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From gene mutations to biomechanical abnormalities and electrophysiological remodeling in hypertrophic cardiomyopathy: exploring the translational approach.

Scientific disciplinary sector, MED-11: Cardiology

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1. Introduction

Cardiomyopathies (CM) are defined as myocardial disorders in which the heart muscle is structurally and functionally abnormal in the absence of coronary artery disease, hypertension, valvular disease, and congenital heart disease sufficient to explain the observed myocardial abnormality.[1] The classification by the European Society of Cardiology adopted a classification of cardiomyopathies based on phenotype, in order to simplify the diagnosis of these complex diseases. The classification includes as main categories the dilated, hypertrophic, restrictive and arrhythmogenic forms, each divided into familial and non-familial.[2]

The clinical presentation of patients with cardiomyopathy is widly variable, ranging from heart failure (HF) symptoms to chest pain, syncope or arrhythmias (including cardiac arrest). However, many cases may remain silent and are diagnosed incidentally or during family screening. On account of the complexity and the limitations of clinical assessment of CM, further investigations are often required following the identification of any given phenotype, in order to define specific subtypes and different underlying disease mechanisms.

Hypertrophic cardiomyopathy (HCM) is the most common heritable heart disease (autosomal dominant tract) with incomplete penetrance and with heterogeneous clinical profile and outcome.[3] The prevalence of HCM is around 1/500 individuals. Recently, preclinical and clinical studies have elucidated the underlying molecular mechanisms and intracellular signaling pathways in HCM and highlighted a number of possible molecular targets.[4]

In the same way genetic testing, identifying the molecular etiology of disease, is a valuable tool for managing inherited cardiovascular disease in patients and families. Genetic testing can improve management of the diseases by distinguishing phenotypic subgroups, identifying disease

mechanisms, and focusing on family care.[5] Mutations in the genes encoding beta-myosin heavy chain (*MYBPC3*) and myosin-binding protein C (*MYH7*) comprise the large majority of genotyped HCM patients. *MYBPC3*- and *MYH7*-related HCM appear indistinguishable at presentation in terms of phenotype and functional characteristics as assessed by contemporary imaging techniques, and genotype-phenotype correlations have been inconclusive. Nevertheless, there are significant molecular differences between the two genes that suggest the possibility of diverse cardiac performance and phenotypic evolution over the very long term. Myosin heavy chain is well known to act as the molecular motor of the heart generating, force and motion by coupling its ATPase activity to its cyclic interaction with actin. Conversely, the exact role of *MYBPC3C* is unresolved, and its role as a tether to control myosin-actin interaction represents a mere hypothesis.[6] Moreover, pathogenic variants in *MYH7* are mainly *missense* and generate significant amounts of abnormal proteins that is incorporated into the sarcomere, with the potential to exert a poison effect on cardiomyocytes.[7] On the other hand, mutations in *MYBPC3* are mainly *frameshift*, causing aberrant splicing, leading to lack or protein production and haplotype insufficiency.[8] Although the mechanism linking haploinsufficiency to HCM development is unresolved, it is assumed that the increased Ca^{+} sensitivity and contractility seen in *MYBPC3*-HCM lead to energy deficiency and compensatory hypertrophy.[9] Data from cohorts with founder mutations and transgenic animal models suggests the tendency of *MYBPC3*-HCM to evolve towards long-term systolic impairment, while no such signal is present in *MHY7*-related disease.[10]

Moreover, the understanding of cellular mechanisms underlying "electrical" profile, arrhythmogenicity and diastolic dysfunction are still limited. For these reasons we previously analyzed the electromechanical profile of cardiomyocytes isolated from myectomy samples of patients with

obstructive HCM. HCM cardiomyocytes showed prolonged action potentials (APs), down regulation of K^+ currents, delayed relaxation due to slower Ca^{2+} transients and elevated diastolic Ca^{2+}, determined by a slight increase of Ca^{2+} current, a marked overexpression of the depolarizing late Na^+ current (I_{NaL}) and a significant reduction of K^+ repolarizing currents.[11]

Imaging techniques play an essential role in the evaluation of patients with HCM.[12] In practice, echocardiography remains the first step and the most cost-effective tool. Exercise echocardiography is commonly performed as a routine test in patients with HCM primarily to measure dynamic LV outflow gradients (LVOT) provoked by physiological exercise and for functional evaluation. However, exercise echocardiography is able to give to clinicians more diagnostic and prognostic information such as blood pressure curve, pulmonary hypertension, mitral regurgitation, transient regional wall motion abnormalities, stress-induced ST segment depression, and abnormalities of coronary flow velocity reserve due to microvascular impairment.[13,14,15] Moreover, electrocardiographic recording during effort are useful not only to evaluate supraventricular and ventricular arrythmias but ECG characteristics such as QTc variability and TQ interval that represent a surrogate of the "electrical diastolic time". Findings suggest that HCM patients exhibit at rest labile repolarization quantified by QT variability analysis.[16]

Despite this wealth of information, many gaps in knowledge remain. Specifically, the response to beta-adrenergic stimulation cardiomyocytes in terms of AP duration (APD) and the association of "electrical" and "mechanic" diastolic impairment in patients with HCM are still not studied. Moreover, the adherence of genetic, pathological substrates and left ventricular (LV) function are still elusive. Better understanding of these links is essential not only to diagnosis and identification of specific

pharmacological targets, but also to evaluate the clinical course of the disease, the risk stratification and prediction of arrhythmias.

2. Aim of the study

I analyze two cohort of HCM patients (as described in Methods paragraph) with the following aims of the study:

1) We determined the effects of isoproterenol (ISO) and high-frequency stimulation on APD, Ca^{2+} transients and force in ventricular myocytes and trabeculae of patients with HCM and the changes of QTc interval and diastolic function occurring during exercise.

2) We hypothesized that the important differences between genetic mutation might become evident in the long-term evolution of LV performance, leading to greater age-related decline in LV systolic function in patients with *MYBPC3*- compared to MHY7-related HCM, and sought to understand whether that may impact outcome.

3. Patients and Method: Part 1

Patient enrolment

Patients for clinical study. In this single-center study, we retrospectively assessed between January 2008 and June 2019 consecutively patients (n=178, >18 years) with a diagnosis of HCM and 81 control subjects underwent standard exercise ECG test (bicycle exercise protocol). The HCM diagnosis was defined as wall thickness ≥ 15 mm in one or more left ventricle (LV) myocardial segments as measured by any imaging technique (echocardiography, MRI, or computed tomography) in the absence of any concomitant pressure or volume overload conditions capable of generating the observed degree of hypertrophic LV remodelling.[17]

We took care to exclude phenocopies such as Anderson Fabry disease and cardiac amyloidosis. Patients with known obstructive coronary artery disease were excluded from the study. Our institutional review board authorized use of this database according to the principles outlined in the Declaration of Helsinki. Deidentified data were used. All study participants gave informed consents to perform exercise echocardiography test.

Patients for cells/trabeculae studies. In vitro studies were performed at the University of Florence. Protocols were approved by the ethical committee of Careggi University-Hospital (2006/0024713; renewed May 2009). We enrolled 23 HCM patients regularly followed by our Cardiomyopathy Unit and consecutively referred to surgical myectomy for relief of drug-refractory symptoms related to LVOT obstruction. Among the 23 patients, 15 agreed to undergo mutational screening in sarcomeric genes. The control cohort comprised 8 patients aged <75 years undergoing heart surgery for aortic stenosis or regurgitation and who required a septal myectomy operation due to the presence of a bulging septum causing symptomatic obstruction. All control patients had septal thickness <15mm and preserved left-ventricular systolic function (ejection fraction >55%).

Clinical study: exercise tests.

Exercise ECG studies were conducted in 178 HCM and 81 control subjects. Routinely used medications (including B-Blockers) were withdrawn before the test. Maximum, symptom-limited exercise tests were performed on a bicycle ergometer in the semisupine position, with stepwise 25-W increments every 2 minutes. Exercise echocardiography was performed under basal conditions and serially every 2 minutes during exercise. Data pertaining diastolic function reserve (E wave, A wave and E' lateral) were routinely collected at peak exercise. Wall motion abnormalities were noted. A 12-lead electrocardiogram was monitored continuously and recorded at

baseline, at each minute during exercise and after exercise. RR, QRS, Tpeak-Tend interval (Tp-e) (Tpeak was identified as the maximum of upright and minimum of inverted T waves, end of the T-wave was found with a return of the trace to the isoelectric line with zero slope),[18] JTp (J point to peak T wave=QT interval-QRS duration- Tpeak-Tend interval), TQ (TQ=RR interval–QT interval), QTc (QTc=QT/RR) and ΔQTc (calculated as difference between QTc durations during rest and exercise) were calculated at rest and at peak exercise in HCM patients and control individuals.[19] QTc was calculated according to the Bazett formula and following the method recommended by the European Heart Rhythm Society.[20] Arterial blood pressure was measured with a sphygmomanometer at baseline and every 2 minutes during exercise and in the post-exercise phase. Abnormal blood pressure response was defined by either a failure of systolic blood pressure to raise >20mmHg or any fall in systolic blood pressure during exercise. Patients were encouraged to perform maximally to achieve their expected heart rate. The maximum predicted heart rate was calculated as 220 minus the patient's age, and heart rate attained was expressed as the percentage of predicted. Exercise was terminated when fatigue, dyspnea, chest pain, clinically relevant arrhythmia or hypotension intervened. Peak exercise was defined as the maximum workload attained before discontinuation. Metabolic equivalents (METs) with 1 MET defined as the energy expended at rest, equivalent to an oxygen consumption of 3.5 ml/kg of body weight/minute, as recommended. [21,22] Notably, we excluded from the analysis all patients who could not achieve maximal or submaximal effort during the stress test. To this end, we excluded patients who did not meet these two criteria: (i) heart rate increased by more than 80% of the heart rate at rest and (ii) peak heart rate was at least 67% of the maximal heart rate calculated with the standard formula 220-age in years.

In a subset of patients and control subjects (45 HCM 36 controls), we performed echocardiography studies during the exercise test (conducted as above), using VIVID E9 echocardiographers (GE Healthcare). Peak instantaneous LVOT gradient was measured at rest (and with the Valsalva maneuver) and during exercise by continuous-wave Doppler interrogation in the apical five-chamber view, taking care to avoid contamination of the waveform by the mitral regurgitation jet.[23] We excluded patients with significant (30 mmHg) LVOT gradient at rest and/or during exercise. LV volume, LV ejection fraction and left atrial volume were measured from the apical view, using the biplane Simpson's rule method. Using M-Mode and 2D, we measured LV diameter, left atrial end-systolic diameter and maximal end-diastolic LV wall thickness. The peak velocity of early (E) and late (A) transmitral flow waves and peak early diastolic mitral annular velocity were measured; moreover we assessed diastolic function using lateral E' and not average for its best correlation with functional capacity and because septal E' is disproportionately reduced in most patients due to the typical localization of asymmetric LV hypertrophy.[24] Four types of LV diastolic function were identified: 0=normal, 1= grade I° diastolic dysfunction; 2= grade II° diastolic dysfunction; 3=grade III° diastolic dysfunction, defined according to existing guidelines. The worsening of diastolic function is considered when all of the following conditions are met during stress echocardiography: average E/e' lateral > 14 and peak TR velocity > 2.8 m/sec. [25]

***In vitro* study**

Tissue processing and cell isolation: Septal specimens from surgical patients were washed with cardioplegic solution and processed within 30 minutes from excision. Endocardial trabeculae suitable for mechanical measurements were dissected and the remaining tissue was minced and

subjected to enzymatic dissociation to obtain viable single myocytes, as previously described.[26]

Single cell studies: Perforated patch whole-cell current-clamp was used to measure membrane potential, as previously described. [Ca^{2+}] variations were simultaneously monitored using the Ca^{2+}-sensitive fluorescent dye FluoForte. Whole-cell ruptured patch voltage-clamp was used to record L-Type Ca^{2+} current and delayed rectifier K^{+} current, using appropriate protocols and solutions.[27]

Intact trabeculae studies: Ventricular trabeculae were mounted between a force transducer and a motor for muscle length control and isometric force was recorded under different stimulation protocols. In brief, we evaluated the inotropic responses to increased pacing frequencies and the kinetics of isometric twitches. Resting sarcomere length was 1.9±0.1 μm.

Drug studies: For experiments on isolated cardiomyocytes and trabeculae, isoprenaline was used at the concentration of 100 nM, unless otherwise specified. Test recordings in presence of the drug were performed after >3 minutes from the beginning of drug exposure. Afterwards, the drug was washed out for >5 minutes and measurements were repeated.

4. Patients and Method part 2

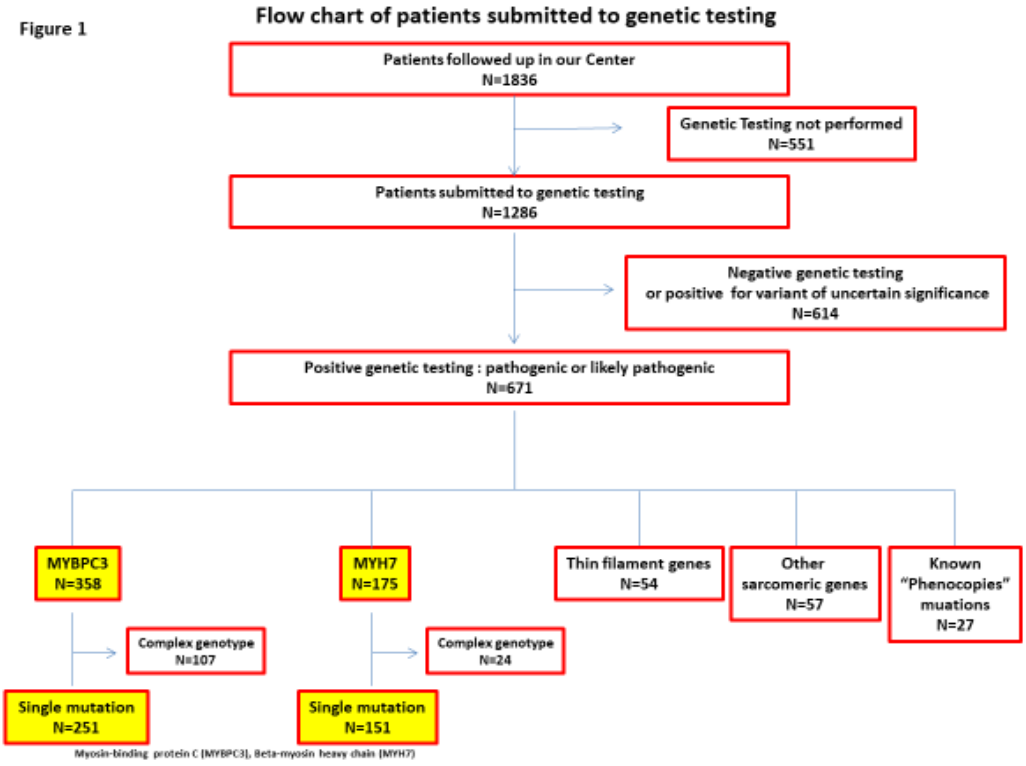
Patient enrolment

The second part of the study included 402 patients with a clinical diagnosis of HCM and pathogenic or likely pathogenic mutations in *MYBPC3* or *MYH7*, consecutively evaluated at our Institution between January 2001 and December 2018. The diagnosis of HCM was based on two-dimensional echocardiographic identification of a hypertrophied (≥15 mm), non-dilated LV, in the absence of another cardiac or systemic disease capable of producing the magnitude of ventricular hypertrophy evident.[28]

Mutational analysis

Following informed consent, genetic testing was performed using established methods available at the time of screening; following the expansion of Next Generation Sequencing (NGS). Details on sequencing and bioinformatics data processing are available elsewhere.[29] All variants were classified as pathogenic (P), likely pathogenic (LP), or of uncertain significance (VUS) following guidelines and standard criteria available at the time of testing. [30] We included in the study only patients with P/LP mutations. Mutations were considered certainly pathogenic if their association to HCM had been previously described in at least two independent studies published on peer-reviewed paper, and if they fulfilled the following, internationally recommended criteria: (i) a non-synonymous variant causing an amino acid change in a residue that is conserved among species, (ii) the variant is not present in healthy control populations, including filtering for 1000 Genomes Project (1KGP), NHLBI Exome Sequencing Project (ESP) and dbSNP with minimal allelic frequency (MAF) <0.05 and (iii) co-segregation with affected family members has been reported in at least one study.[31,32] A mutation fulfilling these three criteria, but not previously described in at least two independent papers, was defined as likely pathogenic. Patients carrying mutations which could not be defined either as certainly or likely pathogenic were not included in this study. Furthermore, patients with complex genotypes associated with a pathogenic or likely pathogenic variant in *MYBPC3* or *MHY7* were excluded from the study (Fig.1).

Figure 1



Echocardiography

Echocardiographic studies were performed using commercially available instruments. LV hypertrophy was assessed by 2-dimensional echocardiography, and the site of maximum wall thickness was identified and measured. LVOT obstruction was considered when a peak instantaneous outflow gradient ≥30 mmHg was estimated with continuous wave (CW) Doppler echocardiography at rest.[33] LV ejection fraction (LVEF) was assessed by biplane Simpson's method and severe systolic dysfunction was defined as an LVEF ≤50% (a cut-off generally used to identify "end-stage" HCM).[34] The definition of diastolic function was previously reported in the first part of the method of the study.

Follow-up and clinical outcomes

Patients were followed-up at yearly intervals or more often if clinically indicated, as previously described.[35] Median follow-up was 9±8 years. Over this period, we documented the development of clinical outcomes

including cardiac arrest, cardiovascular death, HF hospitalization, appropriate implantable cardioverter defibrillator (ICD), shock, resuscitated cardiac arrest, non-fatal stroke, atrial fibrillation and progression to severe congestive symptoms (New York Heart Association [NYHA] class III or IV).

5. Results: part 1

Baseline patient characteristics

Of the 178 HCM study patients (mean age 45±15 years), 63 (35%) were females and 115 (65%) males. 69 patients (39%) and 24 (19%) show respectively a family hystory of HCM and a family history of sudden death. The majority of HCM patients were in NYHA class I or II (Table 1). We identified more than half of patients (61%) with pathogenic or likely pathogenic sarcomere gene mutations.

Table 1: patients characteristics

Patients characteristics of study population			
	HCM (n=178)	Control (n=81)	*P* vs. Ctrl
Age	45±15	45±7	NS
Females	62 (35%)	29 (36%)	NS
Family history of HCM	69 (39%)	0 (0%)	N/A
Family history of sudden death	34 (19%)	0 (0%)	N/A
NYHA class I	114 (64%)	81 (100%)	N/A
NYHA class II	64 (36%)	0 (0%)	N/A
Angina	57 (32%)	0 (0%)	N/A
Syncope	30 (17%)	0%	N/A
NSVT	30 (17%)	0%	N/A
Beta-blockers	116 (65%)	0 (0%)	N/A
Hemodynamic and exercise parameters			
Exercise time, min	11±4	12±5	NS
Peak SBP, mmHg	165±28	160±33	NS
Peak heart rate, beats/min	126±20	137±23	<0.01
% of maximum predicted heart rate	77±13	89±9	<0.0001
Peak METs	6.5±1.6	9.8±1.8	<0.0001

Hypertrophic cardiomyopathy (HCM), New York Heart Association (NYHA), Non sustained ventricular tachycardia (NSVT), Systolic blood pressure (SPB), metabolic equivalents (METs).

Exercise performance and ECG features

Mean exercise time didn't differ between HCM patients and controls (HCM: 11±4 minutes vs controls: 12±4 minutes; p=ns). There was significant difference in the percentage of maximum predicted heart rate achieved between the two groups (HCM: 77±13% vs controls: 89±9%, p<0.0001). 23 HCM patients (13%) showed an abnormal blood pressure response (Table 1). Peak exercise capacity was significantly lower in HCM compared to volunteers, reflecting impaired exercise capacity (HCM: METs 6.5±1.6 vs controls: METs 9.8±1.8) (Table 1). Exhaustion was the most common reason for interruption (90%) and there were no serious complications during or after the test.

Compared to healthy individuals ECG features displayed that HCM patients had both at rest and at peak exercise a prolonged RR (Rest: HCM: $9^4$2±169 vs controls: 865±172; p<0.0001 and exercise: HCM: 473±96 vs controls: 437±79; p<0.003), QRS duration (Rest: HCM: 90±14 vs controls: 87±10; p<0.0001 and exercise: HCM: 93±14 vs controls: 85±10; p<0.0001), JTp interval (Rest: HCM: 239±39 vs controls: 215±38; p<0.0001 and exercise: HCM: 140±35 vs controls: 119±20; p=0.0001), and a Tp-e interval (Rest: HCM: 92±19 vs controls: 77±12; p<0.0001 and exercise HCM: 84±23 vs controls: 65±15; p<0.0001) (Table 2).

Table 2: ECG features at rest and during exercise

	HCM (n=178)	Control (n=81)	*P* vs. Control
RR rest (ms)	942±169	865±172	<0.0001
RR ex. (ms)	473±96	437±79	0.003
QRS rest (ms)	90±14	87±10	<0.0001
QRS ex. (ms)	93±14	85±10	<0.0001
JTp rest (ms)	239±39	215±38	<0.0001
JTp ex. (ms)	140±35	119±20	0.0001
Tp-e rest (ms)	92±19	77±12	<0.0001
Tp-e ex. (ms)	84±23	65±15	<0.0001
QTc rest (ms)	437±35	412±23	<0.0001
QTc ex. (ms)	463±49	408±43	<0.0001
ΔQTc	+27±52	-4±50	<0.0001

JTp= Time from end of QRS to peak T wave; Tp-e= Tpeak-Tend interval
P calculated with Student's T-test (unpaired groups)

Moreover, QTc prolonged during exercise by an average of 26±14 ms from a baseline value of 437±35 ms in HCM group ($p<0.0001$); moreover QTc was significantly prolonged in HCM patients compared to controls both at rest and during exercise (QTc Rest: HCM: 437±35 vs controls: 412±23; $p<0.0001$ and QTc exercise: HCM: 463±49 vs controls: 408±43; $p<0.0001$) with a significant difference in ΔQTc (ΔQTc: HCM: +27±52 vs controls: -4±50; $p<0.0001$) (Table 2). This paradoxical prolongation of QTc during exercise resulted in shortening of the TQ interval ($R^2=0.20$, $p<0.00001$). These changes were less significant in controls ($R^2=0.26$, $p<0.0001$) (Fig. 2).

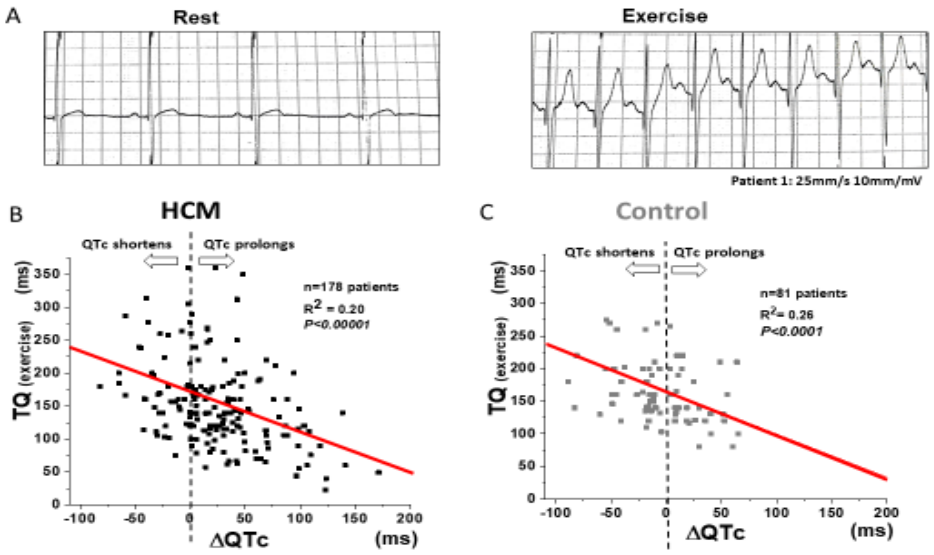


Fig. 2: changes of QT interval during exercise. (A) Representative ECG traces at rest (left) and at peak exercise (right) recorded from an HCM patient (patient 1). **(B-C)** Relationship between TQ interval (time from the end of T wave to the start of QRS) at peak exercise and the variation of corrected QT interval from rest to peak exercise (ΔQTc), in HCM patients (B) and in control subjects (C). Linear fitting is indicated in red.

In patients with HCM, the relationship between TQ and Heart Rate is steeper than controls (HCM: R^2=0.71, p<0.00001, slope=-2.81 vs Controls: R^2=0.80, p<0.00001, slope=-2.11); excessive TQ shortening due to QTc prolongation limited the increase of HR and diastolic time during exercise in HCM patients (Fig. 3).

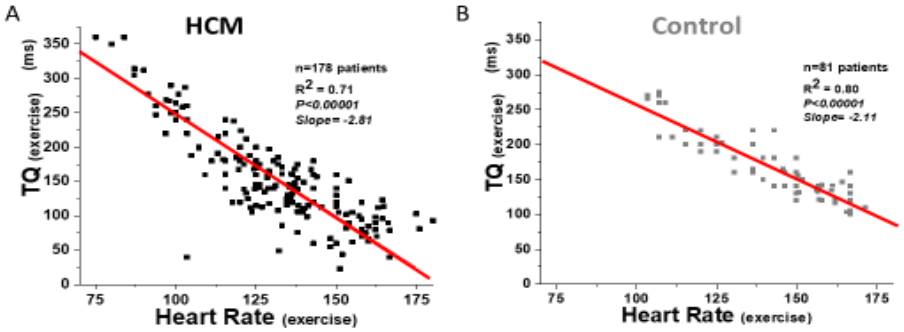


Fig. 3: relation ship between TQ interval and heart rate. (A-B) Relationship between TQ interval (time from the end of T wave to the start of QRS) at peak exercise and peak heart rate, in HCM patients (A) and in control subjects (B). Linear fitting is indicated in red.

Echocardiographic data at rest and during exercise

As expected, at baseline HCM patients showed impaired functional capacity and diastolic dysfunction compared to controls, in particular at baseline HCM patients showed a dilated left atrium (left atrial diameter HCM: 42±7 mm vs controls: 30±5 mm; $p<0.01$), lower E' lateral (HCM: 8.7±3 cm/s vs controls: 13.2±3 cm/s; $p<0.01$) and higher LV filling pressure (E/E' lateral HCM: 9.1±4 cm/s vs controls: 5.7±3 cm/s; $p<0.01$) compared to healthy volunteers (Table 3). At baseline, HCM patients showed a higher LVEF (HCM: 66±8% vs controls: 62±7%; $p<0.01$). As previously mentioned, we excluded from the study patients who developed significant exercise-induced LVOT obstruction (LVOT gradient HCM: 17±7 mmHg vs controls: 7±4 mmHg, $p<0.01$). E and E′ lateral wave velocities progressively increased during exercise in healthy subjects (Controls: E wave: 74±12 cm/sec at rest, E wave 101±12 cm/sec at peak exercise; $p<0.01$), in line with the expected normal diastolic reserve demonstrated by normal value of E/E' during effort. E wave significantly increase from rest also in HCM patients (E wave: 68±15 cm/sec at rest, E wave 125±20 cm/sec at peak exercise; $p<0.0001$), as opposite they displayed higher E/E' ratio, showing elevated LV filling pressure compared to volunteers (E/E' lateral HCM: 9.5±4 cm/s vs controls: 6.3±3 cm/s; $p<0.01$) (Table 3).

Table 3: echocardiographic data

	HCM (n=45)	Control (n=36)	*P* vs. Ctrl
Echocardiographic data at rest			
Maximal LV tickness mm	18±5	8±2	<0.01
LA diameter mm	42±7	30±5	<0.01
LVEDV index ml/mq	54±12	52±10	NS
LVEF (%)	66±8	62±7	<0.01
E wave (cm/s)	68±15	74±12	NS
A wave (cm/s)	62±9	66±10	NS
E/A ratio	1.19±0.8	1.24±0.6	NS
E' lateral (cm/s)	8.7±3	13.2±3	<0.01
E/E' lateral	9.1±4	5.7±3	<0.01
Echocardiographic data at peak exercise			
LVEDV index ml/mq	44±12	46±10	NS
LVEF (%)	71±7	69±7	NS
E wave (cm/s)	125±20	101±12	<0.01
A wave (cm/s)	114±15	84±16	<0.01
E/A ratio	1.17±0.8	1.27±0.6	NS
E' lateral (cm/s)	15.4±4	16.2±3	NS
E/E' lateral	9.5±4	6.3±3	<0.01
LVOT gradient (mmHg)	17±7	7±4	<0.01

Left ventricular (LV), Left ventricular outflow tract (LVOT), Left atrial (LA), Left ventricular end diastolic volume (LVEDV), Left ventricular ejection fraction (LVEF). P calculated with Student's T-test (unpaired groups)

QTc prolongation above 30ms is associated with worsening of diastolic function

Based on universally accepted criteria for classification of diastolic function, in each of the patients who underwent stress echocardiography we classified the degree of diastolic dysfunction at rest and during exercise as described in the methods section.

Based on this classification, all control patients had normal diastolic function at rest and at peak exercise. Among HCM patients at rest, 33/45 (73%) had normal diastole, 10/45 (22%) had grade I diastolic dysfunction, and 2/45 (5%) had grade II diastolic dysfunction. At peak exercise, 23/45 (51%) had normal diastolic function, 15/45 (33%) had grade I diastolic dysfunction and 7/45 (16%) had grade II diastolic dysfunction. Notably, in 12/45 patients (27%) diastolic function worsened during exercise, in 11/45 patients (24%) the already impaired diastole did not further impair, while in the remaining 22/45 patients (49%), normal diastolic function at rest remained normal at peak exercise. To attempt a correlation between

changes of ECG and diastolic function during exercise, we divided patients based on the changes in QTc. We considered “QTc prolongation” where QTC increased by more than 30ms from rest to peak exercise, the remaining being labelled as “unchanged QTc”. Among the 45 HCM patients who underwent stress echo, QTc prolongation occurred in 24 (53%). Notably, QTc prolonged in 10 out of 12 patients where diastolic dysfunction worsened during exercise; according to cross tabulation analysis with Chi-square, there was a significant association between “QTc prolongation” and the worsening of diastolic function during exercise (P=0.015). In line with that, 14 out of 22 patients where diastole remained normal during exercise experienced no increase of QTc. According to Chi-square analysis, there was a significant association between “unchanged QTc” and the maintenance of normal diastolic function during exercise (P=0.025).

In line with that, in the 21 patients with unchanged QTc, average E/e’ ratio at peak exercise was significantly lower than the average E/e’ ratio from the 24 patients where QTc increased (Figure 4)

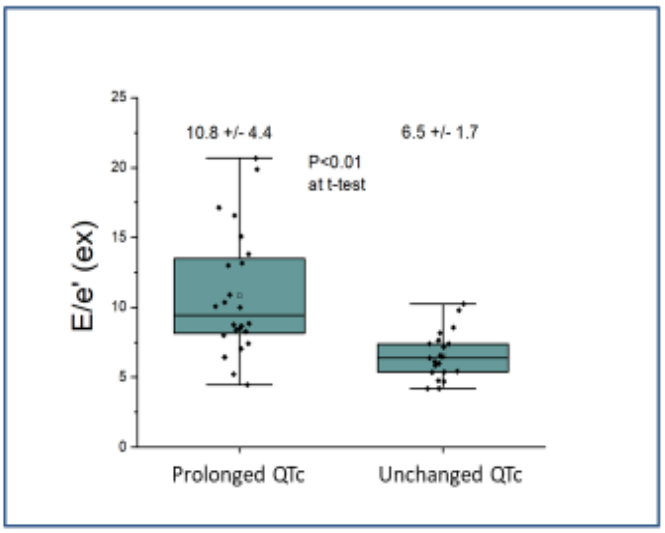


Fig 4: diastolic function and QT variations E/e' ratio calculated at peak exercise in the 24 HCM patients in whom QTc prolongs by more that 30 ms from rest to exercise ("prolonged QTc") and in the remaining 21 patients with no change ("Unchanged QTc").

In vitro results

Effects of β-adrenergic stimulation

The effects of ISO (isoprenaline 100nM) were tested in isolated ventricular cardiomyocytes from obstructive HCM patients who underwent myectomy and control non-failing non-hypertrophic surgical patients, while recording action potentials and calcium transients during regular stimulation at 0.5Hz (Figure 5).

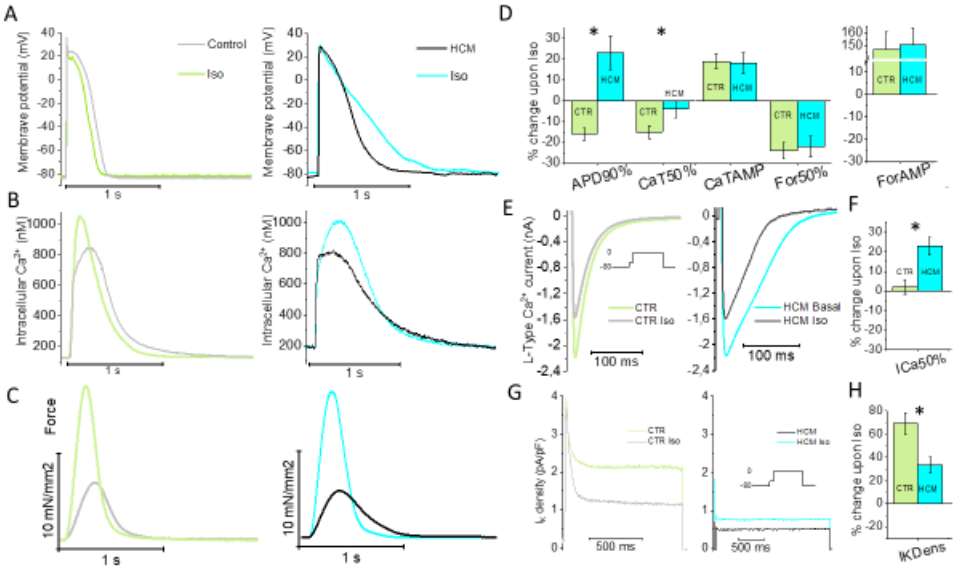


Figure 5. Effects of β-adrenergic stimulation in cardiomyocytes and trabeculae from control and HCM patients. Legend in the next page.

As shown in panels 5A and 5D, ISO led to shortening of APD in control cardiomyocytes (APD at 90% repolarization -$APD_{90\%}$- was 446±32 ms at baseline and 392±21 ms with ISO -p<0.01-, -16±3% on average); on the contrary, ISO prolonged APD in HCM cardiomyocytes ($APD_{90\%}$ was 678±45 ms at baseline and 834±58 ms with ISO -p<0.01-, +23±8% on average). Panels 5B and 5D show that ISO increased the amplitude of Ca^{2+} transients in both CTR and HCM cells by a similar amount (+24±7% in CTR, +23±9% in HCM); however, ISO significantly accelerated the kinetics of Ca^{2+} transients only in CTR myocytes (Ca^{2+} transient duration from stimulus to 50% decay -CaT50%-, was 499±37ms at baseline and 423±29ms with ISO -p<0.01-, -15±3 on average), while CaT50% did not significantly reduce in HCM myocytes (744±53 ms at baseline, 722±57 with ISO, -4±4% on average). The amplitude of isometric force twitches, elicited at 0.5Hz in intact ventricular trabeculae, increased by a similar amount in both CTR and HCM preparations in response to ISO. In particular, twitch force in CTR trabeculae was 5.7±1.6 mN/mm^2 at baseline and 14.6±2.9 mN/mm^2 with ISO (p<0.01, average increase +147±14%); in

HCM muscles, it was 5.2±1.9 mN/mm^2 at baseline and 13.5±3.3 mN/mm^2 with ISO ($p<0.01$, average increase +153±13%). The kinetics of force twitches was accelerated by ISO in both HCM and control trabeculae, by a similar amount (-24±4% in CTR, -22±5% in HCM muscles, Panels 5C and 5D). The duration of 0.5Hz force twitches from onset to 50% relaxation (For50%) was prolonged at baseline in HCM vs. control trabeculae (467±34 ms in CTR vs. 582±53 ms in HCM, $p<0.01$). Despite the acceleration, twitch duration was still prolonged in HCM vs. control trabeculae even in the presence of ISO (349±29 ms in CTR, 458±43 ms in HCM, $p<0.01$).

The abnormal response to ISO in terms of APD in HCM myocytes was paralleled by an abnormal distribution of ion currents. Indeed, as previously shown [2,4], we confirmed that the density of L-type Ca^{2+} current (I_{CaL}) is very slightly increased in HCM vs. control myocytes (at 0mV, I_{CaL} was 6.6±0.3 pA/pF in CTR, and 7.3±0.3 pA/pF in HCM cells, $p<0.05$), while the density of delayed rectifier potassium current (I_K) was instead markedly reduced in HCM cells with regards to controls (at 0mV, 1.42±0.36 pA/pF in CTR, vs. 0.55±0.17 pA/pF in HCM, $p<0.01$). Both currents are expected to increase in response to β-adrenergic stimulation, as L-type Ca^{2+} channels and slow delayed rectifier K^+ channels (I_{Ks} channels) are both modulated by β-receptor signalling. We observed that the amplitude of I_{CaL} similarly increased in response to ISO in CTR and HCM myocytes (+34±12% in CTR, +30±10 % in HCM cells, panel 4E). However, we observed that the kinetics of I_{CaL} inactivation, already slower in HCM vs. CTR cells at baseline, was further prolonged by ISO only in HCM myocytes (panels 4E-4F). In particular, the duration of I_{CaL} from the onset of depolarization to 50% of decay (ICa50%) in CTR cells was 43±12 ms at baseline and 52±14 ms with ISO (+3±6% on average); on the contrary, in HCM myocytes ICa50% was 74±15 ms at baseline ad

increased to 94±13 % with ISO ($p<0.01$, average change was +22±6%). The response of I_K to ISO was also abnormal in HCM myocytes (panels 5G-5H) while I_K density at 0mV increased to 2.34±0.47 pA/pF in CTR cells (+69±10% on average), it only increased to 0.81±0.17 pA/pF in HCM myocytes (+39±7% on average).

Effects of high frequency stimulation (2Hz)

We here compared the response of CTR and HCM ventricular myocytes and trabeculae to an increase of stimulation frequency from 0.5Hz to 2Hz (Fig.6).

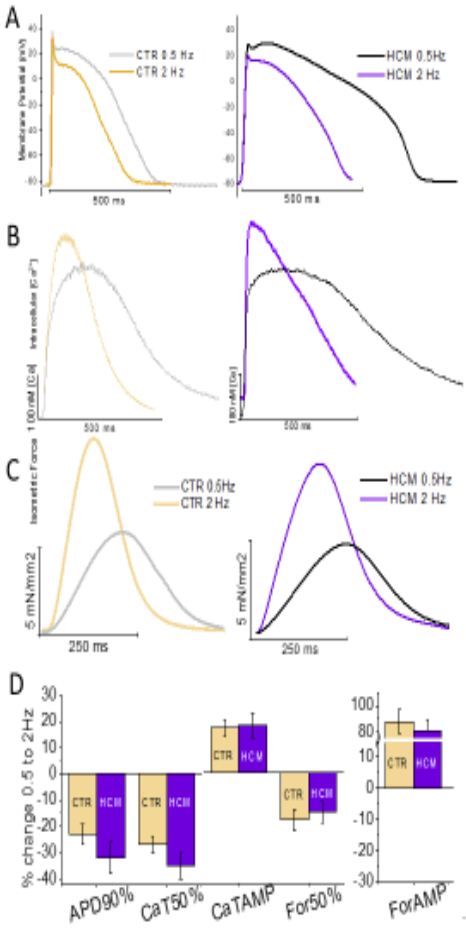


Figure 5. (Previous page). **Effects of β-adrenergic stimulation in cardiomyocytes and trabeculae from control and HCM patients.**
(A) Representative superimposed action potential (AP) traces elicited during 0.5Hz stimulation in control (left) and HCM (right) cardiomyocytes, in the absence and presence of isoprenaline 10^{-7}M (Iso). **(B)** Representative superimposed calcium transients elicited at 0.5Hz stimulation in control (left) and HCM (right) cardiomyocytes, in the absence and presence of Iso. **(C)** Representative superimposed isometric force twitches, elicited during field stimulation at 0.5Hz in control (left) and HCM (right) trabeculae, in the absence and presence of Iso. **(D)** Percentage change of different functional parameters (during stimulation at 0.5Hz) recorded in CTR and HCM ventricular cardiomyocytes and trabeculae in response to Iso. From left: APD90%=Action potential duration at 90% of repolarization; CaT50%= Calcium transient duration from stimulus to 50% of decay; CaTAMP=Amplitude of calcium transients (peak systolic-resting diastolic [Ca]); For50%= force twitch duration from onset to 50% of twitch relaxation; ForAMP= amplitude of force twitches. Average cell data (Means±S.E.M.) from 14 control cardiomyocytes (5 patients) and 34 HCM cardiomyocytes (10 patients). Average force results from 6 control trabeculae (5 patients) and 31 HCM trabeculae (21 HCM patients). **(E)**Representative superimposed L-type calcium current (I_{CaL}) traces elicited at 0 mV from -80mV resting potential in control (left) and HCM (right) cardiomyocytes, in the absence and presence of Iso. **(F)** Average change in the duration of I_{CaL} from the onset of depolarization to 50% of decay (ICa50%) upon Iso administration. Means±S.E.M. from 7 control myocytes (3 patients) and 15 HCM myocytes (6 patients). **(G)** Representative delayed rectifier potassium current (I_K) traces elicited at 0 mV from -80mV resting potential in control (left) and HCM (right) cardiomyocytes, in the absence and presence of Iso. **(F)** Average change in the density of I_K at 0 mV (IKDens) upon Iso administration. Means±S.E.M. from 6 control myocytes (3 patients) and 10 HCM myocytes (3 patients).

Figure 6. Effects of high stimulation frequency in cardiomyocytes and trabeculae from control and HCM patients.
(A) Representative superimposed action potential (AP) traces elicited during stimulation at 0.5Hz and 2Hz in control (left) and HCM (right) cardiomyocytes. **(B)** Representative superimposed calcium transients elicited at 0.5Hz and at 2Hz in control (left) and HCM (right) cardiomyocytes. **(C)** Representative superimposed isometric force twitches, elicited during field stimulation at 0.5Hz and 2Hz in control (left) and HCM (right) trabeculae. **(D)** Percentage change of different functional parameters when changing stimulation frequency from 0.5Hz to 2Hz, as recorded in CTR and HCM ventricular cardiomyocytes. Abbreviations are defined in Figure 4 legend. Means±S.E.M. from 24 control cardiomyocytes (8 patients) and 47 HCM cardiomyocytes (24 HCM patients). Average force results from 6 control trabeculae (5 patients) and 31 HCM trabeculae (21 HCM patients).

APD shortened by a similar amount in CTR and HCM ventricular myocytes when stimulation frequency was increased to 2Hz, highlighting a normal APD rate adaptation in HCM myocardium (panels 6A and 6D). Nonetheless, APD was still longer in HCM vs. CTR myocytes even at 2Hz: average APD90% at 2Hz was 338±21 ms in CTR, vs. 449±27 ms in HCM cells ($p<0,01$). Ca^{2+} transients were also significantly hastened by the increase of stimulation frequency to 2Hz in HCM and CTR myocytes (panels 6B and 6D). Again, Ca^{2+} transients were still slower in HCM vs. control cells even at 2Hz stimulation. Indeed, CaT50% at 2Hz was 341±27 ms in CTR vs. 417±31 ms in HCM ($p<0.01$). The amplitude of Ca^{2+} transients increased by a similar extent in response to 2Hz frequency in HCM and CTR myocytes (panel 6D). However, diastolic Ca^{2+} levels during steady-state stimulation at 2Hz were markedly higher in HCM myocytes as compared with control myocytes, due to a marked increase of diastolic $[Ca^{2+}]$ that occurred only in HCM cells with the increase of pacing rate. Indeed, diastolic $[Ca^{2+}]$ at 2Hz was 112±45 nM in CTR (+12±7% increase with respect to 0.5Hz), vs. 277±60nM in HCM (+76±21% increase from 0.5Hz, $p<0.01$ vs. CTR). Finally, raising stimulation frequency from 0.5Hz to 2Hz in trabeculae led to an increase of both twitch amplitude and velocity (Fig. 6C- 6D), by comparable amounts in HCM and control muscles. However, the duration of force twitches was still prolonged in HCM vs. CTR trabeculae even at 2Hz frequency: For50% at 2Hz was 302±21 ms in CTR vs. 363±24 ms in HCM trabeculae ($p<0.05$). Moreover, as previously shown,[36] the increase of diastolic tension while raising stimulation frequency from 0.5Hz to 2Hz was more pronounced in HCM vs. control trabeculae (+26±18% in CTR, +68±36% in HCM, $p<0.05$), suggesting an increased diastolic tension at high frequencies in HCM myocardium.

6. Results: part 2

Baseline characteristics of *MYBPC3* vs *MYH7* HCM

A total of 251 *MYBPC3* and 151 *MYH7* patients with pathogenic or likely pathogenic variants were included. Patients were diagnosed at a mean age of 39±17 years (yrs). *MYBPC3* patients were more often males (68% vs 56% of *MYH7*, p=0.013) and less frequently obstructive (15% vs 26% of *MYH7*; p=0.005). Clinical status (NYHA class), maximal LV wall thickness (LVWT), distribution of LV hypertrophy and left atrial enlargement did not differ between the two groups (Table 4).

Table 4

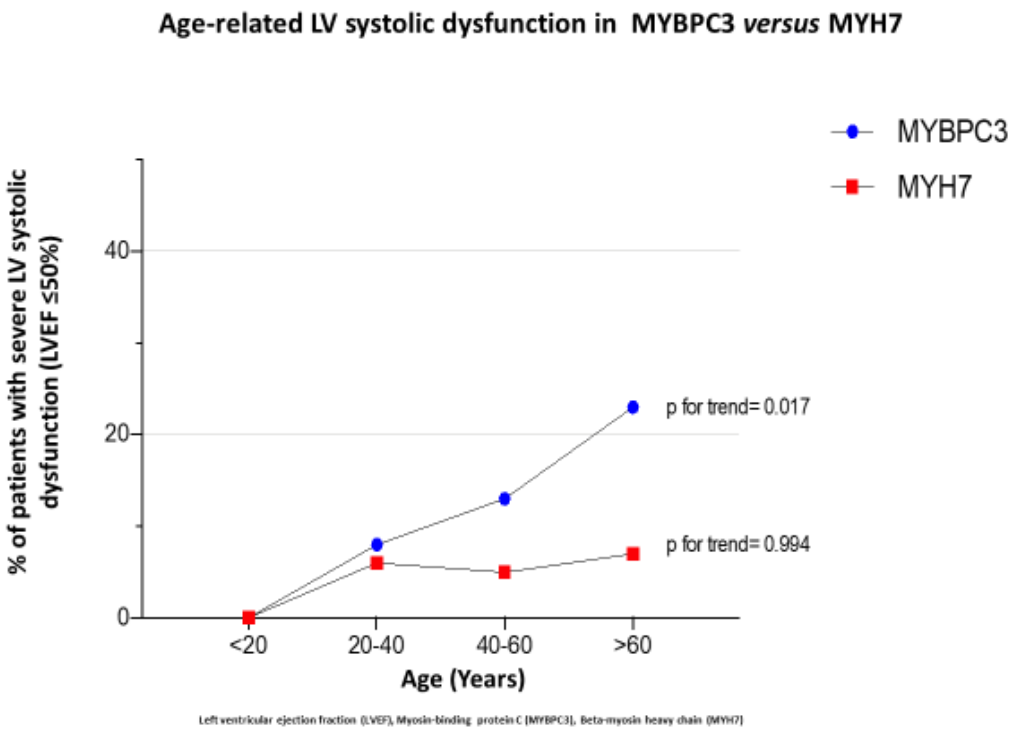
	Full HCM Cohort	MYBPC3	MYH7	P
	n=402	n=251	n=151	
Age at diagnosis (years)	39±17	40±17	39±18	NS
Sex male (n,%)	254 (63)	170 (68)	84 (56)	0.013
BSA (mq, media±DS)	1.9±0.23	1.9±0.22	1.8±0.24	0.263
Age at 1° Echocardiography	43±17	43±17	43±18	NS
NYHA class				
I (n,%)	241(60)	161(64)	80(53)	NS
II (n,%)	127(31)	72(29)	55(36)	NS
III (n,%)	32(8)	16(6)	16(11)	NS
IV (n,%)	2 (1)	2(1)	0	NS
Maximal LVWT (mm,median±DS)	21±6	21±6	21±6	NS
LVOT obstruction (n,%)	76(19)	37(15)	39(26)	0.005
Left atrial volume (ml/mq, median±DS)	43±20	41±18	45±22	NS
LVEDV (ml/mq,median±DS)	52±14	53±15	51±12	NS
LVESV (ml/mq, median±DS)	18±7	18±8	17±6	NS
LVEF (%,median±DS)	67±8	66±8	68±8	0.003
LVEF≤50% (n,%)	16(4)	9(4)	7(5)	NS
Normal diastolic function (n,%)	105(34)	69(36)	36(30)	NS
I° grade diastolic dysfunction (n,%)	117(38)	74(39)	43(346)	NS
II° grade diastolic dysfunction (n,%)	63(20)	34(18)	29(25)	NS
III° grade diastolic dysfunction (n,%)	23(7)	13(7)	10(9)	NS

Body surface area (BSA), New York Heart Association (NYHA), Left ventricular wall thickness (LVWT), Left ventricular outflow tract (LVOT), Left ventricular end diastolic volume (LVEDV), Left ventricular end systolic volume (LVESV), left ventricular ejection fraction (LVEF).

LV systolic function at initial evaluation

MYBPC3 patients displayed lower LVEF compared to *MYH7* (66±8% vs. 68±8%, respectively; p=0.03), as patients with "super-normal" systolic function (LVEF≥70%) were less represented in *MYBPC3* (38% vs 55% in MHY7, p=0.001). However, the proportion of patients with severe LV systolic dysfunction (LVEF≤50%) was similar (4% for *MYBPC3* vs 5% for *MYH7*; p=0.581). Finally, there was a negative correlation between LVEF and age in the *MYBPC3* group (p for trend=0.017) which was totally absent among *MHY7* patients (p for trend=0.994) (Figure 7).

Figure 7

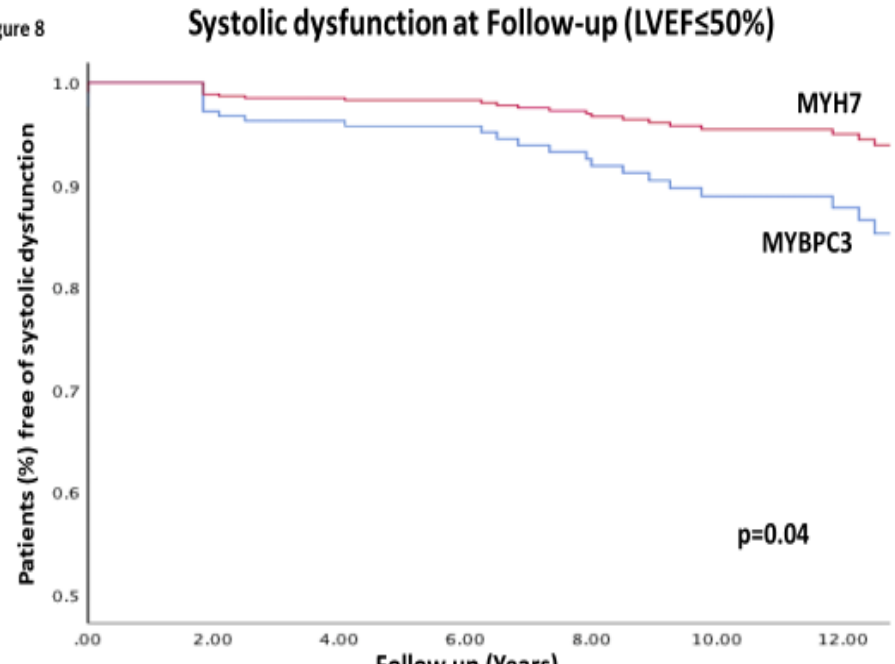


Systolic function at follow up

Overall, patients developed a limited but significant reduction in LVEF during follow-up (*MYBPC3*: 61±11% at FU versus 66±8% at baseline; p<0.001; *MYH7*: 64±9% at FU versus 68±8% at baseline, p<0.0001). In addition, *MYBPC3* patients showed a lower LVEF at final evaluation in

comparison to *MYH7* (61±11% vs 64±9%, p=0.01). The prevalence of patients with "end stage" dysfunction (LVEF≤50%) was higher among *MYBPC3* (15%, vs 5% among *MYH7*; p=0.013). Likelihood of new onset of LVEF≤50% during FU was also higher in the *MYBPC3* group (p=0.040). At Cox multivariable analysis the presence of a *MYBPC3* mutation (HR 2.53 95% CI: 1.09-5.82, p=0.029), age (HR 1.03 95% CI 1.00-1.06, p=0.027) and AF (HR 2.39 95% CI: 1.14-5.05, p=0.020) were independently associated with LVEF <50% at final visit. (Figure 8).

Figure 8



Variables	Multivariate	p
MYBPC3	HR 2.53 95%CI: 1.09-5.82	0.029
Age	HR 1.03 95%CI 1.00-1.06	0.027
AF	HR 2.39 95%CI 1.14-5.05	0.021

Left ventricular ejection fraction (LVEF), Myosin-binding protein C (MYBPC3), Beta-myosin heavy chain (MYH7), Atrial fibrillation (AF)

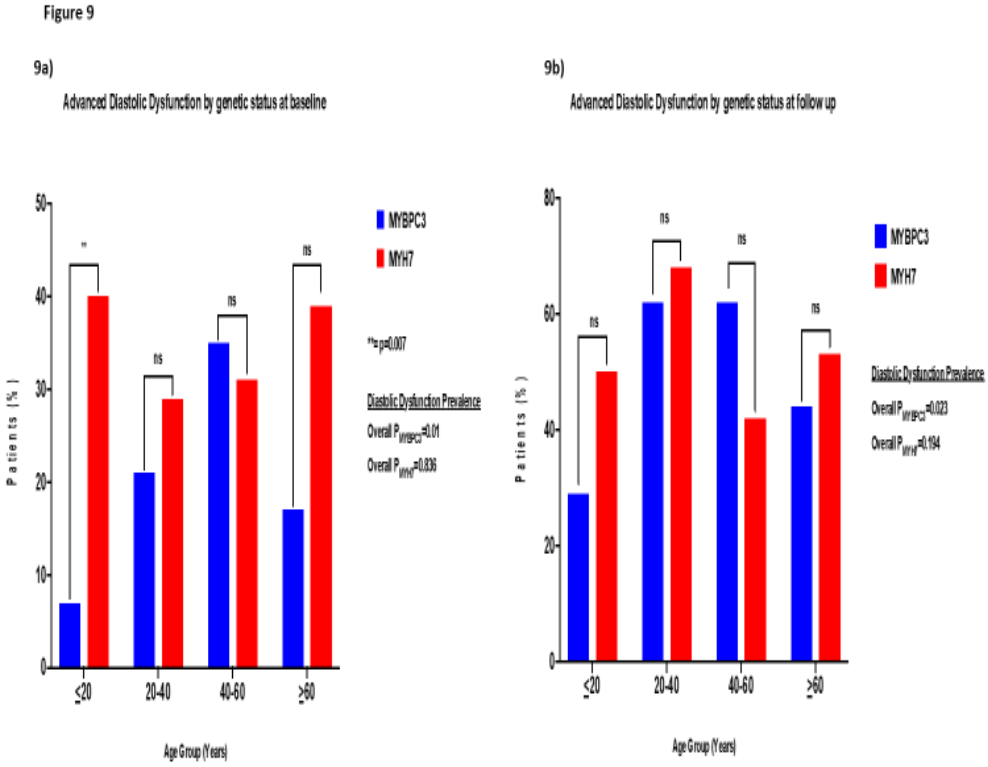
Diastolic function

At baseline, the proportion of patients with diastolic dysfunction grade I, II and III was similar in both groups. In *MYBPC3* patients diastolic function tended to worsen with age (p for trend=0.010), whereas *MYH7* had greater

prevalence of grade II/III dysfunction at baseline. Among patients aged <20 yrs prevalence of advanced diastolic dysfunction was higher in *MYH7* compared to *MYBPC3* patients (Figure 9a).

During follow up, prevalence of grade II/III diastolic dysfunction increased among *MYBPC3* but not among *MHY7* patients. Overall, however, there was no difference in the prevalence of grade II/III dysfunction between *MYBPC3* and *MYH7* by age groups at final visit (p=0.509). (Figure 9b) At Cox multivariable analysis LVOT obstruction (HR 2.77 95% CI: 1.47-5.20, p=0.002) and age (HR 1.02 95% CI 1.00-1.03, p=0.024) were the only predictors of diastolic dysfunction at FU.

Figure 9



Long term outcome

At a mean follow-up (FU) of 9±8 years, no significant differences were observed between *MYBPC3* and *MYH7* patients in terms of functional impairment (NYHA class III -p=0.731- and IV -p=0.625, incidence of AF -p=0.466, stroke -p=0.993, heart failure hospitalization -p=0.967, cardiac

arrest -p=0.093, and cardiovascular death -p=0.206. However, prevalence of NSVT was higher for *MYBPC3* (39% vs 14%, p<0.0001).

7. Discussion

Response of HCM myocardium to β-adrenergic stimulation and high stimulation frequencies: mechanisms and implications

Using cardiomyocytes and trabeculae isolated from the interventricular septum of HCM and control patients, we studied the fine mechanisms underlying the response of healthy and HCM myocardium to β-adrenergic stimulation and high-frequency pacing, conditions that occur simultaneously during physiological exercise, although analysed separately here to distinguish the relative contributions of each. We previously reported an abnormal response to β-stimulation in HCM cardiomyocytes in terms of action potential duration changes[37]. By recording from cells obtained from a large number of different patients, we here confirmed that APs are abnormally prolonged by β-adrenergic stimulation with isoprenaline in HCM (even when stimulation frequency is unchanged (Figure 4), while they slightly shorten in control myocytes. We further assessed the direct effects of β-adrenergic stimulation on sarcolemmal ion currents in HCM vs. control myocytes, and found an increase of both L-Type Ca^{2+} current (I_{CaL}) and slow delayed rectifier potassium current (I_{Ks}), mainly via Protein Kinase A (PKA)-mediated phosphorylation of Cav1.2 and Kv7.1 channel subunits, respectively[38]. Consistently, the amplitude of both Ca^{2+} and delayed rectifier potassium currents were increased by isoprenaline in both HCM and control cardiomyocytes. However, the baseline amplitude of I_{CaL} and I_K currents was different between HCM and control cardiomyocytes, as is their quantitative response to β-stimulus. Indeed, while the density of I_{CaL} is slightly increased in HCM vs. control

cells, due to a slightly higher ion channel protein expression in HCM vs. healthy myocardium[17], the density of I_K was severely reduced in HCM cardiomyocytes, owing to the marked decrease in the mRNA expression of K^+ current ion channel genes in HCM myocardium with respect to control heart muscle. Notably, I_K in ventricular myocytes is determined by the sum of fast and slow delayed rectifier currents (I_{Kr} and I_{Ks}, respectively), that are conducted by two different channel assemblies (Kv11.1 or hERG for I_{Kr}, Kv7.1 or KvLQT1 for I_{Ks}), with similar gating properties and voltage dependency, which collectively mediate ventricular myocyte repolarization and influence the duration of AP plateau. The expression of both *KCNH2* (hERG, I_{Kr}) and *KCNQ1* (KvLQT1, I_{Ks}) genes is reduced in HCM ventricles with respect to controls. However, only I_{Ks} is actively regulated by β-stimulation. In HCM myocytes, we here observed that the relative increase of I_K in response to isoprenaline is lower with respect to control cells. This, combined with the lower density of I_K at baseline, renders I_K deficiency in HCM myocytes even more pronounced under β-stimulation, when I_K density is approximately 1/3 when compared to the current measured in isoprenaline-treated control myocytes. All in all, our results support the idea that the relative contribution of I_K to ventricular repolarization is reduced by β-receptor activation. Conversely, the density of I_{CaL} was physiologically increased by isoprenaline in HCM myocytes, although the kinetics of current inactivation, already slower at baseline in HCM cells, was further delayed by isoprenaline.

During β-stimulation, the increased amplitude and duration of I_{CaL}, the main depolarizing current active during the AP plateau of HCM cells, combined with the insufficient increase of I_K, shifts the balance between depolarizing and repolarizing currents, ultimately leading to delayed repolarization. Despite these abnormal electrophysiological effects, the mechanical response of HCM myocardium to β-agonists appears to be

maintained, in that isoproterenol has both positive inotropic and lusitropic (i.e. acceleration of relaxation) effects in HCM trabeculae. The mechanical effects of β-stimulation are mediated by the PKA-phosphorylation of excitation-contraction coupling proteins, i.e. ryanodine receptors (RyR2) and phospholamban. Combined with the increased sarcolemmal Ca^{2+} entry through the enhanced I_{CaL}, RyR2 phosphorylation potentiates the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and increases Ca^{2+} transient amplitude, ultimately raising twitch force (Fig.4B). Phospholamban phosphorylation enhances the Ca^{2+} transient decrease rate by potentiating Ca^{2+} reuptake to the SR by the SR-Ca^{2+}-ATPase (SERCA), contributing to a faster inactivation of twitch contraction and a swifter relaxation. Such effects appear to be occurring normally in HCM cardiomyocytes, although with longer duration of Ca^{2+} transients vs. control cells[17,20]. (Figure 10)

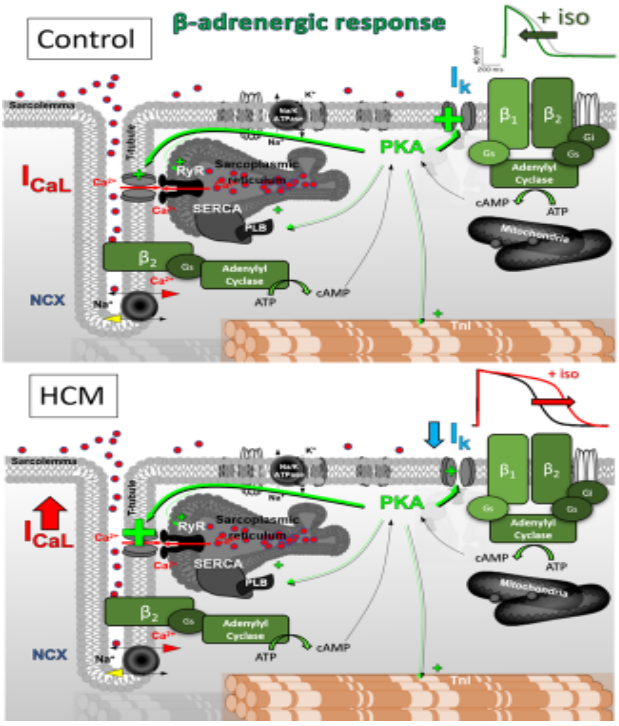


Fig. 10: altered adrenergic response in HCM myocytes. Scheme depicting the altered response of HCM cardiomyocytes to beta-adrenergic stimulation. B-adrenergic receptors activate adenylyl cyclase though Gs subunits, which in turn generate cyclic AMP (cAMP). cAMP activates protein kinase A (PKA), which phosphorylates a numebr of intracellular targets (indicated with green plus symbols), including calcium and potassium channels. Phosphorylation of K and Ca channels lead to increased currents. In control cardiomyocytes (above) the increase of K currents prevails over the increase of Ca current, ultimately leading to shortening of action potentials. In HCM cells (below), instead, K curretns are constitutively reduced while Ca current is even slightly increased. The net effect of β stimulation in HCM cells is therefore a net increase of depolarizing currents driven by the heightened I_{CaL}, ultimately resulting in a longer AP at a given frequency of stimulation.

Finally, PKA-mediated phosphorylation of Troponin-I, causing a decrease of myofilament sensitivity to cytoplasmic Ca^{2+}, further contributes to

accelerate muscle relaxation under β-receptor stimulation. However, as twitch kinetics is slower in HCM vs. control ventricular trabeculae at baseline[17], we observed that twitch duration is longer in HCM samples even under maximal β-stimulation, despite isoprenaline exerted a similar twitch shortening effect in HCM and control muscles. The abnormal AP duration response to β-stimulation in HCM myocytes, combined with the increased intracellular Ca^{2+} load caused by prolonged I_{CaL}, may have deleterious implications in terms of arrhythmic risk. Indeed, we previously observed that isoprenaline causes an increase of both early and delayed afterdepolarizations in HCM myocytes[20], highlighting the risk of exercise- or stress-induced arrhythmias in HCM patients.

We then tested the effects of raising frequency stimulation from 0.5Hz to 2Hz, simulating exercise-induced tachycardia. Notably, increasing pacing rate led to similar changes of functional parameters in HCM and control cardiomyocytes and trabeculae. In parallel, Ca^{2+} transient amplitude slightly increased and transients were significantly shortened while raising pacing rate to 2Hz; again, Ca^{2+} transient duration at 2Hz was still longer in HCM myocytes, compared to controls. Finally, force twitches increased in amplitude and were accelerated by the faster pacing rates in both groups, although twitches were still slower at 2Hz in HCM trabeculae.

The opposite effects of β-stimulation and high frequencies on AP duration in HCM muscle is likely to cause an insufficient shortening of AP duration in response to exercise in patients. However, as Ca^{2+} transients and force twitches appear to shorten near normally in response to both β-stimulation and faster pacing, we expect that exercise to physiologically stimulate accelerated contraction and relaxation of ventricular muscle. However, in these conditions Ca^{2+} transients and twitches remain slower in HCM myocardium, compared to control, suggesting an impairment of diastolic function during exercise.

Clinical correlates: Abnormal electrical and mechanical response to exercise in HCM

In line with the reduced shortening of APs observed in stimulated HCM cardiomyocytes, the QT interval shortened much less during physiological exercise HCM patients, compared to controls (Fig.7), resulting in a prolongation of the QTc (as opposed to no change in healthy individuals). (Figure 11)

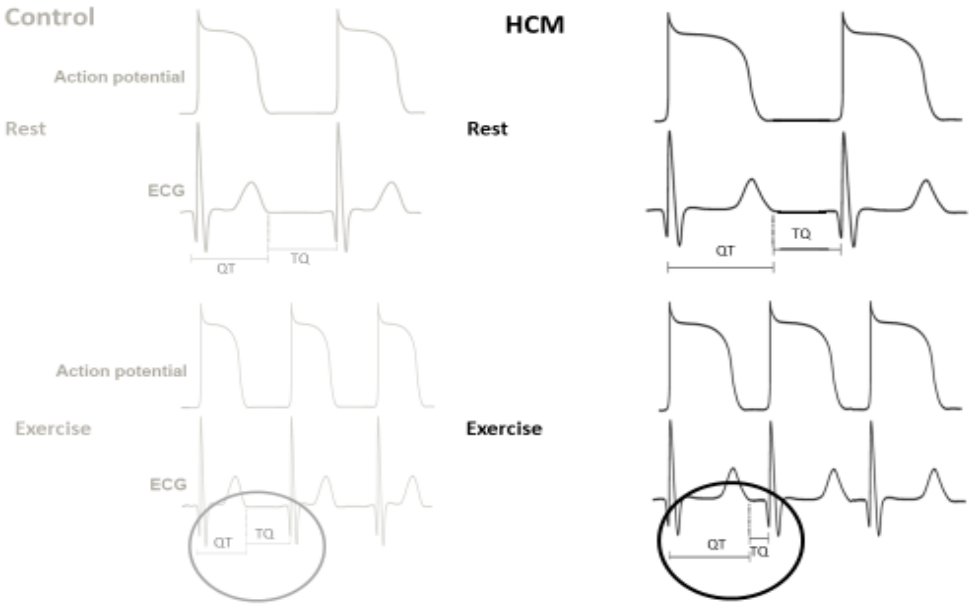


Fig. 11: Action potentials, QT and TQ intervals. Scheme depicting the relationship between action potential duration at rest and at peak exercise and the changes in the duration of QT and TQ intervals during exercise. Notably, the insufficient shortening of APs at peak exercise leads to a lower degree of QT shortening in HCM patients, causing execessive shrinking of the TQ interval (electrical diastole).

These findings are consistent with limited data in the literature addressing the role abnormal repolarization in patients with HCM; specifically, Dritsas et al reported a prolonged QT interval and increased corrected QT dispersion in a small cohort of HCM patients.[39,40,41]

The direct implication of QT prolongation with exercise was a proportional reduction in the TQ interval, i.e. the "electrical diastole" of HCM ventricles, potentially detrimental to the ability of the heart to fully relax at each cycle. TQ shortening at peak exercise was inversely related to

maximal heart rates achieved during exercise and thus appeared to contribute to chronotropic incompetence in HCM patients. Importantly, patients in whom the QTc prolonged by more than 30ms during exercise (and exhibiting the shortest TQ intervals) were more likely to experience a worsening of diastolic function associated with effort, and viceversa. On average, patients with QTc prolongation had greater evidence of diastolic impairment, as shown by higher E/e' ratio by Doppler echocardiography. (Figure 12)

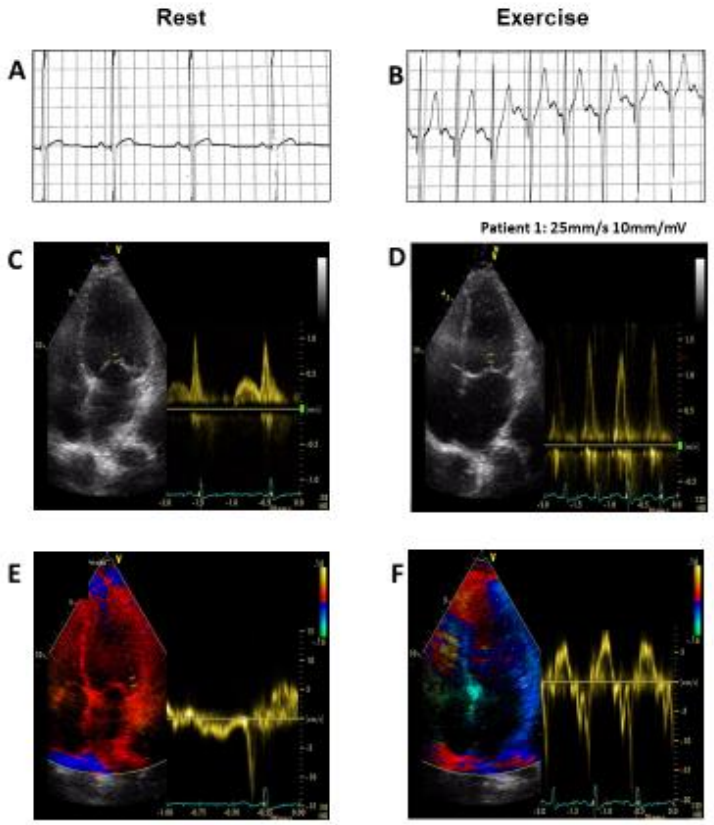


Fig. 12: changes of QT interval during exercise and impaired diastolic reserve in HCM patients. (A-B) Representative ECG traces at rest (A, left) and at peak exercise (B; right) recorded from an HCM patient (patient 1). **(C-D)** Representative 4-chamber apical views and Doppler trasmitral blood flow at rest (C) and at peak exercise (D) in a HCM patient. **(E-F)** Representative color tissue-Doppler images and tissue-Doppler velocity traces of lateral mitral annulus, at rest (E) and at peak exercise (F), recorded in an HCM patient.

Lifetime evolution of LV function in *MYBPC3* vs MHY7 HCM

MYBPC3 and *MYH7* patients were phenotypically indistinguishable;[42,43,44] except for higher male proportion in *MYBPC3* group as confirmed from a large meta-analysis.[45] Clinical presentation and baseline echocardiographic analyses didn't show peculiar characteristics. To our knowledge our study

was the first, with a significant cohort and long follow up, that demonstrated a worsening of LV systolic function in *MYBPC3* during the natural history of the disease highlighting the need to better investigate the pathophysiological mechanism of *MYH7* and *MYBPC3* mutations. We observed that patients harbouring *MYBPC3* mutations were specifically associated with an age-related decline in LV systolic performance, while patients with *MYH7* mutations maintained an hyperdynamic LV and showed a lower incidence of LV systolic dysfunction. We know from survival analyses that both sarcomere+ (SARC) and SARC VUS patients (irrespective of the myofilament involved) have significantly long-term impairment of LV function, earlier onset of events and a higher incidence of the overall composite outcome (cardiovascular death, HF hospitalization and AF occurrence) than SARC− patients.[46,47] More likely SARC+ had reverse septal curvature morphology and more fibrosis compared to SARC negative patients;[48] however, no answer issued from the scientific world about the time course of LV systolic function in the two most frequent genetic mutations in HCM. Contrasting results were available about the pathogenic effects and the clinical course of *MYH7* and *MYBPC3* mutations. Some studies demonstrated a delayed presentation until middle age or old age and a favorable clinical course of *MYBPC3* mutations in comparison to *MYH7*.[49,50,51] Opposite results described a more extensive hypertrophy and worse outcome in *MYBPC3* patients: in particular some founder frameshift mutation in *MYBPC3* gene may evolve in LV systolic dysfunction.[52] Weissler-Snir et al found in patients ≥40 years of age that *MYBPC3* gene mutations was associated with a significant lower LVEF than *MYH7* gene mutations. [53] Moreover a small study involving 27 patients with a founder mutation in *MYBPC3* gene; these patients developed LV dysfunction after the fourth decade.[54]

Diastolic function

In our previous Study we demonstrated a distinctive clinical and biophysical feature characterize HCM associated with thin-filament mutations, at variance with the more common thick-filament disease. Thin-filament HCM was associated with higher likelihood of LV systolic dysfunction leading to functional deterioration, and more frequent occurrence of triphasic LV filling, reflecting profound diastolic dysfunction in comparison to thick-filament mutations.[55] However findings regarding diastolic function between mutations in *MYBPC3* and *MHY7* genes have been inconsistent. Interestingly, a multicenter study of SARC+ patients with HCM or with dilated cardiomyopathy showed that both *MYH7* and *MYBPC3* gene mutations were associated with left atrial dilatation compared with SARC− patients; and surprisingly patients with *MYBPC3* gene mutations had larger atrial sizes compared with those with *MYH7* gene mutations.[56] We found no difference in left atrial volume between the two genes in concordance with the results of Weissler-Snir et al. At baseline patients with *MYBPC3* gene mutations displayed a worsening of diastolic function age related, moreover only in the subgroup of patients with age≤20 years old there was a higher prevalence of advanced diastolic dysfunction in *MYH7* group. Our results displayed that patients harbouring *MYH7* gene mutations had at younger age more advanced diastolic dysfunction and progressive worsening of diastolic impairment but finally, at long term follow up, both mutations showed the similar prevalence of diastolic dysfunction. These results highlighted that *MYH7* mutations started with a more prominent diastolic dysfunction but after the course of life the two genes mutations had the same prevalence of advanced diastolic dysfunction demonstrating that some other factors such as biological senescence, microvascular dysfunction and fibrosis could play a crucial role in the disease. The real diastolic dysfunction and consequent clinical

implication such as HF and arrhythmogenesis in human HCM myocardium were driven by functional alterations at cellular and molecular level that may be targets of innovative therapies.[57]

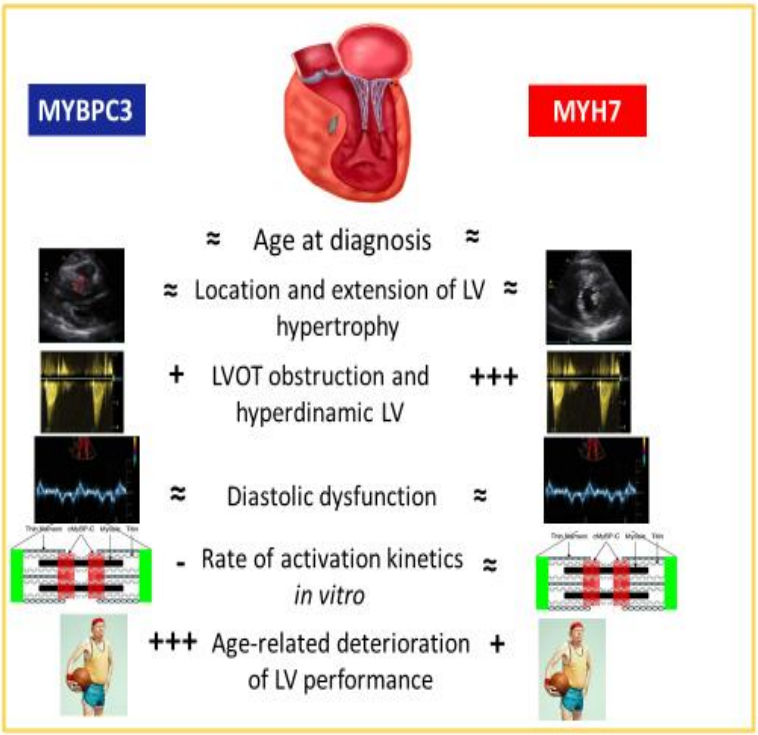
Researches of knockout and transgenic models have demonstrated the role of *MYBPC3* in cross-bridge kinetics, shortening velocity, and myocyte power output.[58,59] *MYBPC3* provided mechanical stability influencing the force activation across the sarcomere and modulate the systolic stiffening. A mutation of *MYBPC3* protein may negatively influence the sarcomeres tension and stiffness that would lead to the disruption of myocytes and trigger a progression to the LV systolic dysfunction.[60] *MYBPC3* gene mutations were more frequently protein truncation that seem to cause a more severe disease phenotype than missense mutations or in-frame deletions.[61]

Outcome-The real strength of our study was the long follow up (9$\pm$8 years) that gave the possibility to appreciate the clinical course of the disease and we did not find any change in the composite outcome except the more frequently episodes of NSVT in *MYBPC3* group that in our idea were independent from the mutation involved and don't correlate with a pathophysiological link. End stage phase was more common in *MYBPC3* group, but we didn't find a different rate of cardiovascular death or HF hospitalization. Recent advances from large international registries have shown the mortality rates of patients with HCM 3-fold higher than that of the US general population at similar ages, but all patients with HCM show a low mortality rate, with rare occurrence of sudden cardiac death compared to earlier descriptions.[62] Moreover, our results confirmed that genetic testing cannot be used in clinical decision making regarding the management strategies for AF. Genotype was not predictive of onset or severity of AF, which appeared rather driven by hemodynamic determinants of atrial dilatation. [63]

Clinical implications

MYBPC3 and *MHY7* mutations showed a similar outcome however they arrived at a common destination through different metabolic and pathophysiological pathway. (Figure 13)

Figure 13



MYBPC3 is a regulatory protein located in the A-band of the cardiac sarcomere and the majority of pathogenic *MYBPC3* variants are premature stop codons or frameshift mutations, frequently associated in absence of protein. The pathomechanism involved haploinsufficiency rather than a poison polypeptide that was more associated with *MYH7* mutations. *MYBPC3* regulated contractility and impaired the sarcomere energetics with a loss of crossbridge cycling inhibition.[64] It lead to deranged phosphorylation of contractile proteins, and reduced maximal force-generating capacity of cardiomyocytes. The enhanced Ca(2+) sensitivity in *MYBPC3* could be related to hypophosphorylation of troponin I secondary to mutation-induced dysfunction.[65] Instead more frequently the pathogenic variants in *MYH7* lead to an aminoacid substitutions in critical residues and

domains that affect the sarcomeric function. For example, the R453C *MYH7* HCM mutation showed an adverse catalytic cycle, and this mutation finally resulted in increased contractility as confirmed by our results of hyperdynamic state in *MYH7* mutations.[66] Increasing evidence suggests that HCM mutations in *MYH7* cause increased energy usage due to a less efficient myosin motor and that this energetic mismatch results in perturbed metabolic state.[67] Moreover *MYBPC3* mutation was associated with compromised LVEF implying that genetic variants of *MYBPC3* encoding mutant structural sarcomere protein could increase susceptibility to left ventricular dysfunction also in a group of patients with coronary artery disease demonstrating that *MYBPC3* may represent a genetic marker for cardiac failure also in non HCM patients.[68] Recently, Mavacamten – MYK-461, a novel allosteric reversible myosin inhibitor was designed to reversibly inhibit β-myosin binding to actin and promote the super-relaxed conformation. PIONEER HCM trial showed encouraging results in HCM patients in terms of LVOT gradient reduction, improved exercise tolerance, and symptomatic benefit.[69,70] In this scenario mavacamten, as a molecular target, reversed the Ca^{2+}-sensitive molecular and cellular changes. The reduction of peak systolic Ca^{2+} with mavacamten treatment represented a novel mechanism that is able to reduce contractility mostly in *MYH7* mutation (following our results) and working synergistically with its direct effect on the myosin motor.[71]

8. Study limitations

This study showed several limitations regarding the cohort of HCM patients analysed, in particular the results of the first part of the study highlighted a “classic phenotype” group with no advance disease status and with only mild diastolic dysfunction. Moreover, while we clinically studied only non-obstructive HCM patients, the cardiac samples we used to obtain

cardiomyocytes and trabeculae were only obtained from obstructive patients undergoing surgical myectomy. Furthermore, we didn't study the correlation between gene mutations and ECG features, but we believed that this analysis was out of the purpose of this study. Additionally, we didn't evaluate a sex-related differences in ECG features and exercise performance. Calculated METs are less robust indexes of exercise capacity than peak V_{O2} values obtained by cardiopulmonary stress testing, but they were easier to obtain and widely usable in clinical practice. Regional wall motion analysis was performed in all, but abnormalities were rare (<5%), whereas ST-T segment abnormalities during effort were more common but notoriously nonspecific in HCM, and were not considered in this study

Finally, due to the low rate of "hard" events during follow-up – a well-established feature of the natural history of HCM, especially in patients with mild disease expression such as those included in this work – the study was not able to demonstrate an association between QTc or other ECG features with outcome and in particular with arrhythmic events. The main implication of these findings is that appropriate knowledge of the molecular determinants of diastolic dysfunction may allow the identification of novel therapeutic targets for symptomatic HCM patients with exercise limitation. In current practice, the use of betablockade is justified by our data. In a future perspective, agents targeting selectively the mechanisms of impaired repolarization reserve may prove beneficial.

The major limitations of the second part of the study is the assessement of LVEF by echocardiography. Nowdays the gold standard to estimate LV volumes and function was the cardiac magnetic resonance; but the utilization of LVEF from cardiac magnetic resonance reduced significantly the patients enrolled in the study and could not give significant results.

9. **Conclusions**

In this era where new precision therapies are growing up in patients with HCM, the knowledge of molecular target and the different pathophysiologic pathway based on genetic defect were pivotal. The differences in lifetime myocardial performance between the two most common HCM-associated genes may help understand mechanisms of progression and tailor long-term preventive strategies. In adult HCM patients, mutations in the *MYBPC3* gene are associated with increased risk of advanced systolic dysfunction age-related during the course of the disease.

In the same way the abnormal balance of inward and outward ion currents in HCM ventricular cardiomyocytes determine a reduced lusitropic response to beta-adrenergic stimulation, due to insufficient APD shortening. In HCM patients, exercise lead to prolongation of QTc, accompanied by shortening in TQ interval, which in turn shortens diastolic filling time, impairs ventricular relaxation and may reduce myocardial perfusion. An abnormal response of APD to beta-adrenergic stimulation cardiomyocytes may underline this phenomenon. This impairment of "electrical" diastole likely subtends reduced exercise tolerance and stress-induced angina in patients with HCM and may constitute a promising therapeutic target. The future perspective of our results had the aim to emphasize the scientific communities to search potential novel therapies which may prevent, delay, or even reverse HCM caused by sarcomeric gene mutations. These include corrections of genetic defects, altered sarcomere function, perturbations in intracellular ion homeostasis, and impaired myocardial energetics.

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