Contractile Dysfunction Irrespective of the Mutant Protein in Human Hypertrophic Cardiomyopathy With Normal Systolic Function

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Background—Hypertrophic cardiomyopathy (HCM), typically characterized by asymmetrical left ventricular hypertrophy, frequently is caused by mutations in sarcomeric proteins. We studied if changes in sarcomeric properties in HCM depend on the underlying protein mutation.

Methods and Results—Comparisons were made between cardiac samples from patients carrying a MYBPC3 mutation (MYBPC3mut; n = 17), mutation negative HCM patients without an identified sarcomere mutation (HCMnn; n = 11), and nonfailing donors (n = 12). All patients had normal systolic function, but impaired diastolic function. Protein expression of myosin binding protein C (cMyBP-C) was significantly lower in MYBPC3mut by 33%, and similar in HCMnn compared with donor. cMyBP-C phosphorylation in MYBPC3mut was similar to donor, whereas it was significantly lower in HCMnn. Troponin I phosphorylation was lower in both patient groups compared with donor. Force measurements in single permeabilized cardiomyocytes demonstrated comparable sarcomeric dysfunction in both patient groups characterized by lower maximal force generating capacity in MYBPC3mut and HCMnn, compared with donor (26.4 ± 2.9, 28.0 ± 3.7, and 37.2 ± 2.3 kN/m², respectively), and higher myofilament Ca²⁺-sensitivity (EC₅₀ = 2.5 ± 0.2, 2.4 ± 0.2, and 3.0 ± 0.2 μmol/L, respectively). The sarcomere length-dependent increase in Ca²⁺-sensitivity was significantly smaller in both patient groups compared with donor (ΔEC₅₀: 0.46 ± 0.04, 0.37 ± 0.05, and 0.75 ± 0.07 μmol/L, respectively). Protein kinase A treatment restored myofilament Ca²⁺-sensitivity and length-dependent activation in both patient groups to donor values.

Conclusions—Changes in sarcomere function reflect the clinical HCM phenotype rather than the specific MYBPC3 mutation. Hypocontractile sarcomeres are a common deficit in human HCM with normal systolic left ventricular function and may contribute to HCM disease progression. (Circ Heart Fail. 2012;5:36–46.)

Key Words: cardiomyopathy • myofilament proteins • mutation • myocardial contraction

Hypertrophic cardiomyopathy (HCM), most often caused by mutations in genes encoding sarcomeric proteins, is a major cause of morbidity and mortality affecting ≈1:500 people worldwide at a relatively young age. It is often characterized by asymmetrical left ventricular (LV) hypertrophy, predominantly involving the interventricular septum, occurring in the absence of other cardiac or systemic disease (such as hypertension or aortic stenosis). Clinical presentation is very heterogeneous in HCM as some patients reach old age with virtually no complaints, while others progress to end-stage heart failure or die at a young age from sudden cardiac arrest. To develop a targeted treatment to prevent or delay HCM, it is highly relevant to understand the pathophysiology of this disease.

Clinical Perspective on p 46

During the last 2 decades, many disease causing mutations have been identified, mainly in genes encoding sarcomeric proteins. Despite improved genetic testing the causal gene mutation remains unidentified in over 40% of HCM patients. Furthermore, the pathophysiological mechanism leading from a
genetic defect to cardiac dysfunction is currently largely unknown. Recent studies have shown that even before hypertrophy is overt, carriers of a HCM-causing sarcomere mutation demonstrate signs of cardiac dysfunction. Echocardiographic strain analysis in preclinical HCM patients with mutations in genes encoding β-myosin heavy chain (MYH7), cardiac myosin binding protein C (MYBPC3), cardiac troponin T (TTN2T), cardiac troponin I (TNNI3), and α-tropomyosin (TPM1) demonstrated a reduced early diastolic velocity. Systolic strain and strain rates were not different from controls in preclinical HCM patients, while systolic function was diminished in HCM patients with manifest ventricular hypertrophy. Cardiac magnetic resonance evaluation of carriers with a Dutch founder mutation in MYBPC3 or a mutation in TPM1 confirmed diastolic dysfunction at the preclinical stage, evident from slower diastolic circumferential strain rate in septal and lateral regions of the LV. Tissue Doppler imaging in MYBPC3 mutation carriers without LV hypertrophy also revealed diastolic abnormalities as a first feature of preclinical HCM. Furthermore, structural abnormalities consisting of crypts were discerned in a large percentage of these preclinical patients. Fibrosis, a hallmark of overt HCM, was not visible in preclinical HCM, but serum level of the profibrotic C-terminal propeptide of type I collagen already was elevated. These data indicate that sarcomeric mutations initiate early modifications in myocardial structure and function.

Recently we have provided evidence for sarcomeric dysfunction in manifest HCM patients with truncating MYBPC3 founder mutations (c.2373dupG and c.2864_2865delCT). The sarcomeric dysfunction included a reduction in maximal force generating capacity and a higher myofilament Ca\(^{2+}\)-sensitivity compared with nonfailing human myocardium, which may be the result of altered sarcomeric protein composition as we observed haploinsufficiency (ie, reduced cardiac myosin binding protein C [cMyBP-C] protein expression) in these patients. Haploinsufficiency was confirmed and extended to HCM caused by missense MYBPC3 mutations. In the present study we investigated if the changes in sarcomeric function and protein composition are specific for the MYBPC3 mutations or are part of the more general clinical HCM phenotype.

To this end sarcomeric protein analysis was combined with force measurements in cardiac cells from patients with LV outflow tract obstruction harboring a MYBPC3 mutation, and in mutation negative HCM patients in whom no mutation was identified on screening of 9 genes that most frequently cause HCM. To investigate if sarcomeric responsiveness to increased cardiac stress (as occurs during exercise) is preserved in HCM patients, force was measured at 2 different sarcomere lengths (ie, Frank-Starling mechanism) and after treatment with protein kinase A (PKA; ie, β-adrenergic receptor stimulation).

Our study showed depressed maximal force, higher Ca\(^{2+}\)-sensitivity, and perturbed length-dependent activation of sarcomeres in HCM patients with normal LV function and impaired diastolic function irrespective of the mutant protein. The high myofilament Ca\(^{2+}\)-sensitivity may in part underlie LV diastolic dysfunction, while the hypocontractile sarcomeres (lower maximal force and impaired length-dependent activation) may contribute to disease progression in HCM. Analysis of sarcomeric protein phosphorylation \(^{15-20}\) and force measurements on single cardiomyocytes \(^{21}\) was performed as described previously.

Data Analysis
Data are presented as means±SEM. Cardiomyocyte force values were averaged per sample. The Shapiro–Wilk test was used to analyze if data from different groups followed a normal distribution. When data were distributed normally and showed equal variances, mean values for MYBPC3\(_{\text{mut}}\), HCM\(_{\text{mn}}\), and donor samples were compared using a 1-way ANOVA followed by a Bonferroni post test. When data were not distributed normally or showed unequal variances, mean values for MYBPC3\(_{\text{mut}}\), HCM\(_{\text{mn}}\), and donor samples were compared using a nonparametric Kruskal-Wallis test, followed by a Dunn Multiple Comparison Test. The effect of Ca\(^{2+}\) on force development was tested in 2-way repeated measures ANOVA using averaged data per heart. Effects of exogenous PKA treatment in single cardiomyocytes were tested with repeated measures 2-way ANOVA, or 2-way ANOVA followed by a Bonferroni post test. \(P<0.05\) was considered significant.

Results
Clinical Characteristics
Hypertrophic obstructive cardiomyopathy was evident from higher septal thickness (21±1 mm in both patient groups; normal value <13 mm) \(^{22}\) and high LV outflow tract pressure gradient (84±4 and 95±7 mm Hg; normal value <30 mm Hg) \(^{23}\) in MYBPC3\(_{\text{mut}}\) and HCM\(_{\text{mn}}\) patients, respectively (Table). LV systolic function was graded as normal (grade 1) in all patients except 1, in whom mild (grade 2) LV systolic dysfunction was observed (MYBPC3\(_{\text{mut}}\) patient 11). In contrast to the normal systolic function, impaired relaxation was observed in all patients (30% stage 1, 60% stage 2, and 10% stage 3 diastolic dysfunction).

Haploinsufficiency and Altered Phosphorylation Pattern in MYBPC3\(_{\text{mut}}\)
Figure 1A shows MYBPC3\(_{\text{mut}}\), HCM\(_{\text{mn}}\), and donor samples separated by 1D-gel electrophoresis stained with SYPRO and ProQ Diamond to determine cMyBP-C protein level (relative to α-actinin) and sarcomeric protein phosphorylation, respectively. A significantly lower cMyBP-C protein level was found in MYBPC3\(_{\text{mut}}\) hearts compared with donor myocardium (Figure 1A). Cardiac tissue was obtained from the LV septum of 17 HCM patients with a MYBPC3 mutation (MYBPC3\(_{\text{mut}}\) group), and 11 mutation negative HCM patients after detailed sequencing of 9 HCM-associated genes (MYBPC3, MYH7, TTN2T, TNNI3, MYL2, MYL3, ACTC, TPM1 and CSRP3; \(^{11}\) HCM\(_{\text{mn}}\) group) that underwent myectomy to relieve LV outflow obstruction (Morrow procedure). The MYBPC3\(_{\text{mut}}\) group comprised patients with truncating mutations c.2373dupG (n=9) and 2864–2865delCT (n=4), and splice site mutations c.927 to 2A>G (n=3) and c.1458 to 1G>C (n=1). Echocardiographic and clinical data of the patients are given in the Table. Nonfailing LV cardiac tissue was obtained from donor hearts (n=12; 13 to 65 years of age, mean 42±5 years; 8/4 male/female). The study protocol was approved by the local ethics committees and written informed consent was obtained.

Protein Analysis and Isometric Force Measurements
An extended version of the methods section is provided as an online supplement (see the online-only supplement).
1B; \( P < 0.0001 \) in 1-way ANOVA), which was not observed in HCMmn. Previous studies indicated ischemia-induced degradation of cMyBP-C at the N-terminus. To exclude that cMyBP-C degradation underlies the lower expression of full-length cMyBP-C in MYBPC3mut, Western blot analysis was performed with a specific antibody directed at the N-terminus of cMyBP-C. No degradation products were observed in any of the samples from the patients (Figure 1C) and donors. Furthermore, absence of smaller cMyBP-C products indicates absence of truncated forms of cMyBP-C (encoded by the mutated allele) in the sarcomeres.

Phosphorylation of cMyBP-C normalized to its own expression level did not differ from donor in MYBPC3mut, while it was significantly lower in HCMmn (Figure 1D; \( P = 0.001 \) in Kruskal-Wallis test). Figure 1E illustrates that, when normalized to \( \alpha \)-actinin, the level of phosphorylated cMyBP-C is independent of cMyBP-C expression in the patient groups and significantly lower in both MYBPC3mut and HCMmn compared with donor.

To further unravel the differences in cMyBP-C phosphorylation between MYBPC3mut and HCMmn, we performed Western blot analysis with phospho-specific antibodies (Figure 2A). The level of Ser273-phosphorylated cMyBP-C was significantly lower in HCMmn compared with donor, while an intermediate value was observed in MYBPC3mut (Figure 2B; \( P = 0.04 \) in Kruskal-Wallis test). Ser282-phosphorylated cMyBP-C also was significantly lower in HCMmn, but unaltered in MYBPC3mut compared with donor (Figure 2B; \( P = 0.04 \) in Kruskal-Wallis test). No significant difference was present in Ser302-phosphorylated cMyBP-C among groups (Figure 2B; \( P = 0.25 \) in 1-way ANOVA).

Phosphorylation of the other PKA target protein, cardiac troponin (cTnI), was significantly lower in both patient groups compared with donor (Figure 3A; \( P < 0.0001 \) in

### Table. Patient Characteristics

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LVOTPG indicates left ventricular outflow tract pressure gradient in mm Hg; ST, septal thickness in mm; CCB, calcium channel blocker; ATII, angiotensin II receptor antagonist; AA, antiarrhythmic agent.
Kruskal-Wallis test). The level of PKA (Ser22/23)-phosphorylated cTnI was significantly lower in both MYBPC3mut and HCMmn compared with donor (Figure 3B; \( P < 0.0002 \) in Kruskal-Wallis test), while signals at the protein kinase C site Thr143 were extremely low in all groups (data not shown), indicating that the lower overall cTnI phosphorylation in patient samples compared with donor is mainly because of lower phosphorylation at PKA sites. cTnI also was separated on a Phos-tag polyacrylamide gel and subsequently stained on Western blots with a cTnI specific antibody (Figure 3C). This analysis yielded the distribution pattern of un- (0P), mono- (1P), and bis- (2P) phosphorylated cTnI. The distribution of the cTnI forms was significantly different among the 3 groups (Figure 3D; tested in 2-way ANOVA; interaction \( P < 0.05 \)). The relative levels of un- and monophosphorylated cTnI were higher in both HCM patient groups compared with donor, while the proportion of bisphosphorylated cTnI was lower, confirming the low overall cTnI phosphorylation observed in these patients with ProQ analysis (Figure 3A).

Figure 4 shows force characteristics at a sarcomere length of 2.2 \( \mu \)m. A lower average Fmax was found in MYBPC3mut (26.4 \( \pm \) 2.9 kN/m\(^2\)) and HCMmn samples (28.0 \( \pm \) 3.7 kN/m\(^2\)), compared with donor (37.2 \( \pm \) 3.7 kN/m\(^2\); \( P = 0.04 \) in 1-way ANOVA). Figure 4A illustrates that the variability in Fmax was relatively large among individual hearts. Likewise, Fpas varied between hearts (Figure 4B) and tended to be somewhat higher in MYBPC3mut (3.5 \( \pm \) 0.6 kN/m\(^2\)) compared with HCMmn (2.4 \( \pm \) 0.3 kN/m\(^2\)) and donor (2.4 \( \pm \) 0.3 kN/m\(^2\)), but the difference was not significant (\( P = 0.68 \) in Kruskal-Wallis test).

Figure 4C shows force-calcium relations for all groups at a sarcomere length of 2.2 \( \mu \)m; force at submaximal [Ca\(^{2+}\)] was normalized to maximal force at saturating [Ca\(^{2+}\)]. The force-calcium relation in both patient groups was shifted to the left compared with donor, indicating a higher myofilament Ca\(^{2+}\)-sensitivity. The relations between force and [Ca\(^{2+}\)] were significantly different among groups (interaction...
The average value for Ca\textsuperscript{2+}-sensitivity (indicated as EC\textsubscript{50}, which is the [Ca\textsuperscript{2+}] required to reach 50% of Fmax) was significantly lower in MYBPC3 mut (2.5\(\pm\)0.2 mol/L) and HCM \textsubscript{mn} (2.4\(\pm\)0.2 mol/L) compared with donor (3.0\(\pm\)0.2 mol/L; Figure 4D; \(P<0.04\) in 1-way ANOVA). In addition, the steepness (nH) of the force-calcium relation was significantly lower in both patient groups (3.1\(\pm\)0.0.2 and 3.0\(\pm\)0.0.2 in MYBPC3 mut and HCM \textsubscript{mn}, respectively) compared with donor (3.7\(\pm\)0.1; Figure 4E; \(P<0.02\) in Kruskal-Wallis test).

Myofilament Length-Dependent Activation

To assess the length-dependent activation of myofilament contraction, force was measured at a sarcomere length of 1.8 and 2.2 \(\mu\)m (Figure 5A) in 43 cells from 13 MYBPC3 \textsubscript{mut} hearts, 36 cells from 8 HCM \textsubscript{mn} hearts, and 32 cardiomyocytes from 9 donor hearts. These measurements were performed in subsets of patients with clinical characteristics similar to the group averages, because of tissue availability. In all groups, an increase in sarcomere length from 1.8 to 2.2 \(\mu\)m increased maximal force, passive force, and Ca\textsuperscript{2+}-sensitivity of the sarcomeres. The relations between force and [Ca\textsuperscript{2+}] at both sarcomere lengths were significantly different among groups (interaction \(P<0.05\) in 2-way repeated measures ANOVA; Figure 5B). Of note, the increase in Ca\textsuperscript{2+}-sensitivity on an increase in sarcomere length from 1.8 to 2.2 \(\mu\)m (ie, \(\Delta EC_{50}\)) was significantly smaller in both patient groups (0.46\(\pm\)0.04 and 0.37\(\pm\)0.05 mol/L in MYBPC3 \textsubscript{mut} and HCM \textsubscript{mn}, respectively) compared with donor (0.76\(\pm\)0.06 mol/L; Figure 5C; \(P=0.002\) in Kruskal-Wallis test).

The resultant of a lower Fmax and higher myofilament Ca\textsuperscript{2+}-sensitivity in patient groups compared with donor is illustrated in Figure 5B. This Figure shows that the sarcomeric properties in patient groups (ie, lower Fmax, higher Ca\textsuperscript{2+}-sensitivity, and lower nH) result in depressed force development at [Ca\textsuperscript{2+}] above 3.3 and 2.5 mol/L at a sarcomere length of 1.8 and 2.2 \(\mu\)m, respectively. In contrast, below this [Ca\textsuperscript{2+}] value force development by the sarcomeres will be somewhat higher in MYBPC3 \textsubscript{mut} and HCM \textsubscript{mn} compared with donor.

Effects of Protein Kinase A

Force measurements were repeated at a sarcomere length of 2.2 \(\mu\)m in 32 MYBPC3 \textsubscript{mut} cardiomyocytes (from 12 hearts), 19 HCM \textsubscript{mn} cells (from 8 hearts), and 24 donor cells (from 8 hearts) after treatment with exogenous PKA to mimic saturated \(\beta\)-adrenergic receptor stimulation. PKA slightly reduced maximal and passive force in all groups (data not shown), while nH was not altered. The minor decreases in force were not significant and may be caused by deterioration of the preparation quality during repeated activations. Treatment of cells with PKA significantly reduced myofilament Ca\textsuperscript{2+}-sensitivity in all groups (\(P<0.0001\) in 2-way repeated measures ANOVA). The PKA-induced reduction in Ca\textsuperscript{2+}-sensitivity of force was significantly larger in MYBPC3 \textsubscript{mut} (0.91\(\pm\)0.11 mol/L) compared with donor (0.47\(\pm\)0.08 mol/L), and intermediate in HCM \textsubscript{mn} (0.73\(\pm\)0.12 mol/L; Figure 6A; interaction \(P=0.009\) in 2-way repeated measures ANOVA). PKA abolished the initial differ-

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**Figure 2.** Site-specific phosphorylation of cardiac myosin binding protein C. A, Western blot analysis with specific antibodies against phosphorylated sites in cMyBP-C. B, Significantly lower phosphorylation was observed at Ser273 and Ser282 in HCM \textsubscript{mn} compared with donor, while no significant differences in phosphorylation at Ser273, Ser282, and Ser302 were found between MYBPC3 \textsubscript{mut} and donor hearts. *\(P<0.05\) versus donor in Dunn Multiple Comparison Test or Bonferroni post test.
ence in Ca\(^{2+}\)-sensitivity of force between patient groups and donor as shown in Figure 6A. Figure 6B illustrates that after PKA, force development was depressed in patient cells compared with donor both at maximal and submaximal [Ca\(^{2+}\)] (interaction \(P=0.005\) in 2-way repeated measures ANOVA).

Interestingly, PKA pretreatment of cells significantly enhanced the sarcomere length-dependent change in Ca\(^{2+}\)-sensitivity (EC\(_{50}\)) in MYBPC3mut (4 hearts, 14 cells) and HCMmn (4 hearts, 15 cells), but not in donor (4 hearts, 12 cells; Figure 7; \(P=0.009\) in 2-way ANOVA). The blunted shift in EC\(_{50}\) on an increase in sarcomere length from 1.8 to 2.2 \(\mu\)m in patient groups compared with donor cells at baseline was absent on PKA pretreatment (Figure 7).

**Discussion**

This study is the first to compare both protein composition and function of the sarcomeres in well-defined HCM patient groups with MYBPC3 mutations and without an identified sarcomere mutation. The observed differences in sarcomere function seem to reflect the clinical HCM phenotype rather than the specific MYBPC3 mutation. Despite normal LV systolic function, the force generating capacity of the sarcomeres was lower and sarcomere responsiveness to an increase in sarcomere length was blunted (ie, reduced length dependent activation). The perturbations in sarcomere function may contribute to HCM disease progression.

**Cardiac MyBP-C Haploinsufficiency is Unique for HCM With MYBPC3 Mutations**

Our study revealed that cMyBP-C haploinsufficiency is not a general feature of HCM. As cMyBP-C exerts a role in myofilament assembly and integrity,\(^{24}\) the lower expression of cMyBP-C may underlie disease pathogenesis by disruption of myocardial structure. HCM patients with a MYBPC3 mutation were younger (\(P=0.05\)) than HCM mutation negative individuals, which suggests that the sarcomere mutation itself is a detrimental modifier of disease progression. In accordance, Olivotto et al\(^{26}\) reported an increased risk to develop severe LV dysfunction and progress to end-stage heart failure in patients with an identified sarcomere mutation (both thin and thick filament mutations) compared with mutation negative patients. Cardiac remodeling, including cardiac myocyte disarray and fibrosis, has been observed in homozygous cMyBP-C knockout mice, in which cMyBP-C was completely absent.\(^{27,28}\) At 11 months of age heterozygous cMyBP-C null mice that only expressed \(\sim75\%\) of normal cMyBP-C content showed asymmetrical septal hypertrophy and signs of fibrosis, while no morphological changes were observed at 3 to 4 months of age.\(^{28}\) Future longitudinal studies should be performed to answer the question if cMyBP-C haploinsufficiency exerts a detrimental effect on the development of hypertrophic cardiomyopathy in patients with MYBPC3 mutations.

**Reduced Phosphorylation of PKA Target Proteins**

Lower phosphorylation of cTnl compared with donor hearts was evident in both HCM groups and may reflect a post-translational phenotype-related protein modification. Low phosphorylation of cTnl compared with nonfailing donor hearts has been observed in previous studies in end-stage failing hearts from patients with idiopathic or ischemic dilated cardiomyopathy,\(^{21,20–31}\) and thus appears to be a general feature of progressive cardiac disease consistent with

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**Figure 3.** Phosphorylation forms of cardiac troponin (cTnl). A, Phosphorylation of cTnl (normalized to \(\alpha\)-actinin) was significantly lower in MYBPC3\(_{\text{mut}}\) and HCM\(_{\text{mn}}\) compared with donor samples. B, Western blot with specific antibodies against the phosphorylated PKA sites Ser22/23 on cTnl revealed significantly lower phosphorylation in both HCM patient groups than in donor. C, Cardiac samples were separated on a Phos-tag acrylamide gel to demonstrate the distribution of un- (0P), mono- (1P), and bis- (2P) phosphorylated cardiac troponin I. Minor differences in sample loading are present, but care was taken to load all samples within the linear range of cTnl detection. D, Both HCM patient groups had relatively higher levels of un- and monophosphorylated cTnl and a lower level of biphosphorylated cTnl compared with donor. \(P<0.05\) versus donor in Dunn Multiple Comparison Test.
down-regulation and desensitization of the β-adrenergic receptor pathway. Western blot analysis confirmed lower phosphorylation at the PKA sites of cTnI in both MYBPC3mut and HCMmn samples compared with donor (Figure 3B). Phosphorylation of the other PKA target protein cMyBP-C also was lower in the HCMmn group, but was relatively high in MYBPC3mut samples (Figure 1D). cMyBP-C has 3 putative phosphorylation sites (Ser273, Ser282, and Ser302) that are substrates for PKA, protein kinase C, and calcium-calmodulin–dependent kinase. Analysis of site-specific cMyBP-C phosphorylation using phospho-specific antibodies against these sites (Figure 2) confirmed the “high” phosphorylation of cMyBP-C in MYBPC3mut, as phosphorylation at all sites did not significantly differ from the donor group. A significantly lower phosphorylation at Ser273 and Ser282 was observed in the HCMmn samples compared with donor hearts. Similarly, a recent study by Copeland et al reported a reduction in total cMyBP-C phosphorylation in HCM heart samples to 39% of the value found in nonfailing myocardium. Our analysis did not reveal a significant difference in phosphorylated Ser302 between MYBPC3mut, HCMmn, and donor samples (Figure 2B). Recent studies indicated that Ser302 is phosphorylated by calcium-calmodulin–dependent kinase, which is activated on an increase in heart rate, rather than by PKA. Hence, the significantly lower total cMyBP-C phosphorylation observed in HCM compared with donor may be the result of reduced PKA-mediated phosphorylation compared with nonfailing donor myocardium. The divergent phosphorylation pattern of the PKA targets, cTnI, and cMyBP-C in the MYBPC3mut samples may be well-explained by an altered stoichiometry between protein level and PKA expression or its activity. This is illustrated in Figure 1E, which shows that the level of phosphorylated cMyBP-C, which reflects kinase activity, is independent of cMyBP-C expression in the patient groups and significantly lower in both MYBPC3mut and HCMmn compared with donor.

Lower Force Generating Capacity and Higher Ca^{2+}-Sensitivity: Common Sarcomere Features of HCM

Our data suggest that low maximal force generating capacity of sarcomeres is a general feature of HCM patients, while passive force is higher only in a few HCM samples with a MYBPC3 mutation (Figure 4). A recent study by Hoskins et al demonstrated a 40% lower maximal isometric force in 6 HCM hearts with mutations in MYBPC3, MYH7, or no identified mutation, while passive stiffness of the cardiomyocytes was not different.
from donor. Thus, the significantly lower maximal force generating capacity appears to be characteristic for the clinical HCM phenotype. Other intrinsic alterations within the sarcomeres, which have been described in diseased myocardium (eg, sarcomeric disarray, oxidative modifications) or a reduction of myofibrillar density may explain the lower maximal isometric force. Interestingly, HCM is regarded as a disease with hyperdynamic LV function, which may evolve to end-stage failure with systolic dysfunction.26 Our patient group showed normal systolic LV function. Therefore, the hypocontractile sarcomeres may not have a large impact on systolic performance, but may contribute to disease progression of HCM.

In vivo studies in preclinical HCM patients reported diastolic dysfunction as one of the earliest signs of cardiac dysfunction.6–9 In our previous studies in patients with diastolic heart failure (ie, heart failure with preserved ejection fraction), we found that passive stiffness is an important determinant of the high LV end-diastolic pressures observed in diastolic heart failure.38 In the present study, we did not find a significantly higher passive stiffness in both HCM patient groups, although Fpas was higher in several of the MYBPC3mut samples compared with nonfailing donor (Figure 4B). A reduction in myofibrillar density, which may be part of HCM myocardial remodelling, would mask stiffening of sarcomeres. In order to separate mutation- from remodelling-related changes in cardiomyocyte function, in our future studies we will investigate myofibrillar density and cardiomyocyte force characteristics in HCM in comparison with patients with aortic stenosis-induced hypertrophy. These future studies will reveal if stiffening of sarcomeres contributes to diastolic dysfunction in (preclinical) HCM patients, and may be a target for therapy in the early stage of HCM.

A higher Ca\(^{2+}\)-sensitivity of myofilaments has been reported previously in end-stage dilated and ischemic human cardiomyopathy,16,30 and has been associated with lower PKA-mediated

Figure 5. Force measurements in Triton-permeabilized single cardiomyocytes. A, Single cardiomyocyte isolated from a MYBPC3mut heart at a sarcomere length of 1.8 and 2.2 \(\mu\)m. Bar indicates 10 \(\mu\)m. Force was measured at 2 sarcomere lengths in cells isolated from 13 MYBPC3mut (43 cells), 8 HCMmn (36 cells), and 9 donor (32 cells) hearts. Cardiomyocyte force values were averaged per sample. B, Absolute force (ie, tension) plotted against \([\text{Ca}^{2+}]\) shows that force development at low \([\text{Ca}^{2+}]\) (below 3.3 and 2.5 \(\mu\)mol/L for a sarcomere length of 1.8 and 2.2 \(\mu\)m, respectively) is higher in patient groups compared with donor, while force development above this \([\text{Ca}^{2+}]\) is depressed in patient groups compared with donor. \(n\) denotes number of cells. C, The length-dependent increase in \([\text{Ca}^{2+}]\)-sensitivity of force was less in MYBPC3mut and HCMmn patient groups compared with donors. \(* P<0.05\) versus donor in Dunn Multiple Comparison Test.
phosphorylation of cTnI and cMyBP-C. Both cMyBP-C and cTnI have a central role in regulating cardiac performance in response to β-adrenergic receptor stimulation as occurs during exercise. Apart from changes in calcium handling (via phosphorylation of L-type calcium channels, ryanodine receptors, and phospholamban), which increase systolic force development, positive lusitropic effects (ie, increased relaxation) are in part mediated via a reduction in myofilament Ca\(^{2+}\)-sensitivity of force generating capacity and Ca\(^{2+}\)-sensitivity of force in all groups (12 MYBPC3mut 32 cells; 8 HCMmn, 19 cells; and 8 donors, 24 cells). The change in Ca\(^{2+}\)-sensitivity of force (ie, ∆EC\(_{50}\)) among groups before PKA was abolished on PKA treatment. B, After PKA treatment, myofilament force development at a sarcomere length of 2.2 μm was lower at maximal and sub-maximal [Ca\(^{2+}\)]\(_i\) in MYBPC3mut and HCMmn, compared with donor. n denotes number of cells. *P<0.05 versus donor; #P<0.05 effect of PKA in Bonferroni post test analyses.

Figure 6. Effect of protein kinase A on myofilament Ca\(^{2+}\)-sensitivity. A, Treatment of cells with exogenous protein kinase A (PKA) significantly reduced Ca\(^{2+}\)-sensitivity of force in all groups (12 MYBPC3mut 32 cells; 8 HCMmn, 19 cells; and 8 donors, 24 cells). The change in Ca\(^{2+}\)-sensitivity of force (ie, ∆EC\(_{50}\)) was largest in MYBPC3mut and smallest in donor hearts. The difference in Ca\(^{2+}\)-sensitivity of force (EC\(_{50}\)) among groups before PKA was abolished on PKA treatment. B, After PKA treatment, myofilament force development at a sarcomere length of 2.2 μm was lower at maximal and sub-maximal [Ca\(^{2+}\)]\(_i\) in MYBPC3mut and HCMmn, compared with donor. n denotes number of cells. *P<0.05 versus donor; #P<0.05 effect of PKA in Bonferroni post test analyses.

Blunted Length-Dependent Activation in HCM

In addition to the regulatory effect of the sarcomeres during increased sympathetic activity, the Frank-Starling mechanism, which provides a beat-to-beat regulating mechanism of systolic performance on enhanced ventricular filling (ie, increased end-diastolic volume), is caused by an increase in both maximal force generating capacity and Ca\(^{2+}\)-sensitivity of the sarcomeres. Again, one of the possible mediating proteins involved in the increased force development on an increase in sarcomere length is cMyBP-C, as the length-dependent activation of force was significantly blunted in muscle strips from cMyBP-C deficient mice. Interestingly, assessment of regional myocardial function with cardiac magnetic resonance imaging indicated blunting of the Frank-Starling mechanism in MYBPC3 mutation carriers compared with healthy individuals, which may involve mutant MYBPC3. However, as similar changes in length-dependent activation of the sarcomeres were observed in patients with and without MYBPC3 mutations, the blunted length-dependent increase in myofilament Ca\(^{2+}\)-sensitivity most likely is related to the secondary HCM disease phenotype. Konhilas et al. showed reduced length-dependent activation in myocytes from mouse myocardium harboring skeletal TnI, which lacks the PKA phosphorylation sites. Moreover, the length-dependent activation was enhanced on treatment with PKA in cardiomyocytes from nontransgenic mice. Likewise, in the present study, incubation with PKA restored the length-dependent increase in Ca\(^{2+}\)-sensitivity in both HCM groups to the response observed in donor (Figure 7). As PKA incubation...
lowered baseline Ca\textsuperscript{2+}-sensitivity, likely via phosphorylation of cTnI, this might restore the margin of Ca\textsuperscript{2+}-sensitivity response and allow for an increase on increased sarcomere length.

Limitations and Clinical Implications
As all HCM patients received drug therapy, we cannot exclude effects of medication on the outcome of our study. Recently, we have shown that β-blocker therapy (bisoprolol) in pigs with a myocardial infarction corrected sarcomeric dysfunction in infarct animals to levels found in sham animals. Moreover, maximal force development of single cardiomyocytes was significantly higher in heart failure patients who received chronic β-blocker treatment compared with patients who did not receive β-blockers. Hence, the lower maximal force of HCM cardiomyocytes compared with donors may be somewhat underestimated because of positive effects of β-blocker treatment. As overall drug treatment was similar in the HCM patient groups we assume that any effect of medication will be equal in both groups.

In conclusion, investigation of protein composition and function of the sarcomeres in a large well-characterized HCM patient group with MYBPC3 mutations and without identified mutations revealed cMyBP-C haploinsufficiency to be unique for HCM patients with MYBPC3 mutations, while lower protein phosphorylation compared with nonfailing myocardium appears common for the clinical HCM phenotype. The hypertrophic cardiomyopathies seem to be characteristic of hypertrophic cardiomyopathy patients, as our previous studies in cardiomyocytes from patients with ischemic and idiopathic cardiomyopathy showed similar maximal force development as observed in nonfailing donor cells. A lower PKA-mediated protein phosphorylation may in part underlie differences in sarcomere function between human HCM and nonfailing hearts, yielding a higher Ca\textsuperscript{2+} -sensitivity and a blunted length-dependent sarcomere responsiveness, which may explain defects in activation and relaxation of the heart muscle in HCM patients with normal LV function and diastolic dysfunction.

Acknowledgments
We thank D.H. Dekkers (Erasmus Medical Center, Rotterdam, The Netherlands) for his assistance with sample collection.

Sources of Funding
This study was supported by an iCaR-VU PhD fellowship (2007 to JvdV), the Seventh Framework Program of the European Union “BIG-HEART,” grant agreement 241577 to JvdV and LC, and a VIDI grant from The Netherlands organization for scientific research (NWO) to JvdV.

Disclosures
None.

References
Familial hypertrophic cardiomyopathy (HCM) is the most frequent inheritable cardiac disease, with a prevalence of approximately 1/500. Clinical presentation is very heterogeneous, as some patients reach old age with virtually no symptoms or events, while others progress to end-stage heart failure or die at young age from sudden cardiac arrest. To develop a targeted treatment to prevent or delay HCM, it is highly relevant to understand the pathophysiology of this disease. HCM is frequently caused by mutations in genes encoding sarcomeric proteins. Mutations in the MYBPC3 gene encoding cardiac myosin-binding protein C (cMyBP-C) represent more than 40% of all HCM cases. Previous in vivo studies in preclinical HCM patients indicate that sarcomeric mutations initiate early modifications in myocardial function. We studied if changes in sarcomeric properties in patients carrying a mutation negative HCM patients. Data from these 2 HCM patient groups were compared with hearts from nonfailing donors. Force measurements in single cardiomyocytes revealed reduced maximal force in both HCM groups compared with healthy heart. Moreover, the length-dependent activation (ie, Frank-Starling mechanism) was blunted in HCM hearts compared with healthy myocardium. A significant reduction in cMyBP-C protein level (cMyBP-C haploinsufficiency) was found only in patients carrying a MYBPC3 mutation. cMyBP-C haploinsufficiency may exert a detrimental effect on HCM development, as average age of these patients was lower compared with mutation negative HCM patients. Overall, we found hypocontractile sarcomeres in HCM patients with normal LV systolic function irrespective of the mutant protein. These perturbations in sarcomere function may contribute to HCM disease progression.
Contractile Dysfunction Irrespective of the Mutant Protein in Human Hypertrophic Cardiomyopathy With Normal Systolic Function

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*Circ Heart Fail.* 2012;5:36-46; originally published online December 16, 2011;
doi: 10.1161/CIRCHEARTFAILURE.111.963702

*Circulation: Heart Failure* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Methods

Myocardial samples
Cardiac tissue was obtained from the LV septum of 17 HCM patients with a MYBPC3 mutation (MYBPC3mut group) and 11 mutation negative HCM patients after detailed sequencing of 9 HCM-associated genes (MYBPC3, MYH7, TNNT2, TNNI3, MYL2, MYL3, ACTC, TPM1 and CSRP3)\(^1\) (HCM\(_{\text{mn}}\) group), that underwent myectomy to relieve LV outflow obstruction (Morrow procedure). The MYBPC3mut group comprised patients with truncating mutations c.2373dupG (n=9) and 2864_2865delCT (n=4) and splice site mutations c.927-2A>G (n=3) and c.1458-1G>C (n=1). Echocardiographic and clinical data of the patients are given in Table 1. LV systolic function was assessed by visual inspection of 2-D echocardiographic images (grade 1 = normal LV function; 2 = mild, 3 = moderate, and 4 = severe systolic dysfunction).\(^2\) Diastolic LV function was graded 0 to 4 as previously described: stage 0: normal LV diastolic function; stages 1 to 4: diastolic dysfunction, stage 1: impaired LV relaxation; stage 2: pseudo-normal LV filling; stage 3: reversible restrictive LV filling; stage 4: fixed restrictive dysfunction.\(^3,4\)

Non-failing LV cardiac tissue was obtained from donor hearts (n=12; 13-65 years of age, mean 42±5 years; 8/4 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.

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.

Protein analysis
Cardiac samples were treated with trichloro acetic acid prior to protein analysis to preserve the endogenous phosphorylation status of the sarcomeric proteins.\(^5\)
SYPRO Ruby and ProQ Diamond Staining of Gradient Gels

To determine cMyBP-C protein level, proteins were separated on 4-15% pre-cast Tris-HCl gels (BioRad) and stained with SYPRO Ruby. The level of cMyBP-C was expressed relative to α-actinin as described previously. The same gels were stained with ProQ Diamond to determine phosphorylation of PKA target proteins (cMyBP-C and cTnI). The phosphorylation status of cMyBP-C and cTnI was expressed relative to SYPRO-stained cMyBP-C and α-actinin, respectively.

Western blot analysis of cMyBP-C and cTnI

To detect truncated or degraded cMyBP-C samples were separated by one-dimensional gel electrophoresis on a 8% polyacrylamide SDS-gel and subsequently transferred to nitrocellulose paper by wet blotting. Specific antibodies were used directed against the N-terminal part of cMyBP-C (residues 2-14) and phosphorylated sites of cMyBP-C (Ser273, Ser282 and Ser302 mouse MYBPC3 sequence; 275, 284 and 304 in the human sequence and against phosphorylated cTnI sites (Ser22/23 and Thr143 in the human sequence) (Cell Signalling, Danvers, MA and Abcam, Cambridge, MA, respectively).

Phos-Tag acrylamide gels

In addition, the recently developed Phos-tag™ acrylamide (FMS Laboratory; Hiroshima University, Japan) was used to visualize phosphorylated cTnI species using alkoxide-bridged dinuclear metal (Mn²⁺) complex as phosphate-binding tag (Phos-tag) molecule. Mn²⁺-Phos-tag molecules preferentially capture phosphomonoester dianions bound to Ser, Thr and Tyr residues. Non-phosphorylated and phosphorylated cTnI species were separated in 1D-PAGE with polyacrylamide-bound Mn²⁺-Phos-tag and transferred to Western blots. Phosphorylated cTnI species in the gel are visualized as slower migration bands compared to the corresponding dephosphorylated cTnI form.

Isometric force measurements

Cardiomyocytes were mechanically isolated from small tissue samples as described previously. Triton-permeabilized cardiomyocytes were glued between a force transducer
and a piezoelectric motor. Force measurements were performed at various [Ca\(^{2+}\)] and sarcomere lengths of 1.8 and 2.2 \(\mu\)m. Passive tension (Fpas) was determined by slackening the cell in relaxing solution by 30%. Maximal calcium activated tension (Fmax, i.e. maximal force/cross-sectional area) was calculated by subtracting Fpas from the total force at saturating [Ca\(^{2+}\)]. Ca\(^{2+}\)-sensitivity is denoted as EC\(_{50}\), i.e. [Ca\(^{2+}\)] at which 50% of Fmax is reached. Force measurements were repeated after incubation of cells for 40 minutes at 20ºC in relaxing solution containing the catalytic subunit of PKA (100 U/mL, Sigma).

**Data Analysis**

Data are presented as means ± S.E.M. Cardiomyocyte force values were averaged per sample. The Shapiro–Wilk test was used to analyze if data from different groups followed a normal distribution. When data were normally distributed and showed equal variances mean values for MYBPC3\(_{mut}\), HCM\(_{mn}\) and donor samples were compared using a one-way ANOVA followed by a Bonferroni post-test. When data were not normally distributed and/or showed unequal variances mean values for MYBPC3\(_{mut}\), HCM\(_{mn}\) and donor samples were compared using a non-parametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test. The effect of Ca\(^{2+}\) on force development was tested in two-way repeated measures ANOVA using averaged data per heart. Effects of exogenous PKA treatment in single cardiomyocytes were tested with repeated measures two-way ANOVA or two-way ANOVA, followed by a Bonferroni post-test. P<0.05 was considered significant.

**References**


