A molecular screening strategy based on β-myosin heavy chain, cardiac myosin binding protein C and troponin T genes in Italian patients with hypertrophic cardiomyopathy

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Background **Mutations causing hypertrophic cardiomyopathy (HCM) have been described in nine different genes of the sarcomere. Three genes account for most known mutations: β-myosin heavy chain (*MYH7*), cardiac myosin binding protein C (*MYBPC3*) and cardiac troponin T (*TNNT2*). Their prevalence in Italian HCM patients is unknown. Thus, we prospectively assessed a molecular screening strategy of these three genes in a consecutive population with HCM from two Italian centres.**

Methods **Comprehensive screening of *MYBPC3*, *MYH7* and *TNNT2* was performed in 88 unrelated HCM patients by denaturing high-performance liquid chromatography and automatic sequencing.**

Results **We identified 32 mutations in 50 patients (57%); 16 were novel. The prevalence rates for *MYBPC3*, *MYH7* and *TNNT2* were 32%, 17% and 2%, respectively. *MYBPC3* mutations were 18, including two frameshift, five splice-site and two nonsense. All were 'private' except insC1065 and R502Q, present in three and two patients, respectively. Moreover, E258K was found in 14% of patients, suggesting a founder effect. *MYH7* mutations were 12, all missense; seven were novel. In *TNNT2*, only two mutations were found. In addition, five patients had a complex genotype [i.e. carried a double *MYBPC3* mutation ($n = 2$), or were double heterozygous for mutations in *MYBPC3* and *MYH7* ($n = 3$)].**

Conclusions **The first comprehensive evaluation of *MYBPC3*, *MYH7* and *TNNT2* in an Italian HCM population allowed a genetic diagnosis in 57% of the patients. These data support a combined analysis of the three major sarcomeric genes as a rational and cost-effective initial approach to the molecular screening of HCM. *J Cardiovasc Med* 7:601–607 © 2006 Italian Federation of Cardiology.**

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Introduction

Hypertrophic cardiomyopathy (HCM) is the most common genetic cardiac disease, characterized by a very heterogeneous morphologic expression and clinical course [1–4]. With an estimated prevalence in the general population of approximately 1 : 500, there are potentially over 110 000 patients in Italy alone [4,5]. Genetic screening is a relatively novel but necessary tool for a definite diagnosis of HCM, particularly in subjects with atypical or borderline phenotypic expression [6,7]. Unfortunately, the genetic background of the HCM phenotype is extremely complex and as yet only incompletely understood [8]. Several sarcomeric and non-sarcomeric protein genes have been associated with the disease, and novel culprit genes are being described every year [9–11]. Thus, a comprehensive molecular screening for HCM represents a technical and economic challenge, and is hardly feasible at any single centre.

The three most common sarcomeric genes involved in HCM were β-myosin heavy chain (*MYH7*), cardiac myosin binding protein C (*MYBPC3*) and cardiac troponin T (*TNNT2*). These genes account for a proportion of disease-associated mutations which was consistently high in Europe and the USA, despite wide fluctuations attributable to the different genetic background. In Europe, this proportion ranged from 57% in France to 26% in Sweden [10]. These genes therefore represent a logical starting point for molecular screening in Italian patients with HCM [7–10]. To test the hypothesis that comprehensive

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screening of *MYBPC3*, *MYH7* and *TNNT2* may allow a favourable yield of genotyped index cases, the present study was undertaken in a consecutive population of Italian HCM patients from two referral centres.

Patients and methods

Patient selection

The study included 88 unrelated index patients aged > 18 years of age, with a confirmed diagnosis of HCM, consecutively seen at the Azienda Ospedaliero Universitaria Careggi and at the Ospedale San Camillo subsequent to the beginning of a systematic genetic screening programme for sarcomeric mutation. Both institutions represent established centres for the management of cardiomyopathies in central Italy [2,4,5,12–16]. The diagnosis of HCM was based on the two-dimensional echocardiographic identification of a hypertrophied, nondilated left ventricle, in the absence of another cardiac or systemic disease capable of producing the magnitude of wall thickening evident [1]. Left ventricular (LV) hypertrophy was assessed by two-dimensional echocardiography, and the site of maximum wall thickness was identified. Peak instantaneous LV outflow gradient was estimated with continuous wave Doppler under basal conditions [1].

Mutational analysis

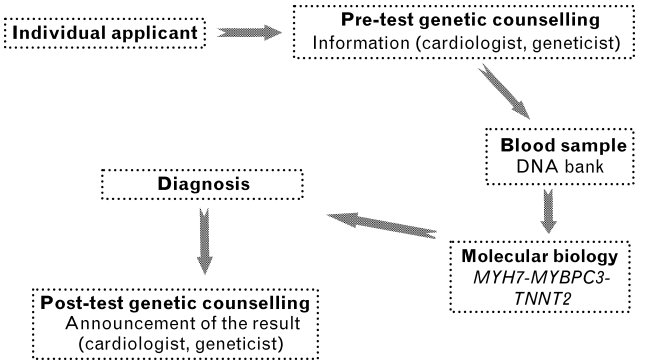
DNA extraction and polymerase chain reaction (PCR)

Following informed consent and complete family history, genomic DNA was extracted from peripheral blood according to QIAamp DNA Blood kit (Qiagen GmbH, Hilden, Germany). In-vitro amplification of all exons of *MYBPC3*, *TNNT2* and *MYH7* was performed by PCR using previously described primers [17–19].

Denaturing high-performance liquid chromatography (DHPLC) analysis and identification of mutations

Patients were screened for mutations in the protein-coding exons and splice sites of the three candidate genes. Sequence variations were detected by DHPLC using the WAVE DNA Fragment Analysis System equipped with a DNAsep column (Transgenomics, San Jose, California, USA). The conditions for DHPLC were developed on the basis of exon-specific melting profiles predicted by NAVIGATOR Software. PCR products were examined for heteroduplexes by subjecting 5 μl of each PCR product to a denaturation step (5 min at 95°C), followed by a renaturing step (30 min at 37°C). The PCR products were then separated through a 5% linear acetonitrile gradient. Commercially available WAVE Optimized buffers (A, B, D) and Syringe Solution (Transgenomics) were used to provide highly reproducible retention times [20,21]. Two melting temperatures were studied for 62 of the 87 amplicons (71%). In the remaining 25, a single temperature was used. DHPLC sensitivity was 95% when compared with automatic sequencing.

Fig. 1



Protocol employed for genetic diagnosis and counselling in the study cohort.

Abnormal DHPLC elution profiles were sequenced on automated dye terminator cycle-sequencing using an ABI Prism 3100 (Applied Biosystems, Foster City, California, USA). Every mutation identified was confirmed by a new PCR, and, whenever possible, by restriction enzyme digestion. According to standard existing guidelines, novel mutations were considered as disease-causing only if they were absent in 200 unrelated chromosomes from

Table 1 Demographic and clinical features of the 88 hypertrophic cardiomyopathy study patients

Feature	*n*(%) or mean ± SD
Institution	
Florence	68 (77%)
Rome	20 (23%)
Female	26 (29%)
Age at diagnosis (years)	42 ± 19
≤ 45 years	46 (52%)
> 45 years	42 (48%)
NYHA class	1.7 ± 0.7
I	39 (44%)
II	35 (40%)
III/IV	14 (16%)
Chest pain	25 (28%)
Syncope	10 (11%)
Atrial fibrillation	26 (29%)
Paroxysmal	16 (18%)
Chronic	10 (11%)
Baseline echocardiographic measurements	
Left atrium (mm)	44 ± 9
LV outflow gradient ≥ 30 mmHg	22 (25%)
Max. LV thickness (mm)	22 ± 6
< 25 mm	65 (74%)
≥ 25 mm	23 (26%)
Septal	85 (97%)
Apical	3 (3%)
LV end-diastolic dimension (mm)	45 ± 7
LV end-systolic dimension (mm)	27 ± 6
Interventions for obstruction/symptoms	
Alcohol septal ablation	7 (8%)
Surgical septal myectomy	4 (5%)
ICD	12 (14%)

ICD, Implantable cardioverter-defibrillator; LV, left ventricular; NYHA, New York Heart Association.

Table 2 Mutational spectrum for *MYBPC3*, *MYH7* and *TNNT2*

Gene	Exon/intron	Mutation	Number of patients	Consequences	Domain
MYBPC3	E4	**E165D**	1	Missense	Pro-Ala region
MYBPC3	E6	**E240D**	1	Missense	Mybp-C motif
MYBPC3	E6	E258K	13	Splice or missense	Mybp-C motif
MYBPC3	E12	**Y340X**	1	Nonsense	Mybp-C motif
MYBPC3	IVS12	**IVS12+1G>A**	1	Splice donor site	Mybp-C motif
MYBPC3	E17	G490R	2	Missense	C3
MYBPC3	E17	G531R	1	Missense	C3
MYBPC3	E17	R502Q	2	Missense	C3
MYBPC3	E17	**Y525S**	1	Missense	C3
MYBPC3	IVS17	E542Q	1	Splice or missense	C3
MYBPC3	E18	M555T	1	Missense	C4
MYBPC3	E23	D770N	1	Splice or missense	C5
MYBPC3	IVS24	IVS24-2A>G	1	Splice acceptor site	C6
MYBPC3	E26	**Y304fs**	1	Frameshift/ter	C6
MYBPC3	E27	Q969X	1	Nonsense	C8
MYBPC3	E30	insC1065	3	Frameshift/ter	C9
MYBPC3	E32	**G1206V**	1	Missense	C9
MYBPC3	E32	**G1206D**	1	Missense	C9
MYH7	E8	**A233S**	1	Missense	*ATP* binding
MYH7	E13	R403Q	1	Missense	Actin binding
MYH7	E16	**L601F**	1	Missense	Actin binding
MYH7	E20	R273C	1	Missense	MYL2-binding
MYH7	E21	A797T	1	Missense	Reactive thiols
MYH7	E22	R869H	7	Missense	S2domain
MYH7	E22	**R858P**	1	Missense	S2domain
MYH7	E22	**K865R**	1	Missense	S2domain
MYH7	E23	**E930Q**	1	Missense	S2domain
MYH7	E30	**T1351M**	1	Missense	Rod domain
MYH7	E30	T1377M	1	Missense	Rod domain
MYH7	E37	**L1769M**	1	Missense	Rod domain
TNNT2	E10	F110L	1	Missense	α-tropomyosin binding
TNNT2	E17	R278C	1	Missense	α-tropomyosin binding

Novel mutations are indicated in bold. MYL2, myosin essential light chain; E, exon; IVS, intervening sequences.

adult healthy controls, and produced a change in a conserved residue among species and isoforms [8,17,22]. Whenever possible, co-segregation of the mutation with the disease was sought in the proband's family. All patients and relatives received pre- and post-test genetic counselling (Fig. 1) [6].

Statistical analysis

Data were expressed as mean ± SD. Chi-square test or Fisher's exact test was utilized, as appropriate, to compare non-continuous variables expressed as proportions. Calculations were performed using SPSS 12.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

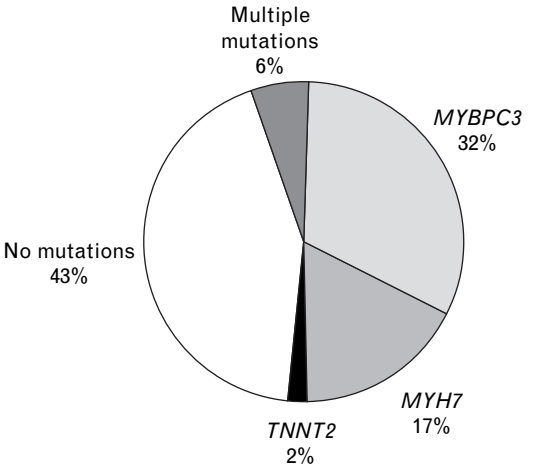
Study population

Clinical and demographic features of the 88 patients with HCM included in the study are shown in Table 1. Twenty-nine percent of patients were female, 48% were older than 45 years at diagnosis, 26% had a maximum LV thickness $\geq$ 25 mm, and 25% had dynamic LV obstruction (outflow gradient $\geq$ 30 mmHg). All 88 index cases were of Italian ethnic origin; most (75%) were born of families based in Florence, other parts of Tuscany, or in immediately adjacent areas of Umbria or Lazio for several generations. The other 25% were originally from other parts of Italy (18% from the South and Islands, 7% from the North).

Molecular screening results

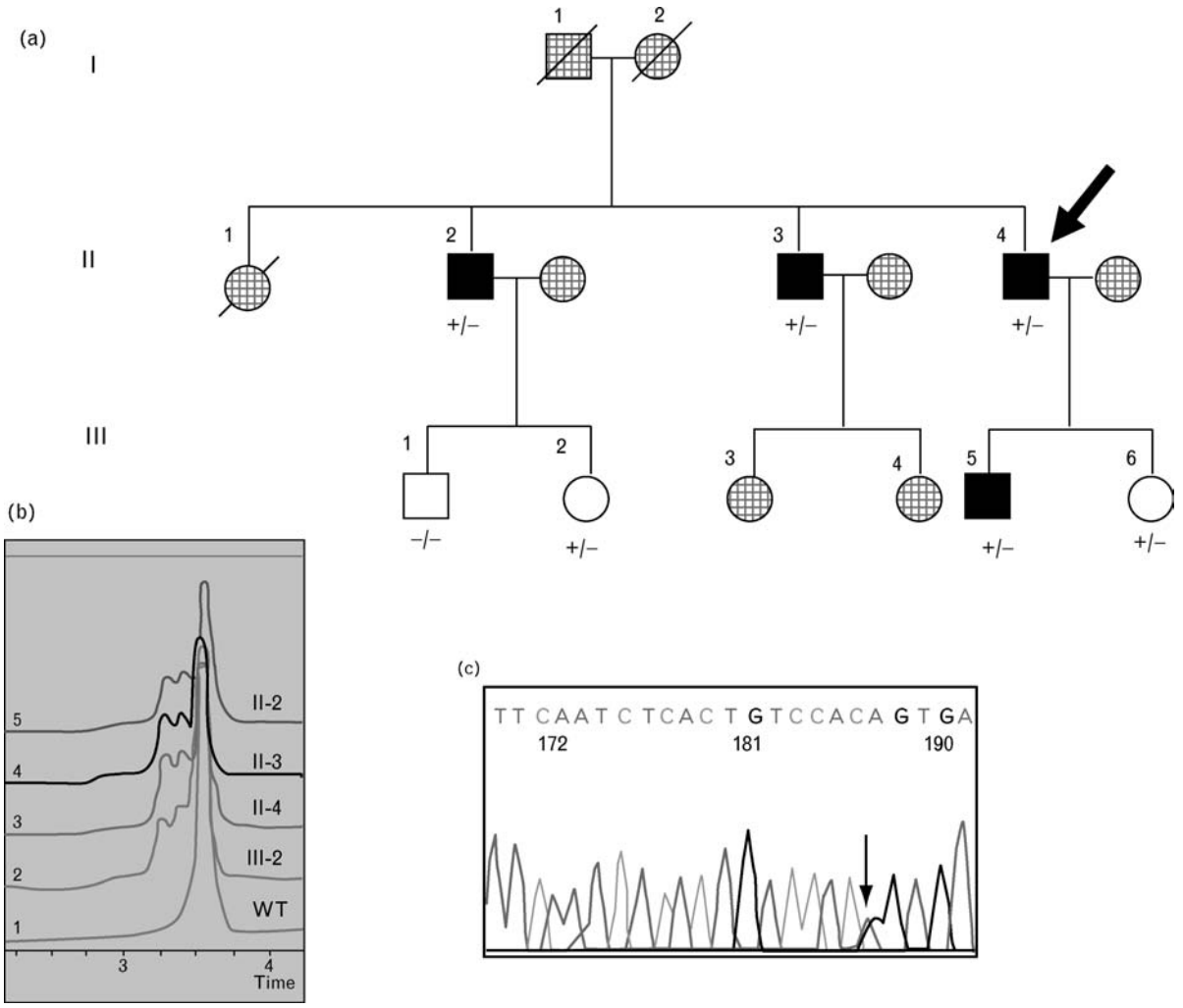
A total of 32 different mutations were identified in *MYBPC3*, *MYH7* and *TNNT2*, in 50 patients (57% of the study cohort); 16 were novel (Table 2 and Fig. 2). The most frequent gene involved was *MYBPC3*, with a

Fig. 2



Distribution of sarcomeric mutations. The relative frequency of each genotype identified in the cohort of 88 unrelated patients is indicated as a percentage of the whole cohort.

Fig. 3



Autosomal dominant transmission with incomplete penetrance in a family with MYBPC3-related hypertrophic cardiomyopathy (HCM). (a) Pedigree: circles = females; squares = males; solid symbols = genetically affected cases with HCM phenotype; open symbols = subjects with negative phenotype; stippled symbols = family members in whom clinical and genetic status was not determined. Symbols +/– and –/– indicate the presence or the absence of the *MYBPC3* mutation, respectively. (b) Abnormal DHPLC profile. (c) DNA sequence analysis showing a mutation in heterozygosity causing a glutamic acid to lysine change in the mutant myosin binding protein C aminoacidic sequence (E258K). DHPLC, Denaturing high-performance liquid chromatography; MYBPC3: myosin binding protein C; WT, wild-type.

32% prevalence. Analysis of *MYBPC3* led to identification of 18 mutations (of which nine were novel) including two frameshift, five splice-site and two nonsense mutations. All mutations were 'private' except insC1065 and R502Q, which were present in three and two unrelated patients, respectively. In addition, E258K, a known HCM-causing mutation [23], was found in 14% of the study cohort (Fig. 3). Half of the mutations ($n = 9$) clustered in two specific domains of the *MYBPC3* coding sequence: the C3, and the MyBP-C motif (Table 2).

The prevalence rates of *MYH7* and *TNNT2* mutations were 17% and 2%, respectively (Fig. 2). Analysis of *MYH7* led to identification of 12 mutations, seven of which were novel. All were missense. The R869H mutation, previously described [17], was found in seven patients (8%). Three mutations were localized in the rod domain, and four in the S2 domain. The other mutations were randomly distributed along the *MYH7* coding sequence (Table 2). In *TNNT2*, only two missense mutations were found, both previously reported (Table 2) [http://genetics.med.harvard.edu/~seidman/cg3/]. Finally, five patients (6%) had a complex-heterozygous status, characterized by two *MYBPC3* mutations (n = 2), or by associated *MYBPC3* and *MYH7* mutations (n = 3). Finally, DNA polymorphisms were found (Table 3).

Table 3 Polymorphisms in coding sequence identified in the 88 patients with hypertrophic cardiomyopathy

Polymorphism	Exon	Nucleotide change	Reference
Polymorphism identified in *TNNT2*			
Ser69Ser	9	TCG/TCA	[27]
Ile106Ile	10	ATT/ATC	[27]
Lys253Arg	17	AAG/AGG	[27]
Polymorphism identified in *MYH7*			
Thr63Thr	3	ACT/ATT	[21]
Ala199Ala	7	GCA/GCG	[21]
Phe244Phe	8	TTC/TTT	[21]
Asp325Asp	11	GAC/GAT	[21]
Gly354Gly	12	GGC/GGT	[21]
Lys365Lys	12	AAG/AAA	[21]
Asp376Asp	12	GAC/GAT	[21]
Ile989Ile	24	ATT/ATC	[21]
Ala1051Ala	25	GCG/GCA	[21]
Ser1491Cys	32	TCC/TGC	[19]
Thr1522Thr	33	ACT/ACC	[19]
Ala1702Ala	35	GCG/GCA	[19]
Polymorphism identified in *MYBPC3*			
Val58Val	4	GTG/ATG	[29]
Val189Leu	5	GTC/ATC	[28]
Ser236Gly	6	ACG/GGC	[28]
Thr262Thr	7	ACC/ACT	[28]
Arg326Gln	12	CGG/CAG	[29]
Gly416Ser	14–15	GGT/AGT	[29]
Gly507Arg	17	GGG/AGG	[29]
Leu545Arg	18	CTG/ATG	[29]
Lys814Lys	25	AAG/AAA	[29]
Val849Val	25	GTC/GTT	[29]
Val896Met	26	GTG/ATG	[29]
Glu1096Glu	30	GAA/GAG	[28]

Frequency of polymorphisms among patients and normal controls was > 0.01. All have been previously reported elsewhere in the literature, as indicated.

Correlations with phenotype

There was ample heterogeneity in the phenotypic expression of HCM, regardless of the culprit gene and site of mutation. When the distribution and prevalence of mutations were analysed with regard to the principal baseline features, a higher combined prevalence of *MYBPC3*, *MYH7* and *TNNT2* mutations was found in patients with maximum LV wall thickness ≥ 25 mm and with age at diagnosis < 45 years (the latter not reaching full statistical significance). Conversely, there was no difference with regard to gender or presence of LV outflow obstruction (Fig. 4).

Discussion

Prevalence of HCM-related mutations in Italian patients

This report represents the first comprehensive screening of *MYH7*, *MYBPC3* and *TNNT2* in Italian HCM patients [8,10,24]. We found that such a molecular screening strategy provided an effective investigational approach, allowing the identification of disease-associated mutations in over half of the patients [8,10]. Prevalence rates for *MYBPC3*, *MYH7* and *TNNT2* were 32%, 17% and 2%, respectively, with a higher yield among patients with maximum LV wall thickness ≥ 25 mm and age at diagnosis < 45 years. Of note, *MYH7* and *MYBPC3* alone accounted for 50% of mutations. These data are in substantial agreement with the existing literature, and relate to the fact that these genes are by far the largest associated with HCM [8,10,17]. Analysis of the relatively small *TNNT2* was included in our screening strategy because its mutations were originally reported as common in HCM patients, and associated with an increased risk of sudden death [1,6,7,18,24,25]. However, more recently, several groups have consistently found a very low prevalence of *TNNT2* mutations among consecutive HCM patients, and the lack of a significant association with a malignant clinical course [8,26].

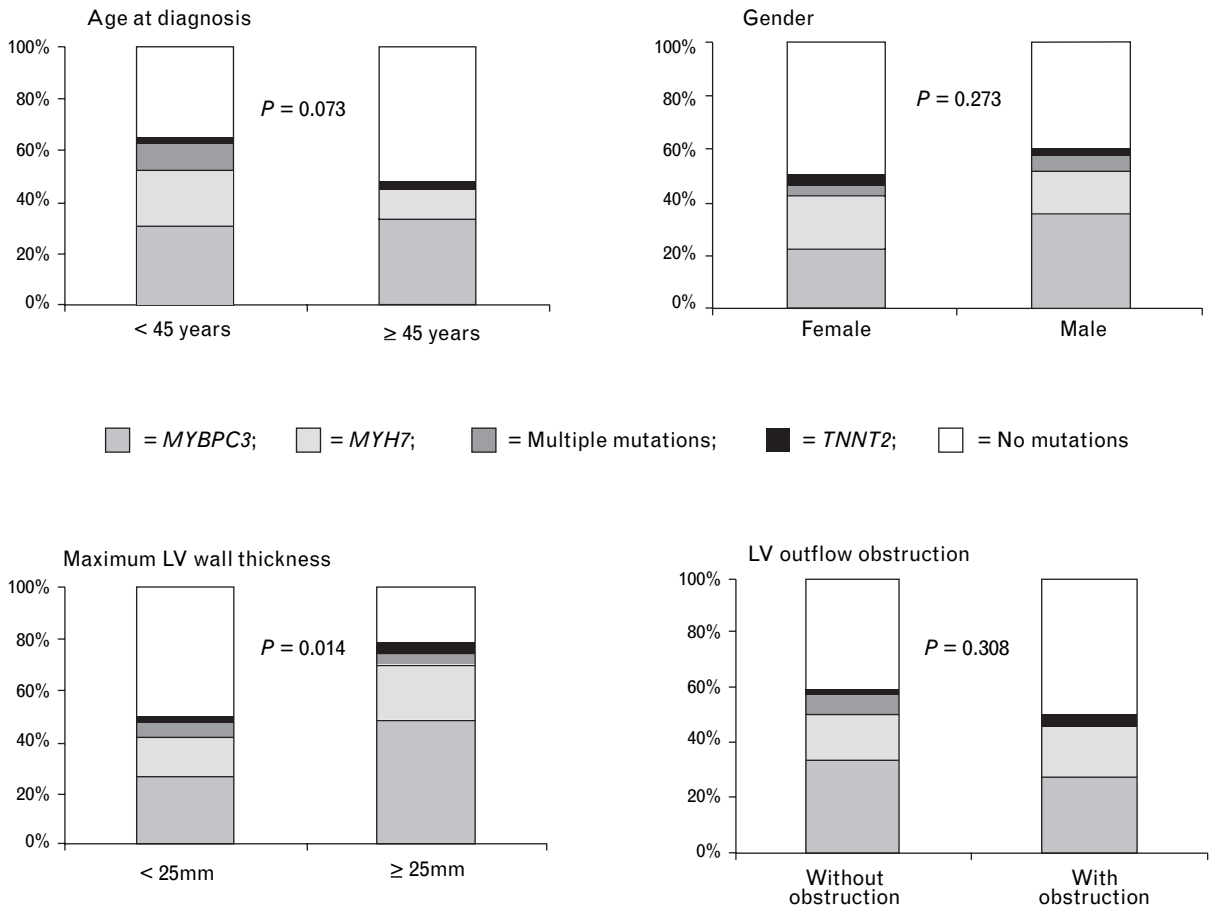
Based on these findings, the planning of an HCM screening programme should consider analysis of *MYBPC3* and *MYH7* as the initial step, whereas *TNNT2* can probably be included in a second line assessment involving the remaining five disease-associated sarcomeric genes, all of which have a relatively small size and a low prevalence of mutations. These include cardiac troponin I (*TNNI3*), α-tropomyosin 1 (*TPM1*), cardiac α-actin (*ACTC*), myosin regulatory light chain (*MYL2*) and myosin essential light chain (*MYL3*). This two-step approach should allow the genotyping of up to 60% consecutive HCM patients in most patient cohorts, such as that reported in the present study [12]. Unfortunately, over one-third of individuals with an HCM phenotype will remain genetically undiagnosed based on current understanding [10,27,28]. This group includes patients with mutations in genes that are very rare (TTN). For example, novel genes such as the human MLP gene (*CRP3*) and Tcap gene (*TCAP*) have only recently been discovered to cause HCM, and others will likely follow [29,30]. Moreover, HCM phenocopies may be caused by rare glycogen storage disease, such as Anderson Fabry disease the recently described *PRKAG2* which encodes the gamma 2 subunit of protein kinase A, or mitochondrial disease such as Danon's disease and MELAS syndrome [31–34].

Genetic heterogeneity of HCM

Over the last decade, it has become clear that comprehensive mutational screening for HCM patients represents an extremely complex, technically challenging and continuously evolving endeavour. In our cohort, mostly comprising patients from central Italian regions, 56% of identified mutations were novel, and most were 'private' (i.e. present only in one index case). Only one *MYBPC3* mutation (E258K), found in 14% of the study patients, suggested a founder effect [35,36].

Such heterogeneity is in agreement with most existing reports [8,10,26,27]. Indeed, our results suggest that the genetic background of Italian HCM patients is similar to that reported in other European countries and in the USA [8,10], and support the rationale for combined international initiatives in this field [7]. The genetic heterogeneity of HCM has been well established by the existing literature and, combined with the vast clinical heterogeneity, represents a major obstacle to

Fig. 4



Differences in the overall prevalence of sarcomeric mutations among the 88 study patients based on the principal baseline features. All comparisons were performed using the chi-square test, assessing the different yield of genetic screening between different patient subgroups. LV, Left ventricular.

genotype–phenotype correlation studies [8,10,26,27]. Ideally, molecular studies on HCM should involve a cooperative network of laboratories, each involved in the assessment of different genes, to optimize the use of available resources. In addition, screening strategies for HCM may greatly benefit from novel technologies such as the DNA-chip, enabling researchers to automatically analyse the presence of several thousand known mutations in a relatively short time [37]. However, this technology requires that most disease-causing mutations are known, whereas the prevalence of novel mutations in HCM is classically high. Therefore, although such a possibility is currently under investigation, it appears to be technically challenging [8,10].

Practical considerations

The molecular diagnosis of mutations associated with HCM has important consequences for patient families because it may allow early diagnosis in individuals who carry the mutation, and exclusion of the disease in those who do not. Therefore, these studies are often charged with unrealistic expectations in those who are unaware of the technical difficulties, time courses and practical implications involved. Therefore, although HCM patients and their families should be encouraged to undergo genetic testing, it is very important that physicians know what to say (and not to say) to those who are referred for molecular studies. First, it should be made very clear that the probability of identifying the culprit mutation in each family is well below 100%, despite recent progress in the field [10]. Moreover, it should be emphasized that genetic studies as yet have no direct influence on the clinical management of patients who already have a diagnosis of HCM, particularly with regard to the delicate issue of sudden death prevention. Finally, patients should be made well aware that these tests are time-consuming, and that results should generally not be expected in the short term. For example, the comprehensive analysis of the three genes for the present study required 13 months. In order to provide correct information to the patients, molecular testing should always be associated with adequate counselling by

dedicated geneticists, preferably in the context of a multidisciplinary team approach.

Conclusions

The first comprehensive evaluation of *MYBPC3*, *MYH7* and *TNNT2* in an Italian HCM population allowed a genetic diagnosis in 57% of a consecutive cohort of unrelated patients. These data support a combined analysis of the three major sarcomeric genes as a rational and cost-effective initial approach to the molecular screening of HCM.

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