Hypertrophic Cardiomyopathy: Pathophysiology, Genetics and Invasive Treatment

Hypertrofische cardiomyopathy: Pathofysiologie, genetica en invasieve behandeling

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HYPERTROPHIC CARDIOMYOPATHY: PATHOPHYSIOLOGY, GENETICS and INVASIVE TREATMENT

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Voor Anne, Floris en Puck

ABBREVIATIONS

ACTC1	Cardiac α-actin
ASA	Alcohol septal ablation
CFR	Coronary flow reserve
ECG	Electrocardiogram
HCM	Hypertrophic cardiomyopathy
HTX	Heart transplant
IVS	Interventricular septum
LAD	Left anterior descending coronary artery
LV	Left ventricle/ ventricular
LVH	Left ventricular hypertrophy
LVOT	Left ventricular outflow tract
MLE	Mitral leaflet extension
MYBPC3	Cardiac myosin binding protein C
MYH7	Beta-cardiac myosin heavy chain
MYL2	Cardiac-regulatory myosin light chain
MYL3	Cardiac-essential myosin light chain
NYHA	New York Heart Association functional class
PTSMA	Percutaneous transluminal septal myocardial ablation
RF	Risk factors
SAM	Systolic anterior movement
S(C)D	Sudden (cardiac) death
TDI	Tissue Doppler imaging
TNNI3	Cardiac troponin I
TNNT2	Cardiac troponin T
TPM1	α-Tropomyosin

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Chapter 1

Introduction

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 (LVH) in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality[1]. Hundreds of mutations scattered among at least 25 putative HCM susceptibility genes encoding various sarcomere, Z-disk, calcium-handling, and mitochondrial proteins are known to cause HCM and are found in up to 60% of cases[2]. Besides the genetic heterogeneity, HCM is also characterized by phenotypic heterogeneity; ranging from negligible to extreme hypertrophy, absent or severe left ventricular outflow tract obstruction, normal longevity or premature sudden cardiac death (SCD), even in patients carrying the same pathogenic HCM mutation[3, 4].

Since the introduction of HCM as a clinical entity in 1958, extensive research has been performed regarding diagnosis, prognosis, therapy and genetics. However, correct diagnosis and optimal management of HCM patients and their asymptomatic affected family members still offers difficulties to the physician.

This thesis describes the findings of HCM patients followed at the ErasmusMC in Rotterdam for years. It is directed towards the different pheno- and genotypical aspects of this disease. Furthermore results of basic sarcomere investigations, pathophysiologic characteristics to explain clinical findings, therapeutic measures both with and without surgery and natural history are described.

The reduction of coronary flow reserve in HCM predisposes to myocardial ischemia, systolic dysfunction and cardiac death[5]. In **chapter 2** we investigated to which extent hemodynamic, echocardiographic, and histological parameters contribute to this reduction. In **chapter 3 and 4** relatively new echocardiographic techniques; Tissue Doppler imaging and speckle tracking are evaluated in HCM patients and in mutation carriers without LVH. In **chapter 5** we go from bedside to bench by studying cardiomyocytes from HCM patients with two known founder mutations in *cardiac myosin binding protein C*[6].

The identification of the genetic defect in one of the HCM genes allows accurate pre-symptomatic 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 SCD, which can be the first manifestation of the disease in asymptomatic persons **(chapter 6 and 7)**[7]. In **chapter 8** the natural history of 3 Dutch founder mutations is described.

The clinical heterogeneity in HCM, even in patients carrying the same underlying pathogenic HCM mutation suggests the presence of disease modifiers[8]. In **chapter 9 and 10** we investigated genetic modifiers in HCM patients carrying one of 3 Dutch founder mutations.

Severely symptomatic patients with obstructive HCM despite maximally tolerated medications are candidates for septal reduction therapy, either surgical or percutaneous[9].

In selected patients with obstructive HCM and elongated anterior mitral valve leaflet septal reduction therapy is combined with mitral leaflet extension, the long-term results of this approach are studied in **chapter 11**. In **chapter 12 and 13** we describe the results of alcohol septal ablation during long-term follow-up and on microvascular function.

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Part I

Pathophysiology of hypertrophic cardiomyopathy

Chapter 2

Coronary flow reserve in hypertrophic cardiomyopathy: relation with microvascular dysfunction and pathophysiologic characteristics

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ABSTRACT

Background

The decrease in coronary flow reserve (CFR) in hypertrophic cardiomyopathy (HCM) predisposes to myocardial ischemia, systolic dysfunction and cardiac death. In this study we investigate to which extent hemodynamic, echocardiographic, and histological parameters contribute to the reduction of CFR.

Methods

In 10 HCM patients (mean age 44 ± 14 years) and 8 heart transplant (HTX) patients (mean age 51 ± 6 years) CFR was calculated in the left anterior descending coronary artery. In all subjects hemodynamic, echocardiographic -and histological parameters were assessed. The relationship between these variables and CFR was determined using linear regression analysis.

Results

CFR was reduced in HCM compared to HTX patients (1.6 ± 0.7 vs. 2.6 ± 0.8, $p < 0.01$). An increase in septal thickness ($p < 0.005$), indexed left ventricular (LV) mass ($p < 0.005$), LV end-diastolic pressure ($p < 0.001$), LV outflow tract gradient ($p < 0.05$) and a decrease in arteriolar lumen size ($p < 0.05$) were all related to a reduction in CFR.

Conclusion

In HCM patients hemodynamic (LV end-diastolic pressure, LV outflow tract gradient), echocardiographic (indexed LV mass) and histological (% luminal area of the arterioles) changes are responsible for a decrease in CFR.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is characterized by a hypertrophied left ventricle (LV) in the absence of apparent causes for hypertrophy such as aortic valve stenosis and hypertension [1]. Angina is frequently present in patients with HCM despite the presence of normal coronary arteries. A reduction in coronary flow reserve (CFR) is a recognized feature in patients with HCM. Indeed, the inadequate increase of myocardial blood flow on demand in HCM predisposes patients to myocardial ischemia, which in turn, can result in syncope, an abnormal blood pressure response to exercise, LV systolic and diastolic function and sudden death [2-6]. Recent studies have shown that severe impairment of the coronary vasodilator response to dipyridamole is strongly associated with "end stage" disease, long-term clinical deterioration and cardiac death in patients with HCM [7,8]. A number of mechanisms are associated with the decrease in CFR, including diastolic dysfunction [9,10], systolic compression of septal coronary branches [11,12], the existence and degree of LV outflow tract (LVOT) gradient [13], small vessel disease of the intramyocardial arterioles [14-16] and inadequate capillary density relative to the increase in myocardial mass [17]. Although all mechanisms have a role in creating an imbalance between oxygen supply and demand, their relative contribution to the decrease in CFR has not yet been evaluated in HCM patients. The aim of the present study is to investigate to which extent each of the aforementioned variables contribute to the reduction of CFR in HCM patients.

METHODS

Patient population

Hemodynamic, echocardiographic, and histological changes in the coronary vascular bed in symptomatic obstructive (LVOT gradient > 50 mm Hg at rest or provocation) HCM patients (N = 10, mean age 44 ± 14 years, range 20-63 years) were compared with a control group of asymptomatic HTX patients (N = 8, mean age 51 ± 6 years, range 40-61 years). In HCM patients septal myocardium was obtained at the time of Morrow myectomy (18). In HTX patients septal myocardium was obtained at the time of routine follow-up right ventricular biopsy 1 to 5 years after HTX. These data were compared with left-sided septal myocardial tissue from victims of traffic accidents (N = 10, mean age 38 ± 19, range 8-58 years) without apparent cardiovascular disease. The institutional review board approved the study and patients gave oral and written informed consent.

Cardiac Catheterization

Cardiovascular therapy was continued during the study in all patients. After intravenous administration of 10.000 IU heparin and 250 mg acetylsalicylic acid right heart

catheterization was carried out with a 7F balloon-tipped flow-directed thermodilution catheter. Left heart catheterization was performed with an 8F double-sensor high fidelity pigtail catheter (Cordis 811-180, Roden, The Netherlands). In each patient, baseline mean pulmonary wedge pressure, LV systolic and end-diastolic pressure, peak LV-aortic gradient, and cardiac index were noted. In addition, coronary angiography was performed according to standard Judkins techniques.

Coronary flow velocity measurements and analysis

After right and left cardiac catheterization, a 0.014-inch Doppler guide wire with a floppy distal end (Cardiometrics, Inc., Mountain View, USA) was introduced through an 8F guiding catheter and positioned at the midsegment of the left anterior descending coronary artery (LAD) to measure Doppler flow velocity at rest and after hyperaemia. In both groups, hearts were paced at a constant heart rate of 100 beats per minute to avoid metabolic vasodilatation during CFR determination. After optimization of the settings of the velocity signal, baseline recordings of flow velocity and perfusion pressure were collected and digitized at a sample rate of 125 Hz for off-line analysis. Maximal hyperaemia was induced by an intracoronary bolus of 18 μg adenosine [19]. The sample volume from the Doppler wire was positioned at a distance of 5.2 mm from the transducer and has an approximated width of 2.25 mm due to a divergent ultrasound beam. After real-time processing of the quadrature audio signal, a fast-Fourier algorithm was used to increase the reliability of the analysis [20]. The flow velocity measurements obtained with this system have been validated in vitro and in an animal model using simultaneous electromagnetic flow measurements for comparison [21]. CFR was defined as hyperaemic divided by basal velocity.

Quantitative angiographic measurements

A validated on-line system operating on digital images (ACA-DCI, Philips, Eindhoven, The Netherlands) was used during the catheterization procedure. With this system, the end-diastolic and end-systolic diameter of the LAD and the proximal part of the first septal branch [22] was determined in the segment of the LAD in which the sample volume of the Doppler wire was located. The derived vessel diameters at the tip of the Doppler guidewire were used to calculate the vessel lumen area assuming a circular shape of the vessel. This vessel area was multiplied with the spatially averaged Doppler velocity (assuming a parabolic profile) to obtain coronary flow (ml/min).

Echocardiography

Two-dimensional echocardiographic studies were performed with commercially available equipment (Toshiba, Tokyo, Japan). Septal and posterior LV wall thickness was measured in end-diastole from both the parasternal short axis and long axis views [23]. LV

mass was calculated according to the Devereux method and subsequently indexed for body surface area[24].

Morphometric analysis of arterioles

The myocardial specimens from HCM patients, HTX patients and victims of car accidents were fixed by immersion in 4% phosphate-buffered formaldehyde. Care was taken that only specimens from the endocardial septal site was utilised for analysis in all three groups. After fixation, the myocardial tissue was processed for paraffin sectioning and staining. For identification and analysis of intramyocardial small arteries an elastic von Giesson staining was used according to Tanaka *et al.* [14]. In brief, each preparation was magnified with a light microscope (Zeiss, Oberkochen, Germany) and digitized for further analysis with a quantitative morphometric system (IBAS system, Kontron, Germany). Only round appearing vessels without side branches were studied. For each arteriole, the wall (intima plus media) and total vessel area were traced. The adventitia was not included in the measurements since the distinction between adventitia and perivascular fibrosis was difficult to visualize. The % luminal area was calculated as L / (W+L) x 100%, where L = lumen area and W = wall area. The external diameter (D) of the intramyocardial coronary arterioles was calculated by the formula: $D = 2 \times \sqrt{((\text{wall} + \text{lumen area}) / \pi)}$. In each subject at least 10 arterioles were identified and used for analysis.

Morphometric analysis of capillaries

In order to elucidate capillary density in the myocardial specimens, sections (4μ) from formalin fixed, paraffin embedded tissue were stained with antibodies directed against the CD-34 epitope (Biogenex, San Ramon, USA) as described below. After deparaffinization and rehydration sections were rinsed with phosphate-buffered saline and incubated with normal goat serum (DAKO, Glostrup, Denmark), antibodies directed against the CD-34 epitope, biolinylated secondary antibodies (Multilink, Biogenex, San Ramon, USA) and peroxidase labelled streptavidin (Biogenex, San Ramon, USA), respectively. The subsequent immunoproduct was visualised with 3.3' diaminobenzidine. In negative controls the primary antibody was replaced by normal mouse serum (DAKO, Glostrup, Denmark). In our experience, staining by Jones' silver methanamine, which was used by Rakusan *et al.* [25] to analyse capillary density in human myocardium, provided suboptimal results, because discrimination of individual capillaries was more difficult. Quantitative morphometric analysis of the sections with an area of 62.500 μm^2 was performed with a specially developed software program applied to a morphometric system (Clemex Techn Inc., Quebec, Canada). We paid special attention that cross-sections were originating from the endo- or midmyocardium, only regions with transversely cut cardiomyocytes were selected and no or only minimal fibrosis was present. For each patient at least five different regions were selected. The number

of cardiac myocytes, and therefore the capillary-myocyte ratio was not determined because of the inability to outline myocytes correctly.

Statistical analysis

Data are presented as mean ± SD. To evaluate differences between groups unpaired student t-test was utilized. The relationship between CFR and hemodynamic, echocardiographic and morphometric variables was evaluated by linear regression analysis. A P-value of <0.05 was considered significant.

Interobserver variability

The % luminal area of the arterioles and the capillary density was independently quantified by two different observers, the variance was < 3% and < 5%, respectively.

Table 1. Demographic, hemodynamic, echocardiographic, and morphometric data of patients with HCM and HTX recipients.

	AGE/ SEX	NYHA	Therapy	IVS (mm)	LVPW (mm)	LV mass index (g/m2)	Cardiac Index (l/min/ m2)	LVEDP (mm Hg)	LVOT-gradient (mm Hg)	% lumen IMSA	Capillaries (n/mm2)	CFR
HCM												
# 1	45 F	II	-	20	11	168	2.8	12	32	-	-	3.10
# 2	59 M	III	B	23	13	202	2.4	28	100	14.9	1379	1.33
# 3	35 F	II	V	30	18	276	3.1	23	124	18.9	849	1.12
# 4	27 F	III	B	25	12	205	2.9	26	100	16.0	864	1.20
# 5	44 F	II	V	20	11	122	3.2	14	100	22.1	1541	2.10
# 6	20 M	II	V	23	11	182	2.9	23	58	27.6	1768	1.22
# 7	41 F	III	B,V	24	18	199	1.8	37	124	19.7	969	0.97
# 8	63 M	III	B	17	13	147	2.3	16	74	18.4	1686	1.13
# 9	62 M	III	V	22	12	184	3.4	20	88	30.7	1496	1.41
# 10	43 M	III	B	22	12	186	2.0	13	84	23.4	2451	1.68
HTX												
# 1	49 M	I	A,C,N,P	11	10	91	3.4	9	-	26.1	1308	3.38
# 2	61 M	I	C,D,N,P	12	10	88	3.2	6	-	26.6	1747	4.01
# 3	40 M	I	A,C,N,P	11	10	82	4.8	10	-	35.3	2079	2.00
# 4	57 M	I	C,D,N,P	11	11	119	4.1	11	-	31.2	2373	2.70
# 5	51 M	I	C,D,N,P	11	10	84	3.4	5	-	31.2	1511	2.25
# 6	55 M	I	A,C,N,P	10	10	73	3.2	7	-	33.7	2050	3.13
# 7	50 M	I	A,C,D,V	10	9	71	2.6	11	-	28.0	2245	1.97
# 8	52 M	I	A,C,N,P	11	10	100	3.9	13	-	31.5	1279	1.72

Therapy: A=aspirin, B=β-blocker, C=cyclosporin, D=dipyridamole, N=nifedipin, V=verapamil, P=pravastatin. CFR=coronary flow reserve, IMSA=intramyocardial small arteries, IVS=interventricular septum, LVEDP=left ventricular enddiastolic pressure, LVmass=left ventricular mass, LVOT-gradient=left ventricular outflow tract gradient, LVPW=left ventricular posterior wall, NYHA=New York Heart Association functional class.

RESULTS

Patient characteristics

Demographic, hemodynamic, echocardiographic, and histological data of individual HCM and HTX patients are presented in Table 1. As seen in Table 2, compared to HTX patients, HCM patients had increased thickness of the septal (2.3 ± 0.3 vs. 1.1 ± 0.1 cm, P <0.0001) and posterior wall (1.3 ± 0.3 vs. 1.1 ± 0.1 cm, P <0.01), and increased indexed LV mass (187 ± 41 vs. 89 ± 15 g/m^2, P <0.0001).

HCM and HTX patients had comparable stroke volume. However, because of lower resting heart rates (70 ± 13 beats vs. 97 ± 13 beats per minute, P <0.005), HCM patients had a lower cardiac index (2.7 ± 0.5 vs. 3.5 ± 0.7 L/min/m2, P <0.01). LV end-diastolic pressure was significantly higher in HCM patients (21 ± 8 mmHg vs. 9 ± 3 mmHg, P <0.001).

None of the HCM and HTX patients had epicardial coronary artery disease or systolic septal perforator artery compression. End-systolic and end-diastolic luminal diameters of the LAD and first septal branch were comparable in HCM and HTX patients.

Table 2. Comparison of patient characteristics between HCM, HTX patients and victims of car accidents.

	HCM patients (N = 10)	HTX patients (N = 8)	Car accidents (N = 10)
Age (years)	44 ± 15	51 ± 6	38 ± 19
Septal wall thickness (mm)	26 ± 3	11 ± 1$^{\#}$	
Posterior wall thickness (mm)	15 ± 4	10 ± 1$^{\$}$	
LV mass index (g/m^2)	187 ± 41	89 ± 15$^{\#}$	
Cardiac Index (L/min/m^2)	2.7 ± 0.5	3.5 ± 0.7$^{\$}$	
LV end-diastolic pressure (mm Hg)	21 ± 8	9 ± 3$^{\#}$	
End-diastolic LAD (mm)	3.3 ± 1.0	3.1 ± 0.5	
End-systolic LAD (mm)	3.1 ± 0.9	3.1 ± 0.5	
End-diastolic SA (mm)	1.5 ± 0.4	1.3 ± 0.2	
End-systolic SA (mm)	1.3 ± 0.4	1.2 ± 0.2	
External Diameter of IMSA (μm)	42.0 ± 7.8	46.1 ± 12.7	49.9 ± 13.4
% Luminal area	21.3 ± 5.2	30.4 ± 3.3	30.9 ± 5.7
Capillary density (number/mm^2)	1,445 ± 514$^{\#}$	1,824 ± 425$^{\#}$	3.883 ± 798
Coronary flow reserve	1.6 ± 0.7	2.7 ± 0.8$^{\$}$	

Data are presented as mean values ± SD. # = P <0.0001. $ = P <0.01. LV = left ventricular. L = lumen area, LAD = left descending coronary artery (diameter), SA = septal artery (diameter), W = wall area.

Histological analysis

As seen in Table 2, the external calibre of the intramyocardial arterioles in HCM patients (42.0 ± 7.8 μm) and HTX patients (46.1 ± 12.7 μm) were not significantly different from those in victims of car accidents (49.9 ± 13.4 μm). In contrast, the % luminal area in HCM patients (21.3 ± 5.2%) was significantly reduced, both compared to HTX patients

(30.4 ± 3.3%, P <0.001) and victims of car accidents (30.9 ± 5.7%, P <0.001). In victims of car accidents the number of capillaries (3,883 ± 798 mm^{-2}) was higher, both compared to HCM patients (1,445 ± 514 mm^{-2}, P <0.0001) and HTX patients (1,824 ± 425 mm^{-2}, P <0.0001). Figure 1 shows an example of capillary density in a HCM patient and a victim of a car accident, respectively. Capillary density in HCM patients tended to be lower than in HTX patients (1,445 ± 514 mm^{-2} vs. 1,824 ± 425 mm^{-2}, p = 0.11).

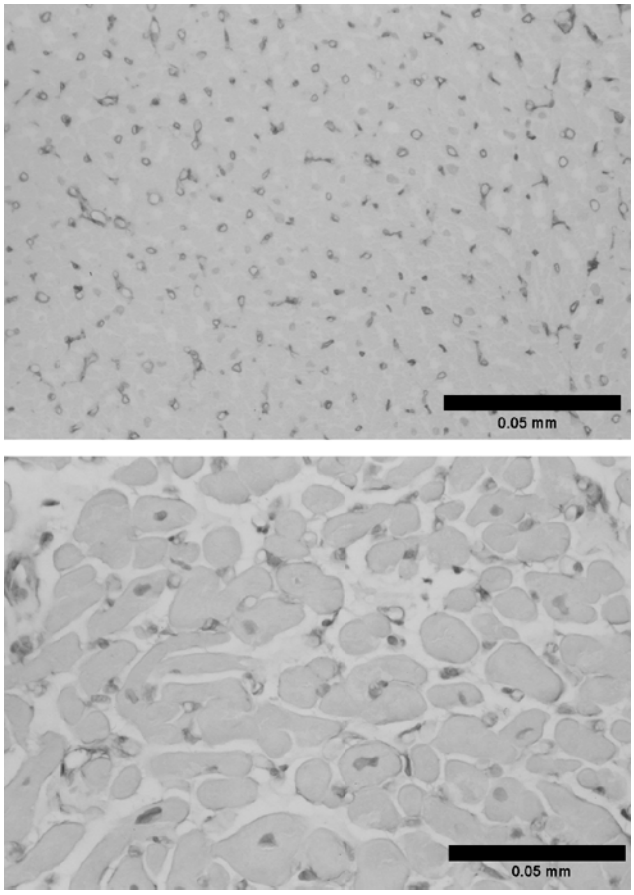

Figure 1:
The capillary density of a car accident victim is represented in the upper panel. In the lower panel the capillary density of a HCM patient is displayed. Note the decrease in the number of capillary vessels in HCM (magnification 40×)

Variables related to CFR

CFR in HCM patients was significantly reduced compared to HTX patients (1.6 ± 0.7 vs. 2.6 ± 0.8, P <0.01). An increase in LV septal thickness (P <0.005), indexed LV mass (P <0.005, Figure 2a), LV end-diastolic pressure (P< 0.001, Figure 2b), LVOT gradient

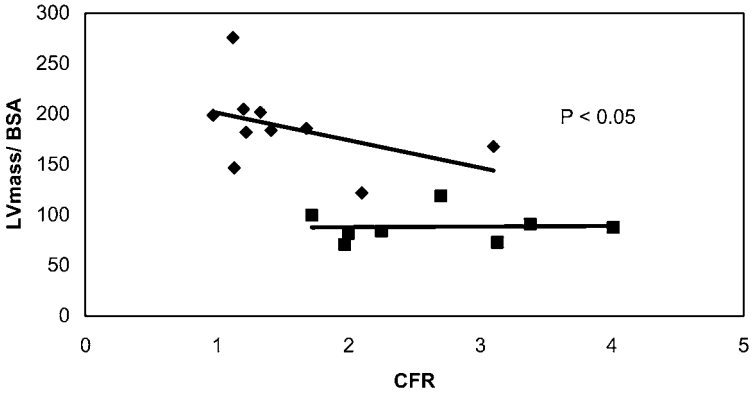

Figure 2A:
The relation between CFR and LV mass corrected for body surface area (LV mass/BSA, g/m^2) in HCM patients (◆) and HTX patients (■).

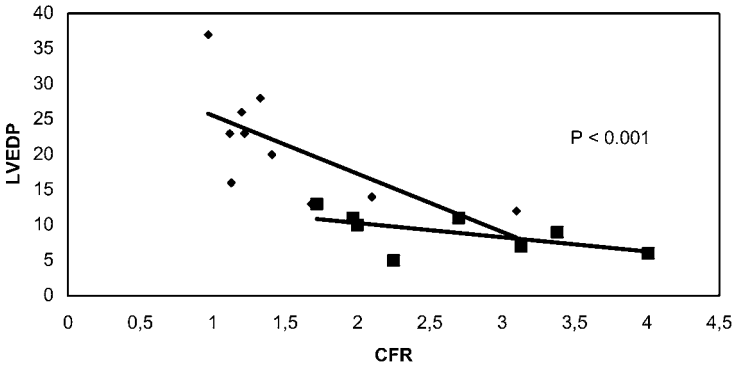

Figure 2B:
The relation between CFR and LV end-diastolic pressure (mmHg) in HCM patients (◆) and HTX patients (■).

(P <0.05), and a decrease in % luminal area of the arterioles (P <0.05) were all related to the reduction of CFR. The reduction in capillary density in HCM patients was not significantly related to the reduction in the vasodilatory reserve (p= 0.10). Multivariate analysis was not performed because of the small patient number.

DISCUSSION

The decrease in CFR is an important phenomenon in HCM, since it predisposes patients to myocardial ischemia and moreover decrements in CFR are a strong predictor of clinical deterioration and death [2-5]. In previous studies the reduction of CFR in HCM patients has been related to single hemodynamic [13], echocardiographic [9, 10] and histological

characteristics [14-17]. This is the first study to relate a combination of hemodynamic (LV end-diastolic pressure, LVOT gradient), echocardiographic (indexed LV mass) and histological (% luminal area of the arterioles) characteristics to the reduction in CFR.

Histological analysis: the role of small vessel disease.

In HCM patients post-mortem histological analysis of LV myocardium has revealed the presence of abnormal intramyocardial arterioles with markedly thickened walls and narrowed lumens [25]. Abnormal intramyocardial arteries are considered one of the possible mechanisms for a reduction in CFR in these patients [13, 14]. Indeed in the present study a positive relationship between the degree of arteriolar dysplasia and the decrement in the CFR was present. Since myocardial tissue was obtained from different sites (LV septal endocardium for HCM patients, right ventricular septal endocardium in HTX patients), this could have affected our results. Nevertheless, Tanaka *et al.* [14] demonstrated that the % luminal area of the intramyocardial arterioles was identical in the right, middle and left thirds of the ventricular septum in HCM hearts and in normal hearts. To evaluate the role of small vessel disease in HTX patients the % luminal area of intramyocardial arterioles in this group was compared to that of normal hearts and no significant differences were found.

Histological analysis: the role of the capillary density

Although small arteries and arterioles mainly control the blood flow of the coronary circulation, the capillary density and distribution are of crucial importance in the process of exchange between blood and tissue [26]. In HCM patients ischemia might be due to an increased oxygen diffusion distance caused by an inadequate growth of capillaries. The existence of this phenomenon has been confirmed in animals with pressure-overload LV hypertrophy (LVH) [27]. However, there are only few studies that have investigated the coronary vascular microcirculation in the normal human population and in patients with pressure-overload LVH. Rakusan *et al.* [25] showed that human LV capillary density is comparable to other mammalian species, and that pressure-overload LVH in children is associated with proportional capillary angiogenesis, whereas in adults, LVH appears to be associated with failure of compensatory angiogenesis. To the best of our knowledge, there are no studies concerning the role of capillary angiogenesis in HCM. In our study, we showed that there was a clear reduction in capillary density in HCM patients compared to patients without cardiovascular disease and HTX patients, although the latter comparison did not reach statistical significance. There was only a weak relation between the reduction in CFR and the decrease in capillary density ($P = 0.11$). However, the patient population could have been too small for differences to appear.

Limitations of the study

The use of HTX patients as control group is debatable; nevertheless Vassali *et al.* showed that CFR is comparable with the normal population 1 to 6 years after transplantation [28]. Importantly none of our HTX patients had signs of acute allograft rejection or epicardial CAD, conditions which are known to diminish CFR [29,30]. From the parameters we studied which could influence CFR, the reduction in capillary density in HTX patients was the most striking difference in comparison with the normal situation. Also, the sample size of the patient populations we studied was relatively small, as a consequence only univariate analysis could be performed and only clear differences in patient characteristics showed significance.

CONCLUSIONS

In HCM patients hemodynamic (LV end-diastolic pressure, LVOT gradient), echocardiographic (indexed LV mass) and histological (% luminal area of the arterioles) changes are responsible for a decrease in CFR (Figure 3).

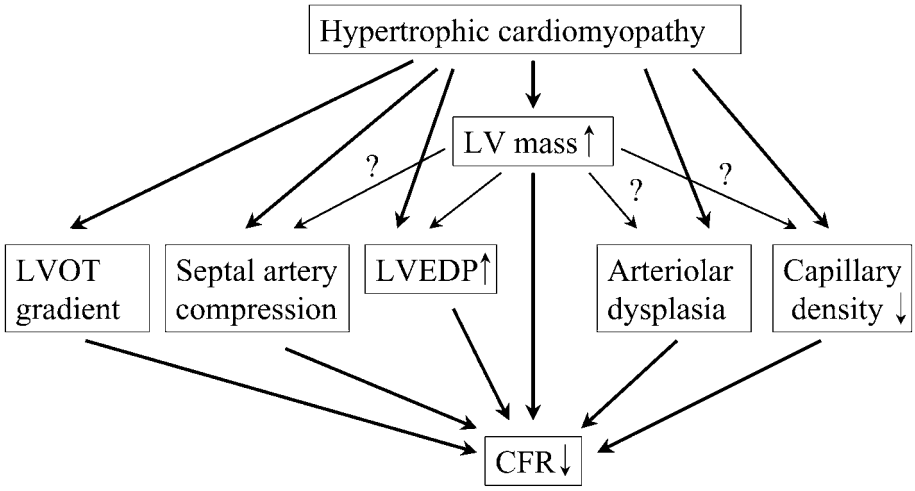

Figure 3:
Mechanisms underlying the reduction in CFR in HCM. LVEDP=LV end-diastolic pressure.

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Chapter 3

Diastolic abnormalities as first feature of hypertrophic cardiomyopathy in Dutch myosin binding protein C founder mutations

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ABSTRACT

Objectives

To test the hypothesis that carriers of Dutch founder mutations in *cardiac myosin binding protein C (MYBPC3)*, without left ventricular hypertrophy (LVH) or electrocardiographic abnormalities have diastolic dysfunction on tissue Doppler imaging (TDI), which can be used for the screening of family members in the Dutch hypertrophoc cardiomyopathy (HCM) population.

Background

TDI is a more sensitive technique for the assessment of left ventricular dysfunction compared to conventional echocardiography.

Methods

Echocardiographic studies including TDI were performed in genotyped HCM patients (genotype positive, G+/ LVH+; n = 27), mutation carriers without LVH (G+/ LVH-; n = 27) and healthy controls (n = 55). The identified mutations in *MYBPC3* in the G+/ LVH+ subjects were c.2864_2865delCT (12 subjects), c.2373dupG (8 subjects) and p. Arg943X (7 subjects). In the G+/ LVH– subjects the following mutations were identified c.2864_2865delCT (11 subjects), c.2373dupG (8 subjects) and p. Arg943X (8 subjects).

Results

Mean TDI-derived systolic (Sa) and early (Ea) and late (Aa) diastolic mitral annular velocities were significantly lower in the G+/ LVH+ subjects compared to the other groups. However, there was no difference between controls and G+/ LVH- subjects. Mean TDI-derived Aa velocities were significantly higher in the G+/ LVH– subjects compared to controls and G+/ LVH+ subjects. Using a cut-off value of mean + 2SD, an abnormal Aa was found in 14 (51%) of G+/LVH- patients. There was no difference between the 3 different mutations.

Conclusions

In contrast to earlier reports mean Sa and Ea velocities were not reduced in G+/ LVH- subjects and TDI velocities were not sufficiently sensitive for determination of the affected status of an individual. Our findings however, do support the theory that diastolic dysfunction is a primary component of preclinical HCM.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is defined by the presence of left ventricular hypertrophy (LVH) in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality (1). In most cases it is a familial disease with an autosomal dominant pattern of inheritance caused by mutations in genes that encode different proteins of the cardiac sarcomere. However, LVH is absent in a significant number of mutation carriers because of the variable penetrance of the mutations and confounding effects of modifier genes, gender and environmental factors. Overall, LVH is neither very sensitive nor very specific in HCM diagnosis (2-4).

An alternative approach to the early diagnosis of HCM is genetic testing, which can identify mutation carriers before development of LVH. However, using the current techniques sarcomeric mutations are identified in 50 to 60 % of HCM patients, making screening of family members by genetic testing impossible in up to 50 % of HCM families (5).

Experimental data suggest that cardiac myocyte contractile function in HCM is reduced and that the hypertrophy is compensatory (6,7). These data, in conjunction with myocyte disarray, the characteristic hallmark of HCM, led to the hypothesis that tissue Doppler imaging (TDI) with its possibility to identify contraction and relaxation abnormalities would be more sensitive for the diagnosis of HCM than conventional echocardiography. This principle has been proven by several studies in both animals and patients carrying different sarcomeric mutations (8-10).

The current study was performed to test the hypothesis that TDI can be used for the screening of family members in the Dutch HCM population. The Dutch HCM population is special because the majority of HCM in the Netherlands is caused by one of three founder mutations in *cardiac myosin binding protein C (MYBPC3)*; c.2373dupG (11), c.2864_2865delCT and p.Arg943X.

METHODS

Study population

At our cardio genetic outpatient clinic systematic family screenings of genotyped HCM patients are performed. The mutation carriers (G+/LVH-; n = 27) were selected from consecutive genotyped family members without major or minor criteria for the diagnosis of HCM on routine echocardiography or ECG as described by McKenna (12).The control group consisted of 55 healthy age and sex matched subjects without cardiovascular disease or diabetes mellitus and with normal left atrial dimension, LV dimension and function. The third group included 27 HCM patients (LV wall thickness ≥ 15 mm) based on one

of the Dutch founder mutations. All subjects did provide informed written consent. The study complies with the declaration of Helsinki and the Erasmus University MC Review Board has approved the study.

Two-dimensional echocardiography

Echocardiographic studies were performed with a Sonos 7500 ultrasound system with a S3 transducer or an iE33 system with a S5-1 transducer (Philips Medical Systems, Best, The Netherlands). The acquired data were digitally stored and subsequently analyzed by an observer, who was blinded for the clinical data. From the second harmonic M-mode recordings, the following data were acquired: left atrial dimension, LV septal and posterior wall thickness, and LV end-diastolic and end-systolic dimension. From the Doppler mitral-inflow pattern, early (E) and late (A) LV filling velocities, the E/A ratio, E-velocity deceleration time and the duration of the A were measured. Pulmonary vein systolic flow, diastolic flow and flow during atrial contraction were also measured. LV ejection fraction was assessed on two-dimensional echocardiography using the bi-plane Simpson method.

Tissue Doppler imaging

TDI was performed by placing the sample volume at the side of the medial, lateral, inferior, anterior, posterior and anteroseptal mitral annulus in the standard apical views. Gain and filter settings were adjusted as needed to eliminate background noise and to allow for a clear tissue signal. To acquire the highest tissue velocities the angle between the Doppler beam and the longitudinal motion of the investigated structure was adjusted to a minimal level. The mitral annular systolic (Sa), early (Ea) and late (Aa) diastolic velocities were recorded end-expiratory at a sweep speed of 100 mm/s and measured using electronic callipers with Enconcert software (Philips, Best, The Netherlands). TDI-derived Sa, Ea, and Aa mitral annular velocities from 6 mitral annular regions were measured. The individual average for the 6 measurements was used for analysis. The different mitral annular regions were also compared. The dimensionless ratio of E/Ea was computed at all corners, this parameter is an index that corrects for the influence of LV relaxation on mitral peak E velocity and provides a good estimate of left ventricular filling pressures in HCM (13).

Diastolic function was subsequently graded 0 to 4 as previously described (14). Based on color Doppler and TDI measurements, normal LV diastolic function (stage 0) was defined as a combination of E/A ratio between 0.75 and 1.50, deceleration time between 150 and 220 ms, pulmonary vein systolic flow > diastolic flow, duration of the pulmonary vein flow during atrial contraction < A-wave duration+30ms, mean Ea>10.0 cm/s, and E/Ea<9. Stage 1 diastolic dysfunction (impaired LV relaxation) was defined as E/A ratio<0.75 and deceleration time >220 ms. Stage 2 diastolic dysfunction (pseudo-normal LV filling) was defined as an E/A ratio between 0.75 and 1.5 with a mean Ea<7 cm/s and E/Ea>15. Stage 3 diastolic dysfunction (reversible restrictive LV filling) was defined as

E/A ratio>1.5 with a deceleration time <150 ms, mean Ea<7 cm/s, and E/Ea>15 reversing to pseudonormal or even impaired relaxation during the Valsalva maneuver. Stage 4 diastolic dysfunction (fixed restrictive) was defined as E/A ratio>1.5 with a deceleration time <150 ms, mean Ea<7 cm/s, and E/Ea>15 without change with Valsalva (14-16).

Statistical Analyses

All statistics were performed using the SPSS 14 for Windows (SPSS Inc, Chicago, IL, USA). Descriptive data were computed as a mean value ± SD. Variables among the 3 groups were compared by ANOVA using the Games-Howell corrections for post-hoc analysis. Statistical significance was defined by P ≤0.05. The Welch and Brown-Forsyth test confirmed the standard statistics.

RESULTS

Study population

The subject characteristics are displayed in Table 1. The mean age and the gender of controls and G+/LVH- subjects were similar. In the G+/LVH+ population, the mean age was significantly higher and there were significantly more males compared with the other two groups. In the G+/LVH- subjects the distribution of the *MYBPC3* mutations was: c.2864_2865delCT in 11, c.2373dupG in 8 and p.Arg943X in 8 subjects. In the G+/LVH+ subjects the distribution of the *MYBPC3* mutations was: c.2864_2865delCT in 12, c.2373dupG in 8 and p.Arg943X in 7 subjects.

Table 1: Subject characteristics

	G+/LVH+ (n=27)	G+/LVH- (n=27)	Controls (n=55)
Age	*†47 ± 12	37 ± 12	37 ± 10
Percentage male	*†63	33	37
Identified mutation			
2864delCT	12	11	0
2373insG	8	8	0
R943X	7	8	0

Between groups Games-Howell-corrected P-value:
*p<0.01 versus controls;
†p<0.01 versus G+/LVH- subjects.
(G = genotype; LVH = left ventricular hypertrophy)

Echocardiographic analysis

All subjects had 2D and Doppler studies satisfactory for analysis. The results of the echocardiographic analysis are displayed in Table 2.

Table 2. Echocardiographic Characteristics of the Study Population

	G+/LVH+ subjects (n=27)	G+/LVH- subjects (n=27)	Controls (n=55)
2 D Echocardiography			
LVEDD (mm)	46 ± 6	49 ± 5	50 ± 5
LVESD (mm)	28 ± 8	30 ± 5	31 ± 5
FS (%)	39 ± 9	38 ± 7	36 ± 7
EF (%)	61 ± 11	61 ± 5	62 ± 7
IVS (mm)	§‡19 ± 5	10 ± 2	9 ± 1
LVPW (mm)	11 ± 2	9 ± 2	9 ± 2
LA (mm)	§‡49 ± 9	36 ± 5	32 ± 5
Diastolic dysfunction			
Stage 0	5	27	55
Stage 1	5	0	0
Stage 2	7	0	0
Stage 3	7	0	0
Stage 4	3	0	0

Between groups Bonferroni- or Games-Howell-corrected P-value:
§p<0.01 versus controls;
‡p<0.01 versus G+/LVH- subjects.

By definition, mean septal and posterior wall thickness were significantly higher in the G+/LVH+ subjects compared with the control group and the G+/LVH-subjects. There were no differences in LV end-diastolic diameter, LV end-systolic diameter, fractional shortening and LV ejection fraction between the three groups. Left atrial dimension was significantly higher in the G+/LVH+ subjects compared with the other two groups.

All patients with definite HCM were in sinus rhythm. None had a LVOT gradient > 50 mmHg. Most patients, 16 (60%) were asymptomatic; 11 (40%) were in NYHA class II. Medical treatment was used by 12 (44%) of HCM patients and consisted of beta blockers in 6 (22%) patients; a combination of a beta blockers and diuretics in 2 (7%) patients; a combination of a beta blockers, diuretics and angiotensin converting-enzyme inhibitors in 3 (11%) patients; amiodarone was used by 1 (4%) HCM patient.

Diastolic function was normal (grade 0) in all controls and all G+/LVH- subjects. In the majority of G+/LVH+ subjects diastolic dysfunction was present.

Mean Sa, Ea and Aa velocities were significantly lower in the G+/LVH+ subjects compared with controls and G+/LVH- subjects. Mean Sa and Ea were not different between controls and G+/LVH- subjects. However, mean Aa velocities were significantly higher in the G+/LVH- subjects compared with controls and G+/LVH+ subjects (Figure 1). Using a cut-off value of mean + 2SD (>97 mm/s), an abnormal Aa was found in 14 (51%) of G+/LVH- patients. G+/LVH+ patients have abnormal regional velocities as compared to G+/LVH- and controls (Figure 2). In the G+/LVH- patients Aa velocity was abnormal in most of mitral annular regions. There was no difference between the 3 different mutations.

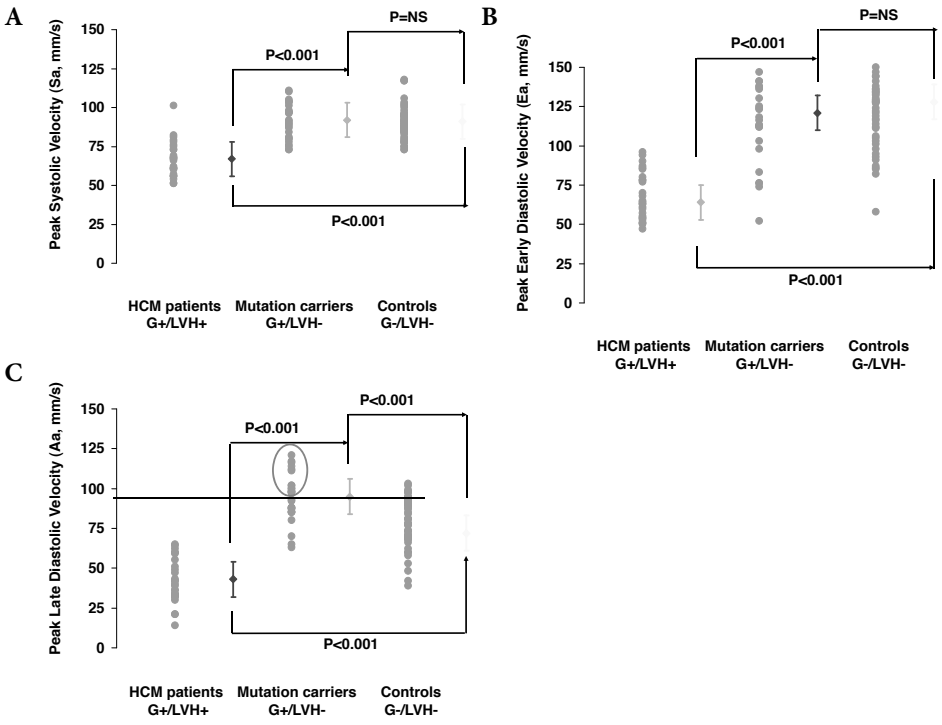

Figure 1. Individual Mean Values of Tissue Doppler Velocity

The individual mean values of the peak early systolic (A), peak early diastolic (B), and peak late diastolic (C) components of longitudinal velocity obtained from tissue Doppler imaging, which are averaged from 6 different mitral annular sites (anterior, anteroseptal, inferior, lateral, posterior, and posteroseptal) in the hypertrophic cardiomyopathy (HCM) patients, family members, and healthy controls. Aa = late mitral annular diastolic velocity; Ea = early mitral annular diastolic velocity;
G+/LVH+ = genotyped hypertrophic cardiomyopathy patients; G+/LVH- = carriers of Dutch myosin-binding protein C founder mutations without left ventricular hypertrophy; NS = not significant; Sa = mitral annular systolic velocity.

DISCUSSION

HCM is a primary disorder of cardiac myocytes, characterized by hypertrophy in abcense of increased external load. In most cases it is an autosomal-dominant disease and routine echocardiography and electrocardiography are used to screen family members (12). However, LVH is absent in a significant number of mutation carriers. Early and accurate phenotypic diagnosis of affected family members by TDI could provide an opportunity to prevent or modify the clinical manifestations of HCM and would complement genetic testing, which is complicated by allelic and non-allelic heterogeneity. In contrast to earlier reports, in our study population mean Sa and mean Ea velocities were not reduced in G+/LVH- subjects compared with controls and could not be used to differentiate the G+/LVH- subjects from the controls. The reasons for this difference could be the underlying

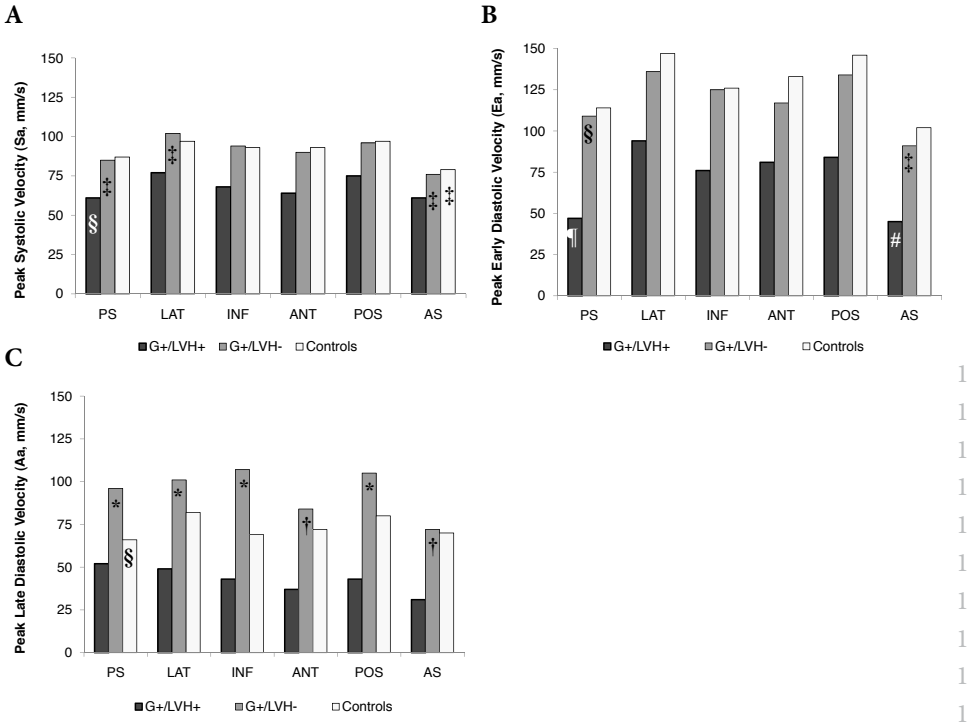

Figure 2: Mean regional values of peak systolic tissue Doppler velocity.
Mean regional values of the 3 components of tissue Doppler longitudinal velocity from 6 mitral annular sites during systole (A), early diastole (B), and late diastole (C) in genotyped hypertrophic cardiomyopathy patients (G+/LVH+), mutation carriers without left ventricular hypertrophy (G+/LVH-), and controls. Mitral annular regions in sequence are as follows: PS = posteroseptal; LAT = lateral; INF = inferior; ANT = anterior; POS = posterior; and AS = anteroseptal. Between-groups Games-Howell–corrected p value: all G+/LVH+ mean values of mitral annular systolic velocity (Sa), early mitral annular diastolic velocity (Ea), and late mitral annular diastolic velocity (Aa),averaged from 6 mitral annular sites and individual sites, are significantly different from other 2 groups ($p < 0.001$). Between mutation carriers and controls: *$p <$ 0.01 mutation carriers versus controls; †$p < 0.05$ mutation carriers versus controls. Within-group Games-Howell–corrected p value:‡$p < 0.01$ versus the other 5 segments; §$p < 0.05$ versus lateral and posterior segments; $p < 0.05$ versus posteroseptal, lateral, inferior, and anterior segments; and #$p < 0.05$ versus lateral, inferior, anterior, and posterior segments.

sarcomeric mutation. Unlike previous studies our study population consisted only of subjects with a truncating mutation in *MYBPC3*, which are known for its later onset of HCM compared with mutations in *beta-myosin heavy chain (MYH7)* (2).

We did however; find an increased Aa velocity in the majority of G+/ LVH– subjects carrying one of three truncating Dutch founder mutations in *MYBPC3*. To our knowledge this is the first study to describe an isolated increase in mean Aa velocity in G+/ LVH- subjects. An increase of mean Aa velocity means that the mitral annulus displacement is increased during late diastole. This could be a very early sign of diastolic abnormality, preceding other signs of myocardial contraction and relaxation abnormalities and LVH (1).

Prediction of genetic abnormalities in patients with a clinical diagnosis of HCM and vice versa has been investigated in several studies (17-19). Fifty to 60 % of HCM patients have abnormalities in the sarcomeric genes (20). Most HCM mutations are found in *MYH7*, which encodes a thick myofilament protein and in *MYBPC3*, which encodes an intermediate myofilament protein. Mutations in both genes cause an indistinguishable disease phenotype in which the ventricular septum has characteristic reverse curve in approximately 40% of cases (20). One of the great benefits of genetic analyses in HCM families is the identification of at-risk family members, which will allow early detection and possible prevention of poor outcome (21).

In the present study *MYBPC3* mutation carriers have, almost all, an increased LV late diastolic lengthening in the form of high Aa peak velocity on TDI. In contrast with HCM mutations in other sarcomeric genes, which are mostly missense mutations, most *MYBPC3* mutations are truncating mutations. Haploinsufficiency is therefore thought to be an important disease mechanism in *MYBPC3*-associated HCM. The 3 described mutations in this study are truncating mutations, thought to lead to a reduction in MYBPC3 protein because of a lack of expression from the mutated allele by the cellular surveillance mechanism of nonsense mediated decay (22). The regulatory role of MYBPC3 on contraction is still controversial. Although it has been shown that removal of the MYBPC3 can increase the velocity of shortening, force output, and force redevelopment in skinned preparation (23-26). Pohlmann et al. investigated consequences of removal of MYBPC3 in ventricular myocytes and left atria from MYBPC3 knockout mice compared with wild type. Both sarcomere shortening and Ca^{2+} transient were prolonged in MYBPC3 knockout mice. Isolated left atria of MYBPC3 knockout mice exhibited a marked increase in sensitivity to external Ca^{2+} and low micro molar Ca^{2+}. The main consequence of removal of MYBPC3 in the MYBPC3 knockout mice was a defect in diastolic relaxation and a smaller dynamic range of cell shortening, both of which likely result from the increased myofilament Ca^{2+} sensitivity (27). MYBPC3 is required for complete relaxation in intact myocytes. Therefore, MYBPC3 might function as a restraint on myosin-actin interaction at low Ca^{2+} and short sarcomere length to allow complete relaxation during diastole. When applying these experimental findings to our findings, the late diastolic abnormality can be a very early sign or pre-clinical phenotypic expression of disease in the G+/LVH- subjects.

CONCLUSIONS

Based on the current study we conclude that TDI velocities are not sufficiently sensitive for determination of affected status of an individual. Our findings however, do support the theory that diastolic dysfunction is a primary component of preclinical HCM.

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Chapter 4

Delayed left ventricular untwisting in hypertrophic cardiomyopathy

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ABSTRACT

Background

Almost all hypertrophic cardiomyopathy (HCM) patients have some degree of left ventricular (LV) diastolic dysfunction. Nevertheless, the pathophysiology remains incompletely characterized. Conceptually, an ideal therapeutic agent should target the underlying mechanisms that cause LV diastolic dysfunction. Assessment of diastolic LV untwisting could potentially be helpful to gain insight into the mechanism of diastolic dysfunction. The purpose of this study was to investigate LV untwisting in HCM patients and control subjects.

Methods

LV untwisting parameters were assessed by speckle tracking echocardiography in 75 consecutive HCM patients and compared to 75 healthy control subjects.

Results

Untwisting at 5%, 10%, and 15% of diastole was lower in HCM patients (all $P < 0.001$) compared to control subjects. Peak diastolic untwisting velocity (-92 ± 32 degrees/sec vs. -104 ± 39 degrees/sec, $P < 0.05$) and untwisting rate from peak systolic twist to mitral valve opening (MVO) (-37 ± 20 degrees/sec vs. -46 ± 22 degrees/sec, $P < 0.01$) were lower, while the normalized time-to-peak diastolic untwisting velocity (17 ± 9 % vs. 13 ± 9 %, $P < 0.05$) was higher in HCM patients. Untwisting rate from peak systolic twist to MVO was negatively correlated to E/A ratio ($R^2 = 0.15$, $P < 0.01$). Peak diastolic untwisting velocity and untwisting rate from peak systolic twist to MVO were increased in mild, but decreased in moderate and severe diastolic dysfunction compared to control subjects.

Conclusion

LV untwisting is delayed in HCM, which probably significantly contributes to diastolic dysfunction.

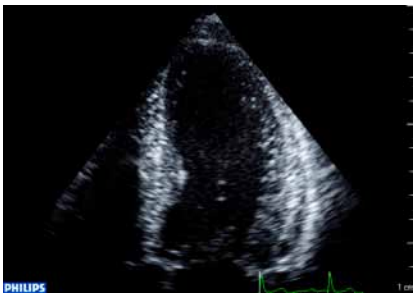
INTRODUCTION

Despite the heterogeneity in the phenotypic expression of hypertrophic cardiomyopathy (HCM), almost all HCM patients have some degree of left ventricular (LV) diastolic dysfunction.[1] The need for objective evidence of diastolic dysfunction has led to an extensive search for accurate, noninvasive methods to quantify its severity.[2] Conceptually, an ideal therapeutic agent should target the underlying mechanisms that cause LV diastolic dysfunction. Given the complex interplay of factors causing diastolic dysfunction in HCM, it should not be surprising that so far no single noninvasive measure has been validated to be accurate.[3] Furthermore, currently available noninvasive measurements (pulsed-wave Doppler flow velocity of the LV inflow, pulmonary veins flow, and mitral annular velocity using tissue Doppler imaging) represent events that occur after mitral valve opening (MVO), thus evaluating the later stages of diastole. Before MVO, in the isovolumic relaxation period, untwist of the obliquely oriented fibres of the LV contributes to the generation of the intraventricular pressure gradient, which leads to LV diastolic suction, a major determinant of early LV filling.[4] LV untwisting can be measured noninvasively using speckle tracking echocardiography.[5, 6] The purpose of this study was to investigate LV untwisting in HCM patients and control subjects and to relate LV untwisting parameters to conventional Doppler-derived parameters of LV diastolic function.

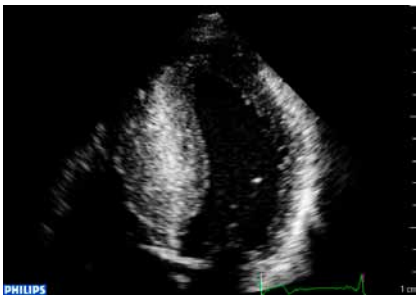
METHODS

Study participants

The study population consisted of 75 consecutive non-selected patients in sinus rhythm with HCM (mean age 42 ± 15 year, 54 men) and good echocardiographic image quality that allowed for complete segmental assessment of LV rotation at both the basal and apical LV level. During the enrolment of these 75 HCM patients, 31 other patients (29%) were excluded because of suboptimal echocardiographic image quality not fulfilling this criterion. These patients were compared to 75 healthy - for age and gender matched - control subjects, without hypertension or diabetes, and with normal left atrial dimensions, LV dimensions, and LV systolic and diastolic function. HCM was characterized morphologically and defined by a hypertrophied, nondilated LV in the absence of another systemic or cardiac disease that is capable of producing the magnitude of wall thickening seen.[7] By consensus reading between two observers, HCM patients could be subdivided into 24 patients (32%) with a typical sigmoidal, and 40 (53%) with a reverse septal curvature (Figure 1).[8] In the remaining 11 patients (15%) the two observers disagreed or an atypical morphology was seen. An informed consent was obtained from all subjects and the institutional review board approved the study.



Sigmoidal septal curvature



Reverse septal curvature

Figure 1:
Examples of a sigmoidal and reverse septal curvature.

Echocardiography

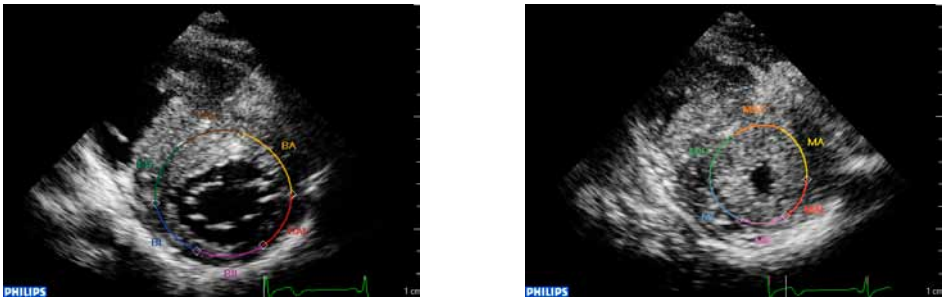
Two-dimensional grayscale harmonic images were obtained in the left lateral decubitus position using a commercially available ultrasound system (iE33, Philips, Best, The Netherlands), equipped with a broadband (1-5MHz) S5-1 transducer (frequency transmitted 1.7MHz, received 3.4MHz). All echocardiographic measurements were averaged from three heartbeats. From the M-mode recordings the following data were acquired: LV end-diastolic septal and posterior wall thickness, and LV end-diastolic and end-systolic dimension. LA atrial volume was measured using the biplane area-length formula and indexed for body surface area. LV ejection fraction was calculated from LV volumes by the modified biplane Simpson rule. LV mass was assessed with the two-dimensional area-length method.[9] LVOT gradient was measured with continuous-wave Doppler in the apical 5-chamber view. LVOT obstruction was defined as a gradient ≥ 30mmHg.[10] From the mitral-inflow pattern, peak early (E-wave velocity) and late (A-wave velocity) filling velocities, E/A ratio, and E-wave velocity deceleration time were measured. Tissue Doppler was applied end-expiratory in the pulsed-wave Doppler mode at the level of the inferoseptal side of the mitral annulus from an apical 4-chamber view. To acquire the highest wall tissue velocities, the angle between the Doppler beam and the longitudinal motion of the investigated structure was adjusted to a minimal level. The spectral pulsed-wave Doppler velocity range was adjusted to obtain an appropriate scale. The timing of the beginning and ending of the isovolumic relaxation time were determined using pulsed wave Doppler. HCM patients were stratified by grade of diastolic dysfunction (grades 1-3): grade 1, abnormal relaxation: E/A ratio < 0.75 and E-wave velocity deceleration time > 240 ms; grade 2, pseudonormal filling: 0.75 < E/A ratio < 1.5 and E-wave velocity deceleration time 140-200 ms; and grade 3, restrictive filling: E/A ratio >1.5 and E-wave velocity deceleration time < 140 ms.[11, 12] In addition, all patients with diastolic dysfunction were required to have an E-wave velocity / peak early diastolic wave velocity of the septal mitral annulus ratio (E/Em ratio) of more than 8. Twenty HCM patients could not

perfectly fulfill all of the criteria for a particular diastolic dysfunction group, and were therefore excluded from this part of the analysis.

To optimize speckle tracking echocardiography, images were obtained at a frame rate of 60 to 80 frames/s. Parasternal short-axis images at the LV basal level (showing the tips of the mitral valve leaflets) with the cross section as circular as possible were obtained from the standard parasternal position, defined as the long-axis position in which the LV and aorta were most in-line with the mitral valve tips in the middle of the sector. To obtain a short-axis image at the LV apical level (just proximal to the level with end-systolic LV luminal obliteration) the transducer was positioned 1 or 2 intercostal spaces more caudal as previously described by us.[13] From each short-axis image, three consecutive end-expiratory cardiac cycles were acquired and transferred to a QLAB workstation (Philips, Best, The Netherlands) for off-line analysis.

Speckle tracking analysis

Analysis of the datasets was performed using speckle tracking echocardiography by QLAB Advanced Quantification Software version 6.0 (Philips, Best, The Netherlands), which was recently validated against magnetic resonance imaging for assessment of LV twist.[14] To assess LV rotation, six tracking points were placed manually (after gain correction) on the mid-myocardium, regardless wall thickness, on an end-diastolic frame in each parasternal short-axis image. Tracking points were separated about 60° from each other and placed on 1 (30°, anteroseptal insertion into the LV of the right ventricle), 3 (90°), 5 (150°), 7 (210°), 9 (270°, inferoseptal insertion into the LV of the right ventricle), and 11 (330°) o'clock to fit the total LV circumference (Figure 2). After positioning the tracking points, the program tracked these points on a frame-by-frame basis by use of a least squares global affine transformation. The rotational component of this affine transformation was then used to generate rotational profiles.



Basal level Apical level

Figure 2:
Examples of positioning of the tracking points at the left ventricular basal and apical level in HCM patients.

Data were exported to a spreadsheet program (Excel, Microsoft Corporation, Redmond, WA) to determine LV peak systolic rotation during ejection, instantaneous LV peak systolic twist (defined as the maximal value of instantaneous apical systolic rotation – basal systolic rotation), and LV untwisting at 5%, 10%, 15%, and 50% of diastole. The degree of untwisting was expressed as a percentage of maximum systolic twist: untwisting = (peak systolic twist – twist at time t) / peak systolic twist x 100%. Furthermore, peak diastolic de-rotation velocity and untwist velocity, and the timing of these parameters were assessed. Peak diastolic de-rotation velocity represents the *peak* velocity of de-rotation, the diastolic reversal of systolic LV rotation, and was determined at both the LV basal and apical level. Peak diastolic untwist velocity represents the *peak* velocity of LV untwisting, the diastolic reversal of systolic LV Untwisting rate was defined as the *mean* diastolic untwisting velocity from peak systolic twist to MVO and calculated as: (twist at MVO – peak systolic twist) / time interval from peak systolic twist to MVO. To adjust for intra- and intersubject differences in heart rate, the time sequence of systolic and diastolic events was normalized to the percentage of systolic and diastolic duration, respectively. End-systole was defined as the point of aortic valve closure. In each study it was verified that the heart rate for the cardiac cycle in which the timing of aortic valve closure was assessed, was the same as the cardiac cycle used for analysis of untwisting.

Statistical Analysis

Measurements are presented as mean ± SD. Variables were compared using Student's *t* test, ANOVA, or Chi-square test when appropriate. Linear regression analysis of peak diastolic untwisting velocity, time-to-peak diastolic untwisting velocity, and untwisting rate from peak systolic twist to MVO against conventional parameters of diastolic function (LA volume, E-wave velocity, A-wave velocity, E/A ratio, Em, and E/Em ratio) was performed. A P value < .05 was considered statistically significant. Intraobserver and interobserver variability for assessment of LV twist by speckle tracking echocardiography in our center are 6% ± 6% and 9% ± 5%, respectively.[15]

RESULTS

Characteristics of the study population

In Table 1, clinical and echocardiographic characteristics of HCM patients and control subjects are shown. LA volume indexed by body surface area, LV mass, maximal LV wall thickness, interventricular septal, and LV posterior wall dimensions were higher, whereas LV end-diastolic and end-systolic dimensions were lower in HCM patients (all P <0.001). Furthermore, E-wave velocity and Em septal were lower, whereas E-wave velocity deceleration time, E/Em ratio, and isovolumic relaxation time were higher in HCM patients.

Table 1. Clinical and echocardiographic characteristics of the study population.

	HCM patients (n = 75)	Control subjects (n = 75)
Age, year	42 ± 15	40 ± 14
Male, n (%)	54 (72)	54 (72)
Heart rate, beats/min	64 ± 11	63 ± 11
Systolic blood pressure, mmHg	126 ± 18	123 ± 14
Diastolic blood pressure, mmHg	77 ± 9	74 ± 8
Echocardiographic characteristics		
Left atrial volume, mL/m^2	53 ± 21†	23 ± 6
IVS_d, cm	2.0 ± 0.5†	1.0 ± 0.2
$LVPW_d$, cm	1.2 ± 0.3†	1.0 ± 0.1
LV-EDD, cm	4.5 ± 0.5†	4.9 ± 0.5
LV-ESD, cm	2.5 ± 0.5†	3.3 ± 0.6
LV ejection fraction, %	60 ± 10	61 ± 7
LV mass, g	305 ± 88†	175 ± 50
Maximal LV wall thickness, cm	2.1 ± 0.5†	1.0 ± 0.2
E-wave velocity, cm/s	65 ± 20*	72 ± 16
A-wave velocity, cm/s	52 ± 20	53 ± 17
E/A ratio	1.5 ± 0.7	1.5 ± 0.6
E-wave velocity deceleration time, ms	195 ± 70*	173 ± 33
Em septal, cm/s	5.6 ± 2.4†	9.9 ± 2.6
E/Em ratio	13.3 ± 7.7†	7.6 ± 2.0
Isovolumic relaxation time, ms	84 ± 23†	70 ± 14

HCM = hypertrophic cardiomyopathy, IVS_d = interventricular septum thickness (diastole), $LVPW_d$ = left ventricular posterior wall thickness (diastole), LV-EDD = left ventricular end-diastolic dimension, LV-ESD = left ventricular end-systolic dimension, E-wave velocity = peak early phase filling velocity, A-wave velocity = peak atrial phase filling velocity, Em = peak early diastolic wave velocity. * P <0.05, † P <0.001 vs. control subjects

LV rotation and twist

HCM patients had higher basal peak systolic rotation (-5.5 ± 2.6 degrees vs. -3.6 ± 2.0 degrees, P <0.001), and comparable apical peak systolic rotation (7.0 ± 3.9 degrees vs. 7.3 ± 2.9 degrees, P = NS), resulting in higher peak systolic twist (11.8 ± 4.6 degrees vs. 10.4 ± 3.2 degrees, P <0.05) (Table 2).

LV de-rotation and untwisting

Untwisting at 5% (12 ± 12% vs. 21 ± 19%), 10% (23 ± 19% vs. 37 ± 23%), and 15% (36 ± 22% vs. 49 ± 21%) of diastole was lower in HCM patients (all P <0.001). Peak diastolic untwisting velocity (-92 ± 32 degrees/sec vs. -104 ± 39 degrees/sec, P <0.05) and untwisting rate from peak systolic twist to MVO (-37 ± 20 degrees/sec vs. -46 ± 22 degrees/sec, P <0.01) were lower, while the normalized time-to-peak diastolic untwisting velocity (17 ± 9 % vs. 13 ± 9 %, P <0.05) was higher in HCM patients (Table 2, Figure 3). Untwisting rate from peak systolic twist to MVO correlated positively to peak systolic twist in HCM patients (R^2 = 0.34, P <0.001) (Figure 4).

Table 2. Left ventricular rotation parameters in hypertrophic cardiomyopathy patients and control subjects

	HCM patients (n = 75)			Control subjects (n = 75)
	All	Septal morphology		
		Sigmoidal (n = 24)	Reverse (n = 40)	
Basal rotation and de-rotation velocity				
Peak systolic rotation, degrees	-5.5 ± 2.6†	-5.5 ± 2.4*	-5.7 ± 2.6†	-3.6 ± 2.0
Peak diastolic de-rotation velocity, degrees/sec	59 ± 21	58 ± 26	60 ± 18	61 ± 26
Normalized time-to-peak diastolic de-rotation velocity, %	17 ± 12	17 ± 12	17 ± 10	14 ± 8
Apical rotation and de-rotation velocity				
Peak systolic rotation, degrees	7.0 ± 3.9	9.2 ± 4.2‡	5.7 ± 3.0	7.3 ± 2.9
Peak diastolic de-rotation velocity, degrees/sec	-57 ± 24$	-66 ± 22	-50 ± 23$	-70 ± 28
Normalized time-to-peak diastolic de-rotation velocity, %	20 ± 15$	22 ± 13*	21 ± 10*	14 ± 11
Twist and untwist				
Peak systolic twist, degrees	11.8 ± 4.6*	14.3 ± 5.0†‡	10.4 ± 3.7	10.4 ± 3.2
Untwisting at 5% of diastole, %	12 ± 12†	10 ± 10*	11 ± 13*	21 ± 19
Untwisting at 10% of diastole, %	23 ± 19†	20 ± 15$	21 ± 20$	37 ± 23
Untwisting at 15% of diastole, %	36 ± 22†	33 ± 22$	35 ± 21$	49 ± 21
Untwisting at 50% of diastole, %	77 ± 15	72 ± 15	79 ± 15	78 ± 14
Peak diastolic untwisting velocity, degrees/sec	-92 ± 32*	-96 ± 30	-91 ± 29	-104 ± 39
Normalized time-to-peak diastolic untwisting velocity, %	17 ± 9*	18 ± 14	17 ± 6	13 ± 9
Untwisting rate from peak systolic twist to MVO, degrees/sec	-37 ± 20$	-46 ± 24§	-31 ± 16$	-46 ± 22

HCM = hypertrophic cardiomyopathy, MVO = mitral valve opening. *P <0.05, $P <0.01, †P <0.001 vs. control subjects; §P <0.05, ‡P <0.01 vs. reverse septal curvature

Relation between LV untwisting and conventional parameters of LV diastolic function in HCM patients.

Untwisting rate from peak systolic twist to MVO correlated positively to A-wave velocity (R^2 = 0.11, P <0.05), and negatively to E/A ratio (R^2 = 0.15, P <0.01) (Figure 5), whereas no relation could be identified between E-wave velocity, Em, E/Em ratio or LA volume indexed by body surface area and any of the LV untwisting parameters. According to grade of diastolic dysfunction, HCM patients with an unambiguous defined grade of diastolic dysfunction (n = 55) could be divided in 13 patients (24%) with grade 1, 36 (65%)

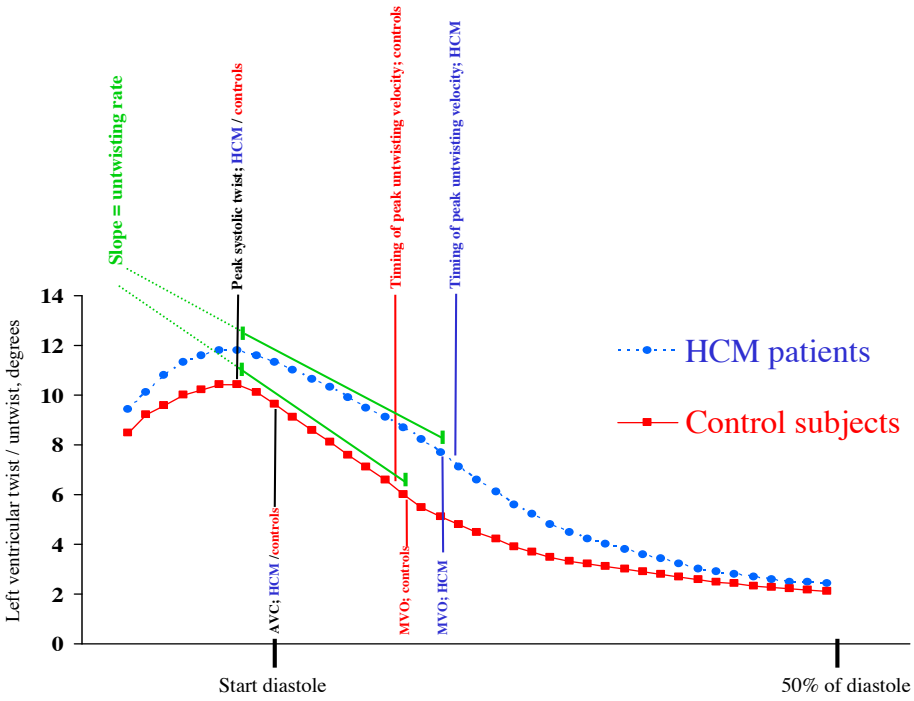


Figure 3:
Schematic left ventricular twist/untwist curves (based on averaged values of peak systolic twist, and twist at aortic valve closure (AVC), mitral valve opening (MVO), and 50% of diastole) in HCM patients andcontrol subjects, highlighting the differences of left ventricular peak systolic twist, untwisting rate, and the timing of peak untwisting velocity.

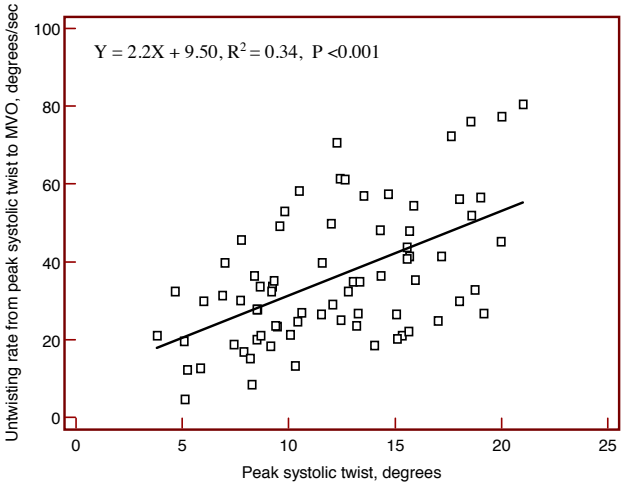


Figure 4:
Linear regression between peak systolic twist and untwisting rate from peak systolic twist to MVO.

with grade 2, and 6 (11%) with grade 3 diastolic dysfunction. Peak systolic twist was higher in HCM patients with grade 1 (13.2 ± 4.9 degrees, P <0.05), whereas it was normal in HCM patients with grade 2 (11.5 ± 5.0 degrees, P = NS) or 3 (11.0 ± 2.6 degrees, P = NS) diastolic dysfunction as compared to control subjects (10.4 ± 3.2 degrees). Peak diastolic untwisting velocity and untwisting rate from peak systolic twist to MVO were higher in grade 1 (-119 ± 35 degrees/sec and -58 ± 27 degrees/sec, respectively), but lower in grade 2 (-82 ± 28 degrees/sec and -30 ± 19 degrees/sec, respectively) diastolic dysfunction as compared to control subjects (-104 ± 39 degrees/sec and -46 ± 22 degrees/sec, respectively, all P <0.05).

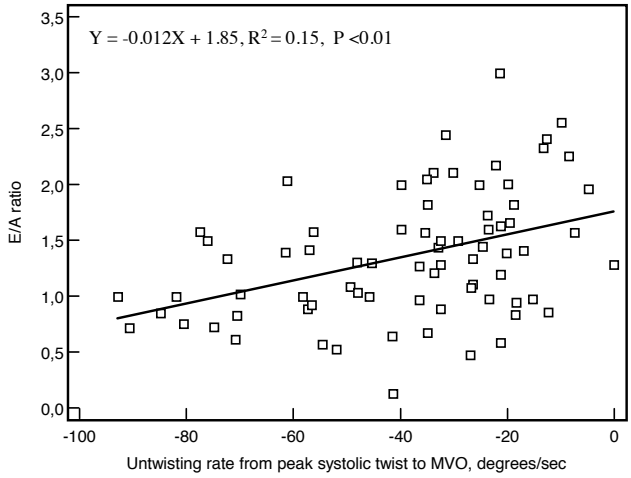


Figure 5:
Linear regression between untwisting rate and the ratio between early and active left ventricular filling velocity (E/A ratio).

Relation between the pattern of LVH and conventional echocardiographic parameters and LV rotation parameters

Maximal LV wall thickness was higher (2.2 ± 0.4 cm vs. 1.8 ± 0.4 cm, P <0.001), whereas LVOT gradient was lower (15 ± 22 mmHg vs. 31 ± 25 mmHg, P <0.01) in HCM patients with reverse compared to sigmoidal septal curvature. Also, a significant LVOT obstruction was less often present in the patients with a reverse septal curvature (5 patients (13%) vs. 10 patients (42%), P <0.05). A-wave velocity (44 ± 14 cm/s vs. 68 ± 20 cm/s, P <0.001) was lower, whereas E/A ratio (1.7 ± 0.8 vs. 1.1 ± 0.4, P <0.01) was higher in HCM patients with a reverse septal curvature. Of the HCM patients, 25 (63%) with a reverse and 19 (79%, P = NS) with a sigmoidal septal curvature had an unambiguous defined grade of diastolic dysfunction. Grade 1 diastolic dysfunction was identified in 4 (16%) vs. 7 (37%), grade 2 in 16 (64%) vs. 12 (63%), and grade 3 in 5 (20%) vs. 0 of the HCM patients

with a reverse vs. sigmoidal septal curvature (all P = NS). The average grade of diastolic dysfunction was 1.6 ± 0.5 in HCM patients with a sigmoidal vs. 2.0 ± 0.6 in HCM patients with a reverse septal curvature (P <0.05). Duration of the isovolumic relaxation time was comparable between HCM patients with a sigmoidal and a reverse septal curvature.

There was a striking difference in apical peak systolic rotation (5.7 ± 3.0 degrees vs. 9.2 ± 4.2 degrees, P <0.01) and peak systolic twist (10.4 ± 3.7 degrees vs. 14.3 ± 5.0 degrees, P <0.01) between patients with reverse and sigmoidal septal curvature, respectively. Furthermore, untwisting rate from peak systolic twist to MVO (-31 ± 16 degrees/sec vs. -46 ± 24 degrees/sec, P <0.05) was lower in HCM patients with reverse septal curvature (Table 2).

DISCUSSION

Echocardiography has been used since its early days to gain insight into the complex pathophysiology of HCM, because it provides a practical and comprehensive assessment of cardiac structure and function.[16, 17] HCM is usually associated with alterations in LV diastolic function, whereas global systolic function is preserved until the later stages of the disease. In the present study delayed LV untwisting, reflecting ineffective diastolic uncoiling of the hypertrophic myocardium, is shown in HCM patients. However, HCM patients with mild or early-stage (grade 1) diastolic dysfunction showed more LV twist and a higher peak diastolic untwisting velocity and untwisting rate from peak systolic twist to MVO.

LV untwisting physiology

In systole, the LV apex rotates counterclockwise (as viewed from the apex), whereas the base rotates clockwise, creating a twisting deformation, originating from the dynamic interaction of oppositely oriented epicardial and endocardial myocardial fibres.[18, 19] The direction of LV twist is governed by the epicardial fibres, mainly owing to their longer arm of movement.[20] Untwisting starts just slightly before the end of systole (marked by aortic valve closure) after the peak of LV twist. The twisting deformation of the LV during systole results not only in ejection but also in storage of potential energy. During the isovolumic relaxation period the twisted fibres behave like a compressed coil that springs open while abruptly releasing the potential energy. This process may be actively supported by still depolarized subendocardial fibres that are – in contrast to the systolic period – now not opposed by active contraction of the subepicardial fibres. Untwist generates expansion of the apex and the intraventricular pressure gradient that helps filling the LV at a low pressure.[4]

LV untwisting in HCM

The current study is the first to show that LV untwisting is delayed in HCM, reflecting ineffective diastolic uncoiling of the hypertrophic myocardium. The higher peak systolic twist in HCM is supposed to store more potential energy and thereby lead to increased LV untwisting. However, whereas subendocardial ischemia[21-23] might be the cause of the increased peak systolic twist by loss of counteraction of the subendocardial fibre helix, it might also lead to loss of the active untwisting normally caused by the subendocardial fibres during early diastole. Furthermore, the impaired compliance of the hypertrophied LV will prevent optimal transformation of the potential energy stored in systolic LV twisting into kinetic energy. Apparently, the factors impairing the process of LV untwisting in HCM outweigh potentially enhancing factors, leading to delayed LV untwisting. Furthermore, Takeuchi et al.[24] found delayed LV untwisting in hypertension patients with versus without LVH. It seems that LVH per se may lead to delayed LV untwisting, irrespective of the cause of hypertrophy. However, in the study by Takeuchi et al., LV twist was not increased in hypertension patients with LVH, in contrast to the HCM patients in the current study. Since LV twist and untwist are tightly coupled, increased LV twist in HCM may be expected to lead to preservation of LV untwisting. Therefore, the delayed LV untwisting found in HCM patients in the current study may be rather surprising. One may hypothesize that there are specific factors in HCM, such as the asymmetrical distribution of hypertrophy and the presence of myocardial fibre disarray, that lead to specific changes in LV rotational mechanics. Future studies, comparing hypertension patients and HCM patients with a similar degree of LVH and LV twist, may be warranted in order to investigate the specific influence of factors related to the cause of hypertrophy on the successfulness of transfer of potential energy stored in systolic LV twisting to diastolic LV untwisting.

LV twist, untwisting rate from peak systolic twist to MVO, and peak diastolic untwisting velocity were higher in HCM patients with mild or early-stage (grade 1) diastolic dysfunction, and lower in the more advanced (grade 2-3) stages of diastolic dysfunction. These data confirm findings of a study by Park et al.[25] in a smaller group of HCM patients with diastolic dysfunction, although it should be noted that grading diastolic function by echocardiography in HCM patients may have limited accuracy.[1] Since the untwisting rate from peak systolic twist to MVO can be increased in the presence of impaired LV relaxation, untwisting rate and relaxation do not rely on a similar mechanism. It has been suggested that increased untwisting rate might be a compensatory mechanism, preventing the need to increase left atrial pressure.[4, 25, 26] Failure to increase untwisting rate might necessitate an increase in left atrial pressure, with the associated detrimental effects.

Influence of the pattern of hypertrophy on LV untwisting

Because of its heterogeneous expression and clinical course,[27, 28] HCM frequently presents uncertainty and represents a management dilemma. Creation of more homogeneous subgroups of HCM patients might be helpful to better predict prognosis. Recently, septal morphology was linked to the underlying genetic substrate, and best predicted the presence of a myofilament mutation.[29] Furthermore, our group has recently shown a phenotype-functional relationship in HCM by relating the pattern of hypertrophy to LV twist.[30] $Twist_{max}$ was higher in HCM patients with a sigmoidal versus a reverse septal curvature due to a higher apical Rot_{max}, a finding confirmed in the current study. This difference could not be explained by regional differences in LV rotation, since in HCM patients with a sigmoidal septal curvature both apical septal and lateral LV rotation were higher as compared to patients with a reverse septal curvature. The current study is the first to relate LV untwisting to septal morphology. In HCM patients with a sigmoidal septal curvature, untwisting rate from peak systolic twist to MVO was normal, whereas it was decreased in HCM patients with a reverse septal curvature. As mentioned in the previous section, LV untwisting rate depends on passive properties and the amount of potential energy stored by systolic LV twist that can be converted into kinetic energy used for LV untwisting, and active properties such as the contribution of still depolarized subendocardial fibres in early diastole. In HCM patients with a reverse septal curvature, less systolic twist, impaired compliance, and subendocardial ischemia may all contribute to a reduced untwisting rate from peak systolic twist to MVO. On the other hand, in HCM patients with a sigmoidal septal curvature, the higher amount of energy stored from increased systolic twist seems to fully compensate for the loss of active LV untwisting and impaired compliance (which may also be less impaired because of the relatively normal apex). The findings of the current study provide insight into the pathophysiology of HCM. Whether measurement of LV untwisting has any added diagnostic or prognostic value should be investigated in future studies.

LIMITATIONS

In the current study, only patients with good echocardiographic image quality that allowed for complete segmental assessment of LV rotation at both the basal and apical LV level, were included. This inclusion criterion led to exclusion of 29% of the patients; a percentage in-line with previous data on the feasibility of speckle tracking by QLAB.[15] However, this limitation may hamper the clinical implementation of LV untwisting.

Direct comparison of HCM patients with hypertension or aortic stenosis patients may reveal the influence of specific factors related to HCM, apart from LVH, on LV untwisting, such as the distribution of hypertrophy or the presence of myocardial fibre disarray.

Unfortunately, there is currently no consensus on the definition of untwisting rate. Both Takeuchi et al.[24] and Park et al.[25] define untwisting rate as a mean velocity during the isovolumic relaxation phase. However, Takeuchi et al. use the mean velocity from end-systolic twist to MVO, whereas Park et al. use the mean velocity from peak twist to MVO, the definition also used in the current study. Even more discrepantly, Wang et al.[31] defined untwisting rate as the peak diastolic time derivative of twist.

Finally, E/A ratio and E/Em ratio are weakly correlated to LV filling pressures in HCM patients.[32] Therefore, conclusions drawn from the presence or absence of correlations between LV untwisting parameters and conventional parameters of LV diastolic function provided in this paper, should be taken with caution. Correlation of LV untwisting to invasively assessed parameters of LV diastolic function may be required in order to provide a definite judgement on the role of LV untwisting in LV diastolic function in HCM.

CONCLUSION

Speckle tracking echocardiography offers novel non-invasive indices to assess LV diastolic function. In HCM patients, delayed LV untwisting is seen, which probably significantly contributes to diastolic dysfunction.

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Chapter 5

Cardiac myosin-binding protein C mutations and hypertrophic cardiomyopathy: haploinsufficiency, deranged phoshorylation and cardiomyocyte dysfunction

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ABSTRACT

Background

Mutations in the *MYBPC3* gene, encoding cardiac myosin-binding protein C (cMyBP-C), are a frequent cause of familial hypertrophic cardiomyopathy (FHCM). In the present study we investigated if protein composition and function of the sarcomere are altered in a homogenous FHCM patient group with frameshift mutations in *MYBPC3* ($MYBPC3_{mut}$).

Methods and Results

Comparisons were made between cardiac samples from *MYBPC3* mutant carriers (c.2373dupG, n=7; c.2864_2865delCT, n=4) and non-failing donors (n=13). Western blots using antibodies directed against cMyBP-C did not reveal truncated cMyBP-C in $MYBPC3_{mut}$. Protein expression of cMyBP-C was significantly reduced in $MYBPC3_{mut}$ by 33±5%. Cardiac MyBP-C phosphorylation in $MYBPC3_{mut}$ samples was similar to the values in donor samples, whereas the phosphorylation status of troponin I (cTnI) was reduced by 84±5%, indicating divergent phosphorylation of the two main contractile target proteins of the beta-adrenergic pathway. Force measurements in mechanically isolated Triton-permeabilized cardiomyocytes demonstrated a decrease in maximal force per crosssectional area of the myocytes in $MYBPC3_{mut}$ (20.2±2.7 kN/m^2) compared to donor (34.5±1.1 kN/m^2). Moreover, Ca^{2+}-sensititivy was higher in $MYBPC3_{mut}$ (pCa_{50}=5.62±0.04) than in donor (pCa_{50}=5.54±0.02), consistent with reduced cTnI phosphorylation. Treatment with exogenous protein kinase A, to mimic β-adrenergic stimulation, did not correct reduced maximal force, but abolished the initial difference in Ca^{2+}-sensititivy between $MYBPC3_{mut}$ (pCa_{50}=5.46±0.03) and donor (pCa_{50}=5.48±0.02).

Conclusions

Frameshift *MYBPC3* mutations cause haploinsufficiency, deranged phosphorylation of contractile proteins and reduced maximal force generating capacity of cardiomyocytes. The enhanced Ca^{2+}-sensititivy in $MYBPC3_{mut}$ is due to hypophosphorylation of troponin I secondary to mutation-induced dysfunction.

INTRODUCTION

Familial hypertrophic cardiomyopathy (FHCM) is the most frequent inheritable cardiac disease with a prevalence of 0.2%.[1,2] FHCM causing mutations are identified in 13 genes encoding sarcomeric proteins.[3] Mutations in the *MYBPC3* gene encoding cardiac myosin-binding protein C (cMyBP-C) represent more than 40% of all FHCM cases.[4] Most *MYBPC3* mutations are predicted to produce C-terminally truncated proteins, lacking titin and/or major myosin binding sites.[4-6] Studies in FHCM patients carrying a *MYBPC3* mutation failed to reveal truncated cMyBP-C protein[7-9] suggesting that *MYBPC3* mutations may lead to haploinsufficiency.

Evidence suggests that the first step in the pathogenesis of FHCM involves mutation-induced sarcomeric dysfunction.[2,3] Myocardial dysfunction in this group of patients has been attributed at least partly to myocyte hypertrophy, disarray and interstitial fibrosis.[10] However, direct evidence for both reduced cMyBP-C expression and sarcomeric dysfunction in *MYBPC3* mutant carriers is missing.

Approximately 35% of the FHCM patients in the Netherlands have founder mutations in the *MYBPC3* gene (c.2373dupG and c.2864_2865delCT)[11] that both encode C-terminally truncated proteins (Figure 1).[8,9] This allowed us to investigate whether truncating mutations in the *MYBPC3* gene alter sarcomeric protein composition and function in a rather homogeneous patient group. Cardiac MyBP-C mRNA and protein expression and phosphorylation status of sarcomeric proteins were analyzed in concert with cardiomyocyte function in *MYBPC3* mutation carriers and compared with non-failing donor samples.

METHODS

Cardiac Biopsies

Cardiac tissue was obtained from the left ventricular (LV) septum of 11 patients with a founder mutation[11,12] in the *MYBPC3* gene encoding cMyBP-C ($MYBPC3_{mut}$: c.2373dupG, n=7; 32-69 years of age, mean 50±5 years; 2/5 male/female; c.2864_2865delCT, n=4; 32-62 years of age, mean 44±6 years; 2/2 male/female), that underwent alcohol ablation or myectomy to relieve left ventricular outflow obstruction. Echocardiographic and clinical data of the patients are given in Table 1. Hypertrophic obstructive cardiomyopathy was evident from increased septal thickness (21±1 mm; normal value <13 mm)[13] and high LVOTPG (78±6 mmHg; normal value <30 mmHg).[14] LV ejection fraction was moderately depressed (44±2%). Both mutations encode for slightly different C-terminally truncated proteins with a theoretical mass of 93 and 116 kDa for c.2373dupG[8] and c.2864_2865delCT[9], respectively (Figure 1).[15,16]

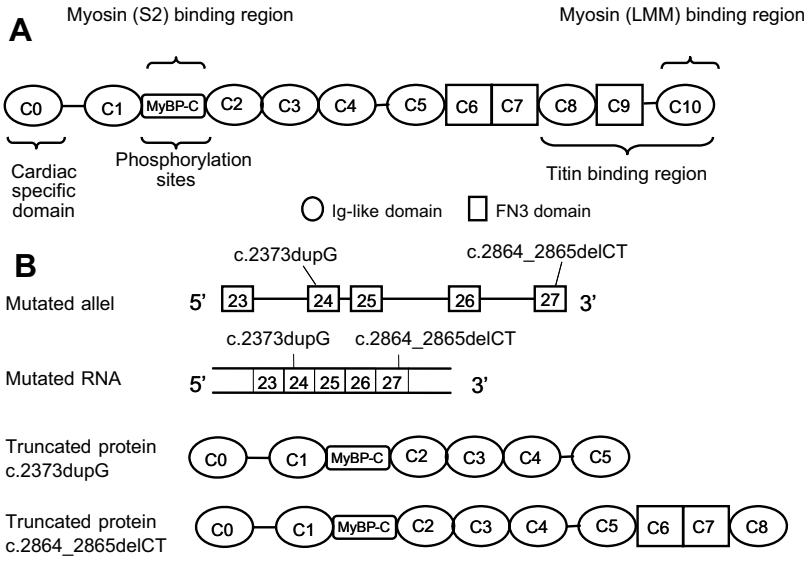

Figure 1.

A: Schematic representation of cMyBP-C structure and MYBPC3 mutations.
Cardiac MyBP-C consists of of 8 immunoglobin (Ig) like and 3 fibronectin (FN3) domains, 15,16 with binding sites for myosin and titin. The N-terminal C0 is specific for cMyBPC, as is the MyBPC like motif between C1 and C2, which contains 3 phosphorylation sites. **B**. Localisation of the mutations in cMyBPC. The c.2373dupG mutation creates a splice donor site, resulting in reading frame shift.8 Consequently, the C-terminus of the protein is altered and terminated after the C5 region, leading to a protein with a predicted weight of ~93 kD. The CT deletion in exon 27 (c.2864_2865delCT)9 creates a premature termination codon in exon 29, which is located 42-bp upstream of the 3'-end. The expected truncated protein of 116 kDa contains 1049 amino-acids including 89 new ones and a termination of the translation at the end of the C8 domain

Table 1. Patient characteristics

Mutation	Age (yrs)	Sex M/F	LVOTPG (mmHg)	ST (mm)	LVEDD (mm)	LVESD (mm)	LVEF (%)	Medication
c.2373dupG	32	F	100	30	40	20	50	BB
c.2373dupG	39	F	60	20	38	21	45	CCB, D
c.2373dupG	45	F	94	20	40	20	50	CCB
c.2373dupG	62	M	64	23	39	22	44	ATII, BB, S
c.2373dupG	44	F	60	17	42	24	43	CCB
c.2373dupG	69	M	74	19	44	30	32	BB, D, S
c.2373dupG	57	F	74	24	41	20	51	BB
c.2864_2865delCT	39	M	116	23	48	26	46	CCB, vitD, Ca
c.2864_2865delCT	44	M	70	20	40	25	38	CCB, BB
c.2864_2865delCT	62	F	67	15	39	24	38	BB, D, S, AA
c.2864_2865delCT	32	F	121	23	44	21	52	BB, CCB, D

M/F, male/female; LVOTPG, left ventricular transaortic pressure gradient; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEF, LV ejection fraction, calculated as (LVEDD-LVESD)/LVEDDx100%. Medication: ATII, angiotensin II receptor antagonist; CCB, calcium channel blocker; BB, beta blocker; D, diuretics; S, statins; vitD, vitamin D; Ca, calcium; AA, anti-arrythmic agent.

Non-failing cardiac tissue from the free LV wall was obtained from donor hearts (n=13; 13-65 years of age, mean 34±5 years; 10/3 male/female) when no suitable transplant recipient was found. The donors had no history of cardiac disease, a normal cardiac examination, normal ECG and normal ventricular function on echocardiography within 24 h of heart explantation. It should be noted that the donor group was slightly younger and included relatively more males compared to the FHCM group.

All samples were immediately frozen and stored in liquid nitrogen. The study protocol was approved by the local ethics committees and written informed consent was obtained.

Quantitative mRNA Analyses

Total RNA was extracted from 5-40 mg of 4 non-failing and 4 FHCM frozen cardiac tissues using the SV Total RNA Isolation kit (Promega) according to the manufacturer's instructions. RNA concentration, purity and quality were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using oligo-dT primers with the Superscript III (Invitrogen) from 50-100 ng RNA. Quantitative determination of wild-type (WT) and mutant cMyBP-C mRNAs was performed by real-time PCR using the TaqMan® ABI Prism® 7900HT sequence detection system (Applied Biosystems) and TaqMan® probes and primers specified as follows (Figure 2A). For the c.2373dupG mutation, primers were designed in exons 23 (F5›-CCT CAC AGT CAA GGT CAT CG-3›) and 25 (R5›-TCC ACC GGT AGC TCT TCT TC-3›). Specific TaqMan® probes were designed to recognize either the WT mRNA in exon 24 (F5›-GAG CCG CCT GCC TAC GAT-3›) or the mutant mRNA at the junction between the smaller exon 24 (-40bp due to the new cryptic donor splice site) and exon 25 (F5›-GCA CAG TAC AGG CTA CAT CCT G-3›). For the c.2864_2865delCT mutation, primers were designed in exons 27 (F5›-AGT GCG GGC ACA CAA TAT G-3›) and 28 (R5›-GGG ATG AGA AGG TTC ACA GG-3›). The WT probe recognized a WT sequence in exon 27 (F5›-TGG AGC CCC TGT TAC CAC C-3›) and the mutant probe recognized a mutant sequence (deleted of CT) in exon 27 (F5›-CTG GAG CCC GTT ACC ACC A-3›). GAPDH was used as endogenous control to normalize the quantification of the target mRNAs for difference in the amount of cDNA added to each reaction. All analyses were performed in triplicates with the software ABI 7900HT SDS 2.2. The mRNA amount was estimated according to the comparative Ct method with the 2-ΔΔCt formula. The amount of both WT and mutant mRNA was reported to the mean of the WT obtained from the 4 non-failing samples for each exon amplification.

Protein Analysis

Cardiac samples (11 $MYBPC3_{mut}$, 8 donor) were treated with trichloro acetic acid prior to protein analysis to preserve the endogenous phosphorylation status of the sarcomeric proteins.[17]

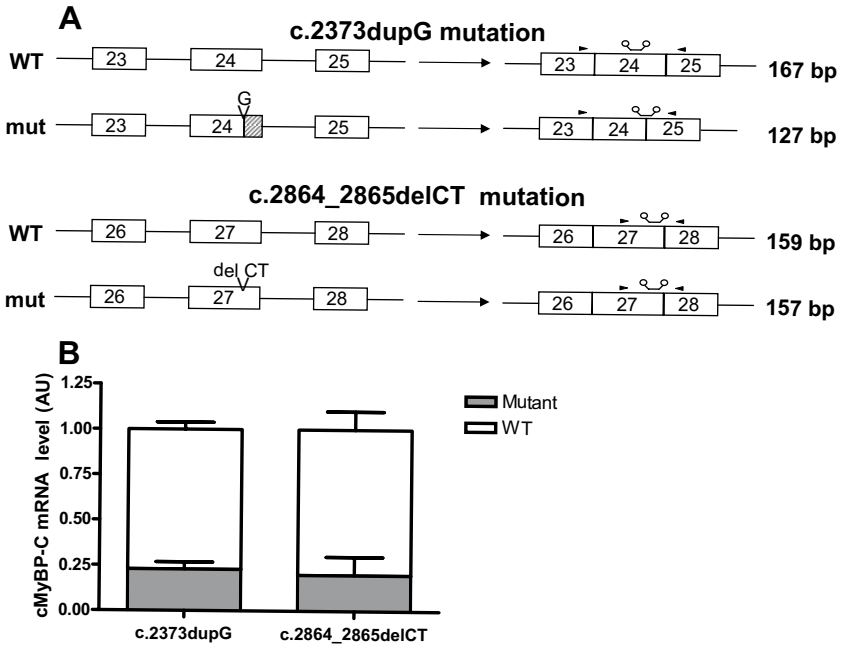

Figure 2: Detection and quantification of mRNA by real-time PCR.
A. Scheme illustrating primers and probes used in real-time PCR using the Taqman system. WT=wild type, MUT=mutant.
B. The amount of mutant mRNA was about 20% of total cMyBP-C mRNAs in both patient groups

Western Immunoblotting

Proteins were separated by one-dimensional gel electrophoresis on a 15% polyacrylamide SDS-gel and subsequently transferred to nitrocellulose paper by wet blotting. Polyclonal antibodies (diluted 1:1000) raised against recombinant C0C2, C5 and C8C9 produced from human cDNA encoding cMyBP-C[15] (Figure 1) were used for detection of cMyBP-C (Dr. S. Winegrad, University of Pennsylvania, Philadelphia, USA). Primary antibody binding was visualized using a secondary goat-anti-rabbit antibody (diluted 1:2000) and enhanced chemiluminescence (Amersham).

To detect truncated cMyBP-C, the two antibodies were used, which are directed to the N-terminal part of cMyBP-C (C0C2) and the middle region of cMyBP-C (C5) (Figure 1). The sensitivity of the two antibodies to detect low amounts of truncated cMyBP-C under the experimental conditions used was assessed using a dilution series of a non-failing donor sample (0.04 µg to 5 µg). The dilution at which the cMyBP-C band was still discernable was defined as the lower detection limit and amounted to 1.6% (0.08 µg) for the C0C2 antibody and 3.2% (0.16 µg) for the C5 antibody (data not shown).

In addition, phosphorylation of cMyBP-C at Ser282 (P-cMyBP-C; dilution 1:1000) and bisphosphorylation of cardiac troponin I (cTnI) at Ser23/24 (i.e. PKA sites, rabbit

polyclonal antibody; dilution 1:500, Cell signaling) were analyzed and normalized to cMyBP-C (C0C2 antibody) and cTnI (8I-7, mouse monoclonal antibody; dilution 1:6000, Spectral Diagnostics), respectively, to correct for differences in protein loading.

SYPRO Ruby and ProQ Diamond Staining of Gradient Gels

Proteins were separated on 4-15% pre-cast Tris-HCl gels (BioRad) and stained with SYPRO Ruby and ProQ Diamond to determine sarcomeric protein levels and phosphorylation, respectively, as described previously.[17] The phosphorylation status of sarcomeric proteins was expressed relative to α-actinin, except when noted otherwise. Protein values of $MYBPC3_{mut}$ are given as fraction (or percentage) of the value found for donor samples, which was set to 1 (or 100%).

Isometric Force Measurements

Cardiomyocytes were mechanically isolated from small tissue samples as described previously.[18] Triton-permeabilized cardiomyocytes were glued between a force transducer and a piezoelectric motor and stretched to a sarcomere length of ~2.2 μm. Force measurements were performed at various calcium concentrations (pCa, $-\log_{10}[Ca^{2+}]$, values ranging from 4.5 to 6.0) as described previously.[18,19] Force measurements were performed in single cardiomyocytes isolated from 10 $MYBPC3_{mut}$ (34 cardiomyocytes) and 13 donor samples (47 cardiomyocytes). On average 2-5 cardiomyocytes were studied for each patient/donor. One $MYBPC3_{mut}$ biopsy was too small to isolate cardiomyocytes and was used for protein analysis only. Cross-sectional area (width*depth*π/4) of the cardiomyocytes determined at a sarcomere length of 2.2 μm was significantly higher in $MYBPC3_{mut}$ (508±67 μm^2) than in donor (374±26 μm^2). Length between the attachments did not significantly differ and amounted to 62±5 μm in $MYBPC3_{mut}$ and 72±4 μm in donor. Passive tension ($F_{passive}$) was determined by shortening the cell in relaxation solution (pCa 9.0) by 30 %. Maximal calcium activated tension (F_{active}, i.e. maximal force/cross-sectional area) was calculated by subtracting $F_{passive}$ from the total force (F_{total}) at saturating $[Ca^{2+}]$ (pCa 4.5). Ca^{2+}-sensitivity is denoted as pCa_{50}, i.e. pCa value at which 50% of F_{active} is reached. Force measurements were repeated after incubation of cells for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of protein kinase A (PKA; 100 U/mL, Sigma) or with the catalytic domain of protein kinase C (PKC; 0.25 U/mL, Sigma).

Data Analysis

Data are presented as means ± S.E.M. Cardiomyocyte force values were averaged per sample and mean values for $MYBPC3_{mut}$ and donor samples were compared using unpaired Student *t*-tests. Effects of PKA/PKC were tested with 2-way ANOVA. $P<0.05$ was considered significant. *Denotes significant difference between $MYBPC3_{mut}$ and donor, † Denotes significant difference before vs. after PKA/PKC treatment.

RESULTS

Reduced Mutant cMyBP-C mRNA Level in FHCM Ventricular Tissue

To investigate whether both WT and mutant cMyBP-C mRNAs were transcribed in FHCM ventricular tissues, real-time RT-PCR was performed using specific Taqman probes and primers for each mutation (Figure 2A). The c.2373dupG mutation was expected to induce a cryptic donor splice site in exon 24 and the skipping of 40 nucleotides (Figure 1B). We therefore designed WT and mutant probes accordingly. Similarly, a specific mutant probe was designed for the 2864-2865 CT deletion in exon 27 and the corresponding WT probe (Figure 2A). Mutant mRNA represented 23% and 20% of the total cMyBP-C mRNA in c.2373dupG and c.2864_2865delCT groups, respectively (Figure 2B). Since both mutations result in a frameshift and a premature termination codon (Figure 1B), the data suggest that both nonsense mutant mRNAs are partially subjected to degradation by the nonsense-mediated mRNA decay (NMD).[20]

Reduced cMyBP-C Protein Level in FHCM Ventricular Tissue

The presence of truncated cMyBP-C in $MYBPC3_{mut}$ patients was examined by Western Immunoblotting. After separation by gel electrophoresis proteins were transferred to nitrocellulose and visualized with Ponceau (Figure 3A). Figure 3B shows results using an antibody directed against the C0C2 region of cMyBP-C. None of the antibodies used (against C0C2, C5 or C8C9) revealed truncated cMyBP-C (predicted mass at 93 or 116 kDa) in any of the samples. Based on the sensitivity of our Western immunoblot analysis, levels of truncated cMyBP-C lower than 1.6% could not be detected. Hence, we cannot completely exclude the presence of trace amounts of truncated cMyBP-C in $MYBPC3_{mut}$. However, overloading of $MYPBC3_{mut}$ samples (40 µg; 8x higher concentration) did not reveal protein bands at the predicted mass (not shown), indicating the trace amounts of truncated protein, if present, would be even less than 0.2%.

To determine the levels of full-length cMyBP-C, proteins were separated by 1D gel electrophoresis and stained with SYPRO Ruby (Figure 3C). The α-actinin intensity was used as loading control. The cMyBP-C/α-actinin protein ratio on the SYPRO Ruby stained gels was 33% lower in $MYBPC3_{mut}$ (n=11) compared to donor (n=8)(Figure 3D). Western immunoblot analysis confirmed reduced level of full-length cMyBP-C (Figure 3B) in $MYBPC3_{mut}$ compared to donor myocardium. On average Western blot data using the C0C2 antibody showed a 23% lower amount of cMyBP-C (normalized to Ponceau-stained actin on the same blot) in $MYBPC3_{mut}$ compared to donor. However, the coefficient of variation of the Western immunoblot analysis was much higher than that of SYPRO Ruby staining (56.3% and 25.8%, respectively). Therefore the SYPRO values are considered to represent the protein levels more accurately.

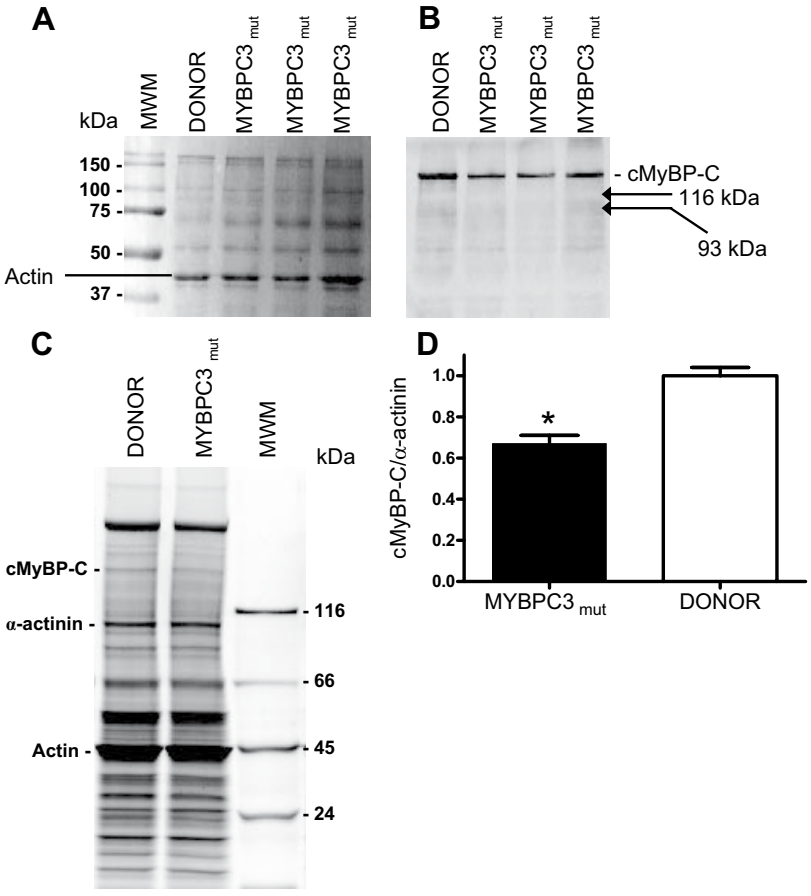

Figure 3: Cardiac MyBP-C protein levels.
A. Western immunoblots were stained with Ponceau to visualize proteins. **B.** Western immunoblot using a C0C2 antibody illustrates reduced expression of full-length cMyBP-C (~140 kD) in cardiac biopsies from MyBP-C_{mut} patients compared to donor (MWM, molecular weight marker). No traces were found of truncated cMyBP-C (theoretical mass: 93 kDa (c.2373dupG); 116 kDa (c.2864_2865delCT)). **C.** Representative cardiac samples on a gradient gel stained with SYPRO Ruby (donor vs $MYBPC3_{mut}$). **D.** Relative expression of cMyBPC is significantly lower in $MYBPC3_{mut}$ (n=11) compared to donor (n=8). *P<0.05.

Deranged Phosphorylation

The SYPRO Ruby stained gels were also stained with ProQ Diamond, which selectively stains phosphorylated serine, threonine and tyrosine residues (Figure 4A). Phosphorylation of cMyBP-C normalized to α-actinin was reduced by 47±7% in the $MYBPC3_{mut}$ compared to donor myocardium (P<0.0001; Figure 4B). Interestingly, phosphorylation of cMyBP-C normalized to its own protein level (determined with SYPRO) was similar between $MYBPC3_{mut}$ and donor myocardium (79±11%; P=0.14; Figure 4C).

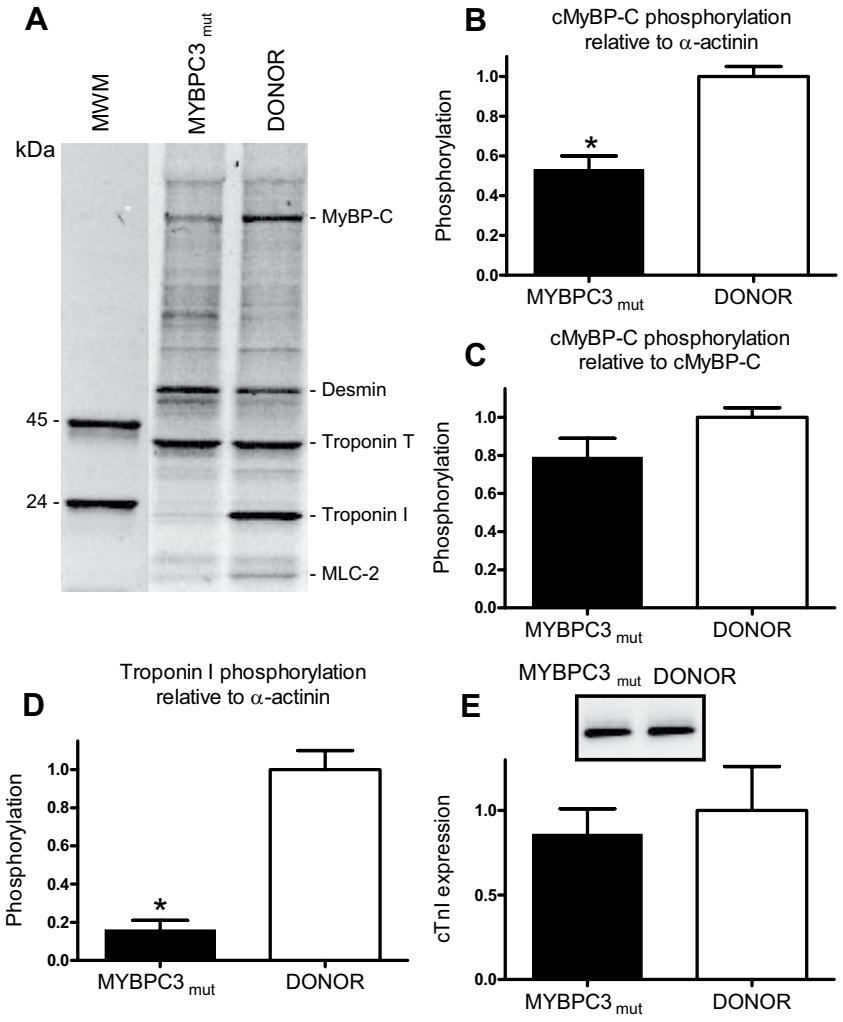

Figure 4: Phosphorylation status of cMyBP-C and cTnI.
A. ProQ diamond-stained gradient gel. **B.** The phosphorylation status of cMyBP-C (relative to SYPRO Ruby stained α-actinin) is lower in $MYBPC3_{mut}$ (n=11) compared to donors (n=8), while phosphorylation of cMyBP-C normalized to its own expression level is comparable between $MYBPC3_{mut}$ and donors (**C**). **D.** The phosphorylation status of cTnI (relative to SYPRO Ruby stained α-actinin) is lower in $MYBPC3_{mut}$ compared to donors. **E.** cTnI relative to Ponceau-stained actin did not differ between $MYBPC3_{mut}$ and donor myocardium *P<0.05

In addition, massive dephosphorylation of troponin I (cTnI) was observed in $MYBPC3_{mut}$ relative to donor by 84±5% (P<0.0001; Figure 4D). As the reduced ProQ Diamond signals for cTnI in $MYBPC3_{mut}$ may be due to reduced cTnI expression, we have analyzed the steady-state level of cTnI by Western blot. The amount of cTnI relative to Ponceau-stained actin did not differ between $MYBPC3_{mut}$ and donor myocardium (Figure 4E).

Hence, the reduced ProQ Diamond signals represent reduced phosphorylation of cTnI in MYBPC3$_{mut}$ compared to donor. Phosphorylation of other sarcomeric proteins desmin, troponin T (cTnT) and myosin light chain 2 (MLC-2) was also significantly reduced in MYBPC3$_{mut}$ by, respectively, 24±8%, 41±7% and 61±4% relative to donor samples.

Western immunoblot analysis (Figure 5) revealed significantly lower bisphosphorylation of PKA-sites (Ser23/24) in cTnI in MYBPC3$_{mut}$ compared to donors, whereas phosphorylation of cMyBP-C at Ser282 was similar in MYBPC3$_{mut}$ and donor myocardium. This confirms the data obtained with ProQ Diamond stain (Figure 4).

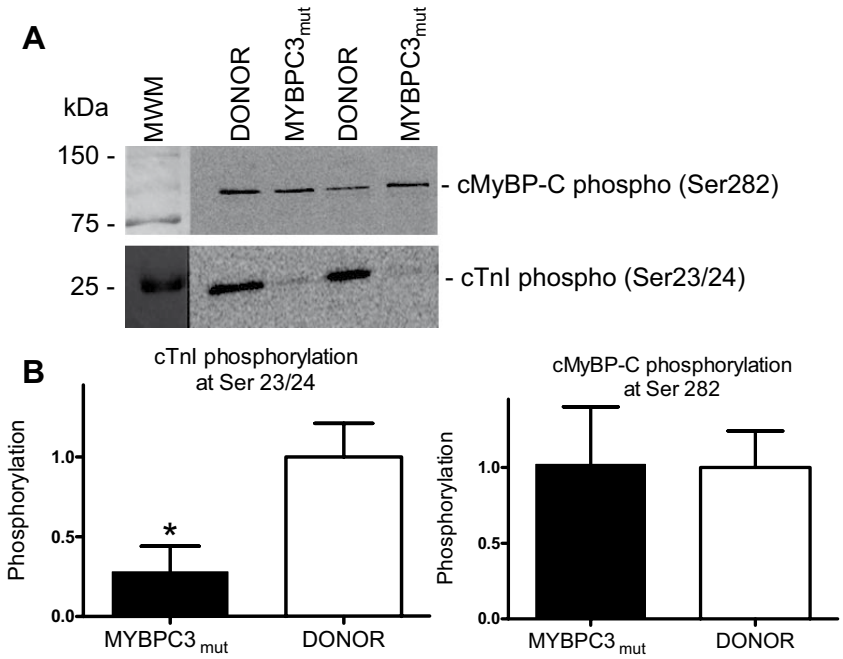

Figure 5:
Western immunoblot analysis using a specific antibody against cTnI phosphorylated at PKA-sites (Ser-23/24) revealed reduced cTnI phosphorylation

Depressed Force Development

Functional implications of the *MYBPC3* mutations were investigated by cardiomyocyte force measurements in c.2373dupG (n=7; 25 cells) and c.2864_2865delCT (n=3; 9 cells). A representative cardiomyocyte and a recording at saturating Ca^{2+}-concentration (pCa 4.5) from a MYBPC3$_{mut}$ patient are shown in Figures 6A,B. Maximal force development (F_{active}) was significantly depressed in MYBPC3$_{mut}$ (20.2±2.7 kN/m^2) compared to donor (34.5±1.1 kN/m^2; n=13; 47 cells)(Figure 6C). Passive force ($F_{passive}$) in MYBPC3$_{mut}$ (3.8±0.7 kN/m^2) was somewhat elevated compared to donor (3.4±0.4 kN/m^2), but the difference was not significant (Figure 6D). Inter-patient variation in F_{active} and $F_{passive}$

in the MYBPC3$_{mut}$ group was larger than in the donor group (Figures 6E,F). F_{active} and $F_{passive}$ were lower in c.2864_2865delCT than in c.2373dupG (ns).

Cells from MYBPC3$_{mut}$ (c.2373dupG, n=4, 9 cells; c.2864_2865delCT; n=2; 6 cells) and donors (n=6; 18 cells) were incubated with protein kinase A,[21] after which F_{active} and $F_{passive}$ measurements were repeated to determine whether βadrenergic stimulation could correct depressed force in MYBPC3$_{mut}$. PKA treatment resulted in a minor decrease in F_{active} in MYBPC3$_{mut}$ (before vs after: 14.2±2.9 and 13.5±2.5 kN/m^2), which was similar to the PKA-effect in donor (before vs after: 27.9±3.5 and 25.8±3.2 kN/m^2)(P=0.03; 2-way ANOVA). Moreover, PKA significantly decreased $F_{passive}$ in both groups (MYBPC3$_{mut}$ before vs after: 2.3±0.3 and 2.1±0.2 kN/m^2; donor before vs after: 3.3±0.5 and 2.7±0.4 kN/m^2)(P=0.001; 2-way ANOVA).

As increased protein kinase C activity/expression has been associated with depressed maximal force generating capacity of myofilaments,[22] force measurements were repeated after incubation with the catalytic domain of PKC. F_{active} in MYBPC3$_{mut}$ (c.2373dupG, n=3, 5 cells) was significantly reduced after PKC by 2.6±0.6 kN/m^2. A similar decrease (3.3±0.7 kN/m^2) was found in cardiomyocytes from donor hearts (n=5; 9 cells). PKC slightly decreased $F_{passive}$ in both groups, although the effect was not significant (data not shown).

Enhanced Ca^{2+}-sensitivity

Ca^{2+}-sensitivity of the sarcomeres was significantly higher in MYBPC3$_{mut}$ (pCa_{50}=5.62±0.04) than in donor cells (pCa_{50}=5.54±0.02). The average force-pCa relationships obtained in MYBPC3$_{mut}$ and donor cardiomyocytes are shown in Figure 7A. Ca^{2+}-sensitivity was similar in c.2864_2865delCT (pCa_{50}=5.63±0.05) and c.2373dupG (pCa_{50}=5.60±0.09). Figure 7B illustrates that inter-patient variation in pCa_{50} was larger in the MYBPC3$_{mut}$ than in the donor group.

Treatment with exogenous PKA significantly reduced Ca^{2+}-sensitivity in both groups. The reduction in pCa_{50} was significantly larger in MYBPC3$_{mut}$ (ΔpCa_{50}=0.18±0.03) than in donor (ΔpCa_{50}=0.06±0.01) cells (Figure 7C). PKA treatment abolished the initial difference in Ca^{2+}-sensitivity between MYBPC3$_{mut}$ and donor (Figure 7D). ProQ-diamond staining of a MYBPC3$_{mut}$ sample which was incubated without (CTRL, control incubation) and with protein kinase A (PKA) showed a 4-fold increase in cTnI phosphorylation, while the increase in cMyBP-C phosphorylation was only small (Figure 7E).

Similar as PKA, PKC significantly reduced pCa_{50} in both groups. However, in contrast to PKA, the PKC-induced shift in pCa_{50} did not significantly differ between MYBPC3$_{mut}$ (ΔpCa_{50}=0.11±0.04) and donor cells (ΔpCa_{50}=0.08±0.01) and thus, does not explain the baseline difference in Ca^{2+}-sensitivity between MYBPC3$_{mut}$ and donor myocardium.

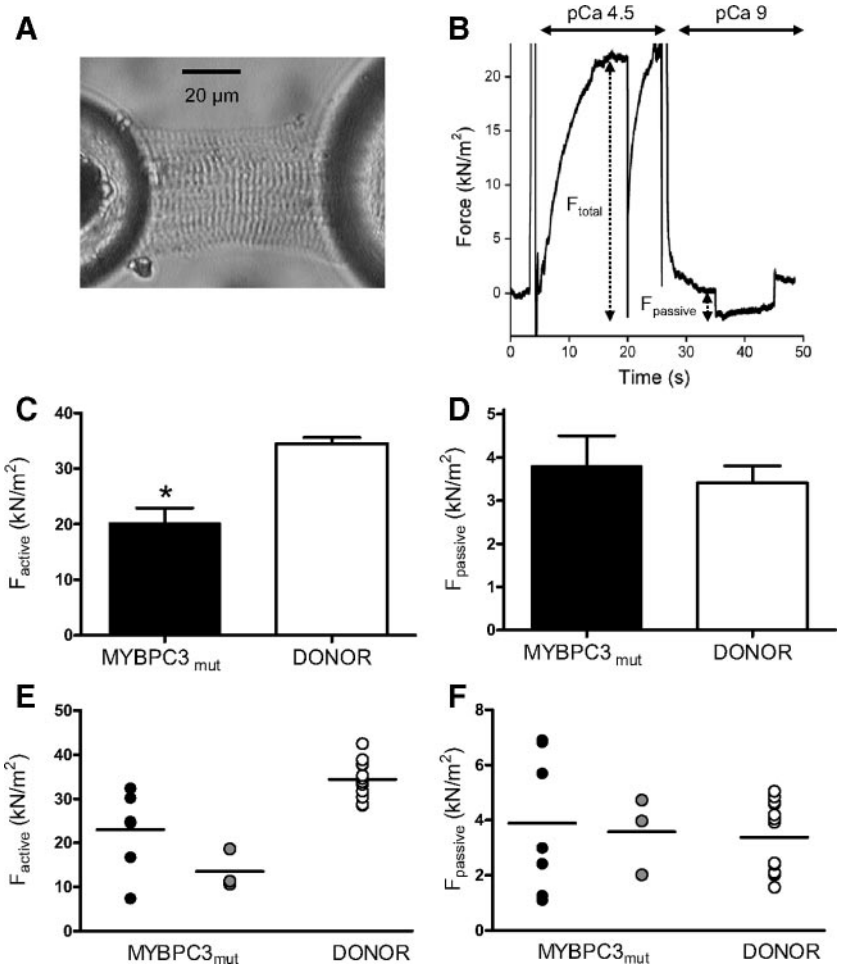

Figure 6:
Force measurements. Permeabilized cardiomyocyte (**A**) and force recording (**B**) of a MYBPC3$_{mut}$ sample. When a maximal steady force level was reached at saturating [Ca2+](pCa4.5), the cell was quickly shortened by 30% to determine the baseline of the force recording and total force (F_{total}). Subsequently, the cell was transferred to relaxing solution (pCa 9.0) and shortened for a period of 10 seconds to determine passive force ($F_{passive}$). **C**. Active force (Factive= F_{total}-$F_{passive}$) in MYBPC3$_{mut}$ was significantly depressed, while $F_{passive}$ (**D**) was slightly but not significantly elevated compared to donor. **E,F**. Graphs illustrating the variation among mean force parameters of individuals among the MYBPC3$_{mut}$ group (c.2373dupG, black symbols; c.2864_2865delCT, grey symbols) compared to the donor group (open symbols). *P<0.05.

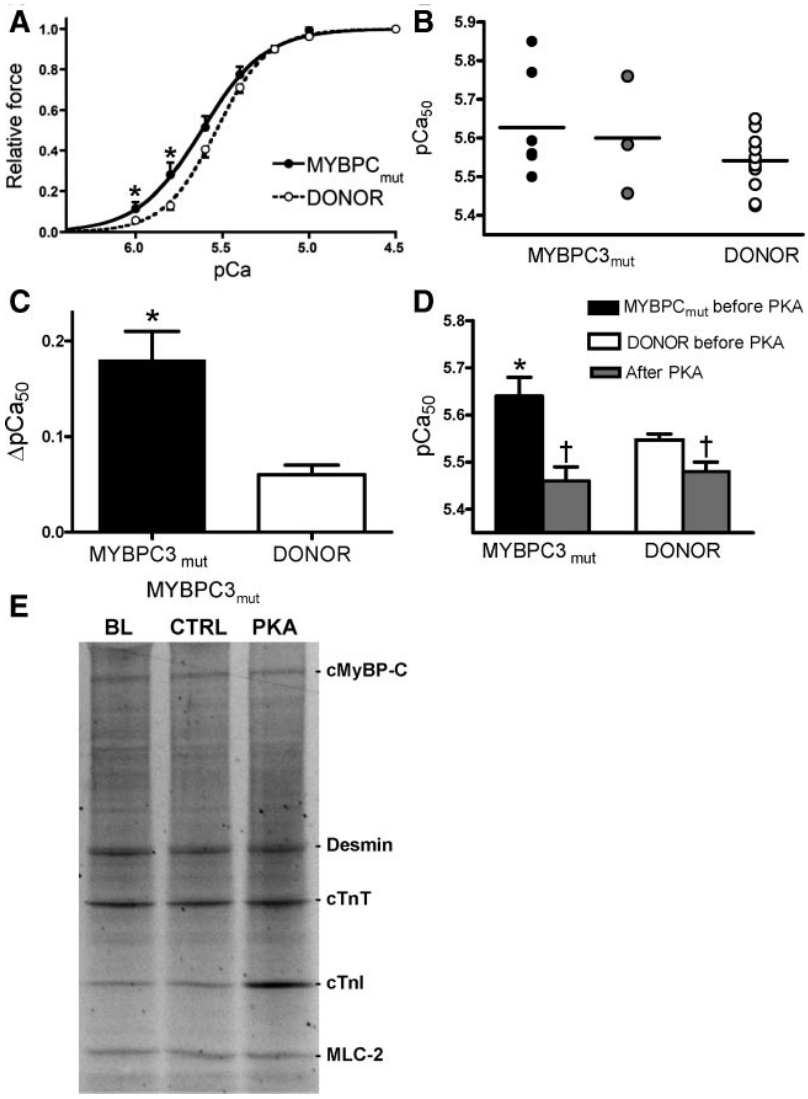

Figure 7:

A. Force measurements were carried out at maximal and submaximal [Ca^{2+}] (pCa range 4.5-6). Force values obtained in solutions with submaximal [Ca^{2+}] were normalized to the maximal force level at pCa 4.5. Force-pCa relations were fit to a modified Hill equation. Ca^{2+}-sensitivity of the sarcomeres (pCa_{50}) was significantly higher in $MYBPC3_{mut}$ than in donor. **B.** Graph illustrating the variation among mean pCa_{50} parameters of individuals among the $MYBPC3_{mut}$ group (c.2373dupG, black symbols; c.2864_2865delCT, grey symbols) compared to the donor group (open symbols). **C.** Treatment of cardiomyocytes with exogenous PKA induced a larger reduction in pCa_{50} in $MYBPC3_{mut}$ than in donor. **D.** PKA treatment abolished the initial difference in Ca^{2+}-sensitivity between $MYBPC3_{mut}$ and donor. *P<0.05 $MYBPC3_{mut}$ vs. donor; † P<0.05 before vs. after PKA treatment. **E.** ProQ-diamond staining of $MYBPC3_{mut}$ myocardium, which was directly frozen (BL, baseline) or incubated without PKA (CTRL, control) or with PKA (PKA; 100 U/mL). Phosphorylation of troponin I (cTnI) increased upon incubation with PKA. Abbreviations: cMyBP-C, myosin-binding protein C; cTnT, troponin T; MLC-2, myosin light chain 2.

DISCUSSION

Our study provides direct evidence for reduced cMyBP-C protein level and contractile dysfunction in a group of FHCM patients with *MYBPC3* frameshift mutations. Consistent with previous studies,[7-9] no truncated cMyBP-C protein was detected, and the amount of full-length cMyBP-C was 33% lower in FHCM compared to donor myocardium. Our data therefore indicate that the pathomechanism involves haploinsufficiency, rather than a poison polypeptide. Using adenoviral gene transfer of cardiomyocytes Sarikas et al.[23] showed rapid and quantitative degradation of truncated forms of cMyBP-C by the ubiquitin-proteasome system (UPS), which could in turn inhibit UPS-mediated degradation of other cellular proteins.[23] Thus, the absence of truncated cMyBP-C, in FHCM patients in the present study suggests that the UPS may degrade truncated cMyBP-C. The full-length C-protein in contrast compensates for the absence of truncated protein. The fact that mutant mRNAs were detected in both FHCM groups supports the involvement of the UPS in the degradation of truncated protein. On the other hand, the lower level of mutant vs WT cMyBP-C mRNA in both FHCM groups suggests partial instability of nonsense mutant mRNA, which could be degraded by the NMD.[20]

The maximum force generating capacity (i.e. F_{active}) of cardiomyocytes from MYPBC3$_{mut}$ carriers was significantly reduced by 42% compared to non-failing myocardium. Recent studies[24-28] revealed an important role for cMyBP-C in cross-bridge kinetics. Loss of cMyBP-C accelerates cross-bridge cycling and impairs kinetics of contraction and relaxation.[24,25,27] Complete knockout of cMyBP-C resulted in profound cardiac hypertrophy and impaired contractile function in mice.[29,30] Surprisingly, transgenic mice harboring only 40% of the normally expressed full-length cMyBP-C did not have left ventricular hypertrophy and showed preserved cardiac function.[26] In contrast, our study shows that an approximately 33% reduction of full-length cMyBP-C level is sufficient to trigger left ventricular hypertrophy and contractile dysfunction in human. Intriguingly, reduced cMyBP-C levels per se does not seem to explain the decline in maximum force in MYBPC3$_{mut}$ as F_{active} did not correlate with the level of full-length cMyBP-C (not shown), but may rather involve reduced expression and altered phosphorylation of cMyBP-C.

Cardiac MyBP-C is phosphorylated by protein kinase A upon adrenergic stimulation.[31] Apart from PKA, cMyBP-C can be phosphorylated by Ca^{2+}-calmodulin dependent kinase (CaMK)[32,33] and protein kinase C (PKC).[34,35] Transgenic mice hearts in which the phosphorylation sites of cMyBP-C were changed to nonphosphorylatable alanines displayed reduced contractility and altered sarcomeric structure indicating that phosphorylation of cMyBP-C is essential for normal cardiac function.[36] Reduced cMyBP-C phosphorylation has been observed in animal models of cardiac hypertrophy and failure[36,37] and in human with end-stage idiopathic and ischemic cardiomyopathy.[21,37] The discrepant phosphorylation levels of cMyBP-C and cTnI in MYBPC3$_{mut}$ are in contrast

with previous observations in non-FHCM (idiopathic and ischemic cardiomyopathy) and donor myocardium, which revealed parallel changes in the main target proteins of the β-adrenergic pathway.[21,37] Hence it is possible that this discrepancy causes contractile dysfunction. As protein kinase A did not correct the reduction in F_{active}, other (mal)adaptive signaling routes are responsible for divergent phosphorylation of cMyBP-C and cTnI and sarcomeric dysfunction. In a recent study, increased protein kinase C expression level in two models of heart failure (pressure overload and ischemic) in rat was associated with reduced maximal force generating capacity of myofilaments.[22] To test whether this applies to human tissue, force measurements were performed in single human cardiomyocytes from $MYBPC3_{mut}$ and donor hearts before and after incubation with PKC. The effects of PKC on cardiomyocyte force parameters ($F_{active,}$ $F_{passive}$ and pCa_{50}) were similar in $MYBPC3_{mut}$ and donor cardiomyocytes, indicating that impaired myofilament function in $MYBPC3_{mut}$ does not seem to be related to a difference in PKC-mediated phosphorylation of myofilament proteins. Interestingly, Yuan et al.[38] revealed differential phosphorylation of cMyBP-C upon myocardial stunning and suggested a role for altered calcium handling and activation of CaMK. Thus, in combination with the evidence presented in the literature, our experiments suggest that the reduced maximal force generating capacity of cardiomyocytes is not a direct consequence of haploinsufficiency, but rather might be caused by differential phosphorylation of cMyBP-C resulting from (mal)adaptive neurohumoral signaling in hearts of $MYBPC3_{mut}$ carriers.

Similarly, the higher Ca^{2+}-sensitivity of force development in $MYBPC3_{mut}$ patients may either be a direct or an indirect consequence of the cMyBP-C haploinsufficiency. Previous studies of FHCM mutations in the thin filament proteins, troponin and tropomyosin, reported enhanced myofilament Ca^{2+}-sensitivity, in contrast to a reduction in Ca^{2+}-sensitivity which is considered characteristic for mutations found in familial dilated cardiomyopathy.[39-41] Robinson et al.[41] proposed that the mutant-induced enhanced Ca^{2+}-sensitivity reflects changes in Ca^{2+} binding affinity, which may directly alter Ca^{2+} transient and trigger hypertrophic signaling routes.[42] Extraction of cMyBP-C by ~30-70% from rat cardiomyocytes resulted in an increase in Ca^{2+}-sensitivity.[43,44] Similarly, a greater myofilament Ca^{2+} sensitivity was found in skinned myocytes at short sarcomere length from cMyBP-C knock-out (KO) mice,[45] and a greater sensitivity to external Ca^{2+} was found in cMyBP-C KO intact atrial tissue.[25] Hence, the frameshift *MYBPC3* mutations inducing cMyBP-C haploinsufficiency may directly increase Ca^{2+}-sensitivity. On the other hand, increased myofilament Ca^{2+}-sensitivity has also been found in end-stage failing human myocardium (idiopathic dilated cardiomyopathy) without known mutations in sarcomeric proteins.[19,46,47] This enhanced Ca^{2+}-sensitivity has been ascribed to hyperactivation of the β-adrenergic signaling pathway in response to reduced cardiac pump function. Chronic activation of the β-adrenergic receptor pathway results in down-regulation and desensitization of the receptors in failing myocardium and a

subsequent parallel reduction in phosphorylation of the PKA-target proteins, cMyBP-C and cTnI.[17,21,37] In healthy myocardium, the main effect of PKA-mediated phosphorylation of cTnI is reduced Ca^{2+}-sensitivity, which contributes to appropriate myocardial relaxation.[27,48,49] Reduced phosphorylation of cTnI and PKA-mediated increase in cTnI phosphorylation and correction of Ca^{2+}-sensitivity to donor values in $MYBPC3_{mut}$ suggest that heart failure-induced β-adrenergic desensitization underlies the increase in Ca^{2+}-sensitivity. Hence, on the basis of our data we postulate that the enhanced Ca^{2+}-sensitivity of sarcomeres in $MYBPC3_{mut}$ is a secondary consequence of the frameshift mutation-induced cardiac dysfunction, which triggers adrenergic hyperactivation. The ensuing defects in β-adrenergic signaling may impair phosphorylation of Ca^{2+} handling proteins and subsequent alterations in cellular Ca^{2+} transient may activate kinases, such as CaMK, involved in differential phosphorylation of cMyBP-C and cTnI.

It should be noted that our data may be confounded by differences in medication and in the origin of LV tissue (septum versus free wall). Moreover, we cannot exclude that age and sex differences affected our analysis. However, the unique *MYBPC3* founder mutations allowed us to characterize contractile properties in a relatively homogenous group of FHCM patients. The combined analysis of sarcomere protein composition and function revealed haploinsufficiency and reduced contractility in patients carrying a frameshift *MYBPC3* mutation. The sarcomeric phenotype in $MYBPC3_{mut}$ is the complex resultant of the mutation and secondary alterations in the sarcomeric phosphoproteome due to maladaptive alterations in neurohumoral signaling and/or Ca^{2+} homeostasis. Therefore our data support the concept that contractile dysfunction is a pivotal link between the mutant sarcomeric protein and pathological hypertrophic cardiomyopathy.

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Part II

Genetics in hypertrophic cardiomyopathy

Chapter 6

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

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is a disease characterized 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 characterized 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 pre-symptomatic 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 cardiologic screening in our HCM population and give an overview of the current literature available on this subject.

INTRODUCTION

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 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 followed and completely genotyped for eight HCM genes.

In the Netherlands a large number of HCM cases is associated with one of three founder mutations (2373insG, R943X and 2864delCT) in the *cardiac myosin binding*

Table 1: Genes identified in familial hypertrophic cardiomyopathy (modified from Ho and Seidman, Circulation 2006;113;858-862).

Gene	Encoding protein	Locus
MyBPC3	Myosin Binding Protein C	11p11.2
MYH7	ß-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

protein C (MYBPC3)[9]. Mutations in the *beta-cardiac myosin heavy chain (MYH7)* and other sarcomere genes are less frequent.

At the cardio-genetics clinic of the Erasmus MC in Rotterdam, the molecular analysis of HCM involves initial screening for the three Dutch founder mutations in the *MYBPC3* gene. In 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 6 months. In families where the molecular defect has been identified, test results for family members are available within 6 weeks.

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[7,10].

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. 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.

Cardiac myosin binding protein-C gene (MYBPC3)

Generally, mutations in the *MYBPC3* are the most common genetic defect in HCM with a prevalence ranging from 20 to 42%[3,11]. In the Rotterdam HCM population 70% (100/142) of the HCM families with an identified mutation had a *MYPBC3* mutation.

The study by Richard et al. showed a relatively benign or intermediate prognosis in 90 % of the families carrying a mutation in the *MYBPC3*[3]. Controversly, others reported that *MYBPC3* associated disease was not statistically different from families with any of the other genetic defects according to several parameters, including age at diagnosis, frequency of surgery or need for implantation of an ICD and the distribution of hypertrophy7. Our own data indicate that the R943X and 2864delCT founder mutations in *MYBPC3* are associated with a malignant prognosis[12].

Beta-cardiac myosin heavy chain gene (MYH7)

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). Generally, mutations the *MYH7* gene are associated with a younger age at diagnosis and a more profound hypertrophy[8,11].

GENETIC COUNSELING, PRESYMPTOMATIC TESTING AND SCREENING OF AT RISK RELATIVES

After the clinical diagnosis of HCM is established at our HCM outpatient clinic, the importance of molecular confirmation, also in patients with no family history of HCM or SD, is discussed with the patient by the cardiologist (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[13]. After informed consent blood samples are drawn for DNA analysis of the proband.

Subsequently, patients are referred to the department of clinical genetics for the ascertainment of 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 counseling by a clinical geneticist and/or a genetic nurse. Expanding the family history allows the identification of more at risk relatives that are subsequently invited for genetic counseling (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 6 weeks. No further cardiologic screening is indicated for relatives without the disease causing mutation. The mutation carriers are referred for cardiologic screening by medical history, clinical examination, echocardiography, exercise-ECG and 24-hours Holter monitoring. 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 2). 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[14]. In relatives fulfilling the diagnostic criteria for HCM we continue with an exercise-ECG and 24-hours Holter monitoring for risk stratification.

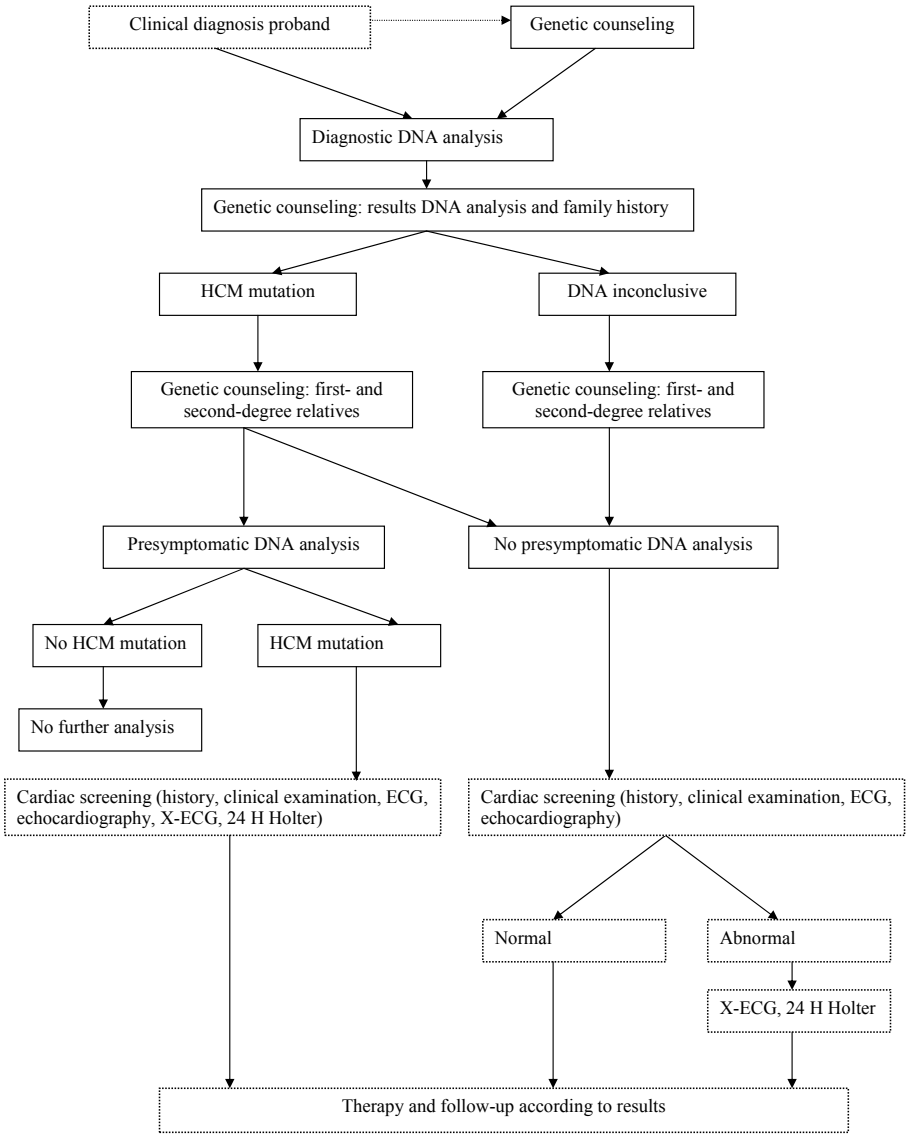

Figure 1:
Flow chart used at the cardio-genetics department in the Erasmus MC University Hospital, showing the multidisciplinary approach by the cardiologist (dashed boxes) and clinical genetic nurses and medical doctors (un-dashed boxes). (X-ECG=exercise test, 24 H Holter= 24 hours Holtermonitoring)

In mutation carriers and in relatives in whom the genotype is not known this cardiac screening is repeated according to the results, and therapy is initiated when necessary. In adult persons repeated screening is indicated after 3 to 5 years when all tests are normal (Figure 1)[15,16].

Table 2: Diagnostic criteria for hypertrophic cardiomyopathy in adult members of affected families (modified from McKenna, Heart, 1997. 77(2): p. 130-2)

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), V3-V6 (≥ 3 mm) or II, III, aVF (≥ 5 mm)	Minor repolarisation changes in LV leads
Abnormal Q (> 40ms or > 25% R wave) in at least 2 leads from II, III, aVF, V1-V4; or I, aVL, V5-V6	Deep S V2 (> 25 mm)

The goal for the next 3 years is to further develop our cardio-genetic outpatient clinic and to identify all affected at risk relatives of the HCM patients in the Rijnmond region.

Screening for HCM in children

Cardiologic screening by medical history, physical examination, ECG and echocardiogram in children of HCM families is performed after the age of 10. Cardiologic screening of younger children will be done when there is suspicion of HCM symptoms in the child, when the family history involves early onset HCM or SD, upon parental request or if the child with suspicion of HCM enrolls in competitive sports.

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 emphasized the potential benefit of testing in HCM[17], 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 to prevent clinical expression child bearing a mutation, combined with possible adverse psychological effects[8,18].

If HCM is diagnosed by echocardiography, the diagnosis may be confirmed by screening for the familial mutation. Pre-symptomatic genotyping of children is, like in other mostly late adult-onset diseases, only done in exceptional cases[19].

Genetic counseling 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 has been identified, there is a possibility for prenatal diagnosis of HCM with chorionic villus sampling as early as 10 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 – 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 cardiological 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[20-22]. In a long-term follow-up study of patients with a mutation in *MyBPC3* disease penetrance was 100% in patients aged > 50 years[20]. Follow-up at regular intervals, including echocardiography, exercise testing and 24-hours 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[23].

In asymptomatic carriers and at risk relatives repeated clinical screening is advised, every 2 years between the age of 10 to 22 years, and every 5 years in older mutation carriers[16].

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 like swimming, brisk walking, biking and skating are permitted[24]. 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[25,26].

Socio-economic consequences of presymptomatic testing for HCM

In an unselected patients population HCM is a benign disease with a incidence of cardiac death of less than 1 % per year[27]. 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[28]. 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[29] and statins[30] showing delay in onset of HCM. In patients with non obstructive HCM treatment with angiotensin II blockade[31] 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[32-34].

TREATMENT OF PATIENTS WITH CLINICAL DIAGNOSIS OF HCM

The HCM patients are treated according to the guidelines[35]. 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[36]. 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 3) had a 4 to 5 % estimated annual SD risk, where as in the absence of risk factors this risk was < 1 % 37. A recent study from the same group showed that LVOT obstruction in asymptomatic patients with no other SD risk markers was not related with increased mortality and did not warrant aggressive interventions[38]. Prophylactic pharmacological therapy with beta-blockers or calcium channel antagonist 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[39]. 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[40,41.]

Table 3: Risk factors for sudden death in patients with hypertrophic cardiomyopathy

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

CONCLUSIONS

The identification of autosomal dominantly inherited genetic defects in a 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 7

Disease penetrance and risk stratification for sudden cardiac death in asymptomatic hypertrophic cardiomyopathy mutation carriers

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ABSTRACT

Aims

To investigate the outcome of cardiac evaluation and the risk stratification for sudden cardiac death (SCD) in asymptomatic hypertrophic cardiomyopathy (HCM) mutation carriers.

Methods and results

Seventy-six HCM mutation carriers from 32 families identified by predictive DNA testing underwent cardiac evaluation including history, examination, electrocardiography, Doppler-echocardiography, exercise testing and 24-hour Holter monitoring. The published diagnostic criteria for HCM in adult members of affected families were used to diagnose HCM.

Thirty-three (43%) men and 43 (57%) women with a mean age of 42 years (range 16-79) were examined; in 31 (41%) HCM was diagnosed. Disease-penetrance was age related and men were more often affected than women ($p = 0.04$). *Myosin Binding Protein C (MYBPC3)* mutation carriers were affected at higher age than *Myosin Heavy Chain (MYH7)* mutation carriers ($p = 0.01$). Risk factors for SCD were present in affected and unaffected carriers.

Conclusion

HCM was diagnosed in 41% of carriers. Disease penetrance was age dependent, warranting repeated cardiologic evaluation. *MYBPC3* mutation carriers were affected at higher age than *MYH7* mutation carriers. Risk factors for SCD were present in carriers with and without HCM. Follow-up studies are necessary to evaluate the effectiveness of risk stratification for SCD in this population.

INTRODUCTION

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 sarcomeric 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 sarcomeric proteins, have been identified[6]. Identification of pathogenic defects in sarcomere genes in familial forms of HCM allows reliable identification of at risk relatives. Accurate and early identification, especially of asymptomatic family members, is important because SCD may be the first manifestation of HCM[7]. Cardiac evaluation of at risk relatives enables early diagnosis and identification of those patients at high risk for SCD. In this study we investigated the outcome of cardiac evaluation and the risk stratification for SCD in asymptomatic HCM mutation carriers, who were identified by predictive DNA testing.

METHODS

Study population

At our outpatient cardio-genetic clinic all HCM patients are offered genetic counseling and genetic testing. DNA analysis consists of direct sequencing of all coding intron-exon boundaries of the following 12 sarcomere and Z-disc genes: Myosin Binding Protein C (MYBPC3), cardiac β-Myosin Heavy Chain (MYH7), cardiac Troponin T gene (TNNT2), cardiac Troponin I gene (TNNI3), cardiac-regulatory Myosin Light Chain (MYL2), cardiac-essential Myosin Light Chain (MYL3), α- Tropomyosin gene (TPM1), cardiac α- Actin (ACTC), cardiac Troponin C (TNNC1), Cysteine and Glycine Rich Protein (CSRP3) and Theletonin (TCAP). If a sarcomeric mutation is identified in the index patient presymptomatic genetic testing is offered to adult first-degree relatives[8]. In 2007 and 2008 a pathogenic HCM mutation was identified in 76 asymptomatic family members of 32 unrelated HCM patients. All subjects did provide informed written consent. The study complies with the declaration of Helsinki and the Erasmus University MC Review Board has approved the study.

Cardiac evaluation

All HCM mutation carriers were referred for cardiac evaluation including medical history, clinical examination, electrocardiography (ECG), Doppler-echocardiography, exercise testing and 24-hour Holter monitoring. Two cardiologists performed medical history and clinical examination. The ECG's were recorded with a Mortara Portrait (Mortara,

Milwaukee, USA) and subsequently scored by a cardiologist, who was blinded for the clinical data. Echocardiographic studies were performed with an iE33 system with a S5-1 transducer (Philips Medical Systems, Best, The Netherlands). The acquired data were digitally stored and subsequently analyzed by an observer, who was blinded for the clinical data. For the diagnosis of HCM the published diagnostic criteria for HCM in adult members of affected families are used (Table 1)[9]. The diagnosis is fulfilled in presence of one major criterion; 2 minor echocardiographic criteria or 1 minor echocardiographic and 2 minor ECG criteria.

Table 1 Diagnostic criteria for HCM in adult members of affected families.

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), V3-V6 (≥ 3 mm) or II, III, aVF (≥ 5 mm)	Minor repolarisation changes in LV leads
Abnormal Q (> 40ms or > 25% R wave) in at least 2 leads from II, III, aVF, V1-V4; or I, aVL, V5-V6	Deep S V2 (> 25 mm)

HCM diagnosis is made in presence of one major criterion; 2 minor echocardiographic criteria or 1 minor echocardiographic and 2 minor electrocardiographic criteria.
HCM = hypertrophic cardiomyopathy, SAM = septal anterior movement, MV = mitral valve, LVH = left ventricular hypertrophy, BBB = bundle branch block, LV = left ventricular. Modified from McKenna, Heart, 1997. **77**(2): p. 130-2

Risk stratification for SCD

The established risk factors (RF) for SCD in HCM were assessed in accordance with guidelines[10]. A history of syncope was defined as an unexplained loss of consciousness. For a positive family history of SCD three different definitions were used: SCD in ≥ 1 relative, regardless of age or degree of relationship, SCD in a first-degree relative < 40 year and SCD in > 1 first-degree relative < 40 year. The exercise test was performed with a Welch Allyn (Welch Allyn, Skaneateles Falls, USA) upright bicycle test using a 20-Watt protocol. An abnormal blood pressure response was defined as a failure to increase systolic blood pressure > 20 mmHg, or a fall in blood pressure during upright exercise testing. The 24-hour Holter monitoring were recorded using the North East Monitoring DR 180 + Pacemaker system (North East Monitoring, Maynard, USA). Nonsustained ventricular tachycardia was defined as a run of three or more consecutive beats at a rate of ≥ 120 beats/min, lasting < 30s during Holter monitoring. Maximal wall thickness was measured on the echocardiogram and counted as a risk factor when ≥ 30 mm[11].

Statistical methods

All statistics were performed using the SPSS 16 for Windows (SPSS Inc, Chicago, IL, USA). Kolmogorov-Smirnov and Shapiro-Wilk tests were used for testing normality of distribution. Descriptive data were computed as a mean value ± SD. Variables among the groups were compared by ANOVA or the Chi square test when appropiate. The homogeneity of variance in the data for HCM and no HCM groups was checked with Levene's test. Statistical significance was defined by $P \leq 0.05$.

RESULTS

Index patients

The 32 index patients had a mean age at diagnosis of 35 ± 14 years (range 3 months – 56 years) and 16 (50%) were men. SCD was the first presentation in 9 (28%) index patients at a mean age of 37 ± 12 (range 20 – 55 years). A septal reduction therapy was performed in 12 (38%) index patients at a mean age of 46 ± 11 (range 23 – 63 years). Most index patients (21, 66 %) had one of the three Dutch founder mutations in *MYBPC3*: c.2373dupG (10 patients), 2864_2865delCT (5 patients) and p.Arg943X (6 patients). Four patients had other *MYBPC3* mutations: IVS 22-2del A (1 patient), IVS5+1G>A (1 patient), 3776delA (1 patient) and Q1061X (1 patient). One (3%) index patient was compound heterozygote for the c.2373dupG and R943X in *MYBPC3*; he was diagnosed with very severe HCM and died at the age of 4 months. Mutations in *MYH7* were present in 4 (13%) patients: T1929M, A326P, H576R and D928V. One (3%) index patient had a mutation in *TNNT2* (p.Arg92Trp) and one (3%) a mutation *TPM1* (E62Q).

Asymptomatic HCM mutation carriers

Seventy-six asymptomatic HCM mutation carriers, 33 (43%) men and 43 (57%) women with a mean age of 40 ± 14 years (range 16-79) were referred for cardiac evaluation and risk stratification for SCD. The majority, 64 (84%) of the carriers had a mutation in *MYBPC3*: c.2373dupG (17 subjects), 2864_2865delCT (17 subjects), p.Arg943X (19 subjects), IVS22-2delA (3 subjects), IVS5+1G>A (5 subjects), 3776delA (2 subjects) and Q1061X (1 subject). *MYH7* mutations were identified in 10 (13%) subjects: A326P (2 subjects), D928V (3 subjects), H576R (2 subjects) and T1929M (3 subjects). One (1%) subject carried the p.Arg92Trp mutation in *TNNT2* and one (1%) subject carried the E62Q mutation in *TPM1*.

Cardiac evaluation

HCM was diagnosed in 31 (41%) asymptomatic HCM carriers, 18 men (58%) and 13 (42%) women with a mean age of 44 ± 14 years (range 22-79). The mean age of the

asymptomatic HCM carriers with HCM was not significantly different from the 45 relatives not meeting the diagnostic criteria for HCM (41 ± 18 years). The majority of asymptomatic relatives without HCM (30, 67%) were women; in our study population men had a significantly higher risk for HCM than women (p = 0.04). There was no significant difference in age between the affected men and women; respectively 44 ± 16 (range 25-71) and 42 ± 18 (range 22-72) years.

MYBPC3 mutations were involved in 23 (74%) of the relatives with HCM: c.2373dupG (8 subjects), 2864delCT (6 subjects), p.Arg943X (7 subjects), IVS22-2delA (1 subject) and IVS5+1G>A (1 subject). Indicating that 35% of carriers of a *MYBPC3* gene defect had HCM. These patients had a mean age of 48 ± 16 years; their age did not differ significantly compared to *MYBPC3* carriers without HCM (41 ± 29 years).

Mutations in *MYH7* were present in 6 (19%) relatives with HCM: A326P (2 subjects), H576R (1 subjects) and D928V (3 subjects). The mean age of the affected relatives with a MYH7 mutation was 30 ± 10 years (range 24-51), their age did not differ significantly compared to *MYH7* carriers without HCM (43 ± 17 years). Mutations in *MYH7* were associated with an earlier age at onset of HCM than mutations in *MYBPC3* in this study (p = 0.01). Indicating that 60% of carriers of a *MYH7* gene defect had HCM, which was 1.7 fold higher as for carriers of a *MYBPC3* mutation. Both relatives carrying a mutation in the *TNNT2* and the *TPM1* gene were affected (Table 2).

Table 2. Cardiac evaluation of HCM mutation carriers.

	HCM	No HCM	P-value
Total population (n=76)	31 (41%)	45 (59 %)	
Men (n=33)	18 (55%)	15 (45%)	
Women (n=43)	13 (30%)	30 (70%)	
Age	44 ± 14	41 ± 28	ns
MYBPC3 (n=64)	23 (36%)	41 (64%)	
Age	48 ± 16	41 ± 29	ns
MYH7 (n=10)	6 (60%)	4 (40%)	
Age	30 ± 10	43 ± 17	ns
TNNT2 (n=1)	1	0	
TPM1 (n=1)	1	0	

HCM = hypertrophic cardiomyopathy; *MYBPC3 = Myosin Binding Protein C*; *MYH7 = cardiac β-Myosin Heavy Chain*; *TNNT2 = cardiac Troponin T gene* and *TPM1 = α- Tropomyosin gene.*

The HCM diagnosis was based on both echocardiographic and electrocardiographic criteria in 16 (52%) carriers, 12 (39%) carriers had only ECG criteria and 3 (9%) carriers only echocardiographic criteria. Two carriers (3%) had a significant obstruction in the left ventricular outflow tract (> 50 mmHg at rest).

In 12 (16%) carriers only minor criteria were present not fulfilling the diagnostic criteria for HCM. Their mean age was 43 ± 14 year (range 23-64) and 7 (58%) were male. Eleven (92%) had a mutation in the *MYBPC3* gene: 2864delCT (4 subjects), 2373insG (2

subjects), R943X (1 subject), 3776delA (1 subject) and IVS5+1G>A (3 subjects). A *MYH7* mutation (H576R) was present in 1 subject (8%).

In 33 (43%) mutation carriers ECG and echocardiography showed no minor or major diagnostic criteria. They had a mean age of 35 ± 11 years (range 16-57), which was significantly lower than the age of the mutation carriers with HCM (p = 0.01). Of these subjects, 11 (29%) were men. The majority, 29 (88%) carried a mutation in *MYBPC3*: 2864delCT (7 subjects), 2373insG (7 subjects), IVS22-2 del A (2 subjects), R943X (11 subjects), IVS5+1G>A (1 subject) and Q1061X (1 subject). *MYH7* mutations were present in 4 (12%): T1929M (3 subjects) and 3776delA (1 subject).

Risk stratification for SCD

None of the carriers experienced syncope. None of the carriers had ≥ 2 first-degree relatives who experienced SCD < 40 years. Four (5%) carriers had one first-degree relative that experienced SCD < 40 years, two with and two without HCM. The majority of carriers, 56 (74%) had a relative who died suddenly regardless the degree of relationship or the age of the relative. Nonsustained ventricular tachycardia was present in 3 (4%) carriers during 24 hour Holter monitoring, two with and one without HCM. During upright exercise test an abnormal blood pressure response was present in 4 (5%) carriers, three with and one without HCM. None of the relatives had a maximal wall thickness ≥ 30 mm (Table 3).

Table 3. Risk factors for sudden cardiac death in HCM mutation carriers.

	HCM	No HCM
NSVT	2	1
Abnormal blood pressure response	3	1
Syncope	0	0
MWT ≥ 30 mm	0	0
Positive family history		
≥ 2 First-degree SCD < 40 years	0	0
1 First-degrees SCD < 40 years	2	2
Any SCD	27	25

HCM = hypertrophic cardiomyopathy; NSVT = non-sustained ventricular tachycardia; MWT = maximal wall thickness; SCD = sudden cardiac death.

DISCUSSION

In our study population HCM was diagnosed in 41% of asymptomatic carriers of a pathogenic mutation in a sarcomere gene. In accordance with previous studies HCM was more frequent in men than women and was related to the genetic defect and age; *MYH7* gene mutation carriers were affected at a younger age than carriers of a mutation in the MYBPC3 gene[12,13]. There was no difference in age between the asymptomatic

carriers with HCM and those without HCM. However, the relatives without any ECG or echocardiographic criteria were significantly younger than the carriers diagnosed with HCM, suggesting an age dependent disease penetrance, which warrants repeated cardiac evaluation up to advanced age.

In half of the affected HCM mutation carriers both ECG and echocardiographic criteria were present to support the diagnosis of HCM. The ECG alone lead to the diagnosis in 39% of the affected HCM mutation carriers. This is in accordance with earlier reports describing the high sensitivity of the ECG in family studies[14].

Genotype-phenotype relations

The majority of subjects in our study population had a mutation in *MYBPC3*; as the result of the three highly prevalent *MYBPC3* founder mutations in the Dutch population[15]. These truncating mutations are thought to lead to a reduction in *MYBPC3* protein because of a lack of expression from the mutated allele by the cellular surveillance mechanism of nonsense mediated decay[16]. Only mutations without a high mortality rate before reproductive age can be transmitted and become founder mutations. This suggests that these mutations have mild effects in the first three decades of life. However when the disease develops, the phenotype can be severe. This is illustrated by the high percentage of families with SCD and with septal reduction therapy in our study population.

Mutations in *MYH7* are generally associated with a younger age of onset, moderate to severe hypertrophy, high penetrance and variable prognosis[17]. In our study population carriers of a *MYH7* gene mutation were affected at a younger age than carriers of a *MYBPC3* gene mutation. However, also families with mild hypertrophy, low penetrance and good prognosis have been described[18]. In our study population, the oldest (64 years) carrier without any abnormality on ECG or echocardiography had a mutation in *MYH7*.

HCM caused by *troponin T* mutations is generally characterized by less hypertrophy and a higher incidence of SCD[19]. In contrast to other sarcomere genes SCD has been reported in carriers of mutations of the *TNNT2* gene without LVH[20].

Mutations in the *TPM1* gene are a rare cause of familial HCM. Some mutations have been associated with a high risk of the development of end-stage disease[21].

In conclusion genotype-phenotype correlations in HCM associated with mutations in sarcomere genes are confounded by phenotypic heterogeneity, with variable clinical expression in patients carrying the same pathogenic mutation, indicating epigenetic or environmental influences in the development of clinical features.

Risk stratification for SCD

Risk stratification for SCD is important in HCM in order to select patients for prophylactic ICD therapy. The annual rate of SCD secondary to HCM ranges from 0.5 to 1.5% per year for most age groups[22]. However, the proportion of HCM patients identified during

family screening are seldom reported in published survival studies[23]. Furthermore, there are currently no data available about the survival rate of unaffected carriers of pathogenic sarcomere mutations or the value of risk stratification for SCD in this specific population.

None of the screened carriers were symptomatic, had a history of syncope or had severe hypertrophy (≥ 30 mm). An abnormal blood pressure response during exercise testing was present in 4 (5 %) of the mutation carriers, in 3 HCM was diagnosed. In HCM patients an abnormal blood pressure response during exercise is present in approximately one third and has prognostic significance, especially in patients younger than 45 years old[24,25]. However, exercise hypotension has also been described in the normal population[26,27]. Non-sustained ventricular tachycardia on Holter monitoring was present in 3 (4%) of the mutation carriers, in 2 HCM was diagnosed. In about one fifth of HCM patients non-sustained ventricular tachycardia is present and has prognostic significance, but in apparently healthy individuals there is no prognostic significance[7,11,27]. The definition of a positive family history for SCD varies throughout literature in terms of the number of SCD, the ages at which SCD occurs and the degree of relationship. Some studies have used strict definitions to define a positive family history, such as premature SCD in ≥ 2 first-degree relatives < 40 years old[11]. Others have used more inclusive criteria, such as ≥ 1 SCD in the family, regardless of age or degree of relationship[7,20]. Possibly, the detailed family histories, which are continuously expanded by ongoing family studies, as ascertained at our outpatient cardio-genetic clinic, enhanced the identification of SCD in the majority of the HCM families in this study. Using the more inclusive criteria this will mean that all these screened family members have at least one RF for SCD and that in the presence of HCM one more RF is sufficient to propose an ICD to these individuals[28]. ICD therapy has been shown to be effective in aborting SCD in high-risk HCM patients, however there are potential adverse effects on morbidity and quality of life, especially in young patients[29,30]. However, the current algorithm used for risk stratification in HCM has limitations. In the first place is has never been evaluated in asymptomatic mutation carriers before. Secondly, SCD also occurs in the absence of conventional criteria for high-risk status[7]. At last, because of the heterogeneous clinical course of HCM and the relatively low annual event rate long-term follow-up studies requiring many years are necessary to establish the natural history of this specific group. The current study is limited by the absence of follow-up data.

Possible additional criteria for the definition of a high-risk status for SCD are the presence of extensive delayed enhancement on contrast-enhanced cardiovascular magnetic resonance (CMR) imaging, left ventricular outflow tract obstruction, myocardial ischemia, specific mutations (troponin T and I), intense physical exertion and atrial fibrillation28. CMR scarring in HCM correlates with the histological finding of fibrosis and thus represents a likely substrate for ventricular tachyarrhythmias[31,32]. A recent study in asymptomatic or mildly symptomatic HCM patients showed that those with delayed

enhancement on CMR had a significantly increased frequency of ventricular tachyarrhythmias on Holter monitoring compared to patients without delayed enhancement on CMR[33]. Before the implementation of CMR in the current risk stratification long-term CMR-based studies in large patient populations are necessary. Because of the risk of SCD during exercise HCM patients are excluded from most competitive sports. According to the Bethesda Conference # 36 unaffected mutations carriers are not precluded from participation in competitive sports. In contrast, the ESC document is more restrictive and excludes these individuals from competitive sports[34]. Because of the fact that mutation carriers can be unaffected up to advanced age, we currently do not exclude them from competitive sports, but evaluate them on a yearly basis.

Follow-up studies are necessary to evaluate the value of risk stratification for SCD in unaffected or mildly affected carriers of a pathogenic sarcomere mutation and to establish the natural history of asymptomatic carriers.

CONCLUSIONS

In 41% of asymptomatic relatives with a sarcomere mutation, HCM was diagnosed. Disease penetrance in HCM mutation carriers is age dependent, warranting repeated cardiologic evaluation. *MYBPC3* carriers were affected at higher age than *MYH7* mutation carriers. Classical RF for SCD were present in asymptomatic mutation carriers with and without HCM. Follow-up studies are necessary to establish the value of risk stratification for SCD in this specific population.

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Chapter 8

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

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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).

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

	Intron	Nucleotide change	Protein	No. index patients	Status	Consequence	Ref.
Exon							
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.

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 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 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.

Table 3. Shared haplotypes of 15 carriers of the p.Arg943X mutation in *MyBPC3*

	1		2		3		4		5		6	
D11S986	157	153	157	153	157	153	157	157	157	157	157	153
D11S4137	268	276	268	268	268	268	268	276	268	272	268	268
D11S1344	278	287	278	280	278	286	278	286	278	284	278	284
492C>T	nd	nd	C	C	C	C	C	C	nd	nd	C	C
IVS4-12insC	nd	nd	-	-	-	+	-	+	nd	nd	-	+
IVS12-24T>C	nd	nd	C	C	C	C	C	C	nd	nd	C	C
R943X	nd	nd	T	C	T	C	T	C	nd	nd	T	C
3288G>A	nd	nd	G	G	G	A	G	A	nd	nd	G	A
IVS33-66C>T	nd	nd	C	C	C	C	C	T	nd	nd	C	C
D11S4109	174	166	174	174	174	170	174	153	174	172	174	166

	7		8		9		10		11		12	
D11S986	157	157	157	157	157	159	157	134	157	135	155	155
D11S4137	268	268	268	268	268	268	268	268	268	268	268	268
D11S1344	278	276	278	280	278	284	278	274	278	274	278	286
492C>T	C	C	C	C	C	C	C	C	C	C	nd	nd
IVS4-12insC	-	-	-	-	+	+	-	+	-	+	nd	nd
IVS12-24T>C	C	T	C	C	C	C	C	C	C	C	nd	nd
R943X	T	C	T	C	T	C	T	C	T	C	nd	nd
3288G>A	G	G	G	G	G	A	G	A	G	A	nd	nd
IVS33-66C>T	C	C	C	C	C	C	C	C	C	C	nd	nd
D11S4109	174	172	174	168	174	172	174	176	174	176	174	166

	13		14		15	
D11S986	157	145	157	135	157	159
D11S4137	268	276	268	268	268	272
D11S1344	278	286	278	274	278	274
492C>T	C	C	C	C	nd	nd
IVS4-12insC	-	-	-	-	nd	nd
IVS12-24T>C	C	C	C	T	nd	nd
R943X	T	C	T	C	nd	nd
3288G>A	G	G	G	G	nd	nd
IVS33-66C>T	C	C	C	C	nd	nd
D11S4109	178	178	178	164	178	170

Extended haplotype analysis using 5 intragenic SNPs and 4 extragenic polymorphic markers flanking the *MYBPC3* gene 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 where 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.

Notably, there was a significant increase in echocardiographic measurements of the left atrium (LA) at follow-up in the p.Arg943X carriers (p=0.0001) 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.

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

	1		2		3		4		5		6	
D11S986	159	161	159	135	159	153	159	157	159	157	159	135
D11S4137	268	268	268	276	268	268	268	279	268	268	268	268
D11S1344	270	284	270	287	270	284	270	284	270	274	270	278
492C>T	nd	nd	C	T	nd	nd	C	C	nd	nd	C	C
IVS4-12insC	nd	nd	-	-	nd	nd	-	-	nd	nd	-	+
IVS12-24T>C	nd	nd	T	T	nd	nd	T	T	nd	nd	T	C
2864delCT	nd	nd	+	-	nd	nd	+	-	nd	nd	+	-
3288G>A	nd	nd	G	G	nd	nd	G	G	nd	nd	G	G
IVS33-66C>T	nd	nd	C	C	nd	nd	C	C	nd	nd	C	C
D11S4109	166	162	166	162	166	170	166	166	166	176	166	166

	7		8		9		10		11	
D11S986	159	157	159	153	155	157	157	155	159	147
D11S4137	268	283	268	285	268	285	272	276	268	268
D11S1344	270	284	270	274	270	286	274	276	282	289
492C>T	C	C	C	T	C	C	nd	nd	C	C
IVS4-12insC	-	-	-	-	-	-	nd	nd	-	-
IVS12-24T>C	T	T	T	C	T	C	nd	nd	C	C
2864delCT	+	-	+	-	+	-	nd	nd	+	-
3288G>A	G	G	G	A	G	G	nd	nd	G	A
IVS33-66C>T	C	C	C	T	C	C	nd	nd	C	T
D11S4109	166	163	166	160	166	172	166	166	172	172

Extended haplotype analysis using 5 intragenic SNPs and 4 extragenic polymorphic markers flanking the *MYBPC3* gene 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 where available for analysis (not shown). nd=not determined

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

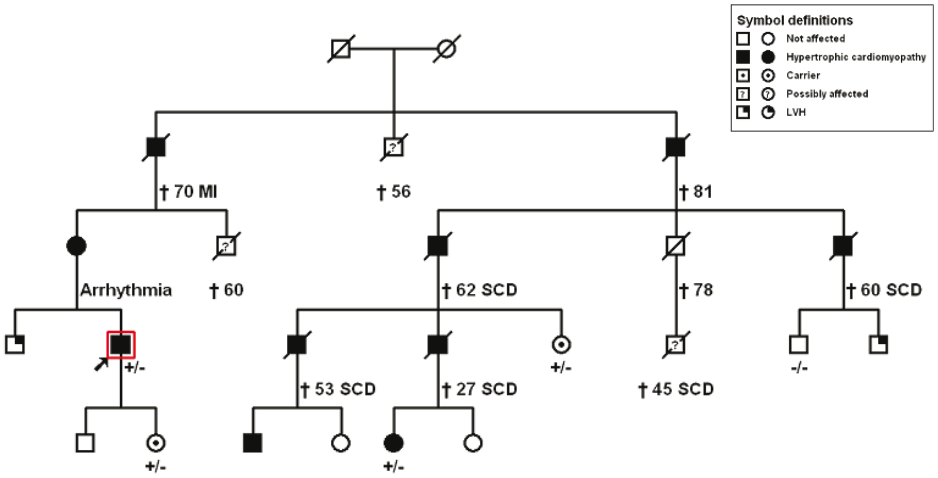

Figure 1:
Family with *MYBPC3* mutation c.2864_2865delCT

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

	LVWT (mm)	IVS (mm)	PW (mm)	LVOTG (mmHg)	SAM (n, %)	MR	LVED (mm)	LA (mm)
p.Arg943X								
First visit	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	NS	NS	NS	NS			NS	0.001
c.2864_2865delCT								
First visit	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	NS	NS	NS	NS			0.05	0.004

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.

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.

Table 7. Prognosis in families with Dutch founder mutations

	Benign n (%)	Intermediate* n (%)	Malignantn (%)	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

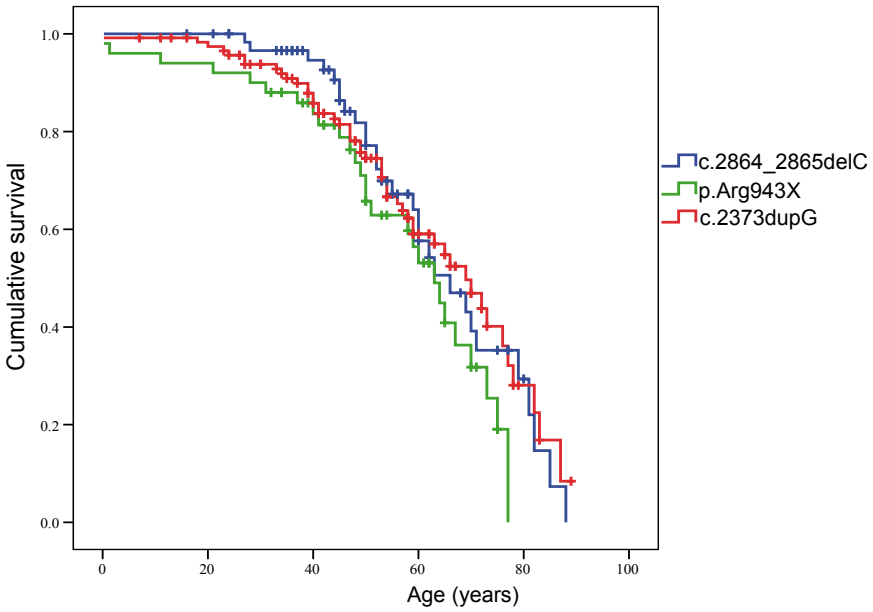

Figure 2:
Survival associated with truncating *MYBPC3* mutations

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 9

Renin-angiotensin-aldosterone system polymorphisms do not affect phenotypic expression of hypertrophic cardiomyopathy in a large set of carriers of Dutch functionally-equivalent *MYBPC3* founder mutations

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Submitted

ABSTRACT

Background

Hypertrophic cardiomyopathy (HCM) is characterized by profound clinical heterogeneity even among patients carrying the same underlying primary pathogenic mutation. In view of its regulatory role in cardiac function, blood pressure, and electrolyte homeostasis, genetic variation in genes encoding components of the renin-angiotensin-aldosterone system (RAAS) is a plausible candidate for modifying phenotypic expression. However, such studies have so far been hampered by small patient numbers and by genetic heterogeneity with respect to the primary genetic defect. In this study we used a large cohort of subjects carrying 3 founder functionally-equivalent truncating mutations in the *MYBPC3* gene, to investigate whether RAAS polymorphisms modulate phenotypic expression as assessed by echocardiography in *MYBPC3*-associated HCM.

Methods

Family based association analysis was performed to analyze the effects of 5 candidate polymorphisms in genes of the RAAS system (*ACE*, *AGTR1*, *CMA*, *AGT*, *CYP11B2*) in 368 subjects carrying one of 3 functionally-equivalent truncating mutations in the *MYBPC3* gene. Interventricular septum (IVS) thickness and Wigle score were assessed by 2D-echocardiography. SNPs in the RAAS system were analyzed for effects on these phenotypes, both separately as well as in aggregate as a pro-left ventricular hypertrophy (pro-LVH) score.

Results

Analyzing the 5 polymorphisms separately for effects on IVS thickness and Wigle score only uncovered 2 modest associations. Carriers of the CC genotype in the *AGT* gene (homozygous T235 on the protein level) displayed lesser IVS thickness as compared to CT and TT genotype carriers. The direction of this effect differed from previous studies which demonstrated pro-LVH effects and greater serum angiotensin levels for this genotype. In addition, the DD polymorphism in the *ACE* gene was associated with a high Wigle score (p=0.01). No association was detected between the pro-LVH score and IVS thickness or Wigle score.

Conclusions

In contrast to previous studies, our study which investigated a large set of HCM patients carrying functionally-equivalent founder mutations in the *MYBPC3* gene, did not uncover marked effects of genetic variation within genes of the RAAS system and expression of HCM.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most common inheritable cardiac disorder with a phenotypic prevalence of 1:500.[1] It is defined by the presence of left ventricular hypertrophy (LVH) in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality.[2] Hundreds of mutations scattered among at least 25 putative HCM susceptibility genes encoding various sarcomere, Z-disk, calcium-handling, and mitochondrial proteins are known to cause HCM and are found in up to 60% of cases.[3] In the Netherlands, approximately one third of all HCM cases are caused by the truncating c.2373dupG, c.2864_2865delCT and c.2827C>T (p.Arg943X) mutations in the myosin binding protein C gene (*MYBPC3*).[4-6] Immunoblotting studies on myocardial tissue from carriers of the c.2373dupG and c.2864_2865delCT truncating mutations have demonstrated absence of the truncated MyBPC-3 protein product coupled to a decreased total (full-length) MyBPC-3 content, strongly suggesting a mechanism of haploinsufficiency.[5] These mutations can therefore be considered to be functionally similar. Although not functionally investigated, the c.2827C>T mutation associated with a premature stop codon at residue 943, encodes for a similarly C-terminally truncated protein, and is therefore also expected to lead to haploinsufficiency.

Extensive phenotypic heterogeneity exists among HCM mutation carriers and even individuals with the same underlying genetic substrate express a broad spectrum of clinical severity.[7, 8] This indicates that the disease course is not solely determined by the nature of the pathogenic mutation but that environmental factors and/or other genetic factors (genetic modifiers) must play an important role in determining the ultimate clinical expression of the disease.

Genetic variants in the renin-angiotensin-aldosterone system (RAAS) have long been considered important candidates for these modifying effects. The RAAS system contributes to LVH through effects mediated by circulating angiotensin as well as local activation of RAAS in the myocardium.[9] Angiotensin (Ang) I, produced from angiotensinogen (AGT), is converted to Ang II predominantly by angiotensin I-converting enzyme (ACE) and possibly by chymase (CMA). Ang II binds primarily to the Ang II type 1 receptor (AGTR1) to promote cell growth and hypertrophy. It is also converted to aldosterone by aldosterone synthase (CYP11B2) which promotes fluid retention and cardiac fibrosis.[10]

A number of studies have suggested a role for genetic variation in genes encoding important components of this pathway (*ACE*, *AGTR1*, *CMA*, *AGT*, *CYP11B2*) in modulation of severity of LVH in HCM patients.[11, 12] Furthermore, a combined pro-LVH profile of 5 polymorphisms in these genes was proposed to underlie a higher degree of LVH among 26 *MYBPC3* mutation carriers from a single family and in a set of 228 unrelated patients that were genotype-negative for myofilament gene mutations.[12, 13]

Studies addressing effects of genetic modifiers in HCM have however been hampered by small patient numbers and by genetic heterogeneity with respect to the primary genetic defect since HCM mutations are often private and HCM patient sets consequently include patients with different causal mutations. In this study we used a large cohort of subjects carrying 3 functionally-equivalent truncating founder mutations in the *MYBPC3* gene, to investigate whether polymorphisms in RAAS modulate disease severity as assessed by echocardiography in *MYBPC3*-associated HCM.

METHODS

Subjects

In the Netherlands, genetic counseling and genetic testing is offered to all HCM patients visiting cardio-genetics outpatient clinics. Upon the identification of the causal mutation in a proband, genetic testing is extended to relatives whenever possible (cascade screening).[14] For this study 368 subjects, including probands and relatives, carrying any one of 3 truncating founder mutations in the *MYBPC3* gene (c.2373dupG, c.2864_2865delCT, c.2827C>T) were selected from 2 university hospitals in the Netherlands; the Amsterdam Medical Centre in Amsterdam and the Erasmus Medical Centre in Rotterdam. All subjects were normotensive (blood pressure < 140/90 mmHg) and did not take medication known to influence the RAAS. All subjects provided written informed consent. The study complies with the declaration of Helsinki and the local review boards of the respective hospitals approved the study.

Echocardiographic evaluation

Echocardiography was performed in all subjects using commercially available equipment. The acquired data were digitally stored and subsequently analyzed by 2 physicians who were blinded to the clinical and genetic data. Interventricular septum (IVS) thickness was measured in diastole from the parasternal short-axis view at the level of the papillary muscles. For relatives ≥16 years a IVS thickness ≥ 13 mm was considered as abnormal.[15] For subjects <16 years IVS thickness was corrected for height and weight and was considered abnormal if the z-score was >2. The extent of hypertrophy was assessed by a semi-quantitative score method developed by Wigle et al. A maximum of 10 points are given: 1 to 4 points for IVS thickness (1 point for IVS thickness between 15-19 mm; 2 points for IVS thickness between 20-24 mm; 3 points for IVS thickness 25-29 mm and 4 points if IVS thickness ≥ 30 mm), 2 points for extension of hypertrophy beyond the level of the papillary muscles (basal two thirds of the IVS), 2 points for extension of hypertrophy to the apex (total IVS involvement), and 2 points for extension of hypertrophy into the lateral wall.[16]

SNP genotyping

The following 5 polymorphisms in the *ACE*, *AGTR1*, *CMA*, *AGT* and *CYP11B2* genes, encoding major components of the RAAS pathway, were genotyped in all subjects. Specifically, these polymorphisms included: (1) an insertion/deletion (I/D) polymorphism in intron 16 of the gene encoding ACE; (2) a polymorphic A/C at position 1166 of *AGTR1* gene; (3) a C>T polymorphism at position −344 of the *CYP11B2*; (4) an A>G polymorphism at position −1903 of the *CMA* gene; (5) a T>C (M235T) polymorphism in the *AGT* gene**.** Patient genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Genotyping was carried out as described previously. Pro-LVH genotypes were defined as described previously, namely as DD-*ACE*, CC-*AGTR1*, AA-*CMA*, CC-*AGT*, and CC-*CYP11B2*. The pro-LVH score was calculated for each patient by adding the number of pro-LVH genotypes present.[12, 13]

Statistical analyses

Phenotypic data for probands and relatives were normally distributed and are reported as mean ± standard deviation. Pedigree information was available for all related subjects. We assumed that each polymorphism-phenotype relationship followed a recessive genetic model as previously reported.[13,14]. Effects on IVS thickness and Wigle score were estimated using a linear mixed model with adjustment for sex, age and proband status, whereas effects on the dichotomous variables IVS thickness ≥ 13 or ≥ 30 mm were estimated using a logistic regression model with adjustment for sex and age. Next to models with a single SNP or the pro-LVH score, a model with all five SNPs and their interactions was also used. To account for the relatedness of study subjects, either the linear mixed model from the Kinship package or the generalized estimation equations from the geepack package in R were used (R foundation for Statistical Computing, Vienna, Austria). P-values < 0.05 were considered significant.

Based on the present sample size (n=368), our study had 90% power to detect a 0.30 mm difference in IVS thickness between the genotype groups and a correlation coefficient of 0.167 (± 3% explained variance) between IVS thickness and the pro-LVH score (α = 0.05 two-sided).

RESULTS

Study Population

DNA and echocardiography data were available for 368 subjects, all carrier of any one of the 3 founder mutations. These included 100 probands and 268 relatives. The age distribution of probands and relatives was similar (Table 1). By definition all probands had an IVS thickness ≥ 13 mm. Extreme hypertrophy (IVS thickness ≥ 30 mm), a known

risk factor for sudden death was present in 9 (10 %) probands[18, 19]. There was a higher frequency of males in the proband group (64% vs. 47%, p = 0.007). LVH (defined as IVS ≥ 13 mm)[16] was present in 107 (40%) of relatives and was extreme in 1 (0.4%) relative.

The distribution of MYBPC3 founder mutation among the study subjects is presented in Table 2. The most common founder mutation was c.2373dupG which was carried by 70% of the individuals. The distribution of RAAS polymorphisms is presented in Table 3.

Table 1: Characteristics of the HCM population studied.

	Probands	Relatives	p-value
N	100	268	
Age	42±14	41±17	0,64
Sex n (% male)	64 (64%)	127 (47%)	0,007
Septum thickness (mm)	22±5	13±4	0.0017
Septum > 13 mm, n (%)	100 (100%)	107 (40%)	
Septum > 30 mm n (%)	9 (10%)	1 (0.4%)	0,0001
Wigle score (0-10)	4.2±2.5	0.7±1.7	2,36E-33

Data depicts mean ± standard deviation, n (%).

Table 2: *MYBPC3* mutation distribution among the HCM population studied.

Mutation	Probands	Relatives
c.2373_2374insG	70	187
c.2827C>T	18	56
c.2864_2865delCT	12	25

Table 3: Number of patients per genotype group for each polymorphism studied.

***ACE*, I/D**	**II**	**ID**	**DD**
probands	29	48	23
relatives	73	125	69
***AGT*, C>T (M235T)**	**TT**	**TC**	**CC**
probands	41	39	20
relatives	123	107	38
***AGTR1*, 1166A>C**	**AA**	**AC**	**CC**
probands	50	41	9
relatives	125	119	24
***CMA1*, -1903 A>G**	**GG**	**AG**	**AA**
probands	26	44	30
relatives	61	133	74
***CYP11B2*, -344C>T**	**TT**	**TC**	**CC**
probands	36	43	21
relatives	96	142	30

Influence of age, sex and proband status

Proband status, age and gender had a significant effect on IVS thickness. Probands displayed a greater mean IVS thickness compared to relatives (22±5 versus 13±4 mm) (Table

1). While in the relatives group, IVS thickness was greater in older individuals; within the proband group, it was smaller in older individuals. On average men had a thicker IVS than women (17±6 mm versus 14±6 mm; p=0.0014). This influence of male sex is especially clear for the relatives (Figure 1).

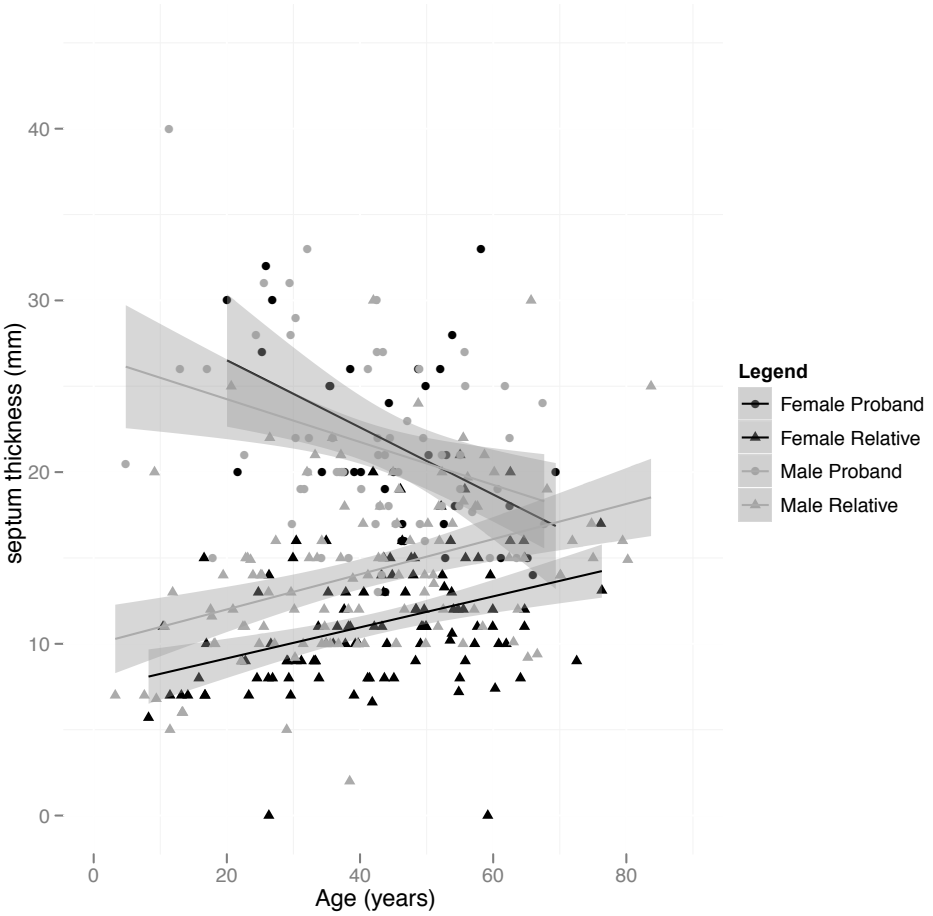

Figure 1:
Scatterplot and lineair regression lines including the 95% confidence intervas, illustrating the effects of sex and age on septum thickness in probands and relatives.

SNP association analyses

The 5 polymorphisms were analyzed both separately, as well as in aggregate - as a pro-LVH score -, for (i) effects on IVS thickness, (ii) association with IVS thickness ≥13mm, (iii) association with IVS thickness ≥30 mm, and (iv) effects on the Wigle score (Table 4).

Analyzing the 5 polymorphisms separately with correction for sex, age and proband status uncovered an effect of the T>C (M235T) polymorphism in the AGT gene on IVS

Table 4: Single SNP association results

Trait	*ACE, I/D*	*AGTR1*, 1166A>C	*CMA*1, -1903 A>G	*AGT*, 704T>C (M235T)	*CYP11B2*, -344C>T	Pro-LVH score												
	I/I+I/D vs. D/D	A/A+A/C vs. C/C	G/ G+A/G vs. A/A	T/T+T/C vs. C/C	T/T+T/C vs. C/C													
	β	SE	P-value	β	SE	P-value	β	SE	P-value	β	SE	P-value	β	SE	P-value	β	SE	P-value
∫ Septum thickness (mm)	0.62	0.6	0.30	0.39	0.87	0.65	-0.58	0.59	0.33	-1.75	0.72	**0.02**	-0.37	0.73	0.61	-0.24	0.3	0.41
∫ Wigle score	0.71	0.28	**0.01**	0.01	0.42	0.98	-0.1	0.26	0.70	-0.52	0.33	0.11	-0.08	0.34	0.82	0.03	0.13	0.80
	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
# Septum thickness > 13 mm	1.23	0.68-2.23	0.49	1.57	0.64-3.83	0.32	0.78	0.49-1.25	0.30	0.95	0.52-1.73	0.86	2.21	0.95-5.14	0.07	1.19	0.93-1.53	0.17
♩ Septum thickness > 30 μμ	1.99	0.63-6.31	0.24	2.44	0.47-12.63	0.29	0.58	0.15-2.27	0.43	0.65	0.08-5.15	0.68	*	*	*	0.93	0.50-1.74	0.82

Data depict association results for the traits studied using a recessive genetic model. P-values < 0.05 are shown in bold text.

∫ The total patient set (n=368) was included in this analysis.

n=207 individuals with septum thickness >13 mm and n=161 individuals with septum thickness <13 mm were included in this analysis.

♩ n=10 individuals with septum thickness >30 mm and n=358 individuals with septum thickness <30 mm were included in this analysis.

* No individuals with septum thickness >30 had the CC genotype.

thickness (Table 4, p=0.02), with the CC genotype (homozygous for threonine at position 235) being associated with less IVS thickness as compared to the other two genotypes (TC, TT). The DD genotype at the ACE I/D polymorphism was associated with a high Wigle score (p=0.01) as compared to the ID and DD genotypes. Interaction analyses between the 5 polymorphisms were negative. No significant interactions were found between the 5 polymorphisms for any of the four outcomes tested.

No association was detected between pro-LVH score and IVS thickness or Wigle score in probands or relatives (Tables 4 and 5).

Table 5 : Septum thickness according to pro-LVH genotype score

Pro-LVH genotype score	Probands <16 yr (n=3)	Probands >16 yr (n=97)	Relatives <16 yr (n=19)	Relatives >16 yr (n=247)	Total Probands (n=100)	Total Relatives (n=268)	Total Population (n=368)
0	26±0	21±5	9±6	13±4	21±5	12±4	15±6
1	-	22±6	8±3	13±5	22±6	13±5	15±7
2	30±14	21±4	9±4	13±4	22±6	13±4	16±7
3	-	18±3	6±0	12±2	18±3	11±3	14±5
4	-	19±2	-	14±0	19±2	14±0	17±3
Total	29± 10	21±5	8±4	13±4	22±5	13±4	15±6
p-value	0.84	0.52	0.44	0.93	0.74	0.80	0.49

Data depict mean ± standard deviation. Yr = years

DISCUSSION

In a large cohort of subjects carrying anyone of 3 functionally-equivalent truncating mutations in *MYBPC3*, we uncovered only minor effects of candidate SNPs in the RAAS system on IVS thickness and Wigle score. These effects were limited to (i) an association of the CC genotype of the *AGT* T>C (M235T) polymorphism with a smaller IVS, and (ii) an association of the DD genotype of the *ACE* I/D polymorphism with a higher Wigle score. There was no effect of the previously described pro-LVH score on IVS thickness or Wigle score.[12, 13]

The T>C polymorphism in the *AGT* gene has been described as a predisposing factor for cardiac hypertrophy in patients with hypertension, in endurance athletes and in sporadic cases of HCM.[20-22] The *AGT* gene encodes angiotensinogen and this polymorphism is associated with elevated angiotensinogen serum concentrations.[23] Although we found an association between the CC genotype of the AGT T>C polymorphism and IVS thickness, it is important to note that the direction of the effect in our patient population was opposite to what is predicted based on these previous studies. The fact that the association with IVS thickness in our study was not reflected in a decreased risk for having an IVS thickness of ≥ 13mm or ≥ 30mm is suggestive of a spurious association. Additionally no association was detected for this polymorphism with Wigle score.

Tissue levels of angiotensin converting enzyme are increased in patients with the DD-*ACE* genotype and this genotype is considered to be a pro-LVH genotype.[24] It may modify the clinical phenotype in a gene-specific manner, one study reported that in *MYBPC3* related HCM an IVS thickness ≥ 30 mm was only present in carriers of the DD-genotype.[13, 24] However, in our study probands with extreme IVS thickness were equally divided among subjects carrying DD-*ACE*, ID-*ACE* and II-*ACE*. In our study the *ACE*-DD genotype only was a significant modifier of the extent of hypertrophy as described by the Wigle score and did not influence IVS thickness.

There was no effect of the combined pro-LVH genotypes in the pro-LVH score on IVS thickness or Wigle score. This is in contrast to the report by Ortlepp et al, describing a single small HCM family (48 individuals, 26 of which mutation carriers) in which cardiac hypertrophy was associated with the burden of pro-LVH genotypes[12]. The absence of a relation between the pro-LVH score and hypertrophy in our much larger set suggests that cardiac hypertrophy in *MYBPC3*-related HCM is not influenced by the pro-LVH score.

IVS thickness in HCM mutation carriers increases progressively with age.[8,9] In our study this was the case for relatives carrying *MYBPC3* mutations but not for probands. The striking difference between probands and relatives is their reason for cardiac evaluation. Probands are being referred because of symptoms, abnormalities at routine physical examination or electrocardiography, i.e. before non-cardiac surgery; whereas relatives are being referred after presymptomatic DNA testing. This will lead to a sampling or referral bias; HCM patients with severe IVS thickening at young age are more likely referred because of symptoms than those with minimal or moderate thickening. The fact that older age groups displayed less extensive IVS thickness than the younger ones also might be due to the fact that probands with larger IVS thickness die at younger age (the so-called healthy survivor phenomenon). Besides this explanation it is known that about 5-10% of HCM patients progress to an end-stage form, which is characterized by systolic dysfunction, dilatation of the left ventricle and wall thinning[25, 26].

Males and females differ in their presentation of HCM, with cohorts usually having a predominance of males.[27, 28] In our study there is a sex effect in the relatives, with men being affected at younger age than women. This is possibly explained by a protective role of estrogens in the hypertrophic response and the evidence that exposure of cardiac myocytes to androgen results in hypertrophy.[29, 30] Furthermore the HCM phenotype is influenced by sex hormone receptor variants.[31] However, in the probands in our study the sex effect is less clear, female tend to be more heavily affected at young age, but there is considerably overlap between males and females (figure 2). This illustrates that there are other, currently unknown modifiers of phenotypic expression in HCM.

In conclusion, we have investigated the role of genetic polymorphisms in genes of the RAAS in patients with HCM in the context of a patient population that was large and homogeneous with respect to the underlying causal genetic defect. Our findings do

not provide support for a marked effect of genetic variation in the RAAS on phenotypic expression of hypertrophy in this disorder. It is therefore unlikely that identification of RAAS polymorphisms may help to risk-stratify HCM mutation carriers and to expect benefit from the prescription of drugs, known to influence the RAAS system on disease expression.

LIMITATIONS

Although the approach to look at these 5 SNPs is plausible we are currently not informed on the effect of other genes and their effect on phenotypic expression.

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Chapter 10

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

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Submitted

ABSTRACT

Objectives

To analyse whether a complex genotype is an important modifier of disease severity in hypertrophic cardiomyopathy (HCM).

Background

Genotype-phenotype studies in HCM have been hampered by the genetic heterogeneity and individual nature of HCM mutations. Disease variability is expected to be partially explained by the effect of additional mutations in sarcomere genes.

Methods

Nine sarcomere HCM genes (*MYH7, MYBPC3, MYL2, MYL3, TNNT2, TNNI3, TNNC1, ACTC1* and *TMP1*) were completely analysed in carriers of Dutch truncating *MYBPC3* founder mutations (c.2373dupG, p.Arg943X and c.2864_2865delCT). Carriers were diagnosed as having "mild" or "severe" phenotype based on the following criteria: age at diagnosis < 25 years, maximal wall thickness ≥ 30 mm, left ventricular outflow tract obstruction > 30 mmHg, sudden cardiac death ≥ 2 first-degree family member < 40 years and the necessity for septal reduction therapy or an implantable cardioverter defibrillator. 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.

Results

A total of 87 carriers were included; 44 with mild and 43 with severe phenotype. No additional pathogenic mutations were found in the nine HCM genes. In total 59 "silent variants" and 9 "missense variants" were identified; none of these variants were significantly associated with a severe phenotype.

Conclusions

The severity of phenotypic expression of HCM in carriers of 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 25 putative HCM susceptibility genes encoding various sarcomere, Z-disk, 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 partially be 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 haplo-insufficiency and are thought to be functionally identical.[11] We completely analysed 9 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.

MATERIALS AND METHODS

Subjects

Subjects were selected from carriers of Dutch founder mutations (c.2373dupG, p.Arg943X and c.2864_2865delCT in MYBPC3), including probands as well as family members, consecutively diagnosed at the cardiogenetics clinic at the Erasmus MC in Rotterdam between 2006 and 2008. All carriers were clinically diagnosed as either having "mild" or "severe" phenotype based on the following criteria: age at diagnosis < 25 years, maximal wall thickness ≥ 30 mm, left ventricular outflow tract obstruction > 30 mmHg, sudden cardiac death ≥ 2 first-degree family member < 40 years and the necessity for septal reduction therapy or an implantable cardioverter defibrillator for primary or secondary prevention of sudden cardiac death. 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 carriers; 41 probands and 46 family members from 52 different HCM 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 9 HCM genes; MYBPC3 *(NM_000256.3), β-myosin heavy chain (MYH7; NM_000257.2), 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 available on request.) Analysis of sequence data was performed using SeqS-cape 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 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 with mild phenotype and 43 with severe phenotype. As expected significantly more probands had a severe phenotype compared with family members. The group with the severe phenotype was diagnosed at a significantly younger age. In this group the maximal wall thickness was significantly thicker and the obstruction in the left ventricular outflow tract was significantly higher compared to the group with a mild phenotype. By definition a positive family history for sudden cardiac death, septal reduction therapy or implantable cardioverter defibrillator implantation were absent in the group with a mild phenotype. Gender was not associated with the severity of HCM. There was no significant difference between the 3 founder mutations in disease severity.

DNA sequence analysis

Complete sequence analysis of nine sarcomere genes in 87 carriers 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

Table 1: Descriptives of the study population

	Total Group	Mild phenotype	Severe phenotype	P-value
Number of carriers	87	44	43	
Probands (n, %)	41 (47%)	9 (20%)	32 (74%)	0.001*
Family members (n, %)	46 (53%)	35 (80%)	11 (26%)	0.001*
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 – 68)	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

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

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). The distribution of heterozygotes and homozygotes was in Hardy Weinberg equilibrium for all sequence variants analysed.

Table 2: Sequence variations in nine sarcomere genes in 87 carriers of a truncating *MYBPC3* mutation.

Gene	Observed variants (nucleotide)	Effect on Protein	Status variants (rs number)
MYBPC3	**p.Val158Met**	**p.Val158 Met**	**rs3729986**
	c.492C>T	p.=	rs3218719
	c.506-12dupC		rs11570050
	c.537C>T	p.=	rs11570051
	c.706A>G	**p.Ser236 Gly**	**rs3729989**
	c.786C>T	p.=	rs11570058
	c.926+25C>A		rs3729991
	c.1091-24T>C		rs2856650
	c.1223+29G>A		rs11570078
	c.1226+30G>A		
	c.1352-21A>C		
	c.1608T>A	p.=	
	c.2308+18C>G		rs3729948
	c.2497C>A	**p.Ala833 Thr**	
	c.2547C>T	p.=	rs3729953
	c.2686G>A	**p.Val896 Met**	**rs35078470**
	c.2737+12C>T		rs3729936
	c.3115C>T	**p.His1039 Tyr**	
	c.3191-21A>G		rs11570115
	c.3288G>A	p.=	rs1052373
	c.3392T>C	**p.Ile1131 Thr**	
MYH7	c.189C>T	p.=	rs2069540
	c.597A>G		rs2069541
	c.732C>T	p.=	rs2069542
	c.895+17G>A		rs45580436
	c.896-17C>T		
	c.975C>T	p.=	rs2231124
	c.1062C>T	p.=	rs735712
	c.1095G>A	p.=	rs735711
	c.1128C>T	p.=	rs2231126
	c.1605A>G	p.=	rs2069543
	c.2733C>T	p.=	
	c.2923-18G>A		rs7157087
	c.2967T>C	p.=	rs7157716
	c.3153G>A	p.=	rs45540831
	c.3337-3dupC		rs45504498
	c.3853+7C>T		rs45467397

Severe phenotype total alleles n=86 (hetero-/homozygotes)	Mild phenotype Total alleles n=88 (hetero-/homozygotes)	P-value #	SIFT	PolyPhen
0	**6 (6/0)**	**0.029**	Not tolerated *	Benign
24 (24/0)	24 (20/2)	1.000		
11 (11/0)	12 (12/0)	1.000		
2 (2/0)	3 (3/0)	1.000		
6 (6/0)	**6 (6/0)**	1.000	Tolerated *	Benign
6 (6/0)	6 (6/0)	1.000		
1 (1/0)	1 (1/0)	1.000		
59 (19/20)	67 (17/25)	0.310		
6 (6/0)	7 (7/0)	1.000		
1 (1/0)	0	0.494		
1 (1/0)	0	0.494		
1 (1/0)	0	0.494		
2 (2/0)	1 (1/0)	0.618		
1 (1/0)	**0**	0.494	Not tolerated *	Benign
1 (1/0)	1 (1/0)	1.000		
1 (1/0)	**0**	0.494	Not tolerated *	Benign
0	1 (1/0)	1.000		
0	**1 (1/0)**	1.000	Not tolerated *	Probably damaging
7 (7/0)	4 (4/0)	0.368		
39 (27/6)	36 (18/9)	0.646		
1 (1/0)	**0**	0.494	Not tolerated *	Possibly damaging
38 (22/8)	46 (22/12)	0.293		
1 (1/0)	1 (1/0)	1.000		
13 (13/0)	22 (16/3)	0.131		
1 (1/0)	0	0.494		
1 (1/0)	0	0.494		
1 (1/0)	1 (1/0)	1.000		
3 (3/0)	7 (7/0)	0.330		
10 (8/1)	7 (7/0)	0.454		
10 (10/0)	9 (9/0)	0.812		
0	3 (3/0)	0.246		
0	2 (2/0)	0.497		
0	3 (3/0)	0.246		
26 (18/4)	29 (21/4)	0.746		
4 (4/0)	1 (1/0)	0.208		
3 (3/0)	8 (8/0)	0.212		
0	1 (1/0)	1.000		

Gene	Observed variants (nucleotide)	Effect on Protein	Status variants (rs number)
	c.3853+21C>T		rs45584435
	c.3853+27T>A		rs2277475
	c.3960G>A	p.=	
	c.3972+15C>T		rs3729820
	c.3973-30A>G		rs7159367
	c.4472C>G	**p.Ser1491 Cys**	**rs3729823**
	c.4520-25C>T		rs45503601
	c.4566T>C	p.=	rs2754155
	c.4644+11_ 4644+12del		
	c.5106G>A	p.=	rs3729830
TNNT2	c.53-11_53-7del		rs45533739
	c.207G>A	p.=	rs3729845
	c.318C>T	p.=	rs3729547
	c.382-94delC		rs35559054
	c.583G>A	**p.Glu195 Lys**	
	c.758A>G	**p.Lys253Arg**	**rs3730238**
TNNI3	c.15-50_15-47dup		rs34226453
	c.25-8T>A		rs3729836
	c.108+21G>A		rs3729837
	c.204G>T	p.=	rs3729711
	c.537G>A	p.=	rs3729841
MYL2	c.132T>C	p.=	rs2301610
	c.274+33_274+34dup		
	c.274+17G>C		
	c.353+20delG		rs3833910
	c.353+46dupC		rs3216817
MYL3	c.*9C>T		
TPM1	c.453C>A	p.=	rs1071646
	c.486T>C	p.=	rs11558747
	c.639+22G>C		rs28730802
ACTC1	c.927T>C	p.=	rs2307493

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.
* 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.
(*MYBPC3* = Myosin binding protein C, *MYH7* = β-myosin heavy chain, *TNNT2* = cardiac troponin T, *TNNI3* = cardiac troponin I, *MYL2* = cardiac-regulatory myosin light chain, *MYL3* = cardiac-essential myosin light chain, *TPM1* = α-tropomyosin, *ACTC1* = cardiac α-actin)

Severe phenotype total alleles n=86 (hetero-/homozygotes)	Mild phenotype Total alleles n=88 (hetero-/homozygotes)	P-value #	SIFT	PolyPhen
1 (1/0)	3 (3/0)	0.621		
26 (22/2)	32 (22/5)	0.424		
1 (1/0)	0	0.494		
1 (1/0)	0	0.494		
28 (20/4)	33 (21/6)	0.528		
0	**1 (1/0)**	1.000	Not tolerated *	Benign
1 (1/0)	1 (1/0)	1.000		
3 (3/0)	2 (2/0)	0.680		
1 (1/0)	0	0.494		
14 (10/2)	18 (18/0)	0.559		
60 (18/21)	58 (22/18)	0.628		
5 (5/0)	5 (5/0)	1.000		
61 (13/24)	62 (20/21)	1.000		
1 (1/0)	1 (1/0)	1.000		
1 (1/0)	**0**	0.494	Tolerated	Benign
0	**1 (1/0)**	1.000	Tolerated	Benign
11 (11/0)	4 (4/0)	0.062		
25 (21/2)	13 (11/1)	0.042		
11 (9/1)	6 (4/1)	0.210		
5 (5/0)	5 (5/0)	1.000		
11 (9/1)	6 (4/1)	0.042		
9 (7/1)	8 (6/1)	0.803		
12 (3/4)	8 (4/2)	0.350		
1 (1/0)	0	0.494		
26 (18/4)	28 (14/6)	0.871		
7 (7/0)	7 (5/1)	1.000		
1 (1/0)	0	0.494		
60 (22/19)	56 (26/15)	0.424		
6 (6/0)	12 (10/1)	0.213		
2 (2/0)	0	0.243		
0	1 (1/0)	1.000		

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 remained 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 group of carriers of a truncating *MYBPC3* founder mutation. No additional pathogenic mutations were found and the identified sarcomere sequence variations could not be associated with either 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 characterized by phenotypic heterogeneity; ranging from negligible to extreme hypertrophy, absent or severe left ventricular outflow tract obstruction, normal longevity or premature sudden cardiac death, even in patients carrying 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 sudden cardiac death, 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 more hypertrophy, and a higher incidence of myectomy and implantable cardioverter defibrillator placement compared to patients with a single 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 haplo-insufficiency, and with identical Kaplan-Meier survival estimates (personal communication).[11] The result of our study illustrates that the clinical heterogeneity of HCM 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. This system contributes to ventricular hypertrophy through effects mediated by circulating angiotensin as well as local activation of renin-angiotensin-aldosteron system in the myocardium.[21] Polymorphisms in this pathway appear to influence the severity of left ventricular hypertrophy 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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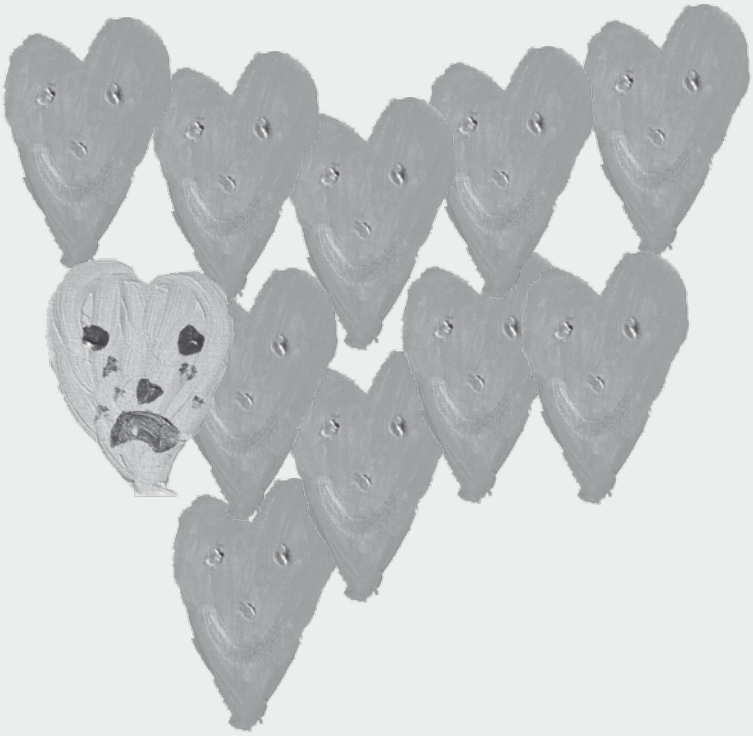
Part III

Invasive treatment in hypertrophic cardiomyopathy

Chapter 11

Long-term benefit after myectomy combined with anterior mitral leaflet extension in obstructive hypertrophic cardiomyopathy



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Submitted

ABSTRACT

Aims

To describe the long-term course after myectomy combined with anterior mitral leaflet extension (MLE) in patients with obstructive HCM and enlarged anterior mitral valve leaflet.

Methods and Results

Between 1991 and 2009, 69 Patients (47.8 ± 15 years, 58% male), underwent myectomy with MLE. Mean follow-up was 8.1 years (1.1 - 19.2 yrs) and included mortality and change in clinical and echocardiographic characteristics. Mortality was compared with matched non-obstructive HCM patients and general population.

NYHA class reduced from 2.9 ±0.3 to 1.3 ± 0.5; LVOT gradient from 94 ± 26 to 9 ± 8 mmHg; mitral valve regurgitation from grade 2.7 ± 0.7 to 0.6 ± 0.8 and SAM grade from 2.2 ± 0.9 to 0.1 ± 0.2 (all $p < 0.001$). There was no operative mortality. The 1-, 5 -, 10-, and 15-year survival was 98%, 94%, 91%, and 86%, respectively; not different from the general population (99%, 97 %, 94%, and 91%, respectively; log-rank $p = 0.25$) or non-obstructive HCM patients (98%, 97%, 89%, and 70%, respectively, log-rank $p = 0.11$).

Conclusion

In selected patients with obstructive HCM myectomy combined with MLE is safe and results in long-term symptom relief and survival similar to general population.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a heterogeneous disease characterized by asymmetrically distributed left ventricular hypertrophy. Up to 70% of patients with HCM have obstruction in the left ventricular outflow tract (LVOT) at rest or during provocation[1]. This obstruction is induced by thickening of the interventricular septum (IVS) and systolic anterior movement (SAM) of the mitral valve. In severely symptomatic patients with obstructive HCM, despite optimal medical therapy, isolated myectomy is the golden standard with excellent perioperative and long-term outcomes[2-5]. HCM can be associated with intrinsic abnormalities of the mitral valve, including increased mitral leaflet area, length, and laxity, as well as anterior displacement of the papillary muscles[6-8], which may predispose to residual SAM and result in suboptimal outcome after isolated myectomy[5, 9]. In the subset of patients with obstructive HCM and enlargement of the anterior mitral leaflet we therefore performed anterior mitral leaflet extension (MLE) in combination with myectomy. We have shown that this approach resulted in favorable short-and mid-term functional and hemodynamic outcome[10, 11]. However, there is no data on long-term outcome of patients with HCM who underwent myectomy combined with mitral valve surgery.

In the current report we present the long-term course after myectomy combined with MLE including survival analysis in comparison with age and gender matched patients with non-obstructive HCM and subjects from the general Dutch population.

METHODS

Patient selection

Patients are selected for invasive therapy at our HCM center on the basis of the following indications: (1) LVOT gradient ≥ 50 mmHg at rest or on provocation and (2) presence of unacceptable symptoms despite maximally tolerated medications consisting of ß-blocking agents and/or calcium channel blockers. Myectomy combined with MLE is performed in patients with enlargement of the anterior mitral valve area (> 12 cm^2), calculated with the formula previously validated by Klues et al[10, 11]. The final decision to perform MLE was made preoperatively by the surgeon after visual inspection and epicardial echocardiography. Between 1991 and 2009, 69 patients underwent myectomy combined with MLE. The study complies with the Declaration of Helsinki. After informed consent, these patients have been prospectively included in a registry, approved by the institutional review committee.

The outcome in the surgery group was compared with an age and gender matched cohort of patients with non-obstructive HCM followed at our HCM outpatient clinic during the same period and with the expected mortality in the general Dutch population.

Surgical Technique

The surgical technique has been described previously[10, 11]. In brief, an autologous pericardial patch is harvested, trimmed of fat and extraneous tissue, immersed for 6 minutes in 0.4 % glutaraldehyde, and then placed in a normal saline bath. After opening the ascending aorta by an oblique incision, myectomy is performed to the left of an imaginary line through the nadir of the right coronary cusp in the beginning with a locally designed electrocautery device, later by excision with scissors and a rongeur[10, 11]. After myectomy anterior MLE is performed. A gap is created in the anterior mitral leaflet through a longitudinal incision, starting at the sub aortic hinge point to the rough zone. Then, an oval autologous pericardial patch, of about 2.5 cm wide and 3 cm long, is grafted across the bending point of the mitral valve where SAM is maximal to stiffen the buckling AMVL. The patch extends the width but not the length of the AMVL, which shifts the centrally attached chordae laterally. As a result, the chordae are stretched and erected, which will enhance leaflet coaptation. Finally, because force produced by blood flow against the leaflet is proportional to its area, the increased leaflet will be pressed posterior, with a decrease in SAM and MR.

The surgical results were assessed with transoesophageal echocardiography immediately after weaning from cardiopulmonary bypass and at a systolic blood pressure of ≥ 100 mmHg. None of the patients had an indication for reinstitution of cardiopulmonary bypass.

Follow-up

The clinical characteristics collected before the intervention included assessment of symptoms, New York Heart Association (NYHA) functional class and prescribed drugs. Physical examination and baseline laboratory studies were performed, including electrocardiography, transthoracic echocardiography, and cardiac catheterization. The echocardiographic data were reviewed by a physician, unaware of the patient's medical history. Echocardiography was performed in the month prior to surgery and after surgery repeated at 1 week, 3 months and at yearly intervals. IVS thickness was calculated at the site of myectomy from the septal width in diastole from both the parasternal short-axis and long-axis views. The severity of the mitral valve regurgitation (MR) was graded on a 0 to 4 scale by color flow Doppler echocardiography. The severity of the SAM of the anterior mitral valve leaflet was determined from the 2D images and was graded on a scale from 0 to 3 depending on the mitral-septal distance (grade 0 indicating no SAM and grade 3 indicating brief or prolonged contact between mitral valve and septum). Peak

LVOT gradient was estimated with Doppler echocardiography by the modified Bernoulli equation ($P = 4v^2$), where P is the pressure gradient and v is Doppler-determined blood velocity.

Follow-up information was obtained at routine visits at the HCM outpatient clinic. For 7 patients follow-up information was collected at their referring cardiologist. Follow-up vital status and cause of death was obtained by reviewing the hospital records, from general practitioners and civil registries. Sudden cardiac death (SCD) was defined as instantaneous and unexpected death within 1 hour after a witnessed collapse in patients who previously were in stable clinical condition, or nocturnal death with no antecedent history of worsening symptoms. Follow-up data were complete for all patients.

Statistical Analysis

Data are expressed as mean ± SD or number (percentage). The normality distribution for continuous data was examined with the Shapiro–Wilk test. Comparison of numerical variables was performed using the two-sided Student's t-test or Wilcoxon rank-sum test, and the chi-square or Fisher's exact tests were used to compare qualitative variables. The p-values are two-sided; $p < 0.05$ was considered statistically significant. The survival analysis model used proportional hazards regression methodology; Kaplan-Meier survival curves were compared using log-rank statistics. End point was all-cause mortality. Long-term survival of patients who underwent myectomy combined with MLE was compared with age- and gender matched patients with non-obstructive HCM and with the expected survival curve for the general Dutch population. This expected survival curve was generated from the database of Statistics Netherlands, which incorporates all-cause mortality (www.CBS.nl). Each patient was matched to an age and –gender matched subject from the Dutch population at the year of study entry.

All statistics were performed using the SPSS 16 for Windows (SPSS Inc, Chicago, IL, USA). The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Baseline characteristics

The baseline characteristics of the 69 patients with obstructive HCM and 69 age and sex-matched patients with non-obstructive HCM are listed in Table 1. Because advanced symptoms refractory to pharmacologic therapy represent the standard indication for operation, patients who underwent myectomy combined with MLE expectedly showed more severe functional disability at study entry; 88% in NYHA class III or IV compared with 4% in the non-obstructive group ($p<0.001$). Echocardiography in the myectomy

combined with MLE group showed significantly more severe hypertrophy and as expected higher LVOT gradient, SAM and severity of MR compared with the non-obstructive HCM group. Four (6%) patients were operated after a failed alcohol septal ablation (ASA). Significant coronary artery disease was demonstrated in a single patient in whom coronary bypass grafting was performed simultaneously during the myectomy combined with MLE procedure.

Table 1. Baseline clinical and demographic data among two cohorts of HCM patients

Parameter	Combined Myectomy (n = 69)	Non-obstructive (n = 69)	p Value
Age (yrs)	47.8 ± 14.7	48.9 ± 13	0.639
Male gender	40 (58%)	40 (58%)	0.833
NYHA			
Mean	2.9 ± 0.3	1.6 ± 0.6	<0.001
I	0 (0%)	45 (66%)	<0.001
II	8 (12%)	21 (30%)	<0.001
III	60 (87%)	3 (4%)	<0.001
IV	1 (1%)	0 (0%)	-
Medications			
Beta-blocker	47 (68%)	22 (32%)	<0.001
Calcium-blocker	41 (59%)	10 (14%)	<0.001
Amiodarone	3 (4%)	4 (6%)	0.882
ACE-inhibitors	0 (0%)	11 (16%)	0.002
Echocardiography			
IVS thickness (mm)	22.7 ± 5.4	19.5 ± 4.7	<0.001
LVEDD (mm)	43.6 ± 5.3	44.8 ± 5.3	0.186
LVOTG (mmHg)	93.5 ± 26.4	10.6 ± 8.7	<0.001
SAM (grade)	2.7 ± 0.7	0.4 ± 0.5	<0.001
MR (grade)	2.2 ± 0.9	0.5 ± 0.7	<0.001
Follow-up			
Duration (yrs)	8.1 ± 6	8.7 ± 5	0.527
Mode of death			
Sudden	2 (3%)	3 (4%)	0.886
Heart failure	3 (4%)	2 (3%)	0.886
Non-cardiac	0 (0%)	5 (7%)	0.076

(HCM = hypertrophic cardiomyopathy; NYHA = New York Heart Association; ACE = angiotensin converting enzyme; IVS = interventricular septum; LVEDD = left ventricular end-diastolic diameter; LVOTG = left ventricular outflow tract gradient; SAM = systolic anterior movement; MR = mitral valve regurgitation)

Clinical benefits of myectomy

Symptomatic relief

The myectomy combined with MLE group experienced substantial symptomatic and hemodynamic improvement after surgery; 61 of 69 (88%) patients were in NYHA functional class III or IV before operation, whereas only 1 of 64 (2%) remaining patients at latest follow-up was in NYHA class III. The mean functional NYHA class was 2.9 ± 0.3 before myectomy combined with MLE and 1.3 ± 0.5 at latest follow-up (Figure 1).

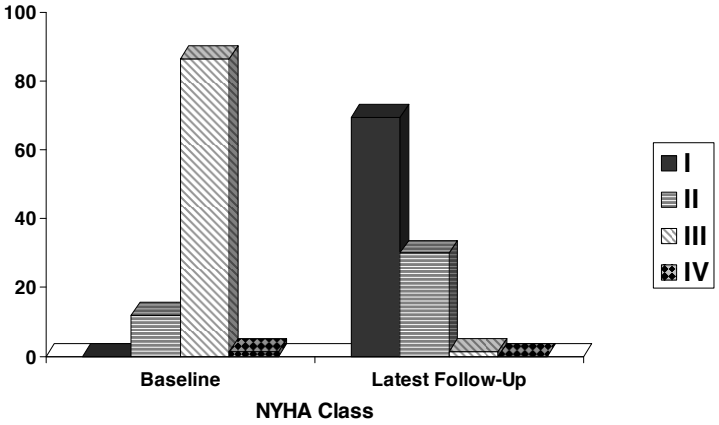

Figure 1:
NYHA functional class at baseline and at latest follow-up in obstructive HCM patients treated with myectomy and MLE

Hemodynamic and echocardiographic changes

Compared with preoperative data, there were significant immediate changes in peak LVOT gradient from 94 ± 26 preoperative to 9 ± 8 mmHg at latest follow-up. MR reduced from grade 2.7 ± 0.7 to 0.6 ± 0.8 and SAM grade from 2.2 ± 0.9 to 0.1 ± 0.2 at latest follow-up (Table 2).

Table 2 Long-term echocardiographic follow-up in 69 patients with obstructive HCM treated with myectomy and MLE

	Preoperative (n = 69)	Latest Follow-up (n = 64)	P-value
IVS, mm	23 ± 5	16 ± 3	<0.0001
LVEDD, mm	44 ± 5	49 ± 7	<0.0001
LVOTG, mmHg	94 ± 26	9 ± 8	<0.0001
MR, grade	2.7 ± 0.7	0.6 ± 0.8	<0.0001
SAM, grade	2.2 ± 0.9	0.1 ± 0.2	<0.0001

(HCM = hypertrophic cardiomyopathy; MLE = mitral leaflet extension; IVS = interventricular septum; LVEDD = left ventricular end-diastolic diameter; LVOTG = left ventricular outflow tract gradient; MR = mitral valve regurgitation; SAM = systolic anterior movement)

Non-fatal events after myectomy and MLE

Two (3%) patients, who were operated after failed ASA already hampering the conduction system, developed complete heart block during surgery. One received a permanent pacemaker, in the other this was combined with an implantable cardioverter defibrillator (ICD) because of the presence of high risk status for SCD. An ICD was implanted in 4 more patients for primary prevention of SCD based on current guidelines[12]. There were no ICD-shocks during follow-up. Atrial fibrillation occurred in 19 (28%) patients during follow-up.

A single (1%) patient was re-operated for ongoing bleeding 6 hours after surgery, for which no specific cause could be identified. Pericardial effusion, requiring subxiphoid surgical approach occurred in 2 (3%) patients in the first two weeks after surgery. Residual obstruction was present in 1 (1%) patient, which was treated by re-myectomy at day 8. Patch-dehiscence requiring repair occurred in 2 (3%) patients, respectively 3 and 7 months after combined surgery. More than 10 years after myectomy combined with MLE 2 (3%) patients were re-operated; 1 (1%) because of central mitral regurgitation caused by left ventricular dilatation and 1 (1%) because of endocarditis (Table 3).

Table 3 Events during 20-year follow-up in 69 patients with obstructive HCM treated with myectomy and MLE

	Latest Follow-up
Death, n (%)	5 (7)
Operative mortality	0 (0)
Sudden cardiac death	2 (3)
End-stage heart failure	3 (4)
Atrial fibrillation, n (%)	19 (28)
Pacemaker, n (%)	1 (1)
ICD, n (%)	5 (7)
Re-operation, n (%)	5 (7)
Residual obstruction	1 (1)
Patch-dehiscence	2 (3)
Central MR	1 (1)
Endocarditis	1(1)

(HCM = hypertrophic cardiomyopathy; MLE = mitral leaflet extension; ICD= implantable cardioverter defibrillator; MR = mitral valve regurgitation)

Survival after myectomy combined with MLE

None of the patients died during surgery or in hospital. Five (7 %) patients died during follow-up; 2 suffered SCD, respectively after 1.9 and 2.8 years. Three patients died of end-stage heart failure, respectively after 2.6, 2.8 and 6.9 years (Table 3).

Survival comparison with the general Dutch population: As seen in Figure 2; the 1-, 5 -, 10-, and 15- year cumulative survival after myectomy combined with MLE was 98%, 94%, 91%, and 86%, respectively and did not differ from the age and gender matched general population (99%, 97 %, 94%, and 91%, respectively; log-rank p = 0.25).

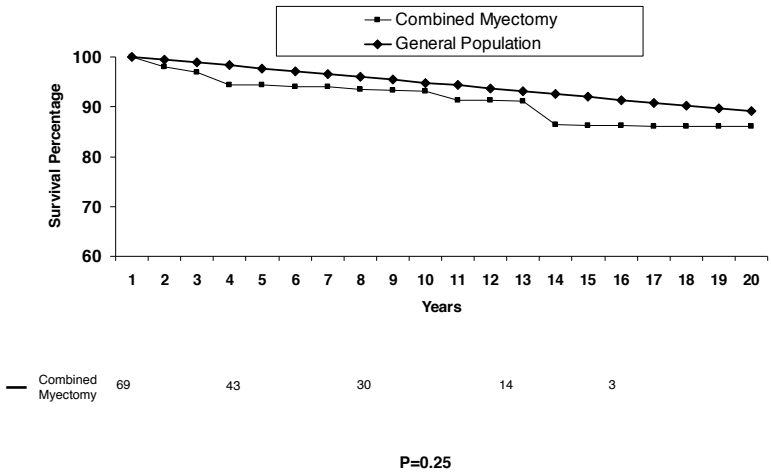

Figure 2:
Survival comparison between obstructive HCM patients treated with myectomy and MLE and age and gender matched subjects from the Dutch population

Survival comparison with non-obstructive HCM: Likewise, the 1-, 5 -, 10-, and 15- year cumulative survival (98%, 97%, 89%, and 70%, respectively, log-rank p = 0.11) of age- and gender-matched non-obstructive HCM patients was not different from the patients who underwent myectomy combined with MLE (Figure 3). The number of cardiac related death in both groups was identical; however in the non-obstructive HCM group more patients died from non-cardiac causes during long-term follow-up.

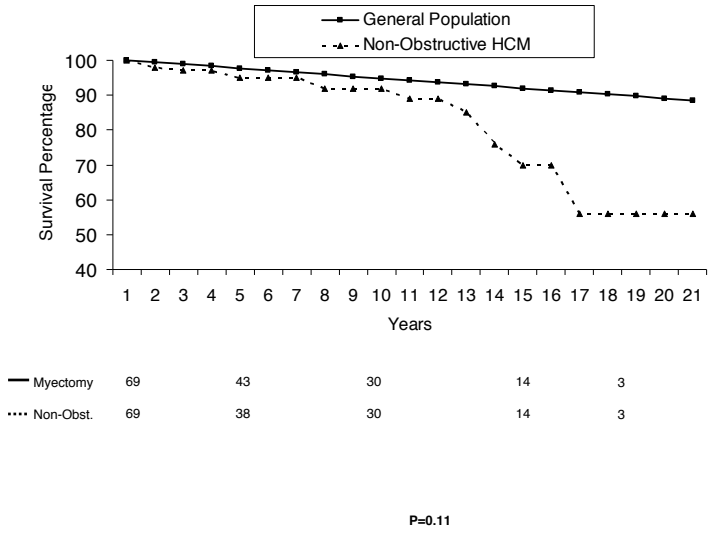

Figure 3:
Survival comparison between obstructive HCM patients treated with myectomy and MLE and age and gender matched non-obstructive HCM patients

DISCUSSION

This study shows that myectomy combined with MLE in selected obstructive HCM patients is a safe and successful procedure leading to long-term symptom relief and comparable survival to age and sex-matched patients with non-obstructive HCM and subjects from the general Dutch population.

LVOT obstruction is an important pathophysiological component of HCM. Clinical studies performed in large HCM populations have identified a consistent relationship between LVOT gradients at rest and heart failure symptoms and cardiovascular events; including overall probability of death due to HCM and progression to NYHA functional class III or IV[13, 14].

Severely symptomatic drug-refractory obstructive HCM patients are potential candidates for invasive septal reduction interventions as surgical myectomy or ASA[13, 14]. However, obstructive HCM can also be associated with a variety of intrinsic abnormalities of the mitral valve, including increased mitral leaflet area[6, 15]. These abnormalities may predispose to residual SAM and result in suboptimal outcome after isolated myectomy[9].

The current study consisted of consecutive patients with enlarged mitral leaflet area and typical SAM as described in previous reports from our institute treated with myectomy combined with MLE[10, 11].

This myectomy combined with MLE has immediate beneficial effects on hemodynamics and NYHA functional class, and these benefits continue in 88 % up to 19 years. At the same time it is a safe procedure; without operative mortality and excellent long-term cumulative survival, equal to general Dutch population or age-and gender matched patients with non-obstructive HCM. This is comparable with the reported long-term survival benefit of obstructive HCM patients who underwent isolated myectomy from the Mayo Clinic and Toronto General Hospital[2, 4]. In the larger Mayo Clinic series 10-year overall survival was 83%, which was equivalent to that expected in the general U.S. population. In the latter study patients who underwent myectomy combined with additional operative procedures were however excluded[4]. Another Mayo Clinic study describes 28 patients who underwent myectomy and mitral valve repair with different techniques with significant improvement in functional class, LVOT gradient, SAM and mitral valve regurgitation at midterm follow-up[16]. A study by Kaple et al reports overall survival after myectomy combined with a number of repair techniques at 1-, 5 - and 10 years of respectively 91%, 81% and 66%; the patients in this study were however significantly (59±14 versus 48±15, P<0.001) older than those in the current study[15]. The current study is to our knowledge the first to describe equivalent survival to the general population in HCM patients undergoing a myectomy combined with MLE.

During long-term follow-up 93 % patients were free of reoperation, which is comparable with the results of combined surgery in obstructive HCM reported by others at

3 years follow-up[15]. Perfect mitral competence (grade 0 MR) was present in 31 (45 %) of patients and only 2 (3 %) had grade 3+ MR at latest follow-up. There were no early valve repair failures in our cohort leading to mitral valve replacement; two (3 %) patients underwent mitral valve repair because of patch-dehiscence. More than 10 years after the initial procedure 2 patients underwent mitral valve replacement combined with aortic valve replacement in one.

In comparison with the age-and gender matched patients with non-obstructive HCM cardiac related mortality in both groups was similar; 5 patients (7%) in each group died during the follow-up period with a mean of 8 years. These numbers are inline with previously reported mortality rates in HCM patients during long-term follow-up[17, 18].

LIMITATIONS

This is a single institution's consecutive experience with myectomy combined with MLE in a relatively small number of selected obstructive HCM patients. These patients were not candidates for isolated myectomy or ASA because of mitral valve abnormalities. We were unable to include a third group of obstructive HCM patients treated by isolated myectomy. This is largely caused by the fact that between 1999 and 2007 almost all patients suitable for isolated myectomy underwent ASA, mostly because of patient preference[19, 20]. Based on the experience in our center with this specific technique there is also a referral bias; especially obstructive HCM patients with enlarged mitral valve leaflets are being referred for surgery. Despite the lack of a statistically significant difference in the Kaplan-Meir survival analysis between patients who underwent myectomy combined with MLE and the general Dutch population, the study may not be powered enough to detect such a difference.

CONCLUSIONS

In selected obstructive HCM patients myectomy combined with MLE is a safe procedure leading to long-term symptom relief. In addition it conveys cumulative survival similar to the general population over 19-year follow-up.

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Chapter 12

Long-term outcome of alcohol septal ablation in patients with obstructive hypertrophic cardiomyopathy: A word of caution

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Circ. Heart Failure 2010; May 1;3(3)362-9

ABSTRACT

Background

The impact of alcohol septal ablation (ASA)-induced scar is not known. This study sought to examine the long-term outcome of ASA among patients with obstructive hypertrophic cardiomyopathy (HCM).

Methods and Results

Ninety-one consecutive patients (aged 54±15 years) with obstructive HCM underwent ASA. Primary study endpoint was a composite of cardiac death and aborted sudden cardiac death (SCD) including appropriate cardioverter-defibrillator (ICD) discharges for fast VT/VF. Secondary endpoints were non-cardiac death and other non-fatal complications. Outcomes of ASA patients were compared with 40 HCM patients who underwent septal myectomy. During 5.4±2.5 years primary and/or secondary endpoints were seen in 35 (38%) ASA patients, of whom 19 (21%) patients met the primary endpoint. The 1-, 5-, and 8-year survival-free from the primary endpoint was 96%, 86%, and 67%, respectively in ASA patients versus 100%, 96%, and 96%, respectively in myectomy patients during 6.6±2.7 years (log rank, *P*=0.01). ASA patients had a ~4 fold increase in the estimated annual primary endpoint rate (4.4% vs. 0.9%) compared with myectomy patients. In a multivariable model including a propensity score, ASA was an independent predictor of the primary endpoint unadjusted HR 5.2: 95% CI 1.2 to 22.1, *P*=0.02; propensity score-adjusted HR 6.1: 95% CI 1.4 to 27.1, *P*=0.02.

Conclusions

This study shows that ASA has potentially unwanted long-term effects. This poses special precaution given the fact that ASA is practiced worldwide at increasing rate. We recommend myectomy as the preferred intervention in patients with obstructive HCM.

INTRODUCTION

Obstructive hypertrophic cardiomyopathy (HCM) is characterized by asymmetrical septal hypertrophy and dynamic left ventricular (LV) outflow tract obstruction.[1] In symptomatic obstructive HCM patients surgical resection (myectomy) of the basal septum relieves LV outflow obstruction with improvement in long-term outcome.[2, 3] Alcohol septal ablation (ASA) is an alternative technique in which ethanol is injected into one or more septal perforator branches of the left anterior descending coronary artery. The efficacy of ASA has been proven with hemodynamic results mimicking those of myectomy.[3-7] The septal morphologic appearance post-ASA is one of a myocardial infarction.[8] It is not well-known whether this may produce an arrhythmic substrate and possibly trigger sudden cardiac death (SCD).[9, 10] Therefore, the present study sought to describe long-term post-ASA outcome.

METHODS

Study Population

The study comprised 91 consecutive patients (aged 54±15 years) who underwent ASA between 1999 and 2007 at the Erasmus MC Rotterdam. Eligibility criteria for ASA were persistent New York Heart Association (NYHA) class III/IV dyspnoea and/or Canadian Cardiovascular Society class III/IV angina, despite standard medical therapy; LV outflow obstruction (gradient ≥50 mm Hg at rest or on Valsalva strain); ventricular septal thickness ≥15 mm and absence of need for surgical correction of mitral valve or coronary artery disease. Once indicated, patients were informed about ASA and myectomy as therapeutic options. The ASA cohort in the present study included all patients (n=31) from our center that were previously described in a multicenter center series.[11] All patients gave written informed consent and the local institutional review board approved this study.

Diagnostic Evaluation

HCM was defined as a hypertrophied and non-dilated LV in the absence of other cardiac or systemic disease that could explain hypertrophy.[1] All patients underwent a standard 2-dimensional echo-Doppler ultrasound examination,[1] and clinical risk assessment including Holter monitoring and treadmill exercise testing.[1]

ASA procedure

ASA was performed as previously described.[5] In brief, using the standard Judkins technique, a 6F pacemaker lead was placed in the right ventricle, a 6F pigtail catheter was positioned into the LV, and a 7F Judkins guiding catheter in the ascending aorta. LV

outflow tract peak systolic gradient was continuously monitored throughout the whole procedure. After initial coronary angiography for localizing the origin of the septal perforating arteries, a 1.5-2.5 x 10 mm balloon catheter was introduced over a 0.014-inch guide-wire into the target perforator artery and inflated. ASA was performed with the assistance of myocardial contrast (1 ml SonoVue, Bracco, Geneva, Switzerland) echocardiography to identify the septal region by the selected septal branch. If no contrast was seen outside the thickened basal septum, 0.5 mL alcohol was injected over 30 seconds followed by saline flush under continuous hemodynamic and ECG surveillance. A successful procedure was defined as absence of a residual invasive dynamic gradient >25 mm Hg. If the target reduction in pressure gradient was not achieved, alcohol injections were repeated after 5 minutes (maximum 2.5 mL) within the same perforator branch. If not successful, the procedure was repeated in a second perforator branch. Once success was achieved, the balloon was deflated, and coronary angiography was repeated to confirm the occlusion of the septal branch and patency of the left anterior descending coronary artery. A temporary pacemaker lead was kept in place for at least 24 hours. A permanent pacemaker was used to treat a persistent high-grade AV block and a cardioverter defibrillator (ICD) implantation was considered at the discretion of the HCM cardiovascular specialist (FJTC).

ICD

ICD implantation for secondary prevention was considered for HCM patients who survived cardiac arrest and for primary prevention for HCM patients who had ≥2 conventional risk factors for HCM-related SCD.[1, 12] In addition, ICD was used as an alternative to conventional pacemaker in a few patients with post-ASA persistent high-grade AV-block. Device implantations were performed according to customary practice, with defibrillation thresholds routinely tested to document successful termination of lethal arrhythmias. For all patients, ICD programming was intended to avoid inappropriate therapy and tailored according to the clinical presentation. Mean ventricular tachyarrhythmia detection rate was 349±18 ms and the mean fibrillation detection rate was 283±15 ms.

Device interrogation was performed on a 3-month basis and otherwise following patients' symptoms. Arrhythmias responsible for triggering defibrillator therapy were identified from the stored intracardiac electrograms.[13] Defibrillator therapy was considered appropriate when triggered by VF or VT. Device therapy was considered inappropriate when triggered by "benign rhythms" with rates exceeding the programmed threshold, such as supraventricular arrhythmias, sinus tachycardia, or device malfunction. Appropriate ICD discharge for lethal arrhythmia (fast sustained VT/VF) was considered equivalent to SCD.

Study endpoints

The primary study endpoint was a composite of cardiac death and aborted SCD including ICD appropriate shocks for fast VT (defined as sustained VT with a rate >260 bpm) or VF. Secondary endpoints included non-cardiac death, procedural failure, and other non-fatal complications. A failed ASA was defined as the need for re-intervention for persistent symptoms due to residual LV outflow tract gradient.[14]

Myectomy patients

Outcome of ASA patients were compared to outcome of 40 consecutive patients with obstructive HCM who underwent modified septal myectomy during the same period of enrollment of the ASA patients.[5].

Follow-up

Two investigators (FJTC, MM) examined all patients at 3, 6 and 12-months and then on yearly basis with complete follow-up to 9 years. Cause of death was documented from hospital records, general practitioners records and civil registries. SCD was defined as instantaneous and unexpected death within 1 hour after a witnessed collapse in patients who previously were in stable clinical condition, or nocturnal death with no antecedent history of worsening symptoms.

Statistical analyses

Qualitative variables were expressed as percentages and quantitative variables as mean (standard deviation). The normality distribution for continuous data was examined with the Shapiro–Wilk test. Comparison of numerical variables was performed using the two-sided Student's t-test or Wilcoxon rank-sum test, and the chi-square or Fisher's exact tests were used to compare qualitative variables. A continuous propensity score analysis was performed to adjust for the intergroup (ASA vs. myectomy) differences in baseline characteristics caused by the selection bias inherent to the nonrandomized nature of the study. A propensity score representing the likelihood of having ASA as opposed to myectomy was calculated for each patient by using a logistic regression analysis that identified variables independently associated with the type of procedure. All variables listed on Table 1 were included in a univariate regression analysis and variables exhibiting a P-value <0.20 were included in a multivariate model. Those variables (P<0.020) were: age, positive risk factors for HCM-related SCD, LV outflow tract peak gradient, of ventricular septum thickness, and LV end-systolic diameter. Kaplan-Meier curves were employed to delineate freedom from death or aborted SCD and compared with log-rank test. In addition, the incidence of post-procedural primary endpoint was further evaluated between groups as unadjusted, adjusted for propensity score in a multivariable regression analysis. The propensity score did not emerge as an independent predictor of

Table 1: Baseline characteristics of the HCM patients

	ASA (n=91)	Myectomy (n=40)	P Value
Demographics			
Age– yrs	54±15	49±15	0.08
Male sex	50(55%)	21(53%)	0.99
NYHA class I/II/III/IV	0/0/90/1	0/0/40/0	-
CCS class III/IV angina	17/2	8/1	-
Coronary artery disease	4(4%)	2(5%)	0.84
Risk factors for HCM-related SCD	*0.6±0.8*	*0.5±0.9*	*0.51*
History of SCD	0	0	-
Spontaneous sustained VT	0	0	-
Family history of SCD	15(16%)	5(13%)	0.86
Non-sustained VT	10(11%)	5(13%)	0.97
Unexplained syncope	11(12%)	4(10%)	0.97
Abnormal blood pressure response to exercise	16(18%)	6(15)	0.87
Ventricular septum >30 mm	2(2%)	2(5%)	0.71
Co-morbidities and past medical history			
Diabetes mellitus	1(1%)	1(3%)	0.93
Systemic hypertension	7(8%)	2(5%)	0.81
Previous stroke	1(1%)	0	0.61
Prior ICD	0	4(10%)	--
Prior permanent pacemaker	0	0	--
Atrial fibrillation	5(5%)	2(5%)	0.67
Beta-blockers	61(67%)	24(60%)	0.57
Calcium antagonists	39(43%)	16(40%)	0.89
Amiodarone	2(2%)	1(3%)	0.78
Angiotensin-converting enzyme inhibitors	6(7%)	2(5%)	0.97
Diuretics	10(11%)	3(8%)	0.83
Echocardiographic data			
Resting LV outflow tract gradient– mmHg	92±25	86±19	0.48
Ventricular septal thickness– mm	23±4	24±5	0.23
LV posterior wall thickness– mm	14±2	14±3	0.99
LV end-diastolic diameter– mm	40±4	39±4	0.19
LV end-systolic diameter– mm	23±5	21±6	0.05
Mitral regurgitation– grade	1.5±0.9	1.7±0.8	0.23
Systolic anterior motion of the mitral valve– grade	2.3±0.9	2.4±0.9	0.56
LV ejection fraction– %	69±6	68±6	0.38
Left atrial diameter– mm	49±9	50±11	0.59

Values are mean±SD or n(%) and otherwise stated; CCS = Canadian Cardiovascular Society; HCM = Hypertrophic cardiomyopathy; ICD = Implantable cardioverter defibrillator; LV = Left ventricular; NYHA = New York Heart Association functional class; VF = VT ventricular fibrillation; VT = Ventricular tachycardia

the primary endpoint, suggesting that differences attributed to the type of intervention by the initial Cox regression analyses were not explained by bias in patient selection on the basis of their baseline characteristics. Results were expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). A P value <0.05 was considered significant. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Baseline characteristics

Table 1 lists baseline data of all patients. In 81 (89%) patients ASA was based solely on patient preference and 10 (11%) patients had a high surgical risk (Table 2).

Table 2. Cited Reasons for Selection of ASA or Myectomy

ASA patients (n=91)	
Patient preference	81(89%)
Co-morbidities	10(11%)
Prior failed myectomy	0
Myectomy patients (n=40)	
Patient preference	2(5%)
Failed ASA	5(13%)
Mitral valve abnormalities	31(78%)
Three-vessel disease	1(3%)
Coronary anatomy not suitable for ASA	1(3%)

Procedural data

Mean number of septal perforator arteries used for ASA was 1.1±0.3. Mean ethanol volume was 3.5±1.5 mL with a larger volume in the early (1st 25 patients) versus late (4.5±1.2mL vs. 2.4±1.0 mL) experience. LV outflow tract peak pressure gradient reduced from 92±25 at baseline to 8±17 mmHg (P<0.001) immediately post-ASA.

Primary endpoint

During 5.4±2.5 years, one or more events were seen in 35 (38%) patients post-ASA (Table 3), of whom 19 (21%) met the primary endpoint. Using Kaplan-Meier survival analysis, the estimated annual rate of the primary endpoint was 4.4% over 8-year (Figure 1). During follow-up 11 (12%) patients died due to cardiac causes in 9 and non-cardiac causes in 2.

Table 3. Study Endpoints Among ASA Patients (n=91)

	Early <30 days	Late ≥30 days	Total
Primary Endpoint (n=19)			
• Total no of patients (percentage)	6(6%)	13(14%)#	19(21%)#
• Cardiac mortality			
-Total	2(2%)	7(8%)	9(10%)
-SCD	1(1%)	6(7%)	7(8%)
-Other cardiac death	1(1%)	1(1%)	2(2%)
• Arrhythmogenic complications			
-Total	4(4%)	7(8%)	11(12%)
-Resuscitated SCD	4(4%)	3(3%)	7(8%)
-Appropriate ICD shocks	0	4(4%)	4(4%)
Secondary Endpoints (n=28)#			
• Non-cardiac death	0	2(2%)	2(2%)
• ICD implantation	8(9%)	8(9%)	16(18%)
• Inappropriate ICD therapy	0	2(2%)	2(2%)
• Acute myocardial infarction	2(2%)	0	2(2%)
• ASA failure	3(3%)	7(8%)	10(11%)
• Permanent pacemaker dependency	4(4%)	0	4(4%)
• Pericardial effusion	1(1%)	0	1(1%)
• Atrial fibrillation	1(1%)	10(11%)	11(12%)

Some patients had more than 1 endpoint.

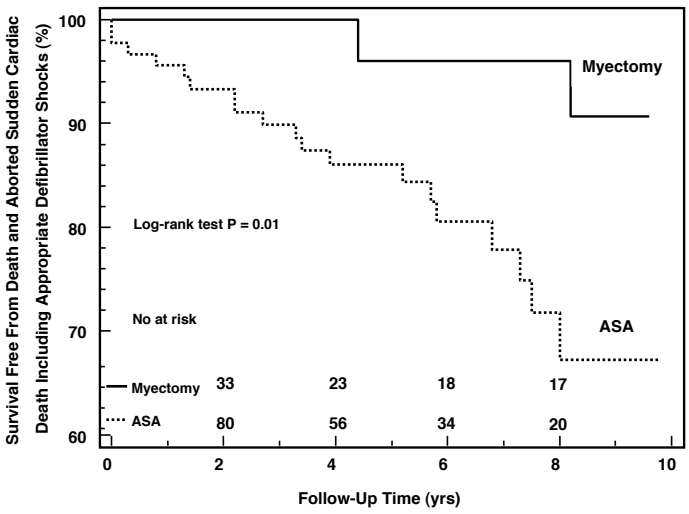

Figure 1:
Survival free from death and aborted SCD including ICD appropriate therapy of patients with obstructive HCM: treated with ASA (n=91) and patients whom were treated with septal myectomy (n=40). Log-rank, *P*=0.01.

In hospital and 30-days primary endpoint

Procedure-related mortality was seen in 2 patients (2%) due to cardiac tamponade and intractable VF, respectively. This latter patient died during the ASA procedure after the first 0.5 mL ethanol injection. Autopsy did not reveal a specific cause. Four (4%) patients survived in-hospital cardiac arrest. One patient had VF on the day post-ASA; the patient refused to receive an ICD and died suddenly 2.3 years post-ASA. One patient had intra-procedural VF and follow-up went uneventful over 7.4 years. One patient survived 2 episodes of in-hospital VF (day 4) and sustained VT 5.6 years post-ASA. One patient survived in-hospital sustained VT with hemodynamic instability on the day post-ASA. In these latter 3 patients an ICD was implanted for secondary prevention.

Long-term (>30 days) primary endpoint

During follow-up, 6 patients (7%) died due to SCD and one patient (1%) died 5.8 years post ASA due to end-stage heart failure. Eleven (12%) patients survived 1 or more episodes of ventricular tachyarrhythmia. In 4 patients the arrhythmia was successfully terminated by ICD discharge, for fast VT/VF 0.3, 0.6, 1.3, and 2.6 years after ICD implantation. Cycle length was 200, 230, 230, 330 ms, respectively (Figure 2). In the remaining 7 patients without ICD cardiopulmonary resuscitation was needed. Of those 7 patients, one later died during follow-up) and 3 patients who survived sustained VT with hemodynamic instability 3.2, 4.4 year and 5.6 years, respectively post-ASA, received an ICD.

Secondary endpoints

Twenty-eight patients (31%) had one or more secondary endpoints.

In hospital and 30-days secondary endpoints (Table 3)

Eight (9%) patients had an ICD implantation. None of these patients had inappropriate discharge during the 30-days follow-up post-ASA. Two patients had non-fatal acute myocardial infarction due to coronary artery dissection and ethanol spill into the left anterior descending coronary artery. These 2 patients were managed successfully and underwent myectomy at a later date. As mentioned before, ASA failure was seen in 3 patients due to technical causes. Four (4%) patients had a high-grade AV block and received a permanent pacemaker. One patient subsequently had extended hospitalization for pericardial effusion due to right ventricular perforation from temporary pacemaker placement. This patient underwent myectomy 2 months later due to unsuccessful reduction of LV outflow pressure gradient.

Long-term (>30 days) secondary endpoints (Table 3)

Two patients (2%) died due to non-cardiac causes (suicide and cancer 2 years post-ASA). Eight (9%) patients had an ICD implantation, of whom 2 patients had inappropriate

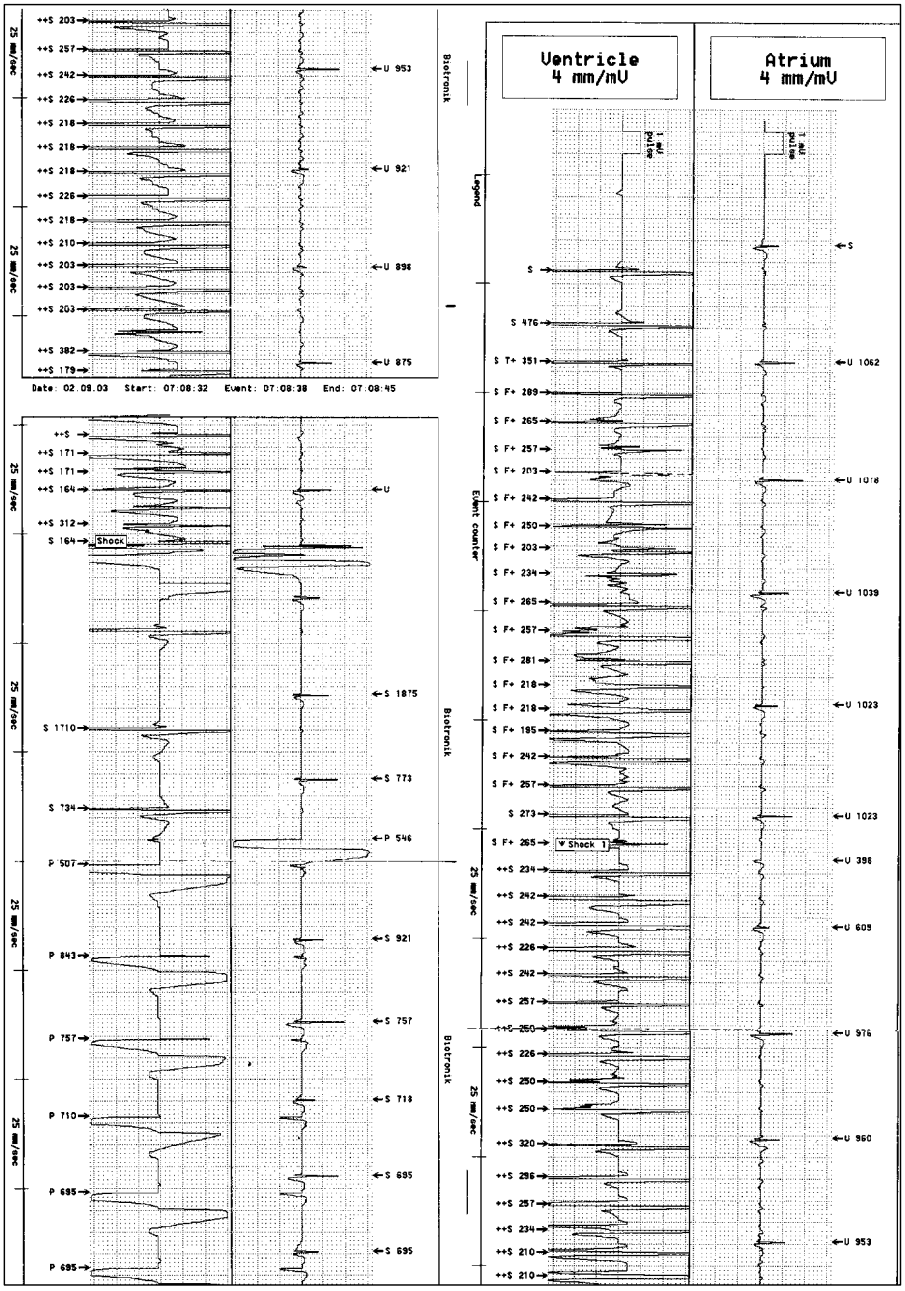

Figure 2:
Appropriate ICD therapy for ventricular arrhythmia with atrio-ventricular dissociation. From top to bottom, bipolar atrial electrogram with marker annotations, and ventricular rate-sensing electrogram with marker annotations are shown. The rhythm before the marker annotation " Shock 1" displays a ventricular tachyarrhythmia (mean ventricular cycle length ~200 ms) detected in the ventricular fibrillation detection zone. The arrhythmia is terminated by shock therapy (marker annotation "Shock"), which restores sinus rhythm with atrio-ventricular sequential pacing.

discharges due to atrial arrhythmias. At the most recent follow-up, 10 (11%) patients had failed ASA, of whom 7 (8%) patients had early successful reduction, but suffered progressive increase in LV outflow gradient associated with recurrence of symptoms. Five patients had repeat ASA and 5 patients underwent myectomy. One or more episodes of atrial fibrillation were reported in 10 (11%) patients.

Conventional risk factors for HCM-related SCD and the primary endpoint

Forty ASA patients (44%) had at least 1 conventional risk factor for HCM-related SCD, of whom 13 (14%) had 2 risk factors. No patient had a history of aborted SCD before ASA. In addition, there was no difference in the number of risk factors between patients with and without the primary endpoint (0.79±0.79 versus 0.54±0.72, *P*=0.21). Likewise, as seen in Figure 3, incidence of primary or secondary end-points was not related to the number of conventional risk factors for HCM-related SCD.

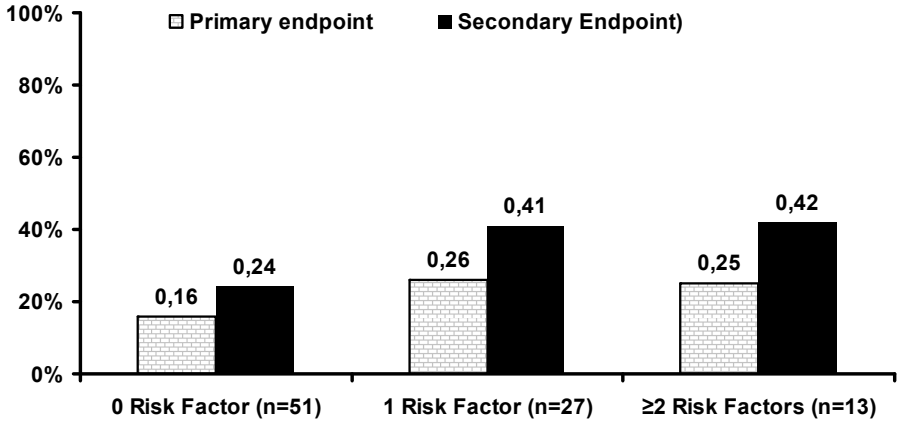

Figure 3:
Graphic display of the percentage of patients with the study endpoints who had 0, 1, or ≥2 risk factors for HCM-related SCD.

Outcome of myectomy versus ASA patients

As seen in Table 1, patients who underwent myectomy had similar baseline characteristics as ASA patients. Also, comparable immediate reductions of LV outflow gradient were seen. There was 98% procedural success in myectomy patients and the in-hospital and 30 days post-operative clinical course went uneventful. During a mean follow-up of 6.6±2.7 years, 2 (5%) patients died and none had aborted SCD. The 1-, 5-, and 8-years survival free from the primary endpoint was 100%, 96%, and 96%, respectively, which was better than seen in the ASA patients (96%, 86%, and 67%, respectively; χ^2=5.9, log-rank *P*=0.01: Figure 1). ASA patients had about 4.8-fold annual rate of the primary endpoint (4.4% versus 0.9%) compared to myectomy patients. A total of 5 (6%) patients

had an ICD implantation in the myectomy group compared with a total of 16 (18%) in the ASA patients and 1 patient had permanent pacemaker for complete AV-block. ICD implantation was for primary prevention indication before (4 patients) and after (1 patient) myectomy. As abovementioned, ICD appropriate shocks were recorded in 4 (25%) of the ICD patients in the ASA group. None of the myectomy patients had an ICD shock during follow-up. Myectomy was repeated in 1 patient due to recurrent obstruction 8 days post-operative and in 1 patient due to dehiscence of the mitral valve patch 6 months post-operative. In both patients post-operative clinical course went uneventful with a follow-up of 8.6 and 3.8 years, respectively.

Cox proportional-hazards regression analysis among the total intervention group (n=131) including baseline variables that are listed on Table 1, type of intervention (ASA versus myectomy), ethanol volume and propensity score showed that only ASA was an independent predictor of the primary endpoint with an unadjusted HR 5.2: 95% CI 1.2 to 22.1, P=0.02; propensity score-adjusted HR 6.1: 95% CI 1.4 to 27.1, P=0.02. Of note large (>2 mL) ethanol volumes were not associated with the primary endpoint (*P*=0.72) (Figure 4). Importantly, only 1 of 10 (10%) ASA patients in whom myectomy was considered a high-risk procedure met the primary endpoint compared to 18 out of the remaining 81 (22%) low-to-moderate risk patients. The combined rate of post-procedural primary and secondary endpoints in myectomy patients was 15% compared with 38% among ASA patients, (χ^2=5.8; adjusted *P*<0.02).

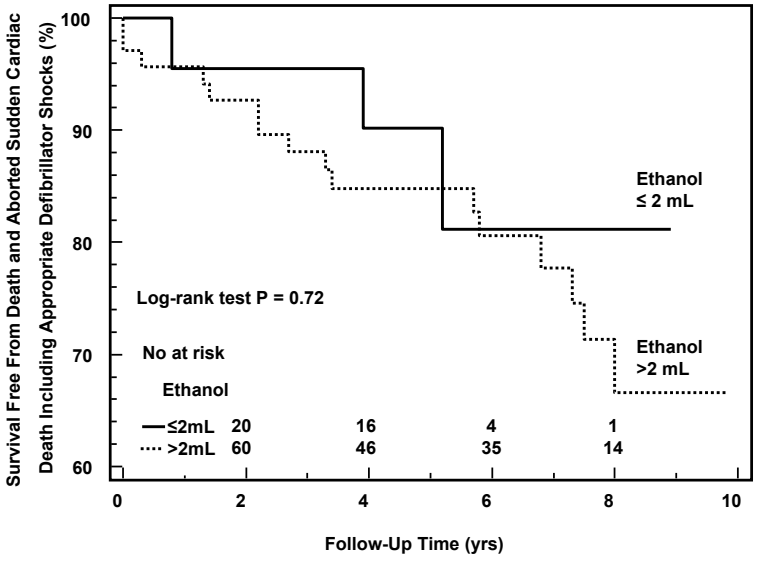

Figure 4:
Survival free from death and aborted SCD including ICD appropriate therapy after ASA of patients treated with ≤2mL (in continous line) and >2mL ethanol (in dotted line).

DISCUSSION

The main finding of our study is that approximately 1 out of 3 HCM patients who underwent ASA had major cardiovascular complications during the procedure and follow-up, including cardiac death or resuscitated SCD in approximately 1 out of 5 patients. Compared with the myectomy patients, ASA patients had a 4.8-fold increase in the estimated annual primary endpoint, respectively.

Of note, the early (30-days) complication rate of ASA patients in our study was similar to the complication rate reported in a meta-analysis in ~3,000 ASA patients with respect to mortality (2.2% versus 1.5%), VF rate (3.3% versus 2.2%), permanent pacemaker dependency (11.0% versus 10.5%), pericardial effusion (1.1% versus 0.6%), and coronary artery dissection (1.1% versus 1.8%), whereas procedural failure occurred less often (3.3% versus 11.1%).15 Importantly, most serious adverse events were late and therefore, ASA carries a long-term risk. Of note, all ASA patients who died suddenly had no ICD, where there was no death among the patients who received an ICD. Since its introduction in 1994, the number of ASA procedures has exceeded the number of surgical procedures by 10- to 35-fold during the same time period worldwide.[16] The notion of our study is to provide a word of caution to the increased number of ASA procedures done.

Arrhythmogenic ASA-induced scar

The potential risk of arrhythmic events due to post-ASA healed myocardial infarction[17] in patients prone to arrhythmias has been a matter of concern since early practice of ASA.[18] Our group described post-ASA regional hyperenhancement on contrast-enhanced magnetic resonance imaging in the basal septum in all patients.[19] Mean infarction size was 20±9 g, corresponding to 10±5% and 31±16% of LV and septal mass, respectively.[19] Frequent episodes of non-sustained VT,[20, 21] and sustained VT and VF[9, 21-24] have been reported in several series early post-ASA. The high arrhythmogenic susceptibility of HCM patients could be attributed to myocardial fiber disarray.[25]

In a large cohort of HCM patients, ICD interventions appropriately terminated VT/VF in 20% of patients with a 10.6% annual intervention rate for secondary prevention after aborted SCD and 3.6% per year for primary prevention of SCD.[26] In a recent study, annual ICD intervention rate was 2.8% over a 3-years period in HCM patients who underwent ASA and ICD implantation for primary prevention of SCD.[27]

The relation between the size of post-ASA infarction and arrhythmogenic risk is not clearly understood. In a recent paper, a lower ethanol volume was an independent predictor of improved long-term survival following ASA,[28] a finding that we could not reproduce. Boekstegers et al. reported that electrophysiologic testing before and 4 to 6 months post-ASA did not suggest enhanced arrhythmogenesis.[29] However, most of the

arrhythmic complications in our series occurred after 4 years post-ASA. Of note, the size of the septal infarction in our series[19] was not different from other series.[17]

ASA versus surgical treatment of obstructive HCM patients

Ideally, a randomized trial comparing myectomy and ASA should be performed to examine whether they are true equivalent therapeutic options. However, such a trial is unlikely to be undertaken.[30] Alternatively, comparison of ASA and myectomy outcomes can be performed by analyzing data from single center registries. Maron et al. found in a small series of ASA patients a 4-fold increase in appropriate ICD intervention rate (10.3% versus 2.6% per year, respectively) compared with patients who had previously undergone surgical septal myectomy.[26] In another report, myectomy patients with an ICD had over 10-fold fewer appropriate ICD discharges (0.2% versus 4.3% annually, respectively) compared to non-operated HCM patients with an ICD.[31] In another report by Sorajja et al. surgical patients had significantly better symptom-free survival compared to ASA (89% versus 71%) over a 4-year follow-up.[32] In our HCM cohort ASA induced relief of LV outflow obstruction with subsequent reduction in LV hypertophy[6, 11] and improvement in clinical status,[6, 11] LV function,[6, 11, 33] and microcirculation.[6] However, the incidence of arrhythmic complications and cardiac death was approximately 4-fold as compared to myectomy patients.

In contrast to the findings described above, Kwon et al. did not suggest excess mortality on long-term follow-up in ASA versus myectomy patients when statistically corrected for the higher risk profile of ASA patients.[34] However, their primary endpoint did not include arrhythmic complications, which occurred in ~half of patients with the primary endpoint in our series. In addition, "mortality" rate was 3-fold increased in the ASA patients compared to myectomy patients (24% versus 8%). Most of the ASA mortality occurred in patients >65 years old.[34] However, a log-rank test between the ASA and myectomy in 28 propensity matched patients was negative,[34] obviously because ASA patients >65 years old were excluded in this analysis. In addition, 6 (11%) of the ASA patients underwent myectomy after failed ASA despite being high-risk candidates.[34] It is not clear to what extent these crossed over patients to myectomy contributed to the mortality in that group. Fernandes et al. recently reported a 1.1% rate of SCD on long-term follow-up post-ASA35 However, contrary to our study 8% of patients were lost to follow-up.[35] In addition, no data on arrhythmic complications were reported.

Risk factors of HCM-related SCD and post-ASA outcome

There is uncertainty as to precisely identify HCM patients at-greatest risk of SCD.[36] In some studies, a single risk factor for SCD was sufficient to justify prophylactic ICD,[26] whereas in other studies well known risk factors were not predictive of ICD therapy.[27] In our study the number of risk factors for SCD in ASA patients was very low and was

not different between patients with and without the primary endpoint. Moreover, the 9 patients who died due to cardiac cause without ICD had no major or minor risk factors for SCD except for 1 patient who had a family history of SCD and had recurrent episodes of non-sustained VT. A logical inference of our risk factor data is that ASA itself is a risk factor for SCD independent of the amount of ethanol, age and co-morbidities.

Of note, appropriate ICD discharges are not equivalent to SCD. In addition, the small sample size, might not rule out completely the possibility that large dosages of alcohol would result in a larger myocardial infarction and thus more of a propensity to develop ventricular arrhythmias.

CONCLUSIONS

The therapeutic choice for ASA is limited by the lack of long-term safety data as compared to myectomy. Since a randomized study between ASA and myectomy is unlikely to be performed we can only rely on registries in single centers. Our data show that ASA is effective but has potentially adverse long-term effects. This poses special precaution given the fact that ASA is practiced worldwide at increasing rates. We recommend myectomy as the preferred treatment of choice in patients with symptomatic obstructive HCM.

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Chapter 13

Effects of successful alcohol septal ablation on microvascular function in patients with obstructive hypertrophic cardiomyopathy

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ABSTRACT

We hypothesized that relief of obstruction in patients with hypertrophic cardiomyopathy (HC) by percutaneous transluminal septal myocardial ablation (PTSMA) improves micro-vascular dysfunction by relief of extravascular compression. Microvascular dysfunction in obstructive HC is related to extravascular compression by increased left ventricular (LV)mass and LV end-diastolic pressure. The study included 14 patients with obstructive HC (mean age 55 ± 12 years, 11 men) who underwent successful PTSMA and 14 healthy volunteers (mean age 31 ± 4 years, 11 men). LV hemodynamics (by Doppler echocardiography) and intramyocardial flow dynamics (by adenosine myocardial contrast echocardiography) were evaluated in healthy volunteers and before and 6 months after PTSMA in patients with HC. LV end-diastolic pressure was estimated from the ratio of transmitral early LV filling velocity to early diastolic mitral annular velocity. PTSMA reduced the invasively measured LV outflow tract gradient (119 ± 35 vs 17 ± 16 mm Hg, $p < 0.0001$) and LV end-diastolic pressure (23 ± 3 vs 16 ± 2 mm Hg, $p < 0.001$). Six months after PTSMA, myocardial flow reserve improved (2.73 ± 0.56 vs 3.21 ± 0.49, $p < 0.001$), but did not normalize compared with healthy controls (vs 3.95 ± 0.77, $p < 0.001$). Also, septal hyperemic endo-to-epi myocardial blood flow ratio improved (0.70 ± 0.11 vs 0.92 ± 0.07, $p < 0.001$). Changes in LV end-diastolic pressure, LV mass index, and LV outflow tract peak systolic gradient correlated well with changes in hyperemic perfusion (all $p < 0.05$). In conclusion, microvascular dysfunction improves after PTSMA due to relief of extravascular compression forces.

INTRODUCTION

The mechanism by which percutaneous transluminal septal myocardial ablation (PTSMA) improves symptoms in patients with obstructive hypertrophic cardiomyopathy (HC) is not well elucidated. Myocardial contrast echocardiography (MCE) has emerged as a promising novel bedside technique that allows quantification of myocardial perfusion at the microvascular level1 and has been validated against several nuclear stress testing techniques.[1–3] We hypothesized that reduction of left ventricular (LV) outflow tract peak systolic gradient after PTSMA would decrease LVmass and wall stress, thereby improving myocardial micro-vascular dysfunction.

METHODS

The study comprised 14 consecutive patients (mean age 55 ± 12 years, 11 men) with obstructive HC and normal epicardial coronary arteries who underwent successful PTSMA and 14 healthy volunteers (mean age 31 ± 4 years, 11 men) who served as a control group. All patients with HC were in New York Heart Association functional class III with or without angina pectoris and had a LV outflow tract peak systolic gradient ≥ 50 mm Hg at rest or provocation. The study protocol consisted of clinical evaluation, standard 2-dimensional echocardiography, and adenosine MCE at baseline and 6 months after PTSMA. All subjects gave informed consent and the hospital review board approved the study protocol.

The PTSMA procedure was performed as previously described.[4] In brief, using standard Judkins technique, a 6F pacemaker lead was placed in the right ventricle, a 6F pigtail catheter was positioned into the LV, and a 7F Judkins guiding catheter in the ascending aorta. LV outflow tract peak systolic gradient was continuously monitored through-out the whole procedure. After initial coronary angiography for localizing the origin of the septal perforating arteries, a 1.5 to 2.5 × 10 mm balloon catheter was introduced over a 0.014-inch guide wire into the target perforator artery and inflated. Contrast agent (SonoVue, Bracco, Geneva, Switzerland) was then injected through the balloon catheter shaft during simultaneous registration of transthoracic 2-dimensional echocardiography to determine the part of the myocardium supplied by the targeted septal artery. If no leakage of contrast occurred into the LV cavity, ethanol was slowly(1 ml/min) injected to a maximum of 5 ml (mean 2.5 ml); 5 minutes after ethanol injection, the balloon was deflated and coronary angiography was repeated. A successful procedure was defined as the reduction in LV outflow tract peak systolic gradient of ≥ 50% of baseline. If the results were not satisfactory, the whole procedure was repeated in another septal branch. LV end-diastolic pressure, LV end-systolic pressure, and LV outflow tract peak systolic gradient were determined at baseline and immediately after PTSMA.

Echocardiography

In accordance to guidelines,[5] all patients underwent a standard 2-dimensional echocardiographic examination with tissue Doppler imaging in spectral mode using a Sonos 7500 ultrasound system (Philips, Best, The Netherlands), including continuous- and pulsed-wave Doppler recording of LV outflow tract peak systolic gradient (at rest and after Valsalva maneuver), mitral inflow, and other Doppler parameters.[6] Mitral regurgitation was graded on a scale from 0 (no regurgitation) to 4 (severe regurgitation). M-mode measurements of septal and posterior wall thickness, and LV dimensions were done in accordance to guidelines.[7] LV end-diastolic and end-systolic volumes and LV ejection fraction (by the modified bi-plane Simpson rule) were calculated from the apical 4-chamber and 2-chamber views. LV mass index was assessed with the 2-dimensional area-length method.[7,8] LV end-diastolic pressure was estimated as E/E'× 1.25+1.9, where E/E' is the dimensionless ratio of transmitral early LV filling velocity to early diastolic tissue velocity of the lateral mitral annulus.[9]

Real-time MCE

All image acquisitions were performed by a single expert sonographer (WBV) as previously described,[10] in accordance to guidelines.[11] Calcium antagonists and ß-blockers were discontinued 48 hours before stress test. MCE was performed using a continuous infusion of contrast agent at a mean rate of 1.0 ml/min. A parallel infusion of 0.9% saline at a rate of 250 ml/h was performed via the same infusion set. The infusion rate was adjusted to obtain maximal opacification of the LV myocardium and to minimize basal attenuation because of intracavitary contrast. Image acquisition was started before contrast injection and during 4 minutes of continuous infusion of contrast agent to allow for steady blood concentration of the micro-bubbles. Images were acquired with the Sonos 7500 ultrasound system equipped with a broadband 1.8 to 4 MHz transducer. Machine settings were adjusted before contrast imaging for optimal gain and color settings and kept constant throughout the study. Contrast image acquisitions were recorded with a low mechanical index (0.1). These settings were interrupted with transient bursts (flash imaging) of high mechanical index (1.8) manually triggered at peak contrast intensity to allow for microbubbles destruction within the LV myocardium. After these flashes were applied, images were recorded for 15 cardiac cycles representing contrast replenishment within the LV myocardium. Image acquisition was done during quiet respiration to minimize cardiac motion and images from the same moment in the respiratory cycle were considered for analysis. All data were digitally stored and sent to a separate workstation for off-line analysis. Stress images were recorded after 8 minutes of adenosine infusion at a rate of 140µg/kg/min. During the stress study, heart rate and blood pressure were recorded every 3 minutes.

Images were analyzed using QLAB (Philips, Best, The Netherlands) quantification software. End-systolic images were selected and fixed-size noninfarcted regions of interest were manually drawn according to segments defined by standard 16-segment model.[12] In addition, regions of interest were placed in the adjacent LV cavity to compensate for intracavitary contrast intensity. Then, contrast time-intensity curves were calculated for each available LV segment as previously described.[1] Logarithmic signal compression was removed, and linearized signal intensity data were expressed in arbitrary units. Absolute myocardial blood flow (MBF) was calculated using the model described by Vogel et al.[2] In brief, absolute MBF is calculated from the formula MBF= rBV×ß/ρÔ = (A/ALV)×ß/ρÔ , where rBV is the relative blood volume pool that can be calculated by dividing the plateau intensity of myocardial contrast (A) by the maximal contrast intensity of the adjacent LV regions of interest (ALV). ß is the exchange frequency or velocity of this blood volume calculated from refill curves after microbubbles flash destruction. ρÔ is the myocardial density in grams and equal to 1.05 g. Myocardial intensity data were corrected for noncontrast signals arising from the tissue by subtracting the signal intensity of the first frame after microbubbles destruction. ß (s-1) was derived from the frames after microbubbles destruction and then was transformed into min-1. Signal averaging of all but the frames during microbubbles destruction including the first one after microbubbles destruction yielded the signal intensity of the LV ALV.

As previously described,[10] myocardial perfusion parameters were calculated by averaging data from available non-infarcted myocardial segments. MBF was calculated in ml.min-1.g-1 from relative blood volume (ml.ml-1) and (s-1), which is converted to minutes. Because at rest MBF is related to the rate-pressure product, corrected at rest MBF was calculated as MBF × rate-pressure product-1 × 10K. MBF reserve was calculated by dividing hyperemic and at rest values. Values of LV rate-pressure product— (LV outflow tract peak systolic gradient + systolic blood pressure) × heart rate— were used for correction.

For evaluation of transmural MBF, 2 regions of interest were manually drawn in the midseptal and midlateral territories, 1 over the subendocardium, 1 over the subepicardium, and a third region of interest in the LV cavity as stated before. MBF values were calculated both at rest and during hyperemia. The subendocardial-to-subepicardial (endo-to-epi) MBF ratio at rest and during hyperemia was calculated.

Statistical analysis

Continuous variables were expressed as mean ± SD and categorical variables were expressed as number (percentage). The chi-square and Fischer exact tests were applied to compare categorical variables. Paired and unpaired t tests were used for the evaluation of effects of PTSMA. Correlations between changes in different parameters induced by PTSMA were assessed using linear regression analysis. Statistical significance was set at $p < 0.05$. All statistics were performed using SPSS version 12.0.2 for Windows (SPSS

Inc., Chicago, Illinois). Size of regions of interest and method of analysis were fixed for accurate comparison between baseline and follow-up MCE data and between observers. To determine the variability in MCE measurements, a second observer performed all MCE measurements before and after PTSMA. For assessment of intraobserver variability, the first investigator reanalyzed the whole study population after 1 month from the first analysis. The variability, expressed as percentage, was calculated for each patient as the absolute difference between 2 measurements divided by the mean of the 2 measurements. Each second analysis was done blinded to other results.

RESULTS

Baseline and 6 months post-PTSMA characteristics of the14 patients with HC are summarized in Table 1. New York Heart Association functional class decreased from 3.0 ± 0.3 to 1.2 ± 0.4 (p<0.0001), and none of the patients had angina pectoris at 6-month follow-up after PTSMA. After-PTSMA medications such as ß-blockers and calcium antagonists were withheld in most patients.

Table 1 Clinical, hemodynamic, and echocardiographic characteristics of the 14 patients with obstructive hypertrophic cardiomyopathy (HC) before and after percutaneous transluminal septal myocardial ablation (PTSMA)

Variable	Pre-PTSMA (n 14)	Post-PTSMA (6 months) (n 14)	p Value
Age (yrs)	55 ± 12		
Men	11 (79%)		
ß-blockers	11 (79%)	1 (7%)	< 0.0001
Calcium antagonists	7 (50%)	1 (7%)	0.04
NYHA functional class	3.0±0.3	1.2±0.4	< 0.0001
Angina pectoris	10 (71%)	0	< 0.0001
LV end-diastolic pressure (mm Hg)	23±3	16±2	< 0.0001
LVOT gradient (mm Hg)	82±16	10±7	<0.001
Heart rate (beats/min)	60±8	60±7	NS
Systolic blood pressure (mmHg)	129±9	132±11	NS
IVS (mm)	23±4	15±3	< 0.001
LVPW (mm)	14±2	11±1	<0.01
LV mass index (g/m^2)	196±45	136±22	<0.001
LVED (mm)	39±3	44±4	<0.001
MR	2.0±0.4	0.5±0.5	<0.0001
SAM	1.0±0.0	0.4±0.5	<0.0001
LV ejection fraction (%)	66±5	60±8	0.03
Mitral E-wave velocity (cm/s)	85±20	75±19	NS
Lateral mitral annular E'-wave (cm/s)	5.0±0.7	6.9±0.9	<0.001
Lateral mitral annular E/E'ratio	18±2	11±2	<0.001

p versus baseline

Immediately after PTSMA, the invasive LV outflow tractpeak systolic gradient decreased from 119±35 to 17±16 mm Hg (p<0.0001). After 6-months follow-up, structural reverse LV remodeling and improvement in diastolic function was evidenced by reduction in LV mass index and improvement in E/E' ratio, respectively. Furthermore, Doppler-estimated and catheter-based measurements of LV end-diastolic pressure (r2=0.78, p<0.001), and LV outflow tract peak systolic gradient (r2=0.83, p<0.001) correlated well.

About 80% of the myocardial segments were available for analysis of myocardial perfusion parameters by MCE. About 70% of the nonanalyzable segments were basal anterolateral. For calculation of the post-PTSMA myocardial perfusion parameters, the infarcted territories (anteroseptal basal segments) were excluded from analysis. The interobserver variability was 10%, 13%, and 18% for MBF, relative blood volume, and ß, respectively. The intraobserver variability was 8%, 8%, and 14% for MBF, relative blood volume, and ß, respectively.

At rest global MBF corrected for LV rate-pressure product improved (0.92 ± 0.13 vs 0.99 ± 0.13 ml.min-1.g-1 p<0.01; Table 2) after PTSMA but did not normalize (1.13± 0.25 ml.min-1· .g-1, p<0.001) compared with healthy controls. Likewise, the hyperemic MBF showed improvement (2.51 ± 0.50 vs 3.18 ± 0.49 ml.min-1.g-1, p<0.001) resulting in improved MBF reserve (2.73 ± 0.56 vs 3.21 ± 0.49 ml.min-1.g-1, p<0.001) after PTSMA but did not reach normal values for hyperemic MBF (4.34 ± 0.78 ml.min-1.g-1, p<0.001) and for MBF reserve (3.95 ± 0.77 ml.min-1.g-1, p <0.001) compared with healthy controls (Figures 1 and 2).

At rest mean relative blood volume improved (0.093 ± 0.018 vs 0.113 ± 0.021 ml.ml-1, p<0.001) after PTSMA but did not normalize (0.138±0.014 ml. ml-1, p<0.001) compared with healthy controls. Likewise, during hyperemia relative blood volume improved (0.107 ±0.024 vs 0.153 ± 0.033 ml.ml-1, p<0.001) after PTSMA but did not normalize (0.188±0.024 ml.min-1, p<0.001) compared with healthy controls. Mean ß decreased at rest (9.7 ± 2.9 vs 8.8 ± 2.7 ml.ml-1, p<0.01) and during hyperemia (24.7 ±6.6 vs 21.5 ±5.7 ml.ml-1, p<0.01) after PTSMA. All myocardial perfusion data are summarized in Table 2.

After PTSMA mean endo-to-epi MBF ratio in the mid-septal region improved during rest (0.84 ±0.12 vs 0.96 ±0.11, p<0.001) and during hyperemia (0.70 ± 0.11 vs 0.92 ±0.07, p<0.001). Furthermore, hyperemic mean endo-to-epi MBF ratio in the lateral wall improved (0.74 ±0.23 to 0.89 ±0.14, p<0.01) after PTSMA (Table 2 and Figure 2).

Linear regression analysis revealed good correlations between percentage change (Δ) LV end-diastolic pressure and Δ hyperemic MBF (r=0.66, p< 0.01), Δ MBF reserve (r=0.71, p<0.01), Δ hyperemic relative blood volume (r = 0.66, p<0.01), and Δ hyperemic endo-to-epi ratio (r = 0.78, p<0.001). Comparable correlations were found between Δ LV mass index and these perfusion variables. In addition, there was an increase in hyperemic endo-to-epi ratio and MBF reserve in almost all patients.

Table 2 Myocardial perfusion data of the 14 patients with obstructive hypertrophic cardiomyopathy before and after percutaneous trasnluminal septal myocardial ablation

Variable	Pre-PTSMA (n = 14)	Post-PTSMA (6 months) (n = 14)	p Value†	Healthy Controls (n = 14)	p Value‡
Resting					
MBF ($ml.min^{-1}.g^{-1}$)*	0.92±0.13	0.99±0.13	0.01	1.13±0.25	<0.001
Relative BV ($ml.\ ml^{-1}$)	0.093±0.018	0.113±0.021	0.001	0.138±0.014	<0.001
BV exchange velocity ($ml.min^{-1}$)	9.7±2.9	8.8±2.7	0.01	7.7±4.2	NS
Endo-to-epi ratio MBF (septal)	0.84±0.12	0.96±0.11	0.001	1.02±0.05	<0.01
Endo-to-epi ratio MBF(lateral)	0.91±0.18	0.94±0.14	0.389	1.05±0.10	<0.01
Hyperemic					
MBF ($ml.min^{-1}.g^{-1}$)	2.51±0.50	3.18±0.49	0.001	4.34±0.78	<0.001
Relative BV ($ml.\ ml^{-1}$)	0.107±0.024	0.153±0.033	0.001	0.188±0.024	<0.001
BV exchange velocity ($ml.\ ml^{-1}$)	24.7±6.6	21.5±5.7	0.01	23.1±6.2	NS
Endo-to-epi ratio MBF (septal)	0.70±0.11	0.92±0.07	0.001	0.99±0.10	NS
Endo-to-epi ratio MBF (lateral)	0.74±0.23	0.89±0.14	0.01	1.07±0.11	NS
Reserve					
MBF ($ml.min^{-1}.g^{-1}$)	2.73±0.56 1	3.21±0.49	0.001	3.95±0.77	<0.001
Relative myocardial BV ($ml.\ ml^{-1}$)	1.15±0.12	1.35±0.17	0.01	1.36±0.20	NS
BV exchange velocity ($ml.\ ml^{-1}$)	2.55±1.36	2.44±1.45	0.231	2.84±1.82	<0.001

*MBF is corrected for rate-pressure-product at rest
† p versus baseline
‡ p versus post-PTSMA
(MBF=myocardial blood flow, BV=blood volume

DISCUSSION

The main finding of our study is that patients with obstructive HC and normal epicardial coronary arteries have impaired vasodilator reserve, in particular in the subendocardium, as evidenced by adenosine stress MCE. After successful PTSMA, a decrease in LV outflow tract peak systolic gradient, LV mass index, and LV end-diastolic pressure and an improvement in vasodilator reserve in non-infarcted myocardium, including the endo-to-epi ratio, were seen. The degree of reduction in LV outflow tract peak systolic gradient, LV mass index, and LV end-diastolic pressure was correlated to the degree of improvement in vasodilator reserve.

It is well known that myocardial perfusion and vasodilator reserve is blunted in patients with obstructive HC.[13–17] However, the effects of PTSMA on myocardial perfusion in

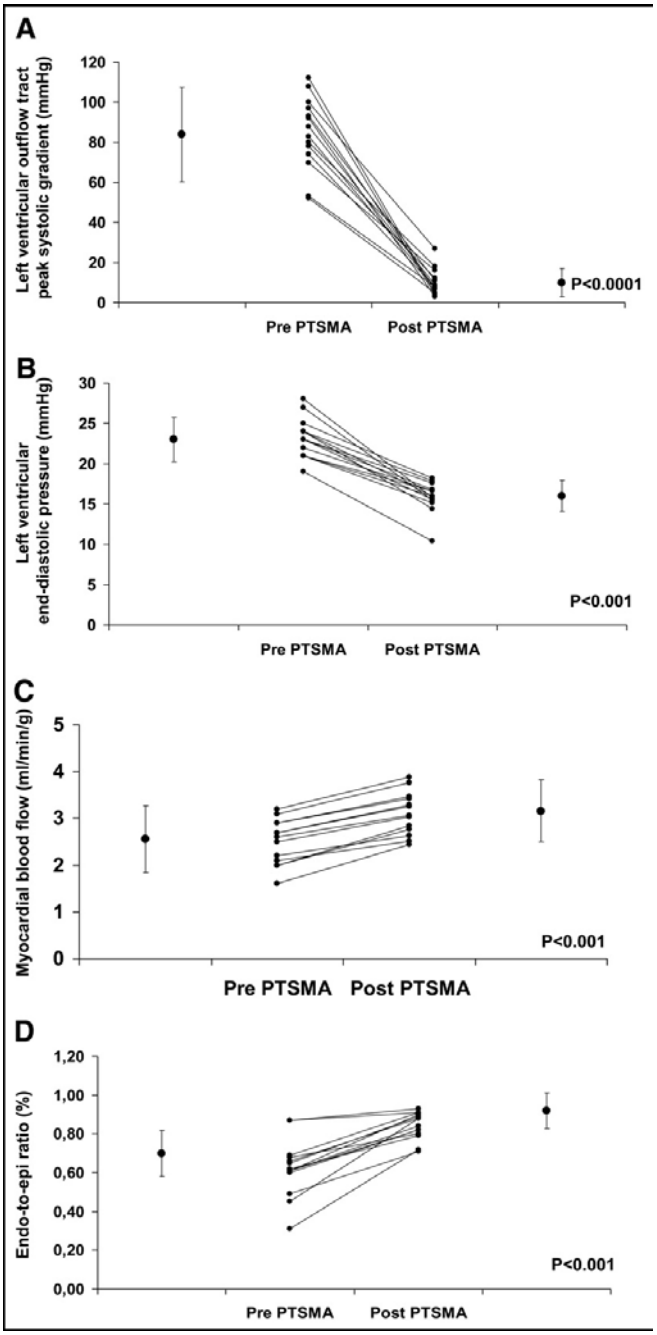

Figure 1:
Changes after ASA in the 14 patients with HCM: *(A)*LV outflow tract pressure gradient, *(B)* LV end-diastolic pressure, *(C)* myocardial flow reserve, and *(D)* hyperemic endo-to-epi ratio

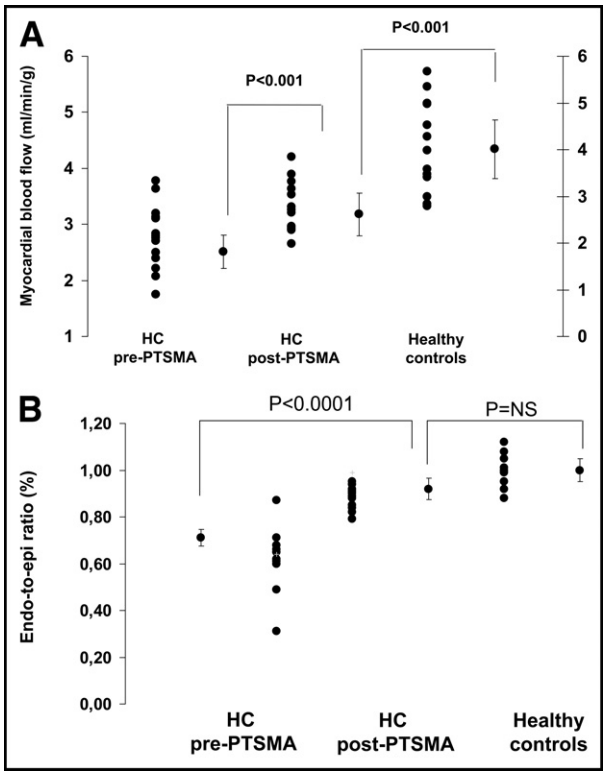

Figure 2:
Baseline and hyperemic changes in MBF *(A)* and septal endo-to-epi ratio *(B)* in patients with obstructive HCM after ASA compared with values from healthy controls.

patients with HC are not well known. In a report of a single patient with nonobstructive HC, redistribution of MBF during pacing was shown by positron emission tomography.[18] In an earlier report we showed that, in patients with obstructive HC, perfusion abnormalities could be reversed after PTSMA, evidenced by improved replenishment velocities derived from the myocardial contrast intensity curves.[19, 20] Quantitative data about myocardial perfusion after PTSMA have not been published. MCE has several advantages compared with competing imaging techniques, such as superior spatial resolution, is free of radiation, and is not limited by contraindications such as claustrophobia and pacing devices. It has the unique property of accurate quantification of the intravascular relative blood volume as a surrogate of capillary density and myocardial blood flow in milliliter per gram per minute.[2]

In patients with HC, both intravascular and extravascular compression forces are possible mechanisms for myocardial perfusion abnormalities.[10] Intravascular (or autoregularity) mechanisms include reduced myocardial capillary density and vascular remodeling resulting from perivascular and myocardial deposition of collagen,[13,21] seen

in histological specimens from patients with HC.[22,23] In addition, patients with HC have stiffened myocardium with impaired LV relaxation and elevated LV filling pressures that may limit diastolic coronary perfusion due to increased extravascular compression.[10,24] The relation between extravascular compression and coronary perfusion is best depicted from the inverse relation between elevated LV end-diastolic pressure and coronary perfusion. Elevated LV end-diastolic pressure produces linear decrements in coronary perfusion by reducing coronary perfusion pressure, which is a result of diastolic systemic pressure and LV end-diastolic pressure. The extravascular compression forces seem to be potentially modifiable factors that can be reversed by interventions such as PTSMA. In some studies early and sustained improvement of LV diastolic function after PTSMA was demonstrated.[25,26] Therefore, it seems possible for PTSMA to improve myocardial flow dynamics. Despite the relatively small number of patients in our study, we showed that relief of LV outflow tract obstruction and improvement in diastolic relaxation with drop in LV end-diastolic pressure were correlated with improvement in hyperemic myocardial perfusion, in particular in the subendocardium. Notwithstanding the relief of extravascular compressing forces, the myocardial perfusion does not normalize after PTSMA. This can be explained by the residual vascular remolding and fibrosis that contributes in limiting myocardial perfusion in patients with HC.

Patients with HC suffer disabling symptoms, such as dyspnea and chest pain. These symptoms have been related to a stiffened myocardium with diastolic dysfunction[27] and perfusion abnormalities.[28] PTSMA has been shown to improve symptoms in selected candidates with severe obstructive HC and refractory symptoms.[29] Such improvement in symptoms may be related to improved diastolic function [25,26,30] and myocardial perfusion. In the present study, for the first time, we have shown that symptomatic improvement may be, at least in part, related to improved myocardial perfusion after PTSMA. Moreover, the correlation between the degree of improvement in vasodilator reserve and the degree of reduction in extravascular compression by increments of LV outflow tract peak systolic gradient, LV mass index, and LV end-diastolic pressure emphasizes our previous finding that suggests a possible causative relation of extravascular compression to myocardial perfusion.

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Part IV

Summary and Conclusions

Chapter 14

Summary and Conclusions

SUMMARY

Ever since the first description of hypertrophic cardiomyopathy (HCM) as a clinical entity in 1958, the disease has intrigued both clinicians and researchers mainly because of the heterogeneity in clinical, morphologic and genetic aspects of the disease.

HCM patients vary in presentation from being lifelong asymptomatic to sudden cardiac death (SCD) at young age, development of heart failure, supraventricular and ventricular arrhythmias and cerebrovascular events. Left ventricular hypertrophy (LVH) can be mild to extreme (> 30 mm) and in most cases is asymmetrical with the septum most heavily affected. However, LVH can also be symmetrical or apical. Up to 70% of patients with HCM have obstruction in the left ventricular outflow tract (LVOT) at rest or during provocation.

Pathophysiology

One of the great challenges in HCM is to predict which patients are more prone to deterioration. The decrease in coronary flow reserve (CFR) is an important phenomenon in HCM, since it predisposes patients to myocardial ischemia and moreover decrements in CFR are a strong predictor of clinical deterioration and death. In **chapter 2** we studied CFR in the left anterior descending artery in 10 HCM patients and compared them with 8 heart transplantation patients to investigate to which extent hemodynamic, echocardiographic and histological parameters contribute to the reduction of CFR. The CFR reduction in HCM patients was related with increased septal thickness, increased indexed LV mass, increased LVOT gradient and the degree of arteriolar dysplasia with thickened walls and narrowed lumen, causing a decrease in % luminal area of the arterioles. A reduction in capillary density was present in HCM patients, but this was not significantly related with the reduction of CFR.

Diastolic dysfunction is present in almost all HCM patients and can be difficult to manage. There have been reports describing diastolic dysfunction detected with tissue Doppler imaging (TDI) in HCM mutation carriers without LVH and this could possibly be used for screening of family members in HCM. The latter would be especially interesting in those HCM families in which presymptomatic DNA testing is not possible because of the lack of identification of an underlying HCM mutation. In **chapter 3** we investigated diastolic function by TDI in carriers of Dutch founder mutations in *cardiac myosin protein C (MyBPC3)*, with and without LVH and compared them to healthy controls. We did find an abnormally (>2SD in normal population) increased late mitral annular diastolic velocity in half of the population of mutation carriers with normal basal ECG and echocardiograms, supporting the theory that diastolic dysfunction is a primary component of preclinical HCM. However, carriers could not be identified from healthy controls based on TDI velocities.

Diastolic dysfunction in HCM is also the subject of **chapter 4**, in which we used speckle tracking to investigate LV untwisting in 75 HCM patients and 75 control subjects. In early diastole, before mitral valve opening, LV untwisting contributes to LV diastolic suction. LV untwisting rate depends on passive properties and the amount of potential energy stored by systolic LV twist that can be converted into kinetic energy used for LV untwisting, and active properties such as the contribution of still depolarized subendocardial fibres in early diastole. In HCM patients with a reverse septal curvature, less systolic twist, impaired compliance, and subendocardial ischemia may all contribute to a reduced untwisting rate. In HCM patients with a sigmoidal septal curvature, LV apical rotation and twist are increased and the higher amount of energy stored seems to fully compensate for the loss of active LV untwisting and impaired compliance.

In **chapter 5** we investigated if protein composition and function of the sarcomere are altered in a homogenous HCM patient group with frameshift mutations (c.2373dupG and c.2864_2865delCT) in *myosin binding protein C* (*MYBPC3).* Comparisons were made between cardiac samples from 4 HCM patients carrying one of 2 mutations, collected during myectomy and 13 cardiac samples from non-failing donors. In the HCM patients protein expression of MYBPC3 was significantly reduced by 33%, indicating that the pathomechanism involves haploinsufficiency, rather than a poison peptide. The maximum force generating capacity of cardiomyocyte was significantly reduced by 42%. This data support the concept that contractile dysfunction is a pivotal link between the mutant sarcomeric protein and HCM.

Genetics

Since the identification of the first HCM gene in 1990 hundreds of mutations scattered among at least 25 putative HCM susceptibility genes encoding various sarcomere, Z-disk, calcium-handling, and mitochondrial proteins are known to cause HCM and are found in up to 60% of cases. The identification of the genetic defect in one of the HCM genes allows accurate pre-symptomatic detection of mutation carriers in a family.

In **chapter 6** we describe the background and our first experience with genetic testing in the HCM population of the Erasmus MC and genetic and cardiologic screening of family members at the cardiogenetic outpatient clinic. A flowchart for clinical and genetic diagnosis and family screening is included. This chapter discusses the importance of identifying autosomal dominantly inherited genetic defects in HCM, thus allowing accurate identification of at-risk relatives. It focuses particular on the phenotypic variability including non-penetrance and late onset demanding repeated cardiac screening.

In **chapter 7** we show that cardiac evaluation with electrocardiogram and echocardiography leads to the diagnosis of HCM in 41% of referred asymptomatic mutation carriers. Disease penetrance is age dependent, warranting repeated cardiologic evaluation. *MYBPC3* carriers were affected at higher age than *MYH7* mutation carriers. Classical risk

factors for sudden cardiac death (SCD) were present in asymptomatic mutation carriers with and without HCM. Risk stratification for SCD is important in HCM in order to select patients for prophylactic implantable cardioverter defibrillator (ICD) implantation. Unfortunately there are currently no data available about the survival rate of unaffected carriers of pathogenic mutations or the value of risk stratification for SCD in this specific population.

In the Netherlands, approximately one third of all HCM cases are caused by the truncating c.2373_2374insG, c.2864_2865delCT and c.2827C>T (p. Arg943X) mutations in the *MYBPC3*. In chapter 5 we demonstrated that the c.2373_2374insG and c.2864_2865delCT mutations lead to haploinsufficiency. Although not functionally investigated, the c.2827C>T mutation which leads to a stop codon at residue 943, is also expected to cause haploinsufficiency. In **chapter 8** we show that these truncating *MYBPC3* mutations also have a similar natural history, with a less favorable disease outcome than previously suggested in *MYBPC3* related HCM.

HCM patients carrying Dutch founder mutations present a genetic homogeneous group with respect to the primary pathogenic HCM mutation and are used to study effects of genetic modifiers in disease expression of HCM. In view of its regulatory role in cardiac function, blood pressure, and electrolyte homeostasis, genetic variation in genes encoding components of the renin-angiotensin-aldosterone system (RAAS) is a plausible candidate for modifying phenotypic expression. The study described in **chapter 9**, however fails to show an important modifying effects of RAAS polymorphisms in a large set of carriers of Dutch founder mutations in *MYBPC3*.

Disease variability in HCM is expected to be partially explained by the effect of additional mutations in sarcomere genes. In **chapter 10** we describe two groups of Dutch founder mutation carriers; one with mild and one with severe phenotype. In these 87 HCM patients nine sarcomere HCM genes (*MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TNNC1*, *ACTC1* and *TPM1*) were sequenced. There were no modifying effects of secondary sarcomere mutations on phenotypic expression in these two groups.

Invasive treatment

Up to 70% of patients with HCM have obstruction in the LVOT at rest or during provocation. This obstruction is induced by thickening of the interventricular septum (IVS) and systolic anterior movement (SAM) of the mitral valve. Severely symptomatic HCM patients with obstruction despite optimal medical therapy are candidates for invasive treatment, either surgically by myectomy or percutaneously by alcohol septal ablation (ASA). HCM is often associated with a variety of intrinsic abnormalities of the mitral valve, including increased enlargement of the anterior mitral leaflet, which may predispose to residual SAM and result in suboptimal outcome after isolated myectomy.

In **chapter 11** we describe the long-term follow-up of 69 HCM patients with enlargement of the anterior mitral leaflet treated with myectomy combined with anterior mitral leaflet extension and show that it leads to long-term symptom relief and survival similar to general population.

In **chapter 12** we describe the long-term results of ASA in 91 HCM patients. During ASA ethanol is injected into one or more septal perforator branches of the left anterior descending coronary artery inducing a myocardial infarction. We show that ASA patients have an increased risk for cardiac death and aborted sudden cardiac death, including appropriate ICD discharges compared with myectomy patients during long-term follow-up.

In **chapter 13** we studied LV hemodynamics by Doppler echocardiography and intramyocardial flow dynamics by adenosine myocardial contrast echocardiography in 14 obstructive HCM patients before and 6 months after ASA compared with healthy volunteers. Six months after ASA myocardial flow reserve improved, but did not normalize compared with healthy controls. We concluded that microvascular dysfunction improves after successful ASA due to relief of extravascular compression forces.

CONCLUSIONS

Microvasular dysfunction plays an important role in HCM since it predisposes patients to myocardial ischemia and is a predictor of clinical deterioration and death. In **chapter 2** we show that CFR reduction in HCM patients is related with increased septal thickness, increased indexed LV mass, increased LVOT gradient and arteriolar dysplasia.

Diastolic dysfunction is present in almost all HCM patients and can lead to symptoms that are difficult to manage. In **chapter 3** we show that diastolic abnormalities are found with TDI in HCM mutation carriers without LVH, however not sufficiently sensitive for determination of the affected status of the individual. The pathofysiology of diastolic dysfunction in HCM is still incompletely understood. In **chapter 4** we investigated LV untwisting parameters in HCM using speckle tracking echocardiography. LV untwisting is delayed in HCM, reflecting ineffective diastolic uncoiling of the hypertrophic myocardium. The increased LV twist found in HCM patients with a sigmoidal septal curvature and the higher amount of energy stored seems to fully compensate for the loss of active LV untwisting and impaired compliance.

The identification of autosomal dominantly inherited genetic defects in HCM, allows accurate identification of at-risk relatives **(chapter 6)**. In asymptomatic mutation carriers the diagnosis of HCM is made in 41% at first cardiologic evaluation. Unfortunately genotype-phenotype relations in HCM are weak, so repeated cardiac screening is necessary. By identifying asymptomatic mildly or non-affected mutation carriers we are

encountering clinical difficulties in management of these subjects. They have not been included in older studies regarding natural history or risk of SCD in HCM on which expert opinions are based for the implantation of ICD or life-style advice **(chapter 7)**.

In the Netherlands, approximately one third of all HCM cases are caused by the truncating c.2373_2374insG, c.2864_2865delCT and c.2827C>T (p. Arg943X) mutations in the *MYBPC3*. These mutations cause haploinsufficiency and have a similar natural history, with more severe disease outcome than previously suggested **(chapter 5, 8)**. HCM patients carrying Dutch founder mutations present a genetic homogeneous group with respect to the primary pathogenic HCM mutation and are used to study effects of genetic modifiers in disease expression of HCM. The HCM phenotype in these patients is not influenced by RAAS polymorphisms or the presence of secondary sarcomere mutations on phenotypic expression in these patients **(chapter 9 and 10)**.

Symptomatic HCM patients with LVOT obstruction despite optimal medical therapy are candidates for invasive treatment. In obstructive HCM patients with enlargement of the anterior mitral leaflet the surgical treatment with anterior mitral leaflet extension leads to long-term symptom relief and survival similar to the general population **(chapter 11)**. Obstructive HCM patients treated with ASA have an increased risk for cardiac death and aborted SCD, including appropriate ICD discharges during long-term follow-up. This is probably caused by the myocardial scar induced by the injected ethanol **(chapter 12)**. ASA should be reserved for those patients with a high surgical risk and possibly be combined with ICD implantation. In **chapter 13** we show that microvascular dysfunction is improved 6 months after successful ASA, due to relief of extravascular compression forces.

Although HCM is a relatively benign disease in most cases, decisions on treatment like ICD-implantation or invasive treatment for LVOT obstruction should ideally be made by a dedicated team in a clinic where all therapeutic options are available.

FUTURE PERSPECTIVES

Genotype-phenotype relations in HCM are still weak and incompletely understood. Future studies are necessary to unravel the mystery how a certain mutation leads to HCM. The functional effects on sarcomere function at the cellular level should be extended further to possibly explain a number of clinical presentations based on an pathophyphysiology.

Modifier genes are likely to play an important role in disease expression. The situation in the Netherlands; with the presence of founder mutations and nation-wide cardio genetic outpatient clinics creates an ideal climate for research.

HCM patients are treated based on their phenotype. Imaging plays a crucial role in determine the phenotype. The value of new echocardiography techniques, like speckle

tracking, in detecting early disease manifestation have to be determined. Cardiac magnetic resonance imaging (CMR) can detect delayed enhancement, which correlates with the histological finding of fibrosis and thus represents a likely substrate for ventricular tachyarrhythmias. Before the implementation of CMR in the current risk stratification for SCD long-term CMR-based studies in large patient populations are necessary. The disease model of HCM seems ideal to study the effects of perfusion and function using PET-CT or PET-MRI machines in the future.

Although current techniques can identify DNA mutations in more than half of the HCM patients, there are still HCM patients and families in which no mutation can be identified. This group of so-called mutation-negative HCM needs further investigation as it can give insight in pathophysiologic aspects of the disease. New generation sequencing, as whole exome sequencing and whole genome sequencing 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 HCM, adding the challenge of determine whether these variants are pathogenic or represent unclassified variants.

Chapter 15

Samenvatting en conclusies

SAMENVATTING

In 1958 werd hypertrofische cardiomyopathie (HCM) voor het eerst beschreven. Bij HCM is er sprake van een verdikking van de hartspier, in afwezigheid van aandoeningen die deze verdikking kunnen verklaren (zoals hoge bloeddruk of aortaklepstenose). Het komt voor bij 1 op de 500 volwassenen en is in de meeste gevallen erfelijk met een autosomaal dominant overervingspatroon.

HCM patiënten kunnen levenslang asymptomatisch zijn, maar aan de andere kant is het ook de belangrijkste oorzaak van plots overlijden van jonge sporters. HCM kan leiden tot de ontwikkeling van hartfalen, ritmestoornissen en herseninfarcten.

De verdikking van de hartspier kan variëren van mild tot extreem (> 30 mm; normale dikte < 13 mm). Meestal is de verdikking asymmetrisch, waarbij het septum het meest verdikt is. De verdikking kan echter ook concentrisch zijn of juist de apex het meeste aantasten. Tot 70% van de HCM patiënten hebben in rust of bij inspanning een obstructie in de linker ventrikel uitstroombaan (LVOT), waardoor het hart hinder ondervindt bij het uitpompen van het bloed naar de aorta.

Uit eerder onderzoek was reeds bekend dat HCM in de meeste gevallen een familieziekte is. In 1990 werd dit bevestigd door het vinden van het eerste HCM-gen. Inmiddels zijn er honderden DNA mutaties gevonden verspreid over ten minste 25 genen, die HCM kunnen veroorzaken. Deze genen coderen voor eiwitten, die deel uitmaken van de hartspiercel. Mutaties in sarcomeer eiwitten komen het meeste voor, maar er zijn ook mutaties beschreven in de Z-schijf, calciumverwerkende -en mitochondriale eiwitten. Mutaties worden gevonden in ongeveer 60% van de HCM patiënten. De genetische verscheidenheid van HCM in combinatie met het feit dat de meeste HCM mutaties zich slechts voordoen in enkele families belemmeren studies naar genotype (aanwezige DNA mutatie)-fenotype (uitingsvorm van de ziekte) relaties.

Pathofysiologie

Een van de grote uitdagingen van HCM is het voorspellen welke patiënten meer kans hebben op achteruitgang en complicaties. Een afname van de coronaire flow reserve (CFR) is een belangrijk fenomeen in HCM, omdat het aanleiding kan geven tot ischemie en een voorspeller is van klinische achteruitgang en cardiale dood. In **hoofdstuk 2** tonen we aan dat de afname van de CFR in HCM patiënten wordt veroorzaakt door een combinatie van hemodynamische, echocardiografische en histologische bevindingen. De CFR afname is gerelateerd aan de toename van de diastolische druk in de linker ventrikel, de obstructie in de uitstroombaan, de verhoogde linker ventrikel massa en mate van dysplasie van de arteriolen.

Bij HCM is er vaak sprake van diastolische disfunctie. In **hoofdstuk 3** tonen we met Tissue Doppler imaging (TDI) aan dat er ook bij HCM mutatiedragers zonder verdikking

van de hartspier sprake is van subtiele veranderingen in de diastolische functie. De veranderingen in TDI snelheden bleken helaas niet voldoende significant om HCM mutatiedragers betrouwbaar te onderscheiden van gezonde vrijwilligers. Speckle tracking echocardiografie is een nieuwe echocardiografische techniek, waarmee de systolische, maar ook de diastolische functie onderzocht kan worden. Het onderzoek beschreven in **hoofdstuk 4** toont aan dat de LV untwisting vertraagd is bij HCM patiënten. LV untwisting treedt op in de vroege diastole voor de opening van de mitralisklep en is voor een deel afhankelijk van de kinetische energie opgeslagen tijdens de twist van het hart tijdens systole. De toegenomen twist in HCM patiënten met een sigmoid septum zorgt voor een compensatie van de vertraagde untwisting. Dit in tegenstelling tot HCM patiënten met een reverse septum, waarbij de twist is afgenomen.

In **hoofdstuk 5** beschrijven we de resultaten van een studie naar het effect van 2 Nederlandse founder mutaties (c.2373dupG en c.2864_2865delCT) in *myosine binding protein C (MyBPC3)* op de eiwitsamenstelling en functie van het sarcomeer. De studie is uitgevoerd in myocardbiopsie materiaal verkregen tijdens chirurgische myectomie. In vergelijking met de controlegroep is de aanwezige hoeveelheid MYBPC3 met 33 % afgenomen en is er zodoende sprake van haploinsufficiëntie. Ook de maximale kracht genererende capaciteit van de hartspiercellen is afgenomen bij mutatiedragers. Contractiele disfunctie lijkt dus een belangrijke rol te spelen in HCM.

Genetica

Het vinden van een HCM mutatie in een HCM patiënt geeft de mogelijkheid eerstegraads familieleden te testen op aanwezigheid van deze mutatie (presymptomatische DNA diagnostiek). Als de mutatie gevonden wordt in een familielid komen de eerstegraads familieleden van deze persoon in aanmerking voor onderzoek (cascade screening). In **hoofdstuk 6** beschrijven we de achtergrond en onze eerste ervaring met cascade screening bij HCM op de polikliniek cardiovasculaire genetica in het Erasmus MC. In **hoofdstuk 7** laten we zien dat in 41% van de asymptomatische familieleden met een sarcomeer mutatie, HCM wordt gediagnosticeerd door middel van echocardiografie en electrocardiogram. De aanwezigheid van klinische tekenen van HCM is afhankelijk van de leeftijd, dit maakt herhaald cardiologisch onderzoek noodzakelijk. Bij *MYBPC3* mutatiedragers waren er op latere leeftijd tekenen van HCM dan bij *MYH7* mutatiedragers.

Het doel van familieonderzoek bij HCM is vroegtijdige diagnostiek om daarmee complicaties in de toekomst te voorkomen. Voor het inschatten van het risico op plotse dood van een HCM patiënt wordt gebruik gemaakt van evaluatie naar de aanwezigheid van bekende risicofactoren voor plotse dood. De bekende risicofactoren zijn een positieve familieanamnese voor plotse dood, extreme verdikking van de hartspier, plots flauwvallen, onvoldoende bloeddrukstijging bij inspanning en de aanwezigheid van ritmestoornissen op een 24-uurs monitor. Deze risicofactoren waren aanwezig bij asymptomatische

dragers met en zonder tekenen van HCM. De waarde van de aanwezigheid van risicofactoren bij mutatiedragers zonder tekenen van HCM moet blijken uit follow-up studies van deze specifieke groep.

In Nederland wordt ongeveer een derde van alle HCM gevallen veroorzaakt door founder mutaties in *MyBPC3* (c.2373_2374insG, c.2864_2865delCT en c.2827C> T (p. Arg943X)). In hoofdstuk 5 hebben we aangetoond dat de c.2373_2374insG en c.2864_2865delCT mutaties leiden tot haploinsufficiëntie. Hoewel niet onderzocht, is het de verwachting dat ook de c.2827C> T mutatie haploinsufficiëntie veroorzaakt. In **hoofdstuk 8** laten we zien dat dragers van deze 3 Nederlandse foundermutaties ook eenzelfde natuurlijk ziektebeloop kennen, wat ernstiger is dan werd voorspeld in eerdere studies.

HCM patiënten met Nederlandse foundermutaties vormen een genetisch homogene groep en kunnen worden gebruikt om onderzoek te doen naar genetische factoren die de ernst van de ziekte beïnvloeden.

Het renine-angiotensine-aldosteron systeem (RAAS) heeft invloed op de hartfunctie, bloeddruk en elektrolytenbalans en is een mogelijke kandidaat voor het beïnvloeden van de genotype-fenotype relatie in HCM. Uit de studie beschreven in **hoofdstuk 9** blijkt echter dat RAAS polymorfismen geen belangrijke rol spelen in de fenotypische expressie van HCM in dragers van Nederlandse foundermutaties in *MYBPC3*.

De ernst van het HCM ziektebeeld bij HCM mutatiedragers wordt naar verwachting gedeeltelijk verklaard door het effect van additionele mutaties in sarcomeer genen. In **hoofdstuk 10** beschrijven we twee groepen met Nederlandse founder mutaties, een met een mild en een met een ernstig fenotype. Er was geen sprake van additionele sarcomeer mutaties in de groep met een ernstig fenotype.

Invasieve behandeling

Tot 70% van de HCM patiënten hebben een obstructie in de linker ventrikel outflow tract (LVOT) in rust of bij provocatie. Deze obstructie wordt veroorzaakt door de verdikking van het interventriculaire septum (IVS) en de voorwaartse beweging van de mitralisklep tijdens systole (SAM). HCM patiënten met obstructie, die ernstige klachten houden ondanks maximale medicamenteuze behandeling komen in aanmerking voor invasieve behandeling, hetzij chirurgisch (myectomie) of percutaan (alcohol septum ablatie (ASA)). HCM is vaak geassocieerd met intrinsieke afwijkingen van de mitralisklep, waaronder een vergroting van het voorste mitralisklepblad. Hierdoor is er een grotere kans op residuele SAM en een suboptimaal resultaat van geïsoleerde myectomie. In **hoofdstuk 11** beschrijven we de lange-termijn follow-up van HCM patiënten met een vergroot voorste mitralisklepblad behandeld met myectomie in combinatie met een mitralisklepplastiek en laten zien dat dit leidt tot langdurige verlichting van de symptomen en overleving vergelijkbaar met de algemene bevolking.

In **hoofdstuk 12** beschrijven we de lange termijn resultaten van ASA. Tijdens ASA wordt alcohol geïnjecteerd in een of meer septale takken van de linker kransslagader, waardoor een hartinfarct wordt geïnduceerd. Het afsterven van een deel van het verdikte septum en het ontstaan van een litteken zorgt voor een afname van de verdikking. Helaas blijkt uit lange termijn follow-up dat er een verhoogd risico is op plotse hartdood of terecht afgaan van de ICD, waarschijnlijk tengevolge van het litteken in het myocard. In **hoofdstuk 13** laten we met contrast echocardiografie zien dat microvasculaire disfunctie verbetert na een succesvolle ASA als gevolg van verlichting van extravasculaire drukkrachten.

CONCLUSIES

Microvasulaire disfunctie speelt een belangrijke rol bij HCM, omdat het predisponeert voor myocardiale ischemie en een voorspeller is van klinische achteruitgang en dood. Het wordt veroorzaakt door de verdikking van het septum, de toegenomen massa van de LV, de obstructie in de uitstroombaan en dysplasie van de arteriolen (**hoofdstuk 2**).

Diastolische disfunctie komt voor bij vrijwel alle HCM patiënten. In HCM mutatiedragers zonder hypertrofie worden met TDI ook veranderingen in de diastole gevonden (**hoofdstuk 3**). Met speckle echocardiografie wordt een vertraagde LV untwisting gevonden in HCM patiënten (**hoofdstuk 4**).

Het vinden van een HCM mutatie in een HCM patiënt maakt nauwkeurige identificatie van at-risk familieleden mogelijk (**hoofdstuk 6**). Bij de eerste cardiologische controle van asymptomatische mutatiedragers wordt bij 41% de diagnose HCM gesteld. De kans op de diagnose neemt toe bij hogere leeftijd, dit maakt herhaalde cardiale screening noodzakelijk. Wat de prognose is van niet-aangedane mutatiedragers en wat de waarde is van de klassieke risicostratificatie moet blijken uit vervolgstudies (**hoofdstuk 7**).

In Nederland wordt ongeveer een derde van alle HCM gevallen veroorzaakt door het foundermutaties in MyBPC3(c.2373_2374insG, c.2864_2865delCT en c.2827C> T (p. Arg943X). Deze mutaties leiden tot haploinsufficiëntie en hebben een vergelijkbaar natuurlijk beloop, wat minder gunstig is dan eerder werd voorgesteld (**hoofdstuk 5, 8**). HCM patiënten met Nederlandse foundermutaties vormen een genetische homogene groep en worden gebruikt om onderzoek te doen naar genetische factoren die de ernst van het ziektebeeld beïnvloeden. De ernst van het ziektebeeld wordt niet beïnvloed door RAAS polymorfismen of additionele sarcomeer mutaties (**hoofdstuk 9 en 10**).

Symptomatische HCM patiënten met LVOT obstructie ondanks optimale medicamenteuze behandeling komen in aanmerking voor invasieve behandeling. In obstructieve HCM patiënten met een vergroot voorste mitralisklepblad leidt myectomie gecombineerd met mitralisklepplastiek tot langdurige verlichting van de symptomen en overleving

vergelijkbaar met de algemene bevolking (**hoofdstuk 11**). Obstructieve HCM patiënten behandeld met ASA hebben een verhoogd risico op levensbedriegende ritmestoornissen tijdens langdurige termijn follow-up. (**hoofdstuk 12**). ASA moet worden gereserveerd voor patiënten met een hoog operatierisico en moet mogelijk worden gecombineerd met ICD-implantatie. In **hoofdstuk 13** laten we zien dat microvasculaire disfunctie is verbeterd 6 maanden na een succesvolle ASA, door afname van de extravasculaire compressie krachten.

Hoewel HCM in de meeste gevallen een relatief goedaardige ziekte is, moeten de beslissingen over de behandeling, zoals ICD-implantatie of invasieve behandeling voor LVOT obstructie idealiter worden gemaakt door een toegewijd team in een kliniek waar alle therapeutische opties beschikbaar zijn.

TOEKOMSTPERSPECTIEVEN

Genotype-fenotype relaties in HCM zijn zwak en onvolledig begrepen. Toekomstige studies zijn noodzakelijk om het mysterie waarom en hoe een bepaalde mutatie tot HCM leidt verder te ontrafelen. Het effect van mutaties op de sarcomeerfunctie moet verder worden onderzocht om meer inzicht te krijgen in de pathofysiologie.

Modificerende genen spelen waarschijnlijk een belangrijke rol in de fenotypische expressie van HCM. De situatie in Nederland, met de aanwezigheid van founder mutaties en cardiogenetica poliklinieken in het hele land, vormt een ideaal klimaat voor dergelijk onderzoek.

HCM patiënten worden behandeld op basis van hun fenotype. Beeldvorming speelt hierin een een cruciale rol. De waarde van de nieuwe echocardiografische technieken, zoals speckle tracking, in het vroegtijdig opsporen van HCM moet worden bepaald. Cardiale magnetic resonance imaging (CMR) kan delayed enhancement detecteren, die correleert met de histologische bevinding van fibrose en waarschijnlijk een substraat is voor ventriculaire ritmestoornissen. Vóór de toevoeging van CMR in de huidige risicostratificatie voor plotse dood zijn lange termijn studies in grote patiënten groepen nodig. Nieuwe gecombineerde beeldvormende technieken als PET-MRI en PET-CT, waarbij perfusie en functie worden gekoppeld lijken veelbelovend voor onderzoek bij HCM patiënten.

Hoewel er met de huidige technieken DNA-mutaties worden gevonden in meer dan de helft van de HCM patiënten, zijn er nog HCM patiënten en families waarin geen mutatie kan worden geïdentificeerd. Deze groep van zogenaamde mutatie-negatieve HCM patiënten moet nader onderzocht worden, omdat het inzicht kan geven in de pathofysiologische aspecten van de ziekte. Nieuwe moleculaire technieken maken het mogelijk om grote aantallen genen tegelijk tegen relatief lage kosten te onderzoeken en dit kan leiden

tot de identificatie van nieuwe genetische defecten in HCM. Het zal hierbij een uitdaging zijn de pathogene mutaties te onderscheiden van unclassified variants.

Part V Epilogue

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Het boek is af! Rest mij nog het moeilijkste, want meest gelezen hoofdstuk. De (overigens fantastische) combinatie van patiëntenzorg, onderwijs, onderzoek en de geboorte van een gezin is op zijn zachtst gezegd uitdagend. Dit proefschrift was er dan ook niet geweest zonder steun en hulp van een groot aantal personen in en buiten het ziekenhuis. Een aantal wil ik hier persoonlijk bedanken.

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Naast de wetenschap is er altijd ruimte voor gezelligheid, met de door jou georganiseerde diners tijdens de buitenlandse congressen voor fellows, laboranten, artsassistenten, technici en cardiologen van de echocardiografie afdeling als hoogtepunten. Je dreigt vaak met je (vervroegde) pensioen, maar ik hoop dat we de komende jaren onze prettige samenwerking kunnen verder zetten!

Mijn promotoren professor dr. M.L. Simoons en professor dr. B.A. Oostra wil ik bedanken voor de gelegenheid die ik heb gekregen dit onderzoek te doen. Beste Maarten, bedankt voor de stimulerende gesprekken en je vertrouwen in een goede afloop. Beste Ben, ik leerde je pas kennen toen het werk bijna klaar was, ik hoop op een fijne verdere samenwerking.

De overige leden van mijn promotiecommissie, dr. C.R. Bezzina, professor dr. A.J.J.C. Bogers, professor dr. E. Sijbrands, professor dr. A.C. Van Rossum en professor dr. F. Zijlstra wil ik bedanken voor hun bereidheid zitting te nemen in de promotiecommissie.

Dr. O.I.I. Soliman, dear Osama, you were often the first to read my papers. And, although Folkert once described you as "angry young man"; your comments were always constructive and inspiring. Thanks for all your help with my research (the use of colours in excel spread sheets is indeed useful!), computer problems and good discussions about cardiology, science and life.

Dit proefschrift is voor een groot deel ontstaan door het samenwerkingsverband met de afdeling klinische genetica. De afgelopen jaren is er door beide afdelingen veel geïnvesteerd om het samenwerkingsverband van deze vakgebieden met een verschillende achtergrond, taal en manier van werken tot een succes te maken. Ik denk dat we erin geslaagd zijn een goede structuur neer te zetten en hiervoor ben ik vooral de betrokkenen van het eerste uur erg dankbaar.

Professor dr. E. Sijbrands, beste Eric, met jouw hulp werd de lokale component binnengehaald waarmee we de cascade screening voor HCM hebben opgezet en de cardiovasulaire genetica poli werd geboren. De verbinding met de afdeling interne geneeskunde was daarmee een feit.

Dr. D.F. Majoor-Krakauer, beste Daniëlle, al zat je grotendeels aan de andere kant van de oceaan, je betrokkenheid was groot en je was altijd snel met je commentaar op de stukken die ik je stuurde, waarvoor dank. Dr. D. Dooijes, beste Dennis, wat heb je een werk verzet in het DNA lab! Veel succes in het UMC Utrecht.

Judith Phefferkorn, genetisch consulente, wil ik bedanken voor het ongelofelijke geduld, waarmee je de families counselt en je grote betrokkenheid bij het wel en wee van onze patiënten. Ook je razendsnelle reactie als ik op zoek ben naar een stamboom of DNA uitslag waardeer ik zeer. Dr. Y.M. Hoedemaekers, beste Yvonne, helaas switchte je van HCM naar non-compaction cardiomyopathie (om vervolgens te ontdekken dat beide door mutaties in sarcomeer genen worden veroorzaakt). Veel succes met je verdere opleiding!

Ten slotte Dr. M.W. Wessels, Drs. I.M.B.H. Van de Laar en Dr. M.A. Van Slegtenhorst. Beste Marja, Ingrid en Marjon; ik verheug me op het verder uitbreiden van de cardiovasculaire genetica samen met jullie.

Ook Drs. I.M.E. Frohn-Mulder, kindercardioloog wil ik bedanken. Beste Ingrid, met jou is er een goede samenwerking met de kindercardiologie van het Sophia kinderziekenhuis ontstaan. Ondertussen zorgen we samen voor hele cardiomyopathie-gezinnen en dit levert leerrijke gesprekken op.

Bij cardiovasculaire genetica mogen de erfelijke ritmestoornissen natuurlijk niet onvermeld blijven en ik ben dan ook erg blij met de betrokkenheid van Dr. J.C.J. Res en Dr. N.M.S. De Groot van de afdeling electrofysiologie.

Zo langzamerhand wordt de tafel tijdens onze vrijdagse cardiovasculaire genetica lunchbespreking steeds voller, een goede afspiegeling van de groei van dit deelgebied en ik wil alle betrokkenen hiervoor bedanken.

Dr. M.J.M. Kofflard, beste Marcel, met plezier ben ik de afgelopen jaren in Dordrecht op bezoek gekomen om echo's te scoren en patiëntengegevens op te halen. De gastvrijheid op de verschillende locaties van het Albert Schweitzer ziekenhuis is groot. Je snelle en goede commentaar op onze gezamenlijke artikelen waardeer ik zeer. Onze samenwerking stopt niet bij het afronden van mijn promotie; ik ben erg benieuwd naar de resultaten van de grote MRI studie.

Drs. P.L. de Jong, beste Peter, jou wil ik bedanken voor het prettige overleg en de grote toewijding waarmee je "onze" HCM patiënten opereert.

Mijn collega cardiologen van de polikliniek, medium care en hartfalen-harttransplantatie afdeling: Dr. K.M. Akkerhuis, Dr. A.H.M.M. Balk, Drs. K. Caliskan, Drs. J.A.A.E. Cuypers, Dr. J.W. Deckers, Dr. T.W. Galema, Dr. M.L. Geleijnse, Dr. R.J.M. van Geuns, professor dr. J.W. Roos-Hesselink, Dr. A.F.L. Schinkel en Dr. M. Witsenburg. Beste Martijn, Aggie, Kadir, Judith, Jaap, Tjebbe, Marcel, Robert-Jan, Jolien, Arend en Maarten bedankt voor de fijne collegiale samenwerking, waarin naast de cardiologie plaats is voor een heleboel meer.

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In de meeste hoofdstukken van dit boek speelt de echocardiografie een essentiële rol; iedere diagnose begint immers bij een goed te interpreteren plaatje. Ik heb het enorme geluk gehad dat de echo's van de HCM patiënten al sinds jaar en dag door Wim Vletter gemaakt worden. Beste Wim, door jou enorme kundigheid en kennis is het zelfs een feestje om meer dan 10 jaar oude VHS-banden te bekijken! Debbie van Dongen wil ik in het bijzonder bedanken voor alle echo's bij de familieleden van onze HCM patiënten. Maar natuurlijk ook mijn dank aan alle andere laboranten; Marianne Blum, Lourus van Haveren, Linda de Jonge, Anne Marie Schets, Anja Seton, Jackie Vletter-McGhie en Ellen Wiegers. Meer dan eens doe ik aanspraak op jullie flexibiliteit ("kunnen jullie nog even…"), waarvoor dank.

Een deel van de hoofdstukken is ontstaan uit vruchtbare samenwerking met andere academische centra. Uit het VU Medisch Centrum wil ik S.J. Dijk, MSc. en Dr. J. van der

Velde bedanken voor de prettige samenwerking. Beste Sabine en Jolanda, met jullie is het HCM onderzoek echt van "bench to bedside" geworden. Ik hoop dat we samen in staat zijn het mysterie van het ontstaan van HCM verder te ontrafelen.

Uit het Amsterdam Medisch Centrum wil ik dr. C.R. Bezzina, dr. I. Christiaans, Drs. I.C. Kolder, en professor dr. A.A. Wilde bedanken voor de inspirerende besprekingen, waaruit hoofdstuk 9 is voortgekomen. Beste Connie, Imke, Iris en Arthur; ik had nooit gedacht dat onderzoek en de Bijenkorf samengaan!

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Mijn zusjes; Jeanne Marie, Jacqueline en Daniëlle; alle drie heel verschillend, maar zo eigen. Bedankt voor alle gezelligheid en het meeleven bij hoogte -en dieptepunten. Dankzij jullie ben ik de afgelopen jaren niet wereldvreemd geworden!

Liefste Martijn, dit boekje was er zonder jou niet geweest. Jij (en niet de fantastische cardiologie in Rotterdam) bracht me bijna 10 jaar geleden terug naar Nederland en daar heb ik nooit spijt van gehad. De afgelopen jaren waren een aaneenschakeling van major life events; trouwen, kinderen krijgen (en opvoeden!), verhuizen, afronden van opleidingen

en nu dus een promotie. In deze (meestal vrolijke) chaos houd jij je hoofd altijd koel. Bedankt voor alles! Hoeveel plezier ik ook beleef aan mijn werk; het allerliefste ben ik bij jou en onze drie schatjes.

Lieve Anne, bedankt voor je hulp bij het maken van de kaft, staat er toch nog een tekening in mama's boek. Lieve Floris, iedere werkdag zwaai je me uit met de woorden: "ga je weer voor nep-dokter spelen?" In jouw ogen is alleen opa een echte dokter, dit boekje zal dat beeld waarschijnlijk niet veranderen. Lieve, kleine Puck, dag en nacht ons zonnetje in huis, een boekje speciaal voor jou om te scheuren!

CURRICULUM VITAE

Michelle Michels is op 24 november 1973 geboren in Amersfoort. Na haar eindexamen in 1992 aan het Bisschoppelijk College te Weert, studeerde zij geneeskunde aan de Katholieke Universiteit Leuven in België. In 1999 heeft ze het doctoraal examen met grote onderscheiding behaald, waarna ze startte met de opleiding Inwendige Geneeskunde in het Universitair Ziekenhuis Gasthuisberg te Leuven (diensthoofd professor dr. J. Fevery). Het tweede opleidingsjaar bracht ze door in het Algemeen Ziekenhuis Stuivenberg te Antwerpen (diensthoofd dr. Verstraelen). Tijdens deze eerste opleidingsjaren werd ze, tegen haar eigen verwachtingen in, gegrepen door de cardiologie. In 2001 verhuisde ze terug naar Nederland en voltooide in 2005 haar cardiologie opleiding in het Erasmus MC in Rotterdam (opleiders professor dr. J.R.T.C. Roelandt en professor dr. M.L. Simoons).

Sinds augustus 2005 is ze als cardioloog werkzaam in het Erasmus MC. Haar aandachtsgebieden zijn cardiogenetica, niet-invasieve beeldvorming en hartfalen.

Michelle is getrouwd met Martijn Vermeulen, octrooigemachtigde en ze hebben drie kinderen: Anne (5), Floris (3) en Puck (1).

SUMMARY OF PHD TRAINING AND TEACHING ACTIVITIES

Name PhD student: M. Michels	PhD period: 2006-2010
Erasmus MC Department: Cardiology	Promotor(s): M.L. Simoons, B.A. Oostra
Research School: COEUR	Supervisor: Dr. J. W. Deckers

1. PhD training

	Year	Workload (hours)
In-depth courses (e.g. Research school, Medical Training)		
- Research Seminars	2007--2009	67.2
Presentations		
- Presentations at international conferences (5)	2006-2010	72
International conferences		
- Congresses and symposia (6)	2006-2010	168
Seminars and workshops (6)	2006-2010	84
Didactic skills		
- Teach the teacher	2007	28
Other		
- NVVC najaarcongres	2006-2008	60
- Hands-on Cardiovasculaire MRI	2009	28
- Ontwikkeling hart	2010	28

2. Teaching activities	Year	Workload (hours)
Lecturing		
- Tutor onderwijs co-assistenten	2006-2009	24.5
- PKV-onderwijs	2009-2010	5
- Journal Club	2006-2010	5
- Echo-onderwijs arts-assistenten	2006-2010	4
- Leerboek onderwijs arts-assistenten	2006-2010	4
- COEUR PhD course and Research Seminar	2007-2009	20

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