

# Multiple Gene Variants in Hypertrophic Cardiomyopathy in the Era of Next-Generation Sequencing

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**Background**—Multiple likely pathogenic/pathogenic (LP/P;  $\geq 2$ ) variants in patients with hypertrophic cardiomyopathy were described 10 years ago with a prevalence of 5%. We sought to re-examine the significance of multiple rare variants in patients with hypertrophic cardiomyopathy in the setting of comprehensive and targeted panels.

**Methods and Results**—Of 758 hypertrophic cardiomyopathy probands, we included 382 with  $\geq 45$  cardiomyopathy genes screened. There were 224 (59%) with  $\geq 1$  rare variant (allele frequency  $\leq 0.02\%$ ). Variants were analyzed using varying sized gene panels to represent comprehensive or targeted testing. Based on a 45-gene panel, 127 (33%) had a LP/P variant, 139 (36%) had variants of uncertain significance, and 66 (17%) had multiple rare variants. A targeted 8-gene panel yielded 125 (32%) LP/P variants, 52 (14%) variants of uncertain significance, and 14 (4%) had multiple rare variants. No proband had 2 LP/P variants. Including affected family members (total  $n=412$ ), cluster-adjusted analyses identified a phenotype effect, with younger age (odds ratio, 0.95; 95% confidence interval, 0.92–0.98;  $P=0.004$ ) and family history of sudden cardiac death (odds ratio, 3.5; 95% confidence interval, 1.3–9.9;  $P=0.02$ ) significantly more likely in multiple versus single variant patients when considering an 8-gene panel but not larger panels. Those with multiple variants had worse event-free survival from all-cause death, cardiac transplantation, and cardiac arrest (log-rank  $P=0.008$ ).

**Conclusions**—No proband had multiple LP/P variants in contrast to previous reports. However, multiple rare variants regardless of classification were seen in 4% and contributed to earlier disease onset and cardiac events. Our findings support a cumulative variant hypothesis in hypertrophic cardiomyopathy.

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**Key Words:** cardiomyopathy, hypertrophic ■ genetic testing ■ genetic variation ■ heart ventricles ■ hypertension

Hypertrophic cardiomyopathy (HCM) is a myocardial disease characterized by left ventricular (LV) hypertrophy in the absence of loading conditions, such as hypertension. With a prevalence of 1 in 200 to 500,<sup>1,2</sup> it is one of the most common cardiac genetic diseases. At least 15 genes have been described in HCM,<sup>3,4</sup> and to date, thousands of variants have been published. Variants in *MYBPC3* (20%–30%), *MYH7* (30%–35%), and *TNNT2* (10%–15%) account for the majority of genotyped cases, followed by variants in *TPMI* (<5%), *TNNI3* (<5%), *ACTC1* (<1%), *MYL2* (<1%), and *MYL3* (<1%). More recently, evidence for variants in other genes, including *ACTN2*, *CSRP3*, *MYOZ2*, *NEXN*, and *TNNC1*, has emerged.<sup>3–6</sup>

## See Clinical Perspective

Genetic testing in HCM is standard clinical practice and a class 1 recommendation for a patient with a clinical diagnosis.<sup>4</sup> Targeted testing refers to inclusion of only the well-established major HCM genes and known phenocopies (ie, *GLA*, *LAMP2*, *PRKAG2*, and *PLN*) whereas comprehensive

panels can include vast numbers of cardiac genes, many having minimal or no evidence of association with an HCM phenotype. Once a genetic diagnosis is established in the proband, cascade genetic testing can be offered to asymptomatic family members, offering a unique opportunity to guide clinical surveillance recommendations.<sup>7</sup> Efforts to identify the underlying genetic cause of disease are, therefore, important but present significant challenges. Rare variation is not uncommon, both in general and disease populations, and increased stringency of variant curation efforts in recent years seeks to ensure that variants are not incorrectly attributed to disease.<sup>8</sup>

We, and others, have shown up to 5% of genetically tested probands will have compound or double heterozygous genotypes,<sup>9–13</sup> and it has been suggested this may confer a more severe phenotype in both animals and humans, including risk of sudden cardiac death (SCD).<sup>14–16</sup> In light of the vast cardiac gene panels that are now commonplace, coupled with increasingly stringent variant interpretation, the landscape of HCM genetic testing is now markedly different. We sought to re-examine the prevalence of multiple variant genotypes in HCM

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and determine the clinical significance using both comprehensive and targeted gene panel approaches.

## Methods

### Consecutive Patient Series

Probands attending a specialized HCM center between 2002 and 2016 who met clinical diagnostic criteria for HCM and provided a DNA sample for comprehensive 45-gene panel testing were included. The proband was defined as the first affected family member who sought medical advice for HCM within our clinic. A clinical diagnosis was made based on a maximal LV wall thickness  $\geq 15$  mm in adults in the absence of a loading condition.<sup>15,16</sup> All aspects of the study were performed according to institutional human research ethics committee approval. Local human research ethics approval gave permission for the study, and all participants gave informed consent.

### Genetic Analysis

DNA was isolated from peripheral blood using a QIAmp DNA blood mini kit (Qiagen, Limburg, NL). The majority of participants ( $n=264$ ) underwent genetic testing using the Illumina TruSight Cardiomyopathy Sequencing Panel, which enriches for 1020 exons spanning 46 cardiomyopathy genes (*TTN* was subsequently excluded from analysis). Genomic DNA (50 ng) was enriched for target exons in strict accordance with the manufacturer's protocol and sequenced on an Illumina MiSeq platform (Ramaciotti Centre for Genomics, Australia). Raw sequence data were analyzed at the Centenary Institute with alignment to the reference genome (hg19) using BWA software, followed by duplicate read removal using Novosort (Novocraft Technologies, Malaysia). The Genome analysis Tool Kit v3.3.0 was used to realign reads around insertions and deletions and recalibrate base quality scores and genotype variants. Single nucleotide variants were required to have (1) a quality by depth score of at least 2.0, (2) a Fisher strand score of  $<60$ , (3) a read position rank-sum score  $>-8.5$ , (4) a mapping quality score of at least 35.0, and (5) a mapping quality rank-sum test score of at least  $-15.0$ . Indels were required to have (1) a Fisher strand score of  $<200$ , (2) a quality by depth score of at least 1.8, (3) a read position rank-sum score  $>-20.0$ , and (4) an inbreeding coefficient score of at least  $-0.8$ . Variants were annotated using the SeattleSeq Annotation Server (<http://snp.gs.washington.edu/SeattleSeqAnnotation138/>).

For 82 participants, exome sequencing was performed as previously described.<sup>17</sup> Only the 45 cardiomyopathy genes (excluding *TTN*) of the Illumina TruSight Cardiomyopathy Sequencing Panel were considered in this analysis. In addition, 39 participants underwent comprehensive genetic testing ( $\geq 45$  genes) by commercial testing laboratories using either massively parallel sequencing or Sanger sequencing-based approaches and variant data from their gene report was included (variants with an allele frequency of  $<1\%$  were reported, allowing equivalent variant lists to be generated). An additional 3 probands with  $\geq 2$  variants identified despite limited Sanger sequencing of  $\leq 7$  genes were included because they could be considered as having  $\geq 2$  variants.

### Single and Multiple Rare Variant Identification

Genetic data were analyzed according to different sized cardiac gene panels to reflect both comprehensive and targeted genetic testing approaches. Genes included in each panel are shown in Table 1. All rare variants (excluding variants in *TTN* and synonymous and intronic changes) with a minor allele frequency of  $\leq 0.02\%$  in the Exome Aggregation Consortium data set (<http://exac.broadinstitute.org/>) and classifications of likely pathogenic/pathogenic (LP/P) and variants of uncertain significance (VUS) were included.<sup>18</sup>

Single (isolated) variants were defined as those seen only in the absence of another potentially LP/P variant within our patient population. Variants seen only in combination with another potentially LP/P variant only (ie, not seen in an HCM proband in isolation in our population) were termed second variants. Variant characteristics between single and second variants were compared.

### Variant Classification

Rare variants were those with an allele frequency of  $\leq 0.02\%$  regardless of their classification. Clinical classifications were performed using in-house criteria (see ClinVar, Agnes Ginges Centre for Molecular Cardiology variant assessment and assertion criteria; [https://submit.ncbi.nlm.nih.gov/ft/byid/djgybgii/mdi-5363\\_505375\\_agnesginges\\_variantassess\\_clinvar.pdf](https://submit.ncbi.nlm.nih.gov/ft/byid/djgybgii/mdi-5363_505375_agnesginges_variantassess_clinvar.pdf)). Key determinants of pathogenicity included rarity ( $\leq 0.02\%$ ) or absence from the Exome Aggregation Consortium data set, previous reports of the variant in  $\geq 2$  additional unrelated patients with HCM (<http://ncbi.nlm.nih.gov/pubmed> and <http://clinvar.com/>), segregation with affected relatives where possible, as well as any

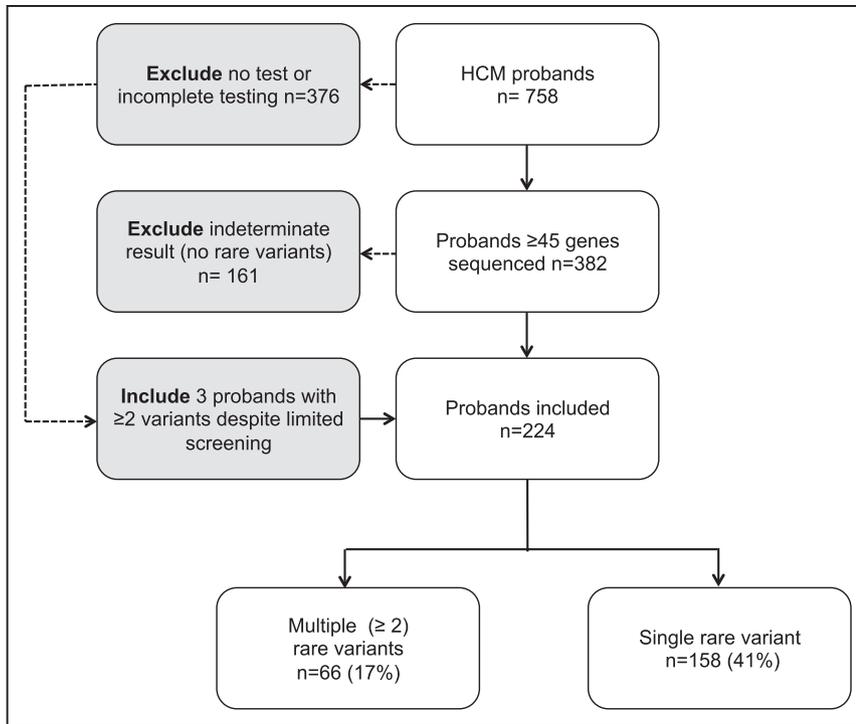
**Table 1. Yield of Rare Variants Based on Different Sized Gene Panels Analyzed in the Study**

| Panel  | Genes  | Yield of Rare Variants Identified in Probands, n (%) |          |          |
|--|--|--|----------|----------|
|  |  | LP/P   | VUS      | Multiple |
| Two-gene panel                                     | <i>MYBPC3, MYH7</i>  | 107 (28)   | 41 (11)  | 12 (3)   |
| Five-gene panel                                    | <i>MYBPC3, MYH7, TNNI3, TNNT2, TPM1</i>  | 123 (32)   | 47 (12)  | 13 (3)   |
| Major HCM genes (8-gene panel)                     | <i>ACTC1, MYBPC3, MYH7, MYL2, MYL3, TNNI3, TNNT2, TPM1</i>   | 125 (32)   | 52 (14)  | 14 (4)   |
| Major HCM genes and phenocopies*                   | <i>ACTC1, MYBPC3, MYH7, MYL2, MYL3, TNNI3, TNNT2, TPM1, PLN*, GLA*, LAMP2*, PRKAG2*</i>  | 127 (33)   | 56 (15)  | 15 (4)   |
| Major and minor HCM genes (15-gene panel)          | <i>ACTC1, ACTN2, CSRP3, MYBPC3, MYH7, MYL2, MYL3, MYOZ2, NEXN, PLN, TNNC1, TNNI3, TNNT2, TPM1, TTR</i>   | 126 (33)   | 66 (17)  | 22 (6)   |
| Major and minor HCM genes and phenocopies†         | <i>ACTC1, ACTN2, CSRP3, MYBPC3, MYH7, MYL2, MYL3, MYOZ2, NEXN, PLN*, TNNC1, TNNI3, TNNT2, TPM1, TTR, GLA*, LAMP2*, PRKAG2*</i>   | 127 (33)   | 70 (18)  | 22 (6)   |
| Comprehensive cardiomyopathy panel (45-gene panel) | <i>ABCC9, ACTC1, ACTN2, ANKRD1, CASQ2, CAV3, CRYAB, CSRP3, CTF1, DES, DSC2, DSG2, DSP, DTNA, EMD, FHL2, GLA, JUP, LAMA4, LAMP2, LDB3, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOZ2, NEXN, PKP2, PLN, PRKAG2, RBM20, RYR2, SGCD, TAZ, TCAP, TMEM43, TNNC1, TNNI3, TNNT2, TPM1, TTR, VCL</i> | 127 (33)   | 139 (36) | 66 (17)  |

HCM indicates hypertrophic cardiomyopathy; LP/P, likely pathogenic or pathogenic; and VUS, variants of uncertain significance.

\*The ideal gene panel for initial HCM proband genetic testing.

†Typical commercial HCM panel.



**Figure 1.** Participant flowchart. HCM indicates hypertrophic cardiomyopathy.

supportive experimental data. Overall agreement among in silico tools and conservation scores was considered a single low-level supportive criterion (Combined Annotation-Detection Depletion, Sorting Intolerant From Intolerant [<http://sift-dna.org/>], Polyphen-2 [Polymorphism phenotyping Ver2 <http://genetics.bwh.harvard.edu/pph2/>], Polyphen-HCM [<http://genetics.bwh.harvard.edu/hcm/>]). Only variants in major HCM and phenocopy genes (ie, 15-gene panel) were classified according to the above criteria as per current guidelines on variant interpretation.<sup>8</sup> Classifications included pathogenic (class V), likely pathogenic (class IV), and VUS (class III). Family segregation of variants was performed where possible.

### Clinical Assessment

Probands and their relatives with a clinical diagnosis of HCM and genetic diagnosis confirming the presence of single or multiple rare variants were included. Clinically unaffected gene carriers were not included. An additional 30 relatives with a clinical diagnosis of HCM and known genotype were included, either in the multiple or single variant gene groups. There were no more than 5 relatives from a single family. Genotype of relatives was based on cascade genetic testing of the identified family variants. Clinical information was obtained by review of the medical record and from the Australian Genetic Heart Disease Registry.<sup>19</sup> An SCD event included sudden death, aborted cardiac arrest, or appropriate implantable cardioverter defibrillator shock for ventricular fibrillation. A 3-generation pedigree was collected by a cardiac genetic counselor as per clinic practice. A positive family history of HCM was defined as  $\geq 2$  individuals in a family with clinical evidence of HCM while a family history of SCD included any sudden death of a relative, at any age, with confirmed or probable HCM based on pre-morbid investigations, death certificate, or post-mortem examination.

### Statistical Analysis

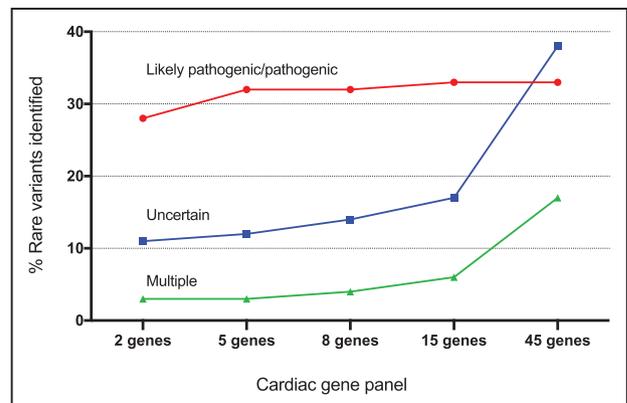
Data were analyzed using Prism (version 6.0), SPSS Statistics (version 22.0), and SAS University Edition (SAS Studio 3.3). Associations between variables and outcome factors were assessed using unpaired *t* tests for continuous data and  $\chi^2$  analysis for categorical data. Logistic regression with generalized estimating equations was used to account for clustering within families when comparing multiple versus single variant patient clinical characteristics. A Kaplan–Meier plot with

log-rank tests for significance was used to assess event-free survival (from all-cause death, cardiac transplant, resuscitated cardiac arrest, or implantable cardioverter defibrillator shock because of ventricular fibrillation; first event only) between patients with single and multiple rare variants in 8 major HCM genes. A  $P < 0.05$  was considered statistically significant.

## Results

### Variant Characteristics

Of 758 HCM probands, 382 had at least 45 genes sequenced and were included (summarized in Figure 1). An additional 3 probands with  $\geq 2$  variants identified in spite of limited Sanger sequencing of  $\leq 7$  genes were included because they could be considered as having  $\geq 2$  variants. Of the total group, 224 (59%) had  $\geq 1$  rare variant identified (Table 1; Figure 2). We identified a total of 151 different variants in 176 probands using the 15 HCM gene panel (Table I in the [Data Supplement](#)). There were 81 (54%) variants classified as LP/P, 50 (34%) variants were novel, and 111 (74%) were missense variants.



**Figure 2.** Yield of likely pathogenic/pathogenic, uncertain, and multiple rare variants by cardiac gene panel size.

**Table 2. Families With Multiple Rare Variants in the 8-Gene HCM Panel**

| Family          | Gene Variants   | Proband  | Family History  |
|-----------------|---|--|---|
| AJV             | <i>MYBPC3</i><br><i>p.Leu994Phe</i><br><i>MHY7</i><br><i>p.Arg652Gly</i>                          | Not index case<br>Age dx: 17<br>Max LVH: 17 mm<br>SCDE: No                         | Father (index case) died awaiting cardiac transplant (carried both variants)<br>Two possibly affected relatives |
| ALK             | <i>MYBPC3</i><br><i>p.Gly5Trp</i><br><i>TNNT2</i><br><i>p.Arg286Cys</i>                           | Index case<br>Age dx: 62<br>Max LVH: 20 mm<br>SCDE: No                             | No family history of HCM or SCD   |
| ARX             | <i>MYBPC3</i><br><i>p.Val219Leu</i><br><i>MYBPC3</i><br><i>p.Trp1112dup</i><br>(phase, in trans)  | Not index case<br>Age dx: 17<br>Max LVH: 14 mm<br>SCDE: No                         | Brother (index case) died suddenly 13 y (carried both variants)<br>Distant relative reportedly affected         |
| BET             | <i>MYBPC3</i><br><i>p.Glu258Lys</i><br><i>MYBPC3</i><br><i>p.Val727Met</i><br>(phase, unknown)    | Index case<br>Age dx: 53<br>Max LVH: 22 mm<br>SCDE: No                             | No family history of HCM or SCD   |
| BGK             | <i>MYBPC3</i><br><i>p.Arg589Cys</i><br><i>MYL2</i><br><i>p.Lys104Thr</i>                          | Index case<br>Age dx: 14<br>Max LVH: 26 mm<br>SCDE: No                             | No family history of HCM or SCD   |
| BJJ             | <i>MYH7</i><br><i>p.Ala797Thr</i><br><i>MYH7</i><br><i>p.Arg807His</i><br>(phase, unknown)        | Index case<br>Age dx: 16<br>Max LVH: 22<br>SCDE: No                                | No family history of HCM or SCD   |
| BKZ             | <i>MYBPC3</i><br><i>p.Ser311Thr</i><br><i>MYBPC3</i><br><i>c.927-9G&gt;A</i><br>(phase, unknown)  | Index case<br>Age dx: 22<br>Max LVH: 24<br>SCDE: No                                | Three affected relatives<br>Reported history of sudden death <35 in relative                                    |
| C <sup>11</sup> | <i>MYBPC3</i><br><i>p.Arg273His</i><br><i>MYH7</i><br><i>p.Arg719Gln</i>                          | Not index case<br>Age dx: 13<br>Max LVH: 20<br>SCDE: No<br>Other: Heart transplant | Three affected relatives (5 possibly affected)<br>Mother died 44 y with heart failure<br>Brother SCD 16 y       |
| FQ              | <i>MYBPC3</i><br><i>p.Arg724Trp</i><br><i>MYBPC3</i><br><i>c.1624+4A&gt;T</i><br>(phase, unknown) | Index case<br>Age dx: 8<br>Max LVH: 19<br>SCDE: No                                 | One possibly affected relative<br>No SCD  |

(Continued)

**Table 2. Continued**

| Family           | Gene Variants   | Proband  | Family History   |
|------------------|---|--|--|
| NM <sup>20</sup> | <i>MYBPC3</i> ;<br><i>p.Gln969*</i><br><i>MYBPC3</i> ;<br><i>p.Arg668His</i><br>(AF>0.02%;<br>phase, in cis)<br><i>MYH7</i> ;<br><i>p.Arg1079In</i> | Not index case<br>Age dx: 48<br>Max LVH: 19<br>SCDE: No  | 4 affected relatives in extended family<br>(3 possibly affected)<br>No SCD                                   |
| PE               | <i>MYBPC3</i><br><i>p.Leu527Pro</i><br><i>MYBPC3</i><br><i>c.1928-2A&gt;G</i><br>(phase, in trans)  | Index case<br>Age dx: Infant<br>Max LVH: 24<br>SCDE: Aborted cardiac arrest<br>ICD shocks for VF | Two affected relatives<br>Two possibly affected<br>Maternal great uncle died suddenly aged 30 y              |
| VN               | <i>MYBPC3</i><br><i>p.Ala693Val</i><br><i>MYBPC3</i><br><i>p.Arg817Gly</i><br>(phase, unknown)  | Index case<br>Age dx: 12<br>Max LVH: 34<br>SCDE: No  | No family history of HCM or SCD  |
| WJ               | <i>MYH7</i><br><i>p.Asp778Val</i><br><i>MYH7</i><br><i>p.Arg1818Trp</i><br>(phase, in trans)  | Not index case<br>Age dx: 30<br>Max LVH: 18<br>SCDE: No  | One affected relative<br>Brother (index case) died suddenly aged 34 y ( <i>MYH7</i> <i>p.Asp778Val</i> only) |
| DW               | <i>MYH7</i><br><i>p.Ile313Phe</i><br><i>MYBPC3</i><br><i>p.Trp792fs*41</i>  | Not index case<br>Age dx: 16<br>Max LVH: 21<br>SCDE: ICD shock for VF                            | One affected relative<br>Father died suddenly, details limited<br>No pedigree available                      |

Index case=first person in the family to present. Proband=first person to present to our clinical service. Genotype of affected relatives shown where data available. Age dx indicates age at diagnosis; HCM, hypertrophic cardiomyopathy; ICD, implantable cardioverter defibrillator; max LVH, maximum left ventricular hypertrophy (mm); SCD, sudden cardiac death; SCDE, sudden cardiac death events; and VF, ventricular fibrillation.

### Comprehensive Versus Targeted Genetic Testing Yield

Using a comprehensive gene panel (45 genes), the yield of patients with LP/P variants was 127 (33%), 126 (33%) for 15 genes, 125 (32%) for 8 genes, and 107 (28%) when considering *MYBPC3* and *MYH7* only. The yield of patients with VUS increased with increasing panel size with 139 (36%) identified using a 45-gene panel, 66 (17%) for 15 genes, 52 (14%) for 8 genes, and 41 (11%) when considering *MYBPC3* and *MYH7* only (Figure 2).

### Multiple Rare Variants

There were 66 (17%) probands with at least 2 rare variants when considering 45 genes, 22 (6%) for 15 genes, 14 (4%) for 8 genes, and 12 (3%) probands had at least 2 rare variants in *MYBPC3* and *MYH7* (Figure 2; Table 2). Of these, 7 (2%)

probands had 3 rare variants. No proband had 2 LP/P variants, rather there were combinations of LP/P and VUS or VUS/VUS. Phase of the variants was established where possible with families demonstrating variants both in *cis* and *trans*, as well as families with double variants in different genes (Table 2; Family pedigrees and information in the [Data Supplement](#)).

### Comparison of Variant Characteristics

There were 107 rare variants identified in the 15-gene panel that were seen in isolation (single variants) and 32 identified only in combination with another variant (second variants). Single variants were more likely to be in the 8 major HCM genes (99 [93%] versus 25 [78%];  $P=0.04$ ), had lower allele frequency in Exome Aggregation Consortium (0.0004% versus 0.001%;  $P=0.03$ ), higher mean Combined Annotation-Detection Depletion score ( $23\pm 7$  versus  $19\pm 6$ ;  $P=0.006$ ), be deleterious using Polyphen-HCM (31/35 [89%] versus 4/9 [44%];  $P=0.01$ ), be classified LP/P (66 [62%] versus 4 [13%];  $P<0.0001$ ), and less likely to be novel (17 [53%] versus 32 [30%];  $P=0.02$ ) compared with variants seen only in combination with a second potentially causative variants (Table 3).

### Impact on Clinical Phenotype

To assess impact on clinical phenotype, we included an additional 30 relatives with a clinical diagnosis of HCM in the single or multiple group depending on how many variants they carried. There was no phenotypic effect observed between individuals with multiple ( $n=70$ ) versus single ( $n=184$ ) rare variants when considering the comprehensive 45-gene panel. However, when comparing individuals with a single ( $n=178$ ) versus those with multiple rare variants ( $n=18$ ) in the 8 major HCM genes, a difference

**Table 3. Characteristics of Variants Seen in Isolation (Isolated Variants) Vs Variants Only Seen in Combination With Causative HCM Variants (Second Variants)**

| Variable                              | Isolated Variant | Second Variant | P Value   |
|---------------------------------------|------------------|----------------|-----------|
| n (%)                                 | 107 (72)         | 32 (22)        | ...       |
| 8 Major HCM genes                     | 99 (93)          | 25 (78)        | 0.04      |
| Missense variant                      | 77 (72)          | 26 (81)        | 0.29      |
| Mean ExAC allele frequency            | 0.0004%          | 0.001%         | 0.03      |
| $\geq 3/4$ in silico tools supportive | 44/77 (58)       | 12/26 (46)     | 0.30      |
| Polyphen-2, n (%)                     | 47/69 (68)       | 13/24 (54)     | 0.22      |
| Polyphen HCM, n (%)                   | 31/35 (89)       | 4/9 (44)       | 0.01      |
| SIFT, n (%)                           | 66/75 (88)       | 21/26 (81)     | 0.35      |
| GERP $\geq 2$ n (%)                   | 101/106 (95)     | 28/32 (88)     | 0.21      |
| Mean CADD score                       | $23\pm 7$        | $19\pm 6$      | 0.006     |
| Novel, n (%)                          | 32 (30)          | 17 (53)        | 0.02      |
| LP/P classification, n (%)            | 66 (62)          | 4 (13)         | $<0.0001$ |

CADD indicates Combined Annotation-Detection Depletion; ExAC, Exome Aggregation Consortium; GERP, Genomic Evolutionary Rate Profiling score; HCM, hypertrophic cardiomyopathy; LP/P, likely pathogenic or pathogenic; and SIFT, Sorting Intolerant From Intolerant.

**Table 4. Cluster-Adjusted Odds of Different Clinical Characteristics in HCM Patients With Multiple Vs Single Variant in the Major 8 HCM Genes**

| Variable           | OR*  | 95% CI    | P Value |
|--------------------|------|-----------|---------|
| Male gender        | 2.49 | 0.79–7.87 | 0.120   |
| Age, y             | 0.95 | 0.92–0.98 | 0.004   |
| Max LVH, mm        | 1.03 | 0.94–1.11 | 0.551   |
| LVESD, mm          | 1.07 | 1.00–1.13 | 0.046   |
| LVEDD, mm          | 1.06 | 0.99–1.14 | 0.086   |
| SCD event          | 1.29 | 0.34–4.90 | 0.710   |
| Family history HCM | 1.56 | 0.56–4.32 | 0.395   |
| Family history SCD | 3.54 | 1.26–9.93 | 0.017   |
| Comorbidities      | 0.24 | 0.07–0.80 | 0.020   |

CI indicates confidence interval; HCM, hypertrophic cardiomyopathy; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVH, left ventricular hypertrophy; OR, odds ratio; and SCD, sudden cardiac death.

\*Adjusted for family membership (cluster adjusted).

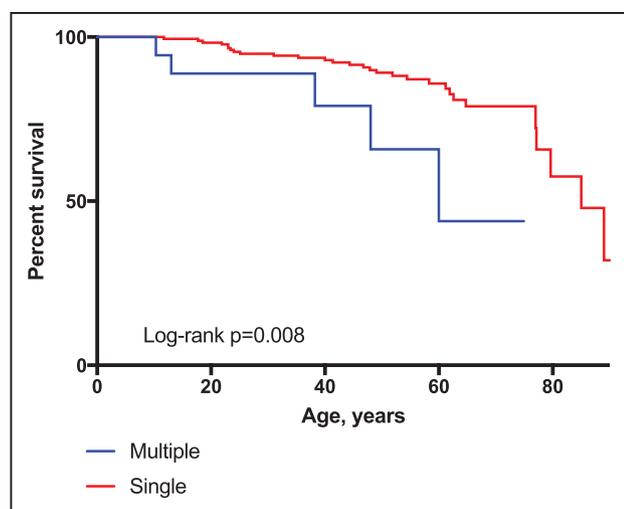
was observed. Multiple variant individuals were younger ( $38\pm 19$  versus  $52\pm 17$  years;  $P=0.0005$ ), had greater LV end-systolic diameter ( $31\pm 10$  versus  $27\pm 7$  mm;  $P=0.03$ ), more likely to have a family history of SCD (7 [39%] versus 27 [15%];  $P=0.01$ ), and less likely to report other comorbidities (4 [29%] versus 76 [63%];  $P=0.02$ ; eg, asthma, diabetes mellitus). There were no differences in LV wall thickness ( $22\pm 7$  versus  $21\pm 6$ ;  $P=0.52$ ) and sudden death events (3 [17%] versus 18 [13%];  $P=0.71$ ). To account for bias potentially introduced by inclusion of additional affected family members, cluster-adjusted analyses were performed. Age, LV end-systolic diameter, family history of SCD, and presence of comorbidities remained statistically significant even after adjusting for family membership (Table 4).

### Event-Free Survival for Multiple Versus Single Variant Patients

When considering an 8-gene panel, multiple variant carriers (relatives and probands) showed worse event-free survival from all-cause death, cardiac arrest, cardiac transplantation, and appropriate implantable cardioverter defibrillator events because of ventricular fibrillation over their lifetime compared with single variant patients (5 of 18 events versus 29 of 178 events; log-rank test  $P=0.008$ ; Figure 3).

### Discussion

The landscape of genetic testing in HCM has evolved in recent years due, largely, to important advances in massively parallel sequencing and increased stringency of variant curation. In a patient population that has undergone comprehensive genetic testing, we have shown no probands carry 2 LP/P variants. The prevalence of multiple variants (including LP/P and VUS, or VUS and VUS) was 4% and was associated with earlier disease onset, greater likelihood of a family history of SCD, and overall worse event-free survival from all-cause



**Figure 3.** Kaplan–Meier plot comparing event-free survival from all-cause death, cardiac transplant, cardiac arrest, and appropriate implantable cardioverter defibrillator shock for ventricular fibrillation between patients with single and multiple rare variants in major (8-gene panel) hypertrophic cardiomyopathy genes (log-rank  $P=0.008$ ).

death, cardiac transplant, and cardiac arrest. An impact on phenotype was not observed when rare variants in less well-established genes were included. Sarcomere variants only ever seen in combination with other LP/P variants (ie, second variants) were statistically more common in population databases. Collectively, our data support a cumulative variant hypothesis, whereby multiple variants identified in key HCM genes contribute to earlier disease onset and worse survival from major cardiovascular events.

Over 10 years ago, our group and others reported experiences of multiple variant genotypes in HCM, noting occurrence in up to 5% with potential for increased disease severity.<sup>9–13</sup> Since 2005, the landscape of HCM genetic testing has changed markedly, and our approach to testing is now much broader and because of this our variant interpretation criteria require greater stringency to make causative assertions, such as LP/P. Typically, variants identified in sarcomere genes would have been considered causative if they fulfilled some basic criteria. In contrast, the latest guidelines for classifying variants written by the American College of Genetics and Genomics<sup>8</sup> include many more criteria and assessment of these often involves multidisciplinary input with a high-level of cardiac genetic expertise. Our finding of a much lower prevalence of multiple LP/P variants when careful curation is applied provides important new data to this field.

Genetic testing for HCM typically now entails an all-encompassing cardiac panel because of increased efficiency and decreasing cost, a practice that has surged into mainstream clinical use. That the technology is available does not mean it is always appropriate and many of the genes included have limited evidence of association with HCM.<sup>3,21,22</sup> Not surprisingly, greater numbers of rare variants are now commonly identified. We show a marked increase in probands identified who have VUS as a direct result of more comprehensive genetic testing. More uncertain results were identified than clinically actionable LP/P results when 45 genes were screened. This impacts on increased uncertainty and

growing complexity of clinical discussions and raises important questions about whether we have tipped the scales to outweighing the obvious benefits of genetic testing.<sup>22</sup> Using targeted panels does have potential drawbacks, for example, 2 probands who had LP/P variants that would have been missed using the 8 gene panel; 1 variant in *PRKAG2* and 1 variant in *PLN*, both established HCM phenocopy genes. The *PRKAG2* variant carrier had an atypical HCM phenotype with LV hypertrophy affecting the posterior wall while the *PLN* carrier had asymmetrical hypertrophy extending to the apex. Both had a positive family history of HCM.

No family had multiple LP/P variants, suggesting that in the setting of more stringent variant curation, the occurrence of multiple LP/P carriers in HCM is rare. The yield of families carrying multiple rare variants in major HCM genes, however, was 4% including both LP/P and VUS. Despite variants having uncertain status, phenotype differences were observed between multiple and single variant patients. This included younger age at disease onset, with multiple variant patients being on average 16 years younger at presentation. Wang et al<sup>23</sup> reported similarly a greater risk of cardiovascular death in multiple variant patients with HCM; however, this was compared with both single and nongene carrier patients. Finally, a positive family history of SCD was more common among patients with multiple variants even after adjusting for family membership. Evaluation of the 14 pedigrees revealed that not all SCD cases carried both variants, however, and when considering SCD events in the patients themselves were no differences between groups, suggesting that multiple rare variants do not predispose to SCD in our population.

A working hypothesis of the underlying genetics of multiple rare variants in sarcomere genes can be proposed based on our data. We suggest that a cumulative variant effect may impact on earlier disease onset and increased severity in terms of major cardiovascular events. Although there are clear examples of single variants that cause severe phenotypes, overall the clinical course for multiple variant carriers was more severe. Importantly, this was true even for variants not necessarily classified as LP/P. Whether the additional second uncertain variant is causative but lacking sufficient evidence to classify it as LP/P, or it acts as a genetic modifier exerting a smaller effect size in combination with another LP/P or even another VUS, remains to be determined. The proposed inverse relationship between variant effect size and rarity in the population<sup>24,25</sup> also lends weight to the latter hypothesis given we have shown there is an incrementally greater frequency of the additional second variants in Exome Aggregation Consortium. A cumulative variant hypothesis is not a new one in cardiac genetics<sup>26</sup> and may explain some of the marked clinical heterogeneity that is characteristic of HCM.

Our findings have important translational implications for cardiac genetic counseling of HCM families. First, we suggest HCM genetic testing in the first instance should be targeted to the 8 major HCM genes, as well as additional phenocopy genes (Table 1). Additional phenocopy genes shown to be important based on our data and others<sup>3,27</sup> include *PRKAG2*, *LAMP2*, *GLA*, and *PLN*. Comprehensive panels should be considered in some circumstances, such as where a family has an atypical phenotype, which may point to other phenocopy or HCM-associated genes (eg, *DES*, *ACTN2*), although with

knowledge that there may be less evidence to assign causation to a variant. Alternatively, genetically elusive cases where there is potential for informative cosegregation studies may be more suited to a comprehensive genetic testing approach although would be ideally performed in a research setting.

In the clinical setting, our findings suggest that a cumulative variant effect may explain some clinical heterogeneity. Specifically, the identification of patients with multiple rare variants in major HCM genes gives rise to earlier onset disease and worse event-free survival from major cardiovascular events. This is true only for rare variants identified in the major HCM genes but regardless of a VUS or LP/P classification. Careful selection of the proband to undergo initial HCM genetic testing, therefore, should be the individual with earliest onset disease, ensuring all variants of interest are identified. Genetic counseling on risk of potential modifier (second) variants should be approached cautiously given the marked uncertainty that segregation of these variants would mean to asymptomatic family members. Furthermore, our data shed light on how the common practice of downgrading variants to likely benign/benign status because of co-occurrence with another LP/P variant (including criteria developed by the American College of Genetics and Genomics)<sup>8</sup> may be inappropriate. Given our finding that these additional second variants have some phenotype impact, we would suggest this does not necessarily apply for rare variants identified in sarcomere genes.

### Conclusions

Patients with HCM with multiple LP/P variants are exceedingly rare in the setting of stringent variant curation approaches. Identification of patients with multiple rare variants in the 8 major HCM genes, whether LP/P or VUS, has clinical implications and may contribute a cumulative variant effect leading to earlier disease onset and worse survival from major cardiac events compared with single gene carriers. Marked increase in VUS identification occurs when comprehensive cardiomyopathy gene panels are used, with no clinical impact, and should be avoided where possible to ensure the overall benefit of HCM genetic testing is maintained. Given the nuances of genetic inheritance in these families, multidisciplinary care in centers with a high-level of expertise and access to cardiac genetic counseling will provide best possible outcomes in patients and families with HCM.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Multiple likely pathogenic/pathogenic variants in hypertrophic cardiomyopathy (HCM) were described >10 years ago with a prevalence of 5% and inferring a more severe phenotype. Given the rapidly changing landscape of HCM genetic testing, including the mainstream use of comprehensive cardiac panels and increased stringency of variant curation efforts, reassessment of multiple variants in HCM was needed. No proband had multiple likely pathogenic/pathogenic variants in contrast to previous reports; however, 4% had multiple rare variants (including likely pathogenic/pathogenic and uncertain variants) in major HCM genes. When considering only major HCM genes, patients with multiple variants had worse event-free survival from all-cause death, cardiac transplantation, and cardiac arrest compared with those with single variants. They were also more likely to be younger and have a family history of sudden cardiac death. The impact on phenotype was not observed when considering multiple variants found from testing larger panels. Using a comprehensive gene panel had no impact on yield of likely pathogenic/pathogenic variants but increased the rate of uncertain variants from 14% to 36%. Given the nuances, management of families in experienced centers will provide the best possible outcomes for patients with HCM and families.

**Multiple Gene Variants in Hypertrophic Cardiomyopathy in the Era of Next-Generation Sequencing**

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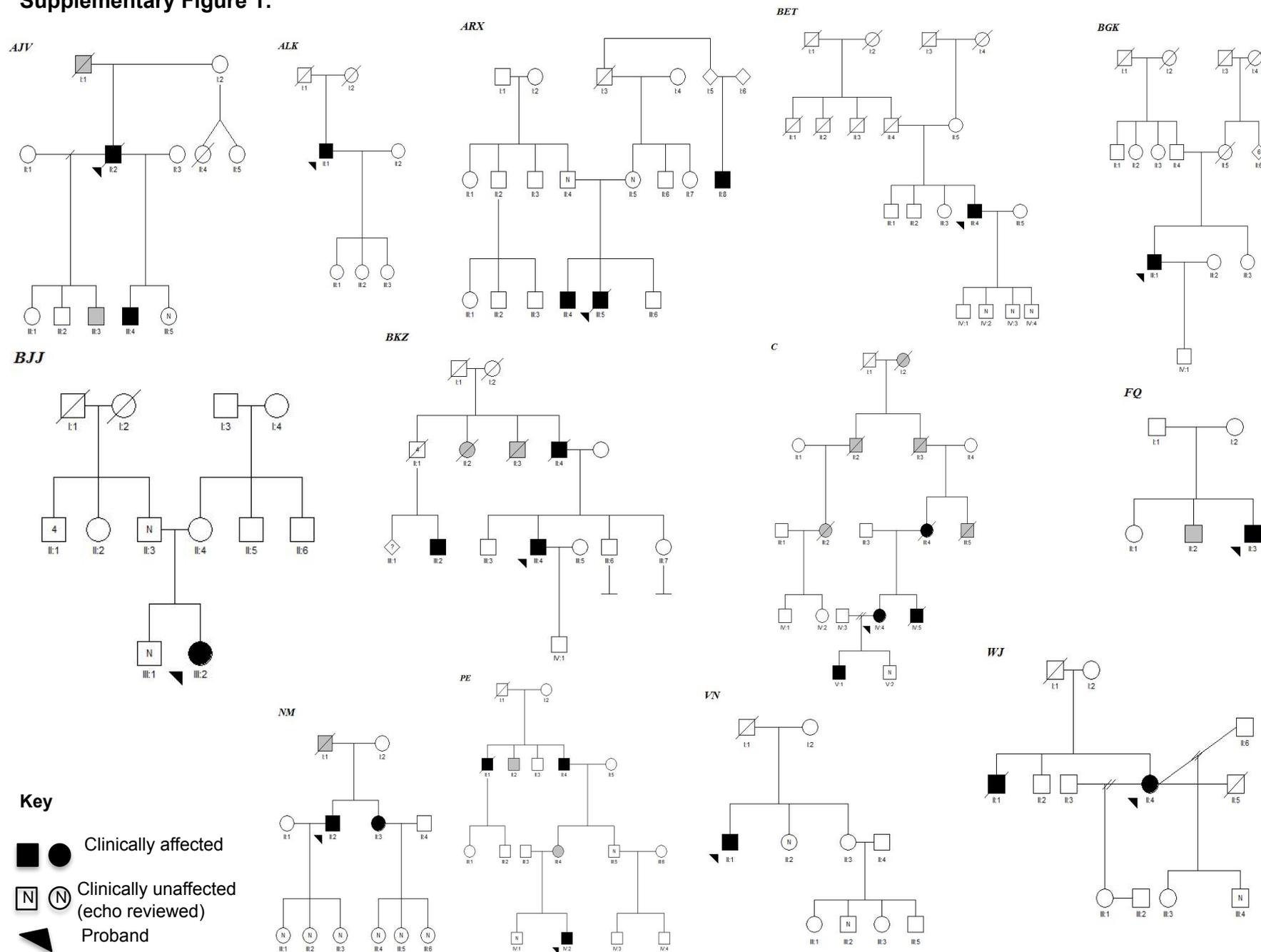
## SUPPLEMENTAL MATERIAL

**TABLE 1: Description of rare variants (<0.02%) identified using a 15-gene panel**

| Characteristic                  | <i>MYBPC3</i>        | <i>MYH7</i>          | <i>TNNT2</i>         | Other                | Total                |
|---------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| n (%)                           | 68 (45)              | 41 (27)              | 9 (6)                | 33 (22)              | 151                  |
| Missense (n,%)                  | 33 (50)              | 40 (98)              | 8 (89)               | 30 (91)              | 111 (74)             |
| Splice (n,%)                    | 12 (18)              | 0                    | 0                    | 1 (3)                | 13 (8)               |
| Nonsense (n,%)                  | 5 (8)                | 0                    | 0                    | 1 (3)                | 6 (4)                |
| Frameshift (n,%)                | 16 (24)              | 1 (3)                | 0                    | 1 (3)                | 18 (12)              |
| Indel (n,%)                     | 2 (3)                | 0                    | 1 (11)               | 0                    | 3 (2)                |
| P/LP (n,%)                      | 43 (63)              | 24 (59)              | 5 (56)               | 9 (27)               | 81 (54)              |
| VUS (n,%)                       | 25 (38)              | 17 (42)              | 4 (44)               | 24 (73)              | 70 (47)              |
| Novel (n,%)                     | 21 (32)              | 11 (28)              | 1 (11)               | 17 (52)              | 50 (34)              |
| ExAC allele frequency (n, mean) | 9 x 10 <sup>-6</sup> | 8 x 10 <sup>-6</sup> | 7 x 10 <sup>-6</sup> | 6 x 10 <sup>-6</sup> | 7 x 10 <sup>-6</sup> |

Abbreviations: Indel (Insertion or deletion) † P/LP (Pathogenic or Likely Pathogenic), ‡ VUS (Variant of uncertain significance)

# Supplementary Figure 1:



**TABLE 2:**

| <b>MYBPC3 rare variants</b> |                                      |                         |                             |                 |                        |                 |
|-----------------------------|--------------------------------------|-------------------------|-----------------------------|-----------------|------------------------|-----------------|
| <b>Gene</b>                 | <b>Genomic location</b>              | <b>cDNA variation</b>   | <b>Amino acid variation</b> | <b>Function</b> | <b>Classification</b>  | <b>Probands</b> |
| MYBPC3                      | Chr11(GRCh37):g.47374186C>A          | c.13G>T                 | p.Gly5Trp                   | Missense        | Uncertain Significance | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47374173C>T          | c.25+1G>A               | p.?                         | Splice          | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47372920del          | c.162delG               | p.Lys54Asnfs*13             | Frameshift      | Pathogenic             | 2               |
| MYBPC3                      | Chr11(GRCh37):g.47372895_47372905del | c.177_187delAGAGGGCACAC | p.Glu60Alafs*49             | Frameshift      | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47371628C>T          | c.442G>A                | p.Gly148Arg                 | Missense        | Uncertain Significance | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47371366G>A          | c.613C>T                | p.Gln205*                   | Nonsense        | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47370092C>G          | c.655G>C                | p.Val219Leu                 | Missense        | Pathogenic             | 2               |
| MYBPC3                      | Chr11(GRCh37):g.47369975C>T          | c.772G>A                | p.Glu258Lys                 | Missense        | Pathogenic             | 4               |
| MYBPC3                      | Chr11(GRCh37):47369411C>T            | c.818G>A                | p.Arg273His                 | Missense        | Uncertain Significance | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47368189GAA>G        | c.913_914delTT          | p.Phe305Profs*27            | Frameshift      | Pathogenic             | 2               |
| MYBPC3                      | Chr11(GRCh37):g.47367917             | c.931T>A                | p.Ser311Thr                 | Missense        | Uncertain Significance | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47367930C>T          | c.927-9G>A              | p.?                         | Splice          | Pathogenic             | 2               |
| MYBPC3                      | Chr11(GRCh37):g.47367758C>T          | c.1090G>A               | p.Ala364Thr                 | Missense        | Likely Pathogenic      | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364621G>T          | c.1302C>A               | p.Tyr434*                   | Nonsense        | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364570A>G          | c.1351+2T>C             | p.?                         | Splice          | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364478CA>C         | c.1359delT              | p.Val454Cysfs*12            | Frameshift      | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364409C>T          | c.1429G>A               | p.Val477Ile                 | Missense        | Uncertain Significance | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364296C>T          | c.1458-1G>A             | p.?                         | Splice          | Pathogenic             | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364270G>C          | c.1483C>G               | p.Arg495Gly                 | Missense        | Likely Pathogenic      | 1               |
| MYBPC3                      | Chr11(GRCh37):g.47364249G>A          | c.1504C>T               | p.Arg502Trp                 | Missense        | Pathogenic             | 4               |

|        |                                 |                      |                  |            |                        |   |
|--------|---------------------------------|----------------------|------------------|------------|------------------------|---|
| MYBPC3 | Chr11:(GRCh37):g.47364248C>T    | c.1505G>A            | p.Arg502Gln      | Missense   | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47364173A>G     | c.1580T>C            | p.Leu527Pro      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47364129C>G     | c.1624G>C            | p.Glu542Gln      | Missense   | Pathogenic             | 3 |
| MYBPC3 | Chr11(GRCh37):g.47364125T>A     | c.1624+4A>T          | p.?              | Splice     | Pathogenic             | 5 |
| MYBPC3 | Chr11(GRCh37):g.47363567G>A     | c.1765C>T            | p.Arg589Cys      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):47362747dup       | c.1838dupA           | p.Asp613Glufs*25 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):47361343T>C       | c.1928-2A>G          | p.?              | Splice     | Pathogenic             | 4 |
| MYBPC3 | Chr11(GRCh37):g.47361239G>A     | c.2030C>T            | p.Pro677Leu      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360945G>A     | c.2078C>T            | p.Ala693Val      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360940C>CTGGG | c.2079_2082dupCCCA   | p.Ile695Profs*14 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360209G>A     | c.2170C>T            | p.Arg724Trp      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360200C>T     | c.2179G>A            | p.Val727Met      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360145T>C     | c.2234A>G            | p.Asp745Gly      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360071C>T     | c.2308G>A            | p.Asp770Asn      | Missense   | Likely Pathogenic      | 1 |
| MYBPC3 | Chr11(GRCh37):g.47360070C>T     | c.2308+1G>A          | p.?              | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359347T>C     | c.2309-2A>G          | p.?              | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359280A>AC    | c.2373dupG           | p.Trp792Valfs*41 | Frameshift | Pathogenic             | 3 |
| MYBPC3 | Chr11(GRCh37):g.47359115C>T     | c.2429G>A            | p.Arg810His      | Missense   | Likely Pathogenic      | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359095G>C     | c.2449C>G            | p.Arg817Gly      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359019T>TA    | c.2524dupT           | p.Tyr842Leufs*41 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359011G>T     | c.2533C>A            | p.Arg845Ser      | Missense   | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47359001G>T     | c.2543C>A            | p.Ala848Glu      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):47357561          | c.2604-2605delTCinsA | p.Ser871Alafs*8  | Frameshift | Pathogenic             | 1 |

|        |                                      |                   |                   |            |                        |   |
|--------|--------------------------------------|-------------------|-------------------|------------|------------------------|---|
| MYBPC3 | Chr11(GRCh37):g.47357429GC>G         | c.2735_2736delGC  | p.Gly912Valfs*138 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47356762T>A          | c.2738-2A>T       | p.?               | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47356716ATG>A        | c.2780_2781delCA  | p.Thr927Ilefs*123 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47356671G>A          | c.2827C>T         | p.Arg943*         | Nonsense   | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47356632CAG>C        | c.2864_2865delCT  | p.Pro955Argfs*95  | Frameshift | Pathogenic             | 5 |
| MYBPC3 | Chr11(GRCh37)g.47356593              | c.2905C>T         | p.Gln969*         | Nonsense   | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47355487G>A          | c.2980C>T         | p.Leu994Phe       | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47355293C>T          | c.3005G>A         | p.Arg1002Gln      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47355233C>T          | c.3065G>A         | p.Arg1022His      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47355108_47355110del | c.3188_3190delTTG | p.Val1063del      | Indel      | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47355103C>T          | c.3190+5G>A       | p.?               | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37)g.47354883dupG          | c.3192dupC        | p.Lys1065Glnfs*12 | Frameshift | Pathogenic             | 2 |
| MYBPC3 | Chr11(GRCh37):g.47354798C>A          | c.3277G>T         | p.Gly1093Cys      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354743A>C          | c.3330+2T>G       | p.?               | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37)g.47354518_47354520dup  | c.3335_3337dupGG  | p.Trp1112dup      | Indel      | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354364C>T          | c.3490+1G>A       | p.?               | Splice     | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354145A>G          | c.3599T>C         | p.Leu1200Pro      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354139C>T          | c.3605G>A         | p.Cys1202Tyr      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354126AC>A         | c.3617delA        | p.Gly1206Valfs*31 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47354119TG>T         | c.3624delC        | p.Lys1209Serfs*28 | Frameshift | Pathogenic             | 2 |
| MYBPC3 | Chr11(GRCh37):g.47353795C>A          | c.3642G>T         | p.Trp1214Cys      | Missense   | Uncertain Significance | 1 |
| MYBPC3 | Chr11(GRCh37):g.47353740G>A          | c.3697C>T         | p.Gln1233*        | Nonsense   | Pathogenic             | 2 |
| MYBPC3 | Chr11(GRCh37):g.47353737C>T          | c.3700G>A         | p.Gly1234Arg      | Missense   | Uncertain Significance | 1 |

|        |                              |                  |                  |            |                        |   |
|--------|------------------------------|------------------|------------------|------------|------------------------|---|
| MYBPC3 | Chr11(GRCh37)g.47353723CAG>C | c.3712_3713delCT | p.Leu1238Glyfs*3 | Frameshift | Pathogenic             | 1 |
| MYBPC3 | Chr11(GRCh37):g.47353686A>G  | c.3751T>C        | p.Tyr1251His     | Missense   | Uncertain Significance | 1 |

| <b>MYH7 rare variants</b> |                             |                       |                             |                 |                        |                 |
|---------------------------|-----------------------------|-----------------------|-----------------------------|-----------------|------------------------|-----------------|
| <b>Gene</b>               | <b>Genomic location</b>     | <b>cDNA variation</b> | <b>Amino acid variation</b> | <b>Function</b> | <b>Classification</b>  | <b>Probands</b> |
| MYH7                      | Chr14(GRCh37):23884311      | c.5452C>T             | p.Arg1818Trp                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23884476C>T | c.5287G>A             | p.Ala1763Thr                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37:g.23884651AC>A | c.5222_5223delTG      | p.Val1741Glyfs*11           | Frameshift      | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23884861G>A | c.5134C>T             | p.Arg1712Trp                | Missense        | Likely Pathogenic      | 1               |
| MYH7                      | Chr14(GRCh37):g.23885041C>A | c.4954G>T             | p.Asp1652Tyr                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23887453C>T | c.4135G>A             | p.Ala1379Thr                | Missense        | Pathogenic             | 3               |
| MYH7                      | Chr14(GRCh37):g.23887464T>C | c.4124A>G             | p.Tyr1375Cys                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23887513G>A | c.4075C>T             | p.Arg1359Cys                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23887522C>T | c.4066G>A             | p.Glu1356Lys                | Missense        | Likely Pathogenic      | 1               |
| MYH7                      | Chr14(GRCh37):g.23889188C>A | c.3592G>T             | p.Asp1198Tyr                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23890178T>C | c.3325A>G             | p.Lys1109Glu                | Missense        | Likely Pathogenic      | 1               |
| MYH7                      | Chr14 (GRCh37)g.23891398C>T | c.3236G>A             | p.Arg1079Gln                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23892791T>C | c.3064A>G             | p.Lys1022Glu                | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):g.23893268C>T | c.2770G>A             | p.Glu924Lys                 | Missense        | Pathogenic             | 1               |
| MYH7                      | Chr14(GRCh37):g.23893300    | c.2738T>C             | p.Ile913Thr                 | Missense        | Uncertain Significance | 1               |
| MYH7                      | Chr14(GRCh37):23893357T>C   | c.2681A>G             | p.Glu894Gly                 | Missense        | Pathogenic             | 4               |
| MYH7                      | Chr14(GRCh37):g.23894048C>T | c.2609G>A             | p.Arg870His                 | Missense        | Pathogenic             | 1               |

|      |                             |           |             |          |                        |   |
|------|-----------------------------|-----------|-------------|----------|------------------------|---|
| MYH7 | Chr14(GRCh37):g.23894079T>C | c.2578A>G | p.Lys860Glu | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g.23894118T>C | c.2539A>G | p.Lys847Glu | Missense | Likely Pathogenic      | 1 |
| MYH7 | Chr14(GRCh37):g.23894494C>T | c.2420G>A | p.Arg807His | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g.23894525C>T | c.2389G>A | p.Ala797Thr | Missense | Likely Pathogenic      | 3 |
| MYH7 | Chr14(GRCh37):23894581      | c.2333A>T | p.Asp778Val | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g.23894965G>T | c.2225C>A | p.Ala742Glu | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g.23894969C>A | c.2221G>T | p.Gly741Trp | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23894983A>G | c.2207T>C | p.Ile736Thr | Missense | Pathogenic             | 2 |
| MYH7 | Chr14(GRCh37):g.23895023G>A | c.2167C>T | p.Arg723Cys | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):23895179C>T   | c.2156G>A | p.Arg719Gln | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23895180G>A | c.2155C>T | p.Arg719Trp | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23895242A>G | c.2093T>C | p.Val698Ala | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23896042C>T | c.1988G>A | p.Arg663His | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23896451T>C | c.1954A>G | p.Arg652Gly | Missense | Likely Pathogenic      | 2 |
| MYH7 | Chr14(GRCh37):g.23896866C>T | c.1816G>A | p.Val606Met | Missense | Pathogenic             | 4 |
| MYH7 | Chr14(GRCh37):g.23896955T>C | c.1727A>G | p.His576Arg | Missense | Likely Pathogenic      | 1 |
| MYH7 | Chr14(GRCh37):g.23898201A>G | c.1370T>C | p.Ile457Thr | Missense | Likely Pathogenic      | 1 |
| MYH7 | Chr14(GRCh37):g.23898247G>A | c.1324C>T | p.Arg442Cys | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23898487C>T | c.1208G>A | p.Arg403Gln | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23898488G>A | c.1207C>T | p.Arg403Trp | Missense | Pathogenic             | 1 |
| MYH7 | Chr14(GRCh37):g.23899071T>C | c.1051A>G | p.Lys351Glu | Missense | Likely Pathogenic      | 1 |
| MYH7 | Chr14(GRCh37):g.23899816T>G | c.952A>C  | p.Thr318Pro | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g.23899831T>A | c.937A>T  | p.Ile313Phe | Missense | Uncertain Significance | 1 |
| MYH7 | Chr14(GRCh37):g23900998C>T  | c.611G>A  | p.Arg204His | Missense | Uncertain Significance | 1 |

| <b>TNNT2 rare variants</b> |  |                       |                             |                 |                        |                 |
|----------------------------|--|-----------------------|-----------------------------|-----------------|------------------------|-----------------|
| <b>Gene</b>                | <b>Genomic location</b>                | <b>cDNA variation</b> | <b>Amino acid variation</b> | <b>Function</b> | <b>Classification</b>  | <b>Probands</b> |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201334766A>T            | c.236T>A              | p.Ile79Asn                  | Missense        | Pathogenic             | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201334425C>T            | c.275G>A              | p.Arg92Gln                  | Missense        | Pathogenic             | 2               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201334389G>A            | c.311C>T              | p.Ala104Val                 | Missense        | Likely Pathogenic      | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201333435G>T            | c.450C>A              | p.Asn150Lys                 | Missense        | Uncertain Significance | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201332505_201332507 del | c.487_489delGAG       | p.Glu163del                 | Indel           | Pathogenic             | 2               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201331078C>A            | c.652G>T              | p.Val218Leu                 | Missense        | Uncertain Significance | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201328372C>T            | c.833G>C              | p.Arg278Pro                 | Missense        | Likely Pathogenic      | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201328372C>T            | c.833G>A              | p.Arg278His                 | Missense        | Uncertain Significance | 1               |
| <i>TNNT2</i>               | Chr1(GRCh37):g.201328349G>A            | c.856C>T              | p.Arg286Cys                 | Missense        | Uncertain Significance | 1               |

| <b>Rare variants in <i>CSRP3</i>, <i>ACTN2</i>, <i>TNNI3</i>, <i>ACTC1</i>, <i>MYL2</i>, <i>MYL3</i>, <i>TPM1</i>, <i>NEXN</i>, <i>PLN</i>, <i>MYL2</i> and <i>TTR</i></b> |                             |                       |                             |                 |                        |                 |
|--|-----------------------------|-----------------------|-----------------------------|-----------------|------------------------|-----------------|
| <b>Gene</b>  | <b>Genomic location</b>     | <b>cDNA variation</b> | <b>Amino acid variation</b> | <b>Function</b> | <b>Classification</b>  | <b>Probands</b> |
| <i>CSRP3</i>   | Chr11(GRCh37):g.19213903G>C | c.93C>G               | p.His31Gln                  | Missense        | Uncertain Significance | 1               |
| <i>CSRP3</i>   | Chr11(GRCh37):19209758T>C   | c.206A>G              | P.Lys69Arg                  | Missense        | Uncertain Significance | 1               |
| <i>CSRP3</i>   | Chr11(GRCh37):19207854del   | c.322delA             | p.Ser108Profs*100           | Frameshift      | Uncertain Significance | 1               |
| <i>CSRP3</i>   | Chr11(GRCh37):g.19206571G>A | c.436C>T              | p.Arg146Cys                 | Missense        | Uncertain Significance | 2               |
| <i>CSRP3</i>   | Chr11(GRCh37):g.19206570C>T | c.437G>A              | p.Arg146His                 | Missense        | Uncertain Significance | 1               |
| <i>CSRP3</i>   | Chr11(GRCh37):g.19204291A>C | c.511T>G              | p.Cys171Gly                 | Missense        | Uncertain Significance | 1               |
| <i>ACTN2</i>   | Chr1(GRCh37):g.236902708G>A | c.983G>A              | p.Arg328Gln                 | Missense        | Uncertain Significance | 1               |

|              |                              |            |             |          |                        |   |
|--------------|------------------------------|------------|-------------|----------|------------------------|---|
| <i>ACTN2</i> | Chr1(GRCh37):g.236906281G>A  | c.1193G>A  | p.Arg398His | Missense | Uncertain Significance | 1 |
| <i>ACTN2</i> | Chr1(GRCh37):g.236908039C>T  | c.1369C>T  | p.Arg457Cys | Missense | Uncertain Significance | 1 |
| <i>ACTN2</i> | Chr1(GRCh37):g.236914861A>C  | c.1748A>C  | p.Glu583Ala | Missense | Uncertain Significance | 1 |
| <i>ACTN2</i> | Chr1(GRCh37):g.236917290A>G  | c.1883A>G  | p.Glu628Gly | Missense | Uncertain Significance | 1 |
| <i>TNNI3</i> | Chr9(GRCh37):g.55666111C>G   | c.370G>C   | p.Glu124Gln | Missense | Likely Pathogenic      | 1 |
| <i>TNNI3</i> | Chr19(GRCh37):g.55665514G>A  | c.433C>T   | p.Arg145Trp | Missense | Pathogenic             | 1 |
| <i>TNNI3</i> | Chr19(GRCh37):g.55665507A>G  | c.440T>C   | p.Val147Ala | Missense | Uncertain Significance | 1 |
| <i>TNNI3</i> | Chr19(GRCh37):g.55665462C>T  | c.485G>A   | p.Arg162Gln | Missense | Likely Pathogenic      | 3 |
| <i>TNNI3</i> | Chr19(GRCh37):g.55663286C>T  | c.550-1C>T | p.?         | Splice   | Uncertain Significance | 1 |
| <i>TNNI3</i> | Chr19(GRCh37):g.55663224C>T  | c.611G>A   | p.Arg204Hi  | Missense | Likely Pathogenic      | 1 |
| <i>ACTC1</i> | Chr15(GRCh37):g.35085622T>C  | c.278A>G   | p.Tyr93Cys  | Missense | Uncertain Significance | 1 |
| <i>ACTC1</i> | Chr15(GRCh37):g.35083455T>A  | c.850A>T   | p.Ile284Phe | Missense | Uncertain Significance | 1 |
| <i>ACTC1</i> | Chr15(GRCh37):g.35083337G>A  | c.968C>T   | p.Ala323Val | Missense | Uncertain Significance | 1 |
| <i>MYL2</i>  | Chr12(GRCh37):g.111356937C>T | c.64G>A    | p.Glu22Lys  | Missense | Likely Pathogenic      | 1 |
| <i>MYL2</i>  | Chr12(GRCh37):g.111351092T>  | c.311A>C   | p.Lys104Thr | Missense | Uncertain Significance | 1 |
| <i>MYL3</i>  | Chr3(GRCh37):g.46902455A>G   | c.152T>C   | p.Ile51Thr  | Missense | Uncertain Significance | 1 |
| <i>MYL3</i>  | Chr3(GRCh37):g.46900985C>T   | c.461G>A   | p.Arg154His | Missense | Likely Pathogenic      | 1 |
| <i>MYL3</i>  | Chr3(GRCh37):g.46900983G>C   | c.463C>G   | p.His155Asp | Missense | Uncertain Significance | 1 |
| <i>TPM1</i>  | Chr15(GRCh37):g.63353123C>T  | c.548C>T   | p.Ala183Val | Missense | Likely Pathogenic      | 3 |
| <i>TPM1</i>  | Chr15(GRCh37):g.63353922G>A  | c.574G>A   | p.Glu192Lys | Missense | Likely Pathogenic      | 1 |
| <i>TPM1</i>  | Chr15(GRCh37):g.63353983A>T  | c.635A>T   | p.Glu212Val | Missense | Uncertain Significance | 1 |
| <i>NEXN</i>  | Chr1(GRCh37):g.78395007G>A   | c.871G>A   | p.Glu291Lys | Missense | Uncertain              | 1 |

|             |                             |          |             |          | Significance           |   |
|-------------|-----------------------------|----------|-------------|----------|------------------------|---|
| <i>NEXN</i> | Chr1(GRCh37):g.78398976G>C  | c.871G>C | p.Asp291His | Missense | Uncertain Significance | 1 |
| <i>PLN</i>  | Chr6(GRCh37):g.118880137T>C | c.53T>C  | p.Ile18Thr  | Missense | Uncertain Significance | 1 |
| <i>PLN</i>  | Chr6(GRCh37):g.118880200T>G | c.116T>G | p.Leu39*    | Nonsense | Pathogenic             | 1 |
| <i>TTR</i>  | Chr18(GRCh37):g.29172958G>A | c.169G>A | p.Ala57Thr  | Missense | Uncertain Significance | 1 |

## Multiple Variant Families

When considering families with two rare variants in 8 HCM genes (Families ALK and BGK) neither reported a family history of disease with no genetic testing undertaken in relatives. In families with two rare variants in *MYBPC3* and *MYH7* (Families AJV, ARX, BET, BJJ, BKZ, C, DW (Pedigree unavailable), FQ, NM, PE, VN, WJ) families BET, BJJ and VN reported no family history of disease. In family AJV the proband (II:2) carried a likely pathogenic variant in *MYH7* p.Arg652Gly as well as a VUS in *MYBPC3* p.Leu994Phe, his maximal wall thickness was 22mm, he had obstructive disease, had undergone myectomy and died awaiting transplant. His son (III:4) with a maximal wall thickness of 17mm also carried both variants. In family ARX, the proband (III:5) died suddenly aged 13 years with a post mortem maximal wall thickness of 20mm. He was found to carry a pathogenic *MYBPC3* p.Val219Leu in addition to a VUS in *MYBPC3* p.Trp112dup. His brother (III:4) aged 19 years carried both variants with a maximal wall thickness of 14mm and an ICD in situ. Their younger brother (III:6) aged 15 years also carried both variants but to date clinical investigations had been within normal limits. Their mother (II:5) was found to carry the pathogenic *MYBPC3* variant but had no clinical evidence of disease (Maximal wall thickness 9mm). Their father (II:6) carried the *MYBPC3* VUS with no clinical evidence of disease (Maximal wall thickness 11mm). In family BKZ, the proband (III:4) presented with symptoms aged 22 years with a maximal wall thickness of 24mm and carried a pathogenic *MYBPC3* variant c.927-9G>A as well as a VUS in *MYBPC3* p.Ser311Thr. No family members had genetic testing. The proband's father (II:4) was diagnosed with HCM and a paternal uncle (II:3) died suddenly aged 16 years playing soccer with probable HCM. In family C the proband (IV:4) carried a pathogenic *MYH7* p.Arg719Gln variant as well as a VUS in *MYBPC3* p.Arg273His.

Her maximal wall thickness was 20mm with an ICD in situ and episodes of atrial fibrillation. Her HCM had progressed to dilated phase and she had undergone heart transplantation. Her brother (IV:5) died suddenly aged 16 years with probable HCM and her mother (III:4) died at 44 years with a maximal wall thickness of 15mm, atrial fibrillation and heart failure. The proband's son (V:1) was found to carry both variants with a maximal wall thickness of 33mm and ICD in situ. Her second son (V:2) carried only the *MYBPC3* variant with a maximal wall thickness of 10mm. In family FQ the proband (II:3) was diagnosed with HCM aged 8 years and was identified to carry a pathogenic *MYBPC3* variant c.1624+4A>T variant as well as a VUS in *MYBPC3* p.Arg724Trp. No family members had undergone genetic testing and the proband's brother (II:2) had borderline echocardiograph findings. In family NM the proband (II:2) carried a pathogenic nonsense variant in *MYBPC3* p.Gln969\* as well as a VUS in *MYH7* p.Arg1079Gln, he had a maximal wall thickness of 16mm and his cardiomyopathy had progressed to the dilated phase. His children (ranging in age from 30-35 years) had normal echocardiograms but had not undergone genetic testing. His sister (II:3) carried both variants and had a maximal wall thickness of 21mm and had an ICD in situ. Her daughter (III:4) aged 40 years carried both variants with no evidence of disease (Maximal wall thickness 7mm). Her second daughter (III:5) aged 37 years carried neither variant with a maximal wall thickness of 9mm and her third daughter (III:6) aged 36 years carried only the pathogenic nonsense variant with a maximal wall thickness of 8mm. In family PE the proband (IV:2) aged 10 years had a maximal wall thickness of 24mm, had a resuscitated cardiac arrest and had received appropriate therapy from his ICD for VF. He carried a pathogenic splice variant in *MYBPC3* c.1928-2A>G as well as a VUS in *MYBPC3* p.Leu527Pro. His mother (III:4) carried only the splice variant with a maximal wall thickness of

12mm. The probands maternal grandfather (II:4) had a maximal wall thickness of 16mm with an ICD in situ. An uncle (II:1) died suddenly aged 30 years with post mortem reporting extensive hypertrophy of the left ventricle. Finally, in family WJ, the proband (II:4) carried two VUS's in *MYH7* p.Arg1818Trp and *MYH7* p.Asp778Val. Her maximal wall thickness was 18mm and she had an ICD in situ. Her clinically unaffected son (III:4, aged 26 years) carried only *MYH7* p.Asp778Val with no clinical evidence of disease (Maximal wall thickness 11mm). Her brother (II:1) died suddenly aged 34 years with cause of death identified as HCM and was found to carry the *MYH7* p.Asp778Val and not the Arg1818Trp variant. A second brother (II:2) carried only the *MYH7* p.Arg1818Trp variant with no clinical details available. Their mother carried only *MYH7* p.Arg1818Trp and reportedly had normal echo screening.