adult isolated NCCM, constituting a relatively young population in adult cardiologic practice. Many patients remain asymptomatic and may be detected due to an asymptomatic heart murmur, or by chance by pre-operative cardiac evaluation or medical assessment for insurance or jobs or because they participated in cardiologic family screening, after a relative had been diagnosed with NCCM.[11, 13] Symptomatic patients may present clinical symptoms of dyspnea, fatigue, (atypical) chest pain and / or (pre) syncope. NCCM may also present as a peripartum cardiomyopathy.[11, 94-96] Review of the literature revealed a male to female ratio of almost 2 to 1.[19] This gender difference can not be fully explained by the occurrence of already known X-linked forms of NCCM.

Different arrhythmias and conduction disorders may occur in NCCM patients (Table 7). None of these arrhythmias is characteristic or pathognomonic for NCCM. Thrombo-embolic events may include stroke (cerebrovascular event or transient ischemic attack), peripheral embolism and mesenterial thrombosis.

Table 7. Arrhythmia and conduction disorders associated with NCCM.

Arrhythmia / conduction disorders associated with NCCM	Reference
Atrial fibrillation	15, 113, 203
Atrioventricular nodal re-entrant tachycardia	204
Bigemini ventricular extra systole	146
Complete atrioventricular block	1, 158, 205, 206
Complete left bundle branch block	109, 146
Giant P-waves and focal atrial tachycardia	207
Long QT syndrome 2	135
Narrow QRS complex	106, 107, 110
Persistent atrial standstill	208
Sick sinus syndrome	209
Sinus bradycardia	153, 210
Supraventriular tachyarrhythmia	7, 113, 130, 146, 211
Ventricular fibrillation	106, 205, 212
Ventricular tachycardia	7, 79, 106, 109, 210
Wolff-Parkinson-White syndrome	2, 7, 146, 210, 213

1.2.6 Differential diagnosis

The definitive diagnosis of NCCM relies on the morphological features of the LV myocardium, as defined by an imaging modality, like echocardiography, MRI, CT or LV angiography. The variability in the extent of physiological trabecularisation may complicate distinction of NCCM from normal physiological left ventricular trabeculations. Especially in the area around the base of the papillary muscles of the mitral valve more trabeculations may be present. However, in the normal heart there is no excessive segmental thickening (due to hypertrabeculation) like in NCCM and the thickness of these physiological trabeculations does not exceed the thickness of the compact layer. Also the area of noncompaction is larger in NCCM than in physiological trabeculations.[13]

Secondary forms of (acquired) NCCM may be the result of hypertension, chronic volume or pressure overload,[97] ischemic heart disease or extreme physical activity (i.e. athletes), leading to NCCM like abnormalities. These are referred to as pseudo-noncompaction cardiomyopathy or a NCCM look-a-like. Hypertensive patients are diagnostically challenging, because of the occurrence of LV hypertrophy due to hypertension. Further studies are needed to confirm whether excessive trabeculation is more prevalent in specific ethnic groups, as suggested by one study.[14]

Furthermore dilated, hypertrophic and ischemic cardiomyopathy may be mistaken for NCCM or vice versa, due to prominent trabculations and or abnormal myocardial thickening. Candida sepsis with intramyocardial abscesses and intramyocardial haematoma may mimic NCCM.[27, 98, 99]

The neuromuscular disorders, syndromes and chromosomal abnormalities mentioned earlier (Table 3-5) should be considered in the differential diagnosis of non-isolated NCCM, especially when NCCM occurs in patients with dysmorphism, growth retardation or skeletal muscle weakness.

1.2.7 Therapy, follow-up and prognosis

Therapy and follow-up

Current guidelines for heart failure, arrhythmias, cardiac resynchronisation therapy and ICD implantation for primary and secondary prevention are applied for NCCM.[100-102] β-Blockers and Angiotensin-converting-enzyme (ACE) – inhibitors are the cornerstones of treatment in the presence of LV dysfunction and/or arrhythmias. Establishing an expert consensus rapport, similar to HCM, based on case reports, small cohorts and clinical registries would be recommended since no randomized trials or studies on management of NCCM have been conducted, and clear-cut evidence-based clinical guidelines for this disorder are therefore missing.[103]

An important issue is the use of prophylactic anticoagulants, in view of frequent thrombo-embolic events. The early case reports and case series emphasised the high risk of thrombo-

embolism and advised routine anticoagulation therapy. However, a review of 22 publications addressing the issue concluded that thrombo-embolic events are rare in NCCM.[104] Fazio et al. came to the same conclusion.[105] Currently, in our hospital anticoagulation therapy is advised only in patients with an ejection fraction less than 40% (cut off arbitrary), paroxysmal or persistent atrial fibrillation and/or previous thrombo-embolic events.

Successful cardiac resynchronisation therapy has been described in several NCCM patients, leading to left ventricular reverse remodelling and an increase in left ventricular function.[106-110]

Heart transplantation has been performed in some NCCM patients with severe heart failure.[3, 11, 23, 111-117] Left ventricular restoration surgery has been reported successful in a single patient.[118]

Treatment with an implantable cardioverter defibrillator (ICD) will be discussed further on.

The indication for cardiologic follow-up depends on individual symptoms and cardiac abnormalities. In asymptomatic patients with preserved LV function, annual or biannual cardiologic follow-up is recommended, including ECG and echocardiography. If necessary, these could be extended with 24-hour-Holter monitoring and exercise-testing. When EF is below 50%, β-blocker therapy and ACE-inhibitors should be prescribed, especially when NCCM is accompanied by hypertension or arrhythmias.

Prognosis

Initially, NCCM was reported to have a grave prognosis.[2,3,12,19,23,119-125] However, the application of new imaging techniques allowing diagnosing NCCM in asymptomatic individuals, suggests that the first observations were influenced by selection of the most severely affected individuals. It has become clear that prognosis of NCCM is as variable as the prognosis in other cardiomyopathies. Even in those with presentation in early childhood, gradual improvement in cardiac function may be observed, although in others evolvement to severe heart failure requiring heart transplantation does occur.[6,88,90,92,126,127] Similarly in some adult patients a rapid deterioration of heart function occurs, whereas in others the disease remains stable up to old age.[89] Malignant arrhythmias leading to sudden cardiac death and heart failure are the main indicators of poor prognosis. The establishment of appropriate risk stratification will be an important issue in the near future in order to identify patients at risk and to help prevent sudden cardiac death.

1.2.8 Risk stratification and indication for ICD

Patients at highest risk for sudden death are patients who previously experienced (aborted) cardiac arrest, ventricular fibrillation and sustained VF. Family history of sudden death, unexplained syncope (especially during exercise), abnormal blood pressure response during exercise tests, frequent premature ventricular beats on the resting ECG and / or non-sustained ventricular tachycardia on Holter monitoring and significantly impaired left ventricular function may be considered risk factors. The results from longitudinal studies and the understanding of underlying disease mechanisms will hopefully help to gain more insight in the risk factors and

allow more appropriate risk stratification.[128] Consensus and guidelines for prophylactic ICD treatment in NCCM patients are also needed. Regular ICD indications include primary and secondary prevention. For secondary prevention, i.e. after a previous episode of aborted cardiac death or collapse due to sustained VT or VF, current ICD guidelines advise ICD implantation. In the Rotterdam NCCM cohort of 67 patients, an ICD was indicated in 42% according the current ICD guidelines (n=28: 21 primary and 7 for secondary prevention). After long-term follow-up, appropriate ICD therapy occurred only in patients with secondary prevention (n=3). Inappropriate ICD therapy occurred in 33% of the patients with primary prevention and in 29% of the patients with secondary prevention.[129] In another study, follow-up of 12 patients who received an ICD showed overall appropriate therapy in 42% in primary and secondary prevention combined.[130] In primary prevention 25% of ICD therapy was appropriate opposed to 50% in secondary prevention.[130] This accentuates the need for further research of appropriate risk stratification of sudden cardiac death in patients with NCCM.

1.2.9 Cardiogenetic aspects

Molecular and cardiologic family screening

Familial NCCM has been estimated to occur in 18 - 71% of adults with isolated NCCM, mostly consistent with an autosomal dominant mode of inheritance, indicating the importance of informing and examining relatives of patients with isolated NCCM.[2, 11, 24, 66, 120, 131-133] Since extensive family studies showed that the majority of affected relatives are asymptomatic, cardiologic evaluation should include all adult relatives irrespective of medical history. Obviously, taking a family history is by itself insufficient to identify familial disease, given the high frequency of asymptomatic disease in families.[11] In families where a pathogenic mutation has been identified, relatives can be offered predictive DNA analysis. In families without a pathogenic mutation cardiac family screening remains the method of choice to identify relatives at risk of developing symptomatic cardiomyopathy, who may benefit from early treatment.

Introducing patients to the probability that their disease is genetic and explaining the significance of DNA testing especially in those who are unaware of familial disease, requests genetic counselling. It is important to explain and discuss risks for relatives and to provide support tailored to individual needs and situations. Whether presymptomatic testing is based on genetic information or cardiac evaluation makes no difference in this respect. Informing relatives includes discussing possible medical, as well as psychological and socio-economical consequences of predictive testing (particularly in countries where genetic discrimination by insurance companies or employers is not prohibited). Asymptomatic relatives, not anticipating to be affected, have to be prepared to cope with a cardiomyopathy or the risk of developing a cardiomyopathy and transmitting the genetic predisposition to their offspring. On the other hand, patients and at risk relatives need to be informed about possible benefits of early diagnosis including suitable treatment and life-style recommendations.

Apart from NCCM other cardiomyopathies may co-occur within families, like hypertrophic and dilated cardiomyopathy, so cardiac screening should aim at identifying all cardiomyopathies. Cardiac screening of relatives may show minor abnormalities not fulfilling NCCM criteria, which may be difficult to differentiate from normal physiologic trabecularisation. Hypothetically these minor abnormalities might develop into NCCM eventually. Longitudinal studies of patients with mild NCCM features are needed to investigate the natural history of these forms of noncompaction.

Genotype-phenotype correlations

Molecular studies of NCCM have thus far shown that there are few recurrent mutations.[10] Therefore it is difficult to establish genotype-phenotype correlations. Additionally, intrafamilial phenotypic variability complicates predictions based on an identified mutation. Multiple (truncating) sarcomere mutations appear to result in a more severe phenotype with childhood onset.[10, 11] Multiple mutations identified in adults, mostly also comprise involvement of a non-sarcomere gene. Adult patients with multiple mutations seem to have more symptoms than adults with a single mutation.[10, 11] These observations may indicate that the combination of a sarcomere mutation and a non-sarcomere mutation causes a less severe phenotype than when a patient has two sarcomere mutations. Mutations in *DTNA* and *TAZ* seem to transfer the strongest predisposition to childhood onset NCCM.

Molecular strategies

The proposed strategies for the molecular and cardiologic evaluation of NCCM are depicted in the flow chart in Figure 8. Extensive genetic screening may lead to the identification of a molecular defect in over 40% of isolated NCCM patients and in half of these patients an *MYH7* mutation is found.[10] *MYH7* gene sequencing should be considered as initial approach, being the most prevalent cause for NCCM in adults and children. Further molecular analyses of the other genes within the NCCM spectrum, which quantitatively have a relatively modest contribution to NCCM morbidity, may be considered when no mutation in *MYH7* can be identified. Sarcomere gene analysis is also warranted in paediatric patients, given the high percentage of sarcomere mutations in this group.[10] When an adult or paediatric patient is severely affected, screening for a second molecular defect is advised, given the high frequency of multiple mutations in NCCM.

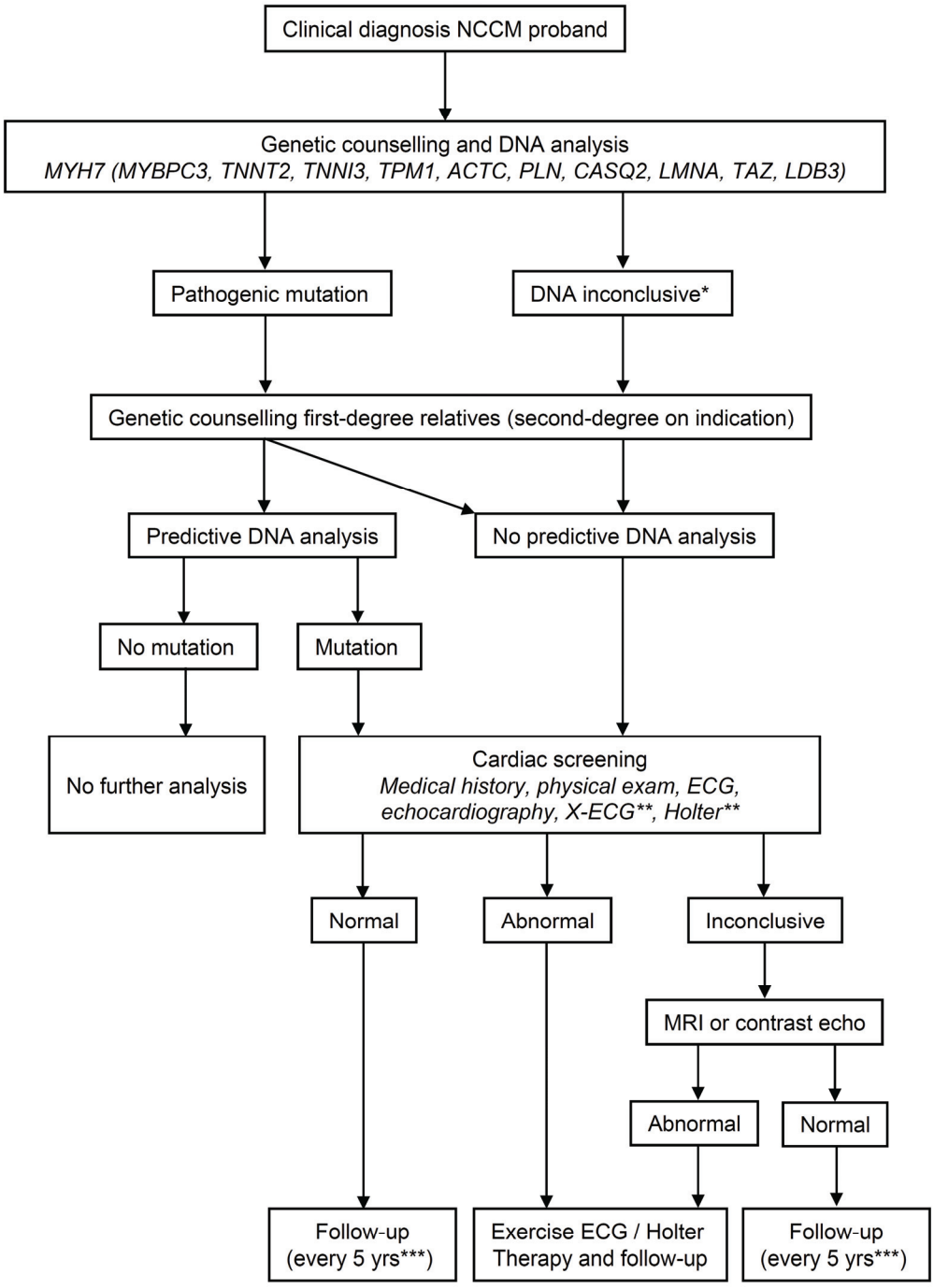


Figure 8. Flow chart for family screening in NCCM
Legend. * including unclassified variants; ** if clinically indicated; *** earlier when symptomatic

Summary

NCCM is a relatively new, genetically heterogeneous, cardiomyopathy. Clinical presentation and prognosis range from asymptomatic disease with no or slow progression, to severe disabling, rapidly progressive cardiac failure. Initial presentation includes the triad of heart failure, (potentially lethal) arrhythmias and /or thrombo-embolism. In adults the majority of NCCM is isolated.

The first clinical presentation of NCCM may occur at all ages, even prenatally. In childhood, clinical features are often more severe and NCCM is frequently associated with congenital heart defects. The echocardiographic diagnostic criteria as proposed by Jenni et al. are convenient in daily practice and currently the most widely applied. The general cardiac guidelines for chronic heart failure and ICD's are suitable and applicable to the NCCM population.

In as much as 41% of isolated NCCM molecular testing may yield a genetic defect, mostly in sarcomere genes. The *MYH7* gene is the most prevalent disease gene. The non-isolated forms of NCCM are caused by a range of different (rare) genetic defects. Until now in half of familial isolated NCCM the genetic defect remains unknown. Genetic defects in a large number of sarcomere and other cardiomyopathy genes and in genes primarily associated with skeletal myopathies indicate that NCCM may result from a wide range of pathophysiologic mechanisms. Shared genetic defects and familial aggregation of NCCM, HCM and DCM indicates that NCCM may be part of a broad spectrum of cardiomyopathies.

The genetic aetiology of NCCM requires that patients and their relatives are offered genetic testing and counselling. This may include (predictive) molecular analysis of relatives, when applicable, and/or cardiac evaluation of at risk relatives, even when they are as yet asymptomatic.

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1.3 Hypertrophic cardiomyopathy

1.3.1 Introduction

Hypertrophic cardiomyopathy (HCM) is characterized by left ventricular hypertrophy in the absence of predisposing cardiac conditions (e.g. aortic stenosis) or cardiovascular conditions (e.g. long-standing hypertension). It is usually asymmetric and involves the intraventricular septum.[1] The right ventricle is occasionally also hypertrophic.

HCM manifestation ranges from asymptomatic disease to progressive heart failure and sudden cardiac death. Symptoms vary from individual to individual even within the same family. Common symptoms include shortness of breath (particularly with exertion), chest pain, palpitations, orthostasis, presyncope, and syncope. HCM frequently manifests during adolescence or young adulthood, although it may also develop in infancy, childhood or later in life.

History

In 1958 Teare was one of the first to describe asymmetrical hypertrophy in nine cases of sudden death at a young age (14-45 years). Two of these cases were siblings.[2] In 1964 Braunwald et al gave a detailed description of the disease based on a thorough analysis of 64 cases, both familial and non-familial.[3] Braunwald characterised idiopathic hypertrophic subaortic stenosis (IHSS) by marked hypertrophy of the left ventricle, involving in particular the intraventricular septum (IVS) and the left ventricular outflow tract. Obstruction of left ventricular outflow tract was observed and the hypertrophy was most often asymmetric, although occasionally concentric hypertrophy could be identified. The papillary muscles were often enlarged and the hypertrophy of the IVS often led to deformation of the mitral valve with a thickened anterior leaflet. His cohort showed an uneven distribution between the sexes, but in familial disease distribution was approximately equal. The women were significantly older than the men. Braunwald concluded that at least in some instances IHSS was congenital, although the disease could also be acquired. Not all patients were symptomatic; the most common symptoms were dyspnoea, chest pain, dizziness and syncope. Heart failure was present in 22% of the patients. Braunwald considered IHSS a slowly progressive disease, although sudden death may occur at any age. Analysis of the familial cases showed that IHSS transmitted as an autosomal dominant trait.[3]

Simultaneously Morrow issued a paper on operative treatment of the disease, describing ventriculo-myotomy in five patients and ventriculomyotomy combined with resection of a portion of the hypertrophied IVS in an additional five patients.[4] All 10 patients came from the Braunwald cohort. Success rate was 90%, although two patients needed a pacemaker. One patient died suddenly, probably due to arrhythmia, eight days post-surgery.[4]

The development of M-mode echocardiography in the 1970s facilitated imaging of the anterior septum and the posterior free wall, and lead to recognition of the asymmetry of the disease,

that was now often called asymmetric septal hypertrophy (ASH). In 1976 Maron was the first to describe a cohort of 46 children suffering from ASH.[5] 31% of the patients died suddenly with an annual mortality rate of 4%. Sudden death could not be predicted by evaluating symptomatology, electrocardiographic (ECG) abnormalities, heart size, left ventricular ejection or upstroke time, magnitude of outflow gradient, or left ventricular end-diastolic pressure. Maron concluded that the clinical and haemodynamic spectrum of ASH in childhood is broad.[5]
Cross sectional echocardiographic imaging, available since the early 1980s, identified hypertrophy in other ventricular segments than the anterior septum.[6, 7]
It was Spirito et al that suggested in 1989 that prognosis in HCM may be less grave than previously considered.[8] Spirito based this suggestion on a study of an outpatient cohort of 25 patients, comparing them with 3404 patients from 78 studies from referral institutions.

Terminology

Since 1958 different terminology to describe HCM has been used. After muscular hamartoma, idiopathic hypertrophic subaortic stenosis (IHSS), muscular subaortic stenosis, asymmetric septal hypertrophy (ASH) and hereditary ventricular hypertrophy, eventually hypertrophic cardiomyopathy (HCM) is the preferred name. In case of obstruction of the left ventricular outflow tract (LVOT) the term hypertrophic obstructive cardiomyopathy (HOCM) is applicable.

Diagnosis

Probands

Conventional two-dimensional echocardiography is reliable in diagnosing HCM by identifying left ventricular hypertrophy (LVH). LVH is often asymmetric but also a diffuse or segmental pattern of LV wall thickening associated with a non-dilated and hyperdynamic LV independent of presence or absence of LV outflow tract obstruction, can be demonstrated.[9] Absence of another cardiac or systemic disease (e.g. hypertension, congenital heart disease or exposure to drugs known to cause cardiac hypertrophy) is required. HCM was thought to be predominantly non-obstructive, only 25% of patients had a sizable resting outflow tract gradient.[10-13] With exercise though, this percentage increases to 70%.[14]
Left ventricular wall thickness (LVWT) smaller or equal to 12 mm is considered normal in adults; LVWT of 13-15 mm is generally classified as borderline LVH. LVWT greater than or equal to 15 mm is enough to establish the clinical diagnosis of HCM in adults.[9] In children diagnosis is made based on a LVWT two or more standard deviations above the normal population mean for body surface area.[15, 16]
Apart from establishing LVH, its degree and its location, echocardiography can also provide details of the atrial cavities, the mitral valve anatomy and function and can assess systolic and diastolic cardiac function. Systolic anterior motion (SAM) of the mitral valve can also be an echocardiographic finding.

Four types of hypertrophic cardiomyopathy can be distinguished:
Type 1: hypertrophy is confined to the anterior segment of the ventricular septum;
Type 2: hypertrophy of both the anterior and the posterior segments of the ventricular septum;
Type 3: involvement of both the ventricular septum and the free wall of the left ventricle;
Type 4: involvement of the posterior segment of the septum, the anterolateral free wall, or the apical part of the septum.[1]

Cardiac magnetic resonance imaging (cMRI) is increasingly used in HCM, especially in patients with poor echocardiographic imaging. Delayed gadolinium hyperenhancement can be used to detect and establish the degree of intramyocardial fibrosis.[17] Figure 9 displays an echocardiographic and a cMRI image of HCM.

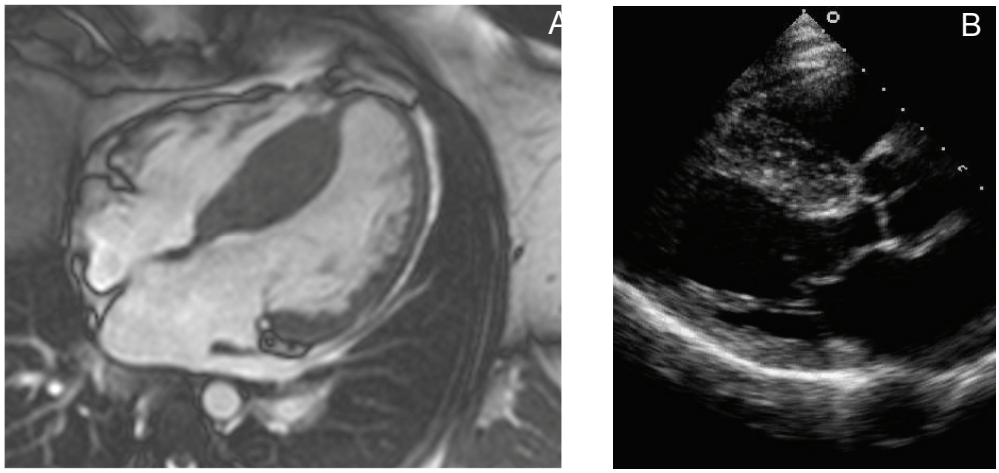

Figure 9. Cardiac MRI (A) and echocardiography (B).

The disease process is not confined to cardiac muscle alone. Primary malformations of the mitral valve apparatus, such as enlargement and elongation of the mitral leaflets and anomalous papillary muscle insertion directly into the anterior mitral leaflet, may lead to dynamic LVOT obstruction and/or muscular midcavity obstruction and are present in at least two thirds of patients.[18, 19]

Adult relatives

Pedigree analysis has revealed that in some families with hypertrophic cardiomyopathy adult relatives who carry the familial pathogenic mutation do not always fulfil conventional echocardiographic criteria.[20] Echocardiographic changes may be mild, sometimes only ECG abnormalities are identified and these relatives are often asymptomatic.[21] Adult carriers may even have a completely normal phenotype.
In 1997 McKenna et al proposed revised diagnostic criteria in members of families with hypertrophic cardiomyopathy, including major and minor criteria based on symptoms, and

electrocardiographic and echocardiographic abnormalities.[22] The criteria are listed in Table 8. Presence of one major criterion, or two minor echocardiographic criteria, or one minor echocardiographic plus two minor electrocardiographic criteria fulfils the diagnosis of HCM in adult relatives of HCM patients.

Table 8. Diagnostic criteria for HCM in adult relatives adapted from McKenna et al.[22]

Major criteria
Echocardiography Anterior septum or posterior wall ≥ 13 mm Posterior septum or free wall ≥ 15 mm Severe systolic anterior motion of the mitral valve (septal-leaflet contact) Electrocardiography Left ventricular hypertrophy and repolarisation changes T-wave inversion in leads I and aVL (≥ 3 mm), V3-V6 (≥ 3 mm), or II, III, aVF (≥ 5 mm) Abnormal Q (> 40 ms or >25% R wave) in at least two leads from II, III, aVF, V1-V4 or I, aVL, V5-6
Minor criteria
Echocardiography Anterior septum or posterior wall 12 mm Posterior septum or free wall 14 mm Moderate systolic anterior motion of the mitral valve (no septal-leaflet contact) Redundant mitral valve leaflets Electrocardiography Complete bundle branch block or (minor) interventricular conduction defect (in LV leads) Minor repolarisation changes in LV leads Deep S in V2 (> 25 mm)
Diagnosis is made when one major criterion or two minor echocardiographic criteria or one minor echocardiographic in combination with two minor electrocardiographic criteria are present.

Children

Charron et al evaluated ECG and echocardiography in the diagnosis of familial HCM in genotyped children.[23] They concluded that HCM was diagnosed in approximately 50% of genetically affected children using conventional ECG and/or echocardiographic criteria (LVWT >95% confidence interval on echocardiography; abnormal Q waves, LVH (voltage >95th percentile), or marked ST-T changes on the ECG).[23] They identified four relevant additional diagnostic criteria (QRS axis, left atrium dimension, intraventricular septum/posterior wall ratio, E/A wave ratio) so that nearly all children considered as healthy carriers of a mutation (based on conventional criteria) could be identified with excellent specificity.[23]

Pathology

Macroscopy

Macroscopically HCM is characterised by cardiomegaly and either asymmetric or symmetric left ventricular hypertrophy.[24] Figure 10 shows gross pathology of HCM. Many patients show LVH that is diffusely distributed, affecting any part of the left ventricle. In approximately 33% however, mild wall thickening is localised to a single segment, including the apical form.[6, 25-27] The apical form was first described in Japanese HCM patients, recently it is increasingly diagnosed in Western HCM patients as well.[28, 29]

In the classical form, the basal anterior septum bulges beneath the aortic valve, leading to narrowing of the LVOT.[2] Anterior displacement of the papillary muscles and the mitral leaflets is seen and beneath the aortic valve formation of a sub-aortic mitral impact lesion of endocardial fibrosis occurs.[30] Few HCM patients show symmetric concentric hypertrophy of the left ventricle combined with a small ventricular cavity.[6, 7, 25]

About 5-10% of HCM patients develop resemblance to dilated cardiomyopathy with LV wall thinning, enlargement of the LV cavity and systolic dysfunction, leading to irreversible heart failure.[31-33]

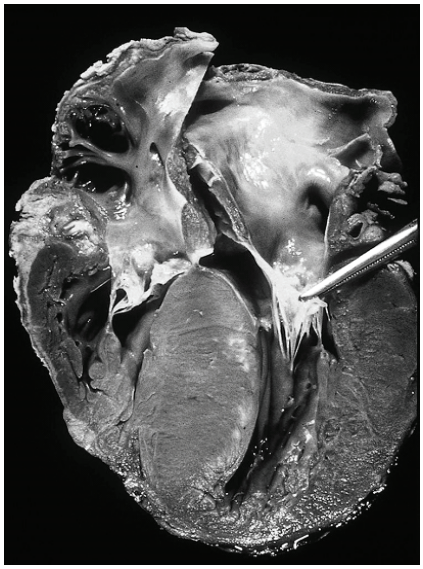

Figure 10. HCM gross pathology.
(Source: univadis.nl)

Microscopy

The triad of myocyte hypertrophy, disarray and interstitial fibrosis is pathognomonic for HCM.[24, 30] Myocyte disarray tends to be regional, with oblique and perpendicular myocyte arrangement around patches of interstitial collagen, the arrangement pattern can be either pinwheel-like or herringbone-like and has no prognostic significance.[30] Figure 11 shows myocyte disarray in HCM. There is hypertrophy, pleomorphism, and hyperchromasia of the myocyte nuclei. Disorganisation of the myofibrillary architecture within the myocyte itself leads to loss of the usual parallel alignment of myofibrils.[30]

Apart from the triad of hypertrophy, disarray and interstitial fibrosis, arterial dysplasia is also a pathological feature seen in HCM. Smooth muscle cell hyperplasia leads to thickening of the wall and narrowing of the lumen of the intramural coronary arteries (Figure 12).[30] Small vessel disease may ultimately lead to fibrosis and may facilitate development of dilated end-stage of HCM.[31]

1.3.2 Epidemiology

HCM is the most common genetic cardiac disorder with an estimated prevalence of 1 in 500 (adult) individuals.[34, 35] In routine cardiologic practice however, HCM is relatively uncommon, affecting approximately 1% of outpatients.[36]

HCM is the leading cause of sudden cardiac death in young athletes in the Unites States, accounting for 25 to 33% of these deaths.[37]

In a United States paediatric population HCM made up 42% of cardiomyopathy cases, corresponding with an annual incidence of 0.47 per 100,000.[38] In Australia 25.5% of paediatric cardiomyopathies can be attributed to HCM, with an annual incidence of 0.32 per 100,000.[39]

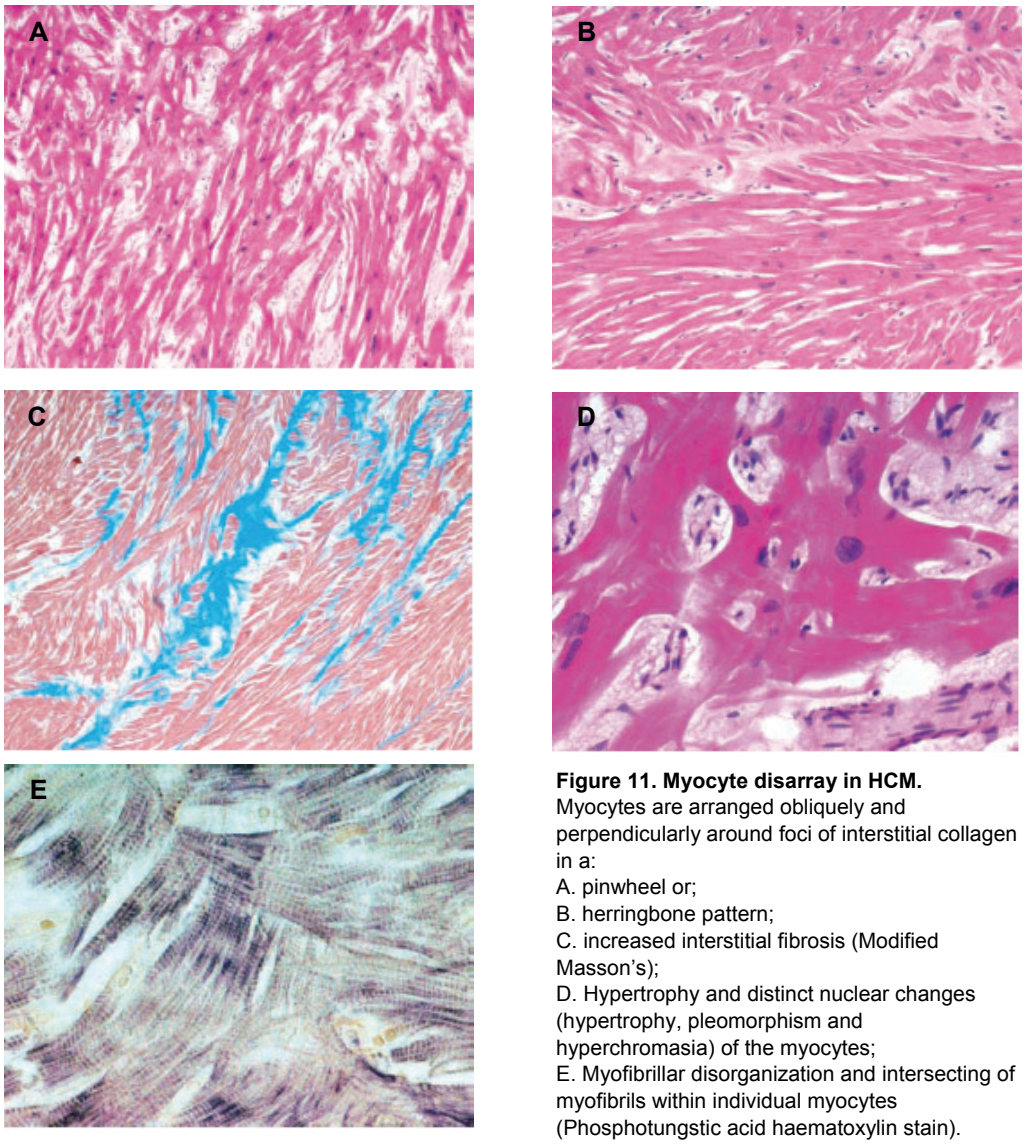

Figure 11. Myocyte disarray in HCM.
Myocytes are arranged obliquely and perpendicularly around foci of interstitial collagen in a:
A. pinwheel or;
B. herringbone pattern;
C. increased interstitial fibrosis (Modified Masson's);
D. Hypertrophy and distinct nuclear changes (hypertrophy, pleomorphism and hyperchromasia) of the myocytes;
E. Myofibrillar disorganization and intersecting of myofibrils within individual myocytes (Phosphotungstic acid haematoxylin stain).

Reproduced with permission from: "The pathology of hypertrophic cardiomyopathy; Hughes; *Histopathology* 2004; 44; 412-427."

1.3.3 Aetiology and molecular genetics

HCM is mainly a disease of the cardiac sarcomere and Z-disc; with mutations in 24 genes, mostly coding for sarcomere or Z-disc proteins, it is genetically heterogeneous. Currently, genetic defects are identified in 60 - 70% of HCM patients.[40] Most genetic defects are inherited as autosomal dominant traits.

Since sarcomere and Z-disc gene mutations are identified in HCM but also in other cardiomyopathies, such as dilated cardiomyopathy (DCM), noncompaction cardiomyopathy (NCCM) and restrictive cardiomyopathy (RCM) and the occurrence of different types of cardiomyopathy within the same family, caused by the same familial mutation, these cardiomyopathies might be part of a spectrum with a shared genetic susceptibility to these different phenotypes. Additional modifying factors, genetic variants or defects, or yet unidentified exogenous or systemic factors might explain the phenotypic variability of cardiomyopathies within families, including variability in age at onset and severity of clinical features.

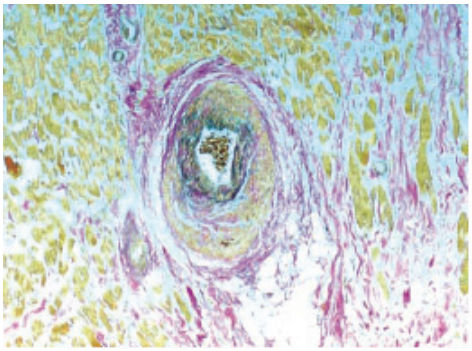

Figure 12. Intramural coronary artery dysplasia in HCM.

There is marked medial hypertrophy and narrowing of the lumen of the intramural coronary arteries with associated myocardial fibrosis (Elastic van Gieson).

Reproduced with permission from: "The pathology of hypertrophic cardiomyopathy; Hughes; Histopathology 2004; 44; 412-427."

Molecular defects in HCM

HCM has been associated with mutations in 24 different genes (Table 9). Defects in these genes (mostly sarcomere and Z-disc genes) are the most prevalent genetic cause occurring in approximately 60-70% of HCM patients.[40]

In a study of 84 paediatric HCM patients, 55% had a mutation in one of the genes *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1*, *MYL3*, *MYL2*, *ACTC, PRKAG2* and *LAMP2*.[41]

Over 500 different mutations in sarcomere genes encoding thick (*MYH7*), intermediate (*MYBPC3*) and thin filaments (*TNNT2*, *TNNI3*, *TPM1*, *ACTC*) have been described. Most mutations are single point missense mutations or small deletions or insertions. Mutations in the *MYH7* and *MYBPC3* genes are the most prevalent, accounting for up to 45 - 65% of HCM in adults and over 75% in children.[40, 41]

In a cohort of 80 HCM patients 5% had multiple gene mutations which may result in a more severe clinical phenotype because of a ''double dose'' effect.[42, 43] Double heterozygousity for truncating sarcomere mutations has been associated with severe congenital forms leading to cardiac death in early infancy.[44-46]

Table 9. Genes associates with hypertrophic cardiomyopathy (HCM).

	Gene	Locus	Protein	Estimated frequency (%)	Reference
Sarcomeric HCM					
Thin filament	*ACTC1*	15q14	α-Cardiac actin	rare	[99]
	TNNC1	3p21.3-p14.3	Cardiac troponin C	rare	[101, 102]
	TNNI3	19p13.4	Cardiac troponin I	< 5	[40, 99, 103, 104]
	TNNT2	1q32	Cardiac troponin T	3 - 5	[40, 99, 103]
	TPM1	15q22.1	α-Tropomyosin	< 5	[99, 103]
Intermediate filament	*MYBPC3*	11p11.2	Cardiac myosin-binding protein C	20 - 30	[40, 99, 103, 105, 106]
Thick filament	*MYH6*	14q11.2-q12	α-Myosin heavy chain	rare	[107]
	MYH7	14q11.2-q12	β-Myosin heavy chain	25 - 35	[40, 99, 103, 108, 109]
	MYL2	12q23-q24.3	Ventricular regulatory myosin light chain	< 5	[40]
	MYL3	3p21.2-21.3	Ventricular essential myosin light chain	rare	[40, 99]
	MYLK2	20q13.3	Myosin light chain kinase 2	rare	[110]
Giant filament	*ANKRD1*	10q23.31	Cardiac ankyrin repeat protein (CARP)	rare	[111]
	TTN	2q24.3	Titin	rare	[112]
Z-disc HCM					
	ACTN2	1q42-q43	α-Actinin 2	rare	[113, 114]
	CSRP3	11p15.1	Cysteine and glycine rich protein	rare	[99]
	FHL1	Xq26	Four-and-a-half LIM domains 1	rare	this thesis
	LDB3	10q22.1-q23.3	LIM domain-binding 3	1 - 5	[112, 114, 115]
	MYOZ2	4q26-q27	Myozenin 2	rare	[116, 117]
	TCAP	17q12-q21.1	Telethonin	rare	[112, 114, 118]
	VCL	10q22.1-q23	Vinculin/metavinculin	rare	[114, 119, 120]
Calcium-handling HCM					
	CALR3	19p13.12	Calreticulin	rare	[121]
	CASQ2	1p13.3-p11	Calsequestrin	rare	[121]
	JPH2	20q12	Junctophilin 2	rare	[122, 123]
	PLN	6q22.1	Phospholamban	rare	[121, 124]
Other	*CAV3*	3p25	Caveolin	rare	[125]

MicroRNA

Recently the role of micro-ribonucleic acids (microRNAs, miRNAs or MiRs) in cardiac development and (hypertrophic) heart disease is under investigation. MiRNAs are single-stranded RNA molecules with a length of 21 to 23 nucleotides. They regulate gene expression at the posttranscriptional level by targeting messenger RNA (mRNA). Almost all miRNAs and especially the miRNA critical region for binding to mRNA (the seed region, spanning 2 to 8 nucleotides from the 5' end of the RNA molecule) are highly conserved in closely related species.[47]

MiRNAs play an important role in cardiac development as well as in orchestrating organogenesis and early embryonic patterning processes.[48, 49] They also seem to play an important role in cardiac remodelling and development of hypertrophy.[50-53] Expression of MiR1 and MiR133 was decreased in patients with HCM.[51]

1.3.4 Pathogenesis

Mutations in different HCM genes affect different mechanisms in the cardiomyocyte, thus leading to changes that may individually cause HCM or may lead to a common cellular disturbance resulting in HCM. Mutations in sarcomere / Z-disc / Calcium-handling genes may have their effect through defective force generation (either by a dominant negative mechanisms where the mutant protein acts as a "poison polypeptide"; by domination of a gain of function of the mutated protein over normal function; or by haploinsufficiency).[54] The mutant protein may interfere with normal spatial myofibrillar arrangement; changes in this arrangement may lead to misshapen cardiomyocytes.[55] Any defect in genes encoding for contractile proteins supports this myofibrillary dysgenesis theory. A similar hypothesis is that the genetic defect, being the primary abnormality, alters myocardial function. This alteration triggers several growth responses leading to myocyte hypertrophy and proliferation of fibrocytes. Environmental and other genetic factors may further enhance this process.[54]

1.3.5 Clinical aspects

There is a great variability in presentation, even within families, ranging from a fully asymptomatic course to sudden cardiac death and severe heart failure necessitating cardiac transplantation. The age of presentation is also highly variable with prenatal and neonatal diagnosis to diagnosis at a high age.[31] HCM most commonly presents during adolescence and young adulthood.[56]

A lot of HCM patients are asymptomatic and may be detected due to a heart murmur, or by chance by pre-operative cardiac evaluation or medical assessment for insurance or jobs or because they participated in cardiologic family screening.[31] Symptomatic patients may present with dyspnea, fatigue, (atypical) chest pain, palpitations and / or (pre) syncope.

Exercise intolerance and dyspnoea, dizziness, presyncope and syncope usually occur despite a preserved systolic function and a non-dilated LV. Diastolic dysfunction with impaired filling due to abnormal relaxation and increased chamber stiffness appear to cause symptoms, and may

lead to elevated left atrial (LA) and LV end-diastolic pressures with reduced stroke volume and cardiac output, pulmonary congestion, and impaired exercise performance with reduced oxygen consumption at peak exercise.[9] Diastolic dysfunction does not appear to play a role in causing symptoms in patients with LVOTO. They appear to be disabled by elevated LV pressures and coexistent mitral regurgitation, as the often dramatic symptomatic improvement after therapeutic reduction of the outflow gradient proves.[9] Chest pain can present as typical or atypical angina pectoris and is probably due to myocardial ischaemia due to arterial dysplasia, as the identification of reversible myocardial perfusion defects, scarring on cMRI or at autopsy prove.

Although 75% of HCM patients do not demonstrate left ventricular outflow tract obstruction (LVOTO) under resting (basal) conditions, provocation of LVOTO by exercise or stress leaves only 30% of HCM patients without obstruction.[11-14] Classic signs indicating LVOTO consist of dynamic systolic ejection murmur, increasing during the Valsalva manoeuvre, and a bifid pulse.

"End-stage" disease with systolic dysfunction, dilation of the LV and heart failure occurs in approximately 10% of HCM patient that already have severe symptoms.[57] In a large study comprising 1259 patients, 3.5% characterised as end stage disease with systolic dysfunction (EF <50% at rest); developing at a wide age range (14 to 74 years), with 45% of patients ≤40 years old.[58]

Arrhythmias

Re-entrant tachycardias, bradyarrhythmias and supraventricular or ventricular tachyarrhythmias may occur in HCM patients, probably due to disruption of the cellular architecture which creates an unstable pro-arrhythmic electrical environment.[59, 60] Atrial fibrillation (AF) is the most common arrhythmia observed in HCM.[61] It occurs in 20-25% of HCM patients and is linked to left atrial dilatation and advanced age.[61, 62] AF in HCM is associated with progressive heart failure, stroke and disease progression.[62] Other supraventricular arrhythmias include tachycardias (SVT), AV block, sinus bradycardia and Wolf-Parkinson-White (WPW). Ventricular arrhythmias include premature ventricular depolarisations, ventricular couplets, non-sustained ventricular tachycardia (NSVT).[63] Ultimately arrhythmias may lead to sudden cardiac death.

1.3.6 Differential diagnosis

Acquired LVH

Several cardiovascular conditions can lead to left ventricular hypertrophy, for instance hypertension, aortic valve stenosis or chronic increase of circulatory volume. In children Kawasaki disease can also lead to LVH.

Physiologic hypertrophy can be caused by LV remodelling due to trained endurance (athlete's heart). Deconditioning of sport activities however should lead to regression of the increased LV mass.[64]

Other conditions leading to acquired LVH are obesity, pulmonary disease, infections, drug abuse or toxicity of medication, radiation, malignancy, connective tissue or granulomatous disease and immunologic or endocrine disease.

Neuromuscular diseases

Muscular dystrophies, myopathies (congenital and/or metabolic) and ataxias can be associated with several types of cardiomyopathy, like HCM, DCM and NCCM. Table 10 represents a list of the neuromuscular disorders that can be identified in HCM patients.

Metabolic diseases

The mechanisms through which metabolic disease can lead to HCM are infiltration (storage disease), defective energy metabolism and cardiotoxicity of intermediary metabolites. They are listed in Table 11.

Syndromes

Several syndromes can be accompanied by HCM. They are listed in Table 12 together with their dysmorphic and congenital traits.

Table 10. Neuromuscular disorders associated with HCM.

Neuromuscular disorders	Gene	Inheritance
Muscular dystrophies		
Becker muscular dystrophy	*DMD*	XR
Duchenne muscular dystrophy	*DMD*	XR
Emery-Dreifuss muscular dystrophy	*FHL1*	XD
Myotonic dystrophy	*DMPK*	XD
Myopathies		
α-B Cristallinopathy (myofibrillar myopathy with cataract)	*CRYAB*	AD
Distal myopathy	*MYH7*	AD
Minicore-multicore myopathy	*SEPN1*	AR
Myofibrillar myopathy	*DES*	AR / AD
Myopathy with postural muscle atrophy	*FHL1*	XR
Nemaline myopathy	*ACTA1*	AD
Ataxias		
Combined Charcot-Marie-Tooth peroneal muscular atrophy and Friedreich ataxia		XR
Friedreich ataxia	*FXN*	AR
Refsum disease	*PAHX / PEX7*	AR

Legend. XR, X-linked recessive; XD, X-linked dominant; AD, autosomal dominant; AR, autosomal recessive

Table 11. Metabolic disorders associated with HCM.

Metabolic disorder	Alternatively	Gene	Inheritance
Alpha-galactosidae A deficiency	Fabry	*GLA*	XD
Carnitine acylcarnitine translocase deficiency		*CACT*?	AR
Carnitine deficiency, primary		*SLC22A5*	AR
Carnitine palmitoyl transferase type II deficiency		*CPT2*	AR
Combined oxidative phosphorylation deficiency type III		*TSFM*	AR
Combined oxidative phosphorylation deficiency type V		*MRPS22*	AR
Complex I deficiency		*NDUFV1-2 / NDUFS1-4, 6-8 / NDUFA1, 11 / NDUFAF2-3 / HRPAP20 / C20ORF7*	AR
Complex I deficiency		*NDUFA1*	XD
Complex I deficiency		*MTND1-6 / MTTS2*	Mt
Complex II deficiency		*SDHA / SDHAF2*	AR
Complex III deficiency		*UQCRB / UQCRQ*	AR
Complex IV deficiency		*COX10 / COX15*	AR
Complex V deficiency		*MTATP8*	Mt
Congenital disorder of glycosilation type Ia	Jaeken	*PM22*	AR
Congenital generalised lipodystrophy type II	Berardinelli-Seip type II	*BSCL2*	AR
Cytochrome C oxidase deficiency		*SCO2*	AR
Glycogen storage disease	HCM with WPW	*PRKAG2*	AD
Glycogen storage disease type II	Pompe	*GAA*	AR
Glycogen storage disease type IIb	Danon	*LAMP2*	XD
Glycogen storage disease type III	Forbes	*AGL*	AR
Glycogen storage disease type IX			XR
GM1 gangliosidosis		*GLB*	AR
Hydroxyacyl-CoA dehydrogenase II deficiency		*HADH*	AR

Continued on next page

Kearns-Sayre syndrome		*MTTL1*	Mt
Long-chain acyl-CoA dehydrogenase deficiency		*ACADL*	AR
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency		*HADHA*	AR
Malonyl-CoA decorboxylase deficiency	Malonic acidemia	*MLYCD*	AR
MERRF syndrome (Myoclonic Epilepsy, Ragged Red Fibers)		*MTTF / MTTH / MTTK / MTTL1 / MTTS1-2*	Mt
Mitochondrial neonatal encephalocardiomyopathy	ATPase deficiency	*ATPAF2 / TMEM70*	AR
Mitochondrial phosphate carrier deficiency		*SLC25A3*	AR
Mitochondrial transfer RNA-Glycine		*MTTG*	Mt
Mitochondrial transfer RNA-Histidine		*MTTH*	Mt
Mitochondrial transfer RNA-Isoleucine		*MTTI*	Mt
Mitochondrial transfer RNA-Leucine	MELAS	*MTTL1*	Mt
Mitochondrial transfer RNA-Lysine		*MTTK*	Mt
Mitochondrial transfer RNA-Valine		*MTTV*	Mt
Mucolipidosis type II		*GNPTAB*	AR
Mucopolysaccharidosis type I	Hurler	*IDUA*	AR
Mucopolysaccharidosis type II	Hunter		XR
Mucopolysaccharidosis type III	Sanfilippo	*SGSH / NAGLU / HGSNAT / GNS*	AR
Multiple acyl-CoA dehydrogenase deficiency	Glutaric academia type II	*EFTA / EFTB / EFTDH*	AR
Muscle glycogen storage disease 0		*GYS*	AR
Phytanic acid oxidation deficiency	Refsum	*PAHX / PEX7*	AR
Tyrosinemia type I		*FAH*	AR
Very-long-chain acyl-CoA dehydrogenase deficiency		*ACADVL*	AR

Legend. XD, X-linked dominant; AR, autosomal recessive; Mt, mitochondrial; AD, autosomal dominant; XR, X-linked recessive

Table 12. Syndromes associated with HCM.

Syndrome	Gene	Inheritance	Features
Amyloidosis II, Indiana type		AD	Neuropathy; sensorimotor polyneuropathy; carpal tunnel syndrome; scleroderma-like atrophic changes of the distal limbs, late-onset cardiomyopathy; congestive heart failure; heart block; onset in fourth to fifth decades; death 14 to 20 years after onset
Amyloidosis III, cardiac type		AD	Cardiomyopathy; congestive heart failure; angina pectoris without significant coronary vessel disease; right bundle branch block, left axis deviation and prolonged P-R interval on ECG; left ventricular hypertrophy; arrhythmias; nephrotic syndrome; purpura; onset in fourth to fifth decades; death in three to six years after onset
Bardet Biedl syndrome		AR	Obesity; rod-cone dystrophy; retinitis pigmentosa; strabismus; cataracts; high arched palate; dental crowding; hypodontia; left ventricular hypertrophy; congenital heart defects; hypertension; hepatic fibrosis; Hirschsprung disease; hypogonadism; renal anomalies; nephrogenic diabetes insipidus; polydactyly; speech disorder / delay; learning disabilities; developmental delay; ataxia
Beckwith-Wiedemann syndrome	*CDKN1C* *NSD1* *H19* *KCNQ10T1*	AD	Generalized overgrowth; hemihypertrophy; metopic ridge; prominent occiput; linear ear lobe creases; posterior helical indentations; macroglossia; cardiomyopathy; omphalocele; diastasis recti; hepatomegaly; pancreatic hyperplasia; overgrowth of external genitalia; cryptorchidism; renal medullary dysplasia; neoplasia
Cardio-facio-cutaneous syndrome	*KRAS* *BRAF* *MEK1* *MEK2*	AD	Short stature; macrocephaly; dolichocephaly; prominent forehead; bitemporal narrowing; micrognathia; posteriorly rotated ears; hearing loss; ptosis; nystagmus; strabismus; down-slanting palpebral fissures; hypertelorism; exophthalmos; epicanthal folds; myopia ; optic nerve dysplasia; absence of eyebrows / eyelashes; short upturned nose; bulbous nasal tip; depressed nasal bridge; cleft palate; high-arched palate; dysplastic teeth; atrial septal defects; pulmonic stenosis; HCM; pectus excavatum / carinatum; splenomegaly; gastroesophageal reflux; hydronephrosis; joint hyperextensibility; clinodactyly; atopic dermatitis; ichthyosis; hyperkeratosis; hemangioma; keratosis pilaris; sparse, curly, slow-growing hair; absence of eyebrows / eyelashes; mental retardation; seizures; cortical / brain stem atrophy; frontal lobe hypoplasia; hypoplastic / absent corpus callosum
Costello syndrome	*HRAS*	AD	Short stature; macrocephaly; low-set ears; hypertelorism; epicanthal folds; downslanting palpebral fissures; strabismus; ptosis; depressed nasal bridge; anteverted nostrils; macroglossia; high-arched palate; short / webbed neck; HCM; pulmonic stenosis; mitral valve prolapse; septal defects; dysrhythmias; tracheo- / bronchomalacia; recurrent pneumothorax; barrel chest; pectus carinatum; hypertrophic pyloric stenosis; renal failure; deep palmar / plantar creases; clubfeet; cutis laxa; brittle nails; sparse / curly hair; mental retardation; cerebral atrophy; hoarse voice; neoplasia
Growth retardation, developmental delay, coarse facies and early death syndrome	*FTO*	XR	Anteverted nostrils; thin vermilion; prominent alveolar ridge; retrognathia; protruding tongue; ventricular septal defect; atrioventricular defect; patent ductus arteriosus; HCM; severe developmental delay; microcephaly; lissencephaly; seizures; Dandy-Walker malformation; short neck; brachydactyly; toenail hypoplasia; neurosensory deafness; umbilical hernia; hypertrophy of the labia; undescended testes; cleft palate; optic disc abnormalities

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Hypertrichotic osteochondrodysplasia		AD	Birthweight > 90th percentile; macrocephaly; prominent forehead; long philtrum; epicanthal folds; long, curly eyelashes; anteverted nares; flat, broad nasal bridge; thick lips; gingival hypertrophy; short neck; cardiomegaly; pericardial effusions; congenital left ventricular hypertrophy; bicuspid aortic valve; patent ductus arteriosus; narrow thorax / shoulders; widened ribs; umbilical hernia; osteoporosis; delayed bone age; platyspondyly; coxa valga; short, broad first toe; lymphedema; congenital, generalized hypertrichosis; mild mental retardation
LDHCP	*LMNA*	Sporadic ?	Lipoatrophy; diabetes, hepatic steatosis; HCM; leukomelanodermic papules; muscular hypertrophy; hypertriglyceridemia
Leopard syndrome	*PTPN11* *RAF1*	AD	Short stature; triangular face; low-set ears; sensorineural hearing loss; hypertelorism; ptosis; epicanthal folds; broad flat nose; cleft palate; short neck; pulmonic stenosis; HCM; subaortic stenosis; complete heart block; bundle branch block; winged scapulae; hypospadia; absent / hypoplastic ovary; unilateral renal agenesis; spina bifida occulta; dark lentigines (mostly neck and trunk); café-au-lait spots
Neurofibromatosis	*NF1*	AD	Macrocephaly; Lisch nodules; glaucoma; hypertelorism; renal artery stenosis; hypertension; HCM; scoliosis; spina bifida; pseudoarthrosis; local bony overgrowth; neurofibromas; cafe-au-lait spots; axillary / inguinal freckling; mental retardation / learning disabilities; aqueductal stenosis; hydrocephalus; neoplasia (optic glioma; meningioma; hypothalamic tumor; neurofibrosarcoma; rhabdomyosarcoma; duodenal carcinoid; somatostatinoma; parathyroid adenoma; pheochromocytoma; pilocytic astrocytoma; malignant peripheral nerve sheath tumors
Noonan syndrome	*PTPN11* *KRAS* *SOS1* *RAF1*	AD	Short stature; triangular face; low-set ears; hypertelorism; downslanting palpebral fissures; epicanthal folds; myopia; micrognathia; high arched palate; low posterior hairline; webbed neck; septal defects; pulmonic stenosis; patent ductus arteriosus; pectus carinatum superiorly / pectus excavatum inferiorly; cryptorchidism; clinodactyly; woolly hair; mental retardation (mild); bleeding tendency; malignant schwannoma
Pallister-Kilian syndrome		SM	Obesity; prominent forehead; full cheeks; long philtrum; micrognathia; deafness; large ears; protruding lobules; external auditory canal stenosis; sparse eyebrows / eyelashes; upslanting palpebral fissures; hypertelorism; ptosis; strabismus; epicanthal folds; cataracts; exophthalmos; flat, broad nasal root; short nose; anteverted nostrils; thin upper lip; protruding lower lip; macroglossia; cleft palate; bifid uvula; delayed dental eruption; short / webbed neck; pericardial agenesis; aortic stenosis; septal defects; HCM; patent ductus arteriosus; aortic coarctation; lung hypoplasia; accessory nipples; diaphragmatic / umbilical / inguinal hernia; omphalocele; intestinal malrotation; anal atresia / stenosis; small scrotum; cryptorchidism; hypospadias / labia majora hypoplasia; absent upper vagina / uterus; cystic / dysplastic kidneys; hypermobile joints; contractures develop with age; clinodactyly; distal digital hypoplasia; postaxial polydactyly; profound mental retardation; seizures
Sensorineural deafness with HCM	*MYO6*	AD	Progressive postlingual sensorineural dafness; HCM

Continued on next page

Table 12. Continued.

Syndrome	Gene	Inheritance	Features
Simpson-Golabi-Behmel-Rosen syndrome	*GPC3*	XR	Tall stature; macrocephaly; preauricular pits / tags; hearing loss; downslanting palpebral fissures; hypertelorism; epicanthal folds; broad flat nasal bridge; short upturned nose; macrostomia; macroglossia; cleft lip / palate; dental malocclusion; cardiac conduction defects; ventricular septal defect; pulmonic stenosis; cardiomyopathy; transposition of great vessels; patent ductus arteriosus; lung segmentation defects; cervical ribs; pectus excavatum; 13 pairs of thoracic ribs; supernumerary nipples; diaphragmatic hernia; umbilical / inguinal hernias; diastasis recti; polysplenia; intestinal malrotation; Meckel diverticulum; hypospadia; cryptorchidism; large cystic kidneys; renal pelvis duplication; vertebral segmentation defects; C2-C3 posterior elements fusion; six lumbar vertebrae; sacral / coccygeal defects; scoliosis; postaxial polydactyly; syndactyly 2nd-3rd fingers / toes; clubfoot; coccygeal skin tags; fingernail hypoplasia; normal to retarded development; corpus callosum agenesis; cerebellar vermis hypoplasia; hydrocephalus; embryonal tumors; Wilms tumor
Syndromic microphtalmia / MIDAS	*HCCS*	XD	Short stature; microcephaly; hearing loss; microphtalmia; sclerocornea; cataract; iris coloboma; retinopathy; septal defects; cardiac conduction defects; HCM; overriding aorta; anteriorly placed anus; hypospadia; linear skin defects; corpus callosum agenesis; hydrocephalus; mental retardation; seizures
Sengers syndrome		AR	Congenital cataract; nystagmus; strabismus; HCM; mitochondrial myopathy; exercise lactic academia; muscular hypotonia; delayed motor development; easy fatigability
Wolf Hirschhorn	Partial 4p monosomy	Sporadic	Growth retardation; microcephaly; cranial asymmetry; posterior midline scalp defects; short philtrum; micrognathia; high forehead; preauricular tags / pits; hearing loss; strabismus; hypertelorism; epicanthal folds; exophthalmus; ptosis; Rieger anomaly; iris coloboma; wide nasal bridge; beaked nose; cleft lip / palate; short upper lip; hypodontia; webbed neck; septal defects; fused ribs; gallbladder absence; accessory spleen; intestinal malrotation; gastroesophageal reflux; hypospadias; cryptorchidism; absent uterus; sacral dimple / sinus; scoliosis; kyphosis; fused / bifid vertebra; hip dislocation; radioulnar synostosis; transverse palmar creases; polydactyly; low posterior hairline; high-arched eyebrows; sparse medial eyebrows; severe mental retardation; hypotonia; seizures; interventricular cerebral cysts; hydrocephalus; precocious puberty

Legend. AD, autosomal dominant; AR, autosomal recessive; XR, X-linked recessive; SM, somatic mosaicism; MIDAS, Microphtalmia Dermal Aplasia, Sclerocornea; XD, X-linked dominant

1.3.7 Therapy and prognosis

Therapy

Treatment of HCM patients depends on the presence of symptoms, LVOTO and risk for SCD. Figure 13 shows a treatment algorithm for HCM.

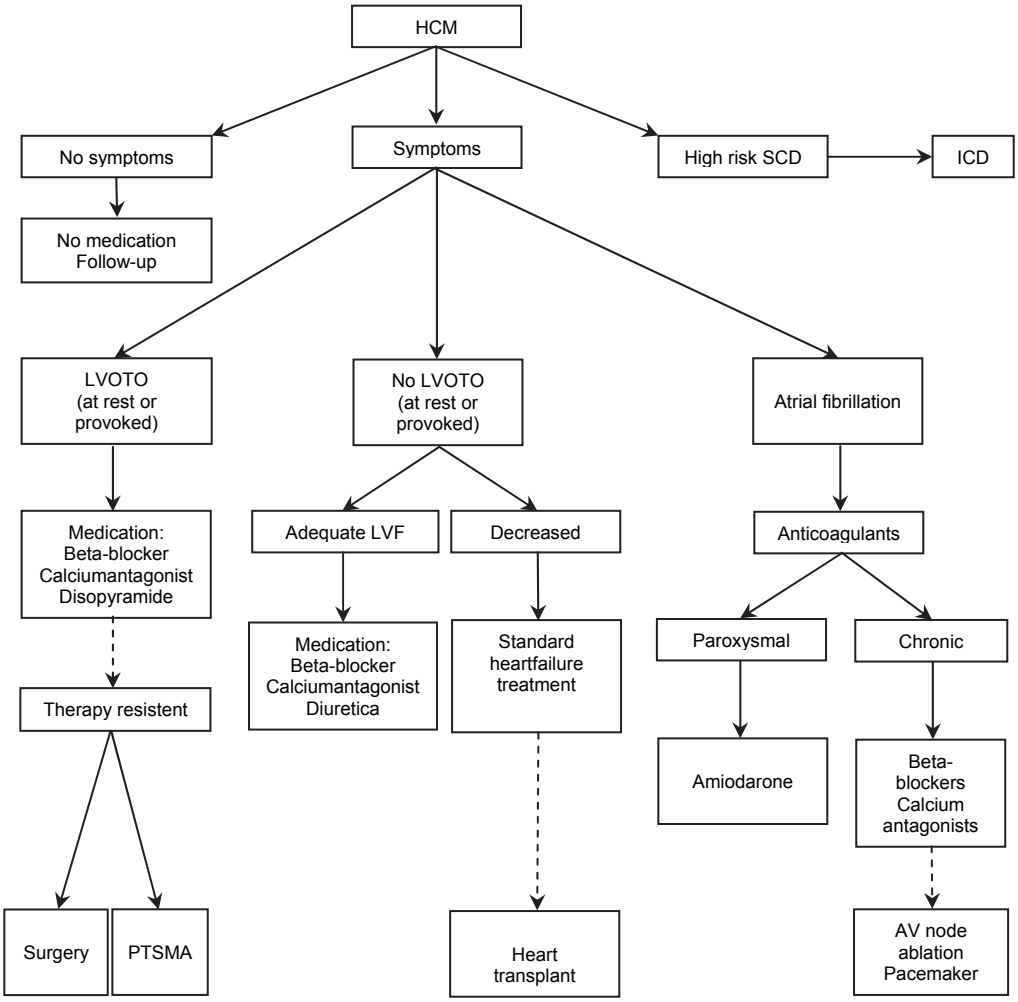

Figure 13. Treatment algorithm or HCM. Treatment depends on symptoms and severity of disease.
Legend.
HCM = hypertrophic cardiomyopathy;
LVOTO = left ventricular outflow tract obstruction;
SCD = sudden cardiac death;
ICD = implantable cardioverter defibrillator;
PTSMA = percutaneous transluminal septal myocardial ablation.

Most asymptomatic patients do not require treatment, although dilemmas may occur when the patient is very young and / or has substantial hypertrophy or other risk factors for SCD. So far no evidence is given for prophylactic therapy in asymptomatic HCM patients or in genotype positive - phenotype negative carriers.
In symptomatic patients the initial therapeutic approach is pharmacological, to relieve dyspnoea and to improve exercise tolerance. It is the only available treatment option for patients without LVOTO and consists of β-blocking agents and calcium antagonists.
A small group of patients with non-obstructive HCM develops systolic dysfunction and heart failure, displaying wall thinning and LV dilatation, due to LV remodelling. Their pharmacological therapy differs from patients without heart failure and comprises conversion to afterload reducing agents such as ACE inhibitors or angiotensin II-receptor blockers or diuretics, digitalis, beta-blockers or spironolactone. Ultimately, patients with end-stage heart failure may become candidates for a heart transplant.
In presence of LVOTO and failure of pharmacological therapy to relieve symptoms the options of myectomy or percutaneous transluminal septal myocardial ablation (PTSMA) remain. Approximately 5% of all HCM patients in non-referral centres qualify for surgery, whereas in tertiary referral centres 30% do.[9] LVOT gradients are equal to or higher than 50 mm Hg, decreasing to less than 10 mm Hg after surgery. During surgery mitral valve abnormalities can also be corrected. Operative mortality is approximately 1-2% and there is over 40 years of follow-up of this technique.[9]
PTSMA is more recent, first reported in 1995 by Sigwart et al, who injected a small amount of absolute alcohol into the first septal branch of the left anterior descending artery to induce a small localised myocardial infarction and so producing a chemical myectomy without open heart surgery.[65] It decreases the LVOT gradient to less than 25 mm Hg and improves exercise capacity with a comparable mortality rate to surgery, 1-2%.[9] The need for postoperative pacemaker therapy is higher than in conventional myectomy, 5-10% opposed to 1-2%.[9]
Atrial fibrillation (AF) can be treated pharmacologically with amiodarone, β-blockers and calcium antagonists and with cardioversion. AV-node ablation is an option when pharmacological therapy fails, as is placement of a pacemaker. As AF can cause thrombo-embolic events, anti-coagulant therapy should be given if there are no contra-indications.

Pregnancy

Pregnancy is usually well tolerated by HCM patients. Maternal mortality is low, occurring mainly in women with high risk clinical profiles.[66] These women should be well monitored throughout their pregnancy and specialised obstetrical care should be provided. Most pregnant HCM patients however can have an uneventful pregnancy and normal vaginal delivery.

Prognosis

Life threatening events in HCM consist of sudden cardiac death, embolic stroke and heart failure. First reports on prognosis mainly concerned HCM populations from tertiary referral

centres with a more severe phenotype and were consequently more pessimistic than nowadays, when more reports of large unselected HCM patients are being described. Currently the overall annual death rate in HCM is estimated to be approximately 1% a year; risk rate for obstructive HCM is approximately 2% per year, with a four-fold greater risk of stroke than in HCM patients without obstruction.[9, 31, 67]

HCM patients with high LVOT gradients are also at higher risk for severe heart failure.[68]

A significant proportion (up to 25%) of HCM patients achieve normal life expectancy (≥ 75 years).[69-72] However, presentation in infancy and young childhood tends to give an unfavourable prognosis.[9]

Patients presenting with a normal ECG at the time of diagnosis often display a less severe phenotype and tend to have better outcomes.[73] Apical HCM appears to have a better prognosis than other HCM types, although it can be complicated by apical ischemia or infarction with aneurysm formation.[27]

1.3.8 Risk stratification and indication for ICD

(Aborted) SCD may be the first symptom of HCM in a previously asymptomatic "healthy" individual. Often there are no signs of warning. SCD may occur at all times, during sleep, early after awakening, in rest or during (or shortly after) physical activity. SCD is more frequent in adolescents and young adults (≤ 35 years) but may also occur above that age. HCM is the most common cause of SCD in young people.[74] Several risk factors and possible risk factors have been identified in HCM; they are listed in Table 13. Having two or more of these risk factors qualifies as a high risk profile; ICD implantation is advisable in these patients. However, approximately 3% of SCD in HCM occurs in patients without any of these risk factors.[75]

Table 13. Risk factors for sudden cardiac death in hypertrophic cardiomyopathy adapted from Maron *et al.* and Sen-Chowdry *et al*.[9, 127]

Major Risk Factors	Possible Risk Factors Gene
Previous cardiac arrest / VF	Co-existent obstructive CAD
Spontaneous sustained VT	LVOTO in rest
Family history (≥1) of SCD	Microvascular ischemia
Non-sustained VT on Holter	Previous PTSMA
Abnormal blood pressure response in exercise	"Burnt out" disease
Unexplained syncope	Diffuse late gadolinium enhancement on cMRI
Maximal LV wall thickness ≥ 30 mm	High risk mutation
	AF
	Intense physical exertion

Legend. AF = atrial fibrillation; CAD = coronary artery disease; cMRI = cardiac magnetic resonance imaging; LV = left ventricle; LVOTO = left ventricular outflow tract obstruction; PTSMA = percutaneous transluminal septal myocardial ablation; SCD = sudden cardiac death; VF = ventricular fibrillation; VT = ventricular tachycardia.

Risk stratification in HCM patients should take place annually or when there is a change in clinical status and should consist of a thorough personal and family history, two-dimensional echocardiography (assessment of LVH and LVOTO), 24-hour ECG monitoring (Holter), and exercise ECG for blood pressure response.[9]

ICD implantation is important in primary (based solely on non-invasive risk factors) and secondary (following cardiac arrest or spontaneous and sustained ventricular tachycardia) SCD prevention. In a multi-centre retrospective study, ICDs appropriately detected and automatically aborted potentially lethal ventricular tachyarrhythmias by restoring sinus rhythm in almost 25% of a high-risk cohort, during a three-year period.[76] In primary prevention appropriate device interventions occurred at a rate of 5% per year and in secondary prevention at 11% per year.[76] ICD functions consist of detecting arrhythmia and delivering appropriate electrical therapy accordingly, but they also provide diagnostic information and antibradycardia pacing. A dual-chamber ICD is therefore preferred in patients with sinus node dysfunction, SVT precipitating VT/VF or AF.[77]

Children

Several studies to identify risk factors for SCD in paediatric HCM patients have been performed. Ostman-Smith et al described SCD in 12.5% of 128 patients and determined that ECG voltages (sum of the R and S waves > 10 millivolts) and septal thickness > 190% of the 95th centile for age were independent predictors of sudden death. Noonan's syndrome and a ratio of the LVW to its cavity in diastole > 0.30 were independent predictors of death due to cardiac failure.[78]

In a population of 91 paediatric HCM patients Decker et al retrospectively assessed whether reported adult risk factors for sudden death are predictive in children with HCM. During a 20-year follow-up four patients had a heart transplant and seven patients died, of which three died suddenly. Extreme LVH and an abnormal blood pressure response to exercise were predictive of non-SCD. Survival analysis predicted an 82% survival over a 20-year period.[79] Since non-SCD was more common than SCD in children, they concluded that the identification of risk factors for non-SCD is at least as important in children as identifying risk factors for SCD. ICD implantation in children to help prevent SCD is safe and has a good medium-term outcome; the occurrence of inappropriate shocks is the most serious problem.[80]

1.3.9 Cardiogenetic aspects

Molecular and cardiologic family screening

Isolated HCM is familial in the majority of cases. Family history is therefore very important. Not only should there be focus on cardiomyopathies and cardiac symptoms such as heart failure, sudden unexplained death, conduction disorders or arrhythmia and thrombo-embolic disease

e.g. stroke, but also on skeletal muscular symptoms, like myopathy, muscle weakness or wheel chair dependence.

Inheritance mode is autosomal dominant in the majority of cases (exceptions are listed in Tables 10, 11 and 12), informing and examining relatives is advisory. Recently Dutch guidelines for genetic diagnostics and genetic counselling in HCM were published.[81] Age of onset and severity of symptoms are highly variable, ranging from prenatal presentation with heart failure to asymptomatic disease at a high age.[31, 82, 83] Since the age of onset is so variable, a single cardiologic examination does not suffice to exclude HCM in a relative. Regular follow up is recommended (Table 14). The proposed strategies for family screening in HCM are depicted in the flow chart in Figure 14. In families where a pathogenic mutation has been identified, relatives can be offered predictive DNA analysis. In families without a pathogenic mutation or in relatives who refuse DNA analysis cardiac family screening remains the method of choice to identify relatives at risk of developing HCM.

Table 14. Screening guidelines for healthy family members adapted from Maron *et al*.[56]

Age	Screening guidelines (physical examination echocardiography and ECG)
< 12 years	Optional
	Highly recommended when:
	Positive family history of paediatric onset of HCM / early HCM-related death or other complications
	Child participates in competitive sports with an intense training program
	Symptoms are present
	Other clinical findings that suggest early LVH are present
12-18 years	Regular evaluation with intervals of 12 - 18 months
> 18 years	Regular evaluation with intervals of 3 - 5 years
	Adjust intervals according to clinical findings and presence of symptoms
Legend. ECG, electrocardiography; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy	

When identifying HCM in a relative additional diagnostics (exercise-ECG and 24-hour Holter monitoring) should be performed to determine the risks of complications.

Similar to all genetic disorders, cardiac or other, genetic counselling is vital to explain patients that their disease is genetic and to explain the importance of DNA testing to reliably identify relatives at risk. Equally, relatives should be well informed about the disease, its risks and possible complications, its heredity and the possibilities for testing, either through DNA analysis when a familial mutation is known or through cardiac screening. Medical, psychological and socio-economical consequences of predictive testing should be discussed elaborately in order for relatives to make a well-informed decision. Apart from HCM other cardiomyopathies may co-occur within families, like noncompaction and dilated cardiomyopathy, so cardiac screening should aim at identifying all cardiomyopathies.

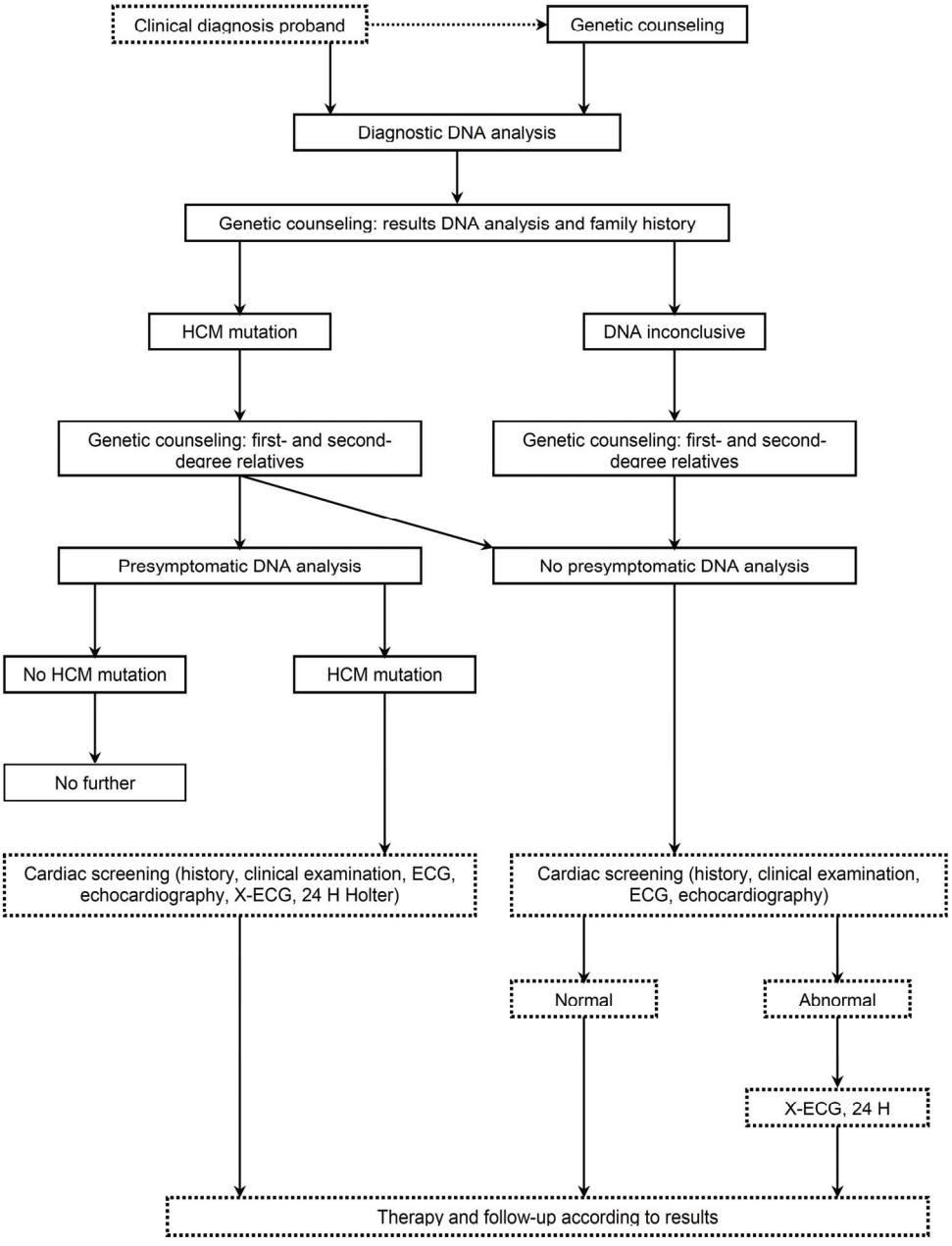

Figure 14. Flow chart for family screening in HCM.[128]

Legend. Multidisciplinary approach by cardiologist (dashed boxes), clinical genetic nurses and medical doctors (un-dashed boxes); X-ECG = exercise electrocardiography; 24 H Holter = 24 hour Holter monitoring.

Genotype-phenotype correlations

After detecting pathogenic mutations that cause HCM, scientists have tried to establish genotype-phenotype correlations. Early observations lead to the belief that specific mutations in the same gene are malignant, while others are benign.[75, 84-90] HCM due to *MYBPC3* was thought to be slowly progressive, with a higher age of onset and milder symptoms, whereas *TNNT2* related HCM was associated with a higher incidence of sudden death, revealing a higher extent of myocyte disarray, although hypertrophy was less severe.[85, 86, 91-93] The major shortcoming of all of these observations was the fact that they were coming form small cohorts of patients originating from a few large families. Genotype-phenotype studies in large cohorts of unrelated patients have indicated that caution is required when assigning particular prognostic significance to any particular mutation.[94-96] Mutations previously thought to be benign were shown to lead to a severe clinical phenotype and “malignant” mutations did not necessarily lead to a severe clinical course. It was also demonstrated that HCM due to *MYBPC3* and *MYH7* may display a similar phenotype, although age of onset may vary.[97, 98] In a large cohort of unrelated Italian HCM patients risk of cardiovascular death, non-fatal stroke, and progression of disease was compared between patients with a genetic defect in one of the genes coding for myofilament proteins and patients without a known causative mutation. In patients with a mutation risk was significantly increased.[99] Several studies have indicated that compound or double heterozygousity, identified in up to 5% of familial HCM, leads to a more severe phenotype with earlier onset and an increase in sudden death, which suggests that a gene-dosage effect might contribute to disease severity.[42, 43, 45, 100]

Molecular strategies

Extensive genetic screening may lead to the identification of a molecular defect in approximately 60-70% of HCM patients, with an *MYH7* or an *MYBPC3* mutation in the majority of cases.[40, 98, 101]

MYBPC3 and *MYH7* gene sequencing should be considered as initial approach, being the most prevalent cause for HCM in adults and children.[41] Further molecular analyses of the other genes within the HCM spectrum (Table 9), which quantitatively have a relatively modest contribution to HCM morbidity, may be considered when no mutation in the *MYBPC3* or *MYH7* gene can be identified.[81] When an adult or paediatric patient is severely affected, screening for a second molecular defect is advised, given the possibility of compound or double heterozygousity in HCM.

Summary

HCM is the most frequent cardiogenetic disorder and is genetically heterogeneous. Clinical presentation ranges from asymptomatic disease, with no or slow progression, to severe, disabling, rapidly progressive cardiac failure. HCM displays a high intra- and interfamilial

variability. It can present with (symptoms of) heart failure or arrhythmias and even SCD. A substantial proportion of HCM patients is asymptomatic. HCM occurs at all ages, from prenatal presentation to onset at a high age.

In up to 70% of HCM molecular testing may yield a genetic defect, mostly in the *MYBPC3* and *MYH7* genes. The genetic aetiology of HCM requires genetic counselling and testing of patients and their relatives, who are offered (predictive) molecular analysis and/or cardiac evaluation.

HCM may also be caused by mitochondrial defects and is associated with several syndromes and neuromuscular disorders.

HCM can have important clinical consequences and may lead to premature death in some patients while a normal life expectancy is reached by many other patients, having mild or no disability. Not all HCM patients require treatment, their prognosis is favourable. When treatment is needed, pharmacological therapy is the first option, followed by myectomy or PTSMA when medication is insufficient to relieve symptoms. Adequate risk stratification is important to determine who will benefit from ICD implantation in order to prevent sudden cardiac death.

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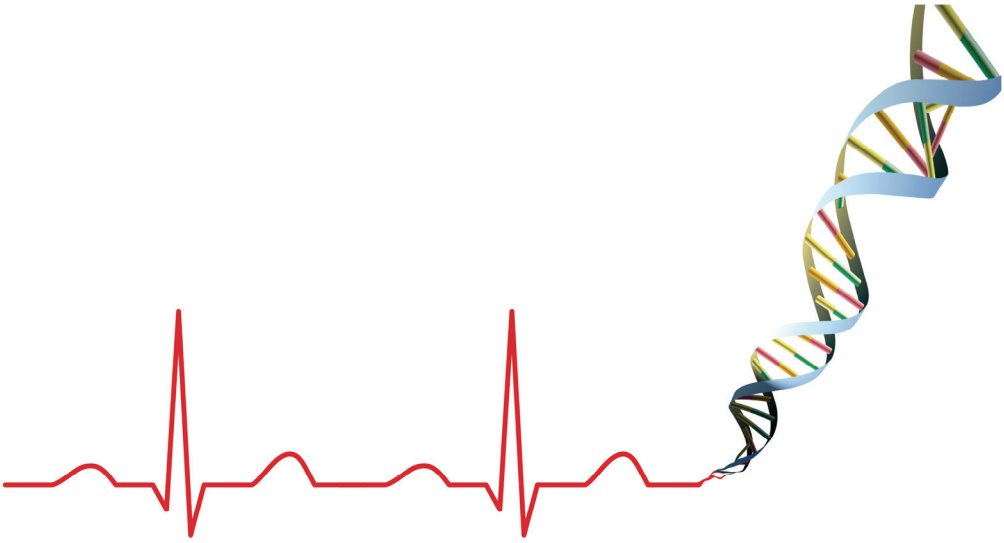
Chapter 2

Cardiac-myosin heavy chain defects in two families with non-compaction cardiomyopathy: linking non-compaction to hypertrophic, restrictive, and dilated cardiomyopathies.

Yvonne M. Hoedemaekers, Kadir Caliskan, Danielle F. Majoor-Krakauer, Ingrid van de Laar, Michelle Michels, Maarten Witsenburg, Folkert J. ten Cate, Maarten L. Simoons, Dennis Dooijes.

Eur Heart J. Nov 2007;28(22):2732-2737



European Heart Journal Advance Access published October 17, 2007

European Heart Journal
doi:10.1093/eurheartj/ehm429

Clinical research

Cardiac β-myosin heavy chain defects in two families with non-compaction cardiomyopathy: linking non-compaction to hypertrophic, restrictive, and dilated cardiomyopathies

Yvonne M. Hoedemaekers[1], Kadir Caliskan[2], Danielle Majoor-Krakauer[1], Ingrid van de Laar[1], Michelle Michels[2], Maarten Witsenburg[3], Folkert J. ten Cate[2], Maarten L. Simoons[2], and Dennis Dooijes[1]*

[1]Department of Clinical Genetics, Erasmus Medical Centre, Dr Molewaterplein 50, 3015GE Rotterdam, The Netherlands; [2]Department of Cardiology, Erasmus Medical Centre, Rotterdam, The Netherlands; and [3]Department of Paediatric Cardiology, Erasmus Medical Centre, Rotterdam, The Netherlands

Received 24 April 2007; revised 27 August 2007; accepted 30 August 2007

KEYWORDS
Non-compaction cardiomyopathy;
β-Myosin heavy chain gene;
Left ventricular non-compaction cardiomyopathy

Cardiomyopathies are classified according to distinct morphological characteristics. They occur relatively frequent and are an important cause of mortality and morbidity. Isolated ventricular non-compaction or non-compaction cardiomyopathy (NCCM) is characterized by an excessively thickened endocardial layer with deep intertrabecular recesses, reminiscent of the myocardium during early embryogenesis.

Aims Autosomal-dominant as well as X-linked inheritance for NCCM has been described and several loci have been associated with the disease. Nevertheless, a major genetic cause for familial NCCM remains to be identified.

Methods and Results We describe, in two separate autosomal-dominant NCCM families, the identification of mutations in the sarcomeric cardiac β-myosin heavy chain gene (*MYH7*), known to be associated with hypertrophic cardiomyopathy (HCM), restricted cardiomyopathy (RCM), and dilated cardiomyopathy (DCM).

Conclusion These results confirm the genetic heterogeneity of NCCM and suggest that the molecular classification of cardiomyopathies includes an *MYH7*-associated spectrum of NCCM with HCM, RCM, and DCM.

Introduction

Non-compaction cardiomyopathy (NCCM), also called isolated ventricular non-compaction (IVNC) or left ventricular non-compaction (LVNC), has recently been recognized as a novel cardiomyopathy.[1–3] It is characterized by an excessively prominent trabecular meshwork and deep intertrabecular recesses, as seen early in human embryogenesis.[4,5] The diagnosis is established by imaging the ventricular walls and cavities, by two-dimensional transthoracic echocardiography with colour Doppler flow, contrast echocardiography, left ventricular (LV) angiography, computed tomography, or magnetic resonance imaging.[4,6–10] The diagnostic criteria, as proposed by Jenni *et al.*,[4] are clinically most convenient and include abnormally thickened ventricular walls with a two-layered structure, consisting of thickened, non-compacted (NC) endocardial myocardium and a thin compacted (C) epicardial myocardium (maximal end-systolic ratio NC/C $\geq$ 2 at parasternal short-axis view) with documentation of perfusion of the deep intertrabecular recesses with colour Doppler flow.

Clinical manifestations include the triad of heart failure, (potentially lethal) arrhythmias, and/or thrombo-embolism, mostly affecting patients at a relatively young age. NCCM is genetically heterogeneous and can be inherited as an autosomal-dominant or X-linked disorder. Thus far no common genetic determinants for NCCM have been identified.[11] A small proportion of familial autosomal-dominant NCCM can be explained by mutations in genes encoding cytoskeletal or cell junction proteins, *LMNA/C*, α-dystrobrevin, and Cypher/*ZASP*.[12–15] In some families with X-linked NCCM, an association was found with mutations in the TAZ gene, which is allelic with Barth syndrome.[16] Mutations in the alpha-cardiac actin (*ACTC*) gene are a rare cause for hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). Recently, a mutation in this gene was

* Corresponding author. Tel: +31 10 4087197; fax: +31 10 4089489.
E-mail address: d.dooijes@erasmusmc.nl

identified in five families with HCM, LVNC, and atrial septal defects (ASDs), originating from the same region in Spain.[17] Additional NCCM loci have been identified on chromosomes 1q43, 5q, and 11p15.[18–20]

At the Cardiogenetics Centre of the Erasmus MC, molecular screening of sarcomeric genes in NCCM patients resulted in the identification of mutations in the sarcomeric cardiac β-myosin heavy chain gene (*MYH7*) in two separate families with autosomal dominantly inherited NCCM.

Methods

Diagnosis

In this study, the internationally acknowledged echocardiographic diagnostic criteria for NCCM were used, consisting of (i) segmental, excessive thickening of the LV wall with a two-layered structure: a thin, compacted epicardial layer and a much thicker, non-compacted endocardial layer with the characteristic appearance of numerous prominent trabeculations (meshwork) and deep intertrabecular recesses, (ii) colour Doppler evidence of deeply perfused intertrabecular recesses, (iii) predominant localization of thickening in the LV apical, mid-lateral, and mid-inferior walls, and (iv) the absence of co-existing cardiac anomalies.[4] A clinical diagnosis of NCCM required compliance to all the four criteria.

Molecular analysis

Genomic DNA from index patients in autosomal-dominant NCCM families A and B was extracted from peripheral blood cells using standard techniques. Using a candidate gene approach, mutation analysis of the *MYH7, TAZ, LMNA/C, MYBPC3, TNNC1, TNNT2, TNNI3, MYL2, MYL3, CSRP3, TCAP, ACTC*, and *TPM1* genes was performed using direct sequence analysis of all coding regions and intron–exon boundaries. Sequence analysis of M13-tagged PCR products was carried out on an ABI3730xl capillary sequencer using Big Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of the method and primer sequences are available on request.) Analysis of sequence data was performed using SeqScape analysis software (V2.5, Applied Biosystems).

Family A

In 2003, a 27-year-old woman presented with symptoms of severe congestive heart failure with progressive dyspnoea, fatigue, and oedema. Echocardiographically she had a moderately dilated LV with severe systolic dysfunction. The apical, lateral, and inferior walls were excessively thickened with prominent hypertrabeculation ('meshwork') with NC/C ratio >2. Her ECG showed sinus rhythm with left bundle branch pattern (QRS width 142 ms). Treatment with diuretics, ACE-inhibitors, β-blockers, and anticoagulants resulted in good clinical improvement. After 4 years of follow-up, she remains asymptomatic with NYHA functional class I. Her LV function also significantly improved with current LV end-diastolic and -systolic dimensions of 60/41 mm and fractional shortening of 32%. The familial history revealed a probably affected 27-year-old sister, who died 6 days after giving birth to her third child; LV function was severely impaired, with substantially dilated LA and LV. She developed clinical features of severe congestive heart failure with ventricular tachycardia and ventricular fibrillation, which could not be resuscitated. Her 73-year-old father was also known with congestive heart failure, but also with coronary heart disease and diabetes. Echocardiography showed typical morphological features of NCCM, with excessively thickened myocardium of the apical, lateral, and anterior LV walls.

Cardiological screening of seven asymptomatic siblings showed typical echocardiographic NCCM features in two sisters, aged 35 and 30, respectively, and two brothers, aged 34 and 49, respectively (*Figure 1*). The asymptomatic daughter of the eldest brother was also diagnosed with NCCM at age 24. Three brothers were free of cardiological features of NCCM.

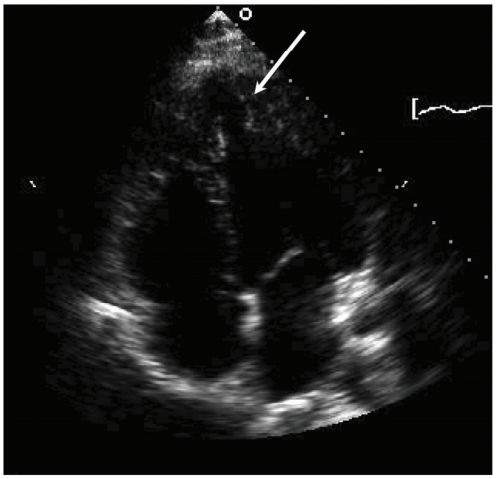


Figure 1 Two-dimensional echocardiographic four-chamber view of the 35-year-old asymptomatic sister of the proband of family A, showing excessive trabeculations of the apical (arrow) and mid-lateral left ventricular walls and mild left ventricular systolic dysfunction. The right ventricle was also excessively trabeculated.

A candidate gene approach, to identify a genetic defect in this family, included screening of known genes associated with DCM and HCM and revealed a missense mutation in the p.Leu301Gln in exon 11 (nucleotide change c.902T>A) of the *MYH7* gene in four NCCM patients of this family (*Figure 2*).[21]

The p.Leu301Gln mutation segregated with the clinical features of NCCM in this family (*Figure 3*). The p.Leu301Gln mutation was not observed on 400 control chromosomes or in 300 HCM patients. p.Leu301Gln is in the functionally important globular head region (subfragment S1; the 'motor domain') of β-myosin heavy chain, a region in which multiple pathogenic mutations have been described. Moreover, this mutation is pathogenic according to a prediction algorithm.[22] No mutations were found in the *TAZ, LMNA/C, MYBPC3, TNNC1, TNNT2, TNNI3, MYL2, MYL3, CSRP3, TCAP, ACTC*, and *TPM1* genes.

Family B

In 2003, a 35-year-old man was hospitalized with severe symptoms of congestive heart failure. At the age of 3 years, he was treated for lymphoblastic leukaemia with chemo- and radiotherapy (cytosine, arabinoside, methotrexate, and prednisone). Two months prior to hospitalization, he experienced progressive dyspnoea, orthopnoea, palpitations, fatigue, coughing, nausea, and vomiting. ECG showed sinus tachycardia with 123 b.p.m., a left bundle branch block (QRS width 154 ms), and biphasic P in V1. Echocardiographic examination revealed NCCM with severe systolic LV dysfunction (*Figures 4A* and *B*). Furthermore, a substantial thrombus was seen in the LV. Treatment with intravenous heparin and coumarine resulted in complete resolution of the LV thrombus after a few weeks (not shown). With diuretics, an ACE-inhibitor, and β-blocker therapy, an excellent clinical response was observed. After 2.5-year follow-up, he remains asymptomatic with moderate LV impairment.

Although chemotherapy-induced cardiomyopathy was primarily considered, the typical echocardiographic features of NCCM warranted screening of first-degree relatives, including both children

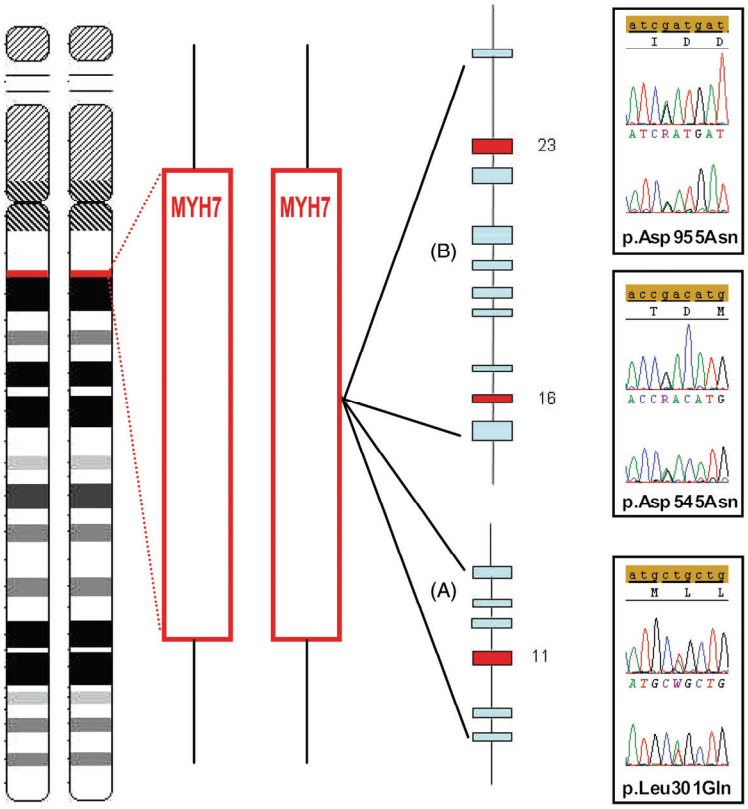


Figure 2 Schematic diagram showing chromosome 14 with the location of the *MYH7* gene (14q12). Mutations were identified on one allele of the *MYH7* gene in exon 11 in family A and in exons 16 and 23 in family B. Sequence traces show the mutations identified in families A and B.

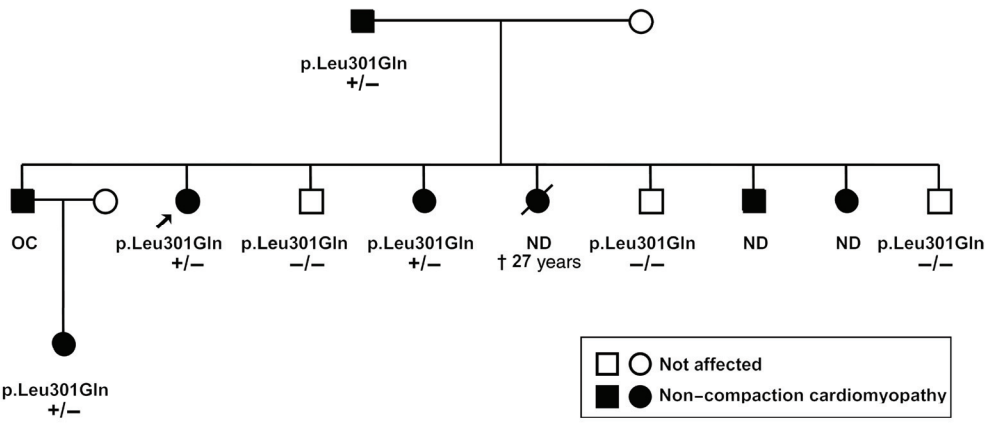


Figure 3 Pedigree of family A. The proband is indicated by the arrow. ND, not determined; OC, obligate carrier; +/−, heterozygous for the p.Leu301Gln MYH7 mutation; −/−, p.Leu301Gln absent.

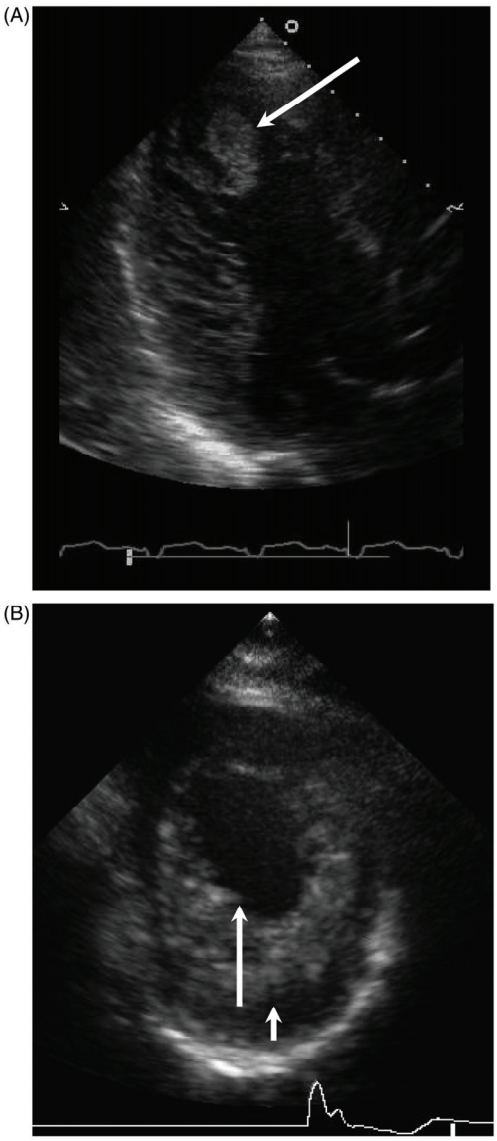


Figure 4 (*A*) Apical three-chamber and parasternal short-axis view of the index showing prominent thickened apical, lateral, and posterior walls of the left ventricle with loose meshwork of trabeculations; the arrow indicates the left ventricular thrombus; (*B*) arrows show the non-compacted to compacted ratio in systole is >2.

of the proband. In the asymptomatic 11-year-old son, echocardiographic examination revealed mild apical LVNC. No cardiac features of NCCM were found in the 14-year-old daughter. In the proband's asymptomatic 32-year-old brother, echocardiography showed moderately impaired systolic LV function with global hypokinesia (ejection fraction 30%) and typical features of non-compaction. Treatment with ACE-inhibitors, anticoagulants, and β-blockers was started. At follow-up, the ejection fraction improved and he remains free of symptoms. Neurological evaluation did not show features of limb girdle or progressive distal myopathy in this family.

Cardiological evaluation of the father, suffering from Alzheimer's disease, similarly revealed NCCM at the apex of the LV. His ejection fraction was 45% at presentation. His ECG showed sinus bradycardia. He is receiving treatment with ACE-inhibitors and anticoagulants. The asymptomatic 72-year-old sister of the father, with a history of diabetes mellitus type II and a coronary bypass, showed hypertrabeculation of the apical, inferior, and posterolateral LV walls, consistent with NCCM. A brother of the father died 2 years after being diagnosed with NCCM, at age 69. Neither of the parents of the father was known to have a heart condition; they both suffered a cerebro-vascular accident; the father died at age 78, the mother at age 75.

DNA analysis of five patients revealed the mutations p.Asp545Asn in exon 16 (nucleotide change c.1633G>A) and p.Asp955Asn in exon 23 (nucleotide change c.2863G>A) of the *MYH7* gene (*Figure 2*).[21] Segregation analysis in this family subsequently demonstrated that these two *MYH7* mutations were on the same allele (*Figure 5*). The p.Asp545Asn and p.Asp955Asn missense mutations were not observed on 400 control chromosomes or in 300 HCM patients. p.Asp545Asn is in the functionally important globular head region (subfragment S1; the 'motor domain') of the myosin heavy chain, a region in which multiple pathogenic mutations have been described. p.Asp955Asn is in the head–rod junction region (subfragment S2), a functionally important region for the interaction with the regulatory domain of myosin-binding protein C (*MYBPC3*).[23] Moreover, each of these mutations is pathogenic according to a prediction algorithm.[22] No mutations were identified in the *TAZ*, *LMNA/C*, *MYBPC3*, *TNNC1*, *TNNT2*, *TNNI3*, *MYL2*, *MYL3*, *CSRP3*, *TCAP*, *ACTC*, and *TPM1* genes.

Discussion

Clinically, familial cardiomyopathies are classified as HCM, DCM, restrictive cardiomyopathy (RCM), and NCCM, also called isolated non-compaction of the left ventricle (IVNC) or left ventricular non-compaction (LVNC) cardiomyopathy. The refinement of cardiac imaging techniques and more awareness among clinicians result increasingly in the recognition of the distinct features of NCCM, where in the past, misclassification may have occurred, particularly when suboptimal (i.e. older) imaging methods were used. Cardiological screening of relatives indicating familial recurrence of NCCM showed that genetic factors are important in the aetiology of this disease. In HCM, sarcomeric gene mutations are the predominant underlying genetic cause. Familial DCM is mainly associated with mutations in cytoskeleton and extracellular matrix genes, in addition to mutations in sarcomeric genes. Rare mutations, mostly in non-HCM/DCM genes, have been identified in a small proportion of familial NCCM. However, so far, no major genetic defect for NCCM has been identified. The identification of mutations in the *MYH7* gene in two separate, large NCCM families suggests that *MYH7* defects may be an important genetic cause for this form of cardiomyopathy. Defects in the *MYH7* gene are a major cause of familial HCM and DCM, and, in addition, *MYH7* mutations were recently shown to be associated with HCM with a restrictive phenotype (RCM).[24] This article is the first to link *MYH7* gene defects to familial NCCM. In family A, we found the single p.Leu301Gln mutation segregating with NCCM. In family B, we identified the missense double-mutation p.Asp545Asn/p.Asp955Asn on the same *MYH7* allele segregating with the disease.

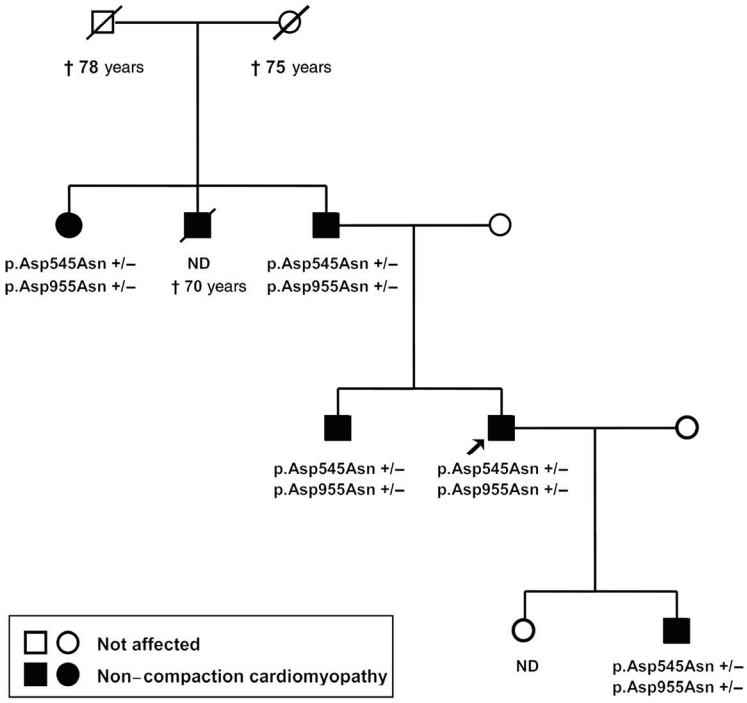


Figure 5 Pedigree of family B. The arrow indicates the proband. ND, not determined; +/−, heterozygous for the p.Asp 545Asn and p.Asp 955Asn MYH7 mutations. † indicates deceased (death at this age).

The majority of mutations in the *MYH7* gene are missense mutations. Truncating mutations in *MYH7*, causing loss of function of one allele, are rarely observed. It is therefore unlikely that haploinsufficiency, i.e. the presence of only one intact allele of the gene, is the main underlying pathophysiological mechanism of *MYH7*-associated disease. This implies that the mutated *MYH7* gene product acts as a 'dominant-negative' protein, perturbing the function of the protein formed by the normal *MYH7* allele.[25,26] This, so-called poison-polypeptide theory, easily accommodates the presence of double mutations on the same *MYH7* allele, in which the second mutation further modifies the function of the mutated protein.[27] Our findings show that a single mutated *MYH7* allele, either carrying one missense mutation or a double missense mutation in *cis*, may result in dominantly inherited NCCM with a variable phenotype.

The major cardiomyopathies are genetically heterogeneous diseases for which the causative genes are partially overlapping. The phenotypic variability of sarcomeric mutations is illustrated by *MYH7* mutations, known to be involved in HCM, DCM, RCM, and NCCM (this study). Whether this means that these are different diseases or rather different manifestations (phenotypes) of the same pathological mechanism is presently not clear. The molecular basis of the phenotypic plasticity of *MYH7* mutations remains unknown but it is likely to be multifactorial. It can be partially explained by the effect of different mutations on structural and regulatory components of the force generation and relaxation complex. Alternatively, effects of mutant proteins on energy homeostasis of the cardiomyocyte may influence disease outcome. The resulting phenotype is likely determined not only by the causal sarcomeric mutation but also by modifier genes, epigenetic factors, and environmental factors.

Thus far it is thought that an intrauterine arrest of myocardial development with lack of compaction of the loose myocardial meshwork is the pathophysiological mechanism behind NCCM.[28] *MYH7* and other sarcomeric gene mutations are well known causes for late onset forms of HCM and DCM, presenting clinical features mostly at adult age. The identification of *MYH7* mutations in familial NCCM and an *ACTC* mutation in HCM with NCCM and ASD,[17] together with the observation of late onset NCCM in a Duchenne patient,[29] suggests that the aetiology of NCCM extends beyond an arrest in embryonic cardiac development (i.e. the possibility of late onset NCCM).

The current findings expand the genetic heterogeneity of NCCM, and the identification of *MYH7* defects in familial NCCM suggests that NCCM may be part of a cardiomyopathy spectrum including HCM, RCM, and DCM.

Regular cardiac follow-up of at-risk relatives is recommended in familial cardiomyopathies associated with sarcomeric defects. Similarly, periodic cardiological screening of unaffected at-risk relatives in familial NCCM could be necessary. Our observation also warrants

molecular screening for *MYH7* and possibly other sarcomeric defects in NCCM patients and has implications for cardiac screening for NCCM features in familial HCM, RCM, and DCM.

Conflict of interest: none declared.

Funding

Y.M.H. receives a Dr. E. Dekker scholarship from the Netherlands Heart Foundation (NHF).

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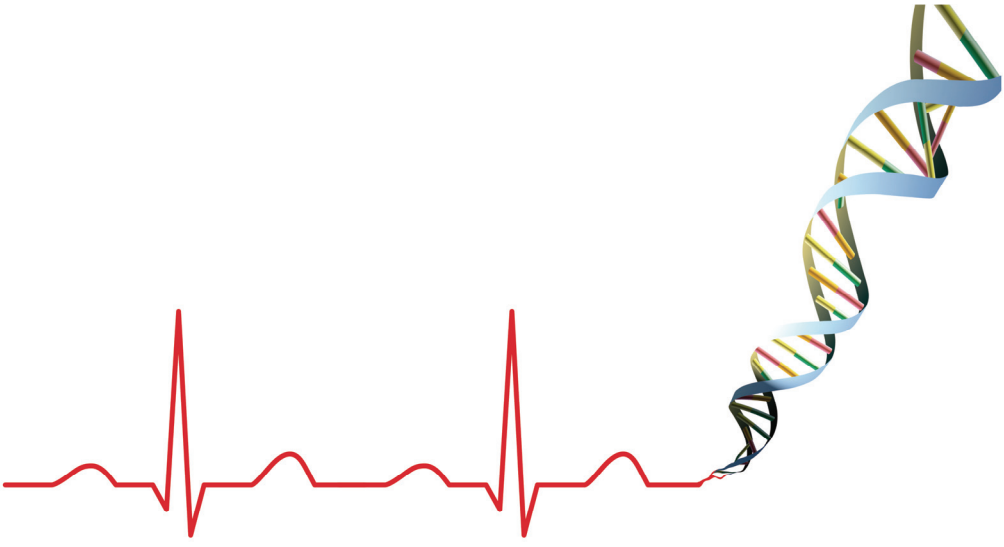
Chapter 3

The importance of genetic counselling, DNA diagnostics and cardiologic family screening in noncompaction cardiomyopathy.

Yvonne M. Hoedemaekers, Kadir Caliskan, Michelle Michels, Ingrid M.E. Frohn – Mulder, Jasper J. van der Smagt, Judith E. Phefferkorn, Marja W. Wessels, Folkert J. ten Cate, Eric J.G. Sijbrands, Dennis Dooijes, Danielle F. Majoor-Krakauer.

Circulation: Cardiovascular Genetics in press



Abstract

Background

Noncompaction cardiomyopathy (NCCM) is a distinct cardiomyopathy featuring a thickened bilayered left ventricular wall consisting of a thick endocardial layer with prominent intertrabecular recesses with a thin, compact epicardial layer. Similarly to hypertrophic (HCM) and dilated cardiomyopathy (DCM), NCCM is genetically heterogeneous and was recently associated with mutations in sarcomere genes. In order to contribute to the genetic classification for NCCM a systematic cardiologic family study was performed in a cohort of 58 consecutively diagnosed and molecularly screened patients with isolated NCCM (49 adults and nine children).

Methods and results

Combined molecular testing and cardiologic family screening revealed that in 67% NCCM is genetic. Cardiologic screening with electrocardiography and echocardiography of 194 relatives from 50 unrelated NCCM probands revealed familial cardiomyopathy in 32 families (64%), including NCCM, HCM and DCM. Sixty-three percent of the relatives newly diagnosed with cardiomyopathy were asymptomatic; of 17 asymptomatic relatives with a mutation, nine had NCCM, in eight non-penetrance was observed, explaining that 44% (14/32) of familial disease remained undetected by ascertainment of family history prior to cardiologic family screening. The molecular screening of 17 genes identified mutations in 11 genes in 41% (23/56) tested probands; in 35% (17/48) adults and 75% (6/8) children. In eighteen families single mutations were transmitted in an autosomal dominant mode. Two adults and two children were compound or double heterozygous for two different mutations. One adult proband had three mutations. In 50% (16/32) of familial NCCM the genetic defect remained inconclusive.

Conclusions

NCCM is predominantly a genetic cardiomyopathy with variable presentation ranging from asymptomatic to severe. Accordingly, the diagnosis of NCCM requires genetic counseling, DNA diagnostics and cardiologic family screening.

Introduction

Noncompaction cardiomyopathy (NCCM) is a cardiomyopathy featuring segmental thickening of the left ventricular (LV) wall with a thin, compact, epicardial layer and an excessively thickened endocardial layer with prominent, deep intertrabecular recesses. Application of the echocardiographic diagnostic criteria for NCCM as postulated by Jenni together with advances in cardiological imaging techniques have enhanced awareness and diagnosis of NCCM.[1] Consequently NCCM was incorporated in the most recent classification of cardiomyopathies as a genetic disease.[2]

Prevalence of NCCM, estimated from retrospective studies, ranges from 4.5 to 26 per 10,000 adult patients referred for echocardiography.[3-5] NCCM was diagnosed in 3.7% of patients with a LV ejection fraction ≤ 45% suggesting that NCCM might not be a rare disorder in adults.[5] In paediatric series NCCM is the most frequent cardiomyopathy after DCM and HCM, comprising approximately 9% of childhood cardiomyopathies.[6] Clinical features include heart failure, arrhythmias and thrombo-embolic events.[3, 7] Familial disease was estimated to occur in approximately 18 - 50% of adults with isolated NCCM, mostly consistent with an autosomal dominant mode of inheritance.[3, 8-13]Intrafamilial phenotypic variability, including NCCM, hypertrophic cardiomyopathy (HCM) and/or dilated cardiomyopathy (DCM) suggests that these cardiomyopathies may be part of a broader cardiomyopathy spectrum. The first association of isolated NCCM with mutations in the cardiac β-Myosin Heavy Chain gene (*MYH7*) was reported in two unrelated Dutch families.[14] NCCM was also associated with mutations in other sarcomere genes (cardiac Troponin T (*TNNT2*) and cardiac α-Actin (*ACTC1*)) in 17% of 63 adult NCCM patients,[15-17] linking NCCM to defects in *MYH7*, *TNNT2* and *ACTC1*, genes encoding sarcomere components that are frequent causes of HCM and DCM, provides additional evidence for a shared genetic susceptibility to NCCM, HCM, and DCM. Reports of mutations in Lamin A/C (*LMNA*), α-dystrobrevin (*DTNA*), Cypher/ZASP or Lim Domain Binding 3 (*LDB3*) and Sodium Channel Type V alpha (*SCN5A)* expanded the genetic spectrum of NCCM.[18-20] Other genetic causes, characteristically in complex childhood NCCM with congenital heart defects or (metabolic) syndromes, include Barth syndrome with mutations in the Tafazzin gene (*TAZ*)[21, 22] and rare chromosomal defects and loci.[23-30] The current study investigates the heredity of NCCM, the spectrum of clinical features and the genetic aetiology of NCCM by combining systematic cardiological family studies with extensive molecular analysis.

Methods

Study population

The study comprised 58 unrelated patients with isolated NCCM; 53 were diagnosed consecutively from 2005-2008 in the Cardiogenetics clinic of the Erasmus MC in Rotterdam, and five in other tertiary referral centres in the Netherlands. All fulfilled the four echocardiographic diagnostic Jenni criteria: a) excessively thickened LV myocardial wall with a two-layered structure comprising a compact epicardial layer (C) and a noncompacted

endocardial layer (NC) of prominent trabeculations and deep intertrabecular recesses; b) maximal end-systolic NC/C ratio > 2 measured at the parasternal short axis; c) colour-Doppler evidence of deep perfused intertrabecular recesses; d) absence of coexisting cardiac anomalies.[1] Subsequently, all patients were referred for genetic counselling, DNA analysis and to initiate family studies as depicted in Figure 1.

Cardiologic family study and molecular analysis

Family studies were initiated by ascertainment of family histories and inviting initially first- and second-degree relatives for genetic counselling. When possible, 'cascade screening' for cardiomyopathies was pursued. Participation of 50 families of probands allowed inclusion of 194 relatives (Table 1). Relatives were referred for cardiologic screening, unless a familial pathogenic mutation had been detected. In that case only mutation positive individuals and relatives refusing DNA testing were examined cardiologically. Informed consent was requested to review medical records from 31 relatives who had cardiologic examinations in other hospitals. Similarly, information was retrieved from the medical records of 13 deceased relatives reported to be affected. Details of the family studies of the probands identified with a genetic defect are presented in the addendum.

Cardiologic Family Study

Cardiologic screening of relatives was performed by two cardiologists (K.C. and M.M.), and included a review of the medical history, physical examination, electrocardiography (ECG; Mortara Portrait, Milwaukee, USA) and two-dimensional echocardiography (iE33 system with a S5-1 transducer; Philips Medical Systems, Best, the Netherlands). If the imaging quality was poor, especially at LV apical or mid-ventricular walls, magnetic resonance imaging (MRI; 1.5 Tesla scanner; Signa CV/*I*, GE Medical systems, Milwaukee, USA) was performed (n=26). Measuring the maximal NC and C with electronic callipers in end-systolic parasternal short axis or apical four-chamber view assessed extent and severity of noncompaction. Relatives were diagnosed with NCCM when complying to the Jenni criteria and were diagnosed with DCM or HCM when meeting the current definitions.[31] When ECG and echocardiography were normal in relatives, NCCM or another cardiomyopathy was excluded. Other cardiologic findings observed in relatives possibly associated with cardiomyopathy included ECG with pathological Q's (>40 ms or >25% of R-waves in at least two leads), left ventricular hypertrophy (LVH), complete bundle branch block, other intraventricular conduction or repolarisation abnormalities.

Molecular study

DNA analysis was performed in 56 probands at the department of clinical genetics and consisted of direct sequencing of all coding regions and intron-exon boundaries of the following genes: *MYH7*, Myosin Binding Protein C (*MYBPC3*), cardiac Troponin C (*TNNC1*), *TNNT2*, cardiac Troponin I (*TNNI3*), cardiac-regulatory Myosin Light Chain (*MYL2*), cardiac-essential Myosin Light Chain (*MYL3*), *ACTC1*, α-Tropomyosin (*TPM1*), Cysteine- and Glycine-rich

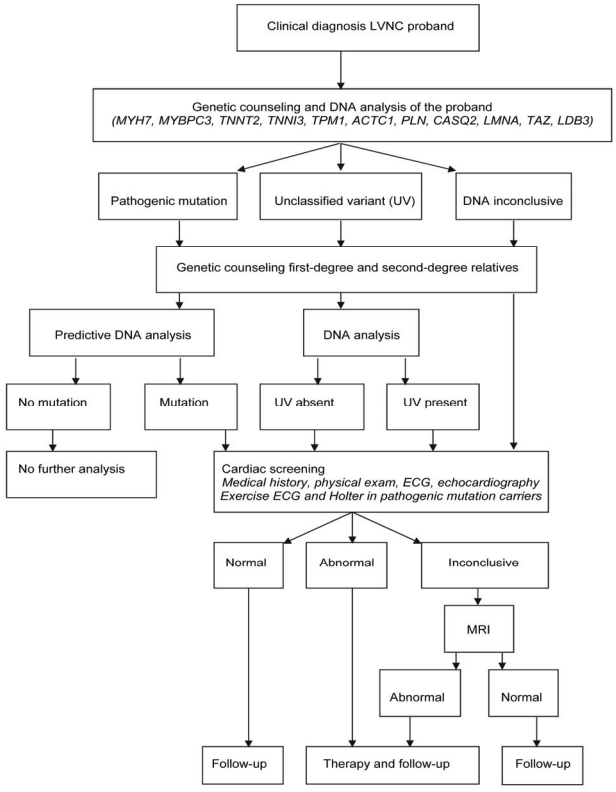


Figure 1. Flow chart for NCCM family screening

protein (*CSRP3*), Theletonin (*TCAP*), Calsequestrin (*CASQ2*), Calreticulin (*CALR3*), Phospholamban (*PLN*), *TAZ*, *LDB3* and *LMNA*. One proband declined DNA analysis and no DNA was available from one patient who died aged 11 days. The parents of this patient were cardiologically unaffected and did not have a mutation.

The mutations previously associated with cardiomyopathy (NCCM or HCM) were regarded as pathogenic. Novel mutations were considered to be pathogenic when they were either truncating, splice-site or de novo mutations or fulfilled the following three criteria: 1. segregation with disease in a family, 2. Absence on 384 ethnically matched healthy control chromosomes and 3. were likely pathogenic according to prediction software (SIFT and PolyPhen).[32] DNA variants not fulfilling these criteria were considered unclassified variants (UV).

Table 1. Descriptives of the NCCM family study.

	Total	Men	Age of onset/screening mean yrs ± SD (range)	Women	Age of onset/screening mean yrs ± SD (range)
Probands	58	30	39 ± 17 (0 – 63)	28	37 ± 19 (0 – 66)
Adults	49	26	44 ± 12 (19 – 63)	23	43 ± 13 (19 – 66)
Children	9	4	7 ± 8 (0 – 17)	5	6 ± 6 (0 – 16)
Participating relatives	194	89	41 ± 21 (0 – 77)	105	43 ± 20 (0 – 78)
Parents	40	20	55 ± 15 (23 – 74)	20	56 ± 15 (23 – 78)
Siblings	64	27	38 ± 18 (3 – 66)	37	43 ± 17 (0 – 71)
Children	41	22	23 ± 15 (0 – 56)	19	33 ± 15 (11 – 47)
Second-degree relatives	43	18	51 ± 19 (15 – 76)	26	48 ± 21 (6 – 74)
Third-degree ≥	6	2	38 ± 1 (37 – 39)	4	41 ± 13 (5 – 55)

Statistics

Statistical analyses were performed by SPSS for Windows 15.0 (SPSS Inc., Chicago, IL). Unpaired student's t-test analysis was used for continuous variables. Descriptive data for continuous variables were presented as mean ± one standard deviation. Chi-square analysis was used for categorical variables. A p-value of <0.05 was considered to be significant.

Results

The cardiologic screening of relatives and the molecular analysis of probands and relatives combined showed that at least 67% (39/58) of NCCM is genetic (Table 2 and 3). The cardiologic family study identified 64% (32/50) of isolated NCCM as familial. Genetic defects were identified in 50% (16/32) of cardiologically confirmed familial NCCM. In 50% (16/32) of familial disease the genetic defect remained unknown. With extensive DNA screening we found a mutation in 41% (23/56) of all tested probands. These results clearly indicate the importance of combining cardiologic family screening for cardiomyopathy with genetic testing of NCCM patients.

Table 2. Cardiologic family studies and genotyping of 58 NCCM probands.

Proband	Cardiologic family screening				Total
	Positive	De novo	Inconclusive	Not performed	
With mutation	16†	1†	3†	3†	23
Without mutation	15†		13	5	33
Without DNA analysis	1†		1		2
Total	32		18*	8	58

*Legend. * including the family of the de novo proband, † genetic NCCM (total 39)*

Family histories reported by probands before DNA testing and cardiologic family studies were performed, failed to identify 44% (14/32) of familial disease. Only nine (53%) of the 17 adult patients with a mutation reported familial disease prior to DNA testing and cardiologic family evaluation. Familial disease was reported correctly by 8/14 (57%) adults without a mutation, and by none of the parents of children with NCCM. Mutations were observed in 6/8 children with NCCM and in 17/48 (35%) of adult probands. NCCM was associated with defects in six sarcomere, two Ca^{2+}-handling, the *LMNA*, *LDB3* and *TAZ* genes in this study. Mutations in sarcomere genes, in particular in *MYH7*, were the most frequent genetic defects: 9/57 adults and 2/9 children (addendum families 1-9 and 18-19). None of the *MYH7* mutation carriers had neuromuscular symptoms. Eighteen (32%) probands (14 adults and four children) had a single mutation consistent with an autosomal dominant mode of inheritance. Two de novo mutations were observed: one in the asymptomatic father of an affected newborn and one in a young patient (addendum families 20-21). Multiple pathogenic mutations occurred in 9% (5/56) of the probands. Two (22%) children (diagnosed at age 4 months and at 7 years) had respectively mutations in *TNNI3* and *TPM1* and two different *MYBPC3* mutations (Figure 2; addendum families 22-23). Complex genotypes in adults constituted respectively of mutations *TNNT2* –

Table 3. Overview of the family studies in families with and without mutations.

Proband		Mutation	Participating relatives (n)	Cardiologically affected relatives (n)	Cardiologically unaffected carriers (n)	Deceased affected relatives (n)
Adult	1	+	12	9		1
	2	+	6	4	2	1
	3	+	3	1		
	4	+	2	0		1
	5	+	2	3	1	2
	6	+	4	0	1	
	7	+	0			
	8	+	1	1		
	9	+	7	3		1
	10	+	1	0	1	1
	11	+	1	0		1
	12	+	2	1		
	13	+	1	0		
	14	+	6	2		
	15	+	0			
	16	+	9	4		2
	17	+	2	1		
Child	18	+	1	1		
	19	+	0			
	20	+	4	1		
	21	de novo	2	0		
	22	+	10	3	2	
	23	+	9	0	4	
Adult	24	-	5	4		
	25	-	8	4		1
	26	-	3	1		
	27	-	5	3		
	28	-	7	2		2
	29	-	3	1		
	30	-	1	1		
	31	-	10	5		
	32	-	4	4		
	33	-	4	2		
	34	-	3	1		
	35	-	5	3		
	36	-	3	1		
	37	no DNA	1	1		1
Child	38	-	3	1		
	39	-	5	1		
Adult	40	-	1	0		
	41	-	1	0		

Continued on next page

Table 3. Continued.

Proband		Mutation	Participating relatives (n)	Cardiologically affected relatives (n)	Cardiologically unaffected carriers (n)	Deceased affected relatives (n)
Adult	42	-	3	0		
	43	-	1	0		
	44	-	5	0		
	45	-	3	0		
	46	-	3	0		
	47	-	3	0		
	48	-	3	0		
	49	-	3	0		
	50	-	3	0		
	51	-	4	0		
	52	-	3	0		
Child	53	no DNA	3	0		
Legend. Numbers 1-23 correspond to families 1-23 and 24-26 to paragraph 1C in the addendum.						

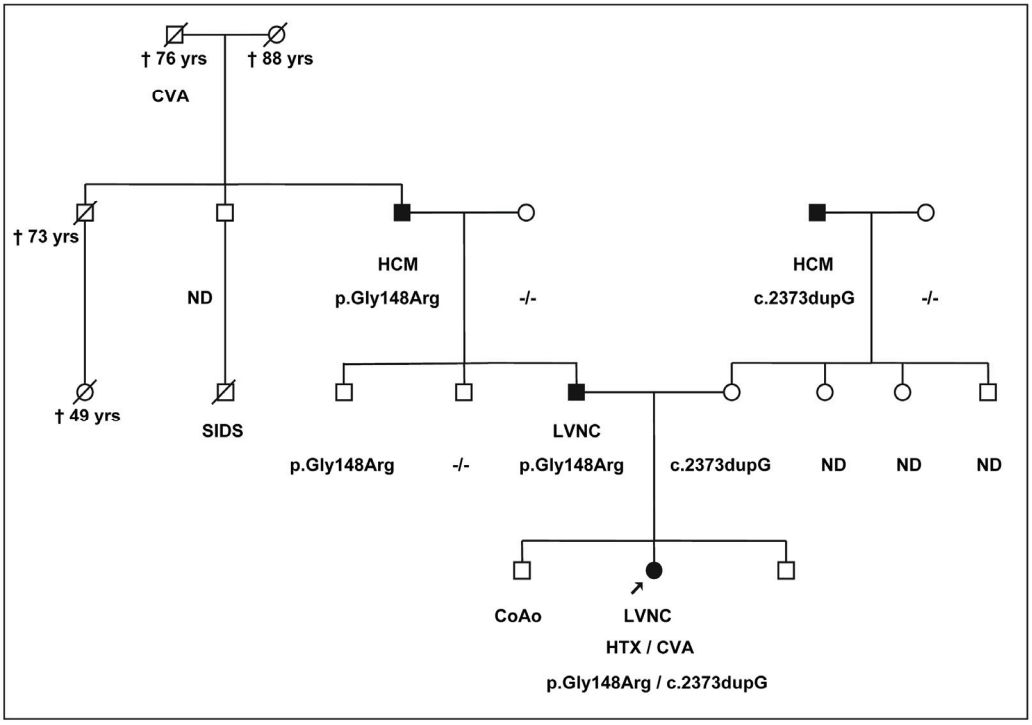


Figure 2. *MYBPC3* p.Gly148Arg and c.2373dupG mutations in one family.
Compound heterozygousity for two different *MYBPC3* mutations in a NCCM patient diagnosed at age seven years from a family where one grandparent was previously diagnosed with HCM (addendum 23). Family studies identified a spectrum of cardiomyopathies: the asymptomatic parents were carriers, NCCM in the father and HCM in the paternal grandfather associated with the p.Gly148Arg mutation, and HCM in the maternal grandfather associated with c.2373dupG. The arrow indicates the proband. († = deceased; CVA = Cerebrovascular Accident; ND = Not determined, i.e. no cardiologic and/or molecular testing; SIDS = Sudden Infant Death Syndrome; CoAo = Coarctation of the Aorta; HTX = Heart transplant).

LDB3 and *LMNA – LDB3*. One adult proband had two *TNNT2* mutations and a *CASQ2* mutation. In five families UV's were observed. Family studies are ongoing to determine the segregation in families and the phenotypic effect of multiple mutations, or UV's especially in families where affected relatives were observed with single mutations (addendum families 14, 16 and 22).

DNA analysis was performed in 61 relatives from 20 families, confirming previous clinical diagnosis of 16 relatives: 12 with NCCM, two with HCM and two with DCM. Four symptomatic relatives (presenting with palpitations, fatigue and shortness of breath) had a mutation and were diagnosed with NCCM by subsequent cardiologic exams. Predictive DNA testing identified a mutation in 49% (17/41) of asymptomatic relatives. Cardiologic evaluation revealed that 53% (9/17) of the asymptomatic carriers had NCCM and eight carriers showed non-penetrance. Results of DNA analysis in relatives endorsed the pathogenicity of the mutation in 17 families. In three families with mutations only unaffected carriers were identified and in three families no cardiologic or DNA family studies have been performed yet (Table 2).

Cardiologic studies

There was no difference in age at diagnosis in adult probands with respect to gender (p=0.4), between adults with one or multiple mutations and those without a mutation (p=0.4) or between the probands and affected relatives (p=0.2). In families with a mutation unaffected adult carriers of a mutation were approximately the same age as the affected carriers (p=0.2). Fifty-six percent of unaffected carriers were older than 40 years, indicating non- or age-dependent penetrance of NCCM.

Similar proportions of adult probands with a single mutation and without a mutation were asymptomatic when diagnosed (29% and 16%; p=0.3) (Table 4). All adult patients with multiple mutations presented with NYHA class II and III. These differences cannot be attributed to a selection bias because clinical diagnosis of NCCM preceded DNA testing.

In nine children NCCM was diagnosed; in four before age one year; in three between 1-10 years and in two between 10 -18 years. The two children with multiple mutations were severely affected with cerebral infarctions and one had a heart transplant at age seven years (Figure 2). All the children were the first in their families to be diagnosed with cardiomyopathy, cardiologic screening and DNA testing indicated familial NCCM in 89% (8/9) of their families; NCCM was diagnosed in 3/17 (18%) parents, and 3/15 (20%) were unaffected carriers. Figure 3 displays cardiac features in an affected asymptomatic parent.

In total cardiologic screening was performed in 145 first-degree, 43 second-degree and six more distantly related relatives (Table 1). Of the 69 (35%) relatives diagnosed with cardiomyopathy 47 had NCCM, five had HCM, 15 DCM and two congestive CM (Table 4). The majority (63%) of the relatives diagnosed with cardiomyopathy was asymptomatic. Severe complications in affected relatives (heart failure, arrhythmia and thrombo-embolic events) were observed more frequently (23%) in families without than in families with a mutation (13%) (OR 2.01, p= 0.36). This may be explained by the fact that we examined two large families without

Table 4. Cardiologic features in NCCM families.

	Mutation*				No Mutation*			
	Probands (23)		Relatives (39)		Probands (35)		Relatives (49)	
	≥ 18 yrs (17)	< 18 yrs (6)	Affected (34)	Other† (5)	≥ 18 yrs (32)	< 18 yrs (3)	Affected (35)	Other† (14)
Age ± SD (yrs)	41 ± 11	6 ± 6	41 ± 21	43 ± 15	46 ± 13	6 ± 10	43 ± 15	48 ± 16
Men	8	2	21	2	19	3	15	8
Presentation								
Asymptomatic	4		25	3	3		24	14
Heart failure	6	6	5		18	2	6	
Arrhythmias	5				6	1	1	1
Thrombo-embolism	1				3		1	
Other‡	1		2		3		1	
NYHA I	5		21	2	5		24	15
II	7		10	1	19		9	
III	5		2		8			
IV								
NC/C ratio ≥ 2	17		25		32		22	
NC/C ratio 1.0–1.9				3				9
ECG abnormal				1				8
LVH	1		4		4			9
Abnormal repolarisation	4				8			
Abnormal Q	1		1		1			1
Bundle branch block	4				9		2	
AV-block			4					
T-wave inversion	1		1		4		1	
Diagnosis								
NCCM	17	6	25		32	3	22	
HCM			2				3	
DCM			6				9	
Congestive CM			1				1	
Family history of CM§	9	0			8	0		
Familial screening‖	15	5			27	3		
Familial CM	13	4			14	2		
Congenital heart defect in relative#	2	2			3	1		

*Legend. * Families with and without mutation; † Other include NC/C ratio between 1.0-1.9 and/or ECG anomalies; ‡ Chest pain (n=4); enlarged heart on X-ray; pre-operative screening; prenatal sonography; cardiac screening before Ritalin use; § Prior to cardiologic family studies; ‖ Cardiologic screening / DNA analysis; # Ebstein malformation; Fallot's tetralogy; Atrial septal defect type II (ASDII) and ventricular septal defect (VSD); aortic coarctation in four families with a mutation. Valvular pulmonic stenosis; ASDII, VSD, pulmonic atresia; patent ductus arteriosus and aortic coarctation in four families without mutation. NYHA = New York Heart Association classification; NC/C ratio: Ratio of noncompacted to compacted wall; LVH = Left Ventricular Hypertrophy; NCCM = Left ventricular noncompaction; HCM = Hypertrophic Cardiomyopathy; DCM = Dilated Cardiomyopathy; CM = Cardiomyopathy*

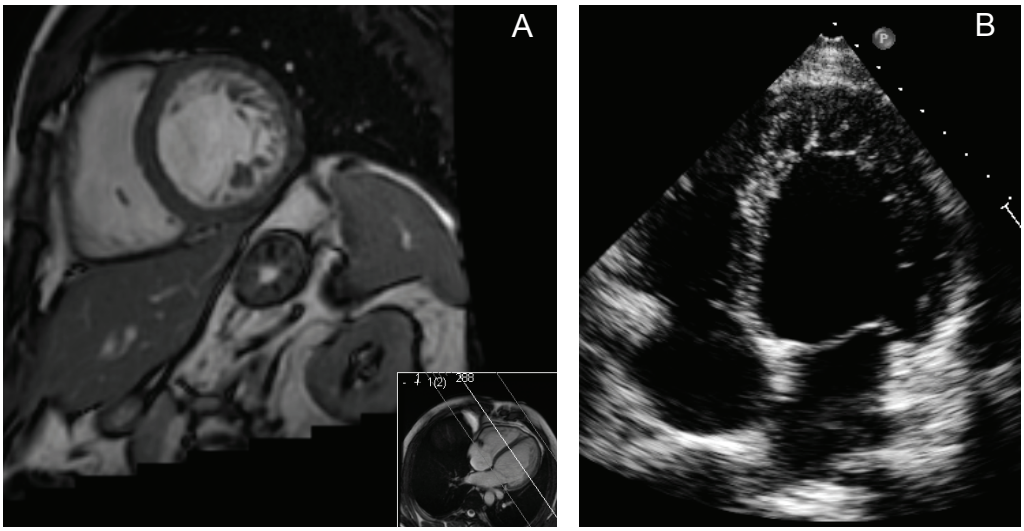


Figure 3. Cardiac MRI (A) and echocardiography (B) illustrating a two-layered myocardium with prominent intertrabecular recesses in the asymptomatic father from Figure 2 with an *MYBPC3* mutation.

mutation with recurrence of a severe phenotype; affected relatives in these families had been diagnosed prior to this study.

In 34% (11/32) of familial disease, familial aggregation of NCCM, HCM and DCM was observed. HCM and/or DCM were diagnosed in four families with a mutation (Figure 2; addendum families 8, 10, 16, 23) and in seven families without a mutation (Table 4).

In seven families congenital heart malformations were diagnosed. In one family with an *MYH7* mutation, NCCM was associated with Ebstein's anomaly and in two families with *MYBPC3* mutations one relative with the mutation had Fallot's tetralogy without NCCM and one had an aortic coarctation but did not have a DNA test. (Figure 2; addendum families 1, 10 and 22). In three families without a mutation NCCM occurred together with a valvular pulmonic stenosis, ventricular septal defect, atrial septal defect type II, pulmonic atresia, patent ductus arteriosus or aortic coarctation in six relatives.

Discussion

The approach of this study was to combine cardiologic family studies with extensive genetic testing to establish a genetic classification of NCCM. The results showed that isolated NCCM is predominantly (67%) a genetic condition, including HCM and DCM in 11 families (34%). The molecular screening of a large number of genes in this study allowed expanding the genetic spectrum of NCCM with novel genetic defects.

Genetic defects were identified in 41% of all patients and in 50% (16/32) of the cardiologically confirmed familial forms and consisted of one or more mutations in 11 different genes, indicating that further studies are needed to find causes for the remaining familial forms of NCCM. Molecular diagnosis of NCCM is important because it offers reliable identification of

asymptomatic relatives at risk. In absence of an identified genetic cause for NCCM, or when relatives decline DNA testing, cardiologic screening remains the appropriate method to identify familial disease.

The proportion of familial disease in this study is higher than reported previously (18%-50%) by studies investigating the prevalence of genetic defects in adult patients or ascertaining family histories of cardiomyopathy.[3, 8-13] The systematic cardiologic family screening showed that the majority (63%) of the affected relatives were asymptomatic, explaining that family histories without cardiologic family studies, failed to identify 44% of familial disease. Intrafamilial variability and incomplete penetrance, including asymptomatic disease, as well as small family size, may contribute to underestimation of familial disease. Therefore cardiologic evaluation of at risk relatives of all NCCM patients is recommended to enhance detection of familial disease, in accordance with the current expert consensus for family screening in HCM.[31] Familial screening for cardiomyopathies is important because early diagnosis in relatives may prevent severe complications. Nevertheless, predictive DNA testing and/or cardiologic evaluation should only take place after relatives have been well informed about possible medical benefits of early diagnosis including suitable treatment and life-style recommendations, as well as psychological and socio-economical consequences of predictive testing (particularly in countries where genetic discrimination by insurance companies or employers is not prohibited).

Similar to other familial cardiomyopathies, familial NCCM showed intrafamilial phenotypic variability, including HCM and/or DCM, reduced expression and reduced penetrance (i*e. clinical symptoms not expressed*).[3, 12, 33, 34] In this study non-penetrance was observed in eight relatives with a mutation ranging in age from 12 to 72 years. The implications of non-penetrance include pursuing cardiologic follow up of unaffected relatives (as depicted in Figure 1). And for families without a mutation, the consequences are that cardiologic screening is recommended. Improved imaging by echocardiography and cardiac MRI has enhanced diagnosis and awareness of NCCM. However, establishing the extent to which physiologic trabeculations are pathologic remains difficult.[35]

Mutations in the sarcomere genes were found in 6/8 tested affected infants and in 17/48 adult probands. Although the number of children included in this study is to small to draw conclusions on the aetiology of childhood disease, molecular testing of sarcomere genes and systemic cardiologic evaluation of first-degree relatives are recommended in early onset NCCM, especially in absence of dysmorphic features or metabolic defects. Congenital heart malformations in NCCM patients should not refer from analysing sarcomere genes. Our results endorse that co-occurrence of NCCM and congenital heart defects with and without sarcomere gene defect is not rare, warranting careful evaluation of the validity of the fourth of the Jenni diagnostic criteria.[15, 17, 36-38]

Two severely affected children and three adults were compound / double heterozygous, indicating that multiple mutations appear to be more prevalent in NCCM (22%) than in HCM (7%) (p=0.15).[39] In HCM double heterozygousity for truncating sarcomere mutations have been associated with severe congenital forms of HCM, inherited in an autosomal recessive mode.[40-

[42] In this study double mutations were also observed in adults with NCCM. The complex genetic defects in adults involved the combination of a sarcomere gene with another gene, suggesting that two sarcomere mutations may cause a more severe phenotype than the combination of a sarcomere mutation and a non-sarcomere mutation. The epigenetic effect of multiple mutations may depend on the specific defects involved. Further studies are needed to investigate the role of additional mutations and determine whether they play a role in the phenotypic variability.

For now, the evidence that sarcomere defects are an important cause for NCCM, together with the occurrence of NCCM, HCM and DCM within families, suggests that these cardiomyopathies represent phenotypic variability within a spectrum and thus require comparable approach with respect to family screening.

The results of cardiological follow-up of families will help to understand the natural history of NCCM, to determine whether NCCM represents a congenital endomyocardial defect or may develop later in life, and eventually to attain recommendations for follow-up of relatives based upon accurate risk classification.[43] The perspective of new studies investigating modifying genetic effects or genome-environment interactions to explain variability and age dependent penetrance of this phenotype is challenging.

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Addendum: Description and pedigrees of the families of NCCM probands with a gene defect.

1. Adult probands

1A. Adult probands, single mutation

Cardiac β-Myosin Heavy Chain (MYH7)

Family 1

NCCM was diagnosed in a 27-year-old woman presenting with progressive dyspnea, fatigue and oedema. In 1993 her sister died aged 27 years of a peripartum cardiomyopathy six days after giving birth to her third child. Cardiological screening of seven asymptomatic siblings revealed NCCM in five: two sisters aged 35 and 30 years, and two brothers aged 34 and 49 years. NCCM was also diagnosed in the 62-year-old father who was in NYHA class II and in the 24-year-old asymptomatic daughter of the eldest brother. DNA analysis showed the p.Leu301Gln mutation in the *MYH7* gene in all tested affected relatives and was excluded in four asymptomatic and cardiologically unaffected relatives. Two asymptomatic children (aged 12 and 15 years) of one of the affected relatives had NCCM, the 12-year-old girl was also diagnosed with an Ebstein malformation; both had the *MYH7* mutation.

Family 2

A 35-year-old man was hospitalised with symptoms of congestive heart failure (extreme fatigue, dyspnea, orthopnoea and palpitations). He was diagnosed with NCCM and severe systolic left ventricular (LV) dysfunction. He had a large thrombus in the left ventricle, which resolved completely with anti-coagulant treatment. Treatment of heart failure was successful and after four-and-a-half years of follow-up he remains asymptomatic with moderate LV dysfunction. In childhood, he had been treated for lymphoblastic leukaemia with chemo- and radiotherapy (cytosine, arabinoside, methotrexate and prednisone). Cardiological family studies revealed NCCM in his asymptomatic son, brother, father and a paternal aunt. The medical records of a paternal uncle who died at age 70 years also indicated NCCM. All the living affected relatives had the p.Asp545Asn and p.Asp955Asn double mutations in cis in the MYH7 gene. Additionally, predictive DNA analysis found both mutations in the asymptomatic and unaffected 15-year-old daughter of the proband and in the unaffected son of the deceased uncle of the proband, confirming the uncle to have been an obligate carrier of the double mutations.

Family 3

A 32-year-old woman with persistent dyspnea following an episode of influenza was diagnosed with heart failure and NCCM. She had the p.Glu1350del mutation in the *MYH7* gene. Her asymptomatic mother had the *MYH7* gene mutation but did not show features of NCCM, although LV function was slightly impaired (Fractional shortening 27%) and her mother had a grade II aortic insufficiency. The asymptomatic father did not carry the mutation and did not show any signs of cardiomyopathy. The asymptomatic brother, an athlete, showed extensive trabeculation of the apex and his ECG showed a non-specific intraventricular conduction delay. Exercise ECG and 24-hrs ECG were normal. He did not have the familial mutation. His cardiac abnormalities are possibly related to athletic performance, or they may indicate involvement of another so far unidentified genetic cause for NCCM in this family.

Family 4

In 2005 NCCM and an *MYH7* mutation (p.Arg1925Gly) were diagnosed in a 50-year-old asymptomatic woman. She was referred for cardiological screening after the sudden death of her brother at age 48 years due to congestive cardiomyopathy confirmed at autopsy. The *MYH7* mutation was excluded in an asymptomatic and cardiologically unaffected 55-year-old sister and the asymptomatic 77-year-old mother of the patient. The father died of lung cancer at age 65 years. The family history suggested that the mutation was inherited from the paternal side because of a reported sudden death at age 64 of one of the paternal aunts. One of her sons showed apical trabeculation at age 37 years, not fulfilling the Jenni criteria and his ECG showed voltage criteria for left ventricular hypertrophy, he refused molecular analysis.

Family 5

Cardiac screening of an asymptomatic 22-year-old woman identified NCCM and an *MYH7* mutation (p.Asn1918Lys). Her father had died aged 59 years after developing a sepsis. He was diagnosed previously with heart failure. The father's sister was also diagnosed with heart failure, and died at the age of 30 years. Three brothers of the father died suddenly, two at age 50 and one at age 63 years. An asymptomatic son and daughter of one of these brothers were diagnosed with NCCM at age 30 and 27 years respectively. They had the *MYH7* mutation. The sole surviving paternal uncle who was diagnosed with NCCM at age 61 years also had the *MYH7* mutation. The asymptomatic sister of the proband had the *MYH7* mutation, showing non-penetrance at age 27 years.

Family 6

A 41-year-old man presented with progressive dyspnea caused by congestive heart failure due to NCCM. An ICD was implanted. DNA analysis showed the p.Tyr1488Cys mutation in *MYH7*. His asymptomatic 9-year-old and 12-year-old sons were screened by a

paediatric cardiologist and found normal, as were his asymptomatic mother and brother. The eldest son carried the *MYH7* mutation, the youngest didn't. The mother and brother refused DNA testing.

Family 7

In 2005 a 61-year-old man had complaints of palpitations and chest pain. Echocardiography showed left ventricular hypertrophy. Two years later NCCM was diagnosed on MRI. He had the p.Leu658Val mutation in *MYH7*. Family history was negative for cardiac disease or sudden death; so far no relatives participated in cardiologic screening or DNA analysis.

Family 8

ECG showed left ventricular hypertrophy which could not be confirmed by echocardiography in a 21-year-old man who had a syncope while jogging. MRI at age 33 years showed evident NCCM with a non-compact to compact (NC/C) ratio of 4.2. After this diagnosis his father, previously diagnosed with HCM at age 17 years, also after syncope while jogging, had an MRI and was subsequently diagnosed with NCCM (NC/C ratio 2.2) at the age of 60 years. He died three years later. Family history reported that the probands paternal grandmother died at 40 years of age and that a sudden death during exercise occurred in a 17-year-old paternal uncle and in a 21-year-old paternal aunt. Cardiologic screening of another paternal uncle shortly before he died at age 64 years showed no abnormalities. The proband and his affected father both had the p.Ile818Asn mutation in *MYH7*.

Family 9

Standard prenatal ultrasound at 20, 22 and 23 weeks gestation showed cardiomegaly with increased wall thickness. Advanced prenatal ultrasound at 23+3 weeks gestation showed cardiomegaly, decreased contractility and increased apical myocardial wall thickness. A foetal cardiomyopathy was suspected, prenatal follow up showed a stable condition of the pump function. Subsequent cardiologic screening identified NCCM in the 34-year-old Turkish father who suffered from fatigue and excessive perspiration. The asymptomatic Turkish mother was unaffected. Their eldest (asymptomatic) son, aged 14 years, was also unaffected; the youngest son, aged 10 years who was also asymptomatic, had NCCM. The paternal mother had been diagnosed with NCCM after having chest pain approximately one year earlier in another medical centre. One of her sisters suffered a sudden cardiac death at the age of 38 years. A pathogenic splice-donor site *MYH7* mutation was identified (c.732+1G>A) in the affected father. At gestational age 37+4 weeks their son was born. DNA analysis confirmed the presence of the *MYH7* mutation. Echocardiography showed evident NCCM with cardiomegaly. Left ventricular function was not impaired and he was discharged form the hospital one day later. Follow-up will take

place at the paediatric cardiology department. The two affected sons both had the *MYH7* mutation. The asymptomatic 28-year-old brother of the father was unaffected, he refused DNA testing; two unaffected sisters of the father, aged 30 and 45 years, did not have the *MYH7* mutation.

Myosin Binding Protein C (*MYBPC3*)

Family 10

NCCM was diagnosed in a 56-year-old woman. In 1980, at the age of 30 years she was diagnosed with an unspecified cardiomyopathy. In 2000 on echocardiography a dilated cardiomyopathy was diagnosed, with a slight mitral valve insufficiency. In 2001, aged 51 years, she suffered a mesenterial thrombosis. She was also diagnosed with myelofibrosis. She had several transient ischemic attacks and a cerebral infarction at age 53 years confirmed by MRI. DNA analysis identified the truncating HCM founder mutation c.2373dupG in the *MYBPC3* gene. She died from progression of myelofibrosis at the age of 59 years. Her father died of a peritonitis aged 87 years. At autopsy his heart showed signs of hypertrophy and dilatation of the left and right ventricle and the founder mutation was confirmed in autopsy tissue. The proband's asymptomatic son inherited the founder mutation. He has Fallot's tetralogy, which was surgically corrected at the age of 14 months and cardiological screening at the age of 31 years did not show features of cardiomyopathy.

Phospholamban (*PLN*)

Family 11

After successful resuscitation due to ventricular tachycardia a 48-year-old woman was diagnosed with NCCM (NC/C ratio was 2.5). She had the p.Arg14del mutation in the *PLN* gene. Her father died aged 62 years. Post mortem analysis showed cardiomyopathy with a thrombus in the left ventricular apex. DNA analysis of the deceased father could not be performed. Cardiologic screening of an asymptomatic 47-year-old sister, without the *PLN* mutation, was normal.

Calsequestrin (*CASQ2*)

Family 12

A 53-year-old man from Surinam had exercise intolerance and was scheduled for an oesophageal-jejunostomy due to complications of a gastrectomy. The gastrectomy, six months previous, was due to gastric necrosis, caused by an arterial thrombo-embolism. Preoperative physical examinations revealed severe LV dysfunction due to NCCM. DNA

analysis identified the p.His244Arg mutation in *CASQ2*. His asymptomatic mother (aged 77 years) and sister (aged 53 years) both had NCCM and the *CASQ2* mutation.

1B. Adult probands, multiple mutations or unclassified variants

***MYBPC3* and a UV in cardiac α-Actin (*ACTC1*)**

Family 13

A 50-year-old man from Surinam presented with shortness of breath and palpitations, especially during stressful events. Cardiological evaluation detected non-sustained ventricular tachycardia and severe LV dysfunction. He was diagnosed with NCCM and received an ICD. DNA analysis identified the mutation p.Ala216Thr in *MYBPC3* and unclassified variant *22C>T in *ACTC1*. Cardiologic examination of an asymptomatic daughter showed no anomalies, she did not have the *MYBPC3* mutation, the *ACTC1* UV has not been tested yet.

Cardiac Troponin T (*TNNT2*) and Lim Domain-Binding 3 (*LDB3*)

Family 14

In 2003 a 45-year-old woman of Moroccan descent presented with exercise related chest pain. Echocardiography showed apical hypertrophy and contrast echocardiography showed typical features of NCCM in the apex of the LV. DNA analysis identified the p.Pro87Leu mutation in *TNNT2* and the p.Asp117Asn mutation in *LDB3*. She had six asymptomatic children who were screened cardiologically. NCCM was diagnosed in two sons (aged 22 and 27 years respectively). One of these sons had the *TNNT2* but not the *LDB3* mutation. The other children declined DNA testing. A third son had supraventricular tachycardia and apical ventricular hypertrophy with trabeculation with a NC/C ratio <2. No signs of cardiomyopathy were detected in three children (aged 18 to 31 years).

TNNT2* and *CASQ2

Family 15

A 35-year-old woman from Curacao was diagnosed with NCCM during pregnancy. Ejection fraction was approximately 20%. She had two mutations in *TNNT2* (p.Arg161His and p.Val225Leu) and one in *CASQ2* (p.His244Arg). So far no relatives have participated in the family study. Therefore it was not possible to determine whether the two *TNNT2* mutations are allelic or not.

Lamin A/C (*LMNA*) and *LDB3*

Family 16

This patient had palpitations at age 30 and was previously diagnosed with limb girdle muscular dystrophy type IB. At age 42 years he was admitted with atrial fibrillation, non-sustained VT's and a total AV block. Cardiac MRI diagnosed NCCM. He received an ICD. He had the c.1608+5G>C mutation in *LMNA* and the p.Asp117Asn mutation in *LDB3*. Family history was positive for dilated cardiomyopathy (two daughters of his paternal grandmother's sister; both confirmed *LMNA* mutation carriers; a son and daughter of one of these daughters; a son and grandson of another sister of the paternal grandmother) and sudden cardiac death (the proband's sister at age 39; the probands father at age 59; a paternal uncle at age 43 and a son of the first sister of the paternal grandmother at age 40 years). The asymptomatic brother of the proband had both mutations, cardiologic evaluation showed a first-degree AV-block with frequent ventricular extrasystoles and left ventricular hypertrophy on ECG. Echocardiography and MRI did not show signs of NCCM, MRI showed an ejection fraction of 47%. One of his sisters, who reported palpitations, carried only the *LMNA* mutation but not the *LBD3* mutation. ECG showed sinus bradycardia and a first-degree AV-block. Echocardiography did not show signs of cardiomyopathy.

Tafazzin (*TAZ*) and a UV in *LMNA*

Family 17

In 2007 a 57-year-old Surinam woman was diagnosed with cardiomegaly on X- ray after referral for persisting coughing. Echocardiography showed NCCM. She had the UV c.1968+26A>G in *LMNA* and the p.Phe128Ser mutation in *TAZ*. One of her sisters was reported with a sudden death at the age of 57 years. Her two sons were asymptomatic, one declined DNA analysis and cardiologic screening; the other was unaffected and didn't have either mutation. A 71-year-old sister, suffering from dyspnea and fatigue, showed significant cardiac enlargement on an X-ray made in Surinam. She had the UV in *LMNA*; the results of the *TAZ* gene analysis are not known yet.

1C. Adult probands with unclassified variants

Three families had only unclassified variants. Although these NCCM families did not have pathogenic mutations they were added here to complete the overview of the results.

UV in *CASQ2*

Preoperative cardiologic screening revealed NCCM in a 56-year-old asymptomatic woman. Cardiologic screening identified NCCM in her asymptomatic 16-year-old son, two asymptomatic brothers, aged 54 and 57 years respectively and in the 20-year-old asymptomatic daughter of the youngest brother. In childhood she was diagnosed with an aortic coarctation that was surgically corrected at the age of four years. She also had a duplicate frontal mitral valve slip. An unclassified variant was identified in *CASQ2* (p.Asp398del) in the proband. This variant did not co-segregate with NCCM in the family.

UV in *CASQ2*

This patient presented with a CVA in the right hemisphere at age 38 years. Following the young-stroke-protocol she had a cardiological examination that revealed NCCM. Her sister was diagnosed with dilated cardiomyopathy at age 21 years. She was one of the first patients in the Netherlands to receive an ICD. She died aged 23 years of complications of an ICD infection. The 66-year-old mother was diagnosed with NCCM after diagnosis was made in her eldest daughter. She had been suffering from dyspnea. Her 65-year-old asymptomatic brother and his 40-year-old asymptomatic daughter were also diagnosed with NCCM; her 55-year-old asymptomatic sister was diagnosed with DCM. Family history further mentioned a paternal nephew who has an ICD, his brother died suddenly at the age of 40 years. The UV p.Asp398del in *CASQ2* has been identified in the mother of the proband but didn't segregate with disease in the family.

UV in *LMNA* and *LDB3*

A 48-year-old man presented with chest pain. Catheterisation showed trabeculation and left ventricular dysfunction. NCCM was confirmed by echocardiography. During admission telemetry showed non-sustained VT's and he received an ICD. DNA analysis showed the unclassified variants c.1968+26A>G in *LMNA* and p.Val118Met in *LDB3.* His 24-year-old daughter, who suffered from syncope, showed LV trabeculation on echocardiography. MRI confirmed NCCM. She has the *LMNA* UV, but not the *LDB3* variant. Her 22-year-old asymptomatic sister was unaffected and carried the *LMNA* UV; the unaffected 53-year-old sister of the proband did not carry the *LMNA* UV. The *LDB3* variant was not tested.

2 Childhood probands

2A. Childhood probands, single mutations

MYH7

Family 18

A four-year-and-nine-month-old girl was diagnosed with NCCM when a cardiac murmur was detected at a paediatric consultation because of her congenital agenesis of the thyroid gland and urinary reflux. DNA analysis identified a nonsense mutation in the *MYH7* gene (p.Tyr266X). The mutation was also identified in the 35-year-old asymptomatic father, who was subsequently screened and diagnosed with NCCM. His father had died suddenly in his sleep aged 60 years.

Family 19

A ten-year-old boy, experiencing persistent fatigue and dyspnea after having had the flu, was diagnosed with heart failure and NCCM. DNA analysis revealed the p.Arg369Gln mutation in *MYH7*. Under medication his left ventricular function improved. Both parents and his twin sister are asymptomatic; they declined molecular and cardiologic testing. Recently, the *MYH7* mutation p.Arg369Gln was also found as a *de novo* mutation in a paediatric NCCM patient presenting with heart failure (Dellefave et al., *Circ Cardiovasc Genet*. 2009; 2:442-449)

Cardiac α- Actin (*ACTC1*)

Family 20

NCCM was diagnosed in a six-week-old girl examined because of failure to thrive and feeding problems. Her heart showed mid-ventricular noncompaction with apical dilatation of the left ventricle and a dilated left atrium with bulging of the atrial septum to the right. Under treatment of an ace-inhibitor and digoxin her condition improved. DNA analysis showed a mutation in the *ACTC1* gene, p.Met271Val. Her asymptomatic father had this mutation and was diagnosed with a dilated LV and RV, with apical, lateral and septal thickening without any evident crypts on echocardiography. On MRI NCCM was evident with a NC/C ratio of 15/3. The paternal grandparents did not have the mutation, identifying it as a pathogenic *de novo* mutation in the father of the proband.

α-Tropomyosin (*TPM1*)

Family 21

A 16-year-old girl was suspected of liver failure. On chest X-ray she had an exceptionally large heart and echocardiography showed a poor cardiac output. She was diagnosed with NCCM. She received a left ventricular assist device before she had a heart transplant at age 17 years. DNA analysis identified the pathogenic *de novo* p.Arg160His mutation in *TPM1:* the mutation was excluded in both parents. Cardiologic screening of her mother was normal; her father had developed ischemic DCM after a major heart infarction eight years earlier, not related to the *TPM1* mutation in the proband.

2B. Childhood probands, multiple mutations

Compound heterozygousity of two mutations in *MYBPC3*

Family 22

A 7-year-old girl presenting with fatigue and vomiting was diagnosed with severe dilated cardiomyopathy. X-ray and ECG showed signs of left ventricular hypertrophy. Four months after diagnosis she had a heart transplantation. The pathological exam of the heart showed a two-layered structure of the left ventricle with excessive trabeculations consistent with NCCM. She suffered a cerebral infarction shortly after being diagnosed with NCCM. DNA analysis revealed two *MYBPC3* gene mutations, the Dutch HCM founder mutation c.2373dupG and a novel mutation, p.Gly148Arg. The founder mutation was identified in the asymptomatic mother, without signs of cardiomyopathy. The maternal grandfather, diagnosed with HCM had the c.2373dupG mutation. The asymptomatic father had the p.Gly148Arg mutation and was subsequently diagnosed with NCCM. The paternal grandfather, suffered from hypertension and atherosclerosis, was diagnosed with HCM at age 76 years and had the p.Gly148Arg mutation. One asymptomatic paternal uncle (age 49 years) had the p.Gly148Arg mutation; cardiologic examination did not show signs of cardiomyopathy. The asymptomatic paternal grandmother and a paternal uncle without the mutation had no signs of cardiomyopathy. Screening of two asymptomatic brothers (ages four and nine years) of the proband did not show signs of cardiomyopathy. The eldest brother had an aortic coarctation which was surgically corrected at age nine years.

Double heterozygousity of mutations in cardiac Troponin I (*TNNI3*) and *TPM1*

Family 23

In 2004 a four-month-old girl was admitted because of failure to thrive, dyspnea and excessive perspiration. Echocardiography diagnosed NCCM. She suffered a cerebral

infarction in the medial cerebral artery area at the age of four months. At age four years and 10 months she died waiting for a heart transplant.

DNA analysis revealed the novel mutations p.Asp180Gly in the *TNNI3* gene and c.241-12_241-11delinsTG in the *TPM1* gene. The *TPM1* mutation was detected in her asymptomatic, cardiologically unaffected father (age 39 years). Her mother (age 37 years) who was also asymptomatic and cardiologically unaffected had the mutation in the *TNNI3* gene. The paternal grandparents were asymptomatic and unaffected. The paternal grandfather had the *TPM1* mutation. This mutation was excluded in the paternal grandmother and an unaffected aunt. In the asymptomatic unaffected maternal grandparents, the *TNNI3* mutation was identified in the grandmother and excluded in the grandfather. A maternal aunt, a professional athlete who did not have the *TNNI3* mutation, had features of noncompaction of the left ventricle with a ratio <2 and a normal ejection fraction. It is unclear whether the attenuated form of trabeculation was primarily related to physical activity or indicates another yet unidentified genetic cause for NCCM in this family.

Table 5. Family studies of genotyped patients.

Probands			Relatives			
			Affected		Unaffected	
Family*	Gene	Mutation	With mutation	No mutation	With mutation	No mutation
1	*MYH7*	p.Leu301Gln	5			2
2	*MYH7*	p.Asp545Asn / p.Asp955Asn	4		2	
3	*MYH7*	p.Glu1350del		1	1	
4	*MYH7*	p.Arg1925Gly				2
5	*MYH7*	p.Asn1918Lys	4		1	
6	*MYH7*	p.Tyr1488Cys			1	1
7†	*MYH7*	p.Leu658Val				
8	*MYH7*	p.Ile818Asn	1			
9	*MYH7*	c.732+1G>A	2			2
10	*MYBPC3*	c.2373dupG	1		1	
11	*PLN*	p.Arg14del				1
12	*CASQ2*	p.His244Arg	2			1
13	*MYBPC3*	p.Ala216Thr (A)‡				1
	ACTC1	c.*22C>T (B)‡				
14	*TNNT2*	p.Pro87Leu (C)‡	1			
	LDB3	p.Asp117Asn (D)‡		1		
15†	*TNNT2*	p.Arg161His (E)‡				
	TNNT2	p.Val225Leu (F)‡				
	CASQ2	p.His244Arg (G)‡				
16	*LMNA*	1608+5G>C (H)‡	3		2	1
	LDB3	p.Asp117Asn (I)‡		3	1	2
17	*TAZ*	p.Phe128Ser (J)‡				1
	LMNA	1968+26A>G (K)‡	1			
18	*MYH7*	p.Tyr266X	1			
19†	*MYH7*	p.Arg369Gln				
20	*ACTC1*	p.Met271Val	1			2
21	*TPM1*	p.Arg160His				1
22	*MYBPC3*	c.2373dupG (L)‡	1		1	
	MYBPC3	p.Gly148Arg (M)‡	2		1	3
23	*TNNI3*	c.241-12_241-11delinsTG (N)‡		1	2	1
	TPM1	p.Asp180Gly (O)‡			2	2

*Legend. * Family number corresponds with pedigree number in Figure 4; † No family studies were performed in probands 7, 15 and 19; ‡ Capitols in brackets refer to capitols in Figure 4.*

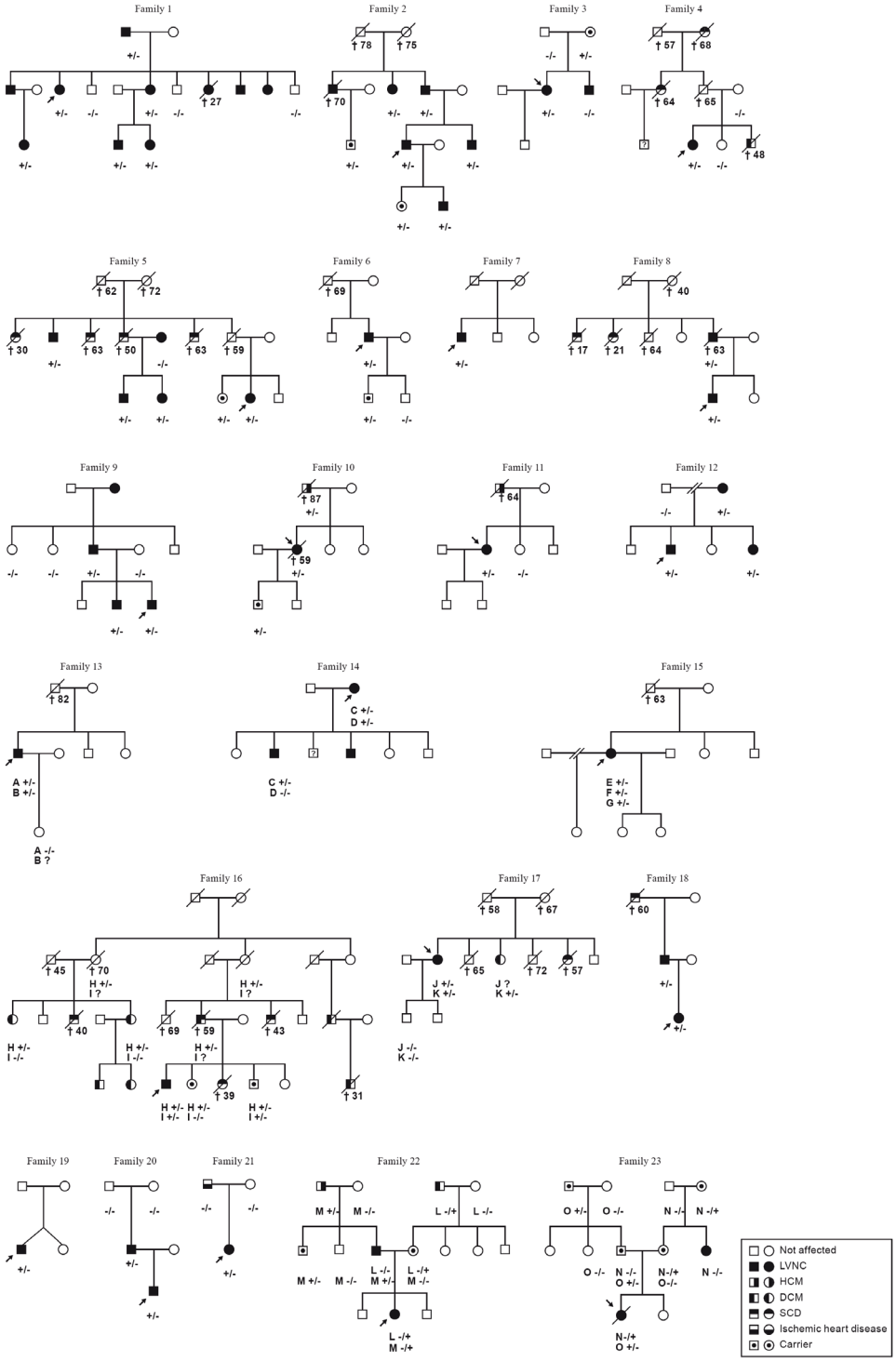


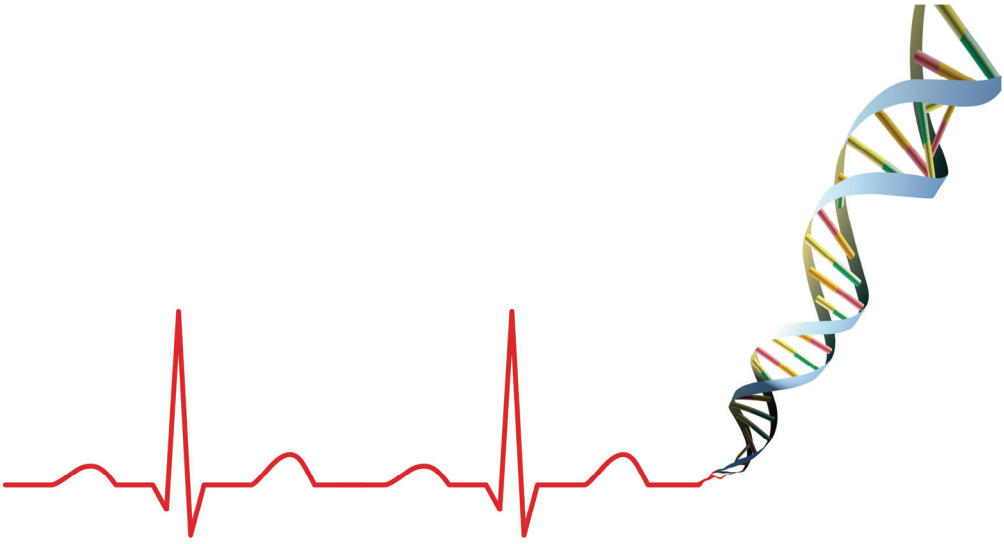
Figure 4. Pedigrees of genotyped families.
Numbers correspond with numbers in the description of the families.

Chapter 4

Noncompaction cardiomyopathy: disease genes, mutation spectrum and diagnostic implications.

Dennis Dooijes; Yvonne M. Hoedemaekers, Michelle Michels, Jasper J. van der Smagt, Raoul van de Graaf, Marianne van Tienhoven, Folkert J. ten Cate, Kadir Caliskan, Danielle F. Majoor-Krakauer.

Submitted



Abstract

Background

Noncompaction cardiomyopathy (NCCM) is characterised by an excessively thickened endocardial layer with deep intertrabecular recesses. Despite partial overlap in causal genes between NCCM and other cardiomyopathies, a genetic classification of NCCM is currently lacking.

Methods and results

Seventeen genes (*MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3*, *TCAP*, *LDB3, CASQ2*, *CALR3*, *PLN*, *TAZ* and *LMNA*) were completely analysed in a cohort of 56 NCCM patients (48 adults and 8 children). These genes encode proteins of the sarcomere, Z-disc, Calcium-handling system and cellular matrix which, when mutated, cause cardiomyopathy.

Twenty-nine mutations, including twenty-one new ones, were identified in 41% of the patients in *MYH7* (11), *MYBPC3* (4), *TNNT2* (3), *TNNI3* (1), *TPM1* (2), *ACTC1* (1), *CASQ2* (2), *PLN* (1), *TAZ* (1), *LDB3* (2) and *LMNA* (1). Thirty-five percent of the adult patients had a mutation. Seventy-five percent of the paediatric patients had a sarcomeric mutation. Eighteen probands had a single mutation, four had two and one had three mutations. Prognosis in families with an identified mutation was worse compared to prognosis in non-genotyped families.

Conclusions

We identified *MYBPC3, TNNI3, TPM1, PLN* and *CASQ2* as novel NCCM genes. Genetically, NCCM classifies primarily as a sarcomeric disease and part of a spectrum including hypertrophic and dilated cardiomyopathy. The high frequency of mutations in adult- and childhood-onset NCCM warrants genetic counselling and molecular analysis, starting with *MYH7*. Identification of the genetic cause allows accurate identification of relatives at risk for cardiomyopathy.

Introduction

Noncompaction cardiomyopathy (NCCM) is characterised by hypertrabeculation and deep intertrabecular recesses of the left ventricular myocardium, reminiscent of the embryonic stages of cardiac development.[1, 2] Cardiac symptoms include heart failure, lethal arrhythmias and/or thrombo-embolic complications. Initially, NCCM was perceived to be an extremely rare disease with a poor prognosis and an estimated prevalence of 0.045% to 0.14%.[3, 4] Recent studies report a prevalence up to 5% depending on the study population and stringency of diagnostic criteria.[5, 6] In childhood, NCCM is the most commonly observed cardiomyopathy after hypertrophic (HCM) and dilated cardiomyopathy (DCM).[7] The echocardiographic as well as MRI diagnostic NCCM criteria presented in recent years are under discussion.[2, 8-11] However, the diagnostic criteria as proposed by Jenni and co-workers remain most widely used.[2]

Whether NCCM should be regarded as a separate cardiomyopathy has not yet been established. After the initial WHO official classification of cardiomyopathies[12], the latest classification updates from the American Heart Association (AHA)[13] and the European Society of Cardiology (ESC)[14] hold different views on NCCM. The ESC classification considers NCCM unclassified since there is currently no consensus whether NCCM is a separate cardiomyopathy or a morphological trait occurring separately or in combination with another cardiomyopathy or congenital cardiac malformations.[14] The AHA regards NCCM as a separate primary genetic cardiomyopathy since the disease predominantly involves the myocardium and has a genetic aetiology.[13]

Rare genetic defects associated with NCCM include mutations in taffazin (TAZ), Cypher/ZASP (*LDB3*) and Lamin A/C (*LMNA*).

The first sarcomeric NCCM mutations were reported in the β-myosin heavy chain (*MYH7*) gene in two families with isolated NCCM.[15] Subsequent studies confirmed the role of *MYH7* defects in the aetiology of NCCM and also implicated sarcomeric cardiac troponin T (*TNNT2*) and cardiac actin (*ACTC1*) as disease genes in NCCM.[16-18] However, a genetic classification for NCCM is currently lacking.

The goal of this study was to establish a genetic classification for NCCM. This is important because a classification including the frequencies of genetic causes and the mutation spectrum is instrumental in directing diagnostic screening efforts, facilitate genotype-phenotype analyses and may contribute to the elucidation of the pathophysiology of this currently not fully understood disease.

We therefore performed a complete analysis of 17 genes, including six genes previously associated with NCCM, in a cohort of 56 unrelated NCCM patients. Because familial aggregation of NCCM, HCM and DCM suggests a shared genetic susceptibility for these forms of cardiomyopathy, eleven additional HCM and DCM genes were selected and analysed as NCCM candidate genes, although such a selection can neither be objective nor complete. Most known sarcomeric cardiomyopathy genes were included and, in addition, some known cardiomyopathy genes from the Z-disc and calcium-handling system were analysed.

Methods

Patients

The study population comprised 56 unrelated patients with isolated NCCM. Coronary artery disease and hypertension were excluded in all patients. Patients were diagnosed from 2005-2008 at the Thorax centre of the Erasmus MC Rotterdam, a tertiary cardiology referral centre and fulfilled all four echocardiographic diagnostic Jenni criteria for NCCM: a) excessively thickened LV myocardial wall with a two-layered structure comprising a compact epicardial layer (C) and a noncompacted endocardial layer (NC) of prominent trabeculations and deep intertrabecular recesses; b) maximal end-systolic NC/C ratio > 2 measured at the parasternal short axis; c) colour-Doppler evidence of deep perfused intertrabecular recesses; d) absence of coexisting cardiac anomalies.[2] All patients were referred for genetic counselling, DNA analysis and to initiate family studies. The study was approved by the institutional ethics committee and all subjects gave informed consent.

Genetic analysis

Complete sequence analysis of all coding regions and intron-exon boundaries of the following genes was performed:

- **Sarcomeric genes**: β-myosin heavy chain (*MYH7*; NM_000257.2), myosin binding protein C (*MYBPC3*; NM_000256.3), cardiac troponin C (*TNNC1*; NM_003280.1), cardiac troponin T (*TNNT2*; NM_000364.2), cardiac troponin I (*TNNI3*; NM_000363.4), cardiac-regulatory myosin light chain (*MYL2*; NM_000432.3), cardiac-essential myosin light chain (*MYL3*; NM_000258.2), cardiac α-actin (*ACTC1*; NM_005159.4), α-tropomyosin (*TPM1*; NM_000366.5).
- **Calcium-handling genes**: calsequestrin (*CASQ2*; NM_001232.3), calreticulin (*CALR3*; NM_145046.3) and phospholamban (*PLN*; NM_002667.3).
- **Z-disc genes**: cysteine- and glycine-rich protein (*CSRP3*; NM_003476.2), theletonin (*TCAP*; NM_003673.2) and LIM domain binding 3/Cypher/ZASP (*LDB3*; NM_007078.2 and NM_001080116.1)
- **Other**: taffazin (*TAZ*; NM_000116.2) and lamin A/C (*LMNA*; NM_170707.2 and NM_005572.3).

Sequence analysis was carried out on an ABI3730xl capillary sequencer using Big-Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of methods and primer sequences available on request.) Analysis of sequence data was performed using SeqScape analysis software (v2.5, Applied Biosystems). Unknown missense variants were considered a mutation when they segregated with disease in a family *and* were not present on 384 healthy ethnically matched control chromosomes *and* were pathogenic according to prediction software.[19, 20] Although this does not formally prove pathogenicity, these three criteria combined were thought to provide the best evidence for pathogenicity. In case of isolated mutations, segregation with

disease could not be used as a criterion. DNA variants not fulfilling these criteria were labelled unclassified variants (UV).

Prognostic classification

The prognosis in families was classified based on the family history at the time of diagnosis.[21] Probands were excluded from the analysis. Prognosis was considered malignant when two or more major cardiac events (MCE, i.e. sudden cardiac death (SCD), stroke, resuscitation or appropriate discharge of an intracardial defibrillator (ICD)), before the age of 60 years were confirmed in a family. One documented MCE in a family led to a classification of intermediate prognosis. Without confirmed familial MCE, prognosis was considered to be benign.

Results

The complete coding sequences of 17 genes; six genes previously associated with NCCM and 11 NCCM candidate genes, were analysed in a cohort of 56 unrelated NCCM patients: 48 adults and 8 children. Disease causing mutations were identified in 23 (41%) patients. Five of these 23 patients (22%) had multiple mutations.

Twenty-nine NCCM mutations, including 21 novel ones, were identified in 11 different genes (Figure 1): *MYH7, MYBPC3, TNNT2*, *TNNI3*, *ACTC1, TPM1, CASQ2, PLN, LMNA, LDB3* and *TAZ*. No mutations were observed in the *MYL2, MYL3, TNNC1, CSRP3, TCAP* and *CALR3* genes. Twenty-two (76%) of the identified mutations were found in sarcomeric genes and seven mutations (24%) were present in genes of the Z-disc, Calcium-handling system and nuclear and mitochondrial lamina (Table 1).

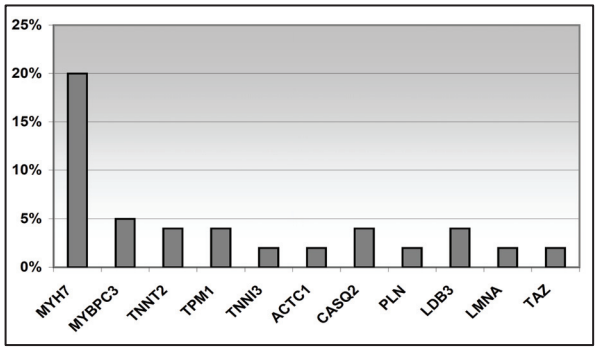


Figure 1. Genetic NCCM in 56 patients

The most frequently involved gene was *MYH7*, mutated in 11/56 (20%) patients demonstrating that this gene is the sole most important genetic factor thus far for NCCM (Figure 1). The *MYBPC3* gene was mutated in 3 patients (5%). Mutations in *LDB3, CASQ2, TNNT2* and *TPM1* each occurred in 2 patients (4%). Mutations in *TNNI3, ACTC1, LMNA, PLN* and *TAZ* were the least frequent in NCCM (2%). As a consequence of complex genotypes, twenty-one mutations were identified in seventeen adult patients and eight mutations were found in six children. The relative contribution of each disease gene to NCCM was analysed by looking at the distribution of the 29 identified mutations in 23 patients (Table 1). Thirty-eight (11/29) percent of the mutations were observed in *MYH7*. Among the patients with an *MYH7* mutation, no complex genotypes were observed. Fourteen percent (4/29) of the mutations were found in *MYBPC3* and 10% of the mutations were in *TNNT2*. Seven percent of the mutations were found in *TPM1, CASQ2* and *LDB3* respectively.

Table 1. Distribution of mutations in 23 genotyped NCCM patients.

Protein function	Gene	Number of mutations found in:					
		Adults (n = 17)	Children (n = 7)	Single Genotype	Complex Genotype	Novel mutations	Total mutations
Sacromere	*MYH7*	9	2	11		10	11 (38%)
	MYBPC3	2	2	2	2	2	4 (14%)
	TNNT2	3			3	3	3 (10%)
	TNNI3		1		1	1	1 (3%)
	ACTC1		1	1		1	1 (3%)
	TPM1		2	1	1	2	2 (7%)
Ca-handling	*CASQ2*	2		1	1	1	2 (7%)
	PLN	1		1			1 (3%)
Z-disc	*LDB3*	2			2		2 (7%)
Nuclear membrane	*LMNA*	1			1		1 (3%)
Mitochondria	*TAZ*	1		1		1	1 (3%)
Total Mutations		20	9	18	11	21	29

Three percent of the mutations were present in *TNNI3, ACTC1, PLN, LMNA* and *TAZ*. Mutations in *TNNT2, TNNI3, LDB3, LMNA* and *TAZ* were only observed in complex genotypes. The six children with a mutation all had a sarcomeric mutation. Non-sarcomeric mutations were only detected in adult NCCM patients (Table 1). The complex genotypes in adult patients comprised combinations of sarcomeric mutations and mutations in non-sarcomeric genes.

NCCM mutations in sarcomere genes

Twenty-two of the 29 (76%) mutations were found in sarcomeric genes (Tables 1 and 2) (Families 1-10, 13-15 and 18-23 of the addendum of chapter 3). Analysis of *MYH7* resulted in the identification of eleven mutations including ten new mutations. Seven *MYH7* mutations were missense mutations. In addition, one previously described double-missense mutation (p.Asp545Asn/p.Asp955Asn *in cis,* Table 3)[15], a splice-site mutation (c.732+1G>A), a single amino acid deletion (p.Glu1350del) and a nonsense mutation (p.Tyr266X) were detected. The p.Tyr266X mutation is predicted to lead to a severely truncated MYH7 protein of 265 amino acids. Alternatively, the mechanism of nonsense mediated decay may interfere with MYH7 protein formation from the mutated allele.[22]

Four *MYH7* mutations, c.732+1G>A, p.Tyr266X, p.Leu301Gln and p.Arg369Gln, cluster in the ATP-ase active site of the globular head-region on the amino-terminal part of MYH7 (Figure 2). Mutations in this region were recently associated with NCCM with or without Ebstein anomaly.[16, 17] The four *MYH7* mutations p.Glu1350del, p.Tyr1488Cys, p.Asn1918Lys and p.Arg1925Gly, were found in the carboxy-terminal rod-region of the MYH7 protein which is normally relatively void of cardiomyopathy mutations. The p.Arg1925Gly mutation was recently also seen in an apparently unrelated NCCM patient, diagnosed after completion of this study.

Table 2. NCCM mutations and unclassified variants in sarcomeric genes.

Gene	Nucleotide	Protein	Consequence	No. of patients	Ref.
ACTC1	**c.811A>G**	**p.Met271Val**	Missense	1	
	**22C>T*		(3'UTR) rs28730667	1	
MYBPC3	**c.442G>A**	**p.Gly148Arg**	Missense	1	
	c.646G>A	**p.Ala216Thr**	Missense	1	
	c.2373dupG	p.Gly791fs	Truncating	2	23
MYH7	**c.732+1G>A**		Splice-donor site	1	
	c.798T>A	**p.Tyr266X**	Nonsense	1	
	c.902T>A	p.Leu301Gln	Missense	1	15
	c.1106G>A	**p.Arg369Gln**	Missense	1	
	c.1633G>A and c.2863G>A *in cis*	p.Asp545Asn and p.Asp955Asn *in cis*	Missense	1	15
	c.1972C>G	**p.Leu658Val**	Missense	1	
	c.2453T>A	**p.Ile818Asn**	Missense	1	
	c.4048_4050delGAG	**p.Glu1350del**	Amino-acid deletion	1	
	c.4463A>G	**p.Tyr1488Cys**	Missense	1	
	c.5754C>G	**p.Asn1918Lys**	Missense	1	
	c.5773C>G	**p.Arg1925Gly**	Missense	1	
TNNI3	**c.539A>G**	**p.Asp180Gly**	Missense	1	
TNNT2	**c.260C>T**	**p.Pro87Leu**	Missense	1	
	c.482G>A	**p.Arg161His**	Missense	1	
	c.673G>T	**p.Val225Leu**	Missense	1	
TPM1	**c.241-12_241-11delinsTG**		Splice-acceptor site	1	
	c.479G>A	**p.Arg160His**	Missense	1	

Legend. Sarcomeric NCCM mutations and unclassified variants identified in this study. Novel mutations are in bold. Unclassified variants are in italics.

Table 3. Sarcomeric NCCM mutations reported in previous studies.

Gene	Nucleotide	Protein	Consequence	Ref.
ACTC1	c.478G>A	p.Glu101Lys		17, 18
MYH7	c.801_803delGAC	p.Asp239del		17
	c.814G>A	p.Arg243His		17
	c.818+1G>A			17
	c.818+3G>C			17
	c.842G>C	p.Arg281Thr		16
	c.840T>C	p.Phe252Leu		17
	c.902T>A	p.Leu301Gln		15
	c.1633G>A and c.2863G>A in cis	p.Asp545Asn and p.Asp955Asn in cis		15
	c.4161C>T	p.Arg1359Cys		17
	c.5382G>A	p.Ala1766Thr		17
TNNT2	c.450C>T	p.Arg131Trp		16

MYBPC3 mutations were identified in three patients, including the Dutch c.2373dupG founder mutation in two patients.[23] One had the p.Gly148Arg mutation on the other *MYBPC3* allele. The Glycine residue at position 148 is conserved in mammals. The *MYBPC3* p.Ala216Thr mutation was identified in another patient. The Alanine residue at position 216 is conserved in most mammal species.

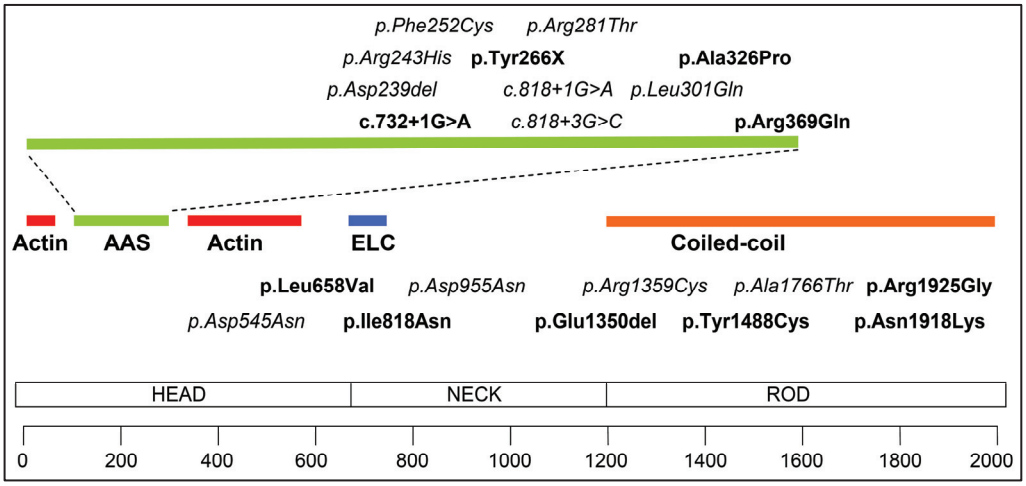


Figure 2. Currently identified NCCM mutations in *MYH7*.
Mutations identified in this study are in bold. AAS: ATPase active site; ELC: myosin essential light chain binding site.

Three *TNNT2* mutations were identified in two NCCM patients. One NCCM patient had the p.Pro87Leu mutation. The Proline at position 87 is conserved up to nematode C. elegans. The other patient had two *TNNT2* mutations, the p.Arg161His mutation and p.Val225Leu. We were unable to determine the phase of the two *TNNT2* variants since no relatives were available for testing. P.Arg161His is likely pathogenic because the Arginine at 161 is highly conserved up to fruit fly and a mutation affecting the same amino acid has been reported (rs45608937; c.481C>T, p.Arg161Cys). The p.Val225Leu mutation affects the relatively weakly conserved Valine residue at position 225 and is predicted to be non-pathogenic.[19, 20]

TPM1 mutations were identified in two patients. The p.Arg160His mutation was identified as *de novo* mutation in a 16-year old NCCM patient who received a heart-transplant at age 17. The Arginine residue at position 160 is conserved in all mammals. The other *TPM1* mutation c.241-12_241-11delinsTG was found in combination with the *TNNI3* mutation p.Asp180Gly in a four months old patient. The *TNNI3* mutation p.Asp180Gly affects the Aspartic acid residue at position 180 which is conserved up to Platypus.

The *ACTC1* mutation p.Met271Val was found in a child that was diagnosed with NCCM one month after birth. The mutation was *de novo* in her father who was diagnosed with a dilated left and right ventricle without evident crypts on echocardiography. Most *ACTC1* amino acid substitutions are poorly tolerated and overall cross-species conservation of protein sequence is one of the highest known. The Methionine at position 271 is conserved up to C. elegans.

NCCM mutations in non-sarcomere genes

Seven of the 29 (24%) mutations were found in non-sarcomeric genes (Tables 1 and 4) (Families 11, 12, 14-17, *A*, *B* and *C* of the addendum of Chapter 3). Analysis of Calcium-handling genes resulted in the identification of p.Arg14del in the phospholamban (*PLN*) gene in

an adult NCCM patient. This mutation was previously associated with hereditary heart failure and dilated cardiomyopathy.[24, 25] *CASQ2* DNA variants were found in four NCCM patients. Two had the p.Asp398del variant, which removes an Aspartic acid residue at the C-terminal part of the CASQ2 protein. This variant was not found in control chromosomes. Although p.Asp398 is part of a conserved stretch of 5 Aspartic acid residues, this part of the CASQ2 protein is weakly conserved between species. This variant did not segregate with disease in the two families of the patients, contesting its pathogenicity.

Table 4. NCCM mutations and unclassified variants in sarcomeric genes.

Gene	Nucleotide	Protein	Consequence	No. of patients	Ref.
CASQ2	c.731A>G	p.His244Arg	Missense rs28730716	2	
	c.1194_1196delTGA	***p.Asp398del***	Amino-acid deletion	2	
PLN	c.40_42delAGA	p.Arg14del	Amino-acid deletion	1	24, 25
CSRP3	*c.*12G>A*		(3'UTR) rs45607943	1	
LMNA	1608+5G>C		Splice-donor site disruption	1	26
	1968+26A>G		Splice-acceptor site?	2	
LDB3	c.349G>A	p.Asp117Asn	(Isoform 4)	2	27
	c.352G>A	*p.Val118Met*	(isoform 1) rs35507268	1	
	c.611A>G	*p.Lys204Arg*	(Isoform 4) rs34423165	1	27
	c.549-4A>G		(Isoform 4) rs45529531	1	
TAZ	**c.383T>C**	**p.Phe128Ser**	Missense	1	

Legend. Non-sarcomeric NCCM mutations and unclassified variants identified in this study. Novel mutations are in bold. Unclassified variants are in italics.

Two NCCM patients had the p.His244Arg mutation in *CASQ2.* The Histidine at position 244 lies in the thioredoxin-like fold of CASQ2 and is highly conserved up to birds. The substitution for Arginine at this position is predicted to be pathogenic.[19, 20] The *PLN* and *CASQ2* mutations are the first mutations in Calcium-handling genes reported to be associated with NCCM. Analysis of *LMNA* resulted in the identification of c.1608+5G>C in a patient with relatives diagnosed with NCCM or DCM. This mutation was associated with limb girdle muscular dystrophy with cardiologic involvement (LGMD1B).[26] In two other NCCM patients, the *LMNA* variant c.1968+26A>G was detected which is predicted to lead to an aberrant splice-acceptor site. Since this variant was also observed twice in control chromosomes it was considered a UV. Analysis of *LDB3* resulted in the identification of the p.Asp117Asn mutation in two unrelated NCCM patients. This mutation was previously shown to be present in cardiac specific Cypher/ZASP isoforms and have a disturbing effect on cellular architecture in transfected C2C12 cells.[27] A taffazin gene (*TAZ*) mutation, p.Phe128Ser, was detected in a 57 years old female patient. There is a large physicochemical difference ('Grantham distance') between Phenylalanine and Serine residues and this mutation was not observed on 384 control chromosomes. Previously reported non/sarcomeric]nccm mutations are listed in Table 5.

Paediatric patients

Eight (14%) of the 56 patients were diagnosed in childhood (<18 years). Six (75%) of these paediatric patients had a sarcomeric mutation, including four single mutations and two complex

sarcomeric genotypes (Tables 1 and 6). Two patients aged four and 10 years had an *MYH7* mutation, one six-weeks old patient had an *ACTC1* mutation and one 16-year-old patient had a *TPM1* mutation.

Table 5. Previously reported non-sarcomeric NCCM mutations.

Gene	Nucleotide	Protein	Consequence	Reference
DTNA	c.362C>T	p.Pro121Leu		31, 34
LMNA	c.368C>T	p.Arg109Trp		30
LDB3	c.349G>A	p.Asp117Asn	Disruption cytoarchitecture	27
	c.587C>T	p.Ser196Leu		27
	c.1876G>A	p.Asp626Asn	Altered PKC binding affinity	34, 35
TAZ	c.157dupC	p.Leu53fs		34
	c.352C>T	p.Cys118Arg		31, 36
	c.589G>A	p.Gly197Arg		37
	c.647-1G>C		Exon 9 skipping	34, 38
	c.777+2T>A		Exon 10 skipping	31, 32, 36

Complex genetic status

Multiple mutations were observed in five of the 23 genotyped NCCM patients: one had three mutations and four patients had two mutations. In addition, two patients had a mutation in combination with a UV (Table 6). One patient had the p.Asp545Asn and p.Asp955Asn *MYH7* mutations. Since these mutations were previously shown to be allelic, the patient was not considered to be of complex genetic status.[15] One paediatric patient had the *MYBPC3* c.2373dupG mutation on one and the p.Gly148Arg mutation on the other *MYBPC3* allele. We have recently found p.Gly148Arg associated with the *MYBPC3* p.Ser311X nonsense mutation *in trans* in two sisters with early onset HCM (onset ~16 years). One patient, diagnosed at the age of 3 months who died at the age of four years, had the *TPM1* mutation c.241-12_241-11delinsTG in combination with the *TNNI3* mutation p.Asp180Gly.

One patient presenting with peripartum cardiomyopathy had two *TNNT2* mutations, p.Arg160His and p.Val225Leu, in combination with a third mutation, the *CASQ2* mutation p.His244Arg. One adult patient had the *TNNT2* p.Pro87Leu mutation and the *LDB3* mutation p.Asp117Asn. An unrelated NCCM patient from a family with LGMD1B had the same *LDB3* mutation p.Asp117Asn in addition to the *LMNA* mutation c.1608+5G>C. His brother, who had ECG abnormalities but no signs of NCCM on echocardiography or MRI, also had these two mutations. Other relatives diagnosed with DCM or LGMD1B had only the *LMNA* mutation.

Table 6. Clinical features of probands with complex genotypes.

Patient	1	2	3	4	5	6	7
Mutation*							
TNNI3	p.Asp180Gly						
TPM1	c.241-12_241-11delinsTG						
MYBPC3		c.2373dupG / p.Gly148Arg	p.Ala216Thr				
ACTC1			*22C>T*				
TNNT2				p.Arg161His / p.Val225Leu	p.Pro87Leu		
CASQ2				p.His244Arg			
LDB3					p.Asp117Asn	p.Asp117Asn	
LMNA						c.1608+5G>C	*c.1968+26A>G*
TAZ							p.Phe128Ser
Age of diagnosis (yrs)	0,33	7	50	34	45	42	57
NYHA†	III	IV	II	III	III	II	II
NC/C ratio‡			2.1	2.2	2.2	2.8	2.2
Cardiovascular events§	CI	CI, HTX	ICD			ICD	

*Legend. *Unclassified Variants are in italics. †NYHA = New York Heart Association classification. ‡NC/C ratio: ratio of noncompacted to compacted wall. §CI = cerebral infarctions; HTX = heart transplant; ICD = implantable cardioverter defibrillator. For completeness also patients with a pathogenic mutation and, in addition, an unclassified variant were included in the table (patients 3 and 7).*

Prognosis

Ascertainment of family histories allowed us to investigate prognosis in 55 families stratified for genotype including a sub classification in *MYH7* and other mutations (Table 7). Sixty-eight percent of non-genotyped families had a benign prognosis and 22% had a malignant prognosis. In contrast, 27% of the genotyped *MYH7* families had a benign prognosis and 45% had a malignant prognosis. Families with non-*MYH7* mutations showed intermediate results, 58% had a benign prognosis and 33% a malignant prognosis. These results indicate that in families with a malignant prognosis the chance to identify a pathogenic (*MYH7*) mutation is increased compared to families with a less severe prognosis. Pathogenic *MYH7* mutations are associated with a more malignant prognosis than mutations in other genes or NCCM caused by yet unidentified causes (non-genotyped) (p=0.112).

Table 7. Prognosis and genotype in NCCM families.

Genotype	Total probands n (%)*	Prognosis		
		Benign n(%)*	Intermediate n(%)*	Malignant n(%)*
MYH7	11 (20)	3 (10, 27)	3 (37, 27)	5 (31, 45)
Other genes	12 (21)	7 (23, 55)	1 (13, 8)	4 (25,33)
Non-genotyped	32 (58)	21 (68, 66)	4 (50, 13)	7 (44,22)
Total	55†	31	8	16

*Legend. Prognosis was determined by the number of major cardiac events (MCE) before the age of 60 yrs in the family. * (% of column, % of row). † Prognosis was not determined in the family of one proband.*

Discussion

To establish a genetic classification and enhance molecular diagnosis for NCCM, a complete analysis of 17 cardiomyopathy genes was performed in a cohort of 56 isolated NCCM patients. Mutations were found in the *MYH7, MYBPC3, TNNT2, TNNI3, ACTC1, TPM1, CASQ2, PLN, LDB3, LMNA* and *TAZ* genes. *MYBPC3, TNNI3, TPM, PLN* and *CASQ2* were identified as new NCCM genes. This is the first report associating the Calcium-handling system genes *PLN* and *CASQ2* with NCCM. Disease causing mutations were identified in 41% of the patients (in 35% of adult patients and 75% of children), making this the highest reported percentage of genotyped NCCM thus far. This is also the first genetic study of NCCM in childhood, indicating that childhood NCCM is predominantly a sarcomeric disease.

Hypertrophic cardiomyopathy is mainly caused by mutations in genes encoding sarcomeric proteins. In large patient series, a pathogenic mutation is identified in up to 65% of the HCM patients by screening the same nine sarcomeric genes that were analysed in this study.[21] The most prevalent disease genes in HCM are *MYBPC3* and *MYH7,* each accounting for approximately 40% of the mutations.[21, 28] The genetic basis of DCM is more heterogeneous and includes, in addition to sarcomeric mutations, mutations in genes encoding proteins of intracellular and extracellular matrix as well as the nuclear and mitochondrial lamina. Extensive molecular analysis in patients reveals a defect in approximately only 30% of DCM.[29] The

present study shows that NCCM, HCM and DCM share a number of genetic causes. Extensive genetic screening results in the identification of a mutation in 41% of the patients, an *MYH7* mutation in half of these patients and only a modest contribution of the other disease genes. Previously described non-sarcomeric genetic causes for NCCM include mutations in the *TAZ, LMNA* and *LDB3* genes.[27, 30, 31] Mutations in these genes have however been shown to be only rare causes of NCCM and their relevance is mostly limited to single families.[32] After the initial report associating sarcomeric *MYH7* mutations with isolated NCCM[15], the importance of *MYH7* mutations was confirmed and the *ACTC1* and *TNNT2* genes were implicated in the aetiology of NCCM.[16-18] Klaasen et al., identified a sarcomeric *MYH7, TNNT2* or *ACTC1* mutation in 17% of NCCM patients.[17] The higher percentage (41%) of genotyped NCCM patients in the present study may partially be explained by the larger number of genes analysed (17 vs. 6) and a higher percentage of paediatric patients in this study. The percentages of *MYH7* mutations in the study of Klaassen et al. and the present study (13% and 20% respectively), indicate that *MYH7* is the most important genetic cause for isolated NCCM in adults as well as in children.[17]

MYH7 mutations are common in familial forms of cardiomyopathy, including HCM, DCM, RCM and distal myopathies with cardiac involvement. Most mutations leading to the different cardiomyopathies are located in the head and neck regions, much more than in the rod region. Remarkably, 50% (10/20, Figure 2) of the *MYH7* mutations currently associated with NCCM, cluster in the ATPase active site in the head region of MYH7. This is an evolutionary well-conserved region of MYH7. As the ATPase active site is required for normal force production, impaired force generation might play a role in the aetiology of NCCM. Six other *MYH7* mutations (30%) associated with NCCM were located in the rod domain of the protein. This light meromyosin (LMM) part of the protein adopts an α-helical coiled coil-structure that forms the core of the thick filament. Mutations in this domain could perturb thick filament assembly which may also be important in the aetiology of NCCM.[33]

Five patients (21%) had multiple mutations. Two patients with complex genetic status were diagnosed in childhood and had a severe presentation. These findings indicate that, especially in patients with a severe presentation and/or early onset of disease, performing a complete molecular screening of all NCCM genes even when a pathogenic mutation has been found is recommended. Ascertainment of complex genetic status is important to determine risk for relatives and may help to understand genotype-phenotype relations. Our results confirm that mutations in non-sarcomeric genes, including mutations in the *TAZ, LMNA* and *LDB3* genes, are relatively infrequent genetic causes for NCCM.[27, 30, 31] Absence of a mutation after genetic screening in approximately half of NCCM could be explained by involvement of other yet unidentified disease genes, undetected mutations in non-analysed gene sequences, diagnostic errors or incomplete sensitivity of the methods used. Alternatively, the possibility of non-genetic forms of NCCM or the involvement of somatic mutations cannot be excluded.

We found that in families of patients in which we identified a mutation, genotype-positive NCCM was associated with a less favourable prognosis. This finding was not statistically significant

due to limited patient numbers. The observation could partly be explained by the presence of non-genetic forms of NCCM among the genotype-negative patients.

Similar to the other cardiomyopathies, NCCM is mainly caused by mutations in genes that encode various structural cardiac proteins. Rather than being a separate disease entity, this study demonstrates that NCCM genetically classifies in the aetiologic spectrum of cardiomyopathies also including HCM and DCM since, sometimes identical, mutations in shared disease genes can lead to these different phenotypes. HCM and NCCM are primarily diseases of the sarcomere but the relative importance of disease genes differs between these two diseases and NCCM mutations are also found in disease genes previously only associated with DCM. The large number of genetic defects identified thus far in isolated NCCM may lead to inclusion of NCCM as a genetic cardiomyopathy in future cardiomyopathy classifications as suggested by the AHA.[33]

In conclusion, we report a systematic molecular genetic screen in a large cohort of isolated NCCM patients. NCCM is predominantly a genetic disease with NCCM associated disease seen in families of 67% of NCCM patients (Chapter 3). Like HCM, NCCM is primarily a disease of the sarcomere. Extensive genetic screening may lead to the identification of a molecular defect in 41% of isolated NCCM patients and in half of these patients an *MYH7* mutation is found. Other disease genes have a relatively modest contribution to the disease. These results indicate that in NCCM, like in the other cardiomyopathies, genetic analysis is recommended. Initial screening of the *MYH7* gene in NCCM patients should be considered as an initial approach, possibly followed by the analysis of the other genes of the NCCM spectrum. In paediatric patients, sarcomeric gene analysis is warranted given the high percentage of sarcomeric mutations in these patients. The identification of genetic causes of NCCM could enhance diagnosis, risk assessment and counselling in NCCM families.

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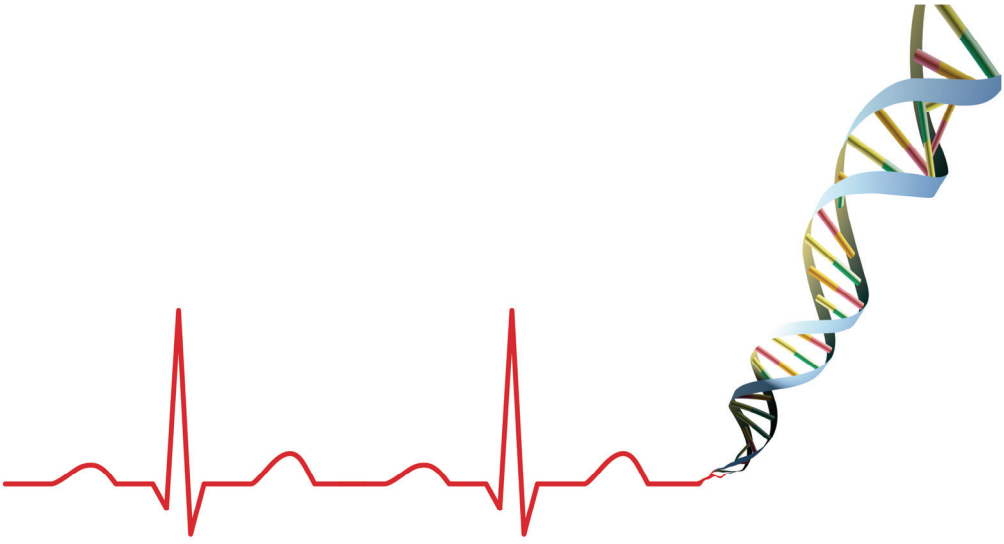
Chapter 5

Prenatal diagnosis of *MYH7* associated noncompaction cardiomyopathy.

Yvonne M. Hoedemaekers, Ernst M. Schoonderwaldt, Ingrid M.E. Frohn-Mulder, Michiel Dalinghaus, Michelle Michels, Kadir Caliskan, Marjon A. van Slegtenhorst, Dennis Dooijes, Titia Cohen – Overbeek, Danielle F. Majoor-Krakauer.

Submitted



Abstract

Introduction

Noncompaction cardiomyopathy (NCCM), characterised by localised endocardial thickening consisting of a meshwork of trabecularisation of the left ventricular wall, can present with heart failure, thombo-embolism and sudden death. The majority of NCCM is isolated and predominantly familial. NCCM was associated with defects in sarcomere genes, particularly the ß-Myosin Heavy Chain (*MYH7*) gene.

Methods and results

We report the first two individual cases of prenatally diagnosed NCCM caused by a mutation in the *MYH7* gene. Biventricular cardiomegaly led to the diagnosis of NCCM at 31 and at 23 weeks of pregnancy respectively. The parents were unaware of familial cardiomyopathy. However, subsequent cardiologic family screening and molecular diagnostics identified *MYH7* cardiomyopathy in several members in both families.

Conclusions

Prenatal NCCM with biventricular enlargement may occur in familial *MYH7* cardiomyopathy. In accordance with previous reports, the prognosis of prenatally diagnosed NCCM predominantly depends on cardiac function and may improve after birth. Since *MYH7* mutations may present with prenatal onset, prenatal ultrasound of pregnancies at risk for *MYH7* cardiomyopathy is recommended. Familial disease may initially present with a prenatal onset, molecular diagnostics and cardiologic screening of first-degree relatives are therefore necessary even in absence of a family history of cardiomyopathy.

Introduction

Noncompaction of the left ventricle or noncompaction cardiomyopathy (NCCM) was first described by Feldt et al in 1969.[1] It is characterised by a prominent trabecular meshwork and deep intertrabecular recesses communicating with the left ventricular (LV) cavity, morphologically reminiscent of early cardiac development. Therefore NCCM was thought to be caused by an arrest of normal embryogenesis of the myocardium.[2, 3] NCCM, alternatively referred to as left ventricular noncompaction (LVNC) in the literature, is the third most prevalent cardiomyopathy in childhood after hypertrophic (HCM) and dilated cardiomyopathy (DCM), with an estimated prevalence of 9% in paediatric cardiomyopathies.[4]

NCCM can present at all ages with heart failure, (potentially lethal) arrhythmia or stroke. A large proportion of NCCM in adults is asymptomatic and is diagnosed by chance at routine medical screening or by family studies with cardiologic or molecular screening, following the diagnosis of cardiomyopathy in a relative. The majority of NCCM diagnosed in adults is isolated. Non-isolated forms of NCCM are more frequent in childhood and co-occur with congenital heart malformations, neurological disorders, as part of a malformation or chromosomal syndrome, or are caused by metabolic or mitochondrial defects.[5] In the majority of patients NCCM, isolated and non-isolated, is hereditary. NCCM appears to be genetically heterogeneous, and is predominantly caused by sarcomere gene defects. A recent study showed that 75% of children diagnosed with isolated NCCM had a mutation in sarcomere genes that are also involved in HCM, DCM and restrictive cardiomyopathy (RCM). Similar to other sarcomere cardiomyopathies, in NCCM genetic and cardiac screening of first-degree relatives is therefore recommended. Absence of a known genetic defect does not preclude a genetic cause of NCCM. In approximately half of familial isolated NCCM diagnosed in adults the genetic defect remains unknown. Shared sarcomere defects and the occurrence of HCM and DCM in families of NCCM patients indicate that, at least some forms of NCCM are part of a broader cardiomyopathy spectrum.

A review of the literature of prenatal diagnosis of NCCM is presented in this study and indicates that none of the previous cases had been genotyped.[6-20] We report the first two prenatal diagnosis of NCCM caused by a sarcomere gene defect.

Patient 1

A 30-year-old healthy Caucasian woman, was referred in her first pregnancy for prenatal ultrasound to the Department of Obstetrics and Prenatal Medicine at our hospital because her husband had been diagnosed with a then unspecified childhood heart condition. He had been discharged from cardiologic follow-up at the age of 14 years. He performed his military service and had a full-time job without suffering from a heart condition.

Prenatal examination

Ultrasound at 20 weeks' gestation revealed a normal four-chamber view of the heart with no evidence of structural or functional abnormalities of the heart (Figure 1). According to protocol a repeat scan was performed at 31 weeks' gestation and showed a severe cardiomegaly with extensive mitral and tricuspid regurgitation. The cardiac-thoracic ratio was 0.7 (>>P95).21 Both ventricles and atria were enlarged, the right ventricle was more dilated than the left ventricle and cardiac contractility was impaired. The myocardium was hypertrophic and showed a spongiform aspect (Figure 2). Amniocentesis was performed and chromosome analysis showed a normal female karyotype; FISH-analysis excluded a 22q11 deletion. There were no signs of intra-uterine infection: screening for TORCH, parvovirus B19, enterovirus, coxackievirus and parechovirus was negative. At repeated examinations at 35^{+6} weeks of gestation the hypertrophic and spongiform aspect was predominantly localized in the myocardial apex; tricuspid regurgitation had increased and was more severe than mitral regurgitation.

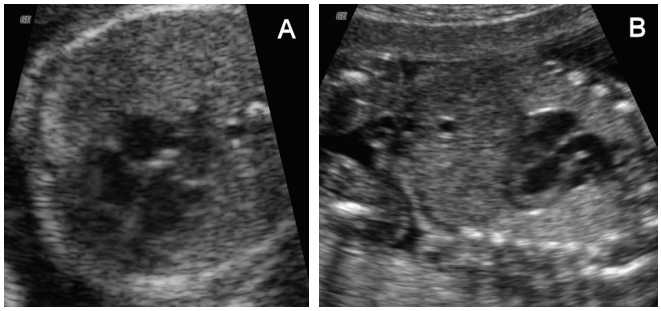


Figure 1. Prenatal ultrasound of patient 1 at 20 weeks' gestation.

A. Normal four-chamber view of the heart.
B. Normal aspect of the left ventricle and aorta.

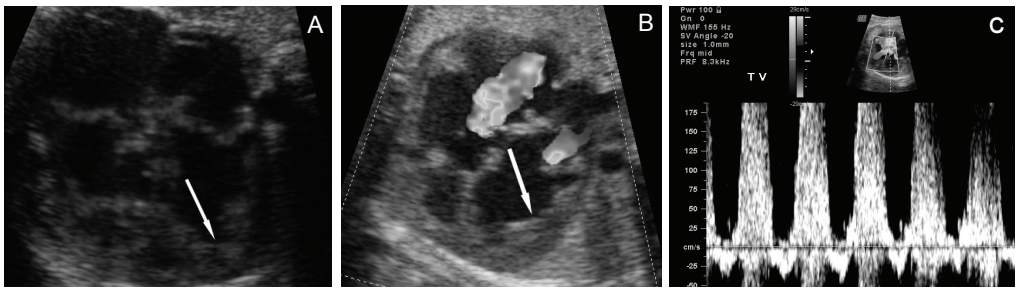


Figure 2. Prenatal ultrasound of patient 1 at 31 weeks' gestation

A. Enlarged heart with hypertrophic and spongy myocardium, particularly in the left apex (arrow).
B. Colour Doppler image of severe regurgitation over tricuspid and mitral valve.
C. Pulsed Doppler image of severe tricuspid regurgitation (> 200 cm/sec)

Postnatal period

Labour was induced at 38^{+2} weeks' gestation due to a deteriorating foetal condition. A girl was delivered with a birth weight of 2990 g (P25) and Apgar scores of 6 and 9 after one and five minutes. Postpartum echocardiography con-firmed noncompaction cardiomyopathy (apical and lateral wall) with biventricular dilatation, impaired left ventricular function (shortening fraction (SF) of 20%), moderate mitral and severe tricuspid regurgitation (Figure 3). The child was treated with continuous positive airway pressure for a few hours and was discharged from hospital after 14 days in good clinical condition. During the following weeks she developed feeding problems and was hospitalised for congestive heart failure at the age of three months with severe mitral valve regurgitation, mild mitral valve stenosis, substantial left atrial dilatation and secondary pulmonary hypertension, reactive to oxygen and nitric oxide. Surgical mitral valvoplasty was performed at four months of age. There was a grade III residual mitral regurgitation one week after the procedure requiring additional intervention. In a third operation a mechanical valve (St. Jude, 17 mm) was implanted in mitral position. Thrombosis of one of the valve leafs necessitated another intervention. LV function gradually improved to near-normal levels after implanting the mechanical valve and increasing ACE-inhibitor and β-blocker medication. Her cardiac situation remains stable at age three years and nine months, still requiring partial tube feeding.

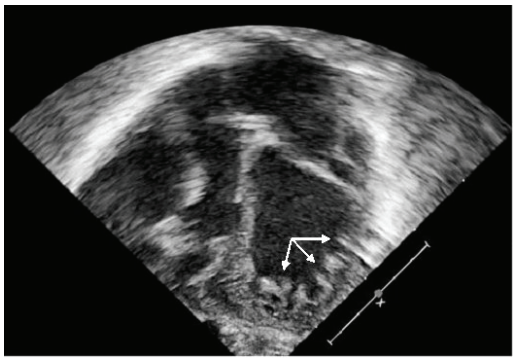


Figure 3. Postnatal echocardiography of patient 1. Four-chamber view with evident noncompaction located in the left ventricular apex (arrows).

Cardiologic family study (Figure 4)

Medical records of the father showed that he suffered of tachypnoea at the age of three months. At the age of six months, he was diagnosed with a dilated LV and mitral valve regurgitation. Differential diagnosis for his dilated LV then included subendocardial fibroelastosis and myocarditis. He was treated with digitalis and prednisone. His condition improved and he was discharged from cardiologic follow up when chest X-ray showed a normal heart-thoracic index and he was without symptoms at age 14 years. Cardiologic screening of the asymptomatic father at age 36 years showed dilated cardiomyopathy (DCM) LV end diastolic diameter 76 mm) with an ejection fraction (EF) of 40%. Cardiologic screening of the paternal grandparents and the mother showed no abnormalities.

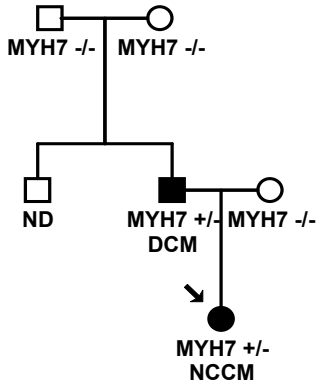


Figure 4. Pedigree of family 1. The arrow indicates the proband. ND: not determined.

Molecular testing

DNA analysis in the father identified mutation p.Cys905Arg in the ß-Myosin Heavy Chain gene (*MYH7*). This mutation was postnatally confirmed in his affected daughter. P.Cys905Arg is a novel *MYH7* mutation (i.e. has not been described previously) and is expected to be pathogenic because a pathogenic mutation p.Cys905Phe has been described on the same amino acid location.[22] The unaffected paternal grandparents did not carry the p.Cys905Arg mutation in the *MYH7* gene confirming that the mutation occurred *de novo* in the affected father.

Patient 2

A 33-year-old woman, gravida 6, para 2, of Turkish descent, was referred at 23 weeks' gestation to our hospital for advanced prenatal imaging after a routine anomaly scan detected a foetal cardiomegaly with increased ventricular wall thickness.

Prenatal examination

Ultrasound at 23 weeks' gestation confirmed biventricular cardiomegaly with a cardio-thoracic ratio >P95 [21], decreased contractility and increased apical myocardial wall thickness. Doppler ultrasound of the tricuspid, mitral, pulmonary valves and aorta did not reveal functional insufficiency. Cardiac structural defects were excluded. Maternal TORCH, parvovirus B19, adenovirus and enterovirus screening was negative. The patient declined amniocentesis. Ultrasound examinations at 28 and 32 weeks' gestation showed a slight decrease in cardiomegaly, unchanged decreased contractility but no evidence of insufficient blood flow. At 36 weeks' gestation signs of noncompaction became visible in the apex (Figure 5).

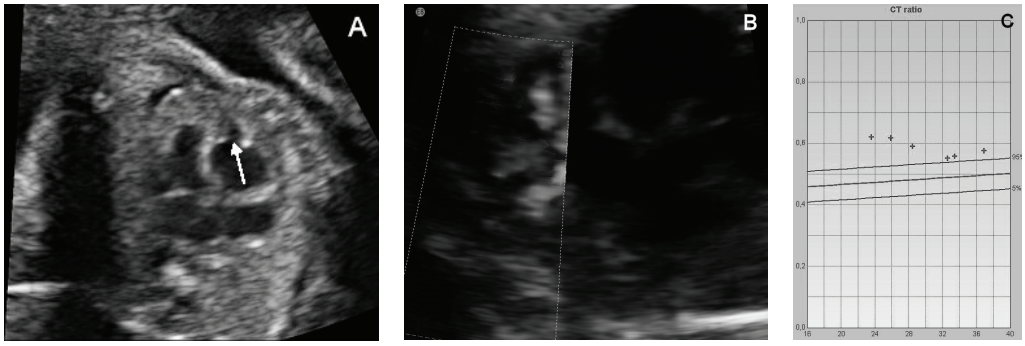


Figure 5. Prenatal ultrasound of patient 2

A. Four-chamber view of the heart at 23 weeks' gestation with increased apical myocardial thickness.
B. Colour Doppler ultrasound of the apical right ventricular wall at 36 weeks' gestation shows filling of the non-compacted myocardium.
C. Graph of cardiothoracic (CT) ratio indicating decrease of cardiomegaly during gestation.

Postnatal period

Labour was induced at 37 weeks and 4 days due to foetal heart rate decelerations. A boy was delivered with a birth weight of 2700 g (>P10), Apgar scores were 9, 10 and 10 after one, five and 10 minutes respectively. Echocardiography showed NCCM of the apex and lateral wall (NC/C ratio 4.4 / 1.8) with cardiomegaly, without impairment of the left ventricular function (SF 30%) (Figure 6). The infant was discharged from hospital on day one after delivery. Cardiologic follow up at three months showed normal cardiac function and dimensions with unchanged NCCM. He was feeding well and growing accordingly.

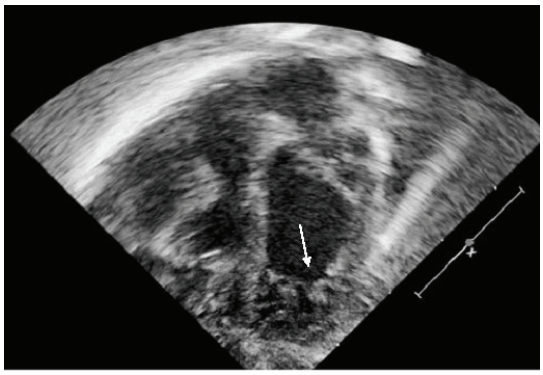


Figure 6. Postnatal echocardiography of patient 2
Four-chamber view with evident thickened endocardium with noncompaction located in the left ventricular apex (arrow).

Cardiologic family study

Subsequent cardiologic screening of the non-consanguineous parents identified NCCM in the 34-year-old father and the 10-year-old asymptomatic brother of the index. The father suffered from exercise dyspnoea. His EF was 36%. The mother, a 14-year-old brother and two paternal aunts were cardiologically unaffected. The paternal grandmother had been diagnosed with NCCM shortly before, at age 62 years after having experienced chest pain. She had an NC/C ratio of 2.1 in the posterolateral, lateral and apical regions of the LV, EF of 23% and left atrial enlargement of 183 ml. One of her sisters suffered a sudden cardiac death at the age of 38 years. The pedigree of this family is depicted in Figure 7.

Molecular testing

A pathogenic *MYH7* splice-site mutation (c.732+1G>A) and a cardiac Myosin Binding Protein C3 gene (*MYBPC3*) unclassified variant (UV) (p.Gln1142Pro) were identified in the affected father. DNA analysis showed the *MYH7* mutation and the *MYBPC3* UV in the index and his affected brother. The *MYH7* mutation was excluded in two unaffected paternal aunts (one had the *MYBPC3* UV) and in an unaffected maternal cousin of the father.

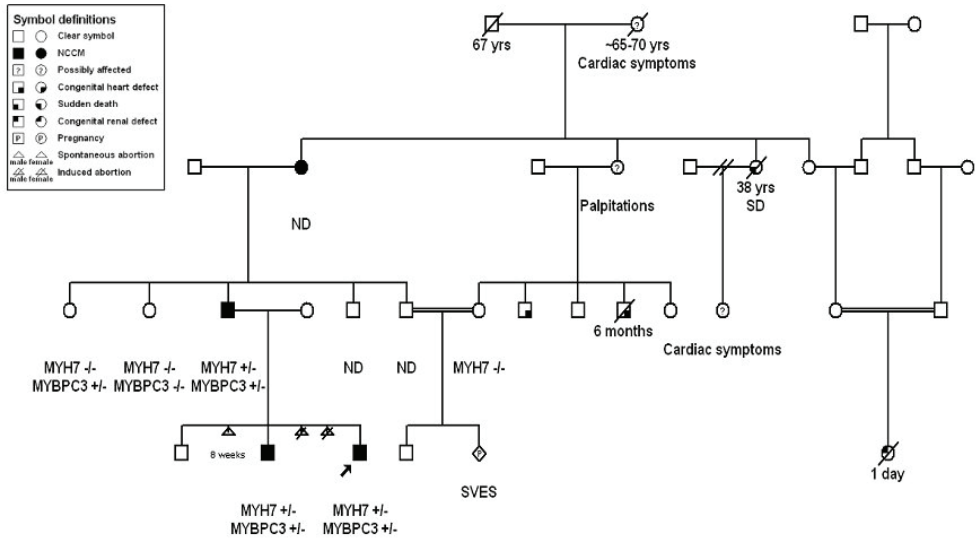


Figure 7. Pedigree of family 2.
The arrow indicates the proband. MYBPC3 = cardiac Myosin Binding Protein C3 gene; MYH7 = ß-Myosin Heavy Chain gene; ND = not determined; SD = Sudden death; SVES = Supra Ventricular Extra Systole.

Discussion

We describe the first two prenatal ultrasound diagnosis of NCCM associated with pathogenic mutations in the gene coding for heavy chain sarcomere protein MYH7. Sarcomere mutations are important causes for HCM, DCM, RCM and NCCM in adults and children. However, to our knowledge, prenatal presentation of NCCM, HCM, DCM, or RCM in families with an *MYH7* or other sarcomere mutation have not yet been reported.

MYH7 mutations are the most common genetic cause for NCCM and show – like other sarcomere cardiomyopathies – variability in age at diagnosis including NCCM diagnosed in childhood. As this report indicates, *MYH7* mutations may also present with prenatal cardiomyopathy. For that reason prenatal ultrasound of pregnancies at risk for cardiomyopathy in families with *MYH7* mutations is recommended, allowing optimal peripartum care in favour of an affected child. Because familial disease may initially present with a prenatal onset, molecular diagnostics and cardiologic screening of first-degree relatives are recommended even in absence of a family history of cardiomyopathy. This way cardiologic surveillance and – if necessary treatment – can be offered during pregnancy in order to reduce risks of peripartum cardiomyopathy in the mother.

Whether prenatal onset of cardiomyopathy in familial NCCM is specific for *MYH7* mutations or whether other sarcomere related cardiomyopathies may also present with prenatal cardiomegaly, arrhythmia or decreased cardiac function has yet to be established.

Previous reports of prenatal diagnosed NCCM illustrate that this diagnose is possible from the 20th week of gestation (Table 1). The majority of the cases were diagnosed between 20 and 26

weeks of gestation (mean 26, median 25 weeks). The first case presented in this paper had normal findings at 20 weeks gestation, indicating that in pregnancies at risk for genetic NCCM, ultrasound should be repeated in the third trimester.

Most cases (17/32) had biventricular involvement; the RV was often more affected than the LV, possibly because of the haemodynamics of the foetal circulation, with a dominant RV. After birth the LV becomes the systemic ventricle, pumping against high pressure, whereas the RV pumps against low pressures after birth. Gradual diminishing of noncompaction of the RV after birth suggests certain reversibility to alterations in haemodynamic conditions. Hypothetically, NCCM may be explained by remodelling of the myocardial tissue, adjusting itself to different circumstances. According to the diagnostic criteria (Jenni) for NCCM, only signs of left ventricular noncompaction are characteristic for NCCM. And although there are no diagnostic criteria for right ventricular involvement in noncompaction cardiomyopathy, right ventricular noncompaction in adults were observed in as much as 38-41% of adult NCCM.[23, 24]

As the review of the literature of prenatally diagnosed NCCM in Table 1 shows, a large proportion of the 32 reported cases were detected in initially diagnosed structural heart defects (16/32). In NCCM families congenital heart defects appear to be more prevalent than in the normal population. So far no aetiological association has been established. Nevertheless, it suggests that further considerations are necessary whether absence of congenital heart defects should remain one of the diagnostic criteria of NCCM.

Arrhythmia (mainly bradycardia), hydrops and reduced foetal movement were among the other indications for advanced ultrasound between 20 - 34 weeks' gestation, leading to the diagnosis of NCCM, indicating that NCCM should be considered in these prenatal conditions.

Genetic risk for cardiomyopathy was the initial reason for prenatal ultrasound in four cases from literature. The importance of screening for cardiomyopathy in parents and siblings was illustrated before: in eleven families of the reported prenatal NCCM cases cardiologic screening of parents and/or siblings was performed showing familial cardiomyopathy in six families (eight cases)[10, 12-14] These results clearly endorse the high prevalence of genetic cardiomyopathy in prenatal NCCM. Molecular diagnostics may explain approximately half of familial postnatal NCCM and - as our report shows - may also find the cause of prenatal NCCM. For that reason DNA testing of NCCM related sarcomere genes is recommended in prenatally diagnosed NCCM, by means of amniocentesis, postnatal blood sampling or, in case of a lethal outcome, at autopsy.

Detection of a molecular defect in NCCM is important because it allows more accurate identification of relatives at risk for cardiomyopathy than cardiologic screening, since a proportion of at risk relatives may show age-dependent penetrance or non-penetrance.

The outcome of prenatal NCCM in previous cases is variable. In complex NCCM, nine of 16 patients died (two terminations of pregnancy; three foetal demises and four neonatal deaths); survival up to four years was described. In isolated NCCM eight of 16 patients died (five terminations; one foetal demise; and two neonatal deaths); survival was described up to three years of age.

Table 1. Part 1. Overview of 32 patients prenatally diagnosed with noncompaction cardiomyopathy, including our two cases.

Patient	GA at diagnosis (weeks)	Involvement		Congenital Heart disease	Arrhythmia / conduction abnormalities	Other features
		LV	RV			
1 (M)	20	+	+			Hydrops
2 (F)	20	+		VSD, ASD	SVT	Mild motor development delay
3 (M)	20	+	-	Ao-LV tunnel		AoR
4 (M)	21			AVSD, DORV, TGV, PS	Heart block	AVVR; heterotaxy; hydrops
5 (F)	21			AVSD, DORV, TGV, PS, PAPVR	Heart Block	Heterotaxy; hydrops
6 (F)	21	+	+	Dextroposition	3^{d}-degree AVB	Omphalocele; polysplenia; clubfeet; systemic venous abnormalities
7 (F)	22	+	+		Bradycardia, SVT	Hydrops
8 (M)$^{\#}$	22	-	+			TR
9 (M)	22	+	+			
10 (M)	22	+		VSD, ASD		Corpus callosum agenesis; Arnold Chiari malformation; facial dysmorphism
11 (M)	23			AVSD, DORV, TGV	Heart block	Heterotaxy; hydrops
12 (M)	23.4	+	+			
13 (F)	24	+	+			TR; single umbilical artery
14 (F)	24	+		PS		TR
15 (M)$^{\#}$	25	-	+			MR, TR, Hydrops
16 (F)	25	+	+			
17 (M)	26	+	+		SVT	Microcephaly; growth retardation; dysmorhic features
18 (M)	26	+	+	AoCo	Bradycardia	Low-set ears
19 (F)	27	+	+		Complete heart block	Multicystic kidney; hydrops
20 (M)	27	+	-	Ebstein, SA		
21 (M)	28			AVSD, DORV	Heart block	AVVR; heterotaxy; hydrops
22	28	-	+		1^{si}-degree AVB, LQT	
23 (F)	29.6	+	+		Bradycardia	MR; TR; omphalocele; absent gallbladder; extrabiliary atresia; enlarged liver; hydronephrosis
24 (M)	30			AVSD, DORV, PA, PAPVR	Bradycardia	AVVR; heterotaxy
25 (M)*	31	+	+			Hydrops

Legend. AoCo = aortic coarctation; AoR = aortic regurgitation; ASD = atrial septal defect;
AVB = atrioventricular block; AVSD = atrioventricular septal defect;
AVVR; atrioventricular valve regurgitation; DA = ductal aneurysm;
DORV = double outlet right ventricle; F = female; GA = gestational age;
LQT = long QT segment; LV = left ventricle; M = male; 46XX / 47XX,+22 = Mosaic trisomy 22;
MR = mitral regurgitation; MVS = mitral valve stenosis;
MYH7 = ß-Myosin Heavy Chain gene; Nt = not tested.

Referral due to	Outcome	Familial	Genetic defect	Ref.
Poor ventricular function	Stable at age 2 yrs	-		13
Abnormal cardiac anatomy	Mild decrease in LVF at age 2 yrs	+		13
Abnormal cardiac anatomy	Stable at age 2 yrs	-		13
Bradycardia; hydrops; abnormal foetal heart	Termination	Nt		20
Bradycardia; hydrops; abnormal foetal heart	Neonatal death	Nt		20
Bradycardia	Foetal demise	Nt		7, 20
Foetal arrhythmia; abnormal ventricular contraction	Termination	Nt		19
Family history	Termination	+		14
Foetal hydrops	Termination	Nt		9
RV hypoplasia	Stable at age 4 yrs	-		13
Bradycardia; hydrops; abnormal foetal heart	Termination	Nt		20
Hypertrophic aspect myocardium	Stable at 7 months	+	*MYH7*	This study
Abnormal foetal heart; single umbilical artery	Stable at age 3 yrs	-		14
Maternal DCM; foetal hydrops	Neonatal death	+		12
Abnormal foetal heart	Termination	+		14
Cardiac enlargement	Stable	-		13
Pericardial effusion	Termination	Nt		14
Reduced foetal movements	Foetal demise	Nt		8
Foetal hydrops; bradycardia	Neonatal death	Nt		11
Increased foetal heart rate; dilated LV	Stable at 3.5 yrs	Nt		18
Bradycardia; hydrops; abnormal foetal heart	Neonatal death	Nt		20
Foetal arrhythmia	Pacemaker; stable at 5 months	Nt		6
Omphalocele	Stable	Nt		11
Bradycardia; hydrops; abnormal foetal heart	Live birth	Nt		20
Family screening?	Foetal demise	+		10

Legend. PA = pulmonary atresia; PAPVR = partial anomalous pulmonary venous return;
PDA = patent ductus arteriosus; PS = pulmonic stenosis; RV = right ventricle;
SA = septal aneurysm; SubAoS = subaortic stenosis; SVT = supraventricular tachycardia;
TGV = transposition of the great vessels; TR = tricuspid regurgitation; US = ultrasound;
VSD = ventricular septal defect;
 # patient 8 and 15 are brothers;
** patient 25 and 31 are half-brother and sister.*

Table 1. Part 2.

Patient	GA at diagnosis (weeks)	Involvement		Congenital Heart disease	Arrhythmia / conduction abnormalities	Other features
		LV	RV			
26 (F)	31.2	+	+	MVS		MR, TR
27 (M)	31.5	+	+			Dysmorhpic features
28	32			VSD, SubAoS	Heart block	Heterotaxy; hydrops
29 (F)	33	+	-		Bradycardia	Hydrops
30	34	+	+	VSD	Bradycardia	Hydrops
31 (F)*	34	+	+		Bradycardia, complete AVB	
32 (F)	35	+	+	ASD, PDA, DA		TR; dysmorphic features

Legend. AoCo = aortic coarctation; AoR = aortic regurgitation; ASD = atrial septal defect;
AVB = atrioventricular block; AVSD = atrioventricular septal defect;
AVVR; atrioventricular valve regurgitation; DA = ductal aneurysm;
DORV = double outlet right ventricle; F = female; GA = gestational age;
LQT = long QT segment; LV = left ventricle;M = male; 46XX / 47XX,+22 = Mosaic trisomy 22;
MR = mitral regurgitation; MVS = mitral valve stenosis;
MYH7 = ß-Myosin Heavy Chain gene; Nt = not tested.

The difference in gestational age at ultrasound diagnosis of NCCM may depend on late onset of NCCM features, like in our first case, or alternatively reflect technical difficulties envisioning the fine noncompacted structure of the heart for instance in case of severe dilatation of the ventricles.

Prognosis of prenatal NCCM seems highly variable, even in cases of isolated prenatal NCCM, ranging from severe progressive disease to postnatal improvement or stabilization of clinical features in asymptomatic infants. This is also illustrated by our two cases, where patient 1 had several interventions, and patient 2 hardly experienced any problems after birth so far. Counselling and making decisions concerning continuation or termination of pregnancy is difficult. In this process, cardiac functioning, presence of additional congenital malformations, family history of cardiomyopathy and if available the molecular defect identified in the family, may all play a role.

In summary, this report endorses that prenatal diagnosis of NCCM may be possible as early as the 20th week of gestation although in some cases NCCM may only become visible later in pregnancy. Prenatal NCCM may be biventricular and prognosis is variable depending among others on cardiac functioning. Prenatal onset of NCCM may be caused by *MYH7* gene mutations and may be the initial presentation of familial cardiomyopathy, therefore warranting molecular testing and also cardiologic (and molecular) screening of the parents especially in case of isolated prenatal NCCM.

Referral due to	Outcome	Familial	Genetic defect	Ref.
Family history	Stable at at 3 yrs	+	*MYH7*	This study
Routine 3^{d} trimester US	Stable at 10 months	Nt		16
Bradycardia; hydrops; abnormal foetal heart	Neonatal death	Nt		20
Reduced foetal movements; uterine contractions	Stable at 2 yrs	Nt		15
Moderate foetal hydrops	Foetal demise	Nt		10
Bradycardia	Neonatal death	+		10
?	?	Nt	46, XX / 47XX,+22	17

Legend. PA = pulmonary atresia; PAPVR = partial anomalous pulmonary venous return;
PDA = patent ductus arteriosus; PS = pulmonic stenosis; RV = right ventricle;
SA = septal aneurysm; SubAoS = subaortic stenosis; SVT = supraventricular tachycardia;
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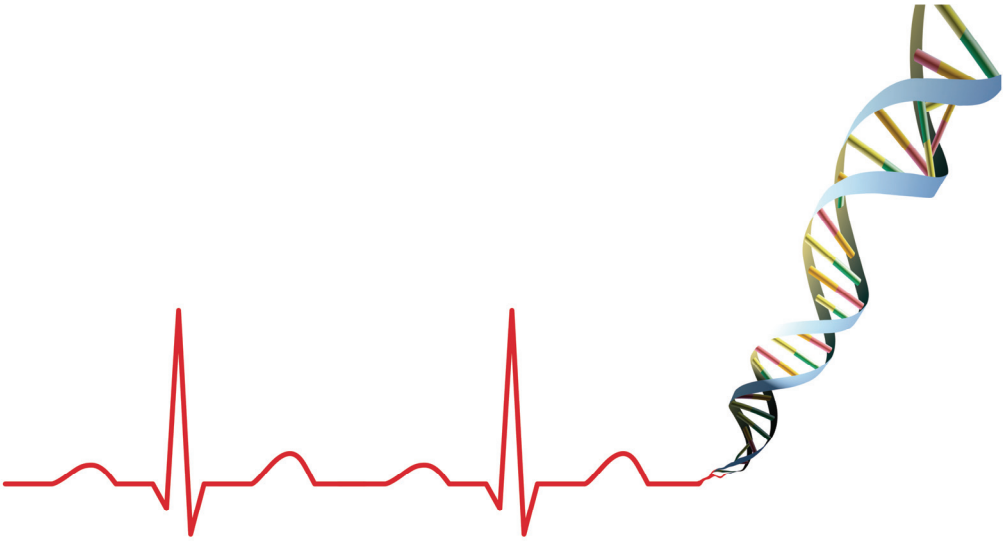
Chapter 6

A new locus for autosomal dominant noncompaction cardiomyopathy is linked to chromosome 4.

Yvonne M. Hoedemaekers, Kadir Caliskan, Rina Keupink-Aarnink, J. Peter van Tintelen, Paul L. van Haelst, Michelle Michels, Lilian R. Parsaulian, Christan F. Rohe, Dennis Dooijes, Ben A. Oostra, Danielle F. Majoor-Krakauer, Aida M. Bertoli Avella.

Manuscript in preparation



Abstract

Background

Noncompaction cardiomyopathy (NCCM) is a genetically heterogeneous cardiomyopathy characterised by left ventricular (LV) prominent trabeculations and deep intertrabecular recesses that communicate with the LV cavity. NCCM in children and adults has been associated predominantly with mutations in sarcomere genes. In approximately half of familial NCCM the genetic defect remains unknown.

Methods and results

Cardiological examination of 20 individuals from an extended Dutch family identified nine patients with NCCM and one patient with dilated cardiomyopathy (DCM). To identify a genetic cause for NCCM in this family we performed a genome-wide linkage analysis using 300K SNP arrays. A maximum LOD score of 3.02 on chromosome 4p15.3 was obtained. The inclusion of a patient that shares a common ancestor with the index case seven generations ago allowed the identification of a small candidate region. This area comprised 5.47 Mb (10.03-15.5 Mb, marker CGR525 until D4S419) on chromosome 4p15.3 and contains 25 genes.

Conclusions

We have mapped a new locus for autosomal dominant NCCM to a 5.47 Mb region on chromosome 4p15.3. Identification of the novel disease gene will facilitate genetic screening and provide fundamental insight into the understanding of NCCM pathogenesis.

Introduction

Noncompaction of the left ventricle or noncompaction cardiomyopathy (NCCM) is a relatively new cardiomyopathy, first described by Feldt in 1969.[1] It is characterized by left ventricular (LV) prominent trabeculations and deep intertrabecular recesses that communicate with the LV cavity.[2, 3] Morphologically NCCM resembles the myocardium during its early cardiac development; it is therefore thought to be caused by an arrest of normal myocardial embryogenesis.[4] Congestive heart failure, thrombo-embolic events and arrhythmias, including sudden cardiac death (SCD), may be the initial presentation. Like other hereditary cardiomyopathies, NCCM shows inter- and intrafamilial variability, ranging from asymptomatic disease in adults to severe congenital forms.[5-7] The majority of NCCM diagnosed in adults is isolated, as defined in the fourth criterion stated by Jenni et al.[8] Non-isolated forms of NCCM are more frequent in childhood and may co-occur with congenital heart malformations, or may be part of a malformation or chromosomal syndrome.[7]

The majority of NCCM is hereditary and appears to be genetically heterogeneous.[9, 10] An important proportion of isolated NCCM in children and adults has been associated with mutations in sarcomere genes.[9, 11] Rare genetic defects include mutations in Tafazzin (*TAZ*), α-dystrobrevin (*DTNA*), Cypher/ZASP (*LDB3*) and Lamin A/C (*LMNA*). Absence of a genetic defect does not preclude a genetic cause of NCCM. In approximately half of familial NCCM the genetic defect remains unknown.[10]

To identify new genetic causes for NCCM we performed linkage analysis in a large NCCM family, linked together through genealogic analysis.

Methods

Clinical studies

This study was performed in the proband and his relatives as depicted in Figure 1. Cardiologic analysis of participating relatives of the proband (VIII:2) was performed at the Cardiogenetic outpatient clinic of the Erasmus Medical Centre in Rotterdam, the department of Cardiology of the Antonius Hospital in Sneek, the department of Cardiology of the Medical Centre Alkmaar and the department of Cardiology of the Isala Clinics in Zwolle, the Netherlands and consisted of echocardiography, electrocardiography (ECG) and physical examination. Additionally 24-hours ambulatory ECG monitoring and exercise ECG were performed in affected relatives. All imaging data were reviewed by KC and MM at the Erasmus Medical Centre. Diagnosis of NCCM was made when the noncompacted endocardial layer to compacted epicardial layer ratio (NC/C) was equal to or larger than two, without structural heart defects, and Colour Doppler imaging visualised blood flow within the intertrabecular recesses.

VI:6 was independently identified as an NCCM patient, she came from the same village as the mother of the proband and could be linked to the NCCM family by genealogical analysis.

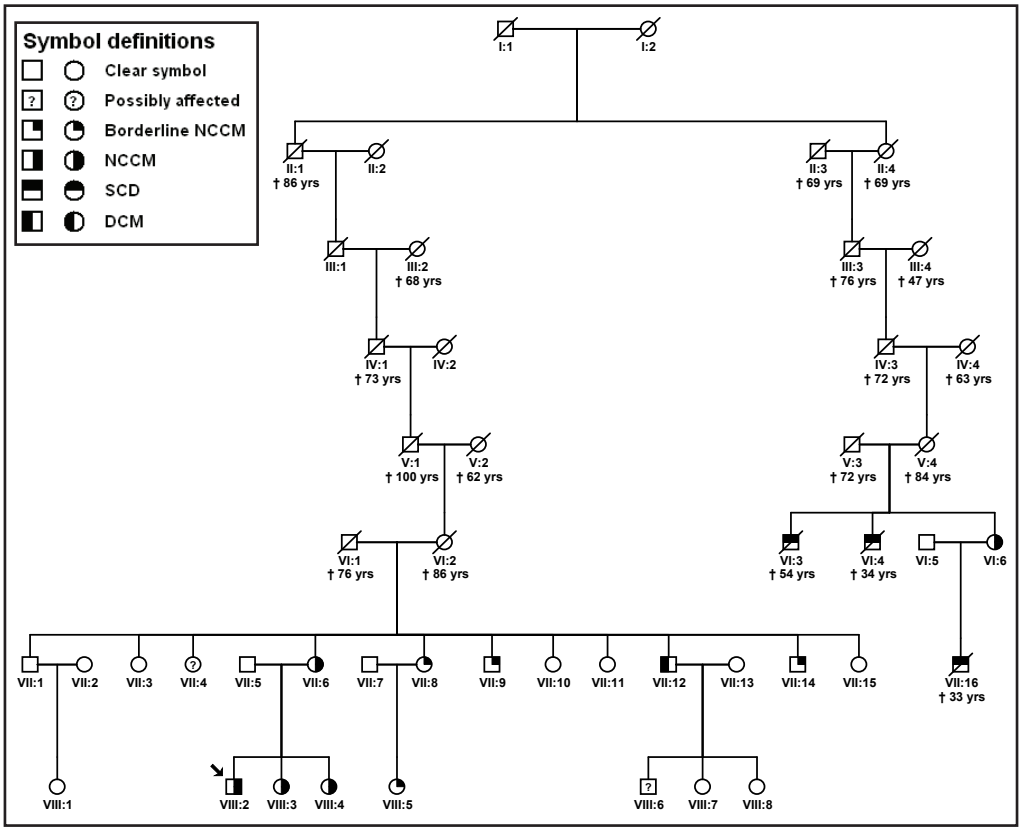


Figure 1. Pedigree. Genealogy identified a shared ancestor seven generations before the proband.

Genealogic analysis

Dutch Civil Registry records publicly available through Genlias (www.genlias.nl), records available on the internet from Frisian archives (www.allefriezen.nl) and private genealogic databases were used for the genealogic study. The resulting pedigree was displayed using Cyrillic 2.1.3 (Cherwell Scientific Publishing Ltd 1997, Oxford, UK).

Molecular studies

Genomic DNA was isolated from peripheral blood using the Puregene DNA purification kit (Gentra Systems) following standard procedures. The genome wide search was conducted using DNA from 12 members of the family including nine patients: eight patients with NCCM (VII:6, VII:8, VII:9, VII:14, VIII:2, VIII:3, VIII:4, VIII:5) and one with dilated cardiomyopathy (VII:12). The Human CytoSNP12 arrays containing 299,671 genome wide tag SNPs were used. Samples were processed according to the manufacturer's instructions (Infinium DNA analysis BeadChip kits, Illumina). The GenomeStudio Data Analysis software (Genotyping module) was used for genotype calling, quality control and to export the data.

Micro-satellite markers

Micro-satellite markers mapping to the identified genomic regions were selected. Polymerase chain reaction (PCR) products were run on an ABI Prism 3130*xl* genetic sequencer (Applied Biosystems) and analyzed using the GeneMapper software v.3.0 (Applied Biosystems). DNAs from 22 available family members were investigated including the newly identified patient, linked through genealogy (VI:6).

Linkage analysis and loci identification

The statistical package EasyLinkage Plus v5.08 [12] designed to perform automated linkage analyses using large-scale SNP data, was used to perform all analyses. All SNPs showing inconsistency in transmission were removed from further analyses. Allegro v1.2c software (incorporated in the EasyLinkage Plus v5.08 package) was used to perform fully automated single point and multipoint linkage analysis. LOD scores were obtained using a dominant model of inheritance, with a penetrance of 90% and a disease allele frequency of 1:1000. A phenocopy rate of 1% was considered. Allele frequencies of genotyped SNPs were set to co-dominant. Map order and inter-SNPs distances were taken from the Illumina website.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.3 to 0.1 centiMorgan (cM), in blocks of 90 and 100 SNPs. Then, single chromosomes showing positive linkage signals were independently analysed under the same conditions and haplotypes were constructed.

The analysis of the micro-satellite data was performed with Merlin (v1.01) to allow the use of an extended genealogical tree that included patient VI:6, (Figure 1) assuming a common ancestor that lived four generations ago.

Graphical visualization of haplotypes to facilitate inspection and analyses was performed with HaploPainter v029.5 [13]; a tool for drawing pedigrees with complex haplotypes.

Results

Clinical studies

VIII:2

The proband was a 36-year-old man without a previous history of cardiac disease, who suffered a sudden cardiac arrest while running a marathon. He was successfully resuscitated and trans-oesophageal and trans-thoracic echocardiography showed moderate LV dysfunction with typical abnormal trabeculation, consistent with noncompaction cardiomyopathy with a NC/C ratio of 2.5 and an ejection fraction (EF) of 45%. ECG showed sinus rhythm of 84 beats per minute and borderline LV hypertrophy pattern with repolarisation abnormalities in anterolateral and inferior leads. Coronary angiography showed no occlusions. He received an implantable cardioverter defibrillator (ICD) for secondary prophylaxis and was treated with an ACE-inhibitor and a β-blocker. His systolic function steadily improved and after three years of follow-up he

remains asymptomatic. No ICD discharges or anti-tachypacing was reported. He is now a father of a healthy girl and boy, they didn't show any abnormalities at prenatal advanced ultrasound; the parents refused cardiologic screening after birth.

VII:6

The mother of the proband had a transient ischaemic attack at the age of 59 years, for which she was evaluated. Duplex of the carotic arteries was normal. Cardiac screening at that time showed a moderately severe LV dysfunction, with an EF of 34% and mild mitral valve insufficiency due to mitral valve prolaps. Repeated echocardiographic screening at the age of 65 years, after her son's cardiac arrest, showed slightly dilated LV (end-diastolic dimension 65 mm and end-systolic dimension of 54 mm) with a fractional shortening (FS) of 17%. Distinct trabeculations were visible in the left ventricular apical, lateral and distal inferior and anterior segments with a NC/C ratio of 3.5 (19/5.5), resembling NCCM. ECG showed sinus rhythm of 68 beats per minute with mild intraventricular conduction abnormalities (QRS width of 110 msec, and a QTc of 421 msec). She experienced fatigue and dyspnea after walking two flights of stairs (NYHA class II) but had no complaints of palpitations or chest pain. Coronary angiography was normal. She received an ICD for prophylactic purposes.

VIII:3

A 33-year-old sister of the proband had a history of arrhythmia and palpitations since the age of 28 years and was diagnosed with mitral valve prolaps. Cardiac evaluation after her brother's sudden cardiac arrest revealed typical features of NCCM with a NC/C ratio of 2.3 and abnormal trabeculations in the left ventricular apical, inferior, inferolateral and posterior segments. EF was 51%. ECG showed sinus bradycardia of 52 beats per minute with normal conduction parameters and repolarisation pattern. Her exercise ECG was normal, but her 24-hour Holter revealed three non-sustained ventricular tachycardia's (VT's). Therefore an invasive electrophysiological study was done for risk stratification; no ventricular arrhythmias could be induced. She was then empirically treated with a β-blocker. After 2.5 years follow up, she was no longer experiencing palpitations and was completely asymptomatic.

VIII:4

A 32-year-old asymptomatic sister of the proband was also diagnosed abnormal trabeculations in the apical, inferior and lateral LV walls, with a maximal NC / C ratio of 2,0. Her EF on MRI was 49%. Her ECG was normal.

VII:8

Of an asymptomatic maternal aunt, aged 64 years, echocardiography showed mildly prominent left ventricular trabeculations with a maximal NC/C ratio of 1.5. MRI showed a NC/C ratio of 1.8 with an EF of 48%. ECG showed no abnormalities.

VIII:5

The daughter of patient VII:8 presented with palpitations at the age of 27 years and was diagnosed with paroxysmal AV-nodal re-entry tachycardia and supraventricular tachycardia (SVT). Echocardiography at that time showed no signs of cardiomyopathy. She was treated with cryo-ablation of the slow pathway twice; afterwards tachycardia could not be induced. After five years of follow up, she had palpitations again, with frequent irregular heart beats. After sudden cardiac arrest of her cousin she was admitted for cardiac re-evaluation. There were prominent trabeculations in the posterior and apical segments. NC/C ratio on echocardiography was 1.5; on MRI it was 1.8 with an EF of 53%. Palpitations were treated with β-blockage therapy.

VII:9

This 59 year old maternal uncle had atypical chest pain and mild hypertension. His ECG showed a left bundle branch block with ventricular extra-systoles. Echocardiography revealed segmental wall motion irregularities inferior, inferoseptal, and anteroseptal. There was hypertrabeculation of the apical and anterior wall, with a NC/C ratio < 2. LVEF was 49%. There was a mild central mitral insufficiency. Follow-up was advised.

VII:12

At the age of 55 years an asymptomatic paternal uncle was diagnosed with dilated cardiomyopathy (DCM) at family screening. Echocardiography showed borderline hypertrophy of the intraventricular septum (12.2 mm) as well as a dilated left ventricle (59 mm) with a mildly impaired function. There are no signs of NCCM. ECG is aberrant showing sinus rhythm with a prolonged QRS duration of 180 ms due to a right bundle branch block and left posterior hemiblock. Stress testing revealed an excellent exercise capacity (116% of expected) in the absence of complaints or arrhythmias. Twenty-four-hour ambulatory ECG was normal. On MRI both ventricles were dilated. LVEF was 45% with an end diastolic volume of 242 ml; RVEF is 33% with an EDV of 231 ml. Because of the normal exercise capacity and the absence of complaints it was decided to postpone invasive diagnostics. Treatment was started with ACE inhibition and follow-up was advised.

VII:14

Family screening showed excessive trabeculations in the apical region, with a NC/C ratio < 2 in another 53 year old paternal uncle. ECG showed no significant abnormalities. The patient was asymptomatic. Follow-up was advised.

VI:6

Patient 10 was cardiologically examined at the age of 76 years after the sudden cardiac death of two of her brothers (aged 54 and 34 years respectively) and of her 33-year-old son. She was asymptomatic. Echocardiography showed noncompaction localised at the anterior wall, septal

and apical regions with a NC/C ratio of 2.5; LVEF was normal. ECG showed normal sinus rhythm with normal conduction and without ST segment abnormalities. She was born in the same small village of approximately 2000 inhabitants as VII:6. Genealogic studies showed the family ties between VI:6 and VII:6 (Figure 1).

VII:4

A 67-year-old maternal aunt with palpitations did not show any abnormalities on her ECG, 24-hr Ambulatory monitoring showed SVT's; her echocardiogram showed some apical trabeculations, with a NC/C ratio <2.

VIII:6

The eldest son of patient VII:11 was asymptomatic. Echocardiography at age 26 years did not show any abnormalities. His ECG showed sinus rhythm with normal PR time and a delayed ventricular activation. QRS width is 95 ms. There was an early repolarisation pattern.
VII:1 (age 69 years); VII:3 (age 68 years); VII:10 (age 58 years); VII:11 (age 56 years); VII:15 (age 60 years); VIII:1 (age 44 years); VIII:7 (age 22 years) and III:8 (age 16 years) were also examined but had no cardiac abnormalities.

Molecular studies

Genome wide linkage analysis (GWLA)

No mutations were found in any of the most prevalent HCM genes (*MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3*, *TCAP*, *LDB3, CASQ2*, *CALR3*, *PLN*, *TAZ* and *LMNA)* in the proband. A genome wide search using 300k SNP arrays was performed. Because incomplete and age-dependent penetrance are common features in most genetic cardiomyopathies, family members with a normal cardiological examination were included in the analysis as "diagnosis unknown".
The linkage analysis revealed linkage to two genomic regions; on chromosome 4p15.3 and chromosome 5 with a multipoint LOD score of 2.1 for each locus. The border of the candidate regions were defined using the SNPs data. On chromosome 4p recombinations observed in patients VIII:2 (upper border) and VIII:4 (lower border) determined the region extending from rs10938692 to rs758327 (8.2-15.5 Mb, NCBI build 37.1). On chromosome 5, the candidate region extended from rs10805760 until rs168825 (24.3-31.7 Mb), based on recombinations observed in patients VII:9 (upper border) and VII:12 (lower border).
We then tested 23 micro-satellite markers mapping to chromosome 4 (3-28 Mb) and chromosome 5 (16-34 Mb) and included the new patient (VI:6, 11^{th} degree relative of the proband). The linkage analysis yielded a maximum LOD score of 3.02 for the locus on chromosome 4 while on chromosome 5 the LOD score was negative (LOD=-0.5).
Haplotype analysis confirmed that all 10 patients were sharing a haplotype on chromosome 4 from marker CGR525 until D4S2960 (Figure 2). The maximum candidate region after

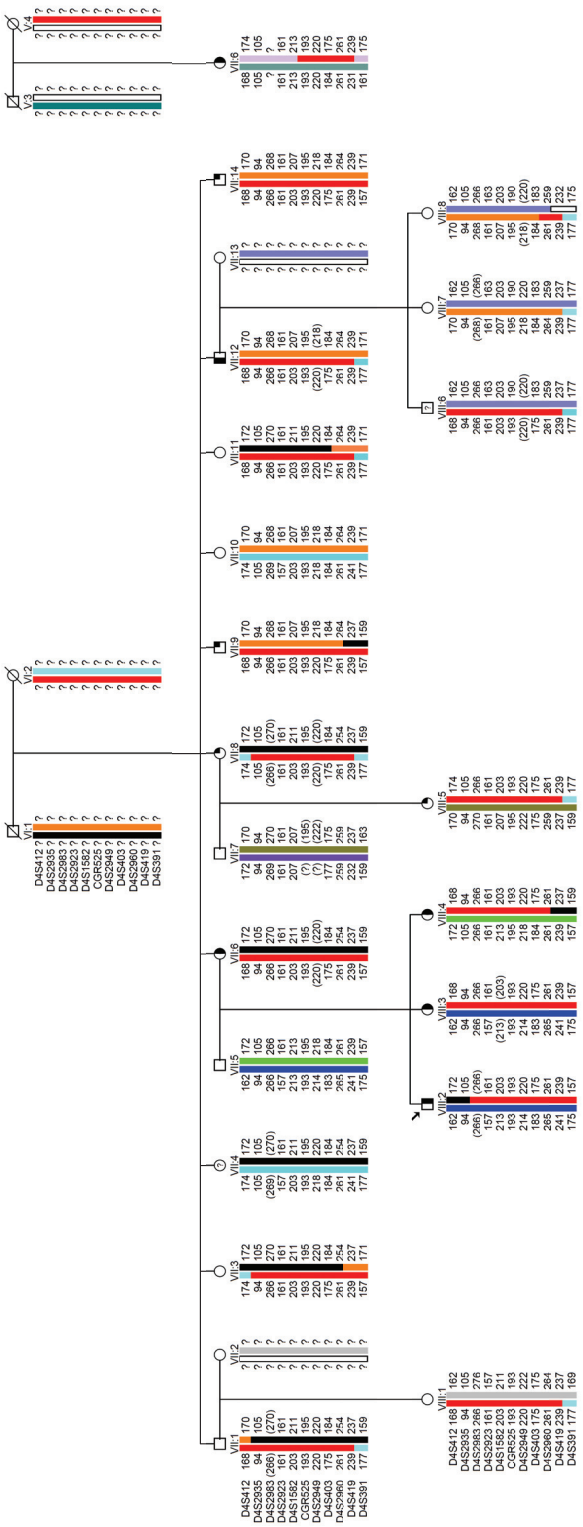


Figure 2. Pedigree with haplotype analysis of chromosome 4
The arrow indicates the proband

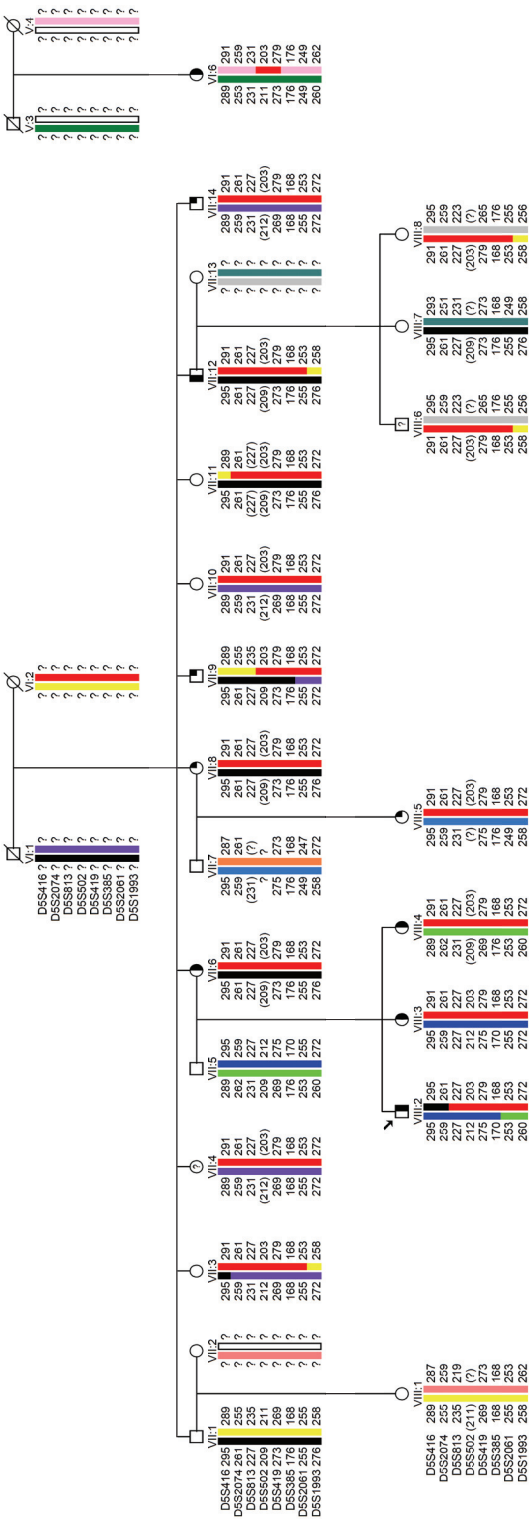


Figure 3. Pedigree with haplotype analysis of chromosome 5
The arrow indicates the proband

combining with the SNP data was reduced to 5.47 Mb (10.03-15.5 Mb) and contained 25 genes from which 13 genes have a "hypothetical or predicted" status with no reference sequence or are pseudogenes (NCBI build 37.1). From the 12 remaining genes, the *RAB28*, *CC2D2A*, *SLC2A9*, *WDR1* and *NKX3.2* gene are considered candidate genes, either because of their expression in the heart or because of their function. Sequence analysis of all these genes is ongoing.

The region on chromosome 5 could not be excluded; we observed allele sharing among the patients in a small region comprising only two markers (D5S502-D5S419, Figure 3). Considering the SNP and micro-satellite data, the maximum region comprised only 1.8 Mb (25.6-27.4 Mb) and 5 genes, the majority are of them also "predicted" or pseudogenes.

Discussion

NCCM is a heterogeneous disorder, so far 14 genes and 2 loci were identified.[5, 9, 11, 14-21] Here we performed a genome wide linkage analysis in a family with autosomal dominant NCCM and identified a novel locus on chromosome 4p15.3 with a maximum LOD score of 3.02. Due to software limitations the linkage analysis was performed assuming that the newly identified patient was sharing a common ancestry only four generation ago. The calculated 3.02 LOD score is therefore lower than the actual LOD score.

We performed extensive haplotype analysis in this genomic area and identified allele sharing among all patients in a region that extended 4.57 Mb and five consecutive microsatellite markers. However, from patient VI:6 no first-degree relatives were available to determine whether these alleles are on the same (maternal) chromosome. On chromosome 5, allele sharing with patient VI:6 was less evident, comprising only two markers. Until the actual segregation of the alleles is confirmed by genotyping of first-degree relatives, the locus on chromosome 5 cannot be excluded. From the known or predicted genes located within the 5.47-Mb disease gene interval on chromosome 4, *RAB28*, *CC2D2A*, and *SLC2A9* were considered candidate genes for NCCM because of their cardiac expression, *NKX3.2* and *WDR1* were considered candidate genes because of their function.

RAS-associated protein 28 (*RAB28*), member of the RAS oncogene family, is a RAS-related GTPase that operates signalling pathways in cell growth, metabolism and organelle trafficking.[22] Mutations in other RAS oncogene family members cause Noonan, Leopard, Costello and cardio-facio-cutaneous (CFC) syndrome and neurofibromatosis (*PTPN11*, *KRAS*, *RAF1*, *SOS1*, *BRAF*, *MAP2K1*, *MAP2K2* and *NF1*). In all these syndromes hypertrophic cardiomyopathy has been identified, making *RAB28* an excellent candidate gene.

Coiled-Coil and C2 Domains-containing protein 2A (*CC2D2A*) is expressed in both foetal and adult heart. Its function is cilia-related.[23] Homozygous mutations in this gene have been associated with Meckel and with Joubert syndrome and with mental retardation in combination with retinitis pigmentosa. Solute carrier family 2 (*SLC2A9*) is a urate transporter mainly expressed in the kidney, and is important for renal urate reabsorption.[24] Lower expression levels were seen in the heart. Mutations in this gene have been associated with hypo-uricemia

and a strong association with serum uric acid concentration was found.[25] There are studies in the literature showing a correlation between increased serum urate and blood pressure, coronary artery disease, and metabolic disorders.[26-28]

WD repeat domain 1 (*WDR1*) or actin-interacting protein 1 (*AIP1*) is a gene encoding for a protein with 9 WD repeats. WD domains are involved in protein-protein interactions. In *C. elegans* AIP1 synergistically promotes sarcomeric actin organisation in striated muscle cells.[29] It is therefore a good candidate gene.

NK3 homeobox 2 (*NKX3.*2) is a member of the homeobox family and is expressed in embryonic tissue. It is associated with skeletal dysplasia.[30] Nishida et al identified *NKX3.2* expression in arterial smooth muscle cells of chicken.[31] Perhaps this gene plays a role in the pathogenesis of NCCM by faulty expression in coronary arteries. Furthermore, another member of the homeobox family, *NKX2.5*, has been shown to be associated with congenital heart disease.[32] Therefore the *NKX3.2* should also be considered.

Similar to other heart muscle disorders, NCCM shows extensive genetic heterogeneity, with sarcomere and Z-disc genes and mutations in *TAZ, LMNA* and *DTNA* have been reported.[5, 9, 11, 14-19] Linkage analysis in the extended family with autosomal dominant NCCM identified one putative NCCM locus. All patients presented with isolated NCCM without congenital heart defects. Four patients did not fulfil all diagnostic criteria established for isolated NCCM because the ratio of noncompacted to compacted myocardium (NC/C ratio) was did not reach the established value for diagnosis. However, there is no mechanical or haemodynamic explanation for the development of NCCM in these patients, there weren't any left ventricular inflow and/or outflow tract abnormalities. It is very likely that the cause of their slight NCCM is genetic, indicating that further studies of NCCM criteria for relatives at risk are needed, similar to HCM.[33]

In conclusion, we have mapped a locus for autosomal dominant NCCM to chromosome 4p15.3 and narrowed the critical region to 5.47 Mb. Sequence analysis of all genes located on this locus is ongoing as is cardiologic family screening of first-degree relatives of patient VI:6.

These findings confirm the genetic heterogeneity of this disorder. Identification of the disease-causing gene will allow genetic screening and provide new insights into the pathogenic mechanism of NCCM development.

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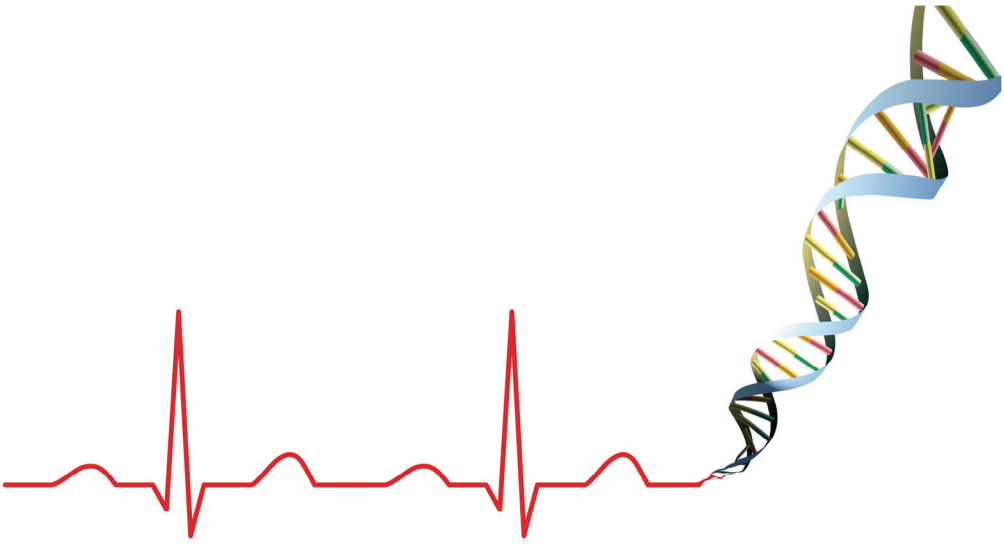
Chapter 7

Compound heterozygousity for truncating mutations in the *MYBPC3* gene cause severe cardiomyopathy with left ventricular noncompaction and septal defects resulting in neonatal death.

Marja W. Wessels, Yvonne M. Hoedemaekers, M. Dalinghaus, Arthur van den Wijngaard, Ingrid M.E. Frohn-Mulder, Ronald R. de Krijger, Patrick J. Willems, Rene F.M.de Coo, Dennis Dooijes.

Submitted



Abstract

Aims

Familial hypertrophic cardiomyopathy is usually caused by autosomal dominant mutations in genes encoding sarcomeric cardiac muscle proteins. The disease mainly affects adults, but young children with severe hypertrophic cardiomyopathy have been reported. We describe here two unrelated neonates with a severe form of hypertrophic cardiomyopathy, and performed molecular studies to identify the genetic defect.

Methods and results

Two unrelated neonates with lethal cardiomyopathy were studied at the clinical, pathological and molecular level. Both patients were compound heterozygous for two common loss-of-function mutations in the *MYBPC3* gene. One of the patients also presented with a ventricular septal defect, whereas the other patient had an atrial septal defect.

Conclusions

Whereas heterozygous mutations in sarcomeric protein genes usually lead to hypertrophic cardiomyopathy with clinical symptoms starting in child- or adulthood, homozygousity or compound heterozygousity for two truncating mutations in the *MYBPC3* gene can cause severe neonatal cardiomyopathy with features of left ventricular noncompaction. Furthermore, mutations in sarcomeric protein genes seem also to be implicated in congenital heart malformations.

Introduction

Hypertrophic cardiomyopathy (HCM) is a major cause of sudden cardiac death in people younger than 35 years of age under physical stress, and a major cause of mortality and morbidity in the elderly, with an estimated prevalence of 1 in 500 individuals according to echocardiographic criteria. In approximately 60% of cases a mutation in one of the sarcomeric contractile protein genes is found.[1, 2] Familial HCM is usually transmitted as an autosomal dominant condition due to heterozygous gene mutations with incomplete penetrance. After the identification in 1990 of HCM-causing mutations in the cardiac β-myosin heavy chain gene (*MYH7*).[3, 4] mutations in genes encoding proteins involved in the sarcomere, cytoskeleton and Z-disc, calcium handling, mitochondrial and lysosomal functions have been associated with HCM.[5, 6] More than 400 different mutations have been found in the genes that encode sarcomeric proteins, such as ß-cardiac myosin heavy chain (*MYH7)*, cardiac myosin-binding protein-C (*MYBPC3*), α-cardiac myosin heavy chain (*MYH6),* regulatory myosin light chain (*MYL2*), essential myosin light chain (*MYL3*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), tropomyosin (*TPM1*), cardiac actin (*ACTC1*), and titin (*TTN*) (see: http://cardiogenomics.med.harvard.edu).

Partly due to the genetic heterogeneity, interfamilial clinical variability in HCM is high. Also intrafamilial variability is considerable, and it has proven difficult to establish good genotype-phenotype correlations in HCM. In addition to the primary genetic defect, the effects of modifier genes or additional mutations in other sarcomeric genes may contribute to the phenotypic expression of HCM.[7] Childhood-onset cardiac hypertrophy is also genetically determined in the majority of cases, and two thirds of familial cases of childhood-onset cardiac hypertrophy are caused by a mutation in one of the sarcomeric protein genes.[8] Here we describe two unrelated children with severe cardiomyopathy, ventricular noncompaction and septal defects, due to compound heterozygousity for truncating mutations in *MYBPC3,* resulting in neonatal death.

Methods

Clinical diagnosis

Two unrelated families with an index patient with severe neonatal cardiomyopathy were studied after informed consent at the clinical, pathological and molecular level. Clinical evaluation included clinical history and physical examination, electrocardiography (ECG) and 2D and M-mode echocardiography. Noncompaction of the left ventricle was diagnosed based upon three echocardiographic criteria defined by Jenni et al.[9] including i) a thick non-compacted (NC) endocardial layer in end systole at the parasternal short-axis views (ratio NC/C >2) with numerous, excessively prominent trabeculations and deep intertrabecular recesses, ii) that are perfused on colour Doppler studies and iii) predominantly apical localisation.

Pathologic studies

Microscopic examination and electron microscopy of cardiac autopsy material of patient 1 was performed with standard techniques.

Molecular analysis

Genomic DNA of the patients was isolated from blood samples. All coding regions and intron-exon boundaries of the *MYBPC3* gene were analysed by direct sequence analysis. Sequence analysis of M13-tagged PCR products was carried out on an ABI3730xl capillary sequencer using Big-Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of methods and primer sequences are available on request.) Analysis of sequence data was performed using SeqScape analysis software (v2.5, Applied Biosystems). In addition to sequence analysis, MLPA analysis of the *MYBPC3* gene was carried out (MRC Holland SALSA MLPA kit P100) to detect possible genomic rearrangements. Exons of the *MYBPC3* gene were numbered 1-34 according to international standards with the Adenine of the translation initiation start site (ATG) numbered +1 and the ATG in exon 1 (www.HGVS.org). Subsequently, using the same techniques, the complete coding regions and intron-exon boundaries of eight other sarcomeric genes (*MYH7, TNNT2, TNNI3, MYL2, MYL3, TNNC, TPM1* and *ACTC1)* were analysed to exclude that additional pathogenic mutations in these genes contributed to the phenotype observed in the patients.

Results

Patient 1

This male patient was born after an uncomplicated pregnancy at 40 weeks gestation with a birth weight of 3110 grams and Apgar scores of 9/9. He was the second child of healthy Dutch non-consanguineous parents. At the age of one week cyanosis was noticed during crying and the child experienced feeding difficulties. At the age of five weeks he was hospitalised because of feeding problems, perspiration and facial cyanosis. X-thorax showed a grossly enlarged heart. Echocardiography revealed a moderately dilated left ventricle with severe systolic dysfunction. The apical wall of the left ventricle was excessively thickened with prominent hypertrabeculation. The left and right atria showed mild dilatation. A small secundum atrial septal defect (ASD) was also present. He was treated for heart failure with Furosemide, Captopril, Carnitine and Digoxin. Viral serology showed no abnormalities. Metabolic screening of urine, including oligosaccharide spot test, was normal. Plasma carnitine and amino acid levels were normal. After stabilisation, the child was released from hospital, but two weeks later he was readmitted to the hospital presenting with a pallor colour and progressive feeding problems. X-thorax and echocardiography revealed further enlargement of the heart (heart-thorax ratio ± 0.7) with severe hypertrophy with a fractional shortening of the left ventricle of less than 10%. The child died from cardiac failure at the age of 12 weeks. Macroscopic examination of the heart revealed severe cardiomegaly and dilatation with a total weight of 115 gram (normal weight at this age: 30 gram). Right ventricular thickening was noted, especially of the LV posterior wall. The anterior wall of the left ventricle was also severely thickened, and showed abnormal trabeculation and multiple intertrabecular recesses as seen in noncompaction cardiomyopathy. The secundum ASD was confirmed. No other congenital malformations were found. Microscopic examination of cardiac tissue (Figure 1A) showed

myofibrillar disarray in both the ventricular septum and the left ventricular wall. No significant amount of interstitial fibrosis was observed. Hypertrophic myocytes with a diameter varying between 20 and 30 micrometers (normal 12 micrometers) and multiple vacuoles on electron microscopy were suggestive of a glycogenosis (Figure 1B). As the echocardiography images and ECG were not suggestive of Pompe disease and urine oligosaccharide analysis was normal, no α-glucosidase enzyme or molecular assay was performed.

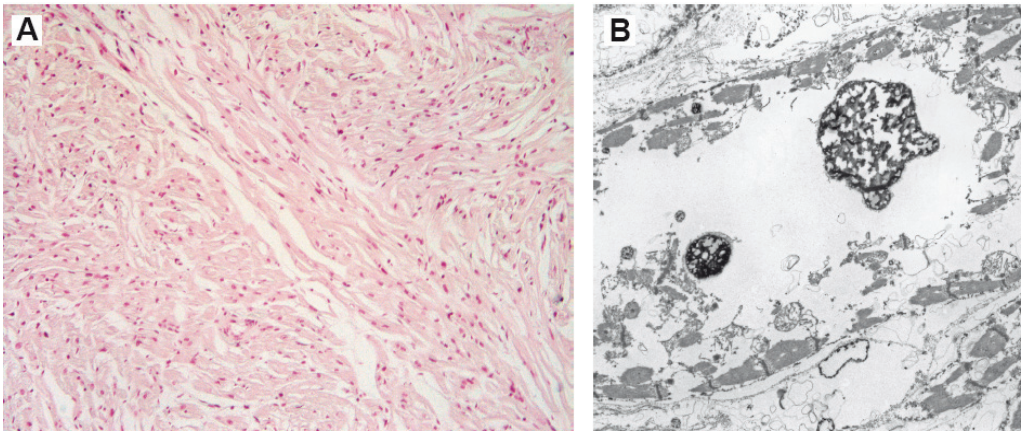


Figure 1. Histopathology

- **A.** Microscopic examination of post-mortem heart muscle from patient 1. Hypertrophic myocytes with myofibrillar disarray typical of HCM due to sarcomeric protein mutations was present, albeit without significant amount of interstitial fibrosis.
- **B.** Electron micrograph showing a large, irregular vacuole

Sarcomere mutation analysis was initiated by screening of the complete coding region and intron-exon boundaries of the *MYBPC3* gene. This analysis revealed that the patient was compound heterozygous for the c.2373dupG mutation and the c.2827C>T (p.Arg943X) mutation. Mutation analysis of additional sarcomeric genes (*MYH7, TNNT2, TNNI3, MYL2, MYL3, TNNC, TPM1* and *ACTC1*) did not result in the identification of additional pathogenic mutations. Large deletions of mitochondrial DNA, and several mitochondrial missense mutations associated with hypertrophic cardiomyopathy were excluded. Mutation analysis in both parents identified the c.2373dupG mutation in the mother and the p.Arg943X mutation in the father (Figure 2).

At the time of diagnosis of cardiomyopathy in their newborn child, both parents had no cardiac symptoms. Echocardiography revealed no abnormalities in the mother at age 32 years and the father at age 31 years. ECG in the father showed mild repolarisation abnormalities (ST-elevation of 0,5 mm in V1 followed by a negative T in V1-V4). Re-evaluation of both parents after seven years, revealed moderate septal hypertrophy (HCM) with an interventricular septum of 14 mm in the mother, and interventricular septal measurements at the upper limit of the normal range (12 mm) in the father. Family history indicated that there were several family members with cardiac symptoms, sudden death, and/or diagnosed with HCM on both sides of the family (Figure 2).

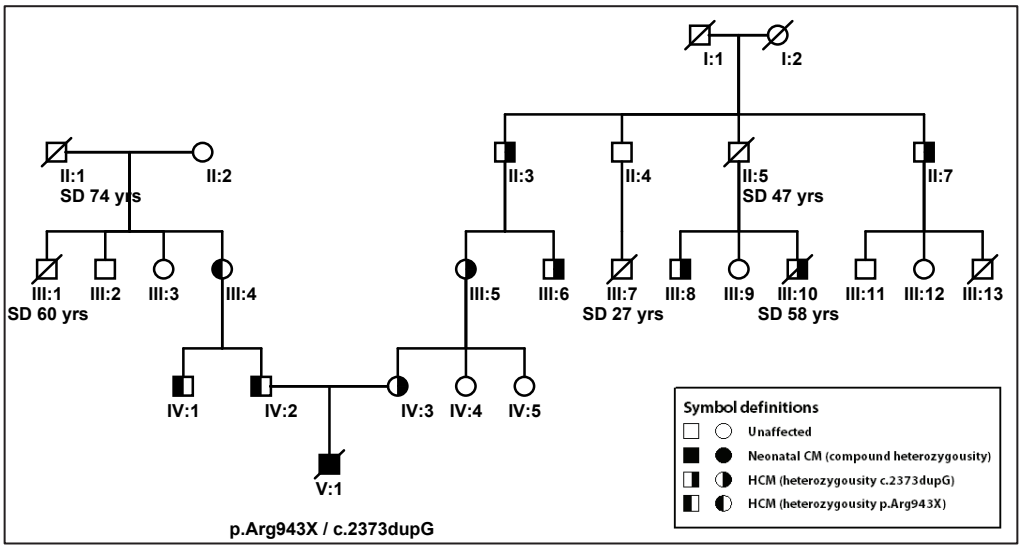


Figure 2. Pedigree of patient 1.
Pedigree of patient 1 who had severe neonatal cardiomyopathy due to compound heterozygousity for the c.2373dupG and p.Arg943X mutations in the MYBPC3 gene. On both sides of the pedigree, there are multiple affected individuals with adult HCM that are heterozygous for one of the mutations.

Patient 2

This male patient was born at 38 weeks gestation with a birth weight of 3345 gr. He was the second child of non-consanguineous parents. He experienced feeding problems in the first weeks of life. At the age of four weeks dyspnoea was noticed and he was referred to the hospital. He was mildly hypotonic. A grossly enlarged heart was observed on the X-thorax and low oxygen saturation blood levels were found. He was referred to the paediatric cardiology centre for further evaluation. Echocardiography revealed moderately dilated ventricles with severe diastolic and systolic dysfunction. Hypertrabeculation with flow perfused intertrabecular recesses was present in both ventricles, including the apical walls (Figure 3). An apical muscular VSD was visualised, although deep recessal flow complicated interpretation of the imaging.

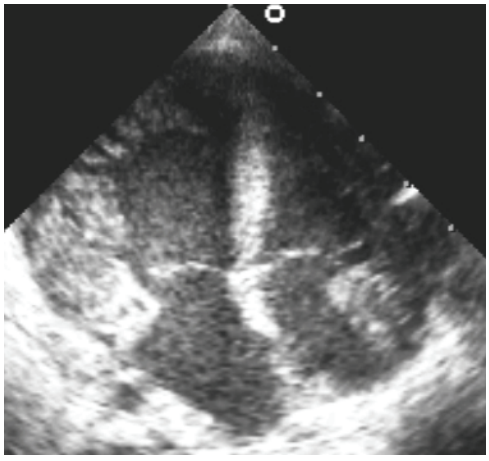


Figure 3. Echocardiography of patient 2.
Echocardiographic studies in patient 2 showing cardiomyopathy with LV non-compaction. The four chamber shows excessive trabeculation (arrows) at the apical right ventricular wall, whereas the left ventricular wall also showed numerous deep trabeculae and recesses.

A muscle CT scan showed no signs of atrophy, and a muscular biopsy revealed no congenital myopathy. Biochemical studies, including lactate, pyruvate, creatine kinase, amino acids, carnitine and sialotransferrines levels, and metabolic screening of urine, including oligosaccharides, were normal. Viral serology showed no abnormalities. The patient was treated for heart failure with Furosemide, Captopril and Digoxin. He deteriorated at the age of 12 weeks, and died.

The complete coding regions and intron-exon boundaries of the *MYBPC3* gene were analysed. Compound heterozygousity for the c.2373dupG and the c.2827C>T (p.Arg943X) in the *MYBPC3* gene was found. Molecular analyses of other sarcomeric genes (*MYH7, TNNT2, TNNI3, MYL2, MYL3, TNNC, TPM1* and *ACTC1*) revealed no additional disease-causing mutation. The parents declined pathological studies of the heart of their deceased child, nor did they decide on further cardiologic evaluation for themselves or pedigree analysis.

Discussion

Here we describe two unrelated newborns with severe cardiomyopathy resulting in neonatal death due to compound heterozygousity for null mutations in the *MYBPC3* gene. Although initially their cardiomyopathies were described as severe atypical HCM, HCM with noncompaction (NCCM) was considered after re-evaluation of serial ultrasounds and pathologic examination. NCCM is a genetically heterogeneous disease associated with mutations in *TAZ(G4.5)*, *DTNA*, *LDB3*, *SCN5A*, and *LMNA* in a limited number of families.[10-14] Recently, NCCM was shown to be due to heterozygous mutations in genes encoding sarcomeric proteins, including ß-cardiac myosin heavy chain (*MYH7)*, alpha-cardiac actin (*ACTC)* and cardiac troponin T *(TNNT2*).[15-19] Here we report that mutations in another sarcomeric protein gene *MYBPC3* also lead to NCCM, as we previously reported.[20] This suggests that NCCM, HCM, dilated cardiomyopathy (DCM) and restrictive cardiomyopathy (RCM) are allelic diseases that share similar pathophysiological mechanisms.

In the majority of patients with familial cardiomyopathy due to a mutation in one of the genes encoding sarcomeric proteins, a single autosomal dominant mutation is found. In contrast our two patients are compound heterozygotes for two truncating *MYBPC3* mutations, suggesting a cumulative effect of these mutations. Mutations in the *MYBPC3* gene, encoding cardiac myosin binding protein C, are one of the most common genetic causes of HCM in many populations, found in approximately 20-40% of individuals with HCM.[21-23] Autosomal dominant mutations in the *MYBPC3* gene, mostly truncating mutations and sometimes missense mutations, give rise to HCM with an age of onset after the third decade and moderate left ventricular hypertrophy.[24] As mutations in *MYBPC3* (and *MYH7*) are the most common genetic cause of familial HCM[8], compound heterozygous or homozygous mutations should be considered in a neonate which presents with severe HCM or NCCM, even in the absence of symptoms in the parents or a negative family history. This is shown here where both patients with severe neonatal cardiomyopathy are compound heterozygotes for the two most frequent HCM mutations in the Netherlands, the c.2373dupG mutation and the p.Arg943X mutation. The c.2373dupG mutation

alone accounts for nearly one-fourth of all HCM cases in the Netherlands, and has previously been shown to be an important founder mutation in the Dutch population and also to be present in other populations.[21, 25, 26] The mutation creates a new aberrant splice donor site leading to skipping of exon 24, resulting in a frame shift after p.Gln791 and a premature stop.[27] No truncated protein product from the c.2373dupG allele could be detected in the sarcomere using antibodies, suggesting that the truncated protein was unstable, or the aberrant transcript was degraded by cell surveillance mechanisms such as nonsense mediated decay.[28] The second mutation found in both our patients is the p.Arg943X mutation. This mutation was identified as an additional founder mutation in the Dutch population (Chapter 9). P.Arg943X is a nonsense mutation in exon 27, leading to a premature stop codon and protein truncation beyond domain C 7 of MYBPC3. Even in the absence of nonsense mediated decay of the mutant *MYBPC3* mRNAs the p.Arg943X mutation is thought to lead to reduction in MYBPC3 due to protein instability and/or loss of the C-terminus of MYBPC3 that binds myosin thick filaments and titin, which is required for normal MYBPC3 incorporation into the A-band of the sarcomere.[29] As a consequence, in our patients no MYBPC3 protein is expected to be incorporated into the sarcomere, which might explain the early and severe presentation of the cardiomyopathy in both patients.

In larger series approximately 3-5 % of HCM patients are compound or double heterozygotes for two disease-causing mutations in the same or different sarcomeric protein genes.[21, 22] However, most of these mutations are missense mutations of which the pathogenic nature is not always easy to establish. In these patients generally a more severe HCM phenotype is seen, characterised by an age of onset around the second decade or in childhood (Table 1).[21, 22, 30-39] In a recent study on sarcomeric protein gene mutations in childhood-onset HCM six out of 84 children (7%) had compound mutations.[8] This suggests that a gene-dosage effect might be responsible for manifestations at a younger age.

Not only clinical features such as the neonatal onset of severe symptoms, but also the pathology of the heart in patient 1 initially suggested a metabolic origin e.g. glycogenosis (Figure 1). It is known that mutations in genes regulating glycogen metabolism, including AMP-activated protein kinase γ_2 (*PRKAG2*) in Wolff-Parkinson-White associated cardiomyopathy, lysosome-associated membrane protein 2 (*LAMP2*) in Danon disease, α-galactosidase (*GLA*) in Fabry's disease, and acid α-1,4-glucosidase (*GAA*) in Pompe's disease can cause left ventricular hypertrophy that mimics HCM.[40] Conversely, metabolic diseases might also be misdiagnosed in patients with HCM due to sarcomeric protein mutations, as shown in our patient. Mutations in genes regulating glycogen metabolism cause myocyte hypertrophy by stimulating glycogen-filled vacuoles but cause neither myocyte disarray nor interstitial fibrosis, which typically occur with defects of sarcomere-protein genes.

Few neonatal cases with severe cardiomyopathy due to homozygous or compound heterozygous truncating mutations in *MYBPC3* have previously been described.[21, 34, 37, 38] A homozygous splice site mutation p.Asp1064GlyfsX38 in *MYBPC3* (leading to a frame shift and premature truncation) was recently described in three neonates with severe neonatal HCM that

Table 1. Patients with cardiomyopathy due to 2 mutations in the *MYBPC3* gene, and/or additional sarcomeric protein genes.*

Gene 1 / Mutation 1	Gene 2/ Mutation 2	Phenotype	Severity	Age at first study	Reference
***MYBPC3* mutation with mutation in other sarcomeric gene**					
MYBPC3 / p.Arg273His	*MYH7* / p.Arg719Gln	HCM	Moderate /severe	35 and 15 yrs	45
MYBPC3 / p.Asp605Asn	*MYH7* / p.Glu894Gly	HCM	**	***	22
MYBPC3 / c.2373dupG	*MYH7* / p.Arg694Cys	HCM	**	***	22
MYBPC3 / p.Glu1096X	*MYH7* / p.Glu483Lys	HCM	Severe	33 and 56 yrs	21, 36
MYBPC3 / p.Ala833Thr	*TNNT2* / p.Arg286His	HCM	**	***	22
MYBPC3 / p.Val256Ile	*TNNT2* / p.Arg92Trp	HCM	**		22
MYBPC3 / p.Arg943X	*TNNI3* / p.Ser166Phe	HCM	**	***	22
MYBPC3 / p.Phe1113Ile	*TPM1* / p.Ile172Thr	HCM	**	***	22
MYBPC3 / p.Arg495Gln	*TNNI3* / p.Arg141Gln	Childhood HCM**		Before 15 yrs	8
Compound heterozygote or homozygote mutations in MYBPC3					
MYBPC3 / p.Pro873His	*MYBPC3* / p.Pro873His	HCM	Moderate /severe	27 yrs	39
MYBPC3 / p.Arg810His	*MYBPC3* / p.Arg810His	HCM	Severe	39 yrs	39
MYBPC3 / p.Arg502Trp	*MYBPC3* / p.Gly5Arg	HCM	*	*	22
MYBPC3 / p.Ala954fs	*MYBPC3* / p.Glu258Lys	HCM	*	*	22
MYBPC3 / p.Ala627Val	*MYBPC3* / p.Ala627Val	HCM	Severe	47 yrs	31
MYBPC3 / p.Pro873His	*MYBPC3* / p.Asp745Gly	HCM	Severe	29 yrs	45
MYBPC3 / p.Glu542Gln	*MYBPC3* / p.Ala851Val	HCM	Severe	34 yrs	45
MYBPC3 / p.Gln76X	*MYBPC3* / p.His257Pro	HCM	Mild symptoms	24 yrs	21
MYBPC3 / p.Thr1028Ser	*MYBPC3* / c.3490+2T→G	Childhood HCM**		Before 15 yrs	8
MYBPC3 / p.Arg502Trp	*MYBPC3* / p.Ser858Asn	Childhood HCM**		Before 15 yrs	8
MYBPC3 / p.Ile154Thr	*MYBPC3* / p.Asp605del	Childhood HCM**		Before 15 yrs	8
Double truncating mutations in MYBPC3					
MYBPC3 / p.Gln76X	*MYBPC3* / p.Gln76X	Neonatal HCM	Death 9 months		21
MYBPC3 / c.1624+1G>A	*MYBPC3* / c.2373dupG	Neonatal HCM	Death 5 weeks	3 days	34
MYBPC3 / p.Arg943X	*MYBPC3* / p.Glu1096fs	Neonatal HCM/**VSD**	Death 6 weeks	2 weeks	34
MYBPC3 / p. Asp1064GlyfsX38	*MYBPC3* / p. Asp1064GlyfsX38	Neonatal HCM/**ASD, VSD**	Death 6 weeks-7 months	1-3 weeks	37, 38
MYBPC3 / p.Arg943X	*MYBPC3* / c.2373dupG	Neonatal HCM/**ASD**	Death 3 months	5 weeks	This study
MYBPC3 / p.Arg943X	*MYBPC3* / c.2373dupG	Neonatal HCM/**VSD**	Death 3 months	4 weeks	This study

Legend.
* The pathogenicity of some of the missense mutations listed here is uncertain
** More severe HCM and highest incidence of myectomy as compared to patients with single *MYBPC3* mutations[22]
*** Diagnosis at a younger age (between 0.2 and 37.4 yrs) as compared to patients with single *MYBPC3* mutations[22]

all died at the average age of 3-4 months in an inbred Old Order Amish pedigree with severe HCM.[37] This homozygous *MYBPC3* mutation was also reported in another cohort of 10 neonates with severe infantile HCM from Old Order Amish descent, suggesting that this mutation is a founder mutation in the Amish.[38] It is remarkable that several of the affected neonates from the Old Order Amish with homozygous truncating mutations in *MYBPC3,* present with septal defects including apical muscular VSD, and ASD and patent ductus arteriosus. Septal defects were also present in the neonates with severe HCM due to compound heterozygous truncating mutations described by Lekanne et al.[34] and the two cases described here. Recently, different congenital heart malformations (septal defects, patent ductus arteriosus, aortic aneurysm and Ebstein anomaly) have been reported in families with mutations in sarcomeric protein genes, including *MYBPC3, MYH6, MYH7, MYH11,* and *ACTC1.*[16, 19, 37, 41-43] This suggests that sarcomeric cardiac muscle proteins are not only involved in cardiomyopathies but also in congenital heart malformations.[44]

In conclusion, the absence of functional MYBPC3 from the sarcomere can lead to a phenotype of severe HCM with features of ventricular noncompaction and septal defects, which appears to be lethal in the postnatal period.

Acknowledgements

We are grateful to the family members of the two patients and the referring physicians for their contributions. The authors kindly thank Dr Koert P. Dingemans for providing a electronic micrograph and Tom de Vries Lentsch for graphical support.

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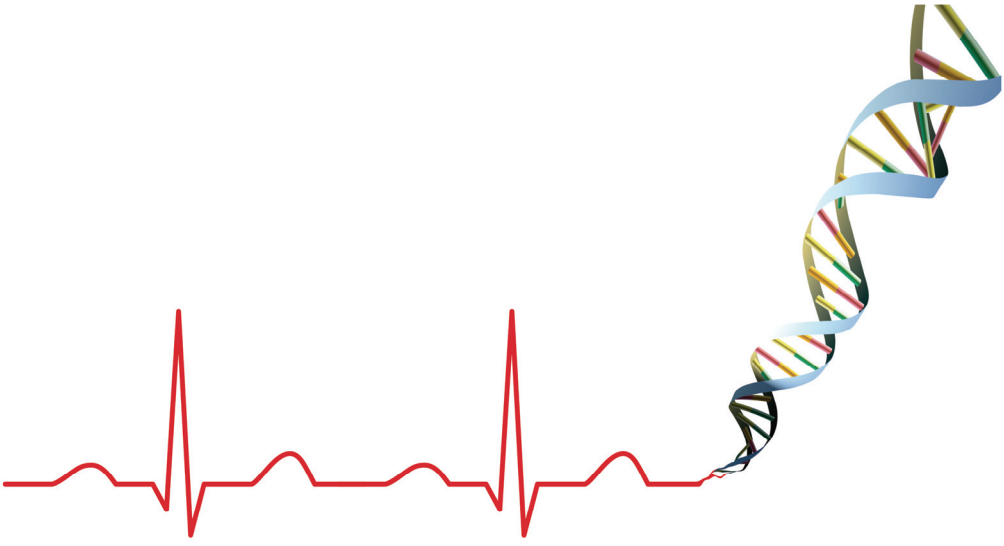
Chapter 8

Familial screening and genetic counseling in hypertrophic cardiomyopathy: the Rotterdam experience.

Michell Michels, Yvonne M. Hoedemaekers, Marcel M.J. Kofflard, Ingrid M.E. Frohn-Mulder, Dennis Dooijes, Danielle F. Majoor-Krakauer, Folkert J. ten Cate.

Neth Heart J. May 2007;15(5):184-190



REVIEW ARTICLE

Familial screening and genetic counselling in hypertrophic cardiomyopathy: the Rotterdam experience

M. Michels, Y.M. Hoedemaekers, M.J. Kofflard, I. Frohn-Mulder, D. Dooijes, D. Majoor-Krakauer, F.J. Ten Cate

Hypertrophic cardiomyopathy (HCM) is a disease characterised by unexplained left ventricular hypertrophy (LVH) (i.e. LVH in the absence of another cardiac or systemic disease that could produce a similar degree of hypertrophy), electrical instability and sudden death (SD).
Germline mutations in genes encoding for sarcomere proteins are found in more than half of the cases of unexplained LVH. The autosomal dominant inherited forms of HCM are characterised by incomplete penetrance and variability in clinical and echocardiographic features, prognosis and therapeutic modalities. The identification of the genetic defect in one of the HCM genes allows accurate presymptomatic detection of mutation carriers in a family. Cardiac evaluation of at-risk relatives enables early diagnosis and identification of those patients at high risk for SD, which can be the first manifestation of the disease in asymptomatic persons.
In this article we present our experience with genetic testing and cardiac screening in our HCM population and give an overview of the current literature available on this subject. (*Neth Heart J* 2007;15:184-9.)

Keywords: hypertrophic cardiomyopathy, genetics

M. Michels
F.J. Ten Cate
Department of Cardiology, Thoraxcentre, Erasmus Medical Centre, Rotterdam, the Netherlands
Y.M. Hoedemaekers
D. Dooijes
D. Majoor-Krakauer
Department of Clinical Genetics, Thoraxcentre, Erasmus Medical Centre, Rotterdam, the Netherlands
M.J. Kofflard
Albert Schweitzer Hospital, Dordrecht, the Netherlands
I. Frohn-Mulder
Department of Paediatric Cardiology, Thoraxcentre, Erasmus Medical Centre, Rotterdam, the Netherlands

Correspondence to: F.J. Ten Cate
Department of Cardiology, Thoraxcentre, Erasmus Medical Centre, PO Box 2040, 3000 CA Rotterdam, the Netherlands
E-mail: f.j.tencate@erasmusmc.nl

The prevalence of unexplained left ventricular hypertrophy (LVH) in the general population is estimated to be 1 in 500.[1,2] Hypertrophic cardiomyopathy (HCM) caused by genetic defects in sarcomere genes may account for up to 60% of cases of LVH. This makes HCM the most common genetic cardiovascular disorder.[3,4] The first HCM gene was identified in 1990;[5] since then more than 11 HCM genes, mostly encoding for one of the sarcomere proteins, have been identified.

HCM is the most common cause of sudden death (SD) in the young and a major cause of morbidity and mortality in the elderly.[6] SD may be the first manifestation of the disease in an asymptomatic individual. Many studies in HCM have shown extensive variability in expression, prognosis and therapy, even within families. Identification of disease-causing genetic defects in familial HCM allows reliable identification of at-risk relatives. Cardiac evaluation of at-risk relatives enables early diagnosis and identification of those patients at high risk for SD.

Genetic studies in HCM

At present, more than 400 disease-causing mutations in 11 genes, mostly encoding one of the myocardial contractile proteins (table 1), have been identified in familial HCM. In approximately 60% of HCM families a sarcomere mutation is identified.[3,7,8] Disease-causing mutations are identified in 60% (142/236) of the HCM families currently being followed and completely genotyped for eight HCM genes in Rotterdam.

At the cardiogenetics clinic of the Erasmus Medical Centre in Rotterdam, the molecular analysis of HCM involves initial screening for the three Dutch founder mutations in the MYBPC3 gene. In the absence of the founder mutations, subsequently the MYBPC3 and the MYH7, cardiac troponin T (TNNT2), cardiac troponin I (TNNI3), regulatory and essential myosin light chain, α-actin, α-tropomyosin are sequenced. DNA sequencing is time consuming and expensive, molecular analysis of all HCM genes will take about six months.

Table 1. Genes identified in familial hypertrophic cardiomyopathy (modified from Ho et al).[17]

Gene	Encoding protein	Locus
MyBPC3	Myosin binding protein C	11p11.2
MYH7	β-myosin heavy chain	14q12
TNNT2	Troponin T	1q32
TNNI3	Troponin I	19q13.4
TPM1	α-tropomyosin	15q22.1
MYL2	Regulatory myosin light chain	12q23-q24.3
MYL3	Essential myosin light chain	3p21.3-p21.2
ACTC	α-actin	15q11-q14
TTN	Titin	2q24.3
MYH6	α-myosin heavy chain	14q12
PRKAG2	Protein kinase A (γ-subunit)	7q36

Generally, mutations in the MYBPC3 are the most common genetic defect in HCM with a prevalence ranging from 20 to 42%.[3,9] In the Netherlands this percentage is higher because of the existence of three founder mutations in MyBPC3.[10] In the Rotterdam HCM population, 70% (100/142) of the HCM families with an identified mutation had an MYPBC3 mutation. Of these families, 65% (65/100) had one of the three founder mutations in MyBPC3; we identified 39 families with the 2373insG mutation, 15 families with the R943X mutation and 11 families with the 2864delCT mutation.

Mutations in the MYH7 gene have been reported frequently, accounting for up to 40% of cases.[3] In the Rotterdam HCM families the contribution of MYH7 mutations was 18% (25/142).

Other HCM genes involved in the Rotterdam population are TNNT2, TNNI3, regulatory myosin light chain and α-tropomyosin; mutations in these genes are found in 2 to 3% of HCM families.

Given the high prevalence of HCM in the general population it is not surprising to encounter compound heterozygousity, especially in patients with severe, early onset of symptoms. Since it has been demonstrated that occurrence of multiple HCM gene mutations in a single patient is associated with a more severe phenotype, we pursue molecular analysis of HCM genes in patients with severe phenotype after the identification of a single mutation.[7,11]

Genotype/phenotype correlation in HCM

HCM is the final common pathway of several different sarcomere defects. There is a continuing debate over the prognostic significance of HCM-causing mutations.[12,13] Several highly penetrant genetic defects have been identified for Mendelian inherited forms of HCM. However, the clinical variability of HCM observed even within families suggests that the disease is at least partially the result of the additive or synergetic effect of risk factors, thus precluding the use of the genotype as the main clinical and prognostic tool for individual patients, at least until the mechanisms underlying the clinical variability are elucidated.

Genetic counselling, presymptomatic testing and screening of at-risk relatives

After the clinical diagnosis of HCM is established at our HCM outpatient clinic, the cardiologist discusses the importance of molecular confirmation, also in patients with no family history of HCM or SD, with the patient (figure 1). Supported by the new classification of cardiomyopathies as proposed by Maron et al. we think that part of the diagnosis of HCM should be molecular classification.[14] After informed consent, blood samples are drawn for DNA analysis of the proband.

Subsequently, patients are referred to the department of clinical genetics to ascertain the family history, allowing the identification of relatives at increased risk for HCM. Initially, we intend to inform first- and second-degree relatives about the occurrence of HCM in the family and invite them for genetic counselling by a clinical geneticist and/or a genetic nurse. Expanding the family history allows the identification of more at-risk relatives who are subsequently invited for genetic counselling (figure 1).

The results of the DNA analysis are conveyed at the department of clinical genetics, where the consequences of these results for the patient and their relatives are discussed. In HCM families in which we were able to identify a mutation in one of the HCM genes, presymptomatic DNA testing is offered to adults at risk. The results of presymptomatic testing of at-risk relatives are received within six weeks. No further cardiac screening is indicated for relatives without the disease-causing mutation. The mutation carriers are referred for cardiac screening by medical history, clinical examination, echocardiography, exercise-ECG and 24-hour Holter monitoring. In 2006, 45 asymptomatic mutation carriers were screened, which led to the

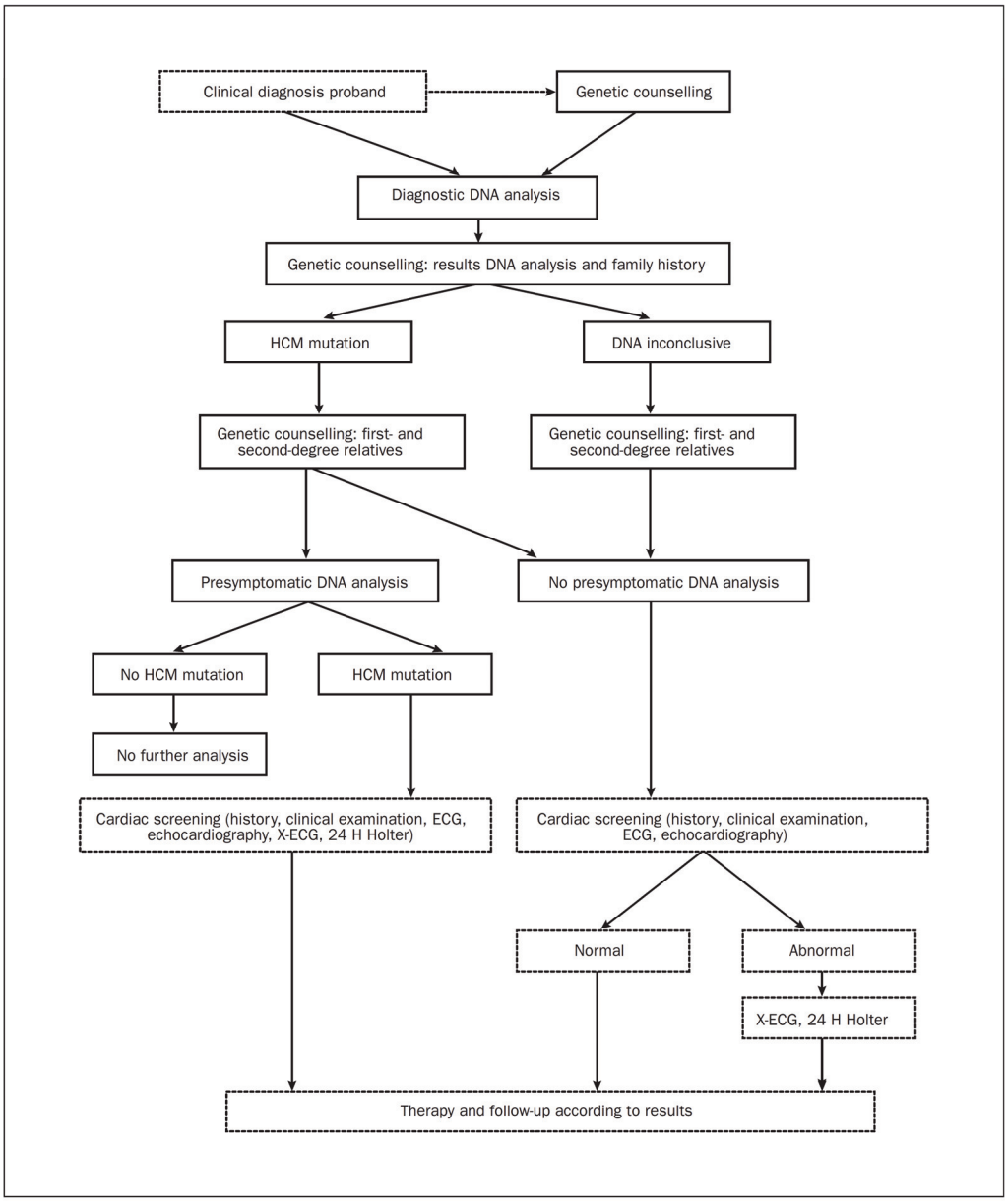


Figure 1. Flowchart used at the cardiogenetics department in the Erasmus Medical Centre showing the multidisciplinary approach by the cardiologist (dashed boxes) and clinical genetic nurses and medical doctors (un-dashed boxes). X-ECG=exercise test, 24 H Holter=24-hour Holter monitoring).

Table 2. Risk factors for sudden death in hypertrophic cardiomyopathy (modified from Elliot et al).[38]

Nonsustained ventricular tachycardia on ambulatory electrocardiography
Syncope
Blood pressure rise <25 mmHg during exercise in patients < 40 years
Family history of sudden death
Left ventricular wall thickness >30 mm

diagnosis of HCM in 16 (36%) of the referred subjects. Subjects carrying a mutation in MYH7 were affected at a significantly younger age than subjects carrying a mutation in MyBPC3. The diagnosis led to lifestyle changes, leading to cessation of competitive sports in seven (16%) subjects and in three (7%) subjects medical therapy was started. According to the established risk factors for SD (table 2) 44 (98%) of referred subjects had a low risk for SD.

Relatives who decline presymptomatic DNA testing and at-risk relatives from families where we were not able to detect a disease-causing mutation are referred for cardiac screening by medical history, clinical examination, electrocardiography and echocardiography. In 1997 McKenna et al. proposed new diagnostic criteria for HCM in adult members of affected families based on echocardiographic and electrocardiographic findings (table 3). The diagnosis would be fulfilled in the presence of one major criterion; two minor echocardiograph criteria; or one minor echocardiographic plus two minor electrocardiographic criteria.[15] In relatives meeting the diagnostic criteria for HCM we continue with an exercise-ECG and 24-hours Holter monitoring for risk stratification.

In mutation carriers and in relatives in whom the genotype is not known, the cardiac screening is repeated according to the results, and therapy is initiated when necessary. In adult persons repeated screening is indicated after three to five years when all tests are normal (figure 1).[16,17]

The goal for the next three years is to further develop our cardiogenetic outpatient clinic and to identify all affected at-risk relatives of the HCM patients in the Rijnmond region.

Screening for HCM in children

Cardiac screening by medical history, physical examination, ECG and echocardiogram in children from HCM families is performed after the age of 10. Cardiac screening of younger children will be done when there is suspicion of HCM symptoms in the child, when the family history includes early-onset HCM or SD, upon parental request or if the child with suspicion of HCM enrols in a competitive sport.

Presymptomatic genetic testing in children remains controversial, since the medical benefit of presymptomatic diagnosis in HCM in childhood has not been established. Some clinicians have emphasised the potential benefit of testing in HCM;[18] however, the only medical implication is a regular follow-up in mutation carriers, which will allow the detection of clinical expression of the disease very early. Others consider presymptomatic testing in children more deleterious than beneficial, because of the lack of efficient treatment

Table 3. Diagnostic criteria for hypertrophic cardiomyopathy in adult members of affected families (modified from McKenna et al).[15]

Major criteria	Minor criteria
Echocardiography	
Anterior septum or posterior wall ≥13 mm	Anterior septum or posterior wall 12 mm
Posterior septum or free wall ≥15 mm	Posterior septum or free wall 14 mm
Severe SAM (septal-leaflet contact)	Moderate SAM (no septal-leaflet contact)
	Redundant MV leaflets
Electrocardiography	
LVH + repolarisation changes	Complete BBB or (minor) interventricular conduction defect (in LV leads)
T-wave inversion in leads I and aVL (≥3 mm), V_3-V_6 (≥3 mm) or II, III, aVF (≥5 mm)	Minor repolarisation changes in LV leads
Abnormal Q (>40 ms or >25% R wave) in at least 2 leads from II, III, aVF, V_1-V_4; or I, aVL, V_5-V_6	Deep S in V_2 (>25 mm)

SAM=systolic anterior motion, LVH=left ventricular hypertrophy, MV=mitral valve, BBB=bundle branch block.

to prevent clinical expression in children bearing a mutation, combined with possible adverse psychological effects.[19]

If HCM is diagnosed by echocardiography, the diagnosis may be confirmed by screening for the familial mutation. Presymptomatic genotyping of children is, as in other mostly late adult-onset diseases, only done in exceptional cases.[20]

Genetic counselling and prenatal diagnosis

In autosomal dominantly inherited HCM, there is a risk of 50% of transmitting the genetic defect to a child. In families where the disease-causing mutations have been identified, there is a possibility for prenatal diagnosis of HCM with chorionic villus sampling as early as at ten weeks of gestation, or by amniocentesis in the 16th week of the pregnancy. However, prenatal diagnosis of HCM is almost never indicated because of the variability in expression of the disease and the small risk of cardiac disease at young age. Although early expression of HCM is extremely rare, pregnant women are offered a structural sonography in the 18th to 20th week of pregnancy.

Recommendations for follow-up of mutation carriers and at-risk relatives

The variability in age at onset of HCM and lack of cardiac pathology in a proportion of known HCM mutation carriers and at-risk relatives mandates long-term follow-up, even when they are asymptomatic at cardiac screening. In the Netherlands these mutation carriers are invited to participate in the Escape study, which is supported by the ICIN and focuses on the prognostic factors of these individuals.

Cardiac follow-up

Age at presentation varies in HCM, even in families carrying the same HCM mutation.[21-23] In a long-term follow-up study of patients with a mutation in MyBPC3 disease penetrance was 100% in patients aged >50 years.[21] Follow-up at regular intervals, including echocardiography, exercise testing and 24-hour Holter monitoring, is therefore mandatory. During echocardiography in these patients we focus on subtle abnormalities and perform tissue Doppler imaging (TDI), since low systolic and diastolic velocities can be a predictor of the development of hypertrophy.[24]

In asymptomatic carriers and at-risk relatives repeated clinical screening is advised, every two years between the age of 10 to 22 years, and every five years in older mutation carriers.[17]

Sport participation

According to the guidelines, all patients with a probable or unequivocal clinical diagnosis of HCM should be excluded from most competitive sports. Some moderate- and low-intensity level recreational sports such as swimming, brisk walking, biking and skating are permitted.[25] For asymptomatic mutation carriers and at-risk relatives there are no compelling data available to prohibit competitive sports, particularly in the absence of cardiac symptoms or a family history of SD. Athletes with preclinical HCM should be carefully examined every 12 to 18 months with electrocardiogram, echocardiography, exercise testing and ambulatory Holter electrocardiogram.[26,27]

Socioeconomic consequences of presymptomatic testing for HCM

In an unselected patient population HCM is a benign disease with an incidence of cardiac death of less than 1% per year.[28] However, these studies on prognosis did not include mutation carriers whose prognosis is suspected to be at least similar and most likely better. Until follow-up studies of mutation carriers are available insurance companies will work with the available studies, which will mean higher insurance premium for life insurance, disability, critical illness, and long-term care insurance.[29] Especially in young patients this can be a reason to postpone presymptomatic testing.

Future perspective in the delay of onset or prevention of HCM

There are promising results from animal studies using calcium channel blockers[30] and statins[31] showing delay in onset of HCM. In patients with nonobstructive HCM, treatment with angiotensin II blockade[32] seems to be beneficial. Studies from our institution have shown that alterations of hypertrophy in HCM patients are influenced by polymorphisms in the renin-angiotensin-aldosterone system genes; these results support the use of agents that influence this system in HCM patients.[33-35]

Treatment of patients with clinical diagnosis of HCM

The HCM patients are treated according to the guidelines.[36] Medical treatment is first-line therapy, traditionally with either β-blockers or nondihydropyridine calcium channel blockers to facilitate diastolic filling and to reduce intracavitary gradients. The negative inotropic effect of disopyramide also may be beneficial in reducing obstructive physiology.[37] Significant (>50 mmHg at rest or >100 mmHg at provocation) intracavitary obstruction with refractory symptoms can be addressed by surgical myectomy or by ethanol septal ablation. It is important to assess the risk of sudden death (SD) in these patients, because the prophylactic implantation of a cardioverter-defibrillator can be defended in some cases. In a study by Elliot et al. several risk factors were identified demonstrating that patients with ≥2 risk factors (table 2) had a 4 to 5% estimated annual SD risk, whereas in the absence of risk factors this risk was <1%.[38] A recent study from the same group showed that left ventricular outflow tract obstruction in asymptomatic patients with no other SD risk markers was not related to increased mortality and did not warrant aggressive interventions.[39] Prophylactic pharmacological therapy with β-blockers or calcium channel

antagonists has frequently been used, but efficacy in SD prevention has not been established. Empiric use of amiodarone has been reported to be associated with improved survival in one observational study with historical controls.[40] Supported by other data we believe that implantation of a cardioverter-defibrillator is the preferred therapy for primary prevention in high-risk patients and for secondary prevention in patients who survived life-threatening arrhythmias.[41,42]

Conclusions

The identification of autosomal dominantly inherited genetic defects in an HCM gene provides new opportunities for accurate identification of at-risk relatives and effective screening of the relatives of HCM patients. The variability in age at onset of symptoms of HCM demands repeated cardiac screening of adults at risk for HCM, whether carriers of known mutations or relatives of families where no genetic defect has been identified. ■

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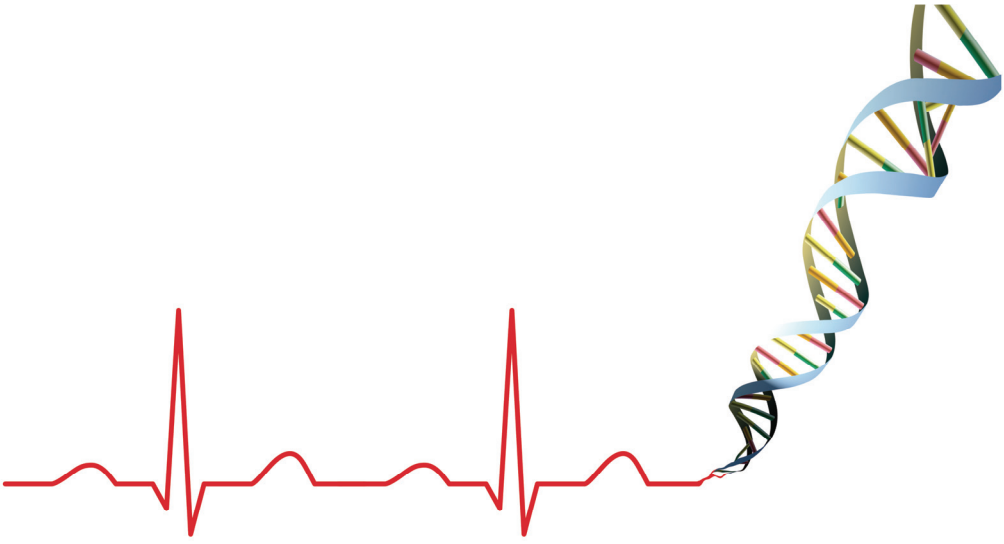
Chapter 9

Natural history of three hypertrophic cardiomyopathy founder mutations in Myosin Binding protein C.

Yvonne M. Hoedemaekers, Michelle Michels, Folkert J. ten Cate, Wout Deelen, Raoul van der Graaf, Marielle Alders, Danielle F. Majoor-Krakauer, Marcel A.M. Mannens, Elena Biagini, Marcel M.J. Kofflard, Dicky J.J. Halley, Dennis Dooijes.

Submitted



Abstract

Background

Hypertrophic cardiomyopathy (HCM) is an autosomal dominantly inherited disease, characterised by incomplete penetrance and variable phenotypic expression. Mutations in 24, mostly sarcomeric, genes are known to cause HCM. Earlier studies have associated mutations in the sarcomere Myosin Binding Protein C gene (*MYBPC3*) with late onset benign HCM. Our aim was to analyse the contribution and natural history of *MYBPC3*-HCM in the Dutch HCM population.

Methods and results

We performed molecular analysis of *MYBPC3* in 327 unrelated Dutch HCM index patients. A genetic variant was identified in 45% (148/327) of the patients. Fifty-one different variants, including 35 novel ones, were found. In addition to the c.2373dupG founder mutation, found in 14.4% of the patients, we identified the two new *MYBPC3* founder mutations p.Arg943X in 6.7% and c.2864_2865delCT in 4.6% of the HCM patients. Family studies show a malignant prognosis in 33%, 38% and 56% of the c.2373dupG, p.Arg943X and c.2864_2865delCT families respectively. Identical survival estimates were computed for all three truncating c.2373dupG, p.Arg943X and c.2864_2865delCT founder mutations.

Conclusion

Truncating *MYBPC3* founder mutations show a comparable natural history likely due to haploinsufficiency.

Introduction

Hypertrophic cardiomyopathy (HCM) is characterised by unexplained left ventricular hypertrophy (LVH), myocardial fibrillar disarray and interstitial fibrosis. HCM has an estimated prevalence of 1:500 in the population. It is the most common cause of sudden cardiac death (SCD) in the young and a major cause of morbidity and mortality in the elderly.[1] Approximately 65% of HCM is hereditary, transmitted as an autosomal dominant trait with incomplete penetrance and high inter- and intra-familial variability.[2]

Thus far 24 different genes have been associated with HCM. Over 400 different sarcomeric mutations have been reported[3], many occurring in single families; founder effects seem to be exceptional.[4-7] In familial HCM, mutations are most frequently observed in the β-myosin heavy chain gene (*MYH7*, 30-40%) and the cardiac troponin-T gene (*TNNT2*, 10-20%). The reported prevalence of mutations in the Myosin Binding Protein C gene (*MYBPC3*) varies between 15-20% [2, 8] to 38-42% [5, 9] from more recent surveys in Finland and France. Genetic defects, with a prevalence of less than 5% include mutations in the *ACTC1, CSRP3, MYH6, MYL2, MYL3, TCAP, TNNC1, TNNI3, TPM1*, *TTN , PLN, CASQ2, MYOZ2* and *CALR3* genes.[2, 8, 10, 11] Mutations in the *MYH7* and *TNNT2* genes are generally missense mutations and appear to be almost completely penetrant by the second or third decade of life. *TNNT2* mutations are associated with a malignant form of HCM with high risk of cardiac failure and sudden death at a young age.[12] In contrast, *MYBPC3* mutations are almost invariably truncating mutations, reported to be associated with slowly progressive hypertrophy, late onset of clinical features and a benign or intermediate prognosis in 90% of the patients.[4, 9, 13, 14]

Previously, the c.2373dupG mutation in the *MYBPC3* gene, demonstrated to create a novel splice donor site within exon 24 of the *MYBPC3* coding sequence,[15] was identified as an ancient *MYBPC3* founder mutation present in a large proportion of HCM patients in the Netherlands.[15, 16]

To establish the contribution of *MYBPC3* mutations in the Dutch HCM population, we performed *MYBPC3* mutation analysis in 327 Dutch unrelated HCM probands. Two additional founder mutations in exon 27 of the *MYBPC3* gene were identified: the p.Arg943X and c.2864_2865delCT mutations, present in 6.7% and 4.6% of the analysed HCM patients respectively.

Although the majority of genetic causes of HCM have been identified, the molecular mediators determining the high inter- and intra-familial variability in clinical expression and cardiac phenotypes remain elusive.[17] Genotype-phenotype studies have been hampered by extensive allelic heterogeneity and the individual nature of the causative sarcomere gene mutations, precluding accurate risk stratification and tailored cardiologic monitoring and management in HCM families. The occurrence of founder mutations in the Dutch HCM population provides the unique opportunity to investigate the genotype-phenotype association in groups of HCM patients sharing the identical molecular sarcomere defect. Clinical signs and symptoms were studied in families with the p.Arg943X and c.2864_2865delCT founder mutations. Prognosis and survival estimates were computed for all three currently known truncating founder

mutations in the *MYBPC3* gene (c.2373dupG, p.Arg943X and c.2864_2865delCT). These results provide the first robust genotype-phenotype relations for individual *MYBPC3* mutations.

Methods

Patients

The study population comprised 327 HCM index patients from the Rotterdam area in the Netherlands. Patients were referred to the Department of Clinical Genetics of the Erasmus MC in Rotterdam, the Netherlands, for genetic counselling and molecular diagnosis of HCM.

Family studies were initiated for all *MYBPC3* mutation patients and included genetic counselling, cardiologic evaluation and (presymptomatic) molecular testing of first and second-degree relatives. Following the identification of the p.Arg943X and c.2864_2865delCT founder mutations, evaluation of phenotypic severity in mutation carriers was performed using history of symptoms (fatigue, palpitations, dyspnoea, chest pain), ECG, exercise ECG, 24-hour ambulatory ECG monitoring, two-dimensional and Doppler echocardiography.

We classified prognosis in families based on family history at the time of diagnosis as described previously.[9] Prognosis was considered malignant when two or more major cardiac events (MCE; i.e. sudden cardiac death (SCD), cardiac-related stroke, resuscitation, appropriate implantable cardioverter defibrillator (ICD) discharge) were documented in a patient's family before the age of 60 years (probands were excluded from the analysis). One reported MCE in the family led to an intermediate prognosis classification and when no MCE had taken place, prognosis was considered benign. In sporadic HCM patients, absence of family history prevented prognostic classification.

Genetic testing

Genomic DNA from 327 HCM index patients was isolated from peripheral blood samples using standard procedures. All coding regions and intron-exon boundaries of the *MYBPC3* gene were analysed using direct sequence analysis (reference sequence NM_000256.3). Analysis PCR products was carried out on an ABI3730xl capillary sequencer using Big-Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of methods and primer sequences are available upon request.) Analysis of sequence data was performed using SeqScape analysis software (v2.5, Applied Biosystems). To detect possible genomic rearrangements in *MYBPC3*, MLPA analysis was performed (MRC Holland kit P100). Exons of *MYBPC3* were numbered 1-34 according to international standards with the Adenine of the translation initiation start site (ATG) numbered 1 and the ATG in exon 1.[18] In case of novel DNA variants, a panel of 384 Dutch control chromosomes was screened to identify common polymorphisms. Unknown missense variants were considered a mutation when they segregated with disease in a family *and* were not present on control chromosomes *and* were pathogenic according to prediction software.[19, 20] Although this does not formally prove pathogenicity, these three criteria combined were thought to provide the best evidence for pathogenicity. In case of isolated mutations, segregation with

disease could not be used as a criterion. DNA variants not fulfilling these criteria were labelled unclassified variants (UV).

Haplotype analysis

Extended haplotype analysis, using five intragenic SNPs and four extragenic polymorphic markers flanking *MYBPC3* (D11S1344, D11S4137, D11S986 and D11S4109), was performed to construct haplotypes segregating with the p.Arg943X and c.2864_2865delCT mutations as described previously.[16] Validity of the constructed haplotypes was checked by segregation analysis in families of patients whose family members were available for testing.

Statistics

Data on survival of 117 mutation carriers from 26 c.2373dupG mutation families, 50 mutation carriers from 16 p.Arg943X families and 62 mutation carriers from 12 c.2864_2865delCT mutation families were used to compute Kaplan–Meier survival curves for each founder mutation separately.

Results

Mutation analysis

Diagnostic *MYBPC3* screening in 327 independent HCM probands identified 51 different genetic variants, including 35 novel ones, in 148 (45%) patients (Table 1), making this the highest percentage of *MYBPC3*-HCM reported thus far. The majority of pathogenic *MYBPC3* mutations were truncating mutations (i.e. nonsense, frame shift or splice site mutations), predicted to lead to shortened or absent protein due to nonsense-mediated decay (NMD).[21] Only three different pathogenic missense mutations were identified in six patients. Twenty-one different unclassified variants (UVs) were identified, mostly missense variants predicted by *in silico* analyses to affect protein function.[19, 21] Several rare and common sequence polymorphisms, both novel and previously published ones, were observed during this study (Table 2).

Some *MYBPC3* mutations were observed frequently. In 47/327 patients (14.4%) the c.2373dupG founder mutation was present.[16] In 22 (6.7%) and 15 (4.6%) of the patients respectively we detected the truncating p.Arg943X (c.2827C>T) and c.2864_2865delCT mutations in exon 27 of *MYBPC3*.

Haplotype analysis

Using extended haplotype analysis, we demonstrated a founder effect for the p.Arg943X and c.2864_2865delCT mutations in the Dutch population; a shared haplotype was identified for the p.Arg943X mutation carriers analysed (Table 3). A different common haplotype segregating with the c.2864_2865delCT mutation was observed (Table 4). Of the 15 p.Arg943X carriers analysed, at least three did not share the common allele for the distal marker D11S4109 and one other patient did not share the common allele for the most proximal marker D11S986. This

Table 1. *MYBPC3* pathogenic mutations and unclassified variants in 327 Dutch HCM patients.

Exon	Intron	Nucleotide change	Protein	No. index patients	Status	Consequence	Ref.
3		**c.312delG***	**p.Ala105fs**	1	MUT	Frameshift in exon 3	
4		**c.442G>A**	**p.Gly148Arg**	2	UV	Missense	
4		**c.478C>G**	**p.Arg160Gly**	1	UV	Missense	
4		c.481C>T	p.Pro161Ser	4	MUT	Missense	16
5		**c.530G>A**	**p.Arg177His**	1	UV	Missense	
5		**c.646G>A**	**p.Ala216Thr**	1	UV	Missense	
	5	**c.655+1G>A**		2	MUT	Affects splice donor site	
6		**c.676_701dup26**	**p. Gly235fs**	1	MUT	Frameshift in exon 6	
6		c.772G>A		1	MUT	Affects splice donor site	32
	7	c.821+1G>A		1	MUT	Affects splice donor site	4
9		**c.897delG**	**p.Lys301fs**	3	MUT	Frameshift in exon 9	
11		**c.913_914delTT**	**p.Phe305fs**	1	MUT	Frameshift in exon 11	
	11	**c.927-2A>G**		4	MUT	Affects splice acceptor site	
12		**c.932C>A**	**p.Ser311X**	2	MUT	Nonsense	
12		**c.961G>A**	**p.Val321Met**	1	UV	Missense	
12		c.977G>A	p.Arg326Gln	2	UV	Missense	5, 14
12		**c.989delC**	**p.Pro330fs**	1	MUT	Frameshift in exon 12	
12		**c.1008C>T†**	**p.=**	1	UV	Silent	
	16	**c.1458-1G>C**		1	MUT	Affects splice acceptor site	
17		c.1468G>A	p.Gly490Arg	1	UV	Missense	31
17		c.1484G>A	p.Arg495Gln	1	MUT	Missense	14, 32
17		**c.1501T>C**	**p.Tyr501His**	1	UV	Missense	
17		**c.1591G>A**	**p.Gly531Arg**	1	UV	Missense	
	17	**c.1624+1G>A**		1	MUT	Affects splice donor site	
18		**c.1765C>G**	**p.Arg589Gly**	1	UV	Missense	
18		**c.1790G>A**		1	MUT	Affects splice donor site	
	18	**c.1791-1G>A**		1	MUT	Affects splice acceptor site	
19		c.1800delA	p.Lys600fs	1	MUT	Frameshift in exon 19	9
19		**c.1827C>G†**	**p.=**	1	UV	Silent	
19		**c.1831G>A**	**p.Glu611Lys**	2	UV	Missense	
	22	**c.2149-9C>A**		1	UV	Affects splice acceptor site	
	22	c.2149-2delA		2	MUT	Affects splice acceptor site	15
23		**c.2164G>A**	**p.Glu722Lys**	1	UV	Missense	
23		**c.2308G>A**		1	MUT	Affects splice donor site	
23		**c.2149-?_2737+?**		2	MUT	Deletion exons 23-26	
24		**c.2346C>T†**	**p.=**	1	UV	Silent	
24		c.2373dupG		47	MUT	Novel splice donor in ex.24	15
25		c.2432A>G	p.Lys811Arg	1	MUT	Missense	9
25		c.2497G>A	p.Ala833Thr	5	UV	Missense	15, 31
25		**c.2542G>A**	**p.Ala848Thr**	1	UV	Missense	
25		**c.2574C>A**	**p.Ser858Arg**	1	UV	Missense	
27		c.2827C>T	p.Arg943X	22	MUT	Nonsense	32
27		c.2864_2865delCT	p.Pro955fs	15	MUT	Frameshift in exon 27	9, 32
27		**c.2869A>T**	**p.Thr957Ser**	1	UV	Missense	
29		c.3181C>T	p.Gln1061X	1	MUT	Nonsense	5
31		**c.3332_3335dup**	**p.Trp1112X**	1	MUT	Nonsense	
31		c.3392T>C	p.Ile1131Thr	3	UV	Missense	15, 31
31		**c.3407_3409delACT**	**p.Tyr1136del**	1	UV	Single amino acid deletion	
	31	**c.3490+1G>T**		1	MUT	Affects splice donor site	
	32	c.3628-41_3628-17del25		2	MUT	Affects splice acceptor site	33
33		**c.3776delA**	**p.Gln1259fs**	5	MUT	Frameshift in exon 33	

** Novel mutations, identified during this study, are in bold.*

† The silent UVs c.1008C>T, c.1827C>G and c.2346C>T likely represent rare polymorphisms, although pathogenic character cannot formally be excluded.

Table 2. *MYBPC3* Polymorphisms in the Dutch population.

Exon	Intron	U91629	Nucleotide change	Amino acid	RefSNP ID (dbSNP129)	Allele freq. (total = 136)
	2	g.2484G>C	c.292+41G>C		rs3729985	<0.01
4		g.3634G>A	c.472G>A	p.Val158Met	rs3729986	0.05
4		g.3654C>T	c.492C>T		rs3218719	0.15
	4	g.3712insG	c.506-46insG			<0.01
	4	g.3746insC	c.506-12dupC		rs11570050	0.20
5		g.3789C>T	c.537C>T		rs11570051	0.04
6		g.5190A>G	c.706A>G	p.Ser236Gly*	rs3729989	0.07
7		g.5788C>T	c.786C>T		rs11570058	0.07
	11	g.7078C>A	c.926+25C>A		rs3729991	0.02
	12	g.9994G>A	c.1091-31G>A		rs7940442	<0.01
	12	g.10001T>C	c.1091-24T>C		rs2856650	0.71
	13	g.10186G>A	c.1223+29G>A		rs11570078	0.07
	15	g.10693A>C	c.1252-21A>C			<0.01
	19	g.12559G>A	c.1897+47G>A		rs11570086	0.03
	23	g.15148C>T	c.2308+18C>G		rs3729948	0.03
25		g.16203C>T	c.2547C>T		rs3729953	0.03
	25	g.16288C>T	c.2602+30C>T			<0.01
26		g.17721G>A	c.2686G>A	p.Val896Met	rs35078470	0.02
	26	g.17784C>T	c.2737+12C>T		rs3729936	0.02
	26	g.17788C>G	c.2737+16C>G			<0.01
	29	g.20294A>G	c.3191-21A>G		rs11570115	0.09
30		g.20412G>A	c.3288G>A		rs1052373	0.38
31		g.20757G>A	c.3413G>A	p.Arg1138His		<0.01
	31		c.3491-29dupC			†
	33	g.21700C>T	c.3815-66C>T		rs2290146	0.28

** The p.Ser236Gly polymorphism was found in coupling with the c.786C>T and c.1223+29G>A polymorphisms.*
† We found every sample tested to be homozygous for c.3491-29dupC when compared to U91629, probably indicating a mistake in the reference sequence rather than a true polymorphism.

accounts for at least three p.Arg943X haplotypes in the population for the small 0.9 cM region tested (Table 3). The 11 tested index patients carrying the c.2864_2865delCT mutation showed a similar pattern. At least three haplotypes exist for the c.2864_2865delCT mutation for the same region indicating a similar age as the p.Arg943X mutation in the population. The high frequencies of both mutations and the observed recombination frequency in the 0.9 cM region between polymorphic markers D11S986 and D11S4109 suggest that both founder mutations p.Arg943X and c.2864_2865delCT arose about 15-20 generations ago in the Dutch population (calculated according to Bergman et al).[22]

Table 3. Shared haplotypes of 15 carriers of the p.Arg943X mutation in *MYBPC3*.

	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
D11S986	157	153	157	153	157	153	157	157	157	157	157	153	157	157	157	157	157	159	157	134	157	135	155	155	157	145	157	135	157	159
D11S4137	268	276	268	268	268	268	268	276	268	272	268	268	268	268	268	268	268	283	268	268	268	268	268	268	268	276	268	268	268	272
D11S1344	278	287	278	280	278	286	278	286	278	284	278	284	278	276	278	280	278	284	278	274	278	274	278	286	278	286	278	274	278	274
492C>T	nd	nd	C	C	C	C	C	C	nd	nd	C	C	C	C	C	C	C	C	C	C	C	C	nd	nd	C	C	C	C	nd	nd
IVS4-12insC	nd	nd	-	-	-	+	-	+	nd	nd	-	+	-	-	-	-	+	+	-	+	-	+	nd	nd	-	-	-	-	nd	nd
IVS12-24T>C	nd	nd	C	C	C	C	C	C	nd	nd	C	C	C	T	C	C	C	C	C	C	C	C	nd	nd	C	C	C	T	nd	nd
R943X (2827C>T)	nd	nd	T	C	T	C	T	C	nd	nd	T	C	T	C	T	C	T	C	T	C	T	C	nd	nd	T	C	T	C	nd	nd
3288G>A	nd	nd	G	G	G	A	G	A	nd	nd	G	A	G	G	G	G	G	A	G	A	G	A	nd	nd	G	G	G	G	nd	nd
IVS33-66C>T	nd	nd	C	C	C	C	C	T	nd	nd	C	C	C	C	C	C	C	C	C	C	C	C	nd	nd	C	C	C	C	nd	nd
D11S4109	174	166	174	174	174	170	174	153	174	172	174	166	174	172	174	168	174	172	174	176	174	176	174	166	178	178	178	164	178	170

Legend. Extended haplotype analysis using 5 intragenic SNPs and 4 extragenic polymorphic markers flanking the MYBPC3 gene (D11S1344, D11S4137, D11S986 and D11S4109) was used to construct haplotypes segregating with the p.Arg943X. Validity of the constructed haplotypes was checked by segregation analysis in the affected families of patients whose family members were available for analysis (not shown). nd = not determined.

Table 4. Shared haplotypes of 11 carriers of the c.2864_2865delCT mutation in *MYBPC3*.

	1		2		3		4		5		6		7		8		9		10		11	
D11S986	159	161	159	135	159	153	159	157	159	157	159	135	159	157	159	153	155	157	157	155	159	147
D11S4137	268	268	268	276	268	268	268	279	268	268	268	268	268	283	268	285	268	285	272	276	268	268
D11S1344	270	284	270	287	270	284	270	284	270	274	270	278	270	284	270	274	270	286	274	276	282	289
492C>T	nd	nd	C	T	nd	nd	C	C	nd	nd	C	C	C	C	C	T	C	C	nd	nd	C	C
IVS4-12insC	nd	nd	-	-	nd	nd	-	-	nd	nd	-	+	-	-	-	-	-	-	nd	nd	-	-
IVS12-24T>C	nd	nd	T	T	nd	nd	T	T	nd	nd	T	C	T	T	T	C	T	C	nd	nd	C	C
2864delCT	nd	nd	+	-	nd	nd	+	-	nd	nd	+	-	+	-	+	-	+	-	nd	nd	+	-
3288G>A	nd	nd	G	G	nd	nd	G	G	nd	nd	G	G	G	G	G	A	G	G	nd	nd	G	A
IVS33-66C>T	nd	nd	C	C	nd	nd	C	C	nd	nd	C	C	C	C	C	T	C	C	nd	nd	C	T
D11S4109	166	162	166	162	166	170	166	166	166	176	166	166	166	163	166	160	166	172	166	166	172	172

Legend. Extended haplotype analysis using 5 intragenic SNPs and 4 extragenic polymorphic markers flanking the MYBPC3 gene (D11S1344, D11S4137, D11S986 and D11S4109) was used to construct haplotypes segregating with the c.2864_2865delCT. Validity of the constructed haplotypes was checked by segregation analysis in the affected families of patients whose family members were available for analysis (not shown). nd = not determined.

Clinical features of the two new MYBPC3 founder mutations

Cardiologic evaluation of 23 mutation carriers from 16 p.Arg943X families and 20 mutation carriers from 12 c.2864_2865delCT families showed patients who developed atrial fibrillation (AF): 22% of the p.Arg943X and 30% of the c.2864_2865delCT carriers**.** Most of these patients underwent several electrical cardioversions. These numbers are comparable with previous reports demonstrating that AF occurs in 25% of HCM patients. A pedigree of a c.2864_2865delCT mutation-family is shown in Figure 1, demonstrating intrafamilial variability of disease. The results of the clinical evaluation of all mutation carriers are summarised in Table 5. Post mortem molecular analysis identified the p.Arg943X mutation in two patients, who suffered a sudden cardiac death at age 11 years and 31 years respectively and were diagnosed at autopsy with severe hypertrophic cardiomyopathy. They had not been diagnosed with HCM during their lives.

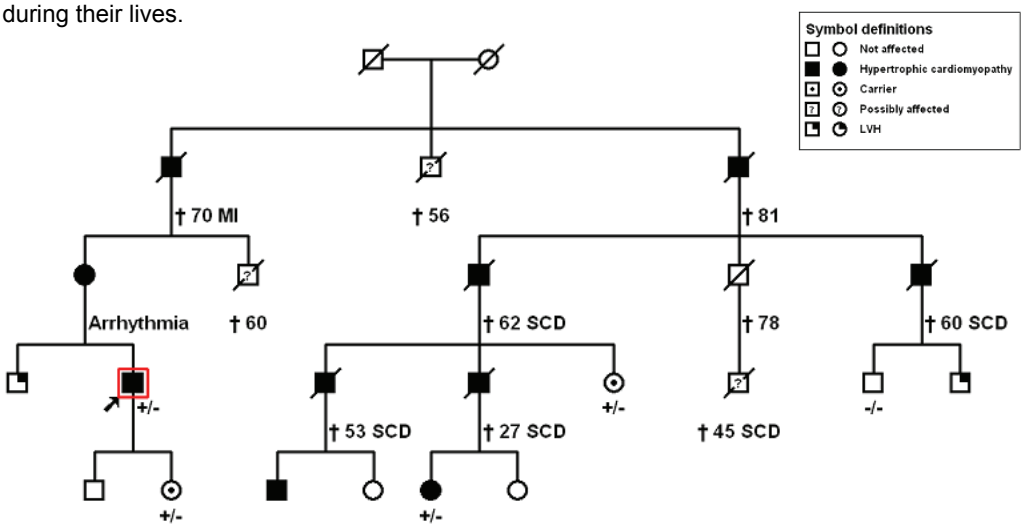


Figure 1. Family with *MYBPC3* mutation c.2864_2865delCT.
Pedigree of a c.2864_2865delCT mutation family illustrating intrafamilial variability. The arrow indicates the proband. MI = Myocardial Infarction.

Notably, there was a significant increase in echocardiographic measurements of the left atrium (LA) at follow-up in the p.Arg943X carriers (p=0.00097) and the c.2864_2865delCT carriers (p=0.004) (Table 6). This may be a reflection of increased left ventricular (LV) filling pressure because of LVH or decreased LV compliance.

Prognosis and Survival in families with founder mutations

Evaluation of available family histories allowed assessment of the natural history, including prognosis and survival, of the three c.2373dupG, p.Arg943X and c.2864_2865delCT *MYBPC3* founder mutations (Table 7). Nine out of 27 (33%) analysed c.2373dupG mutation families had a malignant prognosis and 12 (44%) had an intermediate prognosis. Five of 13 (38%) p.Arg943X families had a malignant prognosis, seven (54%) were intermediate and one was benign (8%). Of nine c.2864_2865delCT families five (56%) families were malignant and four

(44%) were intermediate. A remarkable proportion of patients was unaware of the familial nature of the disease at diagnosis, although SCD had sometimes occurred in one of their (close) relatives.

Table 5. Clinical features in p.Arg943X and c.2864_2865delCT mutation carriers.

	p.Arg943X	c.2864_2865delCT
Number of patients (index patients)	23 (16)	20 (12)
Male / female	16 / 7	12 / 8
Family history		
Positive for HCM	10 (63%)	9 (75%)
Positive for SCD	9 (56%)	7 (58%)
Ascertainment of patients		
Cardiac symptoms	16 (70%)	12 (60%)
Family screening	5 (22%)	3 (15%)
Asymptomatic carriers	7 (30%)	6 (30%)
Age at diagnosis		
Mean ± SD (yrs)	35 ± 16	43 ± 15
< 18 yrs, n	3 (13%)	0
Follow-up duration		
Mean ± SD (yrs)	8 ± 10	12 ± 8
Range (yrs)	0 - 28	2 - 25
Treatment		
No medical treatment	9 (39%)	6 (30%)
B-blocker	4 (17%)	8 (40%)
Other	10 (44%)	6 (30%)
Intervention		
Myectomy	3 (13%)	3 (15%)
PTSMA*	0 (0%)	1 (5%)
ICD	1 (4%)	0 (0%)
NYHA class at first evaluation		
I	14 (61%)	9 (45%)
II	5 (22%)	11 (55%)
III	4 (17%)	0 (0%)
IV	0 (0%)	0 (0%)
NYHA class at follow-up		
I	10 (53%)	13 (65%)
II	7 (37%)	6 (30%)
III	2 (10%)	1 (5%)
IV	0 (0%)	0 (0%)
Complications		
Atrial fibrillation (AF)	5 (22%)	6 (30%)
Mean age AF (+/- SD)	57 (+/- 6.4)	56 (+/- 15.6)
Range age AF	50-64	31-74
Cardiac arrest survival	1 (4%)	0 (0%)
Follow-up		
Unknown	2 (9%)	1 (5%)
Deceased	6 (26%)	4 (20%)
Periodic cardiologic examination	15 (65%)	15 (75%)

*Legend. * PTSMA = Percutaneous Transluminal Septal Myocardial Ablation*
† NYHA = New York Heart Association classification

Table 6. Echocardiographic findings in p.Arg943X and c.2864_2865delCT mutation carriers.

	LVWT[*] (mm) mean ± SD	IVS[†] (mm) mean ± SD	PW[‡] (mm) mean ± SD	LVOT[§] gradient (mmHg) mean ± SD	SAM[‖] n (%)	Mitral valve insufficiency n (%)	LVED[#] (mm) mean ± SD	LA[**] (mm) mean ± SD
p.Arg943X (n=23)								
First evaluation	21 ± 7	20 ± 7	11 ± 3	20 ± 12	10 (43%)	9 (39%)	45 ± 6	41 ± 7
Follow-up	20 ± 5	19 ± 5	12 ± 2	18 ± 14	6 (26%)	13 (57%)	48 ± 7	49 ± 11
P-value	0,366	0,366	0,353	0,487			0,182	0,00097
c.2864_2865delCT (n=20)								
First evaluation	20 ± 4	19 ± 4	11 ± 2	44 ± 34	9 (45%)	8 (40%)	43 ± 7	40 ± 6
Follow-up	19 ± 4	19 ± 4	12 ± 2	32 ± 31	4 (20%)	13 (65%)	46 ± 9	47 ± 6
P-value	0,194	0,365	0,082	0,238			0,046	0,004

[*] LVWT = Left Ventricular Wall Thickness; [†] IVS = Intraventricular Septum; [‡] PW = Posterior Wall; [§] LVOT = Left Ventricular Outflow Tract; [‖] SAM = Systolic Anterior movement; [#] LVED = Left Ventricular End Diastolic size; [**] LA = Left atrial size

Table 7. Prognosis in c.2373dupG, p.Arg943X and c.2864_2865delCT families.

	Benign n (%)	Intermediate* n (%)	Malignant n (%)	Total families n
c.2373dupG mutation	6 (22)	12 (44)	9 (33)	27
p.Arg943X mutation	1 (8)	7 (54)	5 (38)	13
c.2864_2865delCT mutation	0	4 (44)	5 (56)	9
*Legend. * Assessment of prognosis was made based on the occurrence of MCE in available pedigrees*				

Kaplan-Meier survival curves were computed for all three *MYBPC3* founder mutations (Figure 2). The survival curves for all three mutations were not statistically different (p=0.119 for p.Arg943X and c.2373dupG; p=0.134 for c.2864_2865delCT and p.Arg943X and p=0.952 for c.2864_2865delCT and c.2373dupG). Remarkably, the p.Arg943X founder mutation survival curve showed three deaths before the age of 20 years.

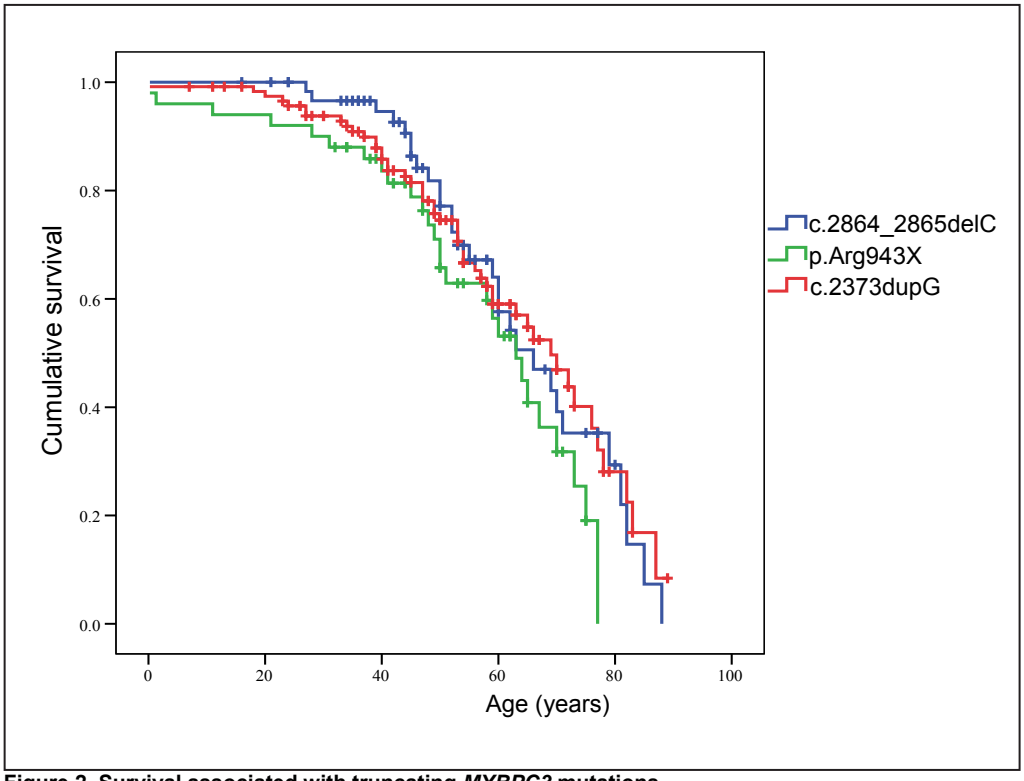


Figure 2. Survival associated with truncating *MYBPC3* mutations.
Kaplan-Meier survival curves for a group of 117 patients from 26 c.2373dupG families, 50 patients from 16 p.Arg943X families and 62 patients from 12 c.2864_2865delCT families.

Discussion

This study describes the molecular analysis of the *MYBPC3* gene in a large cohort of 327 probands with familial or sporadic HCM followed at the adult cardiogenetic outpatient clinic of the Erasmus MC Rotterdam. HCM is a genetically heterogeneous autosomal dominant disease and familial disease is caused by a single pathogenic mutation in one of the 24 currently identified HCM genes. Our approach to analyse a single HCM gene in a large HCM population does not take into account the possibility of multiple pathogenic mutations in additional HCM genes. Double mutations have been reported in literature in 3 to 5 % of genotype-positive patients.[5, 9] However, these percentages are likely to be an over-estimation since in reported HCM patients with double mutations it is often uncertain whether the second mutation is truely pathogenic. In addition, real double-mutation patients appear to have a different disease expression than is seen in classical HCM including very early onset and severe clinical presentation, making it less likely that an important proportion of our 'adult cohort' would constitute double mutation patients.

We report the highest percentage of *MYBPC3*-HCM thus far, emphasising the importance of *MYBPC3* in the aetiology of HCM.[5, 9] The high percentage of *MYBPC3* mutations in the analysed Dutch population can be explained by the presence of three founder mutations: the c.2373dupG, p.Arg943X and c.2864_2865delCT mutations. These founder mutations together are present in 84/148 (59%) of the *MYBPC3*-HCM patients and are found in 84/327 (26%) of the Dutch HCM patients. Based on a population prevalence of HCM of 0.2% it can be estimated that approximately 8320 HCM patients in the Netherlands carry one of the three *MYBPC3* founder mutations (population 16 million). The discovery of three highly prevalent *MYBPC3*-HCM founder mutations in the Dutch population has important implications for molecular HCM screening in this population. The efficacy of initial *MYBPC3* analysis is high, especially when the three founder mutations are tested first.

Most HCM causing mutations are rare and occur in only one or a few families worldwide and founder effects are exceptional.[4-7, 23] Only mutations that do not impose an adverse effect on reproduction may be transmitted repeatedly and become founder mutations. The discovery of strong founder effects for three *MYBPC3* mutations suggest that *MYBPC3*-HCM does not pose a reproductive burden. In other HCM genes, like beta-myosin heavy chain (*MYH7*) and troponin T (*TNNT2*), strong founder effects are not frequently observed. This difference may be explained by the observation that *MYBPC3* associated HCM has in general a 10-year later age-of-onset than *MYH7*-HCM and *TNNT2*-HCM. *MYH7*-HCM and *TNNT2*-HCM may be expressed during the reproductive period and therefore there is evolutionary selective pressure against mutations in these genes. In general *MYBPC3*-HCM is expressed when the biological reproductive period is already over and therefore *MYBPC3* mutations are not counter-selected and founder mutations in this gene are more easily tolerated.

In most populations HCM is highly heterogeneous. Limited numbers of distinct genotypes and families have placed constraints on genotype-phenotype analyses. In the current study, we used family history to analyse clinical phenotype and prognosis associated with two new

founder mutations. In the current study, only 22%, 8% and none of the c.2373dupG, p.Arg943X and c.2864_2865delCT families respectively, were associated with a benign prognosis. Moreover, in 33%, 38% and 56% of the c.2373dupG, p.Arg943X and c.2864_2865delCT families respectively, two or more HCM associated MCE were recorded before the age of 60 years, indicating a malignant course of the disease in these families. However, our results may represent an over-estimation of the malignancy of these mutations since ascertainment bias in patient collection may have played a role because the Erasmus MC is a tertiary referral centre.

The three founder mutations show identical Kaplan-Meier survival estimates. This finding is likely explained by the suggestion from several studies that haploinsufficiency is the pathogenic mechanism for HCM caused by truncating *MYBPC3* mutations.[24-26] Therefore, we hypothesise that most – if not all – truncating *MYBPC3* mutations will have identical survival curves and an identical natural history as the c.2373dupG, p.Arg943X and c.2864_2865delCT mutations.

As reported previously, general measurements of LV function are not disturbed in HCM patients, whereas during clinical follow-up LV remodelling occurs.[27] This is reflected in our study by the LA enlargement during follow-up as a sign of decreased LV compliance and by the high prevalence of AF in this population, reflecting both decrease of LA function and LV remodelling. Also, if detailed LV function measurements such as LV strain are used, it is expected that in mutation carriers a higher prevalence of abnormalities will be identified, compared to when only conventional echocardiography is performed.

Variability in clinical features of HCM presentation is high, even within families. Both environmental and genetic modifying factors may contribute to the intrafamilial clinical variability. The assessment of the influence of possible modifying genes on development of heart failure has been pursued in a large number of studies, yielding contradicting results. In particular, the importance of the well known insertion/deletion (I/D) polymorphism in the angiotensin-converting enzyme (ACE) and several polymorphisms in the Renin-Angiotensin-Aldosterone system (RAAS) have been considered as potential disease modifiers in HCM.[28-31] RAAS plays an important role in normal cardiovascular physiology and disease and the (I/D)-ACE polymorphism has been implicated in ‘hypertension induced’ LVH and HCM. A study in one large family carrying the c.2373dupG mutation reported a correlation between LV mass and number of ‘pro-LVH’ polymorphisms present in each mutation carrier.[29] However, a recent study failed to confirm such a general relationship in a large population of HCM patients; in contrast to *MYH7*-HCM, a significant pro-LVH effect of the DD-ACE genotype could be demonstrated in *MYBPC3*-HCM only.[30] The discovery of three important founder mutations in the Dutch HCM population provides an opportunity to study the role of additional genetic and environmental modifying factors in the clinical presentation of HCM.

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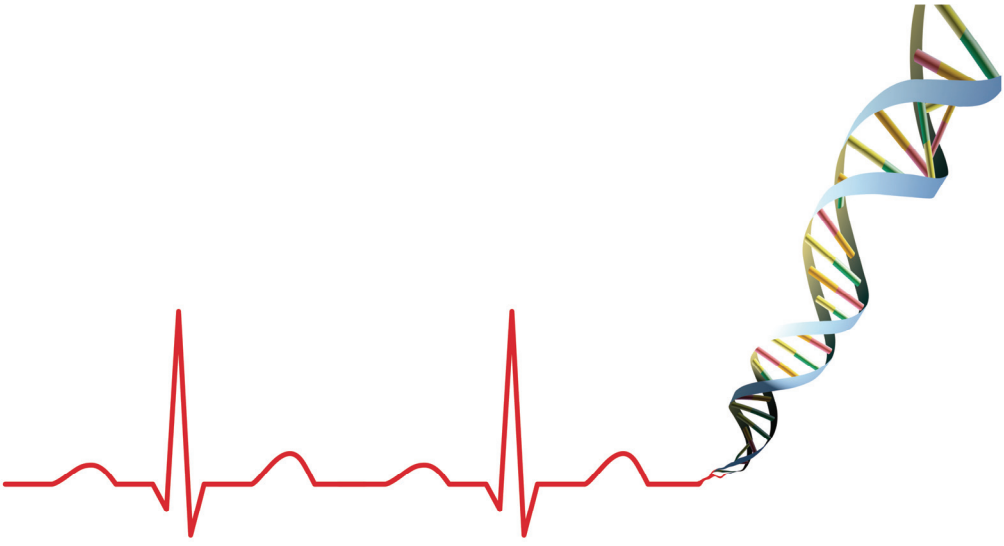
Chapter 10

Four-and-a-half-LIM domain mutations are a rare cause of familial hypertrophic cardiomyopathy.

Yvonne M. Hoedemaekers, Marianne van Tienhoven, Michelle Michels, Judith E. Phefferkorn, Rene F.M. de Coo, Joanne Verheij, Danielle F. Majoor-Krakauer, Folkert J. ten Cate, Dennis Dooijes.

Submitted



Abstract

Background

Hypertrophic cardiomyopathy (HCM) is a genetically heterogeneous cardiomyopathy characterised by unexplained left ventricular hypertrophy. HCM is mostly autosomal dominantly inherited and associated with defects in sarcomere and Z-disc genes. Recently, HCM was observed in families with X-linked myopathies caused by mutations in X-chromosomal Four-and-a-half-LIM domains 1 (*FHL1*), suggesting that *FHL1* may play a role in HCM. We analysed the *FHL1* gene in a selected cohort of 86 HCM patients to investigate its role in HCM.

Methods

From a group of 260 HCM index patients, 86 HCM patients (54 men and 32 women) were selected because no causal HCM mutation could be identified in their HCM genes *MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3* and *TCAP.* Complete sequence analysis of the *FHL1* gene was performed in all 86 HCM index patients.

Results

FHL1 mutations were identified in two HCM patients; a nonsense mutation in a woman with neuromuscular symptoms and a missense mutation in a neurologically asymptomatic man. Family studies showed intrafamilial variability in cardiologic and neurologic symptoms and in age of onset (including incomplete penetrance).

Conclusion

FHL1 mutations are an infrequent cause (<1%) of HCM, characterised by X-linked dominant inheritance. *FHL1* analysis is recommended in male and female HCM patients, especially when family history shows X-linked inheritance or is positive for myopathy or muscular dystrophy. Absence of family history does not exclude *FHL1* cardiomyopathy given the intrafamilial variability and incomplete penetrance of HCM and neuromuscular disease. Cardiologic family studies are recommended in *FHL1* disease.

Introduction

Hypertrophic cardiomyopathy (HCM) is a genetically heterogeneous disease characterised by unexplained left ventricular hypertrophy. It is the most common genetic cardiac disorder with an estimated prevalence of 1:500 individuals. Patients can be asymptomatic, or have symptoms of heart failure, angina or arrhythmia. It is a major cause of sudden cardiac death in the young.[1]

Numerous disease-causing mutations have been identified in 24 genes. Currently, genetic defects are identified in approximately 60% of HCM patients, depending on the number of genes analysed.[2] Mutations are mainly identified in sarcomere and Z-disc genes and cause autosomal dominant HCM in the majority of cases. X-linked HCM or HCM phenocopies are less frequent and may occur in Fabry and in Danon disease.[3-5] Isolated HCM has been recognised in female and male carriers of both Fabry and Danon disease.[6-8] Fabry disease is a lysosomal storage disorder, caused by deficiency of Xq22 *GLA*-encoded α-galactosidase A with multisystemic involvement, including renal, neurological, ocular, skin, and cardiac manifestations. The classical form is characterised by early multisystemic manifestations. Atypical forms with late-onset isolated renal or cardiac manifestations may occur in men with some residual plasma enzymatic activity or in female carriers. Primary deficiency of *LAMP2*-encoded lysosomal-associated membrane protein 2 on Xq24 leads to Danon disease. It is characterised by intracytoplasmic vacuoles containing autophagic material and glycogen in cardiac and skeletal muscle cells. The phenotypic expression of Danon's disease is variable and includes cardiomyopathy and skeletal myopathy, sometimes with conduction defects, Wolff-Parkinson-White syndrome, and/or mental retardation.

The Four-and-a-half-LIM domains 1 gene (*FHL1*) is located on Xq26; mutations in this Z-disc gene have been shown to cause a variety of X-linked myopathies like X-linked dominant scapuloperoneal myopathy (SPM, MIM 300695); X-linked myopathy with postural muscle atrophy and generalised hypertrophy (XMPMA, MIM 300696); reducing body myopathy (MIM 300717); rigid spine syndrome (RSS) and Emery-Dreifuss muscular dystrophy (EDMD, MIM310300).[9-13] Recently *FHL1* mutations were also suggested to be associated with HCM and dilated cardiomyopathy (DCM).[10, 12]

FHL1 consists of eight exons. Exons one and two are thought to be non-coding; the other exons are transcribed into three major protein isoforms. FHL1 proteins consist mainly of double zinc finger motifs (LIM domains), each zinc finger containing highly conserved cysteines and histidines which are held together by one zinc ion.[14, 15] FHL1A, composed of 4.5 LIM domains, is the main isoform and is predominantly expressed in skeletal muscle and shows intermediate expression in cardiac muscle.[16-18] FHL1A has been implicated in sarcomere assembly by interacting with myosin binding protein-C (MYBPC3).[19] The two other isoforms, FHL1B and FHL1C, composed of 3.5 and 2.5 LIM domains respectively, expressed to a lesser extent in cardiac and skeletal muscles, are also expressed in brain (FHL1B) and testis (FHL1C).[16, 17, 20]

To explore the role of *FHL1* mutations in sporadic and familial HCM we analysed the *FHL1* gene in 86 unrelated HCM index patients in whom a causal mutation in 11 known HCM genes was absent.

Methods

260 unrelated HCM index patients were diagnosed between 1993 and 2007 at the department of cardiology of the Erasmus MC in Rotterdam, the Netherlands, according to current diagnostic criteria by Maron *et al*.[21] Eighty-six patients from this cohort were selected for this study because consecutive screening of the genes *MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3* and *TCAP* did not result in the identification of a causative mutation. Women were included because of the possibility of X-linked dominant inheritance of HCM as is seen in Fabry and Danon disease. No selection was made according to family history of HCM or neuromuscular disorders. Complete sequence analysis of coding regions in exons 3-6 and 8 (NM_001449.4) and intron-exon boundaries of the *FHL1* gene was performed.

Sequence analysis of M13-tagged PCR products was carried out on an ABI3730xl capillary sequencer using Big-Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of methods and primer sequences available on request.) Analysis of sequence data was performed using SeqScape analysis software (v2.5, Applied Biosystems). Mutations were considered pathogenic when they were either truncating or, alternatively, when they segregated with disease in a family *and* were not present on 279 healthy control chromosomes *and* were pathogenic according to prediction software.[22, 23]

Results

A novel *FHL1* mutation was found in two of the 86 (2.3%) HCM patients, in whom causal mutations in 11 known HCM genes had been previously excluded (Table 1); in one woman with neurologic symptoms and in one cardiologically and neurologically asymptomatic male HCM patient. These results indicate a frequency of around 1% (2/260) in the general HCM population and of 1/32 (3.1%) in women and of 1/54 (1.9%) in men after exclusion of the most frequent genetic causes for HCM. The p.Gln270X (c.808C>T) mutation in patient 1 is a nonsense mutation that disrupts the second zinc finger of the fourth LIM domain (Figure 1). The p.Cys188Phe (c.563G>T) mutation is a missense mutation, pathogenic according to prediction software[22, 23], that affects the first cysteine residue of the second zinc finger of the third LIM domain (Figure 1). This cysteine residue is conserved from humans to zebrafish (Figure 2) and substitution of this residue for a phenylalanine likely destabilises the structure of the third LIM domain. The p.Gln270X mutation affects isoform FHL1A and the p.Cys188Phe mutation affects isoforms FHL1A and FHL1B (Figure 3).

Patient 1 is a female patient who was diagnosed with HCM at the age of 64 years with an interventricular septum (IVS) of 15 mm without signs of obstruction; diastole was pseudo-normal and there was a mild mitral regurgitation. She was under cardiologic surveillance since the age of 56 years, after the sudden cardiac death (SCD) of her 18-year-old son while playing soccer. He was diagnosed with HCM at autopsy. She had an abnormal electrocardiogram (ECG) with repolarisation disturbances without echocardiographic signs of HCM. From the age of 37 years on she suffered from pain in her shoulders, had four operations of shoulder

Table 1. ***Clinical and molecular characteristics of two HCM patients with an FHL1 mutation.***

	Patient 1	Patient 2
Clinical characteristics		
Sex	Female	Male
Age of onset (yrs)	64	32
Initial presentation	Cardiac screening after SCD of son	Heart murmur
NYHA	II	I
IVS (mm)	15	17
ECG	Negative T in V1-3	Abnormal Q in AVL, negative T in V3-6
24-hour ECG	Short periods of atrial tachycardia	np
Neurological symptoms	Shoulder pain with abduction restriction	None
	Nocturnal calf muscle cramps	
	Food passage problems	
Family history for		
HCM / SCD	+ / +	- / -
Neurological disease	+	-
Familial disease		
Cardiologic	+	np
Neurologic	+	np
Molecular characteristics		
Exon	8	6
Nucleotide	c.808C>T	c.563G>T
Protein	p.Gln270X	p.Cys188Phe
Consequence	Nonsense, disruption fourth LIM domain.	Missense, disruption third LIM domain.
	Presumed effect on FHL1A	Presumed effect on FHL1A and FHL1B

Legend. NYHA: New York Heart Association classification; IVS: intraventricular septum; ECG: electrocardiography; SCD: sudden cardiac death; np: not performed.

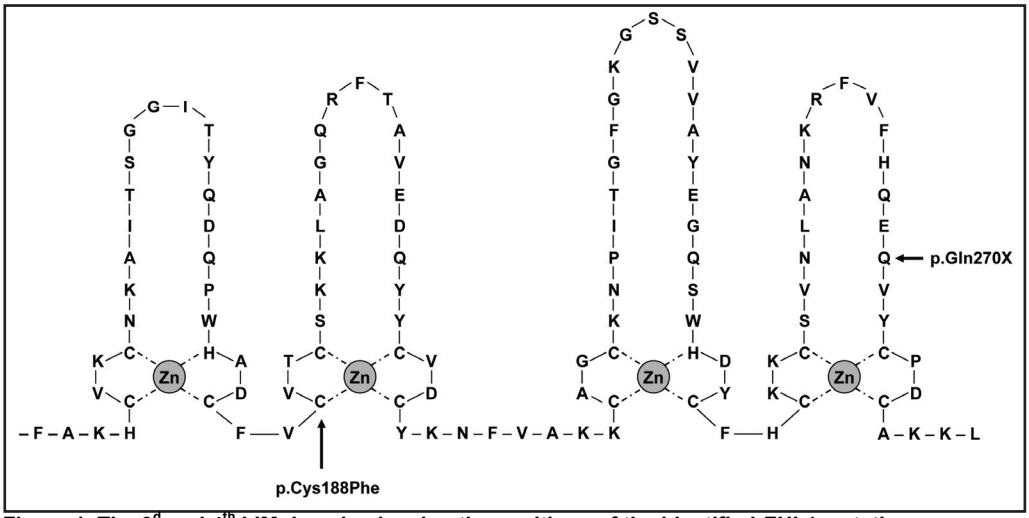


Figure 1. The 3[d] and 4[th] LIM domain showing the positions of the identified *FHL1* mutations.

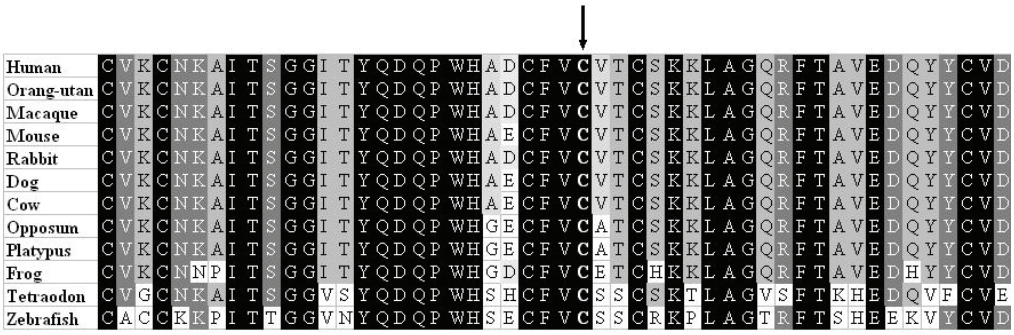


Figure 2. Alignment of amino acids of the 3[d] LIM domain.
Black background shows conservation throughout all species. The arrow shows the cysteine on position 188.

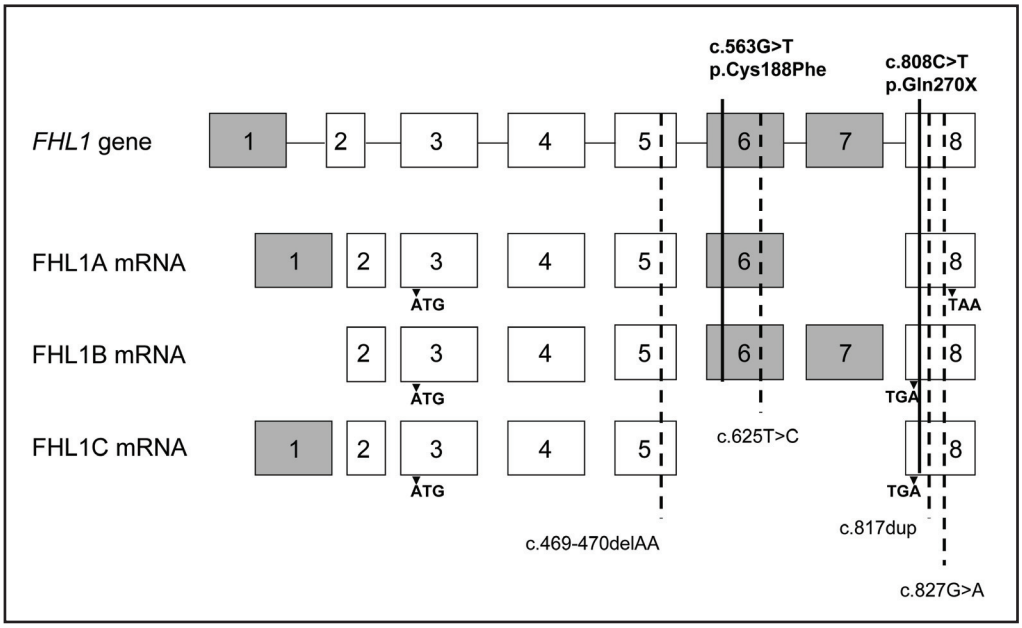


Figure 3. Distribution of the HCM-related mutations along the *FHL1* gene and corresponding RNA isoforms (adapted from Guneau *et al*).[12]
FHL1 consists of eight exons; exons 1 and 2 are non-coding, exons 3 to 8 are alternatively spliced, giving rise to three isoforms: FHL1A, FHL1B and FHL1C. Translation of all three isoforms starts with ATG on exon 3 and ends with TAA and TGA respectively on different positions on exon 8. Exons 1, 6 and 7 are alternatively spliced in the different isoforms (grey). Mutation p.Cys188Phe (patient 2), located on exon 6, affects isoforms FHL1A and FHL1B; mutation p.Gln270X (patient 1) is located on exon 8 positioned behind the stop codon of the FHL1B and FHL1C isoforms and thus only affects FHL1A. Mutations in bold were identified during this study, dotted lines represent mutations previously reported in EDMD *FHL1* families with isolated HCM.[12]

ligaments and was eventually diagnosed with fibromyalgia. At the age of 66 years she was seen in the neurological outpatient clinic with difficulties of moving food from the pharynx to the stomach. Neurologic examination showed no weakness of facial or shoulder girdle muscles but a restriction in shoulder abduction at 120° at both sides was noted. There were no signs of limb

girdle or distal muscle weakness. Serum creatine kinase was normal. There were no signs of hypermobility or of skin hyperextensibility or scapular winging. There were no chewing problems. During the night she had complaints of easily elicited muscle cramps in her calf muscles. Muscle biopsy performed from the quadriceps muscle demonstrated aspecific myopathic features with central nuclei and subsarcolemmal positioned vacuoles. Electron microscopy was not performed.

The pedigree of family 1 is shown in Figure 4. At autopsy of IV:2 a heart weight of 760 grams was noted (normal ~350 grams), with hypertrophy of the left and right ventricular wall and asymmetric hypertrophy of the IVS. Microscopic examination of the heart demonstrated architectural distortion with myocyte disarray with variable hypertrophy of the cardiomyocytes and interstitial fibrosis (Figure 5). In DNA from post-mortem material, the p.Gln270X mutation in *FHL1* was identified. The family history revealed HCM in IV:3 at age 28 years. A maternal uncle (II:3) was reported with an unspecified muscle dystrophy and was wheelchair bound from the age of 35 years onwards; he died from heart failure at the age of 78 years. Medical records were not available. Molecular analysis identified the p.Gln270X mutation in the *FHL1* gene in IV:3, III:4, and in III:9, the 59-year-old unaffected daughter of II:3, confirming that the uncle with muscular dystrophy had been an obligate carrier of the mutation. Three of her four asymptomatic daughters were identified as carriers. Cardiologic examination showed no signs of HCM at the ages of 29, 32 and 36 years respectively. The mutation was absent in the unaffected son of the proband (IV:1) and in a niece (IV:4).

Patient 2 was diagnosed with HCM at the age of 32 years with an IVS of 17 mm. He was referred to the cardiologist because a heart murmur was detected when he suffered form pneumonia. The patient did not have signs of a neuromuscular disorder. Family history was negative for HCM and neuromuscular disorders, relatives declined cardiologic and molecular screening.

Discussion

FHL1 mutations were identified in <1% of HCM patients (2/260). In a selected cohort of 86 HCM patients in which more prevalent genetic causes for HCM were excluded, *FHL1* mutations were found one male and one female patient, indicating a frequency of 1/32 (3.1%) in women and of 1/54 (1.9%) in men in this population. Guneau et al previously showed isolated HCM in four EDMD families to be more frequent in female (7) than male carriers (5).[12]

Since we also detected a pathogenic mutation in a woman diagnosed with HCM at age 64 years, we conclude that *FHL1* mutations are associated with X-linked dominant inheritance of HCM and can be distinguished from autosomal dominant HCM by the absence of father to son inheritance. X-linked dominant HCM is also seen in Fabry and in Danon disease. The prevalence of Fabry can be as high as 12% in female patients with late-onset HCM.[7] Danon disease often presents at a young age with hypertrophic or dilated cardiomyopathy accompanied by skeletal myopathy and Wolff-Parkinson-White syndrome. However, isolated cardiomyopathy has been described in female carriers.[5, 6] The difference between *FHL1*- HCM

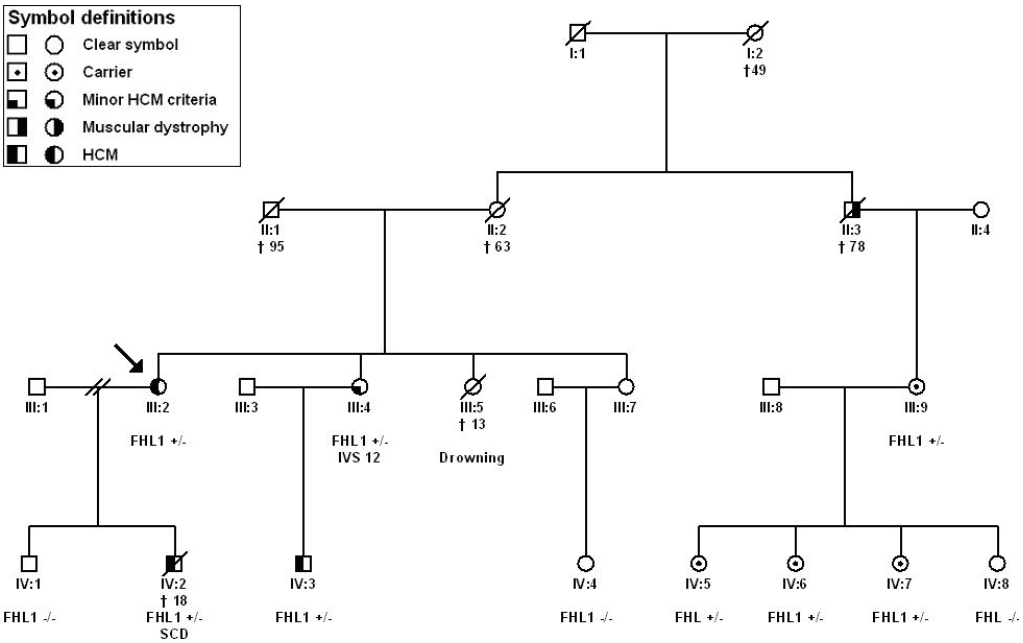


Figure 4. Pedigree of patient 1.
The arrow indicates the proband. HCM: hypertrophic cardiomyopathy; *FHL1*: four-and-a-half LIM domains 1 gene; IVS: interventricular septum; SCD: sudden cardiac death

and HCM associated with Fabry and Danon disease is that microscopy of *FHL1*- HCM shows the typical characteristics of sarcomere-related autosomal dominant HCM, whereas Fabry and Danon disease patients show microscopic inclusions typically associated with lysosomal storage disorders.

FHL1 codes for four-and-a-half-LIM domains 1 which is localised in the sarcolemma, sarcomere and nucleus of muscle cells.[16-18, 20] *FHL1* interacts with MYBPC3 and may compete with myosin for binding to MYBPC3.[19] Myosin thick filament formation was impaired in FHL1 knockdown experiments in mouse myoblast cells (C2C12 cells) and was associated with reduced incorporation of MYBPC3 into the sarcomere, suggesting that FHL1 has a role in sarcomere assembly.[19] This could explain the neuromuscular disorders associated with *FHL1* mutations although in EDMD patients none of the identified *FHL1* mutations seemed to have an impact on sarcomere formation in myoblasts.[12] Human cardiac muscle tissue has not been studied so far. Cardiac FHL1

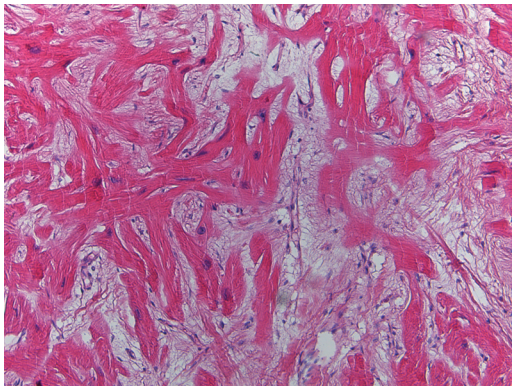


Figure 5. Post-mortem microscopy of relative IV:2 of patient 1.
Note the myocyte disarray, with oblique and sometimes perpendicular orientation of the cardiomyocytes, interstitial fibrosis and irregular, polymorphic and hyperchromatic nuclei.

deficiency in knockout mice resulted in a blunted hypertrophic response to biomechanical stress.[24] The discrepancy between the weakened hypertrophic reaction in *FHL1* knockout mice and HCM in human *FHL1* mutation carriers might be explained by differences in FHL1 expression in human and mouse cardiac muscle. All mutations identified in *FHL1* so far are unique mutations that may have different effects on FHL1 function, similar to different mutations in the N2-B region of titin / connectin leading to either an increase of titin-FHL2 interaction causing HCM, or a decrease in titin-FHL2 interaction causing dilated cardiomyopathy.[25] Since there are three FHL1 isoforms, differences in expression or function between the three isoforms may also play a role in the different observed phenotypes. In addition, truncated FHL1 proteins may act as poison-polypeptides and cause HCM through a dominant-negative effect.

Functional studies on human cardiac tissue might establish which mechanism leads to HCM in *FHL1* mutations carriers.

Mutations that have previously been identified in EDMD (missense, truncating and suppression of stopcodon) were located on exons 5, 6 and 8; seven of the eight EDMD mutations affect the FHL1A isoform, four also affect the FHL1B isoform, two also affect FHL1C and one mutation solely affects FHL1C.[12] Mutations described in reducing body myopathy, XMPMA, SPM and RSS are missense mutations often affecting highly conserved cysteines and are mainly located in exon 5 and to a lesser extent in exon 4, affecting all three isoforms. One mutation in XMPMA is located on exon 6 and affects the FHL1A and FHL1B isoforms.[9-11, 13] Cardiac involvement in *FHL1* mutation carriers has been seen in both women and men and consists of HCM and DCM with or without arrhythmia. Isolated cardiac disease without neuromuscular involvement in both women and men has been described in EDMD families by Gueneau et al. [12] The mutations in these families are located on exon 5 (frameshift), exon 6 (missense) and exon 8 (one frameshift and one missense mutation) (Figure 3). No specific genotype-phenotype relationships can be established yet, based on the present knowledge. Family studies are therefore recommended in all *FHL1* families, especially since *FHL1* mutations have been associated with an increased risk of SCD, as demonstrated in family 1 and in previous studies.[12] Molecular and cardiologic family screening allows accurate identification of relatives at risk of developing HCM and/or SCD. In relatives who refuse DNA analysis, neurological evaluation may establish their risk-status, even in the absence of a cardiac phenotype.

Neurological family studies of *FHL1* HCM families are necessary to establish whether *FHL1* is linked to isolated HCM without neuromuscular disease. For now cardiologic and neurologic examinations remain recommended in *FHL1* families.

In summary, *FHL1* mutations are an infrequent cause of HCM with cardiologic and neurologic intrafamilial variability and incomplete penetrance. When X-linked dominant inheritance is suspected, the chance of finding *FHL1* defects may be higher, especially when family history is positive for myopathy or muscular dystrophy. In these cases *FHL1* analysis is recommended.

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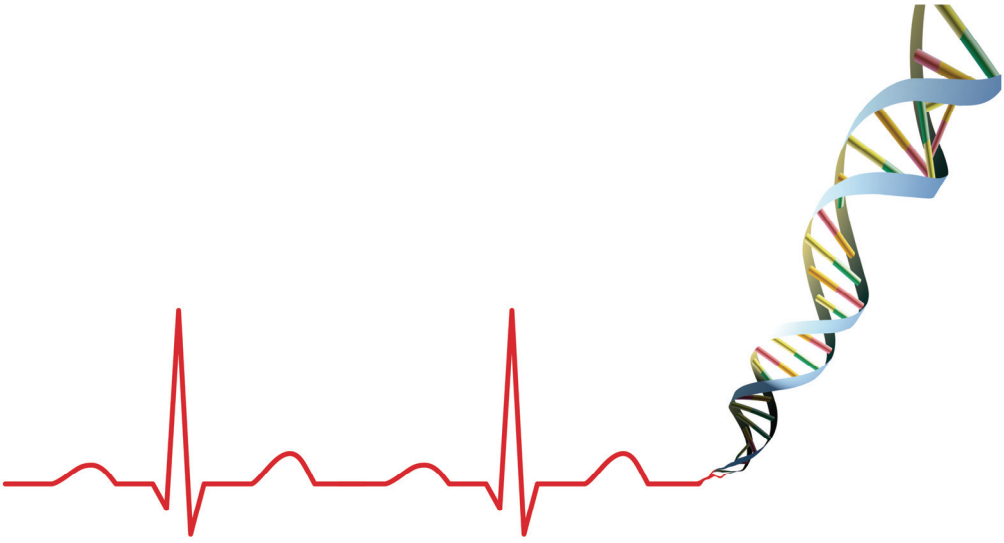
Chapter 11

Complex sarcomeric genetic status is not an important modifier of disease severity in MYBPC3 associated hypertrophic cardiomyopathy.

Michelle Michels, Yvonne M. Hoedemaekers, Marianne van Tienhoven, Osama I.I. Soliman, Danielle F. Majoor-Krakauer, Folkert J. ten Cate, Dennis Dooijes.

Submitted



Abstract

Background

Hypertrophic cardiomyopathy (HCM) is the most common inheritable cardiac disorder and is characterised by marked genetic and clinical heterogeneity. Genotype-phenotype studies have been hampered by the genetic heterogeneity of HCM and uniqueness of HCM mutations. Disease variability is expected to be partially explained by the effect of additional mutations in sarcomere genes.

Methods and results

To analyse whether a complex genotype is an important modifier of disease severity we completely analysed nine sarcomere HCM genes (*MYH7, MYBPC3, MYL2, MYL3, TNNT2, TNNI3, TNNC1, ACTC1, TMP1*) in 87 subjects carrying truncating *MYBPC3* founder mutations as primary genetic HCM defect. The subjects were clinically diagnosed as either having a mild or severe phenotype based on age of diagnosis, maximal wall thickness, left ventricular outflow tract obstruction, positive family history of sudden cardiac death and the necessity for septal reduction therapy or an implantable cardioverter defibrillator.
No additional mutations were seen in the group with a severe phenotype compared to the group with a mild phenotype.

Conclusion

The severity of phenotypic expression of HCM in subjects with a truncating *MYBPC3* mutation is not primarily dependent on the modifying effects of secondary sarcomere mutations.

Introduction

Hypertrophic cardiomyopathy (HCM) is the most common inheritable cardiac disorder with a phenotypic prevalence of 1:500. It is defined by the presence of left ventricular hypertrophy in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality. Hundreds of mutations scattered among at least 24 putative HCM susceptibility genes encoding various sarcomere, Z-disc, calcium-handling, and mitochondrial proteins are known to cause HCM and are found in up to 60% of cases.[1-5] The genetic heterogeneity of HCM together with the fact that most HCM mutations are unique and occur in single families, have hampered studies establishing genotype-phenotype relations. The observed variability in disease severity may be partially explained by complex genotypes i.e. the effect of additional mutations in sarcomere genes.[6-9]

In the Netherlands, approximately one third of all HCM cases are caused by the truncating c.2373dupG, p.Arg943X and c.2864_2865delCT founder mutations in the myosin binding protein C gene (*MYBPC3*).[10] These mutations lead to haploinsufficiency and are thought to be functionally identical.[11] We completely analysed nine sarcomere genes in 87 patients from this group, homogeneous with respect to the primary HCM defect, to analyse whether a complex sarcomere genotype is a modifier of disease severity in *MYBPC3* associated HCM.

Methods

Subjects

Subjects were selected from our cohort of carriers of Dutch founder mutations (c.2373dupG, p.Arg943X and c.2864_2865delCT in *MYBPC3*), including probands as well as family members. All subjects in the cohort were clinically diagnosed as either having “mild” or “severe” phenotype based on the following criteria: age at diagnosis < 25 years, maximal wall thickness (MWT) ≥ 30 mm, left ventricular outflow tract obstruction (LVOTO) > 30 mmHg, sudden cardiac death (SCD) ≥ 2 first-degree family member < 40 years and the necessity for septal reduction therapy or an implantable cardioverter defibrillator (ICD) for primary or secondary prevention of SCD. A mild phenotype was defined as the absence of any of these criteria and a severe phenotype was defined as the presence of ≥ 2 criteria. Based on these definitions 87 subjects, from 52 different families, were included in the current study. For the diagnosis of HCM in family members the published diagnostic criteria for HCM in adult members of affected families were used.[12]

Genetic analysis

Complete DNA sequence analysis was performed of all coding regions of nine HCM genes; β-myosin heavy chain (*MYH7*; NM_000257.2), myosin binding protein C (*MYBPC3*; NM_000256.3), cardiac troponin C (*TNNC1*; NM_003280.1), cardiac troponin T (*TNNT2*; NM_000364.2), cardiac troponin I (*TNNI3*; NM_000363.4), cardiac-regulatory myosin light chain (*MYL2*; NM_000432.3), cardiac-essential myosin light chain (*MYL3*; NM_000258.2), cardiac α-actin (*ACTC1*; NM005159.4), α-tropomyosin (*TPM1*; NM_000366.5).

Sequence analysis was carried out on an ABI3730xl capillary sequencer using Big-Dye Terminator v 3.1 chemistry (Applied Biosystems). (Details of methods and primer sequences are available on request.) Analysis of sequence data was performed using SeqScape analysis software (v2.5, Applied Biosystems). All identified sequence variations were subjected to statistical analysis.
Nomenclature for the description of sequence variants was used according to the recommendations of the Human Genome Variation Society (www.HGVS.org).

Statistical methods

All statistics were performed using the SPSS 16 for Windows (SPSS Inc, Chicago, IL, USA). Descriptive data were computed as a mean value ± SD. Variables among the groups were compared by ANOVA. Statistical significance was defined by $P \leq 0.05$.

Results

HCM mutation carriers

The descriptives of the study population are displayed in Table 1. A total of 87 subjects were included in the study; 44 had a mild phenotype and 43 a severe phenotype. The group with the severe phenotype was diagnosed at a significantly younger age. In this group MWT was significantly thicker and the gradient in the LVOT was significantly higher compared to the group with a mild phenotype. By definition a positive family history for SCD, septal reduction therapy or ICD implantation were absent in the group with a mild phenotype. There was no significant difference in gender or in the presence of the three founder mutations between the two groups.

Table 1. Subject characteristics.

	Total group	Mild phenotype	Severe phenotype	P-value
Number patients	87	44	43	
Men (n, %)	48 (55%)	25 (57%)	23 (53%)	0.5
HCM diagnosis (n, %)	65 (75%)	22 (50%)	43 (100%)	0.001
Age at diagnosis (yrs, mean ± SD) (range)	38.4 ± 13.5 (11 - 68)	44.7 ± 10.9 (29 – 64)	35.7 ± 13.7 (11 – 65)	0.006
MWT (mm, mean ± SD) (range)	18.7 ± 7.2 (7 - 40)	15.1 ± 5.9 (7 - 29)	23.8 ± 5.3 (15 – 40)	0.001
LVOT (mmHg, mean ± SD) (range)	32.7 ± 37.7 (3 – 130)	8.6 ± 5.4 (3 – 29)	57.9 ± 39.7 (5 – 130)	0.001
Family history SCD (n, %)	18 (21%)	0 (0%)	18 (41%)	0.001
Septal reduction therapy (n, %)	26 (30%)	0 (0%)	26 (59%)	0.001
ICD (n, %)	9 (10%)	0 (0%)	9 (20%)	0.001
c.2373dupG (p.Trp792fs) (n, %)	47 (54%)	22 (50%)	25 (58%)	0.5
c.2827C>T (p.Arg943X) (n, %)	24 (28%)	12 (27%)	12 (28%)	0.5
c.2864_2865delCT (p.Pro955fs) (n, %)	16 (18%)	10 (23%)	6 (14%)	0.3

Legend. HCM = hypertrophic cardiomyopathy; MWT = maximal wall thickness; LVOT = left ventricular outflowtract; SCD = sudden cardiac death; ICD = implantable cardioverter defibrillator

DNA sequence analysis

Complete sequence analysis of nine sarcomere genes in 87 subjects with a truncating *MYBPC3* mutation resulted in the identification of 68 different sequence variations (Table 2). Forty-seven different sequence variations were found in the *MYBPC3* and *MYH7* genes. No or only a single sequence variant was observed in the *TNNC1*, *MYL3* and *ACTC1* genes.

Except for the c.2373dupG, p.Arg943X and c.2864_2865delCT founder mutations in *MYBPC3*, no additional clearly pathogenic mutation was found in the nine HCM genes. Fifty-nine variants were identified that were either in intronic sequences or did not have an effect on protein composition (i.e. 'silent variants'). For none of these 59 variants a significant difference in distribution could be demonstrated between the mildly affected group and the severely affected group (Table 2).

A total of nine different missense variants (i.e. variants with an effect on protein composition) were identified. Missense variants are of interest because of their potential modifying effect on protein function and disease expression in HCM. In total, six missense variants were identified in *MYBPC3*, one missense variant in *MYH7* and 2 missense variants in *TNNT2*. The missense variants were analysed using the SIFT and PolyPhen prediction algorithms[13, 14]. The predictions by SIFT were mostly of low confidence and the predictions of PolyPhen suggested seven missense variants to be benign and not have an adverse effect on protein function. Only the p.His1039Tyr and p.Ile1131Thr variants in the *MYBPC3* gene, observed in a mildly affected and a severely affected subject respectively were predicted by PolyPhen to have a deleterious effect on protein function.

There was no significant difference between the total number of missense variants (10) in the severely affected group and the total number of missense variants (15) in the mildly affected group (p=0.389). When the frequent polymorphism p.Ser236Gly in *MYBPC3*, present in equal numbers in both groups, is excluded from the analysis the difference remains non-significant (p=0.249).

The heterozygous p.Val158Met variant in the *MYBPC3* gene was found six times in the group with the mild phenotype and was absent in the group with severe phenotype (p=0.029). However, after correction for multiple testing this difference was found to be non-significant.

Discussion

Secondary sarcomere mutations did not explain the difference in disease severity in this cohort of HCM subjects with a truncating *MYBPC3* founder mutation. No additional pathogenic mutations were found and the identified sarcomere sequence variations could not be associated with a mild or a severe phenotype. We therefore conclude that severity of phenotypic expression of HCM is not primarily dependent on the modifying effects of a secondary sarcomere mutation or sequence variant on top of the primary genetic defect. HCM is characterised by phenotypic heterogeneity; some carriers do not develop cardiomyopathy, in others negligible to extreme hypertrophy, absent or severe left ventricular outflow tract obstruction, normal longevity or premature sudden cardiac death occurs, even in patients carry-

Table 2. Syndromes associated with NCCM / hypertrabeculation.

Gene	Observed variants (nucleotide)	Effect on protein	Status variants (rs number)	Severe phenotype total alleles n=86 (hetero-/ homozygotes)	Mild phenotype total alleles n=88 (hetero-/homozygotes)	P-value[#]	Sift	Poly-phen
ACTC1	c.927T>C	p.=	rs2307493	0	1 (1/0)	1.000		
MYBPC3	**p.Val158Met**	**p.Val158Met**	**rs3729986**	**0**	**6 (6/0)**	**0.029**	NT*	Benign
	c.492C>T	p.=	rs3218719	24 (24/0)	24 (20/2)	1.000		
	c.506-12dupC		rs11570050	11 (11/0)	12 (12/0)	1.000		
	c.537C>T	p.=	rs11570051	2 (2/0)	3 (3/0)	1.000		
	c.706A>G	**p.Ser236Gly**	**rs3729989**	**6 (6/0)**	**6 (6/0)**	1.000	T*	Benign
	c.786C>T	p.=	rs11570058	6 (6/0)	6 (6/0)	1.000		
	c.926+25C>A		rs3729991	1 (1/0)	1 (1/0)	1.000		
	c.1091-24T>C		rs2856650	59 (19/20)	67 (17/25)	0.310		
	c.1223+29G>A		rs11570078	6 (6/0)	7 (7/0)	1.000		
	c.1226+30G>A			1 (1/0)	0	0.494		
	c.1352-21A>C			1 (1/0)	0	0.494		
	c.1608T>A	p.=		1 (1/0)	0	0.494		
	c.2308+18C>G		rs3729948	2 (2/0)	1 (1/0)	0.618		
	c.2497C>A	**p.Ala833Thr**		**1 (1/0)**	**0**	0.494	NT*	Benign
	c.2547C>T	p.=	rs3729953	1 (1/0)	1 (1/0)	1.000		
	c.2686G>A	**p.Val896Met**	**rs35078470**	**1 (1/0)**	**0**	0.494	NT*	Benign
	c.2737+12C>T		rs3729936	0	1 (1/0)	1.000		
	c.3115C>T	**p.His1039Tyr**		**0**	**1 (1/0)**	1.000	NT*	Probably damaging
	c.3191-21A>G		rs11570115	7 (7/0)	4 (4/0)	0.368		
	c.3288G>A	p.=	rs1052373	39 (27/6)	36 (18/9)	0.646		
	c.3392T>C	**p.Ile1131Thr**		**1 (1/0)**	**0**	0.494	NT*	Possibly damaging
MYH7	c.189C>T	p.=	rs2069540	38 (22/8)	46 (22/12)	0.293		
	c.597A>G		rs2069541	1 (1/0)	1 (1/0)	1.000		

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MYH7	c.732C>T	p.=	rs2069542	13 (13/0)	22 (16/3)	0.131	
	c.895+17G>A		rs45580436	1 (1/0)	0	0.494	
	c.896-17C>T			1 (1/0)	0	0.494	
	c.975C>T	p.=	rs2231124	1 (1/0)	1 (1/0)	1.000	
	c.1062C>T	p.=	rs735712	3 (3/0)	7 (7/0)	0.330	
	c.1095G>A	p.=	rs735711	10 (8/1)	7 (7/0)	0.454	
	c.1128C>T	p.=	rs2231126	10 (10/0)	9 (9/0)	0.812	
	c.1605A>G	p.=	rs2069543	0	3 (3/0)	0.246	
	c.2733C>T	p.=		0	2 (2/0)	0.497	
	c.2923-18G>A		rs7157087	0	3 (3/0)	0.246	
	c.2923-18G>A		rs7157087	0	3 (3/0)	0.246	
	c.2967T>C	p.=	rs7157716	26 (18/4)	29 (21/4)	0.746	
	c.3153G>A	p.=	rs45540831	4 (4/0)	1 (1/0)	0.208	
	c.3337-3dupC		rs45504498	3 (3/0)	8 (8/0)	0.212	
	c.3853+7C>T		rs45467397	0	1 (1/0)	1.000	
	c.3853+21C>T		rs45584435	1 (1/0)	3 (3/0)	0.621	
	c.3853+27T>A		rs2277475	26 (22/2)	32 (22/5)	0.424	
	c.3960G>A	p.=		1 (1/0)	0	0.494	
	c.3972+15C>T		rs3729820	1 (1/0)	0	0.494	
	c.3973-30A>G		rs7159367	28 (20/4)	33 (21/6)	0.528	
	c.4472C>G	**p.Ser1491Cys**	**rs3729823**	**0**	**1 (1/0)**	1.000	NT* Benign
	c.4520-25C>T		rs45503601	1 (1/0)	1 (1/0)	1.000	
	c.4566T>C	p.=	rs2754155	3 (3/0)	2 (2/0)	0.680	
	c.4644+11_4644+12del			1 (1/0)	0	0.494	
	c.5106G>A	p.=	rs3729830	14 (10/2)	18 (18/0)	0.559	

Continued on next page

*Legend. Total number of alleles of 68 analysed sequence variations in 9 analysed sarcomere genes. (No sequence variations were seen in the TNNC1 gene.) dbSNP build 130. Missense variants are in bold. # After correction for multiple testing (p = 0.0007 is significant), no significant differences in sequence variations are observed between the severe and mild patient populations. NT: Not tolerated; T: Tolerated; * This substitution may have been predicted to affect function just because the sequences used were not diverse enough. There is low confidence in this prediction.*

Table 2. Continued.

Gene	Observed variants (nucleotide)	Effect on protein	Status variants (rs number)	Severe phenotype total alleles n=86 (hetero-/ homozygotes)	Mild phenotype total alleles n=88 (hetero-/homozygotes)	P-value[#]	Sift	Poly-phen
MYL2	c.132T>C	p.=	rs2301610	9 (7/1)	8 (6/1)	0.803		
	c.274+33_274+34dup			12 (3/4)	8 (4/2)	0.350		
	c.274+17G>C			1 (1/0)	0	0.494		
	c.353+20delG		rs3833910	26 (18/4)	28 (14/6)	0.871		
	c.353+46dupC		rs3216817	7 (7/0)	7 (5/1)	1.000		
MYL3	c.*9C>T			1 (1/0)	0	0.494		
TNNI3	c.15-50_15-47dup		rs34226453	11 (11/0)	4 (4/0)	0.062		
	c.25-8T>A		rs3729836	25 (21/2)	13 (11/1)	0.042		
	c.108+21G>A		rs3729837	11 (9/1)	6 (4/1)	0.210		
	c.204G>T	p.=	rs3729711	5 (5/0)	5 (5/0)	1.000		
	c.537G>A	p.=	rs3729841	11 (9/1)	6 (4/1)	0.042		
TNNT2	c.53-11_53-7del		rs45533739	60 (18/21)	58 (22/18)	0.628		
	c.207G>A	p.=	rs3729845	5 (5/0)	5 (5/0)	1.000		
	c.318C>T	p.=	rs3729547	61 (13/24)	62 (20/21)	1.000		
	c.382-94delC		rs35559054	1 (1/0)	1 (1/0)	1.000		
	c.583G>A	**p.Glu195Lys**		**1 (1/0)**	**0**	0.494	T	Benign
	c.758A>G	**p.Lys253Arg**	**rs3730238**	**0**	**1 (1/0)**	1.000	T	Benign
TPM1	c.453C>A	p.=	rs1071646	60 (22/19)	56 (26/15)	0.424		
	c.486T>C	p.=	rs11558747	6 (6/0)	12 (10/1)	0.213		
	c.639+22G>C		rs28730802	2 (2/0)	0	0.243		

*Legend. Total number of alleles of 68 analysed sequence variations in 9 analysed sarcomere genes. (No sequence variations were seen in the TNNC1 gene.) dbSNP build 130. Missense variants are in bold. [#] After correction for multiple testing (p = 0.0007 is significant), no significant differences in sequence variations are observed between the severe and mild patient populations. NT: Not tolerated; T: Tolerated; * This substitution may have been predicted to affect function just because the sequences used were not diverse enough. There is low confidence in this prediction.*

ing the same pathogenic HCM mutation.[15] According to literature compound or double heterozygotes are detected in 3 - 5% of genotype-positive patients.[6-8] These patients appear to have a more severe phenotype and an increased incidence of SCD, suggesting a gene-dose effect might contribute to disease severity.[7, 16] Compound heterozygousity for truncating *MYBPC3* mutations causes severe neonatal HCM leading to death in the first weeks or months of life.[17] This is probably the result of the lack of functional MYBPC3 protein (i.e. human *MYBPC3* knockouts). Since the youngest patient in our cohort was 11 years old it is not surprising that we did not find compound heterozygousity for two truncating mutations in *MYBPC3*. Double heterozygousity, when two pathogenic mutations in two *different* genes are present and at least one functional copy of the *MYBPC3* gene is still active, has been described in adult patients without being responsible for a lethal phenotype at a young age.[6, 8] In a large cohort from the Mayo Clinic, patients with two mutations in different genes were significantly younger at diagnosis, had the most hypertrophy, and the highest incidence of myectomy and ICD placement compared to patients with one mutation.[7] Based on these data additional analysis of sarcomere genes in the presence of a severe phenotype was recommended, posing important implications for genetic counselling and pre-symptomatic testing of family members.[8] However, the current study suggests otherwise for specific *MYBPC3* founder mutations, unless severe hypertrophy or end stage heart failure is present at a young age. It remains to be established whether these findings extend to other *MYBPC3* and sarcomere mutations.

All three described Dutch founder mutations are functionally identical truncating mutations, leading to haploinsufficiency, and have identical Kaplan-Meier survival estimates (Chapter 9).[11] The result of our study illustrates that the clinical heterogeneity of HCM is in this group is more likely to be modified by environmental factors as well as by other non-sarcomere genetic factors than by multiple mutations in sarcomere genes.[18-20] The most important subgroup of non-sarcomere polymorphisms studied to date involve the major components of the renin-angiotensin-aldosteron system (RAAS). The RAAS system contributes to ventricular hypertrophy through effects mediated by circulating angiotensin as well as local activation of RAAS in the myocardium.[21] Polymorphisms in the RAAS pathway appear to influence the severity of LVH in HCM patients carrying *MYBPC3* mutations.[18, 22] Proteins involved in hypertrophic pathways or mediators of calcium signalling in cardiomyocytes are other promising candidates as modifier genes.[23-25] Further studies are needed to identify modifier genes in HCM and to elucidate the molecular mechanisms by which they influence cardiac hypertrophy; as this could possibly lead to new prognostic markers and new therapeutic targets for HCM.

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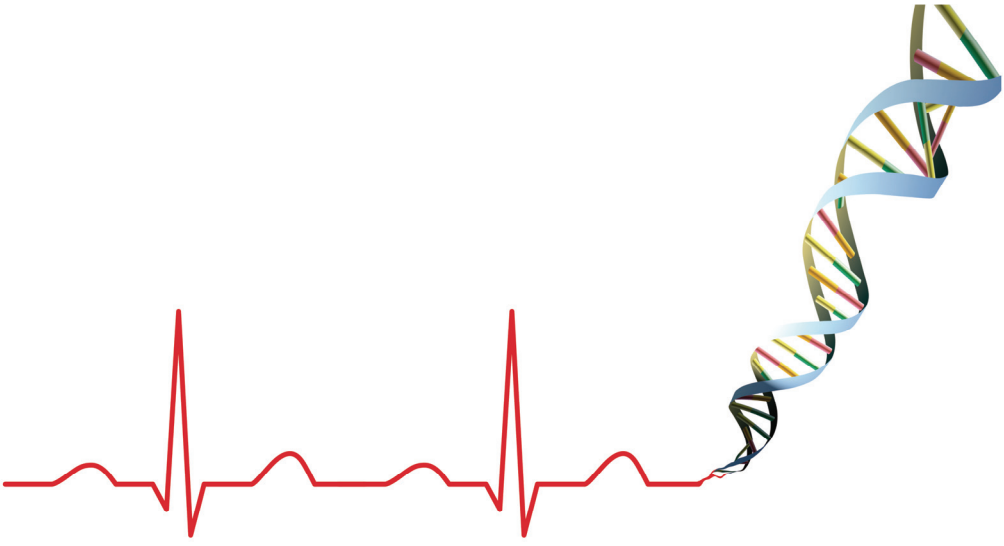
Chapter 12

Cardiac aldosterone in subjects with hypertrophic cardiomyopathy.

Wenxia Chai, Yvonne M. Hoedemaekers, Ron H.N. van Schaik, Marianne van Fessem, Ingrid M. Garrelds, Jasper Saris, Dennis Dooijes, Folkert J. ten Cate, Marcel M.J. Kofflard, A.H. Jan Danser.

J Renin Angiotensin Aldosterone Syst. Dec 2006;7(4):225-230



Paper

Cardiac aldosterone in subjects with hypertrophic cardiomyopathy

Wenxia Chai, Yvonne M Hoedemaekers,* Ron HN van Schaik,† Marianne van Fessem,† Ingrid M Garrelds,* Jasper J Saris,* Dennis Dooijes,* Folkert J ten Cate,‡ Marcel MJ Kofflard,‡ AH Jan Danser*

Key words: aldosterone, hypertrophy, polymorphism, angiotensin, gender

Departments of Pharmacology, *Clinical Genetics, †Clinical Chemistry and ‡Cardiology, Erasmus MC, Rotterdam, The Netherlands.

Correspondence to: Professor AH Jan Danser, Department of Pharmacology, Room EE1418b, Erasmus MC Dr. Molewaterplein 50 3015 GE Rotterdam, The Netherlands Tel: +31 10 408 7540 Fax: +31 10 408 9458 E-mail: a.danser@erasmusmc.nl

Accepted for publication 7th November 2006

JRAAS 2006;**7**:225–30

Abstract

Left ventricular (LV) hypertrophy in subjects with hypertrophic cardiomyopathy (HCM) is variable, suggesting a role for modifying factors. Here, we determined whether aldosterone modulates hypertrophy in HCM. Cardiac and/or plasma aldosterone were measured in organ donors and HCM patients. The effect of the aldosterone synthase (*CYP11B2*) C-344T polymorphism on LV mass index (LVMI) and interventricular septum thickness (IVS) was determined in 79 genetically independent subjects with HCM. Aldosterone in HCM hearts and plasma was similar to that in normal hearts and plasma. In HCM women, no associations between *CYP11B2* genotype and any of the measured parameters were observed, whereas in HCM men, LVMI increased with the presence of the T allele. Similar T allele-related increases were observed for IVS. Multiple regression analysis revealed that the T allele-related effect on IVS occurred independently of renin, the ACE I/D polymorphism, the AT_1-receptor A/C[1166] polymorphism and the AT_2-receptor A/C[3123] polymorphism. In conclusion, circulating and cardiac aldosterone are normal in HCM, thereby arguing against selectively increased cardiac aldosterone production in HCM. Thus, the association between the *CYP11B2* C-344T polymorphism and hypertrophy in HCM most likely relates to the T allele-related increases in circulating aldosterone. This finding raises the need for studies determining the benefit of aldosterone blockade in HCM.

Introduction

Hypertrophic cardiomyopathy (HCM) is a genetic disease characterised by unexplained cardiac and myocyte hypertrophy, interstitial fibrosis and myocyte disarray.[1] Mutations in at least 11 different sarcomeric proteins have been identified as the primary defect.[2] Yet, even if patients have identical causative genotypes, they still vary considerably in phenotype.[3] Other factors, genetic as well as environmental, may therefore modify the phenotypic expression of the mutated gene. Angiotensin II (Ang II), the end-product of the renin-angiotensin system (RAS), is among these factors. It modulates cardiac hypertrophy in HCM both via growth-stimulatory Ang II type 1 (AT_1) receptors and via growth-inhibitory[4] Ang II type 2 (AT_2) receptors.[5,6] Its effects are gender-specific, and occur independently of the circulating RAS.

A recent study suggests that aldosterone, like Ang II, acts as a major link between sarcomeric mutations and cardiac phenotype in HCM.[7] According to this study, the myocardial aldosterone levels in humans with HCM are four-fold elevated, and aldosterone provokes expression of hypertrophic markers in rat cardiomyocytes and of collagens in rat cardiac fibroblasts. The latter effects occurred in a mineralocorticoid receptor (MR)-dependent manner, as they could be blocked by the MR antagonist spironolactone.[7,8] MR do occur in the human heart,[9] and aldosterone, like Ang II,[10] may be produced in the heart, particularly in HCM subjects because their cardiac aldosterone synthase (*CYP11B2*) messenger ribonucleic acid (mRNA) levels are seven-fold increased as compared to age- and gender-matched normal donor hearts.[7] However, aldosterone synthase expression in the heart is six orders below that in the adrenal,[11] and thus cardiac aldosterone production is still controversial.[11-14] The mere fact that the *CYP11B2* C-344T polymorphism associates with left ventricular mass (LVM)[15] may well be explained on the basis of the T allele-related increase in circulating aldosterone levels.[15-19]

It was the aim of the present study to first verify whether the cardiac aldosterone levels are elevated in HCM subjects. Secondly, we investigated the role of aldosterone as a modulator of hypertrophy in HCM, by determining the relationship between the *CYP11B2* C-344T polymorphism and cardiac hypertrophy in HCM patients.

Methods

Patients

All studies were approved by the internal review board and patients gave informed consent. One-hundred and seventeen Caucasian patients with HCM (age 21–81 years) visiting the HCM Clinic at the Erasmus MC between 1994 and 1997 for a routine follow-up were included. HCM had been diagnosed on the basis of echocardiographic criteria showing a non-dilated, hypertrophied left

Journal of the Renin-Angiotensin-Aldosterone System
(Including other Peptidergic Systems)

December 2006
Volume 7
Number 4

ventricle (any wall thickness >15 mm) in the absence of known causes of left ventricular hypertrophy (LVH).[20] DNA quality and quantity allowed genotyping in 81 patients. Of these patients, 31 had a sporadic form of HCM and 36 had at least one other affected first degree family member. The family history of HCM was unknown in 14 patients. To avoid potential bias introduced by the presence of genetically dependent samples (relatives), we randomly selected one patient per family. This resulted in a final cohort of 79 genetically independent patients, of whom five were receiving an angiotensin-converting enzyme (ACE) inhibitor, (ACE-I) 22 a β-adrenergic antagonist, 39 a calcium-channel blocker (CCB) and nine a diuretic.

LV tissue was obtained from 8 HCM subjects with obstructive hypertrophy (3 men, 5 women, age 36±3 (range 16–57) years) undergoing septal myectomy[21] and from 12 heart-beating organ donors (5 men, 7 women, age 39±3 (range 17–54 years), who died of non-cardiac causes (9 cerebrovascular accident, 1 head trauma, 2 brain hypoxia) <24-hours before the heart was taken to the laboratory. Tissue pieces (1–10 g) were kept at -70ºC.

Echocardiographic methods

Two-dimensional echocardiography was performed with commercially available equipment (Toshiba Sonolayer). Images were recorded on videotape for off-line analysis by two physicians who were blinded to the genotyping results. Interventricular septal thickness (IVS) and LVM were determined as described before.[5] LVM was indexed (LVMI) to body surface area (BSA). Peak LV outflow tract gradient at rest was estimated using the modified Bernoulli equation.[5] Since echocardiographic measurement of LVMI may not truly reflect the extent of hypertrophy and the involvement (or lack thereof) of the distal (apical) half of the septum or lateral wall, the extent of hypertrophy was also assessed by a semi-quantitative point score (range 0–10) method developed by Wigle *et al.*[22]

Biochemical measurements

Prorenin and renin were quantified in peripheral venous blood using an immunoradiometric assay kit (Nichols Institute).[23] Prorenin and renin are expressed as mU/L, using the human kidney renin standard MRC 68/356 as a reference. ACE activity was measured with a commercial kit (ACE Color).[24] Aldosterone was measured by solid-phase radioimmunoassay (DPC) in plasma and LV tissue. To extract aldosterone from cardiac tissue, LV tissue was homogenised 1:2 in methanol. The supernatant was collected after a 15-minute centrifugation at 3,000 rpm at 4ºC, vacuum dried, and dissolved in water. Recovery of ^{125}I-aldosterone added to cardiac tissue prior to the homogenization procedure was >70%, and values were not corrected for incomplete recovery. The detection limit was 25 pg/mL in plasma and 10 pg/g wet weight, and levels below the detection limit were taken to be equal to the detection limit.

Genetic analysis

Peripheral leukocytes were used to isolate genomic DNA in H_2O using the QIAamp Bloodkit (QIAGEN Inc.). The aldosterone synthase gene (*CYP11B2*) C-344T polymorphism was determined according to Barbato *et al.*[16] Polymerase chain reaction (PCR) amplifications were carried out in a 50 µl reaction volume, using 10 ng of genomic DNA. Each reaction contained 1 x PCR buffer II (Perkin Elmer), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each of the deoxynucleotide triphosphates (Roche), 1.25 U of Amplitaq Gold (Perkin Elmer) and 40 pmol each of forward primer 5-CAGGAGGAGACCCCATGTGAC-3 and reverse primer 5-CCTCCACCCTGTTCAGCCC-3'. PCR consisted of initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 67°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. For restriction fragment length polymorphism (RFLP) determination, the PCR product (10 µl) was digested with *Hae III* for two hours at 37°C. The fragment sizes were analysed on a 4% agarose gel with ethidium bromide staining. The PCR-RFLP was validated by direct sequencing of PCR product from a heterozygote sample, using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on a ABI 310 capillary sequencer (Applied Biosystems). The ACE I/D polymorphism, the AT_1-receptor A/C^{1166} polymorphism, and the AT_2-receptor A/C^{3123} polymorphism were determined as described before.[5,6]

Statistical analysis

Data are expressed as means±SEM or geometric mean and range. Analysis was performed with the SPSS 11.0 statistical package. Hardy–Weinberg equilibrium was tested by X^2 test. Univariate and multiple regression analyses were conducted to determine the percentage of explained variance in LVMI and IVS that is accounted for by the genotypes of the candidate modifier genes and other variables. In the multiple regression analysis the RAS gene polymorphisms, age, peak LV outflow tract gradient and renin concentration were tested as independent variables. Prorenin and ACE were excluded from this analysis because of their high correlations with renin ($r=0.68$, $p<0.001$) and ACE genotype ($r=0.39$, $p=0.003$), respectively.

Results

Cardiac aldosterone levels in HCM patients (27 (10–233) pg/g, n=8) were not different from the aldosterone levels in hearts of organ donors (26

Table 1
Characteristics of HCM patients according to aldosterone synthase genotype.

Parameter	Genotype		
	CC (n=10)	CT (n=45)	TT (n=24)
Sex, M/F	7/3	24/21	14/10
Age, y	48±5	51±2	48±4
BSA, m^2	1.88±0.05	1.81±0.03	1.88±0.04
IVS, mm	19.9±1.9	21.5±0.6	22.1±1.0
LVMI, g/m^2	152±16	178±8	169±10
Wigle score, 1–10	5.8±0.7	6.5±0.3	6.2±0.5
Gradient, mmHg	33.6±8.6	54.8±6.0	53.0±8.3
Prorenin, mU/L	289±119	198±19	181±15
Renin, mU/L	43.1±18.0	23.4±2.0	22.3±2.2
ACE, U/L	11.7±1.0	9.8±0.3	10.0±0.5

BSA = body surface area; IVS = interventricular septum thickness; LVMI = left ventricular mass index; gradient = peak left ventricular outflow tract gradient.

(10–481) pg/g, n=12). No differences were observed between men and women (data not shown). Plasma aldosterone levels in 8 HCM patients (age 54±5 (range 34–73) years) were 33 (25–70) pg/mL.

Table 1 lists the characteristics of the HCM patients by *CYP11B2* genotype. Frequencies of the C and T allele (0.41 and 0.59, respectively) were similar to previously reported numbers in normal white populations,[15-17] and genotype frequencies were in agreement with Hardy–Weinberg equilibrium. The percentage of patients taking ACE-Is, β–adrenergic antagonists, CCB or diuretics did not differ between the various groups (data not shown). When analysing all subjects together, no genotype-related differences were observed with regard to any of the measured parameters (table 1), in full agreement with a previous study.[25] However, subdivision of the population according to gender (table 2) revealed that IVS was significantly higher (p=0.01) in men carrying 1 or 2 T alleles than in male CC homozygotes. Similar trends were observed for LVMI (p=0.06) and Wigle score (p=0.08), and both parameters did not differ between CT and TT men. No T allele-related effects were observed in women, nor did any of the other parameters correlate with the presence of the T allele in either men or women. Univariate regression analysis showed that the *CYP11B2* genotype accounted for 16.3% of the variability of IVS in men (r=0.40, p<0.01). Multiple regression analysis revealed that this effect occurred independently (β=3.7±1.7; p=0.04) of renin, the ACE gene I/D polymorphism, the AT_1-receptor gene A/C[1166] polymorphism, the AT_2-receptor A/C[3123] polymorphism, age and peak LV outflow tract gradient.

Discussion

The present study shows that aldosterone, like Ang II, is among the factors that modify the phenotypic expression of the mutated gene in HCM. IVS was higher in male HCM subjects carrying the *CYP11B2* T allele, and similar observations were made for LVMI and Wigle score. The lack of significance with regard to the latter two parameters relates to the fact that our male population, although large enough to detect a T allele-related increase in IVS of 2 mm or more (power 90%, 5% significance level), was too small to detect significant increases in LVMI and Wigle scores of similar magnitude. To achieve this goal, the patient number should have been three times as large. Thus, given the current population size, the borderline significance of the findings on LVMI and Wigle score can, at most, be interpreted as supportive for the findings on IVS.

The T allele-related effect on IVS occurred independently of the RAS. Contrary to our expectation, and opposing the recent study by Tsybouleva *et al.* on this subject,[7] the LV tissue levels of aldosterone in subjects with HCM were not significantly different from those in age-matched controls.

Paper

Table 2
Characteristics of male and female HCM patients according to aldosterone synthase genotype.

Parameter	Genotype			
	Men		Women	
	CC	CT+TT	CC	CT+TT
n	**7**	**38**	**3**	**31**
Age, y	46±6	47±2	51±3	54±3
BSA, m²	1.88±0.05	1.81±0.03	1.88±0.04	1.88±0.04
IVS, mm	17.6±1.0	21.9±0.7*	25.3±5.8	21.5±0.9
LVMI, g/m²	137±9	178±9†	183±53	172±9
Wigle score, 1-10	5.0±0.8	6.4±0.3°	7.7±1.5	6.3±0.5
Gradient, mmHg	33.3±12.3	51.0±6.5	34.3±13.2	57.8±7.8
Prorenin, mU/L	346±167	206±20	91±4	173±16
Renin, mU/L	51.3±25.4	24.7±1.9	14.4±1.0	20.5±2.4
ACE, U/L	14.7±2.0	10.1±0.4	8.6±0.0	9.6±0.3

BSA = body surface area; IVS = interventricular septum thickness; LVMI = left ventricular mass index; gradient = peak left ventricular outflow tract gradient. *p=0.01, †p=0.06, °p=0.08 vs. CC men.

Our human cardiac aldosterone levels are of the same order of magnitude as the levels reported by Gomez-Sanchez *et al.*[11] and Chai *et al.*[14] in the rat heart. These authors observed a close relationship between the levels of aldosterone in the heart and in blood plasma, and they concluded that, at least in the rat, cardiac aldosterone is largely, if not completely, of adrenal origin. In fact, the rat heart displayed a large capacity to accumulate circulating aldosterone, allowing blood-derived aldosterone to reach cardiac tissue levels that are up to 10 times higher than the aldosterone levels in blood.[13,14] The plasma levels of aldosterone in subjects with HCM, like those of renin, are in the (low) normal range (~30–200 pg/ml),[7,26,27] both in this study and a previous study,[7] and thus no alteration in the cardiac aldosterone content of HCM subjects would be expected if cardiac aldosterone were exclusively of adrenal origin. Based on cardiac *CYP11B2* messenger ribonucleic acid measurements however, Tsybouleva *et al.*[7] have suggested that cardiac aldosterone is of local origin. In contrast with this conclusion, their cardiac aldosterone levels were < 0.1 pg/g protein, i.e. > 2 orders of magnitude below the levels that are minimally expected based on the presence of blood in cardiac tissue.[28,29] Thus, conclusive evidence for cardiac production of aldosterone in subjects with HCM is lacking. Future studies should further investigate this issue, also taking into consideration the morphology of HCM and whether the hypertrophy obstructs output flow or not.

The association between *CYP11B2* genotype and cardiac hypertrophy in HCM parallels the association between the *CYP11B2* T allele and LVM in subjects with essential hypertension.[15] It also extends a previous study on this polymorphism in HCM subjects which showed no significant T allele-related increase in LVMI.[25] The latter study did not evaluate the gender-specificity of the effect. In addition, since the influence of the *CYP11B2* genotype, like that of the ACE I/D genotype, will depend on the gene mutation that determines the primary defect,[30] *CYP11B2* T allele-related effects do not necessarily apply to all HCM populations.

According to most[15-19] (but not all)[17,31] studies, the *CYP11B2* T allele is associated with elevated plasma aldosterone levels, while serum aldosterone associates with the variability of LVM in both healthy controls and subjects with hypertension.[32] Thus, a picture arises in which the T allele results in elevated plasma levels of aldosterone, which subsequently affect cardiac hypertrophy, not only in subjects with essential hypertension,[32] but also in subjects with HCM. Indeed, there is ample evidence suggesting that circulating aldosterone acts as a pro-inflammatory, hypertrophic and profibrotic factor in the heart.[7-8,33-34] In further support of this concept, MR

occur in the human heart[9] and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity associates directly with LVM in essential hypertension.[35] 11β-HSD2 inactivates cortisol and corticosterone, thereby preventing these glucocorticoids from stimulating MR. The 11β-HSD2 levels are relatively low in the heart,[9] whereas the glucocorticoid concentrations in blood are several orders of magnitude above those of aldosterone. Thus, activation of cardiac MR by circulating aldosterone is possible only in the presence of sufficiently high 11 β-HSD2 activity. Importantly, the above scenario does not require the cardiac aldosterone levels to be elevated in HCM subjects as compared to controls, since the genotype distribution in our HCM population was similar to that in normal white populations.[15-17]

Two independent investigations have recently shown that the *CYP11B2* C-344T polymorphism is in strong linkage disequilibrium with polymorphisms of the nearby *CYP11B1* gene.[36,37] Since the latter polymorphisms predict activity of the cortisol-producing enzyme 11β-hydroxylase, an alternative explanation of our findings is that reduced 11β-hydroxylase activity rather than increased aldosterone levels underlies the T allele-related cardiac hypertrophy.[38] Future studies, involving aldosterone, 11-deoxycortisol and cortisol measurements in serum and/or urine of HCM subjects,[39] should address this possibility.

The effect of the T allele on cardiac hypertrophy occurred independently of circulating renin, the ACE gene I/D polymorphism, the AT_1-receptor gene A/C^{1166} polymorphism and the AT_2-receptor A/C^{3123} polymorphism. Since both AT_1- and AT_2-receptors modulate cardiac hypertrophy in HCM, most likely in an opposite manner,[5,6] it appears that aldosterone exerts additive effects on top of Ang II,[8] despite earlier studies suggesting that aldosterone exerts its effects via Ang II or AT_1-receptors and vice versa.[40-42] This conclusion is in full agreement with the renin-independent associations between LVM, *CYP11B2* genotype and 11β-HSD2 activity in hypertension.[15,35] The gender-specificity of the association in our study is more difficult to explain, and, given the low number of CC women, should be interpreted with care. In particular, the apparently opposite findings in women as compared to men (i.e., IVS, LVMI and Wigle score being higher in CC women *vs.* CT+TT women) are due to one CC woman with an exceptionally large heart. Without this woman the findings in women would have been identical to those in men. Nevertheless, gender-related differences might occur. For instance, estrogen may mimic or antagonise some of the effects of aldosterone,[43] whereas the well-known gender-related differences in renin and angiotensinogen[27,44] will affect, through Ang II, the biosynthesis of aldosterone.

Conclusion

The levels of aldosterone in heart and blood plasma of HCM subjects are in the low normal range, thereby arguing against the concept of selectively increased cardiac aldosterone production in HCM. This does not exclude the possibility that aldosterone modulates cardiac hypertrophy in HCM. In fact, the *CYP11B2* T allele-related increase in cardiac hypertrophy in HCM subjects fully parallels similar observations in subjects with hypertension, and the simplest explanation of these observations is that they are related to the elevated circulating aldosterone levels in T allele carriers, both in hypertension and HCM. Interestingly in this regard, the MR antagonist spironolactone was found to reduce the extent of myocyte disarray and to reverse interstitial fibrosis in a transgenic mouse model of human HCM mutation (cTnT-Q92 mice), despite the fact that these mice did not display elevated cardiac aldosterone levels.[7] Combined with the results from the present study, these data raise the need for studies determining the benefit of MR blockade in HCM.

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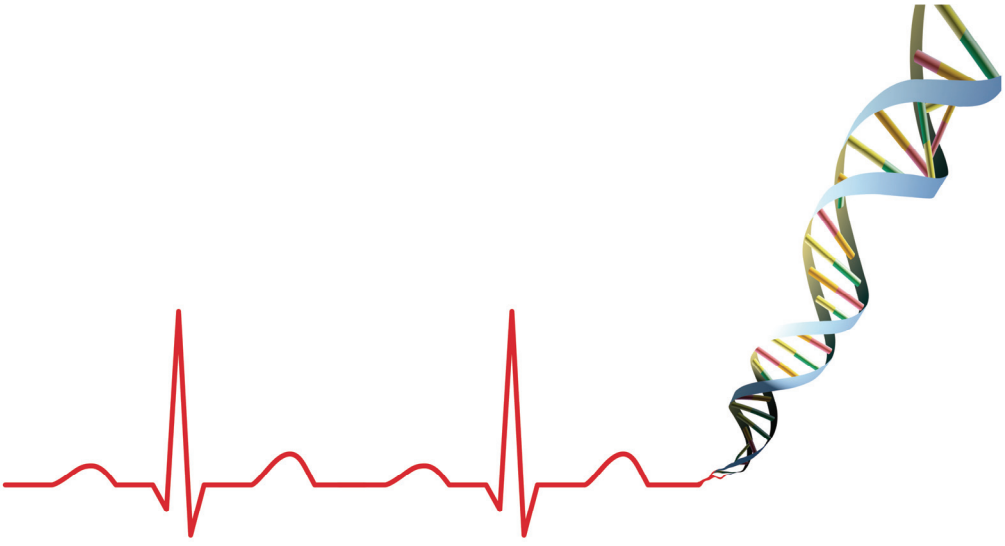
Chapter 13

General discussion



General Discussion

The aim of this dissertation was to investigate the genetics of cardiomyopathies, in particular noncompaction cardiomyopathy (NCCM) and hypertrophic cardiomyopathy (HCM). With cardiologist Dr. Folkert ten Cate's experience in HCM of many years' standing, the Thorax Centre outpatient-clinic of the Erasmus Medical Centre in Rotterdam became a tertiary referral centre for HCM with renowned expertise in natural history and treatment of HCM patients. The large "Rotterdam" HCM population provided the opportunity to start the research projects described in this dissertation, and to identify a new HCM gene and investigate genotype-phenotype relationships. Interest in cardiomyopathies further extended to NCCM, and the relatively large number of NCCM patients referred nationwide to cardiologist Kadir Caliskan allowed establishing a genetic classification for NCCM.

Funding by the Dutch Health Insurances ("Locale component") enabled to achieve a multidisciplinary cardiogenetic out-patient clinic, where cardiologist and clinical geneticists work together to facilitate genetic diagnosis, genetic counselling and family screening. In the mean time a growing number of genetic causes for cardiomyopathies were identified, improving molecular diagnostics. The first part of this thesis focused on the genetic aspects of NCCM, the second part concerned HCM.

NCCM

Since NCCM is a relatively newly discovered cardiomyopathy, little was known of the genetic origin and aetiology of NCCM. The first objective of the NCCM study was to investigate the prevalence of genetic NCCM by extensive cardiologic family studies of NCCM probands and simultaneous molecular analysis of 17 genes (*MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3*, *TCAP*, *LDB3, CASQ2*, *CALR3*, *PLN*, *TAZ* and *LMNA*) in the probands. This way we identified genetic NCCM in the majority of cases (67%; 39/58). In thirty-two probands (16 with a genetic defect, 15 without a genetic defect and one in whom no genetic analysis was performed) cardiologic family studies identified cardiologically affected relatives, indicating genetic NCCM. Additionally, one proband had a *de novo* mutation, three had a mutation without any affected relatives and three had a mutation but cardiologic screening of relatives did not take place. Approximately half of the probands with familial disease did not report familial disease prior to the cardiologic family study (47%). Therefore, ascertainment of family history alone proved to be insufficient to detect familial disease.

Although our estimates of the role of genetics in NCCM are higher than in previous report, underestimation of the true proportion of genetic disease may have occurred. There are four possible causes for underestimation of familial disease in our cardiologic family study. First, compliance in the family study of first-degree relatives was 52%; of the 277 first-degree relatives identified to be possibly at risk for NCCM, 145 participated in the cardiologic study. Since our study showed that the majority of affected relatives was asymptomatic, and family history cannot reliably detect familial disease, relatives will have to be examined by a

cardiologist to establish whether they are affected. Screening more relatives will most likely lead to detection of more cases of familial NCCM. Secondly, some families were small with few first-degree relatives to include in our study. Perhaps the proband in these families had a genetic form of NCCM but the relatives did not have the genetic defect and were therefore unaffected. When the family is larger, chances of finding an affected relative are higher. Thirdly, we encountered 20 relatives with clinical features not meeting current diagnostic criteria for NCCM as stated by Jenni.[1] Neither did they fulfil criteria for HCM or DCM.[2] We could not include these relatives as affected, neither were they unaffected. These relatives may have reduced penetrance or an early stage of cardiomyopathy that eventually might develop into NCCM, HCM or DCM. Revised criteria for diagnosis in relatives, as those proposed for HCM, distinguishing major and minor diagnostic criteria for NCCM are needed for accurate family screening.[3] Follow-up studies of these probably affected relatives are necessary to validate the significance of minor abnormalities. Fourthly, non penetrance was observed in eight unaffected carriers of a familial mutation. It is possible that similar to HCM and DCM, late-onset NCCM may occur in relatives. Follow-up studies of unaffected and possibly-affected relatives will determine whether they will develop NCCM.

Taking into account the compliance of relatives, the “affected” relatives not fulfilling NCCM criteria, non-penetrance and possible age-dependent penetrance, the actual percentage of familial disease in NCCM may be even higher than the 67% we reported. In literature previous studies reported 33% - 44% familial disease.[4-10] Careful evaluation of the methodology of these studies may explain why there are such substantial differences. Our results originate from a combined cardiologic and molecular family study. None of the previous studies performed cardiologic screening of relatives as systematically and as extensively as in our study, nor did they perform extensive molecular studies.[4-10] One study did not present the proportion of probands where family screening revealed familial NCCM; instead the proportion of affected relatives was presented (25%).[4] Another study used questionnaires reporting 44% familial disease.[7] Other studies relied on retrospective review of medical records of probands to assess occurrence of familial disease, which is very likely to lead to under-ascertainment. As our study showed, at least 47% of the probands is unaware of familial disease. One study that did perform molecular analysis (in six sarcomere genes: *MYH7*, *TNNT2*, *TNNI3*, *ACTC1*, *MYL2* and *MYL3*) identified mutations in 17% but did not perform family studies.[11] It is therefore difficult to compare the results of our study with the previous reports.

The next objective of this study was to establish a genetic classification for NCCM by identifying the genes associated with isolated NCCM. Since different phenotypes (NCCM, HCM and DCM) may occur within one family and mutations in some sarcomere genes can cause HCM as well as DCM, our hypothesis was that genes involved in HCM / DCM may also play a role in NCCM. For that reason extended molecular diagnostics of 17 genes previously associated with NCCM and/or with HCM and DCM, were performed systematically in 48 unrelated adults and eight children with NCCM. This way the molecular genetic cause for NCCM was identified in 23 patients (41%). The *MYH7* gene is the most prevalent genetic cause for NCCM identified so far,

accounting for 20% of all tested probands (11) and for 48% of the probands in whom a mutation was identified. Apart from *MYH7*, mutations were also identified in *MYBPC3* in three patients (four mutations), *TNNT2* in two patients (three mutations), *TPM1* in two patients, *TNNI3* in one patient who also had a *TPM1* mutation, *ACTC1* in one patient, *CASQ2* in two patients (one in combination with two *TNNT2* mutations), *PLN* in one patient, *TAZ* in one patient, *LDB3* in two patients, (once combined with a *TNNT2* mutation, once with an *LMNA* mutation). Single mutations were identified in 18 patients; complex genotypes, identified in adults and in children, consisted of two mutations in four patients and three mutations in one patient. In addition to these genes we also identified a new locus associated with NCCM on chromosome 4. We can conclude that genetics play an important role in NCCM. By finding 11 genes associated with NCCM and identifying a new NCCM locus, a large step has been taken in identifying the genetic causes of NCCM. These genes are mainly of the sarcomere and Z-disc, similar to the genes associated with HCM. Establishing the genetic causes facilitates accurate identification of relatives who are at risk of developing NCCM or a related cardiomyopathy.

Combining the results of the family and molecular studies showed that the majority of NCCM, with or without a mutation, is inherited in an autosomal dominant mode and that clinical features and age at onset in NCCM are highly variable, even within families. One of the most important findings was that the majority of relatives diagnosed with NCCM by family screening was asymptomatic (63%). In addition we observed non-penetrance in eight cardiologically unaffected mutation carriers (age range 12 - 72 years). These observations have major implications for recommendations for follow-up of relatives and for cardiologic family screening. In families without a mutation, asymptomatic relatives can only be identified as affected through cardiologic examination. However, in case of non-penetrance, continuation of follow-up of unaffected relatives is necessary and further family screening is recommended (as depicted in flow chart in Chapter 3). If a molecular defect is found in a family, predictive molecular testing will identify the asymptomatic relatives at risk for cardiomyopathy, and may exclude those who do not need cardiologic screening. Asymptomatic disease seems to occur frequently in adults with NCCM, and was also found in probands (20%). These probands were diagnosed by chance for instance during pre-operative screening or after an X-ray due to persistent coughing revealed an enlarged heart. Apparently it is possible to have a cardiomyopathy without any symptoms. It is likely that relatives with symptoms of cardiomyopathy are picked up earlier when they seek medical care.

Phenotypic variability in NCCM

In our family study we identified NCCM at all ages; even prenatal presentation was seen in two cases (Chapter 5). Disease severity was also variable; some patients died at a young age, some required heart transplants and some patients were coincidentally diagnosed and completely asymptomatic. Phenotypic variability can even be so extreme that a mutation carrier might not even develop a cardiomyopathy at all. For this phenomenon there is no good explanation, there are only theories. Numerous additional modifying factors, either genetic or

environmental or both, probably play a role in phenotype development. Some modifying factors may have a protective effect, others may cause more damage. Currently there is not enough evidence that additional genetic factors play a large role in the variability of the severity of NCCM and HCM. In our NCCM study five patients with a complex genetic status all presented with NYHA classification II or higher, whereas some patients with a single mutation were asymptomatic. Two of the multiple mutation patients presented at a young age (four months and seven years respectively); nevertheless in single mutation patients, four patients presented at an early age (ranging from one month to 17 years). According to these data a second (and third) mutation does not necessarily lead to early-onset disease nor is a second mutation always identified in early-onset disease. Numbers of this study are too small too draw any final conclusions on genotype-phenotype relationships in NCCM, especially since most mutations are unique. Similarly, there was no evidence for additional genetic factors playing a role in disease severity in our HCM double heterozygousity study (Chapter 11). Limitations of these studies are that we cannot actually exclude a second or third genetic factor, since we did not sequence the whole genome. Perhaps even a non-coding part of the DNA will be of importance as was recently established in coronary artery disease in mice.[12]

Congenital heart defects and NCCM

In our NCCM study we identified structural heart defects in eight families, in families with as well as in families without a mutation. Some of the relatives with a structural heart defect also had NCCM. In congenital heart disease over 25 genes have been identified, such as ligands-receptor genes (e.g. *NOTCH1*, *JAG1* and *PTPN11*), transcription factor genes (e.g. *GATA4*, *NKX2.5*, *TBX1*) and also several sarcomere genes (*ACTC1*, *MYBPC3*, *MYH6*, *MYH7* and *MYH11*).[13-19] Because of the typical trabecularisation reminiscent of early embryological cardiac development, it has been suggested that NCCM is caused by an arrest of normal embryogenesis of the myocardium.[5, 20] Identifying sarcomere defects in NCCM as well as in structural heart disease may indicate that NCCM indeed has its origins in defective myocardial embryogenesis. This may also explain the co-occurrence of structural heart defects in NCCM. Possible late-onset disease however, as seen in HCM, will be more difficult to explain if this hypothesis is correct. Evidence for late-onset NCCM has not been established yet. Non-penetrance however, as observed in our study population indicates otherwise. We also do not know if patients who are diagnosed in adulthood had NCCM in their early infancy. Follow-up of unaffected relatives is needed to establish the occurrence of late-onset NCCM.

HCM

HCM is one of the most prevalent genetic cardiac disorders, in adults and children, affecting approximately 1 in 500 people. In the last decade, more than 24 genes involved in HCM have been identified, accounting together for approximately 60 - 70% of HCM patients. The *MYBPC3* and the *MYH7* gene are the most prevalent causes for autosomal dominantly inherited HCM. X-linked forms of HCM or phenocopies of HCM have been described, for instance in Fabry and

Danon disease. The frequency of Fabry in the HCM population is currently investigated. The highest prevalence reported so far was 12% in female patients with late-onset HCM.[21]
In some patients HCM occurs as a part of a complex syndrome, especially in childhood. Among these complex causes are malformation syndromes, neurological, mitochondrial and metabolic disease and chromosomal defects (as listed in Table 10-12 of chapter 1). Systematic research of the prevalence of non-sarcomere causes of HCM in adults is currently lacking.
The presently known 24 genes do not explain HCM in 30 - 40% of the patients. Some of these patients show evident familial disease, others may display sporadic HCM. In this thesis we describe the identification of a novel genetic cause for HCM. New candidate genes for HCM may be found in sarcomere and Z-disc genes that have not been tested in an HCM population yet. One of these genes is the *FHL1* gene, a Z-disc gene located on the X-chromosome. Additional to this gene being a Z-disc gene, the *FHL1* gene has been implicated in several neuromuscular disorders, some of these patients also displayed cardiomyopathy.[22] Family studies even revealed isolated HCM without neuromuscular disease in male and female relatives in these families.[22] The *FHL1* gene was therefore an excellent candidate, so we analysed this gene and identified mutations in the *FHL1* gene as a new genetic cause for HCM. Since HCM was also present in women with an *FHL1* mutation we can conclude that this type of HCM inherits as an X-linked dominant trait. Although the exact frequency of *FHL1*-associated HCM remains to be established, in a family with X-linked inheritance (no father to son transmission), especially combined with presence of neuromuscular disease, it is advisory to sequence the *FHL1* gene.

Founder mutations

In the Netherlands genetic HCM features the presence of three founder mutations in the *MYBPC3* gene (c.2373dupG; p.Arg943X and c.2864_2865delCT), causing approximately 25% of Dutch HCM and thus making the *MYBPC3* gene the most prevalent HCM-causing gene in the Netherlands. The three founder mutations are all truncating mutations, most likely causing haploinsufficiency. The genetically homogenous HCM population provides an excellent opportunity to evaluate genotype phenotype correlation and investigate the role of potential genetic modifiers on the variability of the phenotype.

Genotype - phenotype correlation in HCM

Our study showed a similar natural history of founder-mutation-related HCM in the Netherlands, with a malignant prognosis in 33-56% of the founder mutation families and almost identical survival estimates (Chapter 9). Early diagnosis of HCM may prevent a malignant course of the disease, it is therefore important to perform family studies (cascade screening). Naturally, careful genetic counselling is required in order to inform each relative before DNA analysis, so they can make in informed decision concerning presymptomatic testing.

Modifiers in the founder population

Variability in disease severity and age of onset is observed within and between families. Modifying factors may explain this variability. In order to identify possible modifiers, we analysed 87 HCM patients with an *MYBPC3* founder mutation for additional defects in 11 HCM genes (*ACTC1*, *CSRP3*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TCAP*, *TNNI3*, *TNNC1*, *TNNT2* and *TPM1*) to analyse whether a complex genotype plays a role in disease variability. We did not find additional defects in the group of more severely affected HCM patients. Nevertheless, the co-occurrence of two truncating mutations in the *MYBPC3* gene, resulting in a human *MYBPC3* knockout, does constitute a very severe phenotype with death in early infancy, as shown in the two cases in Chapter 7 and also in literature.[23] We cannot exclude that other, yet unknown, genetic causes of cardiomyopathy, play a role in the observed variability within the founder population.

Other HCM modifiers

To determine whether aldosterone is a modifying factor in HCM we studied the influence of the C-344T polymorphism in aldosterone synthase (*CYP11B2*) on left ventricular mass index (LVMI) and intraventricular septum (IVS) thickness in 79 unrelated HCM patients. In male HCM patients LVM and IVS increased with presence of the T-allele in *CYP11B2* (p-value 0.06 and 0.01 respectively). This effect was not seen in women; since the very small number of women without a T-allele no definite conclusions about the gender difference should be made.

Overlapping features of NCCM and HCM

The cardiologic NCCM family study revealed that in eight of the 50 screened families, NCCM as well as HCM and DCM were diagnosed in relatives of NCCM probands. HCM and DCM occurred in families with an identified genetic defect and in families without such a defect. This may be coincidental but previous studies also identified different cardiomyopathies within one family, indicating that NCCM belongs to a spectrum of cardiomyopathies with HCM and DCM.[15, 24-26] This observation raises the question, why does one person develop NCCM and another HCM or DCM? We observed that even the same mutation in a gene may result in different phenotypes within one family. Most NCCM mutations that were identified are unique. Except for the identification of a Dutch *MYBPC3* founder mutation in our NCCM cohort: we identified the c.2373dupG mutation twice in our NCCM cohort, once as a single mutation in an adult and once in combination with another *MYBPC3* (p.Gly148Arg) mutation in a 7-year-old girl. As previously mentioned, the *MYBPC3* c.2373dupG mutation is the most prevalent disease-causing mutation in the Dutch HCM population (14.4%, Chapter 9); approximately 4600 Dutch HCM patients carry the c.2373dupG mutation (population 16 million, HCM prevalence 0.2%). Despite this high prevalence of c.2373dupG patients, NCCM was not reported in c.2373dupG carriers so far. It is possible, that the p.Gly148Arg mutation in *MYBPC3* in the girl with two mutations attributes to the NCCM phenotype; her father carried the p.Gly148Arg and had NCCM whereas her mother, carrying the c.2373dupG mutation, did not show a cardiomyopathy

phenotype (yet); the maternal grandfather carried the c.2373dupG mutation as well and he was diagnosed with HCM. In the adult NCCM patient with only the c.2373dupG mutation we were unable to identify a second genetic defect, however it can not be excluded that she carries a second mutation in a currently unknown NCCM gene.

Similar to the *MYBPC3* c.2373dupG mutation being identified in NCCM and HCM, the *MYH7* p.Asp545Asn and p.Asp955Asn *in cis* mutations were recently identified in a DCM family in Groningen and in a DCM family in Amsterdam. It is highly probable that these families are distantly related, because the odds of developing two identical *MYH7* missense mutations *in cis* in three independent families seem slim. So far we were not able to link these three families by genealogy.

For now there is no explanation for the difference in phenotypes in patients carrying the exact same mutations. Nevertheless, it seems likely that additional genetic factors do play a role. Given the fact that quite a large proportion of NCCM and a smaller proportion of HCM are currently still genetically unexplained, perhaps the unidentified genetic factors in these patients also play a role as second genetic defect in families where a causative mutation has already been identified.

Molecular mechanisms of the NCCM – HCM spectrum

Different mutations in the same genes may lead to different phenotypes, because they may involve different pathogenic pathways. It is likely that the nature and location of the specific mutation dictates phenotypic variability. The most frequently mutated gene in NCCM was the *MYH7* gene with 11 mutations and this gene is the second most frequent cause for HCM in the Netherlands. Therefore the *MYH7* gene is suitable for having a closer look at the different nature and locations of the mutations. In our study, seven of the *MYH7* mutations identified in NCCM patients were missense mutations; additionally a double missense-mutation, a slice-site mutation, a single amino-acid deletion and a nonsense mutation were identified. Four *MYH7* mutations (the splice-site, the nonsense and two missense mutations) are located in the ATP-ase active site of the globular head-region on the amino-terminal part of the MYH7 protein. Of all NCCM-related *MYH7* mutations that have been identified, half are located in this region.[11, 16] Four other *MYH7* mutations in our study (three missense mutations and the single amino-acid deletion) were found in the carboxy-terminal rod-region of the MYH7 protein. In this region few cardiomyopathy mutations have been identified. However, *MYH7* mutations associated with HCM are mainly missense mutations and have been identified throughout the *MYH7* gene.[27] In conclusion, the current insight of pathogenetic mechanisms of specific mutations in the *MYH7* gene is insufficient to explain phenotypic variability. Clinical and molecular data support that NCCM, HCM and DCM belong to a cardiomyopathy spectrum. However, insight into the genetic and / or other factors playing a role in phenotype differences remains scant. Understanding of the pathogenetic mechanisms that lead to NCCM, HCM and DCM may help in identifying these additional modifying factors.

Heterogeneity of NCCM and HCM

Isolated forms of NCCM and HCM are genetically heterogeneous, and since these clinically distinct forms of cardiomyopathy share a number of disease-causing genes, mainly coding for sarcomere and Z-disc proteins, overlapping pathophysiological mechanisms are suspected. Figure 1 clearly demonstrates the heterogeneity of NCCM and HCM and the shared genetic causes. Members of the same family of genes (for instance sarcomere or Z-disc genes) will most likely share common pathways leading to cardiomyopathy. NCCM and HCM may also occur as part of genetic neuromuscular diseases or malformation syndromes. The RAS pathway seems to play an important role in syndromal HCM and NCCM (Noonan, Leopard, Costello, cardio-facio-cutaneous syndrome and neurofibromatosis). Apparently there are different pathways leading to cardiomyopathy.

In some of the (rare) complex disorders NCCM and HCM may occur in different patients, suggesting an aetiologic overlap, also between complex forms of NCCM and HCM. However a more prevalent genetic cause for NCCM and HCM involved in the occurrence of cardiomyopathy in complex disease cannot be excluded. For instance, one patient in our study was a girl with Down syndrome and NCCM who was diagnosed with an *MYH7* mutation (p.Ala326Pro). She could not be included in Chapter 3 and 4 since she didn't fulfil the fourth Jenni criterion of absence of co-existing cardiac anomalies because she had an atrioventricular septal defect, as often seen in Down syndrome. It is very well possible that NCCM or HCM in some of the syndromes listed in Figure 1 are caused by a gene defect in an additional (sarcomere or Z-disc) gene, unrelated to the syndrome. Obviously, a search for a cardiomyopathy-related gene defect may not be performed when a structural heart defect or an evident syndromal cause is present, assuming that the cardiomyopathy is secondary. In this way, a diagnosis of genetic familial cardiomyopathy may be overlooked.

We may conclude that even in the presence of structural heart defects, a syndrome or other disorders, separate molecular diagnostics for cardiomyopathy may be appropriate, especially when there is a family history of cardiac symptoms.

Sarcomere and Z-disc

The sarcomere is the contractile unit of muscle cells and contains different filament protein complexes. The thick filament system, composed of myosin heavy chain and myosin essential and regulatory light chain, is connected from the M-line to the Z-disc by giant filament titin (Figure 2). Intermediate filament myosin-binding protein C binds the thick filament to thin filament component actin. The thin filament is formed by actin monomers that bind to nebulin; troponin C, troponin I, troponin T and tropomyosin complete the thin filament. Titin is present in both thick and thin filament and gives stability and structure to the sarcomere. The main component of the Z-disc is α-actinin 2, other Z-disc proteins are cysteine-and-glycine-rich protein, LIM domain-binding 3, four-and-a-half-LIM domains 1, myozenin 2, telethonin and vinculin/metavinculin.

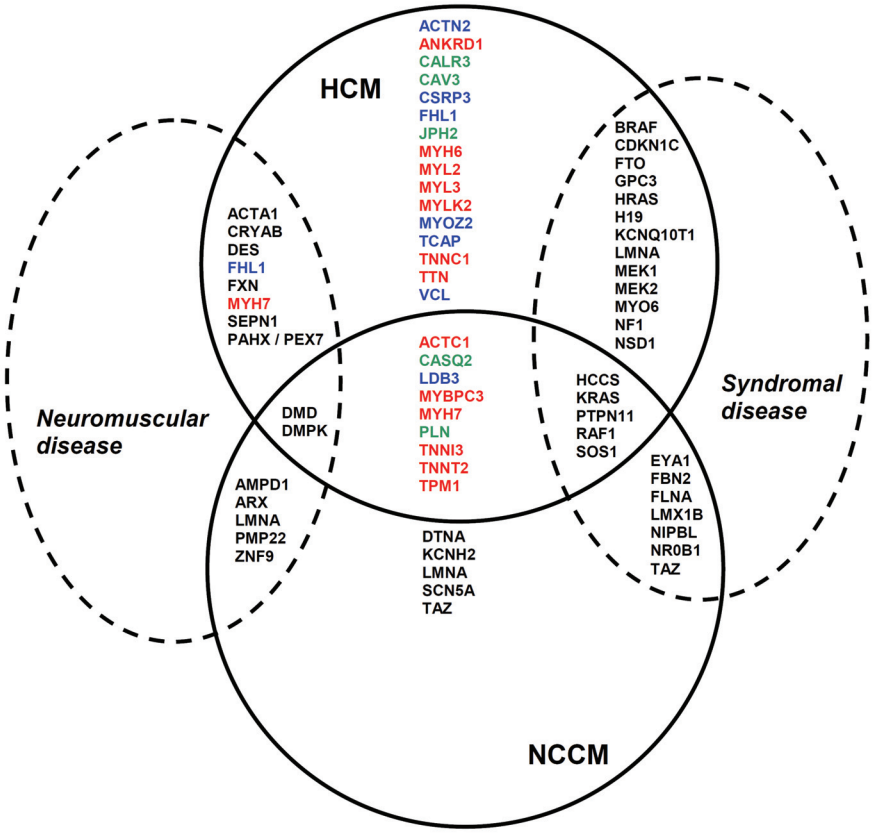


Figure 1. Heterogeneity and overlap of disease-causing genes in HCM and NCCM and in neuromuscular and syndromal disease. Red displays sarcomere genes, blue Z-disc genes and green calcium-handling genes. *ACTA1*: α-Skeletal actin; *ACTC1*: α-Cardiac actin; *ACTN2*: α-Actinin 2; *AMPD1*: Adenosine monophosphate deaminase 1; *ANKRD1*: Cardiac ankyrin repeat protein; *ARX*: Aristaless-related homeobox; *BRAF*: V-RAF murine sarcoma viral oncogene homolog B1; *CALR3*: Calreticulin; *CASQ2*: Calsequestrin; *CAV3*: Caveolin; *CDKN1C*: Cyclin-dependent kinase inhibitor 1C; *CRYAB*: α-B Crystallin; *CSRP3*: Cysteine and glycine rich protein; *DES*: Desmin; *DMD*: Dystrophin; *DMPK*: Dystrophia myotonica protein kinase, *DTNA*: α-Dystrobrevin; *EYA1*: Eyes absent 1; *FBN2*: Fibrillin 2; *FHL1*: Four-an-a-half LIM domains; *FLNA*: Filamin A; *FXN*: Frataxin; *GPC3*: Glypican 3; *HCCS*: Holocytochorme C synthase; *HRAS*: V-HA-RAS Harvey rat sarcoma viral oncogene homolog; *H19*: H19 gene; *JPH2*: Junctophilin 2; *KCNH2*: Potassium channel, voltage-gated, subfamily H, member 2; *KCNQ10T1*: KCNQ1-overlapping transcript 1; *KRAS*: V-KI-RAS2 Kirsten sarcoma viral oncogene homolog; *LDB3*: LIM domain-binding 3; *LMNA*: Lamin A/C; *LMX1B*: LIM homeobox transcription factor 1; *MEK1*: Mitogen-activated potein kinase kinase 1; *MEK2*: Mitogen-activated potein kinase kinase 2; *MYBPC3*: Cardiac myosin-binding protein C; *MYH6*: α-Myosin heavy chain; *MYH7*: β-Myosin heavy chain; *MYL2*: Ventricular regulatory myosin light chain; *MYL3*: Ventricular essential myosin light chain; *MYLK2*: Myosin light chain kinase 2; *MYO6*: Myosin VI; *MYOZ2*: Myozenin 2; *NF1*: Neurofibromatosis 1; *NIPBL*: Nipped-B-like; *NR0B1*: Nuclear receptor subfamily 0, group B, member 1; *NSD1*: Nuclear receptor-binding Su-var, enhancer of zeste, and trithorax domain protein 1; *PAHX*: Phytanoyl-CoA hydroxylase; *PEX7*: Peroxisome biogenesis factor 7; *PLN*: Phospholamban; *PMP22*: Peripheral myelin protein 22; *PTPN11*: Protein-tyrosin phophatase, nonreceptor-type 11; *RAF1*: V-RAF-1 Murine leukemia viral oncogene homolog 1; *SCN5A*: Sodium channel, voltage-gated, type V, α-subunit; *SEPN1*: Selenoprotein N1; *SOS1*: Son of sevenless, drosophila homolog 1; *TAZ*: Taffazin; *TCAP*: Telethonin; *TNNC1*: Cardiac troponin C; *TNNI3*: Cardiac troponin I; *TNNT2*: Cardiac troponin T; *TPM1*: α-Tropomyosin; *TTN*: Titin; *VCL*: Vinculin/metavinculin; *ZNF*: Zinc finger protein.

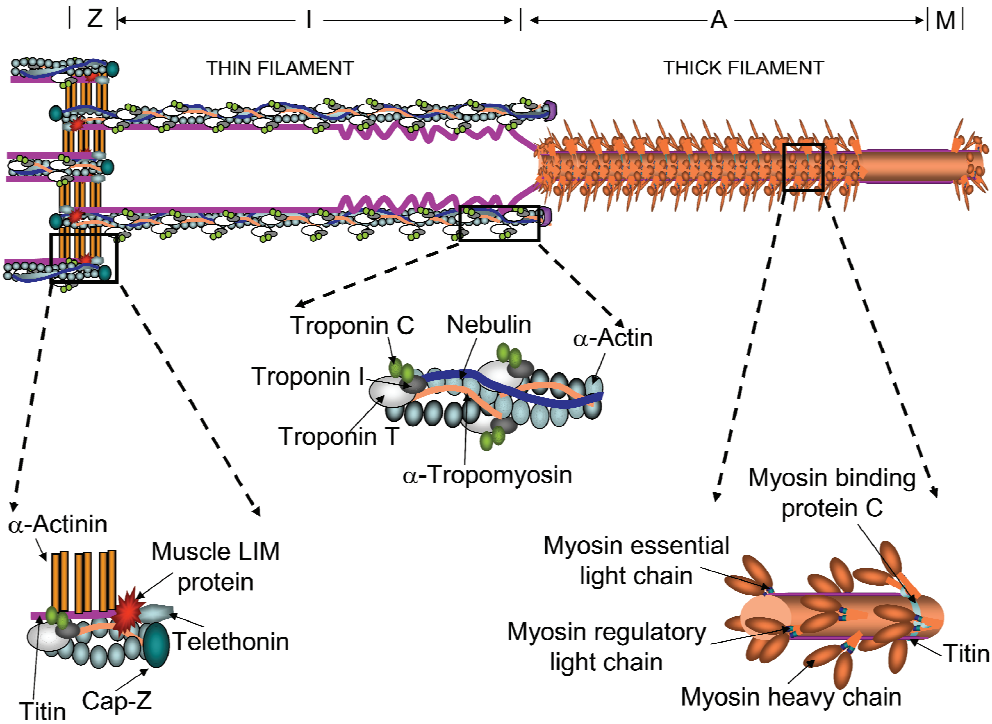


Figure 2. The sarcomere and its proteins. Z, I, A and M represent corresponding sarcomere zones and bands.
Reproduced with permission from: "Diagnostic yield, interpretation and clinical utility of mutation screening of sarcomere encoding genes in Danish hypertrophic cardiomyopathy patients and relatives; Andersen et al; Human Mutation 2009;30:363-370"

Contraction occurs after release of calcium into the cardiomyocyte, after which the thick filament slides in between the thin filaments. In almost all sarcomere and in many Z-disc genes mutations causing cardiomyopathy have been identified. Several mechanisms have been suggested through which cardiomyopathy occurs in patients with such a gene defect. The defective allele may act as a poisonous peptide, interfering with the normal allele. Alternatively a gain of function dominates normal function. A third mechanism is haploinsufficiency, in which the remaining normal allele provides insufficient protein to perform the function required. Hypertrophy or noncompaction may be compensatory reactions to one of these mechanisms.

Daily practice

Although many questions currently remain unsolved, identifying genetic defects in NCCM and HCM is important enabling family studies and accurate identification of who is at risk for NCCM or HCM and, equally important, who can be excluded from cardiologic follow-up.

In NCCM the *MYH7* gene is the most frequently mutated gene, our recommendation is to analyse this gene first. Additionally *MYBPC3*, *TPM1, TNNT2*, *TNNI3*, *ACTC1*, *CASQ2*, *PLN*, *TAZ*, *LDB3* and *LMNA* sequencing gives a yield of approximately 40%. In HCM in the

Netherlands it is recommended to screen for the Dutch founders c.2373dupG, p.Arg943X and c.2864-2865delCT in the *MYBPC3* gene first since approximately 25% of the Dutch HCM population carries one of these three founder mutations. When no founder mutation is identified the rest of *MYBPC3* should be sequenced, followed by *MYH7* and *TNNT2*. Screening of additional sarcomere and Z-disc genes will lead to a total yield of approximately 60-70%. When family history indicates X-linked inheritance and/or when family history is positive for neurological disease, the *FHL1* gene should be sequenced.

When no mutation can be established in the NCCM or HCM proband, periodic cardiologic family screening is recommended to identify relatives at risk. Identifying new affected relatives in its turn will help facilitate future linkage studies in order to find novel genetic causes for cardiomyopathy.

Future studies

To establish whether NCCM may develop at a later age, similar to HCM and DCM, follow-up of unaffected relatives in NCCM families is necessary, especially in mutation carriers without a phenotype. NCCM was thought to originate from an arrest in embryonic development since the phenotype is very similar to embryonic cardiac development. Establishing late onset NCCM and non-penetrance of NCCM may invalidate this theory, at least in some cases. Follow-up is equally important to establish whether currently “affected” relatives not fulfilling diagnostic criteria will eventually develop NCCM or another cardiomyopathy. Knowledge regarding this special group of neither affected nor unaffected relatives will be invaluable to determine reliable diagnostic NCCM criteria for relatives. Thirdly, follow-up will help to establish accurate risk stratification.

With new molecular techniques (e.g. next generation sequencing) to identify mutations more unclassified variants will be exposed. This will pose a problem since it is difficult to establish whether an unclassified variant is pathogenic or not. Conservation throughout different species, prediction programs and segregation with disease are easily available. Nevertheless, they are not always conclusive. RNA studies in splice-site mutations may give us more insight in those mutations. For missense mutations such techniques are not readily available. Since the majority of NCCM and HCM patients does not provide us with cardiac tissue, functional studies of specific mutations will currently only be possible in patients who had a heart transplantation or a biopsy.

In search of new candidate genes for NCCM and HCM additional sarcomere and z-disc genes should be sequenced. Genes important for cytoarchitecture, nuclear envelope structure and cardiac ion-channel genes (“DCM-genes”) might add to the genetic classification. Additionally linkage studies may reveal different associated genes and array-CGH may detect submicroscopic genomic aberrations. No doubt in the future sequencing of the whole genome of the patient will become possible, technically and financially. This will shift genetics from monogenetic disease to genetically more complex aetiologies, and will possibly help to unravel the protective effects that cause non-penetrance in familial disease.

Finally, the *MYBPC3* founder mutation population is extremely valuable in the search for genetic modifiers. However, it will take a joint effort of the different cardiogenetic clinics in the Netherlands to detect all approximately 4600 c.2373dupG mutation carriers and to perform a large-scale research, with sufficient statistical power to detect associations with modifiers. The Dutch GENCOR database (www.gencor.nl) will play a major role in national research. GENCOR was developed in 2004 by the Interuniversity Cardiology Institute of the Netherlands (ICIN) to establish a national registry for patients (and their affected relatives) with familial heart diseases such as cardiomyopathies and channelopathies, in whom a causative mutation was identified; to facilitate prevalence studies of hereditary heart diseases in the Netherlands and to promote research and improve diagnostics and therapeutic possibilities. This database will be invaluable in the search for all Dutch founder mutation carriers and will facilitate large-scale modifying factor studies. Identifying novel genetic causes and modifiers will give a better understanding of pathogenesis and may lead to new therapeutic strategies for NCCM and HCM.

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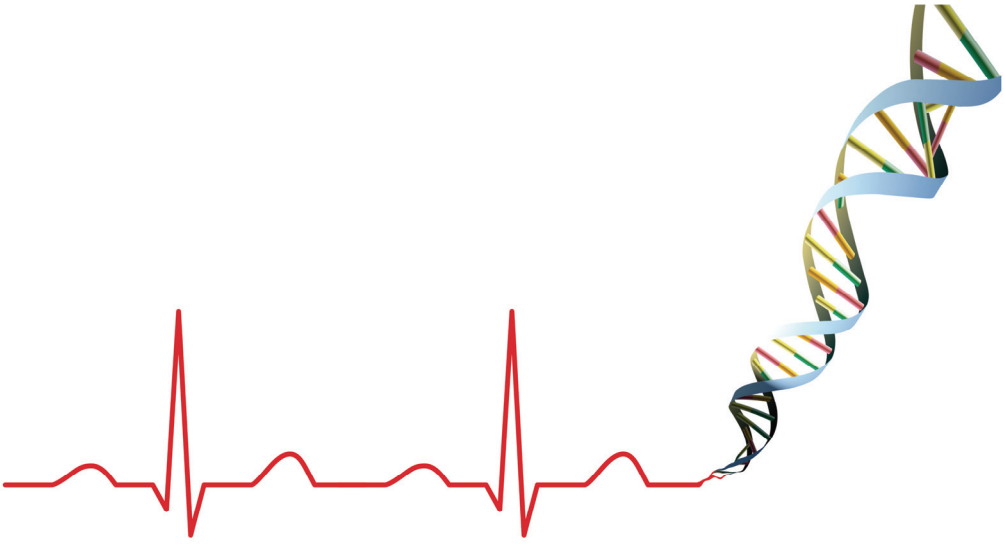
Summary

Samenvatting



Summary

In 1949 the first report of familial cardiomegaly appeared. Over 60 years later, insight in the aetiology, especially the genetics of cardiologic disorders, has increased tremendously. With the discovery of many genes involved in (mostly autosomal dominantly inherited) familial cardiomyopathies, arrhythmias, congenital heart disease and cardiovascular disorders the cardiogenetic field is rapidly expanding. These discoveries offer increasing understanding of pathogenesis. But above all, this knowledge is of importance for patients and their families, enabling accurate identification of relatives at risk of developing a cardiogenetic disorder. Subsequently, an increasingly large number of patients, and their relatives, are currently referred for genetic counselling and DNA diagnostics. At present the most frequently counselled cardiogenetic disorders in Rotterdam are the cardiomyopathies, mainly hypertrophic cardiomyopathy (HCM) and noncompaction cardiomyopathy (NCCM). The focus of this thesis lies on genetic and family studies of NCCM, in the first part, and HCM, in the second part of this thesis.

Chapter 1 presents a general overview of the prevalence, clinical features, clinical and molecular diagnostics of these cardiomyopathies and describes the goal of this study: identification of familial NCCM and its genetics causes, finding additional genes involved in hypertrophic cardiomyopathy en establishing genotype-phenotype relationships. The first part of this chapter describes NCCM, a relatively newly classified (by the American Heart Association and the European Society of Cardiology) and genetically heterogeneous cardiomyopathy. It is characterised by a two-layered structure of the myocardium, with a thin compact epicardial layer and a thickened endocardial layer (at least twice as thick as the epicardial layer) with prominent trabeculations. Clinical presentation varies among patients. In some (asymptomatic) patients NCCM is detected by chance, other patients may present with (aborted) sudden cardiac death. Prognosis is also variable, from slow progression to rapidly developing severely disabling cardiac failure. Treatment consists of medication to improve cardiac function (mainly β-blockers and ACE-inhibitors). Non-penetrance may occur in carriers of disease causing mutations. The clinical features of NCCM may occur at all ages, even prenatally. In adults the majority of NCCM is isolated. In childhood, the course of the disease is often more severe and NCCM is frequently associated with congenital heart defects, metabolic or neuromuscular disease or chromosomal aberrations. In isolated NCCM molecular testing may yield a genetic defect, mostly in sarcomere genes, responsible for the production of sarcomere proteins. Sarcomere proteins play an important role in myocardial contraction.

The second part of the first chapter describes HCM, the most frequent cardiogenetic disorder. HCM is characterised by left ventricular hypertrophy (left ventricular wall thickness ≥ 15 mm) and is mostly asymmetric with hypertrophy of the intraventricular septum. HCM displays a high intra- and interfamilial variability. It can present with (symptoms of) heart failure or arrhythmias and even SCD. A substantial proportion of HCM patients however, is asymptomatic. HCM

occurs at all ages, from prenatal presentation to onset at a high age. HCM is genetically heterogeneous. In up to 70% of HCM molecular testing may yield a genetic defect, mostly in the *MYBPC3* and *MYH7* genes. In part of familial HCM no genetic defect can be detected with current knowledge. Genetic counselling and (predictive) molecular analysis and/or cardiac evaluation of patients and their relatives is important. Figure 14 of chapter 1 shows the process of genetic counselling and family studies in HCM.
HCM may also be caused by mitochondrial defects and is associated with several syndromes and neuromuscular disorders. HCM can have important clinical consequences and may lead to premature death in some patients while a normal life expectancy is reached by many other patients, having mild or no disability. Not all HCM patients require treatment, their prognosis is favourable. When treatment is needed, pharmacological therapy is the first option, followed by myectomy or PTSMA when medication is insufficient to relieve symptoms. To prevent sudden cardiac death adequate risk stratification and subsequent management is important.

In **chapter 2** we describe the first two families with pathogenic mutations in the sarcomeric cardiac β-myosin heavy chain gene (*MYH7*) causing autosomal-dominant NCCM. This is the first report of the association of *MYH7* with NCCM. The *MYH7* gene is one of the most frequent causes of HCM worldwide and has also been associated with restricted cardiomyopathy (RCM), and dilated cardiomyopathy (DCM). These results confirm that the genetic heterogeneity of NCCM includes *MYH7*-associated disease, and suggest that *MYH7*-associated NCCM may be part of a cardiomyopathy spectrum with HCM, RCM, and DCM.

Chapter 3 describes the results of the cardiogenetic family study in a cohort of 58 consecutively diagnosed unrelated patients with isolated NCCM (49 adults and nine children). Combined molecular testing and cardiologic family screening revealed that in at least 67% NCCM is genetic. Cardiologic screening with electrocardiography (ECG) and echocardiography of 194 relatives from 50 unrelated NCCM probands revealed familial cardiomyopathy in 32 families (64%), including NCCM, HCM and DCM. Molecular diagnostics, described in more detail in the next paragraph, identified a pathogenic mutation in half of the patients with familial NCCM. The majority (63%) of the relatives newly diagnosed with cardiomyopathy in this study was asymptomatic. Of 17 asymptomatic relatives with a mutation, nine had NCCM, and in eight relatives non-penetrance was observed, explaining that 44% (14/32) of familial disease remained undetected by ascertainment of family history prior to cardiologic family screening. In 41% of the tested probands molecular screening of 17 genes identified mutations in 11 genes. In 50% (16/32) of familial NCCM the genetic defect remained inconclusive. In conclusion, we identified that NCCM is predominantly a genetic cardiomyopathy with variable presentation. Since early diagnosis in (asymptomatic) relatives may be important, NCCM requires genetic counselling, DNA diagnostics and cardiologic family screening. A flow chart for this purpose is presented in Figure 1.

In **chapter 4** extensive molecular studies provided a genetic classification of NCCM. Seventeen genes (*MYH7*, *MYBPC3*, *TNNC1*, *TNNT2*, *TNNI3*, *ACTC1*, *MYL2*, *MYL3*, *TPM1*, *CSRP3*, *TCAP*, *LDB3*, *CASQ2*, *CALR3*, *PLN*, *TAZ* and *LMNA*) were completely analysed in a cohort of 56 unrelated NCCM patients (48 adults and eight children). These genes encode proteins of the sarcomere, Z-disc, Calcium-handling system and cellular matrix which, which had all previously been associated with other genetic forms of cardiomyopathies. Twenty-nine mutations, including 21 new ones, were identified in 23 (41%) of the patients. The *MYH7* gene was the most prevalent genetic cause for NCCM identified so far, accounting for 11 (20%) of all tested probands and for 48% of the probands in whom a mutation was identified. Mutations in *MYBPC3* were identified in four patients (7%), *TNNT2* in three (5%), *TPM1*, *CASQ2* and *LDB3* in two patients each (4%) and *TNNI3*, *ACTC1*, *PLN*, *TAZ*, and *LMNA* in one patient each (2%). Thirty-five percent of the adult patients had a mutation and 75% percent of the paediatric patients had a sarcomeric mutation. Mutations previously associated with cardiomyopathy (NCCM, HCM or DCM) were regarded as pathogenic. Novel mutations were considered pathogenic when they were either truncating, splice-site or de novo mutations or fulfilled the following three criteria: segregation with disease in a family; absence on 384 ethnically matched healthy control chromosomes; likely pathogenic according to prediction software (SIFT and PolyPhen). Eighteen probands had a single mutation and five patients had a complex genotype. *MYBPC3*, *TNNI3*, *TPM1*, *PLN* and *CASQ2* were identified as novel NCCM genes. These results showed that genetically, NCCM primarily classifies as a sarcomeric disease and is part of a sarcomere gene-associated spectrum including hypertrophic and dilated cardiomyopathy.
Furthermore, investigating prognosis in families with an identified mutation indicated that in families with an identified mutation the prognosis was worse compared to non-genotyped families. In conclusion the high frequency of mutations in adult- and childhood-onset NCCM confirms the importance of molecular analysis in NCCM. Starting with the most frequent genetic cause, the *MYH7* gene, is recommended.

Chapter 5 reports on the first two cases of prenatal ultrasound diagnosis of *MYH7*-associated NCCM. Prenatal ultrasound examinations revealed biventricular cardiomegaly leading to the diagnosis of NCCM at 31 and at 23 weeks of pregnancy respectively. There was no family history of cardiomyopathy in the two families, although one of the fathers was reported with an unspecified cardiac condition in childhood, he had been discharged from follow-up at the age of 14 years. Since maternal cardiomyopathy may require special measures during delivery and to identify the cause of the prenatal cardiomyopathy, both parents were cardiologically screened and molecularly analysed. In both families *MYH7* cardiomyopathy was identified. Subsequent family studies identified more affected relatives, who were mostly asymptomatic. Post partum follow up of the two prenatal cases and a review of the literature showed that prognosis of prenatally diagnosed NCCM predominantly depends on cardiac function and may improve after birth. We concluded that *MYH7* mutations may present with prenatal onset with biventricular enlargement and therefore prenatal ultrasound of pregnancies at risk for *MYH7*-NCCM is

recommended. Since familial disease may initially present with a prenatal onset, molecular diagnostics and cardiologic screening of first-degree relatives is necessary even in absence of a family history of cardiomyopathy.

Currently, the genetic defect in approximately half of familial NCCM is unknown. In **chapter 6** a large multi-generation family with an autosomal dominantly inherited cardiomyopathy presenting alternatively with NCCM and DCM is described. Cardiological examination of 20 individuals from this Frisian family identified nine patients with NCCM and one patient with DCM. No causative genetic defect was identified by sequencing the 17 cardiomyopathy genes as described in chapter 4. To identify the genetic cause for NCCM / DCM in this family a genome-wide linkage analysis was performed using 300K SNP arrays. A maximum LOD score of 3.02 on chromosome 4p15.3 was obtained. The inclusion of the tenth patient that shares a common ancestor with the index case seven generations ago allowed the identification of a small candidate region, leading to the mapping of a new locus for autosomal dominant NCCM to a 5.47 Mb region on chromosome 4p15.3. Identification of the novel disease gene is ongoing and will facilitate genetic screening in this family and provide fundamental insight into the understanding of NCCM pathogenesis.

Chapter 7 describes the clinical, pathological and molecular findings of two unrelated neonates with complex cardiac pathology, including noncompaction cardiomyopathy. These two patients were compound heterozygous for two loss-of-function mutations in the *MYBPC3* gene (founder mutations) that are common in the Dutch HCM population (the founder mutations are described in more detail in chapters 9 and 10). One of the patients had a ventricular septal defect, whereas the other patient had an atrial septal defect. Heterozygous mutations in sarcomeric protein genes usually lead to hypertrophic cardiomyopathy with clinical symptoms starting in child- or adulthood. Homozygousity or compound heterozygousity for two truncating mutations in the *MYBPC3* gene can cause severe neonatal cardiomyopathy with features of left ventricular noncompaction.

Chapter 8 presents the experience with genetic testing and cardiac screening of HCM patients and their relatives at the cardiogenetic outpatient clinic at the Erasmus MC. A flowchart for clinical and genetic diagnosis and family screening of HCM is included. This chapter discusses the importance of identifying autosomal dominantly inherited genetic defects in HCM, thus allowing accurate identification of at-risk relatives and effective screening of relatives of HCM patients. It focuses in particular on the phenotypic variability including non-penetrance and late onset of HCM symptoms demanding repeated cardiac screening of adults at risk for HCM, whether they are carriers of a known mutation or relatives of families without an identified genetic defect.

Previous studies have associated mutations in the sarcomere Myosin Binding Protein C gene (*MYBPC3*) with late onset benign HCM. In **chapter 9** molecular analysis of *MYBPC3* was performed in 327 unrelated Dutch HCM index patients, leading to the identification of a genetic variant in 45% of the patients. In addition to the c.2373dupG founder mutation, that was present in 14.4% of the patients, two new Dutch *MYBPC3* founder mutations were identified: p.Arg943X in 6.7% and c.2864_2865delCT in 4.6% of the HCM patients. Family studies showed a malignant prognosis in 33%, 38% and 56% of the c.2373dupG, p.Arg943X and c.2864_2865delCT families respectively. Estimates of survival were similar for these three truncating founder mutations, suggesting a comparable natural history most likely due to haploinsufficiency of the three truncating *MYBPC3* founder mutations.

HCM often is an autosomal dominantly inherited disorder caused by defects in sarcomere and Z-disc genes. Recently, HCM was observed in families with X-linked myopathies caused by mutations in X-chromosomal Z-disc gene Four-and-a-half-LIM domains 1 (*FHL1*), suggesting that *FHL1* may play a role in HCM. **Chapter 10** describes complete sequence analysis of the *FHL1* gene in 86 HCM patients to investigate the role of *FHL1* in HCM. From a group of 260 HCM index patients, 86 HCM patients (54 men and 32 women) were selected because no causal HCM mutation had been identified in *MYH7*, *MYBPC3*, *TNNC1*, *TNNT2*, *TNNI3*, *ACTC1*, *MYL2*, *MYL3*, *TPM1*, *CSRP3* and *TCAP*. *FHL1* mutations were identified in two HCM patients; a nonsense mutation in a woman with subtle neuromuscular symptoms (muscle biopsy revealed aspecific myopathic changes) and a missense mutation in a neurologically asymptomatic man. Family history was positive for sudden cardiac death and HCM in the female patient. Subsequent family studies showed intrafamilial variability in cardiologic and neurologic symptoms and in age of onset and included incomplete penetrance in the family of the female HCM patient. No family studies of the male patients were performed (yet). These results showed that *FHL1* mutations are an infrequent cause (<1%) of HCM. *FHL1* associated HCM is characterised by X-linked dominant inheritance. For that reason *FHL1* analysis is recommended in male and in female HCM patients, especially when family history suggests an X-linked inheritance pattern or when family history is positive for myopathy or muscular dystrophy. Absence of a positive family history does not exclude *FHL1* cardiomyopathy given the intrafamilial variability and incomplete penetrance of HCM and neuromuscular disease, family studies are therefore recommended.

Intrafamilial variability of disease severity and age of onset is characteristic for HCM but its cause remains unknown. It has been hypothesised that additional genetic or environmental factors play a role in HCM phenotype. Additional risk factors are difficult to determine because of the unique status the identified mutations. The high prevalence of three truncating founder mutations in *MYBPC3* in the Netherlands provided an opportunity to investigate the cause of variability in disease severity among carriers of identical genetic defects. **Chapter 11** describes the results of the study on the effect of additional mutations in sarcomere genes on disease

variability in carriers of founder mutations. In order to analyse whether a complex genotype is an important modifier of disease severity nine sarcomere HCM genes (*MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TNNC1*, *ACTC1* and *TPM1*) were sequenced in 87 HCM patients with a previously identified truncating *MYBPC3* founder mutation as their primary genetic HCM defect. The HCM patients were clinically categorised with a mild or severe phenotype based on age of diagnosis, maximal wall thickness, left ventricular outflow tract obstruction, positive family history of sudden cardiac death and the necessity for septal reduction therapy or an implantable cardioverter defibrillator. There was no difference in the number of additional mutations in the group with a severe phenotype compared to the group with a mild phenotype. Therefore we concluded that the severity of phenotypic expression of HCM in subjects with a truncating *MYBPC3* mutation does not primarily depend on the modifying effects of secondary sarcomere mutations. Other, yet unknown, genetic causes of cardiomyopathy playing a role in the observed variability within the founder population cannot be excluded.

In **chapter 12** the role of aldosterone as a modifying factor in HCM was investigated. Cardiac and / or plasma aldosterone were measured in 79 genetically independent HCM patients and in 12 organ donors without cardiac pathology. The effect of the aldosterone synthase (*CYP11B2*) C-344T polymorphism on LV mass index (LVMI) and intraventricular septal thickness (IVS) was determined the HCM patients. Aldosterone in HCM hearts and plasma of eight HCM patients was similar to that in normal hearts and plasma. In HCM women, no associations between *CYP11B2* genotype and any of the measured parameters were observed, whereas in HCM men, LVMI increased with the presence of the T allele. Similar T allele-related increases were observed for the IVS. Multiple regression analysis revealed that the T allele-related effect on IVS occurred independently of renin, the ACE I/D polymorphism, the AT1-receptor A/C1166 polymorphism and the AT2-receptor A/C3123 polymorphism. In conclusion, circulating and cardiac aldosterone are normal in HCM, thereby arguing against selectively increased cardiac aldosterone production in HCM. Thus, the association between the *CYP11B2* C-344T polymorphism and hypertrophy in HCM most likely relates to the T allele-related increases in circulating aldosterone. This finding raises the need for studies determining the benefit of aldosterone blockade in HCM.

In the general discussion in **chapter 13** the results of this thesis and comments on their significance and implications for daily clinical practice are presented. NCCM and HCM are two highly heterogeneous forms of cardiomyopathy that partly share their genetic aetiology. In NCCM the *MYH7* gene is the most frequently mutated gene, our recommendation is to analyse this gene first. Additionally *MYBPC3*, *TPM1*, *TNNT2*, *TNNI3*, *ACTC1*, *CASQ2*, *PLN*, *TAZ*, *LDB3* and *LMNA* sequencing gives a yield of approximately 40%. For HCM in the Netherlands it is recommended to screen for the Dutch founders c.2373dupG, p.Arg943X and c.2864-2865delCT in the *MYBPC3* gene first since approximately 25% of the Dutch HCM population carries one of these three founder mutations. If this analysis does not reveal a pathogenic

mutation, the rest of the *MYBPC3* gene should be sequenced, followed by *MYH7* and *TNNT2*. Screening of additional sarcomere and Z-disc genes will lead to a total yield of approximately 60-70%. When family history indicates X-linked inheritance and / or when family history is positive for neurological disease, the *FHL1* gene should be sequenced. Approximately half of familial NCCM and a third of HCM are currently still genetically unexplained. Establishing the genetic causes facilitates accurate identification of relatives who are at risk of developing NCCM, HCM or a related cardiomyopathy and is therefore important. Carriers of a pathogenic mutation or relatives from NCCM or HCM families without an identified genetic defect are advised to undergo periodic cardiologic examinations to establish an early diagnosis and start necessary therapy, striving to diminish the risk of serious complications within cardiomyopathy families. New candidate genes may be found in sarcomere and Z-disc genes that have not been tested in NCCM and HCM. Genes involved in DCM, coding for proteins involved in nuclear envelope structure, cytoarchitecture and cardiac ion-channels, may also play a role in NCCM and DCM. New molecular techniques will make simultaneous sequencing of great numbers of genes possible at relatively low costs and may lead to the identification of novel genetic defects in NCCM and HCM, adding the challenge of determining whether these variants are pathogenic or represent unclassified variants with an unknown effect. Novel genetic defects will give more insight into the involved pathways in the pathogenesis of cardiomyopathies.

Samenvatting

In 1949 verscheen de eerste publicatie over het familiair voorkomen van een vergrote hartspier. Nu, meer dan 60 jaar later, is het inzicht in de erfelijkheid van cardiomyopathieën enorm toegenomen. Vele genen werden inmiddels ontdekt, die betrokken zijn bij het optreden van – voornamelijk autosomal dominant overervende – vormen van cardiomyopathie en hartritmestoornissen. Deze ontdekkingen bieden in toenemende mate inzicht in de ontstaanswijze. Maar bovenal zijn de ontdekkingen van belang omdat deze de mogelijkheid van gerichte en nauwkeurige diagnostiek bij patiënten bieden, waarna op betrouwbare wijze kan worden vastgesteld welke familieleden een verhoogd risico lopen op de betreffende aandoening. De kennis over de erfelijkheid van de hartziekten heeft ertoe geleid dat steeds meer cardiologische patiënten en hun familieleden worden verwezen voor erfelijkheidsonderzoek, erfelijkheidsadvisering en DNA diagnostiek. Nu zijn de cardiomyopathieën de meest voorkomende erfelijke cardiologische aandoeningen waarvoor erfelijkheidsadvisering plaatsvindt in Rotterdam met in het bijzonder de hypertrofische cardiomyopathie (HCM) en de noncompaction cardiomyopathie (NCCM). Het eerste deel van deze dissertatie beschrijft de resultaten van de onderzoeken naar de erfelijkheid van NCCM en het tweede deel gaat over de genetische onderzoeken van HCM.

Hoofdstuk 1 is de algemene inleiding van de dissertatie en beschrijft het doel van de studie: het identificeren van familiaire NCCM en de genetische oorzaken, het vinden van additionele genen die betrokken zijn bij het ontstaan van HCM en het vaststellen van verhoudingen tussen genotype en fenotype. Daarna volgt een overzicht van de prevalentie, de klinische en moleculaire (DNA) diagnostiek van NCCM en vervolgens HCM.
NCCM is een relatief recent door de American Heart Association en de European Society of Cardiology geclassificeerde cardiomyopathie waarvan al enkele, voornamelijk zeldzame, erfelijke oorzaken bekend waren. NCCM wordt gekenmerkt door een twee-lagige structuur van de hartspier, waarbij de buitenste laag dun en compact is en de binnenste laag verdikt is (minimaal twee maal zo dik als de compacte laag) met het voorkomen van trabekels. De klinische presentatie van NCCM kan van patiënt tot patiënt verschillen. Bij sommige patiënten wordt NCCM bij toeval bij een medische keuring of preoperatief onderzoek ontdekt, zonder dat er hartklachten zijn. Maar NCCM kan ook een hartstilstand of ernstig hartfalen tot gevolg hebben. Ook het beloop van NCCM kan sterk verschillen: van niet of langzaam progressief tot een snelle achteruitgang. De behandeling bestaat uit medicijnen die de pompfunctie van het hart ondersteunen (voornamelijk β-blockers en ACE-remmers). NCCM komt op alle leeftijden voor en kan zelfs al voor de geboorte worden vastgesteld met echografisch onderzoek. Bij volwassenen komt NCCM meestal geïsoleerd voor, dat wil zeggen zonder andere (aangeboren hart-) afwijkingen. Bij kinderen met NCCM zijn er vaker complexe ziektebeelden; zoals het voorkomen van NCCM samen met een aangeboren hartafwijking, of een erfelijke stofwisselingsziekte, een neuromusculaire ziekte of een chromosoomafwijking. Bij patiënten

met een geïsoleerde NCCM wordt vaker een ziekteveroorzakende (pathogene) mutatie in een sarcomeer gen gevonden (zie hoofdstuk 2-4), genen die verantwoordelijk zijn voor de productie van sarcomeer eiwitten. Deze eiwitten spelen een belangrijke rol in het samentrekken van het hart.

Het tweede deel van hoofdstuk 1 beschrijft HCM, de meest voorkomende erfelijke hartziekte. HCM is een, meestal asymmetrische, verdikking van de hartspier (≥ 15 mm), vooral het tussenschot tussen de twee hartkamers is verdikt. Binnen en tussen HCM families is een grote variabiliteit in ernst van het ziektebeeld. HCM kan aanleiding geven tot klachten van hartfalen of hartritmestoornissen en zelfs plotse dood. Een aanzienlijk deel van de HCM patiënten heeft echter geen klachten. HCM komt op alle leeftijden voor, prenatale presentatie als ene uiterste en het ontstaan van HCM op hoge leeftijd als andere uiterste zijn mogelijk. Ook HCM is genetisch heterogeen. Tegenwoordig kan in ongeveer 70% van de HCM patiënten een genetisch defect worden aangetoond, voornamelijk in sarcomeer genen *MYBPC3* en *MYH7*. Bij een deel van de familiare vormen van HCM kan momenteel nog geen gen defect worden aangetoond. Erfelijkheidsvoorlichting en -onderzoek bij HCM patiënten en hun familieleden, die in aanmerking komen voor diagnostisch of voorspellend DNA en / of cardiologisch onderzoek, is belangrijk. De werkwijze voor een dergelijk erfelijkheids- en familieonderzoek wordt weergegeven in Figuur 14 van hoofdstuk 1.

HCM kan ook veroorzaakt worden door defecten in de mitochondrieën. Tevens kan HCM voorkomen bij verscheidene syndromen en spierziekten. HCM kan belangrijke klinische consequenties hebben en kan leiden tot vroegtijdig overlijden, terwijl andere patiënten een normale levensverwachting bereiken zonder of met weinig lichamelijke beperkingen. Niet alle HCM patiënten hebben therapie nodig, deze patiënten hebben een gunstige prognose. Wanneer wel therapie noodzakelijk is, is medicatie de eerst aangewezen optie, indien nodig gevolgd door een open hart operatie waarbij een stukje van de verdikking wordt weggesneden (myectomie) of een alcohol-ablatie, waarbij door middel van het inspuiten van alcohol lokaal een klein hartinfarct wordt opgewekt in de verdikte hartspier, opdat dit stukje hartspierweefsel afsterft en de hartspier dunner wordt. Om plotse dood te voorkomen is adequate risico stratificatie nodig om te bepalen wie hier een hoog risico op heeft opdat deze patiënten tijdig behandeld kunnen worden.

In **hoofdstuk 2** beschrijven we de eerste twee families waarin pathogene mutaties in het sarcomeer gen β-myosine zware keten (*MYH7*) autosomaal dominant overervende NCCM veroorzaken. Dit was de eerste keer dat *MYH7* mutaties met NCCM in verband werden gebracht. Mutaties in het *MYH7* gen zijn de meest frequente oorzaken van HCM wereldwijd en kunnen ook dilaterende en restrictieve cardiomyopathie (DCM, RCM) veroorzaken. Deze resultaten geven aan dat de genetische heterogeniteit van NCCM *MYH7*- geassocieerde ziekte omvat en suggereert dat *MYH7*-geassocieerde NCCM deel uit maakt van een cardiomyopathie spectrum met HCM, RCM en DCM.

In **hoofdstuk 3** staan de resultaten van het cardiogenetische familie onderzoek in een cohort van 58 patiënten met geïsoleerde NCCM (49 volwassenen en negen kinderen). Met het toepassen van gecombineerd moleculair onderzoek en cardiologisch familieonderzoek vonden wij dat in minimaal 67% van de patiënten NCCM genetisch was. Voor het cardiologische familieonderzoek werd een cardiologisch onderzoek met elektrocardiografie (ECG of hartfilmpje) en echocardiografie verricht van 194 familieleden van 50 NCCM index patiënten. Hierbij werd in 32 families (64%) een familiaire vorm van hartspierziekte (NCCM, HCM en DCM) aangetoond. Met moleculair onderzoek, dat in de volgende paragraaf in meer detail beschreven wordt, werd in de helft van de patiënten met familiaire NCCM een pathogene mutatie gevonden. Het merendeel (63%) van de familieleden, die een cardiomyopathie bleken te hebben, had geen hartklachten. Door het combineren van voorspellend DNA onderzoek en cardiologisch onderzoek van de familieleden kon worden vastgesteld dat van 17 asymptomatische volwassen dragers er negen NCCM hadden en acht geen tekenen van cardiomyopathie hadden (non-penetrantie). Het hoge percentage asymptomatische dragers in de families kan verklaren waarom bij 44% (14/32) van de familiaire vormen van NCCM de familiegeschiedenis die werd afgenomen voorafgaand aan het cardiologische familieonderzoek negatief of niet informatief was. Concluderend kunnen we vaststellen dat NCCM overwegend een genetische cardiomyopathie is met een variabele presentatie. Omdat vroeg diagnostiek van (asymptomatische) familieleden belangrijk kan zijn is erfelijkheidsadvisering en -onderzoek voor de NCCM patiënten en hun familie nodig. In dit hoofdstuk wordt een stroomdiagram gepresenteerd voor de genetische en cardiologische diagnostiek in NCCM families en wordt besproken wat de voor- en nadelen van voorspellend onderzoek bij familieleden kunnen zijn en dat het van belang is dat de familie daarover ingelicht worden.

Hoofdstuk 4 geeft de genetische classificatie van NCCM op basis van uitgebreide moleculaire studies Zeventien genen (*MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3*, *TCAP*, *LDB3, CASQ2*, *CALR3*, *PLN*, *TAZ* en *LMNA*) werden volledig geanalyseerd in een cohort van 56 NCCM patiënten (48 volwassenen en acht kinderen). Deze genen coderen voor sarcomeer en Z-disk eiwitten, Calcium regulerende eiwitten en eiwitten die een functie hebben in de cellulaire matrix; al deze genen werden eerder al geassocieerd met (andere) genetische hartspierziekten. Negenentwintig mutaties, inclusief 21 nieuwe, werden geïdentificeerd in 23 (41%) van de onderzochte NCCM patiënten. Erfelijke veranderingen in het *MYH7* gen waren de meest frequente genetische oorzaak voor NCCM en werden vastgesteld in 11 (20%) van alle geteste index patiënten en in 48% van de patiënten bij wie een mutatie werd vastgesteld. Mutaties in *MYBPC3* werden in vier patiënten aangetoond (7%), *TNNT2* in drie (5%), *TPM1*, *CASQ2* en *LDB3* elk in twee patiënten (4%) en *TNNI3*, *ACTC1*, *PLN*, *TAZ*, en *LMNA* elk in één patiënt (2%). Bij 35% van de volwassen patiënten werd een mutatie vastgesteld. Bij 75% van de kinderen werd een sarcomeer mutatie aangetoond. Mutaties die eerder geassocieerd werden met hartspierziekten (hetzij NCCM, hetzij HCM of DCM) werden als ziekteveroorzakend beschouwd. Nieuwe mutaties werden als ziekteveroorzakend

beschouwd wanneer ze tot een ingekort (truncerend) eiwit leiden of aanleiding geven tot veranderingen in de opbouw van het eiwit, wanneer ze *de novo* waren (nieuw ontstaan in de patiënt) of wanneer ze voldeden aan de volgende drie criteria: segregatie met het ziektebeeld binnen een familie, afwezigheid in een controle panel van 384 gezonde controle chromosomen en voorspeld pathogeen waren volgens predictie programma's SIFT en PolyPhen. Achttien index patiënten hadden een enkele mutatie, in vijf patiënten werd een complex genotype aangetoond (meerder mutaties al dan niet in hetzelfde gen). De genen *MYBPC3*, *TNNI3*, *TPM1*, *PLN* en *CASQ2* werden voor het eerst geassocieerd met NCCM. Deze resultaten laten zien dat NCCM voornamelijk een ziekte is die veroorzaakt wordt door veranderingen in sarcomeer eiwitten en daarmee behoort tot het spectrum sarcomeer - geassocieerde cardiomyopathieën waartoe ook een deel van de erfelijke vormen van HCM en DCM behoren. Tevens werd onderzocht of er een verschil is in prognose in families met een aangetoond gen defect en de families waarin (nog) geen erfelijke aanleg kon worden aangetoond. Hieruit bleek dat het ziektebeloop in families met een aangetoonde mutatie ongunstiger was. Concluderend kunnen we stellen dat de hoge mutatie frequentie in volwassenen en kinderen met NCCM het belang van moleculaire diagnostiek bij NCCM bevestigt. Het verdient aanbeveling om met het analyseren van de meest frequente oorzaak voor NCCM, het *MYH7* gen, te beginnen.

Hoofdstuk 5 beschrijft de eerste twee patiënten bij wie de diagnose *MYH7*-geassocieerde NCCM prenataal door middel van een echo werd vastgesteld. Prenataal echo onderzoek toonde biventriculaire cardiomegalie (vergroting van zowel de linker als de rechter hartkamer) en noncompaction bij een zwangerschapsduur van respectievelijk 31 en 23 weken. Aanvankelijk was niet bekend of er cardiomyopathie in de families voorkwam, hoewel bij een van de vaders op jonge leeftijd een aspecifieke hartaandoening werd vastgesteld, waarvoor hij sinds zijn 14^{e} jaar uit cardiologische controle was ontslagen. Om te weten of de moeders een cardiomyopathie hadden, waarvoor bij de bevalling speciale maatregelen nodig zouden zijn, en om de oorzaak voor het optreden van de prenatale cardiomyopathie te vinden, werden de beide ouders cardiologisch onderzocht en werd er een DNA onderzoek bij de ouders verricht. Uit deze onderzoeken bleken dat in beide families een *MYH7* cardiomyopathie voorkwam. Uitbreiding van het familieonderzoek toonde later aan dat er nog meer aangedane familieleden waren die veelal asymptomatisch waren. Vervolgonderzoek na de geboorte van de twee patiënten en een overzicht van de literatuur geven aan dat de prognose van prenataal vastgestelde NCCM voornamelijk afhankelijk is van de pompfunctie van het hart en dat deze na de geboorte kan verbeteren. We concluderen dat *MYH7* mutaties zich prenataal kunnen presenteren met biventriculaire noncompaction cardiomyopathie. Daarom wordt prenataal echo onderzoek in zwangerschappen met een risico op *MYH7*-geassocieerde NCCM geadviseerd. Anderzijds is, aangezien familiaire ziekte zich initieel prenataal kan presenteren, DNA en cardiologisch onderzoek geïndiceerd van eerstegraads familieleden wanneer er bij een prenataal echo onderzoek NCCM wordt ontdekt, ook als de familieanamnese blanco is voor cardiomyopathie.

Momenteel wordt in ongeveer de helft van de familiaire vormen van NCCM geen gendefect aangetoond. In **hoofdstuk 6** wordt een grote familie met een autosomaal dominant overervende cardiomyopathie beschreven die voornamelijk bestaat uit NCCM maar waar ook DCM is vastgesteld. Cardiologisch onderzoek van 20 familieleden uit deze Friese familie toonde negen patiënten aan met NCCM en één patiënt met DCM. Er werd geen erfelijke aanleg aangetoond in de 17 cardiomyopathie genen zoals beschreven in hoofdstuk 4. Om de genetische oorzaak voor NCCM / DCM in deze familie te identificeren werd een genoom-breed koppelingsonderzoek uitgevoerd met 300K SNP arrays. Aanvankelijk werd een maximale LOD score van 3.02 verkregen op chromosoom 4p15.3. De inclusie van de tiende patiënt die een gemeenschappelijke voorouder deelt met de index patiënt zeven generaties terug leidde tot de identificatie van een kleine kandidaat regio. Zo werd een nieuw gebied voor autosomaal dominante NCCM vastgesteld met een grootte van 5.47 Mb op chromosoom 4p15.3. Analyses ter identificatie van het nieuwe ziekte gen worden momenteel uitgevoerd. Identificatie van het gendefect maakt genetisch testen van familieleden binnen deze familie mogelijk en zal nieuwe inzichten in de ontstaanswijze van NCCM geven.

Hoofdstuk 7 beschrijft de klinische, pathologische en moleculaire bevindingen van twee pasgeborenen met een structurele hartafwijking en NCCM. Deze twee patiënten hadden allebei twee verschillende mutaties in het *MYBPC3* gen: de zogenaamde founder mutaties voor HCM, die in meer detail in de hoofdstukken 9 en 10 worden besproken. Eén van de patiënten had een ventrikel septum defect; de ander had een atrium septum defect. Een enkele erfelijke verandering in een gen dat codeert voor een sarcomeer eiwit (heterozygote mutatie) leidt meestal tot hypertrofische cardiomyopathie waarbij de klachten en klinische kenmerken zowel op kinderleeftijd als op volwassen leeftijd kunnen ontstaan. Twee dezelfde (homozygotie) of twee verschillende truncerende mutaties (compound heterozygotie) in het *MYBPC3* gen kunnen ernstige cardiomyopathie met tekenen van noncompaction veroorzaken die zich op zeer jonge leeftijd (neonataal) presenteert.

In **hoofdstuk 8** wordt de ervaring van de cardiogenetica polikliniek van het Erasmus MC met het genetisch testen en cardiologisch onderzoek in HCM families beschreven. Een stroomdiagram voor het stellen van de klinische en genetische diagnose en het daaropvolgende familieonderzoek wordt gepresenteerd. In dit hoofdstuk wordt het belang van het identificeren van een autosomaal dominante erfelijke aanleg voor HCM besproken. Het vaststellen van een erfelijke aanleg maakt identificatie van familieleden die risico lopen mogelijk en faciliteert een effectieve screening van familieleden. Tevens worden de variabiliteit in het ziektebeeld, inclusief non-penetrantie, (hierbij heeft de erfelijke aanleg niet tot het ziektebeeld geleid) en het ontstaan van HCM op latere leeftijd en de consequenties daarvan voor het screenen besproken. Omdat een betrouwbare risico classificatie voor asymptomatische familieleden ontbreekt en HCM ook op latere leeftijd nog kan ontstaan is het van belang om

cardiologisch onderzoek periodiek te laten plaatsvinden, zowel voor mutatie dragers als voor familieleden van HCM patiënten bij wie geen pathogene mutatie kan worden vastgesteld.

In eerdere studies werd beschreven dat bij mutaties in het sarcomeer gen Myosine Bindend Proteïne C gen (*MYBPC3*) HCM op latere leeftijd ontstaat en relatief mild kan verlopen. In **hoofdstuk 9** worden de resultaten van moleculaire analyse van het *MYBPC3* gen in 327 Nederlandse HCM index patiënten beschreven. Bij 45% van de patiënten werd een verandering in het *MYBPC3* gen aangetoond waaronder de al bekende c.2373dupG founder mutatie, in 14,4% van de patiënten. Ook werden twee nieuwe Nederlandse *MYBPC3* founder mutaties geïdentificeerd: de p.Arg943X mutatie in 6,7% en de c.2864_2865delCT in 4,6% van de HCM patiënten. Het opnemen van de familiegeschiedenissen met de prognose en overleving in de families met deze drie founder mutaties toonde dat in 33%, 38% en 56% van de respectievelijk c.2373dupG, p.Arg943X en c.2864_2865delCT families de prognose slecht was. Overlevingscurves van deze drie truncerende founder mutaties waren nagenoeg identiek, suggererend dat er een vergelijkbaar natuurlijk beloop is dat waarschijnlijk veroorzaakt wordt door haplo-insufficiëntie (onvoldoende eiwit productie, met verlies van functie als gevolg).

HCM erft vaak autosomaal dominant over er wordt voornamelijk veroorzaakt door mutaties in sarcomeer en Z-disk genen. Recentelijk werd HCM ook vastgesteld in families met X-gebonden spierziekten veroorzaakt door mutaties in het Z-disk gen, gelegen op het X-chromosoom, Four-and-a-half-LIM domains 1 (*FHL1*). Deze bevindingen suggereren een rol voor het *FHL1* gen in het ontstaan van HCM. **Hoofdstuk 10** beschrijft de resultaten van onderzoek naar de rol van het *FHL1* gen in HCM. Voor dat doel werd DNA analyse van het *FHL1* gen in 86 HCM patiënten (54 mannen en 32 vrouwen) verricht. Deze patiënten werden geselecteerd uit een groep van 260 HCM index patiënten omdat bij hen geen pathogene mutatie werd aangetoond in de HCM genen (*MYH7, MYBPC3*, *TNNC1*, *TNNT2, TNNI3, ACTC1, MYL2*, *MYL3*, *TPM1, CSRP3* en *TCAP). FHL1* mutaties werden vastgesteld in twee HCM patiënten; een nonsens mutatie (leidend tot een getrunceerd eiwit) in een vrouw die achteraf ook subtiele spierklachten had en bij wie een spierbioptie aspecifieke myopathische veranderingen aantoonde. De familiegeschiedenis van deze patiënte was positief voor plotse hartdood en HCM. Cardiologisch en neurologisch familieonderzoek toonde variabiliteit van symptomen en leeftijd waarop de klachten begonnen aan. Ook waren er dragers in de familie die (nog) geen HCM hadden. De andere patiënt, een man zonder neuromusculaire klachten, had een missense mutatie (een enkele aminozuurverandering in het eiwit). In de familie van de mannelijke HCM patiënt werd (nog) geen familie onderzoek verricht. De resultaten laten zien dat *FHL1* mutaties een zeldzame oorzaak (<1%) van HCM zijn. *FHL1* geassocieerde HCM wordt gekenmerkt door X-gebonden dominante overerving. Analyse van het *FHL1* gen wordt dan ook geadviseerd in zowel mannen als vrouwen met HCM, zeker wanneer de familie geschiedenis doet vermoeden dat de aandoening X-gebonden is (er is dan geen overerving van vader op zoon) en/of wanneer er neuromusculaire problemen in de familie voorkomen. Afwezigheid van een familie

geschiedenis van HCM of spierziekte sluit een *FHL1* cardiomyopathie niet uit gezien de incomplete penetrantie van HCM en neuromusculaire klachten. Familie onderzoek wordt daarom ook aanbevolen bij deze geslachtsgebonden erfelijke vorm van HCM.

Het verschil in ernst en in leeftijd waarop klachten optreden in HCM families is karakteristiek voor deze ziekte en de oorzaak hiervoor is onbekend. De hypothese is dat er naast de pathogene mutaties andere genetische of omgevingsinvloeden een rol spelen bij het optreden van HCM. Deze additionele risicofactoren zijn moeilijk te onderzoeken omdat de mutaties in de meeste families uniek zijn. Het vaak voorkomen van drie truncerende *MYBPC3* founder mutaties in Nederland biedt de gelegenheid om in grotere genetisch homogene patiëntengroepen naar de modificerende risicofactoren onderzoek te doen. In **hoofdstuk 11** worden de resultaten van onderzoek naar het effect van additionele mutaties in sarcomeer genen op de ernst van het ziektebeeld bij dragers van een founder mutatie beschreven. Om te analyseren of een complex genotype een invloed heeft op de ernst van HCM werden negen sarcomeer genen (*MYH7, MYBPC3, MYL2, MYL3, TNNT2, TNNI3, TNNC1, ACTC1* and *TPM1*) onderzocht in 87 HCM patiënten bij wie eerder een truncerende *MYBPC3* founder mutatie als primair genetisch HCM defect werd vastgesteld. De HCM patiënten werden op grond van klinische kenmerken geclassificeerd in een mild of in een ernstig fenotype gebaseerd op leeftijd van diagnose, maximale wand dikte, obstructie van linker kamer uitstroombaan, een positieve familie geschiedenis voor plotse hartdood en de noodzaak voor een operatie of ablatie of het implanteren van een inwendige defibrillator. Wij vonden geen verschil in het aantal additionele mutaties in de groep met een ernstig ziektebeeld in vergelijking met de groep met een mild ziektebeeld. De ernst van het ziektebeeld in HCM patiënten met een truncerende *MYBPC3* mutatie wordt niet beïnvloed door een modificerend effect van een tweede sarcomeer mutatie. Andere, momenteel nog onbekende, genetische oorzaken van cardiomyopathie kunnen mogelijk de variabiliteit binnen de founder HCM populatie verklaren.

In **hoofdstuk 12** wordt de rol van aldosteron als modificerende factor in HCM onderzocht. In dit onderzoek werden de concentraties van cardiaal en / of plasma aldosteron vergeleken in 79 HCM patiënten en in 12 orgaan donoren zonder cardiale pathologie. Ook werd het effect van het aldosteron synthase (*CYP11B2*) polymorfisme (een niet-pathogene variatie in het DNA) C-344T op de linker kamer massa index (LVMI) en de dikte van het tussenschot tussen de hartkamers (intraventriculair septum of IVS) onderzocht. Het aldosteron in de harten en het plasma van acht HCM patiënten was vergelijkbaar met dat in de harten en plasma van de personen zonder cardiomyopathie. In vrouwen met HCM was er geen verband tussen het *CYP11B2* genotype en de cardiomyopathie parameters. Mannelijke HCM patiënten met dit T allel hadden een hogere LVMI en een dikker tussenschot. Multipele regressie analyse toonde aan dat het T allel - gerelateerde effect op het tussenschot onafhankelijk was van renine, het ACE I/D polymorfisme, het AT1-receptor A/C1166 polymorfisme and the AT2-receptor A/C3123 polymorfisme. Circulerend en cardiaal aldosteron zijn normaal in HCM, wat pleit tegen een

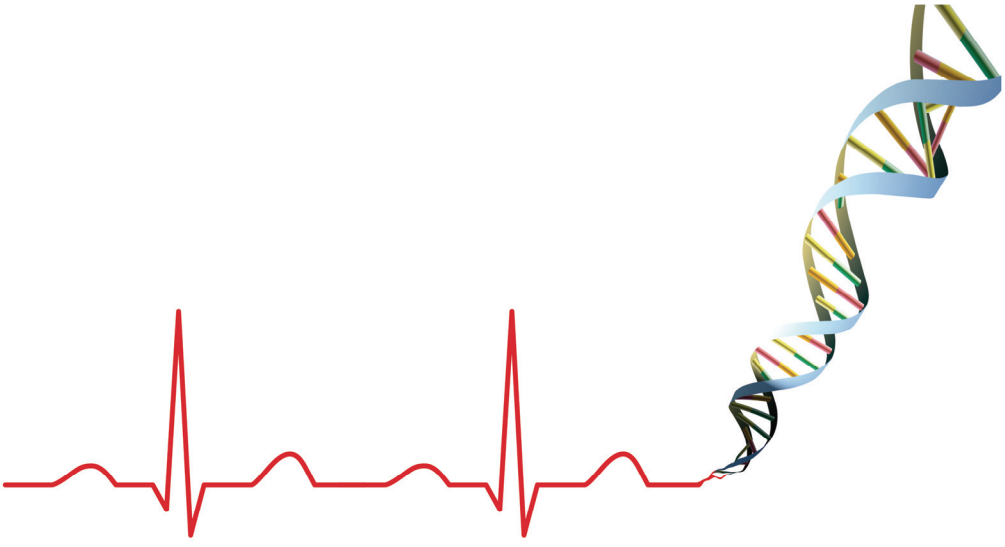
selectief verhoogde cardiale aldosteron productie in HCM patiënten. Het verband tussen het *CYP11B2* C-344T polymorfisme en hypertrofie in HCM heeft waarschijnlijk te maken met de T allel - gerelateerde verhoging in circulerend aldosteron. Deze bevindingen geven de noodzaak aan van aanvullende studies naar de gunstige uitwerking van aldosteron blokkade als therapie in HCM patiënten.

In de algemene discussie in **hoofdstuk 13** worden de resultaten van dit proefschrift besproken en wordt aangegeven wat het belang is van de bevindingen en hoe ze in de praktijk te gebruiken zijn. NCCM en HCM zijn twee heterogene vormen van cardiomyopathie, met een gedeeltelijk overlappende genetische etiologie. In NCCM is het *MYH7* gen het meest frequent gemuteerde gen. Daarom geldt het advies dit gen het eerst te analyseren. Aansluitend DNA onderzoek van de genen *MYBPC3*, *TPM1, TNNT2*, *TNNI3*, *ACTC1*, *CASQ2*, *PLN*, *TAZ*, *LDB3* en *LMNA* leidt tot het vinden van een erfelijke oorzaak voor NCCM in ongeveer 40% van de patiënten. Wat betreft HCM in Nederland wordt aanbevolen eerst de Nederlandse founder mutaties c.2373dupG, p.Arg943X en c.2864_2865delCT in het *MYBPC3* gen te analyseren aangezien ongeveer 25% van de Nederlandse HCM populatie één van deze drie pathogene founder mutaties heeft. Indien deze analyse geen pathogene mutatie aantoont wordt aansluitend geadviseerd de rest van het *MYBPC3* gen te onderzoeken, gevolgd door *MYH7* en *TNNT2*. Screenen van additionele sarcomeer en Z-disk genen zal een uiteindelijke opbrengst leveren van ongeveer 60 tot 70%. Als de familiegeschiedenis past bij een X - gebonden overerving en / of wanneer er neuromusculaire ziekten in de familie voorkomen wordt geadviseerd het *FHL1* gen te onderzoeken.
Ongeveer de helft van NCCM en een derde van HCM kunnen momenteel nog niet moleculair verklaard worden. Identificatie van een pathogene mutatie in een familie maakt een nauwkeurige identificatie van familieleden die risico hebben op NCCM en / of HCM mogelijk. Bij personen die drager zijn van een pathogene mutatie of bij personen uit families met NCCM of HCM waarin geen genetisch defect kan worden vastgesteld, wordt periodiek cardiologisch onderzoek geadviseerd teneinde een eventuele cardiomyopathie tijdig te diagnosticeren en, indien noodzakelijk, te behandelen. Hiermee wordt getracht het risico op ernstige complicaties binnen cardiomyopathie families te verkleinen.
Het ligt in de lijn van de verwachtingen dat er over niet al te lange tijd nieuwe cardiomyopathie genen zullen worden gevonden bijv. in andere dan tot nu toe onderzochte sarcomeer en Z-disk genen. Genen die betrokken zijn bij het ontstaan van DCM zouden ook een rol kunnen spelen in het ontstaan van NCCM en HCM. De snelle introductie van nieuwe moleculaire technieken, waarmee grote aantallen genen tegelijkertijd gescreend kunnen worden tegen relatief lage kosten zullen leiden tot het identificeren van nieuwe genetische defecten in NCCM en HCM, maar er zullen ook een heleboel varianten ontdekt worden waarvan de betekenis niet duidelijk is. Het zal een uitdaging zijn deze varianten te classificeren en erachter te komen welke pathways betrokken zijn bij het ontstaan van cardiomyopathieën.

Curriculum Vitae

PhD Portfolio Summary

Publications



Curriculum Vitae

Yvonne Hoedemaekers was born on November 22nd 1975 in Venray, the Netherlands. She studied Gymnasium β at the "Boschveld College" in Venray and graduated in 1994. She had a stroke of luck as she was able to start her medical studies at the "Katholieke Universiteit Nijmegen" in August 1994. Despite her addiction for sneak previews and film marathons and despite her volunteer work as member of the board of Amnesty International Nijmegen she passed all her exams without any difficulties. Her interests were in paediatrics leading to internships at the departments of paediatrics at the "Spittaal Ziekenhuis" in Zutphen and at the UMC St. Radboud in Nijmegen, where she also did her scientific internship researching the follow-up of medulloblastomas at the department of paediatric oncology. In January 2001 she obtained her medical degree in Nijmegen. From February until September 2001 she worked as a resident at the department of paediatrics at the UMC St. Radboud in Nijmegen. In October 2001 she entered the Maxima Medical Centre in Veldhoven, again working at the department of paediatrics. She enjoyed her job tremendously, especially the care for pretermly born babies at the neonatal intensive care unit she found extremely rewarding. Nevertheless, encountering Tirza, a neonate with 68 chromosomes, led to a career switch from paediatrics to clinical genetics. After spending 26 months working in Veldhoven, she came back to Nijmegen in December 2003 to start working as a resident at the department of Anthropogenetics of the UMC St. Raboud in Nijmegen. Getting used to the lack of constantly beeping beepers and hectic weekend, evening and night shifts took very little time, although the lack of actually treating patients is still felt until this day. Unfortunately, specialisation options were slim in Nijmegen, leading to another location switch. She moved to "far-away" Rotterdam in July 2004, to start as a resident at the department of Clinical Genetics of the Erasmus Medical Centre. From the start her focus was on Cardiogenetics and she spent her first year co-working as a resident at the department of Cardiology. She obtained a scholarship from the Dutch Heart Foundation in January 2007, and the research described in this thesis began. In January 2010 she started her training at the Erasmus Medical Centre to become a clinical geneticist.

PHD Portfolio Summary

Name PhD student	Yvonne Maria Hoedemaekers
Erasmus MC Department	Clinical Genetics
Research Schools	Medisch Genetisch Centrum Zuid-West Nederland – MGC COEUR
PhD period	2006-2010
Promotors	Prof.dr. M.L. Simoons Prof.dr. B.A. Oostra
Date of public defence thesis	June 25th, 2010
Thesis title	Genetic aspects and family studies of noncompaction and hypertrophic cardiomyopathy

General academic skills (4.3 ECTS)

- Biomedical English Writing and Communication, Erasmus MC Rotterdam, 2008.
- Basic Life Support, Erasmus MC Rotterdam, 2009.

In-depth courses (5.5 ECTS)

- Workshop browsing genes and genomes with Ensemble III. MolMed postgraduate school / dept. of Bio Informatics / research school MGC, Erasmus MC Rotterdam, 2008.
- Daycourse Clinical Genetics Diagnostics: “How do I analyse my gene”. MolMed postgraduate school / research school MGC, Erasmus MC Rotterdam, 2008.
- COEUR PhD training course Congenital Heart Disease, Erasmus MC Rotterdam, 2009.
- European School of Medical Genetics, 23^{d} course, Bologna, Italy, 2010.

Oral presentations (8.3 ECTS)

- Erasmus MC Science day, 2006, Rotterdam (First prize): Novel myosin binding protein C founder mutations may confer severe hypertrophic cardiomyopathy.
- Assistenten LOG, 2006, Leiden: Genetic analysis of Hypertrophic Cardiomyopathy Genes in Noncompaction Cardiomyopathy Patients: could NCCM be a Phenotypic Variant of HCM?
- COEUR research seminar, 2007, Rotterdam: Clinical Genetics of Cardiomyopathies.
- Training course for Dutch departments of nuclear medicine, 2007, Utrecht: Cardiogenetics: “heart for heredity”.
- UK / Dutch Clinical Genetics Societies Spring Conference, 2008, Liverpool, United Kingdom: Familial noncompaction cardiomyopathy: a novel genetic cardiomyopathy.
- Cardiogenetics evening course for cardiologists of the Rijnmond region, 2008, Rotterdam
- Fellow symposium on paediatric cardiology and intensive care, 2009, Rotterdam: Genetic aspects of cardiomyopathy.
- ICIN workgroup on hereditary heart disease, semi-annual meeting, 2009, Utrecht: Familial noncompaction cardiomyopathy (I): cardiologic features in adults and children.

- Assistenten LOG, 2009, Maastricht: Familial noncompaction cardiomyopathy: counselling, DNA diagnostics and cardiologic family studies.
- COEUR Research seminar, 2009, Rotterdam: Genetics of hypertrophic cardiomyopathy.
- Department of Cardiology UMC Utrecht, 2010, Utrecht: Familial noncompaction cardiomyopathy: counselling, DNA diagnostics and cardiologic family studies.
- LOG on Cardiogenetics, 2010, Utrecht: Noncompaction cardiomyopathy, an overview.
- UK / Dutch Clinical Genetics Societies Spring Conference, 2010, Amsterdam: Prenatal diagnosis of *MYH7* associated noncompaction cardiomyopathy.
- Department of Cardiology St. Antonius Hospital, 2010, Sneek: Familial noncompaction cardiomyopathy: counselling, DNA diagnostics and cardiologic family studies.

Poster presentations (1.2 ECTS)

- **Hoedemaekers YM**, Biagini E, Deelen W, Meijers – Heijboer HJ, Halley DJJ, ten Cate FJ, Dooijes D. *55th Annual meeting of the American Society of Human Genetics, 2005 Salt Lake City, USA.* Myosin Binding protein C R943X and 2864delCT founder mutations may confer relatively severe hypertrophic cardiomyopathy.
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Majoor – Krakauer DF, Dooijes D. *56th Annual meeting of the American Society of Human Genetics, 2006, New Orleans, USA.* Genetic analysis of Hypertrophic Cardiomyopathy Genes in Noncompaction Cardiomyopathy Patients: could NCCM be a Phenotypic Variant of HCM?
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Majoor – Krakauer DF, Dooijes D. *European Human Genetics Conference, 2006, Amsterdam.* Genetic analysis of Hypertrophic Cardiomyopathy Genes in Noncompaction Cardiomyopathy Patients: could NCCM be a Phenotypic Variant of HCM?
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Michels M, Dooijes D, Majoor – Krakauer DF. *57th Annual meeting of the American Society of Human Genetics, 2007, San Diego, USA.* Familial Noncompaction Cardiomyopathy: A Novel Genetic Cardiomyopathy.
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Michels M, Majoor-Krakauer DF, Dooijes D. *Keystone Symposium on Cardiac Hypertrophy, 2008, Copper Mountain Resort, USA.* Sarcomeric Defects: A Major Cause of Noncompaction Cardiomyopathy.
- van Tienhoven M, **Hoedemaekers YM**, Michels M, ten Cate FJ, Majoor – Krakauer DF, Halley DJJ, Dooijes D. *Keystone Symposium on Cardiac Hypertrophy, 2008, Copper Mountain Resort, USA.* Disease severity in MYBPC3 associated hypertrophic cardiomyopathy is not primarily dependent on compound heterozygousity for a sarcomeric mutation.
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Majoor – Krakauer DF, Dooijes D. *Florence International Course on Advances in cardiomyopathies; 5th meeting of the European myocardial and pericardial diseases workgroup of the European Society of Cardiology, 2008, Florence, Italy.* Sarcomeric Defects: A Major Cause of Noncompaction Cardiomyopathy.

- **Hoedemaekers YM**, Caliskan K, Majoor – Krakauer DF, Dooijes D. *Science day Dutch Heart Foundation, 2008, Amsterdam.* Family and genetic study of noncompaction cardiomyopathy.
- **Hoedemaekers YM**, Caliskan K, ten Cate FJ, Michels M, Majoor – Krakauer DF, Dooijes D. *Dutch Society of Human Genetics spring meeting, 2009, Veldhoven.* Noncompaction Cardiomyopathy; mutation spectrum, distribution of disease genes and implications for diagnostic strategies.
- **Hoedemaekers YM**, Caliskan K, Michels M, Frohn – Mulder I, van der Smagt JJ, Phefferkorn JE, ten Cate FJ, Dooijes D, Majoor – Krakauer DF. *Dutch Society of Human Genetics spring meeting, 2009, Veldhoven.* Familial noncompaction cardiomyopathy: genetic and cardiologic features in adults and children.
- Phefferkorn JE, **Hoedemaekers YM**, Frohn – Mulder I, Wessels MW, Dooijes D, Majoor – Krakauer DF. *Dutch Society of Human Genetics spring meeting, 2009, Veldhoven.* Familial Hypertrophic Cardiomyopathy in Children.
- **Hoedemaekers YM**, Caliskan K, Michels M, Frohn – Mulder I, van der Smagt JJ, Phefferkorn JE, ten Cate FJ, Dooijes D, Majoor – Krakauer DF. *European Society of Cardiology conference, 2009, Barcelona, Spain.* Familial noncompaction cardiomyopathy: genetic and cardiologic features in adults and children.
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- **Hoedemaekers YM**, Caliskan K, Michels M, Frohn – Mulder I, van der Smagt JJ, Phefferkorn JE, Wessels MW, ten cate FJ, Sijbrands EJG, Dooijes D, Majoor – Krakauer DF. *The Second Cardiff Symposium on Clinical Cardiovascular Genetics, 2009, Cardiff.* Familial noncompaction cardiomyopathy: genetic counseling, DNA diagnostics and cardiologic family screening.

International conferences (9.9 ECTS)

- 55th Annual meeting of the American Society of Human Genetics, 2005, Salt Lake City, USA.
- 56th Annual meeting of the American Society of Human Genetics, 2006, New Orleans, USA.
- European Human Genetics Conference, 2006, Amsterdam.
- 57th Annual meeting of the American Society of Human Genetics, 2007, San Diego, USA.
- Keystone Symposium on Cardiac Hypertrophy, 2008, Copper mountain Resort, USA.
- UK / Dutch Clinical Genetics Societies Spring Conference, 2008, Liverpool, UK.
- 5th meeting of the European Myocardial and Pericardial Diseases Workgroup of the European Society of Cardiology, 2008, Florence, Italy.
- First European Dysmorphology Club, 2010, Amsterdam.
- UK / Dutch Clinical Genetics Societies Spring Conference, 2010, Amsterdam.

Seminars and workshops (3.9 ECTS)

- COEUR research seminar on Cardiac Hypertrophy and Cardiomyopathies, 2007, Erasmus MC Rotterdam.
- COEUR research seminar on Genetics of Cardiovascular Disease, 2007, Erasmus MC Rotterdam.
- Spring Conference Dutch Anthropogenetics Society (NAV), 2007, Veldhoven.
- Cardiogenetics evening course for cardiologists of the Rijnmond region, 2008, Rotterdam.
- Dutch Heart Foundation Science Day, 2008, Amsterdam.
- Spring Conference Dutch Society of Human Genetics (NVHG), 2009, Veldhoven.
- ICIN Knowledge Day, 2009, Utrecht.
- CVOI Electrophysiology, 2009, Utrecht.
- Cardiogenetics evening course for cardiologists of the Rijnmond region, 2008, Rotterdam.
- COEUR research seminar on Left Ventricular Hypertrophy and Genetics, 2009, Erasmus MC Rotterdam.

Total ECTS: 33.1

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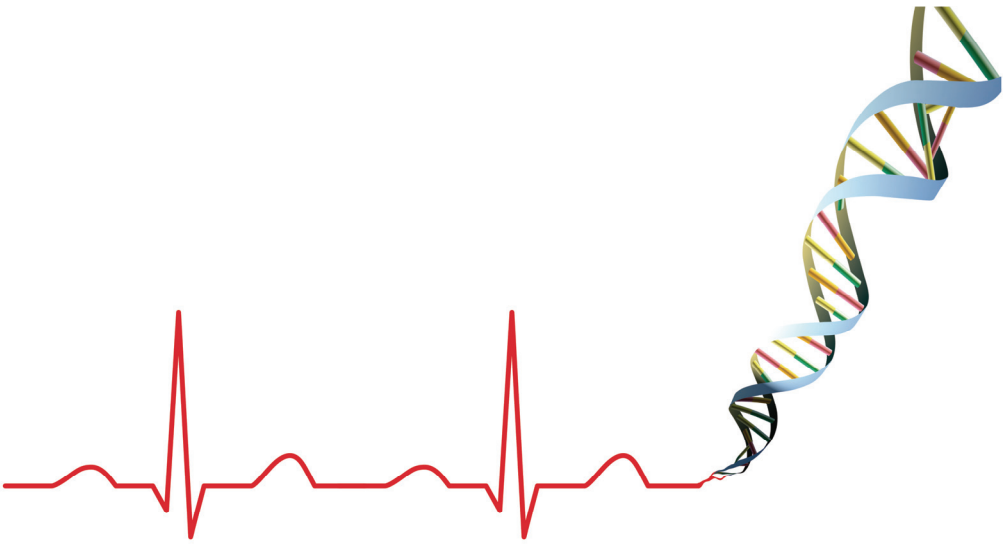
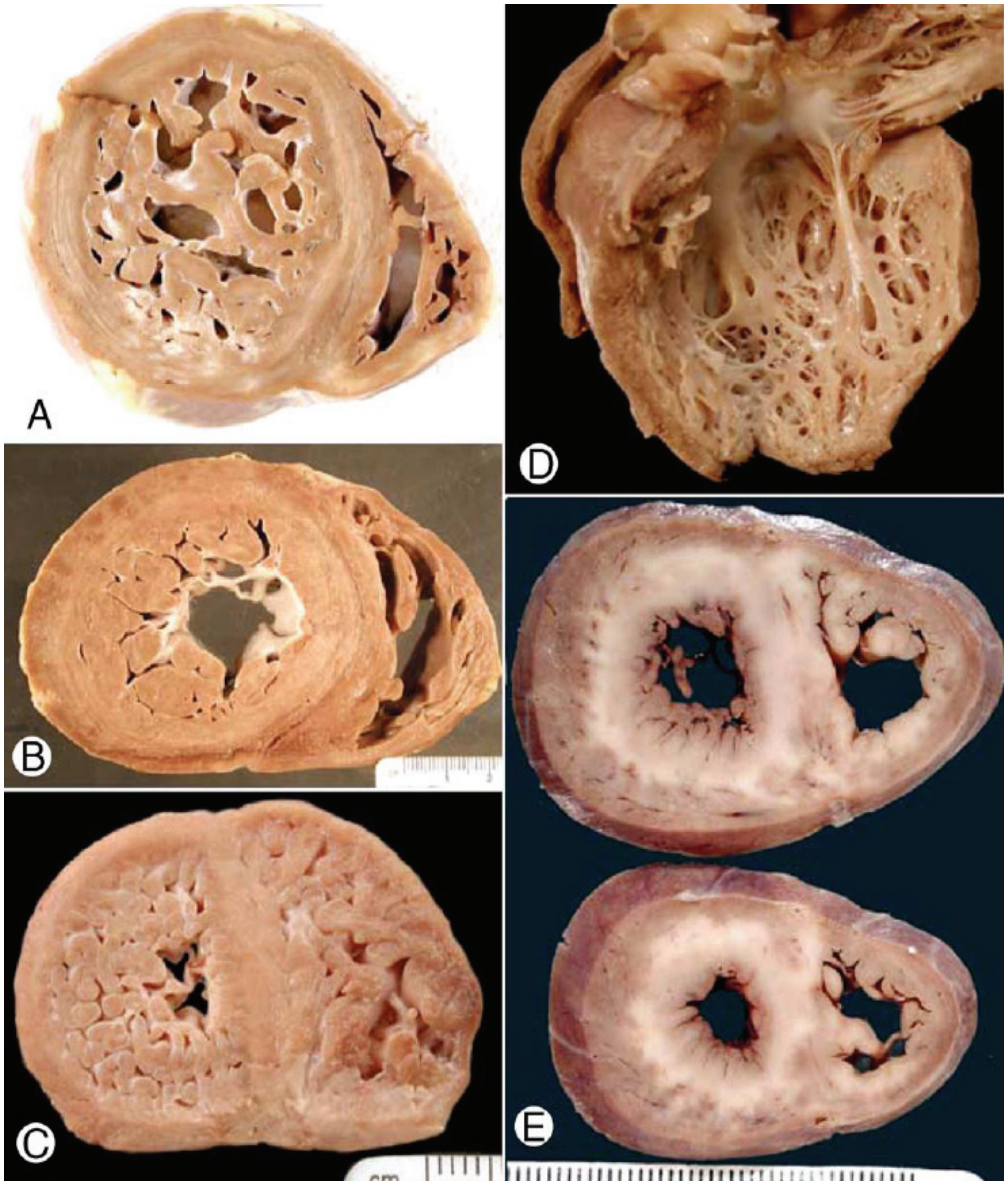
Colour figures



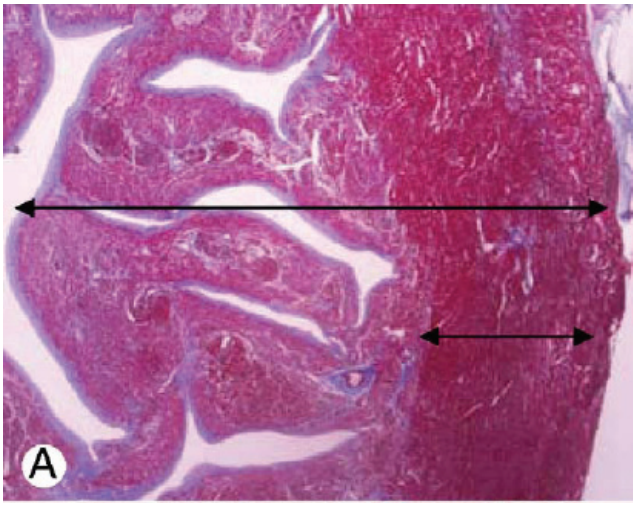


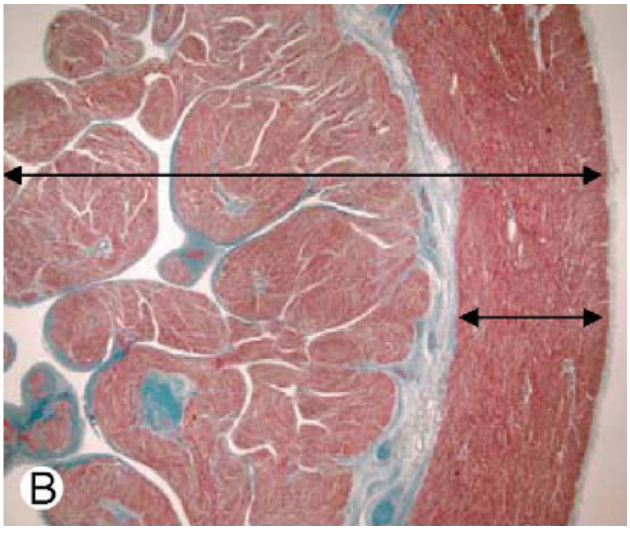
Chapter 1: Figure 1

NCCM gross pathology with a variety of NCCM patterns:

A. Anastomosing broad trabeculae
B. Coarse trabeculae resembling multiple papillary muscles
C. Interlacing smaller muscle bundles resembling a sponge
D. Trabeculae viewed en face
E. Subtle NCCM on gross section, requires histological confirmation

Reproduced with permission from "Left ventricular noncompaction: a pathological study of 14 cases; Burke et al; Human Pathology 2005;36;403-411"





Chapter 1: Figure 2

Histological features in NCCM:

The ratio of noncompact versus compact myocardium is larger than two.

A. Relatively smooth endocardial surface (left) with anastomosing broad trabeculae
B. Polypoid pattern of trabeculae; prominent fibrous band separating the noncompact from the compact myocardium

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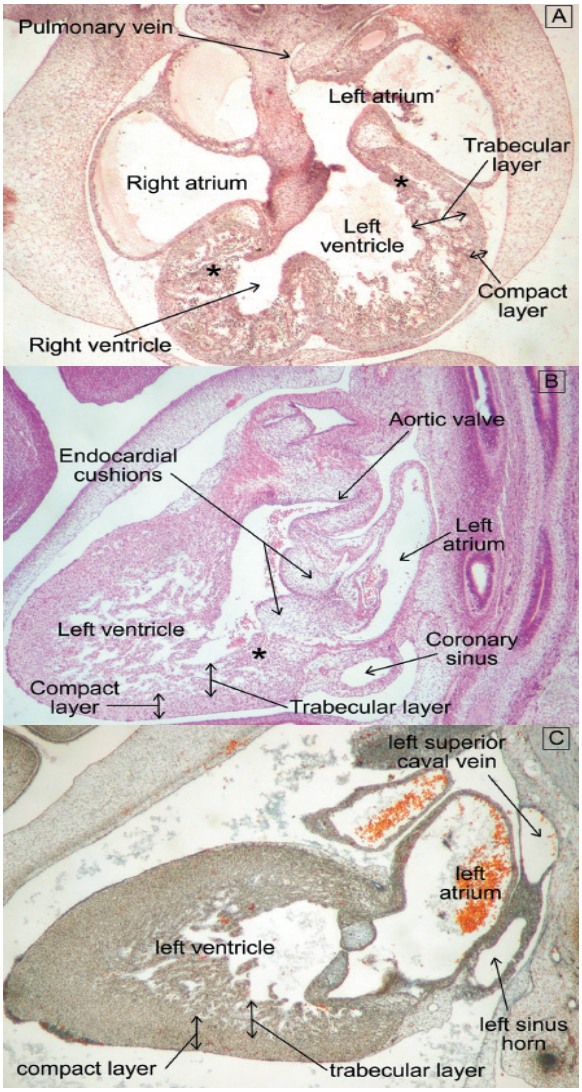
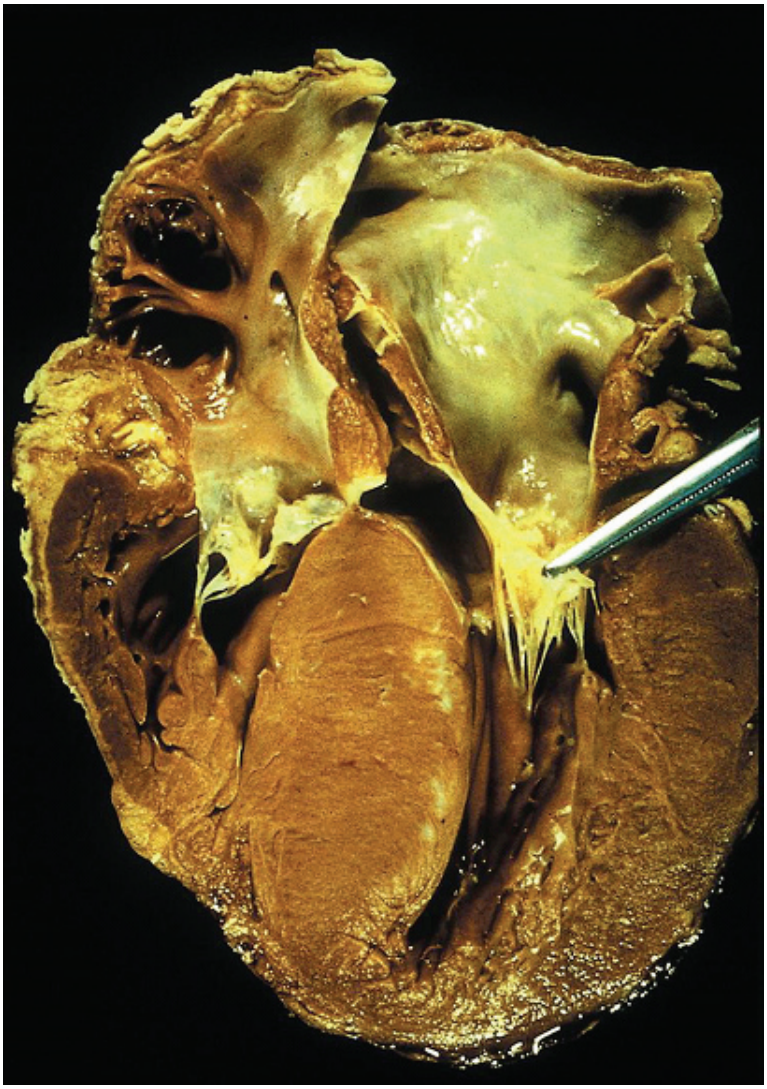


Chapter 1: Figure 3

Human embryos at Carnegie stage 16 (A), stage 18 (B) and after closing of the embryonic interventricular foramen (C)

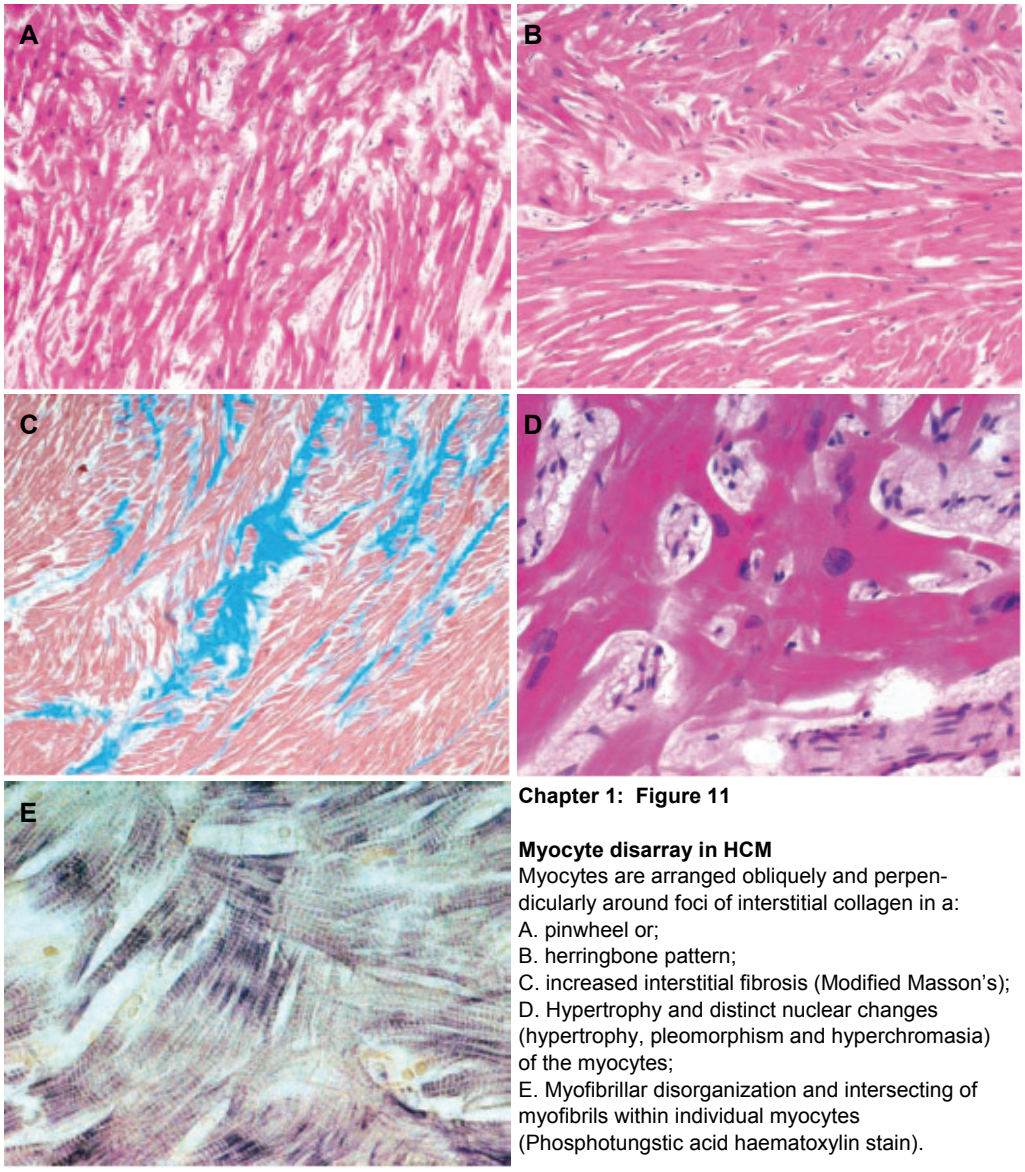
During development there is an extensive trabecular layer forming the greater part of the ventricular wall thickness compared to the extent of the compact layer. The trabecular layer becomes compacted and forms the papillary muscles of the atrioventricular valves (*).

Reproduced with permission from:
"The morphological spectrum of ventricular noncompaction; Freedom et al; Cardiology in the Young 2005;15:345-364"



Chapter 1: Figure 10

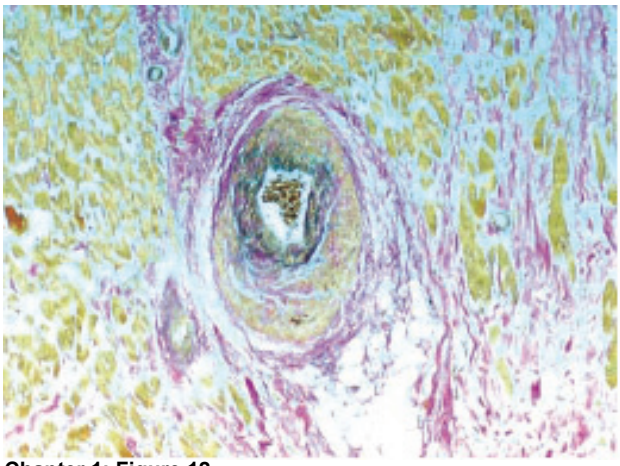
HCM gross pathology *(Source: univadis.nl)*



Chapter 1: Figure 11

Myocyte disarray in HCM
Myocytes are arranged obliquely and perpendicularly around foci of interstitial collagen in a:
A. pinwheel or;
B. herringbone pattern;
C. increased interstitial fibrosis (Modified Masson's);
D. Hypertrophy and distinct nuclear changes (hypertrophy, pleomorphism and hyperchromasia) of the myocytes;
E. Myofibrillar disorganization and intersecting of myofibrils within individual myocytes (Phosphotungstic acid haematoxylin stain).

Reproduced with permission from: "The pathology of hypertrophic cardiomyopathy; Hughes; Histopathology



Chapter 1: Figure 12

Intramural coronary artery dysplasia in HCM

There is marked medial hypertrophy and narrowing of the lumen of the intramural coronary arteries with associated myocardial fibrosis (Elastic van Gieson).

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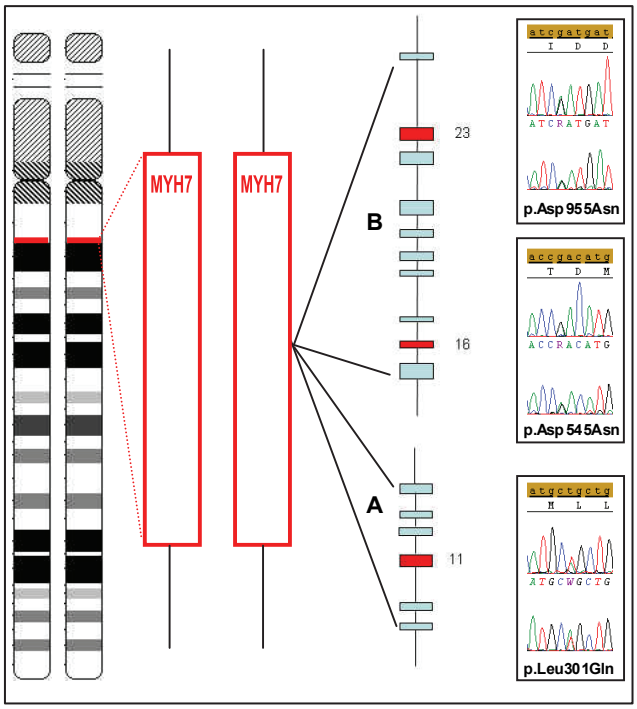
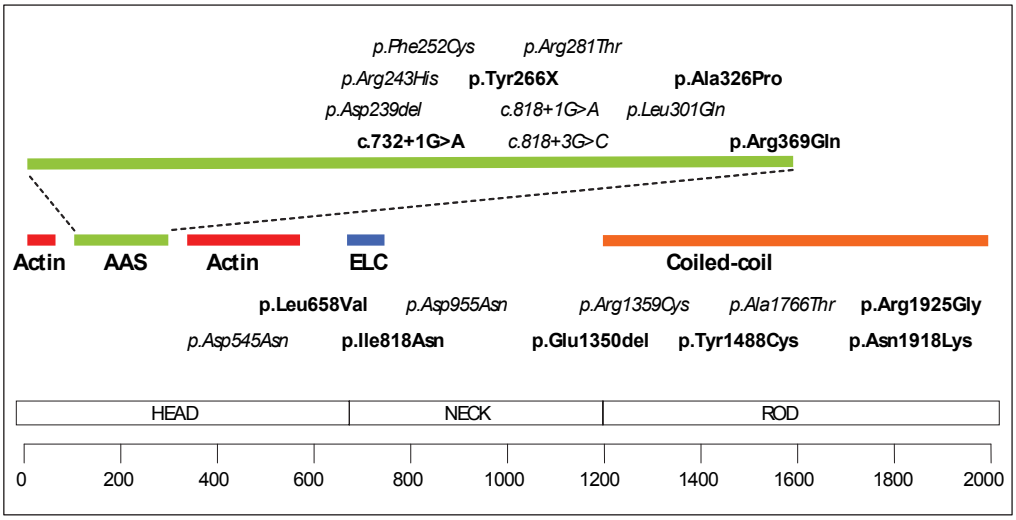
"The pathology of hypertrophic cardiomyopathy;
Hughes; Histopathology 2004; 44; 412-427."



Chapter 2: Figure 2

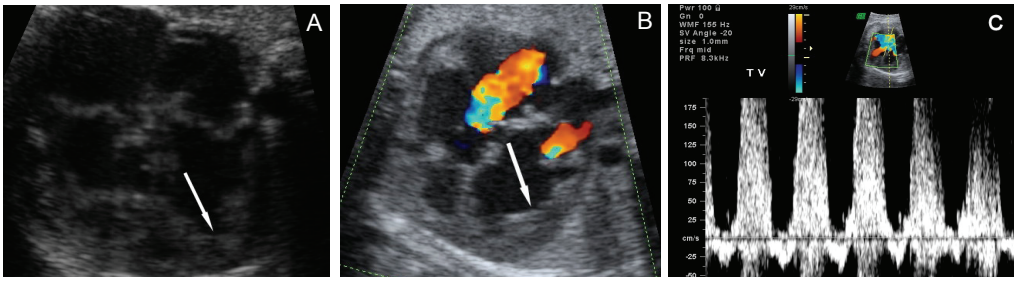
Schematic diagram showing chromosome 14 with the location of the *MYH7* gene (14q12)

Mutations were identified on one allele of the *MYH7* gene in exons 11 and 16/23 in families A and B respectively. Sequence traces show the mutations identified in families A and B.



Chapter 4: Figure 2

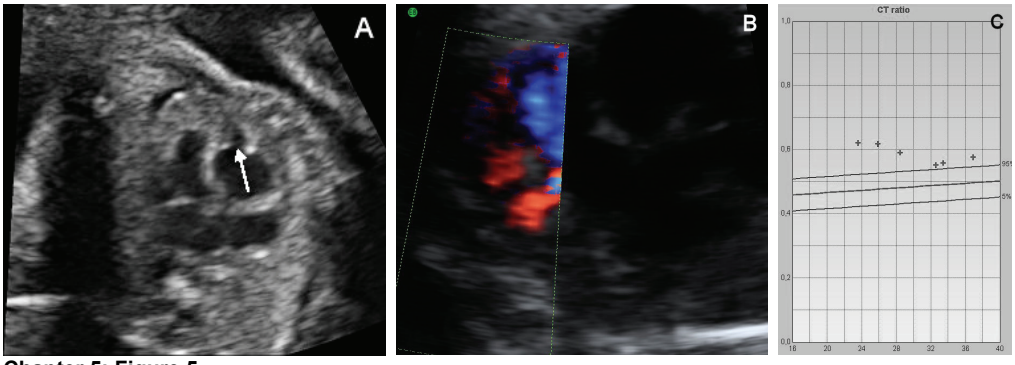
Currently identified LVNC mutations in *MYH7*
The mutations identified in this study are in bold. AAS: ATPase active site; ELC: myosin essential light chain binding site.



Chapter 5: Figure 2

Prenatal ultrasound of patient 1 at 31 weeks' gestation

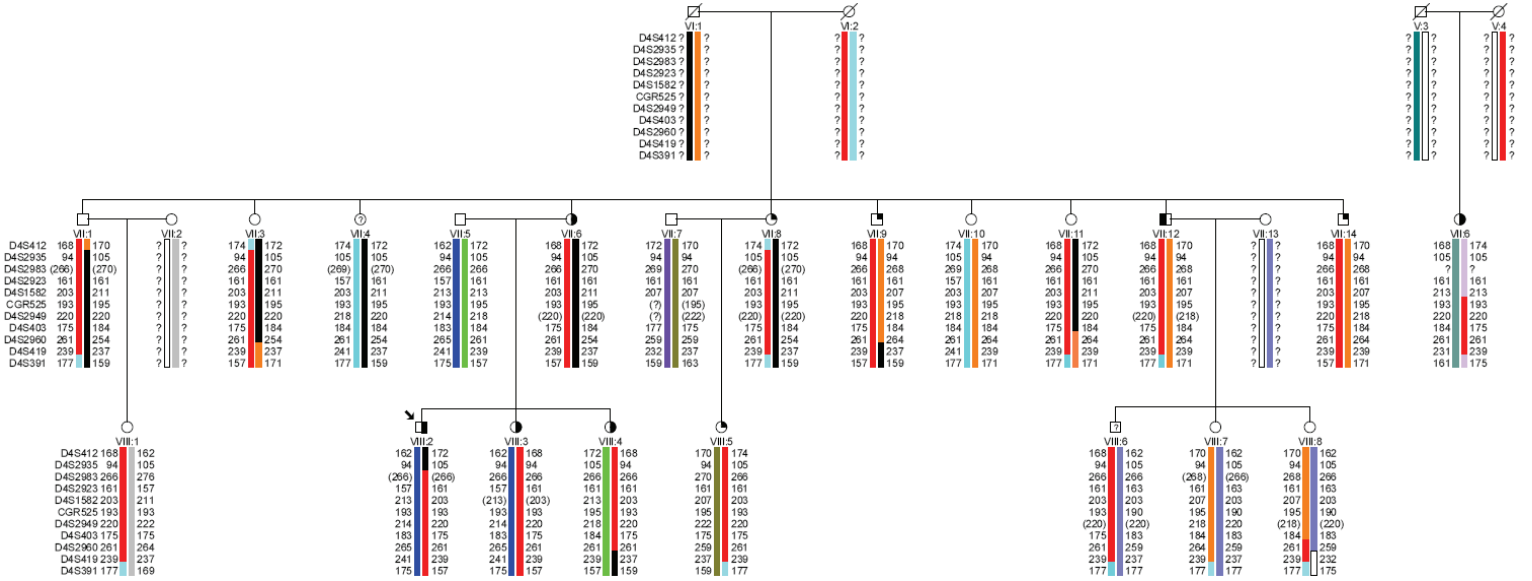
A. Enlarged heart with hypertrophic and spongy myocardium, particularly in the left apex (arrow).
B. Colour Doppler image of severe regurgitation over tricuspid and mitral valve.
C. Pulsed Doppler image of severe tricuspid regurgitation (> 200 cm/sec)



Chapter 5: Figure 5

Prenatal ultrasound of patient 2

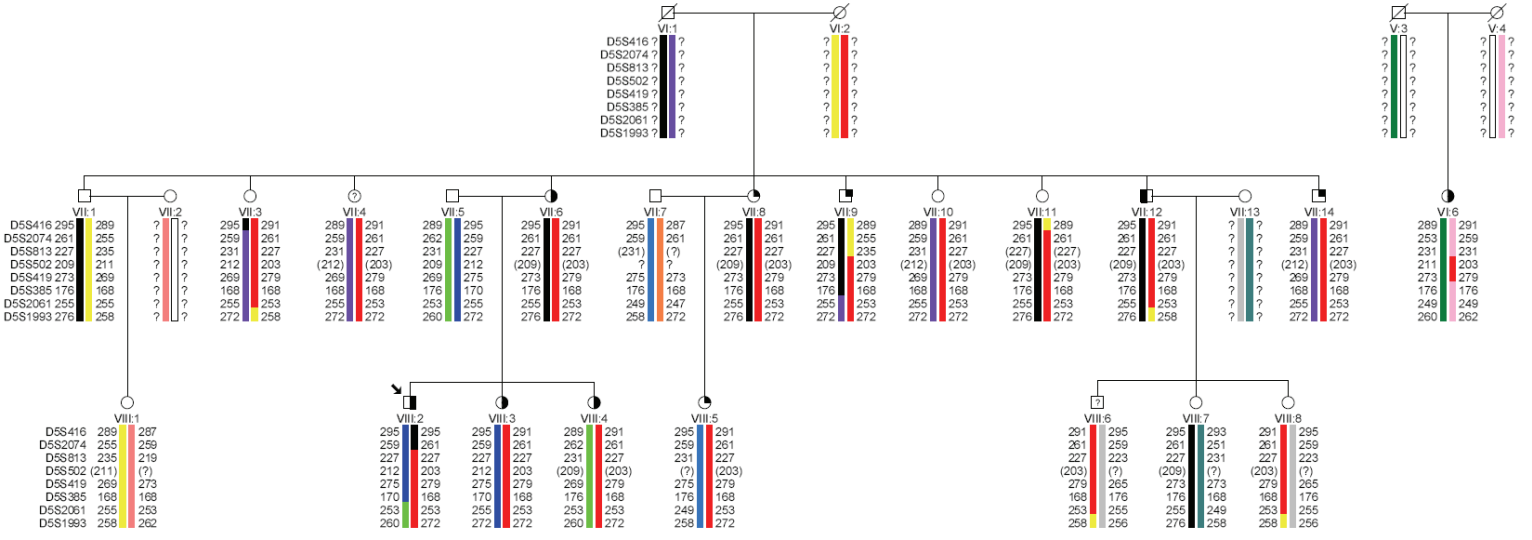
A. Four-chamber view of the heart at 23 weeks' gestation with increased apical myocardial thickness.
B. Color Doppler ultrasound of the apical right ventricular wall at 36 weeks' gestation shows filling of the non-compacted myocardium.
C. Graph of cardiothoracic (CT) ratio indicating decrease of cardiomegaly during gestation.



Chapter 6: Figure 2

Pedigree with haplotype analysis of chromosome 4

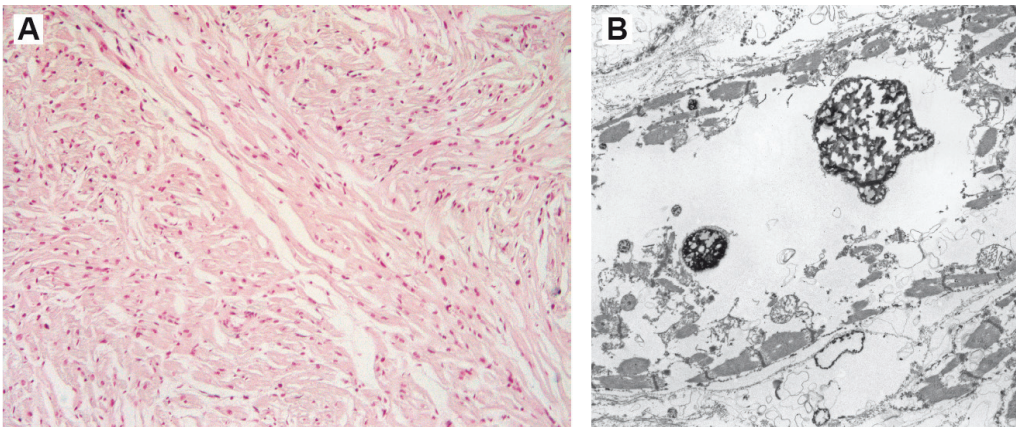
The arrow indicates the proband



Chapter 6: Figure 3

Pedigree with haplotype analysis of chromosome 5

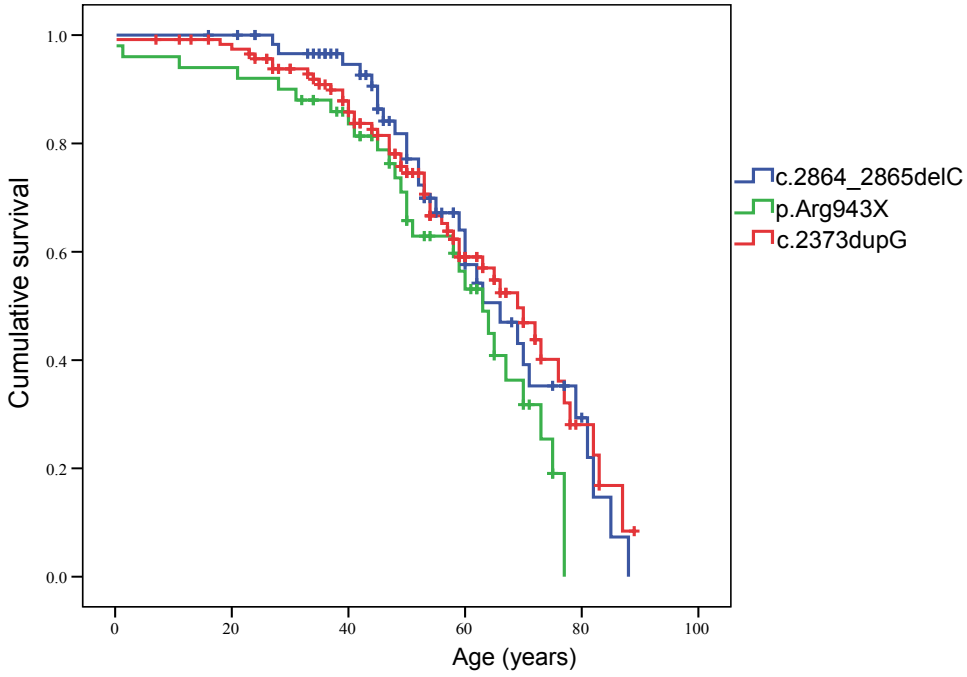
The arrow indicates the proband



Chapter 7: Figure 1 Histopathology

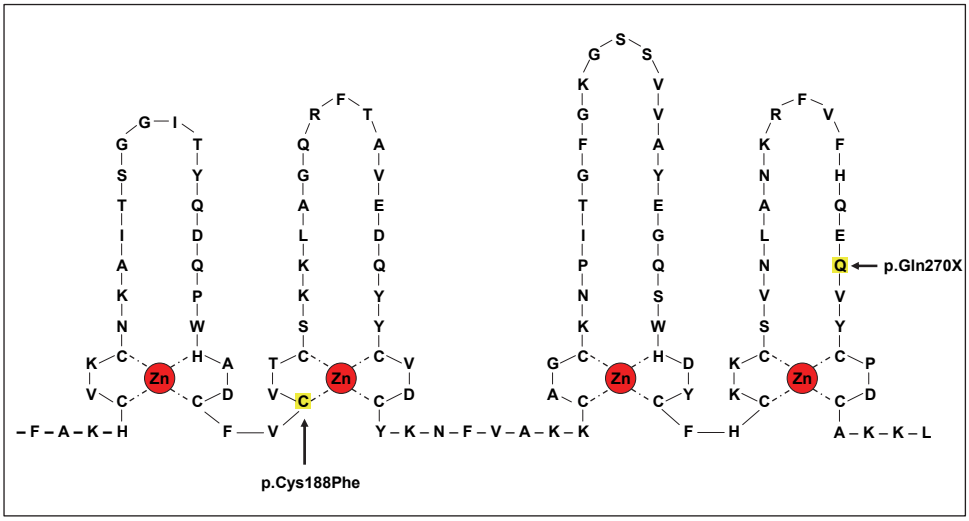
A. Microscopic examination of postmortem heart muscle from patient 1. Hypertrophic myocytes with myofibrillar disarray typical of HCM due to sarcomeric protein mutations, was present, albeit without significant amount of interstitial fibrosis.

B. Electron micrograph showing a large, irregular vacuole



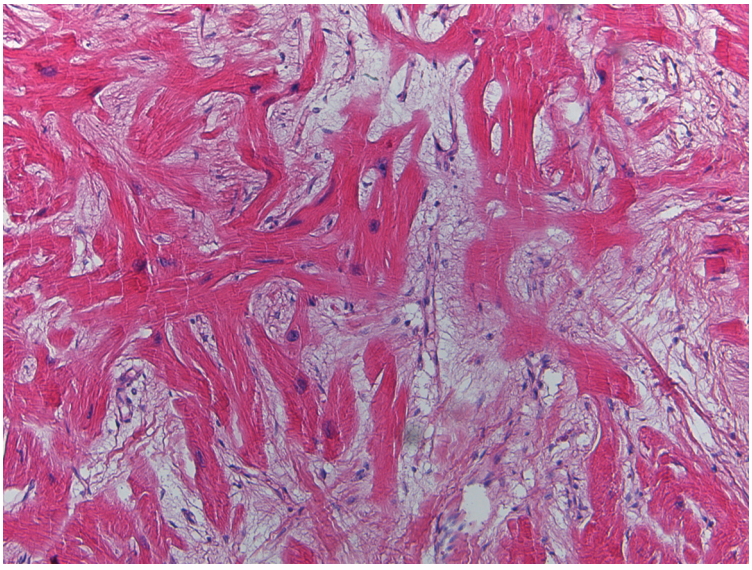
Chapter 9: Figure 2

Survival associated with truncating *MYBPC3* mutations.
Kaplan-Meier survival curves for a group of 117 patients from 26 c.2373dupG families, 50 patients from 16 p.Arg943X families and 62 patients from 12 c.2864_2865delCT families.



Chapter 10: Figure 1

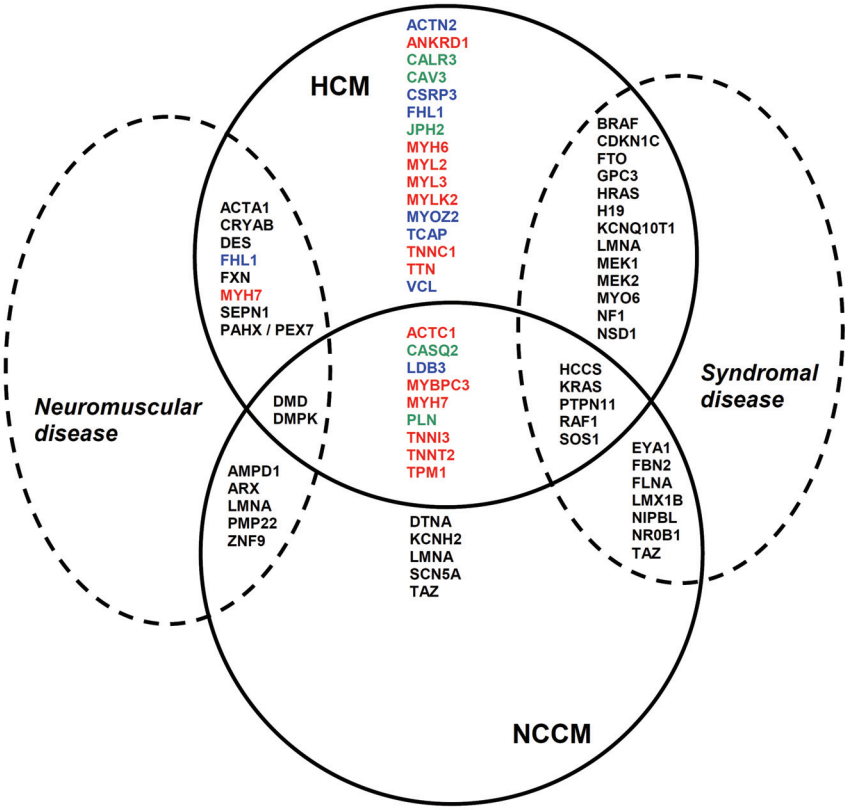
The 3[d] and 4[th] LIM domain showing the positions of the identified *FHL1* mutations.



Chapter 10: Figure 5

Post-mortem microscopy of relative IV:2 of patient 1.

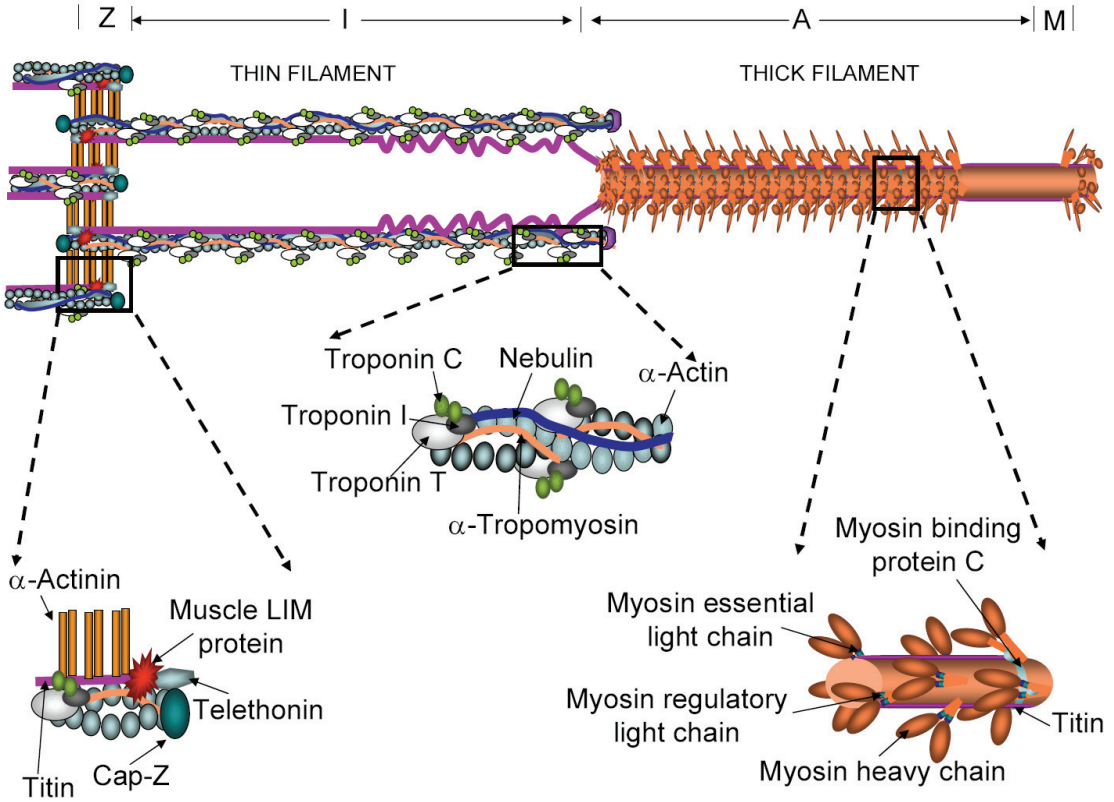
Note the myocyte disarray, with oblique and sometimes perpendicular orientation of the cardiomyocytes, interstitial fibrosis and irregular, polymorphic and hyperchromatic nuclei.



Chapter 13: Figure 1

Heterogeneity and overlap of disease-causing genes in HCM and NCCM and in neuromuscular and syndromal disease.

Red displays sarcomere genes, blue Z-disc genes and green calcium-handling genes. *ACTA1*: α-Skeletal actin; *ACTC1*: α-Cardiac actin; *ACTN2*: α-Actinin 2; *AMPD1*: Adenosine monophosphate deaminase 1; *ANKRD1*: Cardiac ankyrin repeat protein; *ARX*: Aristaless-related homeobox; *BRAF*: V-RAF murine sarcoma viral oncogene homolog B1; *CALR3*: Calreticulin; *CASQ2*: Calsequestrin; *CAV3*: Caveolin; *CDKN1C*: Cyclin-dependent kinase inhibitor 1C; *CRYAB*: α-B Crystallin; *CSRP3*: Cysteine and glycine rich protein; *DES*: Desmin; *DMD*: Dystrophin; *DMPK*: Dystrophia myotonica protein kinase, *DTNA*: α-Dystrobrevin; *EYA1*: Eyes absent 1; *FBN2*: Fibrillin 2; *FHL1*: Four-an-a-half LIM domains; *FLNA*: Filamin A; *FXN*: Frataxin; *GPC3*: Glypican 3; *HCCS*: Holocytochorme C synthase; *HRAS*: V-HA-RAS Harvey rat sarcoma viral oncogene homolog; *H19*: H19 gene; *JPH2*: Junctophilin 2; *KCNH2*: Potassium channel, voltage-gated, subfamily H, member 2; *KCNQ10T1*: KCNQ1-overlapping transcript 1; *KRAS*: V-KI-RAS2 Kirsten sarcoma viral oncogene homolog; *LDB3*: LIM domain-binding 3; *LMNA*: Lamin A/C; *LMX1B*: LIM homeobox transcription factor 1; *MEK1*: Mitogen-activated potein kinase kinase 1; *MEK2*: Mitogen-activated potein kinase kinase 2; *MYBPC3*: Cardiac myosin-binding protein C; *MYH6*: α-Myosin heavy chain; *MYH7*: β-Myosin heavy chain; *MYL2*: Ventricular regulatory myosin light chain; *MYL3*: Ventricular essential myosin light chain; *MYLK2*: Myosin light chain kinase 2; *MYO6*: Myosin VI; *MYOZ2*: Myozenin 2; *NF1*: Neurofibromatosis 1; *NIPBL*: Nipped-B-like; *NR0B1*: Nuclear receptor subfamily 0, group B, member 1; *NSD1*: Nuclear receptor-binding Su-var, enhancer of zeste, and trithorax domain protein 1; *PAHX*: Phytanoyl-CoA hydroxylase; *PEX7*: Peroxisome biogenesis factor 7; *PLN*: Phospholamban; *PMP22*: Peripheral myelin protein 22; *PTPN11*: Protein-tyrosin phophatase, nonreceptor-type 11; *RAF1*: V-RAF-1 Murine leukemia viral oncogene homolog 1; *SCN5A*: Sodium channel, voltage-gated, type V, α-subunit; *SEPN1*: Selenoprotein N1; *SOS1*: Son of sevenless, drosophila homolog 1; *TAZ*: Taffazin; *TCAP*: Telethonin; *TNNC1*: Cardiac troponin C; *TNNI3*: Cardiac troponin I; *TNNT2*: Cardiac troponin T; *TPM1*: α-



Chapter 13: Figure 2

The sarcomere and its proteins
Z, I, A and M represent corresponding sarcomere zones and bands.

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Femke heeft nu al een enorm netwerk van hoogopgeleiden.

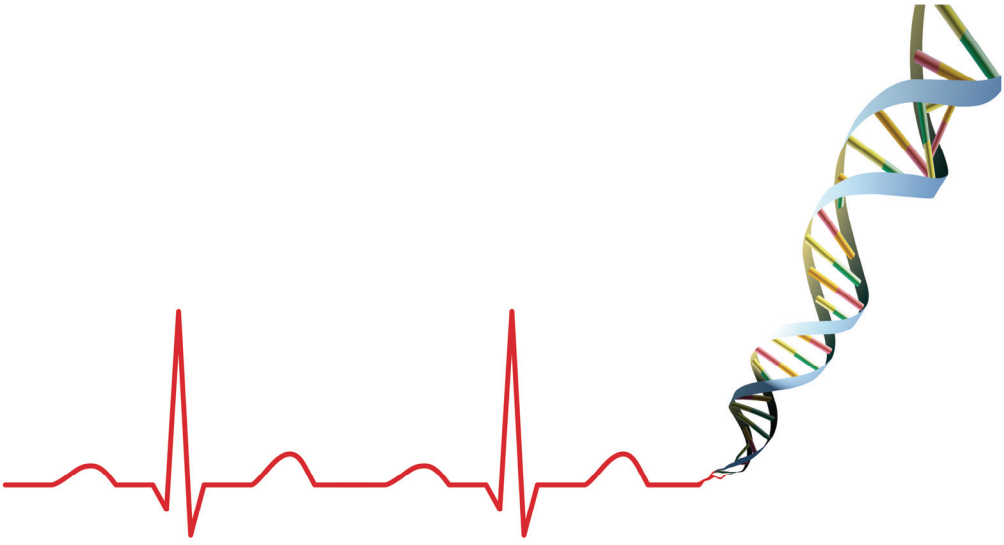
Femke is pas 4 jaar. Toch kent ze haar hele leven al tientallen artsen, wetenschappers, doctorandussen en andere hoogopgeleiden. En zij kennen haar. Want ze heeft cardiomyopathie, een ongeneeslijke hartspierziekte.

Een kleine 1.000 kinderen in Nederland lijdt aan cardiomyopathie. Een ernstige, vaak aangeboren hartspierziekte. Een te groot deel van hen sterft op jonge leeftijd. Of kan alleen verder leven na een harttransplantatie. Er is helaas nog weinig bekend over cardiomyopathie bij kinderen. De Stichting Hartedroom wil dit veranderen en financiert wetenschappelijk onderzoek naar kindercardiomyopathie. Onder meer om de behandeling en levenskwaliteit van deze zeer zieke kinderen te verbeteren. Kijk op **www.hartedroom.nl** voor meer info en donaties. Steun ons. Elke bijdrage telt. Echt.

Stichting Hartedroom

Steun onderzoek naar cardiomyopathie: rek.nr. 24.93.33.333 t.n.v. Stichting Hartedroom

Dankwoord



Dankwoord

Als ik iets geleerd heb tijdens mijn promotie onderzoek dan is het wel dat het leven vol uitdagingen zit. Grote uitdagingen en kleine uitdagingen… Zie hier mijn laatste uitdaging, het dankwoord, misschien wel het belangrijkste en mogelijk het meest gelezen deel van mijn proefschrift…

Ten eerste wil ik alle patiënten en familieleden hartelijk danken voor hun medewerking en de getoonde interesse voor mijn onderzoek. Zonder u had dit proefschrift er niet gelegen.

Dan mijn co-promotoren: Danielle Majoor – Krakauer en Dennis Dooijes. Beste Danielle, jouw grenzeloze inzet is van onschatbare waarde geweest, van de aanvraag voor de subsidie van de Hartstichting tot de laatste bladzijde van mijn proefschrift ben je ongelofelijk betrokken geweest. Bovendien heb je de structuur voor de cardiomyopathie counseling en het familieonderzoek vorm gegeven. Hartelijk dank voor al je inspanningen! Beste Dennis, je hebt de cardiogenetische DNA diagnostiek in Rotterdam op de kaart gezet. Dankzij jouw inspanningen was de uitgebreide diagnostiek van de cardiomyopathie patiënten mogelijk. Met je vertrek naar Utrecht heb je zo een grote nalatenschap in Rotterdam achtergelaten, waar niet alleen ik, maar het hele cardiogenetica team je dankbaar voor is. De discussies, onder andere over diagnostiek en etiologie, zijn uitermate leerzaam geweest voor mijn academische vorming. Dank voor al je hulp!

Mijn promotoren: Prof.dr. Oostra, beste Ben, de begeleiding van de laatste loodjes was zeer waardevol. Ik heb bewondering voor de rust waarmee je me altijd onmiddellijk te woord stond in tijden van nood en ik wil je ook nog graag expliciet bedanken voor het vertrouwen dat alle deadlines daadwerkelijk gehaald zouden worden en dat mijn boekje op tijd af zou komen. Prof.dr. Simoons, beste Maarten, hartelijk dank voor uw tijd en het meegaan in de in uw ogen wellicht wat onorthodoxe manier van het afronden van mijn proefschrift.

De leden van de leescommissie, Prof. van Langen, Dr. Balk en prof. Wilde. Beste Irene, Aggie en Arthur, maar liefst ruim een week voor de deadline kreeg ik bericht dat mijn proefschrift goedgekeurd was. Hartelijk dank voor deze snelle beslissing en de genomen moeite.

Op één been kun je niet lopen… Zo heeft ook dit proefschrift twee benen (nee, geen poten), de afdelingen cardiologie en klinische genetica.

Te beginnen met het linkerbeen, de cardiologie. Mijn onderzoek was niet mogelijk geweest zonder de inspanningen van de cardiologen met wie ik nauw samen gewerkt heb. Geachte Dr. ten Cate, beste Folkert, de huidige opzet van de cardiogenetica poli was nooit zonder u ontstaan. Met uw uitgebreide ervaring in de diagnostiek en behandeling van HCM is het Thorax

Centrum van het Erasmus MC een tertiair verwijs centrum voor HCM geworden, wat heeft geleid tot de grote huidige Rotterdamse HCM populatie. Beste Kadir Caliskan, ik kende je al vanuit je laatste jaar in opleiding, toen je me uitlegde hoe ik een ECG moest beoordelen. Het lijkt allemaal zo lang geleden… Zonder jouw verwijzingen waren er in ieder geval geen NCCM patiënten voor mij. Samen hebben we er toch maar een mooi cohort van gemaakt! Beste Michelle Michels, je bent altijd snel in je antwoord, altijd bereid om een CD-tje van elders te (her)beoordelen. Fijn dat je Kadir kon helpen met het zien van de vele NCCM familieleden en dank voor de snelle berichtgeving in Elpado en je inspanningen voor de modifier paper! Beste Tjebbe Galama, dank voor de ruimte die de klinische genetica op de poli kreeg. Jan Res, Agnes Muskens, Eric Sijbrands en Janneke Langendonk, zonder jullie is de cardiogenetica bespreking op vrijdag niet meer compleet! Eric, dank je wel voor je steun en interesse in mijn onderzoek en mijn presentaties. Alhoewel ik de cursus kritisch literatuur lezen helaas niet volledig heb kunnen volgen was het zeer waardevol. Prof. Roos – Hesselink, beste Jolien, dank voor je getoonde interesse in mijn onderzoek en voor de fijne samenwerking bij mijn niet - cardiomyopathie patiënten. Wim Vletter, hartelijk dank voor het maken van de vele echo's. Kitty, altijd behulpzaam, dank voor het inplannen van alle patiënten. Celeste, bij afwezigheid van Kitty kon ik altijd bij jou terecht. De dames van de functie en de Holter afdeling: Cindy Macnack, Anouschka van Moorselaar, Irma den Adel, Conny de Ruiter, Istrelia Duijzer, Memento Almeida, Mariette Mijnlieff, Ramona Beversluis, Donna Bode, Lenne Stok – Hoogerwerf, Angela Peterse, Petra de Haan en Jeanette Steenhorst, hartelijk dank voor het bloed prikken, het afnemen van fietstesten en het aanhangen van Holters bij de vele cardiomyopathie patiënten en hun familieleden. Ook de dames achter de balie op de poli cardiologie: Corrie Scheepers, Rina de Kruijff, Anita van der Jagt, Henny Kroos, Hanneke van der Most, Miriam van der Hoeven, Conny Roos en Marianne Grauss, dank voor jullie hartelijkheid, gezelligheid en medeleven. Tijdens mijn jaar op de poli cardiologie heb ik lief en leed gedeeld met Susan Valk, Lucia Jewbali, Tabitha Valentijn, Natascha Rabatic, Raemon Baak, Jawed Polad en Chris van der Lee. Bedankt hiervoor! Hartfalenverpleegkundigen Ellen Klaassen en Ymkje Hendriksma, bedankt voor jullie blijvende interesse en voor jullie hulp in het "vangen van Kadir". Ron van Domburg, hartelijk dank voor je hulp bij het last-minute statistiek werk! Beste Willeke van der Bent, mijn promotie moet een ware nachtmerrie voor je zijn geweest, zonder de gebruikelijke structuur van een cardiologische promotie. Tirza Springeling, bedankt voor het leveren van de plaatjes voor mijn kaft.

Ook de kindercardiologen waren van groot belang in mijn onderzoek. Beste Ingrid Frohn – Mulder, Michiel Dalinghaus, Lennie van Osch – Gevers, Wim Helbing, Frederik du Plessis en (inmiddels overgestapt naar de congenitale poli bij de volwassenen) Maarten Witsenburg, dank voor het beoordelen van de kinderen die verwezen werden en dank voor het verwijzen van de kinderen bij wie als eerste in hun familie een NCCM werd vastgesteld. Ingrid en Michiel, hartelijk dank voor jullie tijd en hulp met het schrijven van de artikelen. Ingrid, je kwam er zelfs voor op je vrije woensdag naar het ziekenhuis, helemaal geweldig!

Daar ik zelf rechtsbenig ben, betreft het rechterbeen van mijn proefschrift de klinische genetica. Beste Fred Petrij, niet alleen vroeg je me regelmatig hoe het onderzoek ging, je vroeg ook hoe het met mij ging. Die vraag was zelden een overbodige luxe, dank voor je luisterend oor. Beste Anneke Maat – Kievit, ook van jouw kant regelmatig vragen van interesse, of alles volgens planning en naar wens liep. Dank ook voor de ruimte die ik kreeg sinds het begin van mijn opleiding voor het afronden van de “laatste loodjes”. Ook de andere stafleden wil ik graag bedanken voor hun begrip en steun, Jeanette Hoogeboom, Grazia Mancini, Marleen Simon, Alice Brooks, Margriet Collée, Anja Wagner, Rogier Oldenburg, Grétel Oudesluijs, Marieke van Dooren (je was zo trots toen ik de wetenschapsdag gewonnen had…), Marieke Joosten (ja, je staat nu ook in het rijtje bazen…), Robert-Jan Galjaard, Lutgarde Govaerts (dank voor je voortdurende interesse in mijn onderzoek) en natuurlijk Yolande van Bever, dank je voor de ruimte die je voor me hebt gecreëerd en dat je mijn mentor wilde zijn. Beste Marja Wessels, dank voor het meedenken en schrijven, ik heb de samenwerking altijd als prettig ervaren. Ingrid van de Laar, je had een belangrijke bijdrage in de eerste NCCM familie bij wie we een mutatie vonden, dank hiervoor! Fijn dat je na je opleiding als staflid aan de slag kon op de afdeling, je bent een aanwinst voor het cardiogenetica team!

Ook de artsen op de afdeling wil ik graag bedanken voor hun steun en interesse. Beste Lieke Berger, Marije Meeuwissen, Renske Oegema, Laura van Loon, Danielle Bosch en Shimriet Zeidler, dank voor jullie betrokkenheid. Dan mijn kamergenoten, wat zou ik zonder jullie gemoeten hebben. Wanneer tot 10 tellen niet genoeg was leerden jullie me nog een keer tot 10 te tellen. Beste Rogier Kersseboom, je bent de nodige Y-factor tussen al die X-factors… Beste Karin Diderich, tijdens je zwangerschapsverlof is het wel erg stil op onze kamer… Beste Judith Verhagen, ook jij bent een ware aanwinst voor het cardio-team, met jou kan ik lachen en huilen… Beste Anja Kattentidt, dank voor de gezelligheid in Liverpool en Bologna! En natuurlijk Esther de Theije – Kors, wat een krachtige vrouw ben jij! De hele afdeling put moed uit jouw positieve instelling. Ik blijf hopen op een medisch wonder…

Inmiddels vertrokken maar tegelijk met mij begonnen in Rotterdam, Nienke Verbeek, je was een fijne collega, jammer dat je niet in Rotterdam bent gebleven! Ook Ingrid Krapels, met roots in Rotterdam, jij bent in Maastricht ook de cardio ingerold en dat heeft je geen windeieren gelegd! Dank je wel voor je interesse in mijn onderzoek. Eveneens vertrokken maar van vitaal belang geweest voor mijn carrière, Hanne Meijers – Heijboer, nu professor aan de VU in Amsterdam, je nam me aan voor de cardiogenetica, dank zij jou raakte de bal aan het rollen. Hartelijk dank hiervoor!

Alle genetisch consulenten, Annemarieke Otto, Charlotte Castenmiller (je trouwjurk poll houdt de hele afdeling bezig), Desiree de Jongh, Ingrid van Kessel, José van Montfoort – Kroes, Judith Prins – Cornelisse, Lydia Overbeek en Margreethe van Vliet, dank voor jullie steun en interesse. Conny van de Meer, je hebt een hoop productie gedraaid in de cardiogenetica, het is fijn samenwerken met je! Judith Phefferkorn, hoe kan ik jou ooit genoeg bedanken! Je bent mijn steun en toeverlaat. Het lijkt wel gisteren dat je je in de cardio-wereld stortte, inmiddels ben je de spil van de cardiomyopathie counselingen geworden. In de loop van de jaren hebben we

een hoop meegemaakt met toch wel als hoogtepunt het congres in Florence, waar je de schrik van je leven kreeg toen ik je bij kaarslicht iets wilde vragen… …of je mijn paranimf wilde zijn! Je zei (natuurlijk) ja en daar ben ik erg blij mee. Dank je wel voor alles!
Samantha Riedijk, super dat iedereen, patiënt en counselor, bij jou terecht kan! Je bent een enorme motivator en weet iedereen enthousiast te krijgen. Dank je wel voor wie je bent! Uiteraard vullen Prof. Tibben en Kathleen Vanheusden het psychologen team perfect aan. Aad, altijd de rust zelve en Kathleen, jammer dat je weggaat!
Het datateam, Aart Leegwater, Anja Nieuwlaat, Mayra de Man en, inmiddels ook vertrokken, Sintyha Buddha. Wat een luxe dat jullie de cardio-stambomen maken en updaten. Anja, de database die je hebt gemaakt is van groot wetenschappelijk belang, hieruit kunnen we in de toekomst nog eindeloos putten! Sintyha, jammer dat je wegging, je inzet was reuze en het was altijd gezellig met jou! Aart, je bent altijd lekker snel met de stambomen, dank je wel hiervoor! Mayra, ik ga je kiprecept echt een keer uitproberen, waarschijnlijk als dit oeuvre dan eindelijk bij de drukker ligt…
Beste Marlies Veldhuizen, Liesbeth Middelkoop – Joppe, Marjolijn de Jonge, Gisla Damrie, Isabel Abazie – van de Boogert, Wilma Verbrugh (dank je wel voor het bloedprikken bij de patiënten), Joke Kruithof – van de Bergen, Agnes Jansen – Spelt, Daphne ‘t Hart, Hellen de Bruin – van de Wende, Ruud Voorburg, Gulay Aydogan, Dilek Gönül – Sever en Elly Kranendonk, zonder jullie ligt de hele sectie counseling stil! Beste Gisla, altijd bereid toch nog even dat spoedje weg te werken, ik wil je graag hartelijk bedanken voor je inzet! Hans Blanksma, je bent al weer een tijdje bij ons weg, hopelijk heb je het naar je zin op je nieuwe plek. In ieder geval bedankt voor je hulp. De poli-dames, Corry van de Weegen – Pompstra en Serena Rosman, jullie maken het verschil op de Westzeedijk! Corry, je bent een ware moederkloek die haar weidse vleugels als een warme deken over de afdeling legt! Serena, ik heb bewondering voor jouw levensinstelling en kan alleen maar tegen je zeggen: “hand in hand, kameraden!!!”
Ellen Crepin en Elisabeth Huijskens, op de Westzeedijk zaten we nog op dezelfde kamer, het was gezellig met jullie. Esther Buunk, ook jij bedankt voor je interesse.
Marjoleine van Berckel Bik, wat een aardverschuiving toen jij wegging! Gelukkig ben je niet helemaal weg, hartelijk dank voor al je regelwerk op de afdeling.
Technische ondersteuning is ook geen overbodige luxe op de afdeling, als de V-schijf er weer eens uit ligt of Thunderbird moeilijk doet. Om de een of andere reden lijkt dat vaker op een maandag voor te komen (is hier sprake van het “maandag-syndroom”?), maar Ton Verkerk, Mario Redeker en Pim Visser staan altijd klaar om het euvel te verhelpen. Dank hiervoor! En natuurlijk Tom de Vries Lentsch, ondanks je vele bezigheden vond je altijd de tijd om “nog even” een figuur voor me aan te passen of een poster of de kaft van dit proefschrift in elkaar te zetten. Je tips en hulp bij de lay-out van dit boekje waren heel welkom! Ruud Koppenol, dank voor je hulp bij het verwerken van alle foto’s, je geduld wordt zeer gewaardeerd! Ook wil ik graag de altijd vrolijke Jane Pique bedanken voor het schoonhouden van onze werkplekken, zelfs als heel schoonmakend Nederland staakt!

Naast (de omvangrijke) sectie counseling bestaat de afdeling klinische genetica natuurlijk ook uit de diagnostische laboratoria. Zonder DNA diagnostiek had ik niet zulke mooie resultaten behaald. Marjon van Slegtenhorst, fijn dat jij de cardio diagnostiek voor je rekening hebt genomen nadat Dennis wegging. Het is goed zaken doen met je (en ook altijd weer gezellig), je bent altijd zo snel in het antwoorden van je mail. Martina Wilke, als kamergenoot van Marjon moest je me vaak tolereren, sorry voor de vele onderbrekingen! Hetzelfde geldt voor Dr. Halley, beste Dicky, toen je nog met Dennis een kamer deelde op de 24e heb ik ook jou meerdere malen moeten storen. Dank je wel voor je geduld en voor je interesse. Beste Marianne van Tienhoven, gedeelde smart is halve smart! Dank je wel voor je altijd snelle reactie als ik overzichtsgegevens nodig had en bedankt voor je hulp met het maken van de figuren. Onze lunches zijn altijd om naar uit te kijken! Raoul van de Graaf, dank je wel voor je waardevolle hulp met de diagnostiek. Wilma Mouthaan, dank je wel voor je vrolijkheid en je behulpzaamheid, we zullen je allemaal missen.
Ook op het research lab werd hard meegewerkt aan mijn proefschrift. Dear Aida Bertoli – Avella, thank you for all your time and patience and for our always enjoyable meetings. Lilian Parsaulian, thank you so much for your valuable work on the linkage study. Hetzelfde geldt uiteraard voor Christan Rohe, hartelijk dank! Beste Jeanette Lokker, hartelijk dank voor al je hulp rondom alle promotie bureaucratie! Het uitprinten van mijn proefschrift was een hele klus, dank hiervoor!

Natuurlijk was er ook samenwerking met andere afdelingen. Beste Titia Cohen – Overbeek en Ernst Schoonderwaldt, dank voor jullie hulp, ik heb veel van jullie geleerd over prenatale cardiomyopathieën. René de Coo, dank voor je neurologische inzichten en de informatieve tramritjes. Joanne Verheij, veel dank voor je snelle hulp en de prachtige PA-plaatjes! Prof. Danser, beste Jan, dank voor je betrokkenheid. David Alexander, thank you for your inspirational course, I hope you will not be too disappointed by my writing…

Cardiogenetisch Nederland is klein, maar stiekem groeit deze groep heel hard. Teveel mensen om allemaal apart te noemen, dus dank ik de hele ICIN werkgroep voor erfelijke hartziekten hartelijk. Elke vergadering is weer een bron van inspiratie. Een aantal mensen wil ik graag apart noemen. Uit Groningen: Peter van Tintelen en Rina Keupink hartelijk dank voor jullie enthousiasme en de bergen werk die jullie verzet hebben in de linkage studie! Ook Paul van Haelst verdient een zeer eervolle vermelding! Zonder jou had ik niet afgeweten van dat verre familielid! Uit Utrecht: Jasper van der Smagt, ook jij bent altijd enthousiast geweest over mijn onderzoek en stuurde patiënten in. Helaas werd je getroffen door een ernstige ziekte, veel sterkte in het herstel! Eveneens uit Utrecht: Bert Baars, dank voor het getoonde vertrouwen om mij een hoofdstuk te laten verzorgen in het cardiogenetica boek, ik ben erg benieuwd naar het totale resultaat. Uit Nijmegen: Carlo Marcelis, toen ik wegging uit Nijmegen gaf je me een heel pakket cardiogenetische literatuur mee, dat was mijn initiatie… Dank je wel! Uit Leiden: Anneke van Mil, die gezamenlijke familie die we hebben is helaas nog niet opgelost, maar we gaan

dapper verder! Uit Amsterdam: Imke Christiaans, jij mag een dag voor mijn verdediging de hete kastanjes vast uit het vuur halen. Veel succes met jouw verdediging! Wanda Hermans – van Ast, dank voor je hulp met de ICIN adressen!

Dan zijn we nu aangekomen bij het hart van mijn dankwoord. Het is maar goed dat het promoveren zelf niet door een medisch-ethische commissie getoetst hoeft te worden. Gezien de sociale deprivatie die kenmerkend is voor het leven van een promovendus zou het promoveren al gauw verboden worden… Bij deze dan ook eerst mijn welgemeende excuses aan familie en vrienden voordat ik met mijn dankwoord verder ga…

Lieve Eddy, wat heb je allemaal moeten doorstaan? Ik heb je tot wanhoop en zelfs tot (letterlijk) koken gedreven (het was heel goed te eten, misschien voor herhaling vatbaar?)… Ik ben je ongelofelijk dankbaar voor je steun, voor het geven van perspectief en voor je nuchtere kijk op de wetenschappelijke wereld. Dat je zoveel tijd hebt gestoken in de lay-out van mijn boekje geeft duidelijk je betrokkenheid aan. Te quiero mucho, met jou wil ik oud en rimpelig worden!

Lieve pap en mam, mijn dankbaarheid is eindeloos. Voor jullie stimulatie, steun en begrip, voor jullie medeleven en meedenken. Niet alleen in mijn promotie traject, maar in mijn hele leven. Voor wie jullie zijn, voor wat jullie van mij gemaakt hebben (zowel nature als nurture…) en voor wat jullie allemaal voor mij gedaan hebben. Jullie zijn er altijd voor me geweest, ik kan altijd op jullie rekenen. Miljoenen schapen (nee, dat is niet overdreven…) zijn onvoldoende om mijn liefde en dankbaarheid uit te drukken!

Lieve Lilian, mijn grote zus… Dank je wel voor je andere kijk, voor je betrokkenheid en voor je zeer rustgevende massages! Je betekent veel voor mij! En samen met Stefan heb je toch maar even twee prachtige meiden op de wereld gezet. Lieve Sara en Eva, ik zie jullie veel te weinig naar mijn zin, maar geniet met volle teugen van elke keer. Hoogste tijd voor een nieuw dagje Rotterdam met jullie!

Lieve Miriam. Mijn jongste zusje… mijn paranimf (je moet toch wat bij gebrek aan behoefte aan een ceremoniemeester…). Je bent geweldig! Zo sterk, zo kordaat. Hoogzwanger met een man uitgezonden naar Afghanistan, petje af! Don, dank je wel dat je uit de buurt van de bermbommen bent gebleven en heelhuids terug kwam! Ook jullie hebben samen voor een prachtige uitbreiding van de familie gezorgd. Lieve Nynke, je bent nog klein maar het is nu al alsof je er altijd al was. Je glimlach is er een uit duizenden! Jou de fles geven was een zegen in hectische tijden…

Lieve Mariska, beste Peter. Wat een geluk dat jullie onze buren zijn. Maris, je bent altijd erg betrokken geweest. Dank je wel voor je meedenken en voor je luisterend oor en natuurlijk voor je paella!

Bao Chau en Louis, Judith en Koen en Hilde en Robert, dank je wel voor jullie begrip. Ik ben ernstig in gebreke gebleven maar kom zeker alsnog op kraambezoek!

Elly en Jork en Karin en Erik, dank jullie voor jullie interesse en steun. Elly en Jork, bedankt voor de MSN-sessies, altijd net op het moment dat ik het nodig had! Karin, je ging me voor… Als eerste in onze generatie van de familie promoveerde je. Dank je wel voor het delen van je ervaringen.

Dan is het nu tijd om het humane achter me te laten en nog een paar speciale exemplaren uit het dierenrijk te bedanken… Guusje, als ik jou aai daalt mijn bloeddruk spontaan! Je voorliefde voor riet is op zijn zachtst gezegd vreemd te noemen maar wel uiterst vermakelijk! Sukke, je bent zo lekker zacht, en met je geklets maak je er een vrolijke boel van. Ondanks jullie voorkeur voor mijn toetsenbord kan ik me geen andere katten wensen. Fokke, ik mis je… En wie het kleine niet eert… ook de eendjes in de sloot wil ik graag bedanken. Voor de welkome rust die jullie me gaven als ik jullie hele broden toe wierp.

Toch nog even terug naar de mensenwereld, last but not least: Eduard Douwes Dekker. Zelfs je pseudoniem is mooi, Multatuli. Uitkijkend over de van Oldenbarneveltstraat in het centrum van Rotterdam, hebben zijn wijze woorden mij zeer aangesproken: “Van de maan af gezien zijn we allen even groot”. Woorden die ik zowel letterlijk als figuurlijk heb geïnterpreteerd en waar een grote troostende waarde vanuit gaat.