Circulation

ORIGINAL RESEARCH ARTICLE

Low Penetrance Sarcomere Variants Contribute to Additive Risk in Hypertrophic Cardiomyopathy

Joshua K. Meisner, MD, PhD*; Aaron Renberg, BS*; Eric D. Smith, MD; Yao-Chang Tsan, PhD; Brynn Elder, BS; Abbey Bullard; Owen L. Merritt; Sean L. Zheng, PhD, MRCP; Neal K. Lakdawala, MD; Anjali T. Owens, MD; Thomas D. Ryan, MD; Erin M. Miller, MS; Joseph W. Rossano, MD; Kimberly Y. Lin, MD; Brian L. Claggett, PhD; Euan A. Ashley, MRCP, Dphil; Michelle Michels, MD, PhD; Rachel Lampert, MD; John C. Stendahl, MD; Dominic Abrams, MD, MRCP; Christopher Semsarian, MBBS, PhD; Victoria N. Parikh, MD; Matthew T. Wheeler, MD, PhD; Jodie Ingles, MPH, PhD; Iacopo Olivotto, MD; Sharlene M. Day, MD; Sara Saberi, MD, MS; Mark W. Russell, MD; Michael Previs, PhD; Carolyn Y. Ho, MD; James S. Ware, PhD, MRCP; Adam S. Helms, MD, MS

BACKGROUND: Classically, hypertrophic cardiomyopathy (HCM) has been viewed as a single-gene (monogenic) disease caused by pathogenic variants in sarcomere genes. Pathogenic sarcomere variants are individually rare and convey high risk for developing HCM (highly penetrant). Recently, important polygenic contributions have also been characterized. Low penetrance sarcomere variants (LowSVs) at intermediate frequencies and effect sizes have not been systematically investigated. We hypothesize that LowSVs may be common in HCM with substantial influence on disease risk and severity.

METHODS: Among all sarcomere variants observed in the Sarcomeric Human Cardiomyopathy Registry (SHaRe), we identified putative LowSVs defined by (1) population frequency greater than expected for highly penetrant (monogenic) HCM (allele frequency $>5\times10^{-5}$ in the Genome Aggregation Database, gnomAD) and (2) moderate enrichment (>2×) in patients with HCM compared with gnomAD. LowSVs were examined for their association with disease severity and clinical outcomes. Functional effects of selected LowSVs were assessed using induced pluripotent stem cell–derived cardiomyocytes. Association of LowSVs with HCM-adjacent traits in the general population was tested using UK Biobank cardiac magnetic resonance imaging data.

RESULTS: Among 6045 patients and 1159 unique variants in sarcomere genes, 12 LowSVs were identified. LowSVs were collectively common in the general population (1:350) and moderately enriched in HCM (aggregate odds ratio, 14.9 [95% CI, 12.5–17.9]). Isolated LowSVs were associated with an older age of HCM diagnosis and fewer adverse events. However, LowSVs in combination with a pathogenic sarcomere variant conferred higher morbidity (eg, composite adverse event hazard ratio, 5.4 [95% CI, 3.0–9.8] versus single pathogenic sarcomere variant, 2.0 [95% CI, 1.8–2.2]; $P<0.001$). An intermediate functional impact was validated for 2 specific LowSVs—*MYBPC3* c.442G>A (partial splice gain) and *TNNT2* c.832C>T (intermediate effect on contractile mechanics). Cardiac magnetic resonance imaging analysis of the general population revealed 5 of 12 LowSVs were significantly associated with HCM-adjacent traits without overt HCM.

CONCLUSIONS: This study establishes a new class of low penetrance sarcomere variants that are relatively common in the population. When penetrant, isolated LowSVs cause mild HCM. In combination with pathogenic sarcomere variants, LowSVs markedly increase disease severity, supporting a clinically significant additive effect. Last, LowSVs also contribute to age-related remodeling even in the absence of overt HCM.

Key Words: cardiomyopathies ■ cardiomyopathy, hypertrophic ■ genetic variation ■ induced pluripotent stem cells ■ penetrance ■ sarcomeres

Correspondence to: Adam S. Helms, MD, MS, 1500 E Medical Center Dr, Ann Arbor, MI 48019. Email adamhelm@med.umich.edu
*J.K. Meisner and A. Renberg contributed equally.
Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.124.069398.
For Sources of Funding and Disclosures, see page 796.

Circulation is available at www.ahajournals.org/journal/circ

ORIGINAL RESEARCH ARTICLE

Clinical Perspective

What Is New?

- We identified a new class of low penetrance sarcomere gene variants.
- Low penetrance sarcomere gene variants are collectively common in the population (1:350) but carry only a 2% to 12% risk of causing hypertrophic cardiomyopathy (HCM) in a given individual, in contrast with the high risk associated with pathogenic HCM-causing sarcomere variants.
- Low penetrance sarcomere gene variants typically cause mild HCM when present alone but significantly worsen disease when combined with additional variants, supporting additive genetic risk models in HCM.

What Are the Clinical Implications?

- Incorporating low penetrance sarcomere gene variants into genetic testing interpretation may improve risk assessment and individual patient management in HCM.

Nonstandard Abbreviations and Acronyms

CMB	cardiac muscle bundle
CMR	cardiac magnetic resonance imaging
gnomAD	Genome Aggregation Database
HCM	hypertrophic cardiomyopathy
HCM_{Low}	HCM with a single low penetrance sarcomere variant
$HCM_{MultiPath}$	HCM with >1 pathogenic or likely pathogenic sarcomere variant
HCM_{Path}	HCM with a single pathogenic or likely pathogenic sarcomere variant
$HCM_{Path+Low}$	HCM with both a low penetrance variant and a pathogenic sarcomere variant
$HCM_{SarcNeg}$	HCM with negative genetic testing for sarcomere gene variants
iPSC	induced pluripotent stem cell
iPSC-CM	induced pluripotent stem cell–derived cardiomyocyte
IQR	interquartile range
LowSV	low penetrance sarcomere variant
MAF	minor allele frequency
MyBP-C	myosin binding protein C
***MYBPC3*tv**	truncating *MYBPC3* variant
OR	odds ratio
PathSV	pathogenic or likely pathogenic sarcomere variant
SHaRe	Sarcomeric Human Cardiomyopathy Registry
VUS	variant of uncertain significance

Hypertrophic cardiomyopathy (HCM), affecting 1:500 in the population, has been considered an autosomal dominant inherited condition since the first genetic causes were described in the 1990s.[1–6] Individually rare, pathogenic variants in sarcomere genes are, collectively, the most common genetic cause of HCM, and they account for ~50% of patients with HCM in the multicenter Sarcomeric Human Cardiomyopathy Registry (SHaRe).[7] Pathogenic sarcomere variants (PathSVs) have been defined primarily based on rarity in the general population and presence of the variant within affected family members.[8,9] Patients with HCM caused by PathSVs, on average, have worse outcomes.[7] Yet marked clinical variability exists across individuals, even when they carry identical PathSVs, as we showed in our analysis of *MYBPC3*-associated HCM.[10] Thus, knowledge of PathSVs alone does not allow refined risk prediction, likely because of additional genetic and nongenetic factors that affect disease risk.

On the other end of the spectrum from disease caused by single PathSVs, HCM has been recently shown to have substantial polygenic contributions.[11–16] The polygenic variants contributing to HCM are defined as "risk alleles" with small effect size (eg, relative risk <2) and are individually insufficient to cause HCM.[17] Through recent genome-wide association studies and polygenic risk scores in HCM, risk alleles have been shown to both (1) have an additive effect in causing HCM in patients without PathSVs and (2) contribute to disease severity risk in the presence of PathSVs.[11–13,15,16] The contribution of multiple risk alleles partially explains the nonfamilial form of HCM for whom prospective family screening is often negative.[18,19] However, these genome-wide association studies to date do not fully account for the residual risk in HCM.

In between pathogenic variants (ie, large effect) and risk alleles (ie, small effect), the presence of "low penetrance" variants has recently been proposed to further refine genetic risk models in autosomal dominant disorders.[17] By definition, low penetrance variants have an intermediate effect size compared with pathogenic variants and risk alleles.[17] Low penetrance variants in sarcomere genes have been suggested[20–22] but have not been systematically assessed and correlated with clinical outcomes. Establishing the wider presence and clinical impact of this class of variant would further improve genetic risk stratification in HCM.

Here, we leverage the scale, longitudinal follow-up, and genetic curation of SHaRe to define a new class of low penetrance sarcomere variants (LowSVs) (Figure 1). For 2 of these variants (*MYBPC3* c.442G>A and *TNNT2* c.832C>T), we validated functional effects using human induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs).

METHODS

A detailed methods section is available in the Supplemental Material. SHaRe is a multicenter registry of high-volume HCM

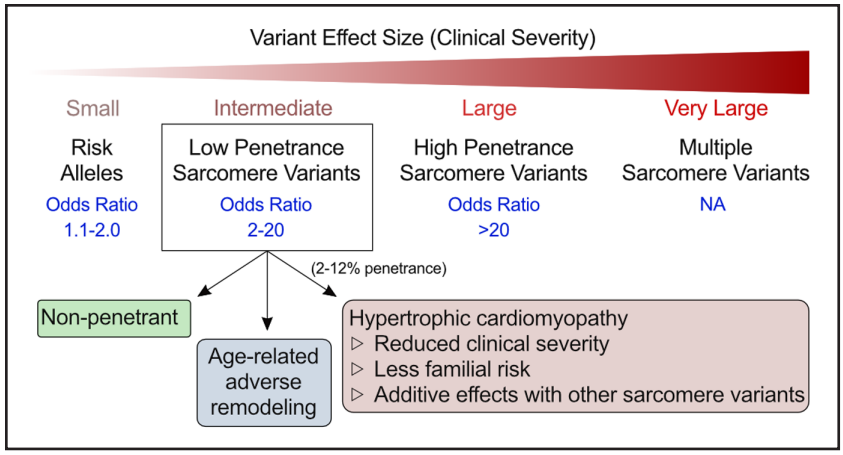

Figure 1. Hypertrophic cardiomyopathy (HCM) disease severity and penetrance are driven by the differing effect size in sarcomere gene variants.
The spectrum of variant effect size spans highly penetrant, pathogenic sarcomere variants (PathSVs) to risk alleles that alone do not cause HCM but contribute to additive risk. Odds ratio ranges for HCM cases vs controls are shown for each variant class. Low penetrance sarcomere variants (LowSVs) cause milder HCM when penetrant (in 2%–12% of individuals), but markedly increase HCM severity when present in combination with PathSVs.

centers of phenotypic, genetic, and clinical outcomes data on patients with HCM with retrospective and prospective elements. The structure of the registry, methods of data collection, and data curation have previously been described.[7] Participation in SHaRe was independently approved by the institutional review board at each center, and de-identified data were used to construct the central registry. Classification of variant pathogenicity was determined based on accepted standards, as previously described.[7,10] If conflicting classifications were present between sites, SHaRe investigators with expertise in variant curation assigned a SHaRe-adjudicated classification. Data for this study were exported February 2023. We defined LowSVs agnostically based on a combination of population prevalence from the Genome Aggregation Database (gnomAD) v4.0 and enrichment in SHaRe relative to population frequency data. We used the following criteria: (1) minor allele frequency (MAF) $>5\times10^{-5}$ in gnomAD v4.0 and (2) odds ratio (OR) for enrichment in SHaRe relative to gnomAD v4.0 >2.0 and with a 95% CI lower bound exceeding 1.0 and P value <0.05 by Fisher exact test.[7,9,23,24] Outcomes were assessed as class aggregates without adjustments in P values for individual variants. Level of evidence was defined by replication in a separate dataset and functional studies per ClinGen recommendations.[17] Counts per specific variants for odd ratios and significance excluded additional family members as identified in SHaRe. The MAF threshold was selected as the established maximally credible allele frequency for a causative variant and has been used previously in variant classification within SHaRe.[7,23,24] *MYBPC3* variant c.1504C>T (p.R502W) is an archetypal pathogenic variant with relatively high population frequency, and, similar to previous methods, this variant was separated as a reference compared to identified LowSVs.[24–26] Otherwise, variants defined as "pathogenic" or "likely pathogenic" were considered in a single category (PathSV) distinct from variants of uncertain significance (VUSs) or likely benign variants. After reclassification of LowSVs, patients with both a PathSV and 1 or more VUSs or with multiple VUSs were excluded from outcomes analyses. Composite outcomes were defined as outlined in the detailed methods (Supplemental Material).

Functional validation studies were performed using human iPSC-CMs. Patient induced pluripotent stem cells (iPSCs) were generated as part of a protocol approved by the University of Michigan Institutional Review Board and the University of Michigan Human Pluripotent Stem Cell Research Oversight Committee. iPSC-CM differentiation and functional quantification were performed using our previously published techniques.[27–29] Briefly, iPSCs were differentiated into iPSC-CMs using modulation of the WNT pathway, followed by metabolic purification.[27–29] Validation experiments on the functional effect of the *MYBPC3* c.442G>A variant on splicing and protein level were performed on purified iPSC-CM monolayers from a patient-derived iPSC line containing this variant in trans with a truncating variant. Specifically, splice variant quantification of the *MYBPC3* c.442G>A variant was performed using RNA sequencing (GSE274124). Additive effect of the *MYBPC3* c.442G>A variant on MyBP-C (myosin binding protein C) protein content was quantified by mass spectroscopy compared with 2 control, 2 patient, and gene-edited heterozygous truncating variant lines. Functional effects of the *TNNT2* c.832C>T variant on contractility were determined using lentiviral expression in iPSC-CMs to enable control for both line and batch variability. We generated lentiviral constructs to express wild-type (control) *TNNT2*, *TNNT2* R278C, and *TNNT2* R286C in iPSC-CMs, and positively transduced iPSC-CMs were selected by puromycin resistance. All lentiviral constructs included a C-terminal FLAG epitope tag to verify transduction and protein expression. These iPSC-CMs were then used to generate cardiac tissues using our previously published cardiac muscle bundle (CMB) technique.[28] Briefly, transduced and selected iPSC-CMs were assembled into CMB tissues on micropatterned elastomer substrates that mimic the elasticity of the heart (~8 kPa stiffness). Rectangular micropatterning on these substrates induces long-axis alignment of myofibrils and a uniaxial contractile direction, both of which enhance contractile function and increase resolution of contractile assays.[28] Further details are described in the Supplemental Methods.

The UK Biobank, a population-based prospective cohort with extensive genetic and phenotypic data collected on approximately 500 000 individuals aged 40 to 69 years recruited from across the United Kingdom between 2006 and 2010, was used as a separate orthogonal dataset to assess replication of the previously identified LowSVs in SHaRe with HCM and association with a priori selected quantitative cardiac magnetic resonance imaging (CMR) traits. HCM in the UK Biobank cases was identified from self-report clinical data (hospital admissions and death registry) and CMR (left ventricular maximum wall thickness >15 mm). Cases with HCM were excluded from single LowSV association testing with quantitative CMR traits. Replication of LowSVs identified in SHaRe with HCM and associated with quantitative CMR traits was performed using the genome-wide regression test implemented by Regenie.[30]

Statistical Analysis

SHaRe clinical data through February 2023 were analyzed. Continuous variables are presented as mean±SD if normally distributed or as median (interquartile range [IQR]) if deviating from the normal distribution unless otherwise noted. Categorical variables are presented as counts and percentages. Between-group comparisons were evaluated statistically using *t* test or χ^2 tests as appropriate with Bonferroni correction for multiple comparisons. Time-to-event analysis was performed using the Kaplan-Meier method and cumulative incidence function of events with statistical significance assessed by pairwise Breslow analysis. Survival analyses were limited to age 60 years, similar to previous analyses.[7] Multivariable Cox proportional hazards modeling was performed to evaluate the association of baseline variables on the rate and timing of the events of interest and reported as hazard ratios. A *P* value <0.05 was considered significant. Statistical analyses were conducted using IBM SPSS Statistics (version 29) with data presentation through GraphPad Prism (version 9).

RESULTS

Identification of LowSVs in SHaRe

From all patients in SHaRe with a diagnosis of HCM who underwent genetic testing (n=6045), we identified 1159 unique variants in sarcomere encoding genes (*ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2*, *TPM1*; Table S1). Of these 1159 variants, 145 unique variants were found to have a population frequency exceeding expected for the established maximally credible allele frequency for autosomal dominant cardiomyopathy (MAF $>5\times10^{-5}$) (Table S1).[7,23,24] Of these 145 variants, 12 variants met criteria for designation as LowSVs (see Methods), including relative enrichment in SHaRe and insufficient evidence to be considered PathSVs using accepted criteria.[8,9] The relative enrichment across all variants in SHaRe and the 12 identified LowSVs is shown in Figure 2A and 2B, and details of each LowSV are shown in Table 1 (further details in Table S1). A subset of 4 of 12 LowSVs were replicated in the UK Biobank HCM dataset, despite the smaller cohort size (n=637 HCM cases; Table S6) and 9 of 12 were replicated from a large 10 400-patient dataset described in McGurk et al.[20] Overall strength of evidence for each LowSV was assessed using criteria from the ClinGen Working Group for low penetrance and risk alleles[17] with 12 individual variants having strong or very strong evidence of being a true LowSV (level of evidence definitions in Supplemental Methods). Cumulatively, these 12 LowSVs are present in 126 patients (2.1%) with HCM in SHaRe. The identified LowSVs have a general population prevalence of 0.14% (1 of 350 individuals in gnomAD). As expected for variants with lower disease penetrance, the putative LowSVs had relatively low levels of enrichment (OR, 3.2–16.1) with an aggregate OR for all 12 LowSVs of 14.9 (95% CI, 12.5–17.9) for HCM cases in SHaRe versus gnomAD. In contrast, archetypal pathogenic variants exhibited a greater degree of enrichment in HCM. For example, the pathogenic *MYBPC3* variant c.1504C>T (p.R502W) had an HCM enrichment OR of 42.3 (95% CI, 32.5–55.1; *P*<0.0001 versus LowSV enrichment). Because *MYBPC3* truncating variants (*MYBPC3*tv) are, together, the most common genetic cause of HCM and are pathogenically similar,[10] we also calculated the relative enrichment for all *MYBPC3*tv in SHaRe, resulting in an OR of 79.1 (95% CI, 71.7–87.3; *P*<0.0001 versus LowSV enrichment). Although penetrance cannot be directly determined from SHaRe, we can approximately estimate penetrance of LowSVs in first-degree relatives of affected individuals using the relative ORs of LowSVs to *MYBPC3*tv's given that the penetrance of *MYBPC3*tv's in HCM relatives is known to be ~55%.[31] The relative OR for LowSVs is 0.19 (ie, 14.9/79.1 from above). Multiplying this relative ratio by 55% yields an approximate average penetrance estimate of 10% (range, 2%–12%). Using the approach described by McGurk et al,[20] the aggregate penetrance of incidentally found LowSVs is estimated at 1.3% (95% CI, 1.0%–1.8%; range for individual LowSVs, 0.6%–3.2%). The identified LowSVs predominantly include variants previously classified in ClinVar and SHaRe as VUS, likely benign, or conflicting evidence (Table 1).

AlphaMissense[32] and SpliceAI tools[33] were used to identify potential effects of the identified LowSVs on protein structure or splicing, respectively (Table 1). AlphaMissense[32] classified 8 of the 12 LowSVs with high pathogenicity scores consistent with predicted disruption of protein structure or function. SpliceAI[33] predicted the *MYBPC3* c.442G>A, *MYBPC3* c.1224-52G>A, and *MYBPC3* c.3065G>C variants to result in a gain of an alternative splice acceptor or donor site, which is consistent with previous splice assays for *MYBPC3* c.442G>A and *MYBPC3* c.1224-52G>A.[34,35]

Patients With LowSVs in Isolation Exhibit Milder HCM

Characteristics of patients in SHaRe stratified by variant type are shown in Table 2. A total of 86 patients (1.5%) carried a LowSV (HCM_{Low}) in isolation (without an additional PathSV). We previously showed that earlier age of diagnosis is associated with greater disease severity in the SHaRe cohort.[7,36] Here, we found that patients with HCM_{Low} were diagnosed with HCM at an older age compared with patients with a single PathSV (HCM_{Path}) (48 IQR [32, 56] versus 38 IQR [24, 51] years; *P*<0.001; Figure 2C). The age of diagnosis was modestly younger in HCM_{Low} compared with genetic test negative ($HCM_{SarcNeg}$; Figure 2C; adjusted *P*=0.03). Consistent with lower penetrance, a family history of HCM and sudden cardiac death were both less frequent in HCM_{Low} than HCM_{Path} (family history of HCM 33% versus 64%; *P*<0.001; family history of sudden cardiac death 4%

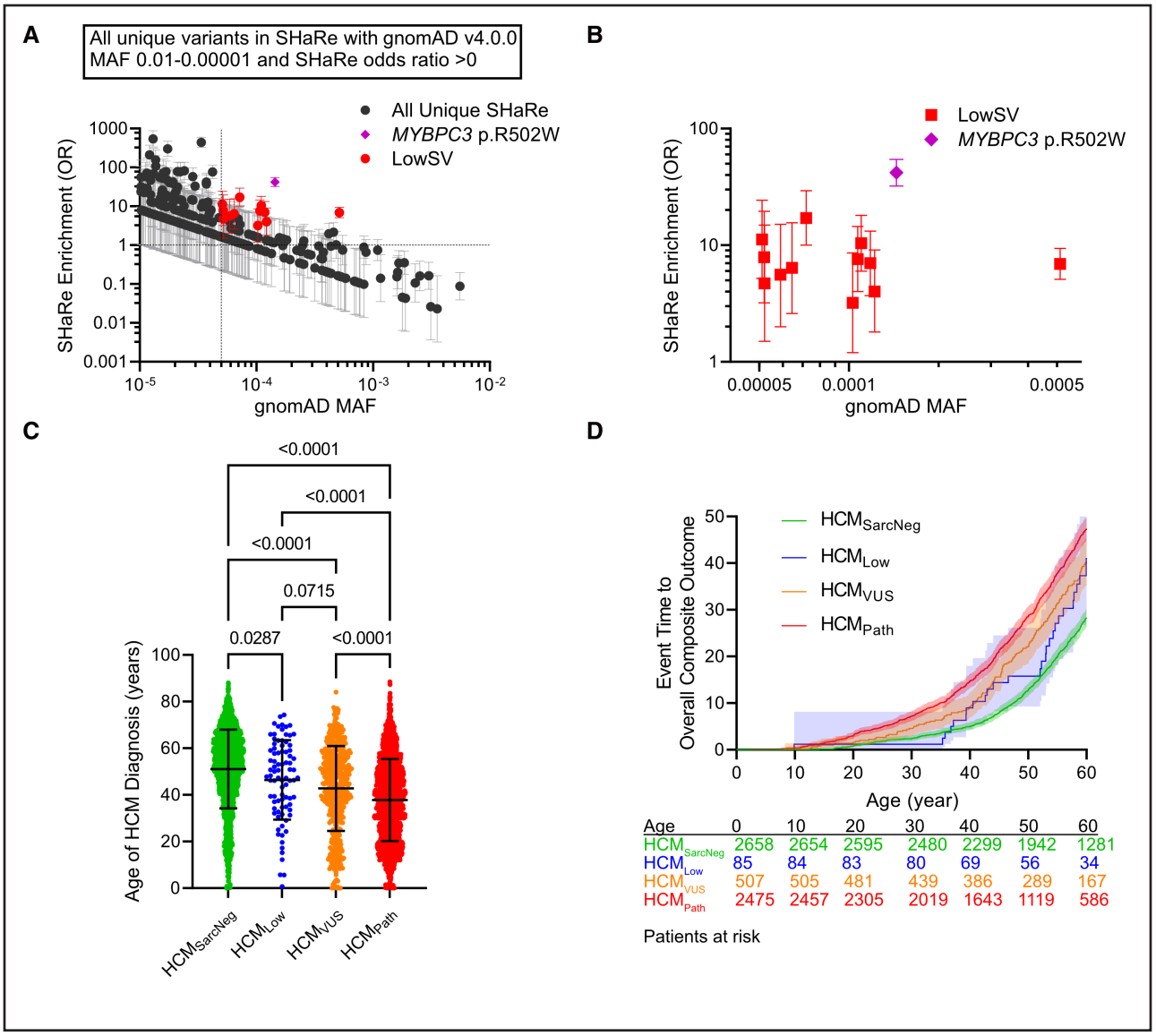

Figure 2. Isolated lower penetrant sarcomere variants (LowSVs) are associated with mild, lower penetrance hypertrophic cardiomyopathy (HCM).

A, Depicts the enrichment OR of all unique variants found in patients with HCM in the SHaRe registry with a MAF of 1×10^{-2} to 10^{-5} in the general population (gnomAD) (error bars represent 95% CI). Those with MAF $>5\times10^{-5}$ and enrichment with lower bound >1 were classified as LowSVs (red) with reference high frequency known pathogenic variant *MYBPC3* p.R502W (purple). **B**, Isolated depiction of LowSVs and *MYBPC3* p.R502W demonstrate that LowSVs have a more moderate enrichment within patients with HCM compared with reference pathogenic variant *MYBPC3* p.R502W, consistent with lower disease penetrance. **C**, Depicts age of onset of HCM, a marker of disease severity.[7] Patients with HCM with an isolated LowSV (HCM_{Low} blue) demonstrate an intermediate, mild increase in disease severity with age of onset between patients with HCM that are genotype negative ($HCM_{SarcNeg}$ green) and those that carry a variant of uncertain significance (HCM_{VUS}, orange) with significantly later age of onset compared with patients with HCM with a pathogenic or likely pathogenic sarcomere variant (HCM_{Path}, red) (*P* value by 1-way ANOVA with Bonferroni correction). **D**, Kaplan-Meier curves of overall composite outcome (defined as sudden cardiac death, resuscitated cardiac arrest, appropriate implantable cardioverter-defibrillator therapy, cardiac transplant, left ventricular assist device implantation, New York Heart Association [NYHA] class III or IV heart failure, atrial fibrillation, stroke, or all-cause mortality). Similar to age of onset, Kaplan-Meier curves demonstrate patients with HCM_{Low} have an intermediate effect on time to the composite outcome compared with $HCM_{SarcNeg}$ and HCM_{Path} ($P<0.001$ $HCM_{SarcNeg}$ vs HCM_{VUS} or HCM_{Path}, $P=0.04$ HCM_{Low} vs HCM_{Path}, $P=0.08$ HCM_{Low} vs $HCM_{SarcNeg}$ by Breslow, univariate Cox regression noted in Table S4). Shaded area denotes 95% CI.

versus 17%; $P=0.02$; Table 2). A male bias was highest in HCM_{Low} and $HCM_{SarcNeg}$ groups (both 65%) versus HCM_{Path} (57%; both $P<0.001$; Table 2).

Patients were followed for a median duration of 5.4 [IQR 2.0, 10.2] years across the complete cohort. The duration of follow-up was similar for patients with HCM_{Low} and HCM_{Path} (Table 2). We compared composite major adverse events (death, transplant, cardiac arrest, ventricular arrhythmia, or New York Heart Association class III/IV heart failure; Table S4) with $HCM_{SarcNeg}$, a group we previously reported

Table 1. Low Penetrance Sacromere Variants (LowSVs) with Enrichment in SHaRe >2.0 and Minor Allele Frequency >5×10-5 in gnomAD v4.0

Genomic variant	Protein variant	Previous classification		Variant counts (individuals/total) MAF		Enrichment (95% CI)	*P* value	AlphaMissense		SpliceAI	Level of evidence
		ClinVar	SHaRe	SHaRe	gnomAD			Score	Classification	Type, score >0.05	
MYBPC3 c.2429G>A	p.Arg810His	Conflicting	LP	14/6045 2.32E-03	116/1613846 7.19E-05	16.1 (9.3–28.1)	<0.00001	0.65	LP	…	Strong - REP†
MYH7 c.3981C>A	p.Asn1327Lys	LB	LB	7/6045 1.16E-03	82/1606190 5.11E-05	11.4 (5.2–24.6)	<0.00001	0.96	LP	…	Strong - REP†
MYBPC3 c.442G>A	p.Gly148Arg	Conflicting	VUS	13/6045 2.15E-03	172/1561288 1.10E-04	9.8 (5.6–17.2)	<0.00001	0.44	Ambiguous	Acceptor gain 0.94	Strong - FS, REP†
MYBPC3 c.1224-52G>A	…	P/LP	VUS	5/6045 8.27E-04	79/1522824 5.19E-05	8.0 (3.2–19.7)	0.00056	…	…	Acceptor gain 0.99	Strong - REP*
MYH7 c.976G>C	p.Ala326Pro	Conflicting	VUS	10/6045 1.65E-03	173/1613828 1.07E-04	7.7 (4.1–14.6)	<0.00001	0.26	LB	…	Strong - REP†
TNNT2 c.832C>T	p.Arg278Cys	Conflicting	LB	40/6045 6.62E-03	827/1611210 5.13E-04	6.5 (4.7–8.9)	<0.00001	1.00	LP	…	Very strong - FS, REP*†
MYBPC3 c.1828G>C	p.Asp610His	Conflicting	VUS	5/6045 8.27E-04	103/1598984 6.44E-05	6.4 (2.6–15.8)	0.0014	0.98	LP	…	Very strong - REP*†
MYL3 c.170C>A	p.Ala57Asp	Conflicting	VUS	9/6045 1.49E-03	190/1614056 1.18E-04	6.3 (3.2–12.4)	<0.00001	0.65	LP	…	Strong - REP†
MYBPC3 c.3065G>C	p.Arg1022Pro	Conflicting	LP	3/6045 4.96E-04	84/1611220 5.21E-05	4.8 (1.5–15.1)	0.028	0.96	LP	Donor gain 0.15	Very strong - REP*†
MYBPC3 c.2618C>A	p.Pro873His	VUS	LB	3/6045 4.96E-04	90/1528202 5.89E-05	4.2 (1.3–13.3)	0.0066	0.87	LP	…	Limited
MYBPC3 c.1321G>A	p.Glu441Lys	Conflicting	LB	6/6045 9.93E-04	197/1613118 1.22E-04	4.1 (1.8–9.2)	0.0044	0.78	LP	…	Strong - REP†
MYBPC3 c.1813G>A	p.Asp605Asn	Conflicting	LB	4/6045 6.62E-04	164/1593218 1.03E-04	3.2 (1.2–8.7)	0.039	0.16	LB	…	Limited
Total	…	…	…	1.97E-02	1.42E-03	…	…	…	…	…	…

gnomAD v4.0 reference GRCh38 reference genome. *P* value calculated by Fisher exact test, OR and 95% CI by Woolf Logit method. Variant allele counts limited to 1 per family ID. LB indicates likely benign; LP, likely pathogenic; and VUS, variant of uncertain significance.

Level of evidence support qualifiers denoted as FS, supporting functional studies, and REP, replication in independent dataset.

*Replication based on UK Biobank.

†Replication based on McGurk et al.[20]

to have fewer adverse events, as the reference.[7,37,38] Compared with patients who were $HCM_{SarcNeg}$, overall composite adverse events occurred in patients with HCM_{Low} at a comparable rate (mean event-free survival age, $HCM_{SarcNeg}$, 62 years [95% CI, 61–62] versus HCM_{Low}, 60 years [95% CI, 57–63]; *P*=0.08; Figure 2D). In contrast, individuals with HCM_{Path} exhibited a significantly higher rate of overall composite adverse events (mean event-free survival age, HCM_{Path}, 56 years [95% CI, 55–57] versus HCM_{Low}, 60 years [95% CI, 57–63]; *P*=0.04; Figure 2D).

LowSVs Exhibit Additive Effects in Combination With PathSVs

We next assessed whether LowSV in the presence of an additional PathSV is associated with more severe HCM, consistent with an additive effect. Patients with a combination of an LowSV and a PathSV variant ($HCM_{Path+Low}$) were diagnosed with HCM at an earlier age than HCM_{Path} (median, 27 [13, 34] versus 38 [23, 51] years [IQR]; adjusted *P*=0.04). Age of diagnosis was more similar in patients with $HCM_{Path+Low}$ and patients with HCM with 2 or more PathSVs ($HCM_{MultiPath}$; 27.1 versus 28.5 years; adjusted *P*=0.20; Figure 3A). We also observed differences in clinical characteristics in the $HCM_{Path+Low}$ group. For example, there was a trend toward no male bias in $HCM_{Path+Low}$ (48% male versus 65% in $HCM_{SarcNeg}$; unadjusted *P*=0.09 versus $HCM_{SarcNeg}$; Table 2). Additive exposures contributing to HCM (ie, male sex, diabetes, and hypertension, equally weighted)[39] were also fewer in $HCM_{Path+Low}$ (Table 2; adjusted *P*<0.0001 versus $HCM_{SarcNeg}$). These data could suggest a lesser relative influence of nonsarcomere risk factors for HCM development in the presence of additive effects from sarcomere variants. At first echocardiogram, individuals with $HCM_{Path+Low}$ had a lower mean left ventricular ejection fraction (59%±15%; *P*<0.05 for $HCM_{Path+Low}$ versus $HCM_{SarcNeg}$, HCM_{Low}; Table S3).

A left ventricular ejection fraction 50% to 60% in patients with HCM is predictive of future progression to overt systolic dysfunction as a late-stage complication of HCM.[38]

Table 2. Demographics of Patients With HCM Within SHaRe Delineated by Variant Classification

Variable	Variant classification						
	$HCM_{SarcNeg}$	HCM_{Low}	HCM_{VUS}	HCM_{Path}	$HCM_{Path+Low}$	$HCM_{MultiPath}$	Total
Column vs group adjusted $P<0.05$*	**(n=2694)**	**(n=86)**	**(n=515)**	**(n=2509)**	**(n=21)**	**(n=75)**	**(n=5900)**
Age of HCM diagnosis (years)	53.6 [42.0, 63.2] *VUS, Path, Low+Path, MultiPath	47.8 [34.9, 59.0] *Path, Low+Path, MultiPath	45.1 [31.6, 56.3] *Path, Low+Path,MultiPath	38.2 [23.5, 51.0] *Low+Path	27.1 [13.3, 34.4] …	28.5 [13.7, 47.8] …	46.6 [31.0, 58.5]
Family history of HCM	632 (23.5%) …	28 (32.6%) …	198 (38.4%) *SarcNeg	1593 (63.5%) *SarcNeg, Low, VUS	7 (33.3%) …	46 (61.3%) *SarcNeg, Low, VUS	2504 (42.4%)
Family history of sudden cardiac death	183 (6.8%) …	4 (3.7%) …	55 (10.9%) *SarcNeg	421 (16.8%) *SarcNeg, Low, VUS	6 (19.4%) *SarcNeg	15 (20.0%) *SarcNeg, Low	684 (11.6%)
Follow-up duration (years)	4.6 [1.5, 8.8] …	4.5 [1.3, 7.1] …	4.9 [1.7, 9.2] …	6.4 [2.5, 11.9] *SarcNeg, Low, VUS	6.9 [0.9, 15.3] …	6.2 [2.0, 10.9] …	5.4 [2.0, 10.2]
Sex							
Female	933 (34.6%)	30 (34.9%)	188 (36.5%)	1069 (42.6%)	11 (52.4%)	33 (44.0%)	2264 (38.4%)
Male	1761 (65.4%) …	56 (65.1%) …	327 (63.5%) …	1440 (57.4%) *SarcNeg	10 (47.6%) …	42 (56.0%) …	3636 (61.6%)
Proband	2584 (97.6%) *VUS	83 (96.5%) *Path	471 (92.9%) *Path	1967 (78.9%) …	18 (85.7%) …	63 (84.0%) …	5186 (88.9%)
Diabetes	310 (11.5%) *Path	10 (11.6%) …	38 (7.4%) …	151 (6.0%) …	0 (0.0%) …	4 (5.3%) …	513 (8.7%)
Hypertension	1449 (53.8%) *Low, VUS, Path, Low+Path, MultiPath	31 (36.0%) …	207 (40.2%) *Path, Low+Path	750 (29.9%) …	1 (4.8%) …	19 (25.3%) …	2457 (41.6%)
Aggregate risk factors							
Male + diabetes+ hypertension	1.3±0.8 *VUS, Path, Low+Path, MultiPath	1.1±0.8 *Low+Path	1.1±0.8 *Path, Low+Path	0.9±0.7 …	0.5±0.6 …	0.9±0.7 …	1.1±0.8
Race							
Asian	104 (3.9%) …	1 (1.2%) …	24 (4.7%) …	73 (2.9%) …	0 (0.0%) …	1 (1.3%) …	203 (3.4%)
Black	135 (5.0%) *Path	0 (0.0%) …	27 (5.2%) *Path	68 (2.7%) …	0 (0.0%) …	2 (2.7%) …	232 (3.9%)
White	2180 (80.9%) …	77 (89.5%) …	403 (78.3%) …	2175 (86.7%) *SarcNeg, VUS	19 (90.5%) …	66 (88.0%) …	4920 (83.4%)
Other or not reported	275 (10.2%) *Path	8 (9.3%) …	61 (11.8%) *Path	193 (7.7%) …	2 (9.5%) …	6 (8.0%) …	545 (9.2%)

Data presented as count (% of variant population), median [interquartile range], mean±SD.

*t Tests (for continuous variables) and chi-square tests (for proportions) were performed for comparison between classifications with Bonferroni multiple test correction. Adjusted P value <0.05 for two-way comparison with each listed group (note: for brevity, each significant two-way comparison is listed only once in the table).

Patients with $HCM_{Path+Low}$ also exhibited a significantly higher adverse event rate compared with patients with HCM_{Path} (Figure 3B; Kaplan-Meier adjusted $P<0.001$ between HCM_{Path} and $HCM_{Path+Low}$; hazard ratio [95% CI]; Table S5; $HCM_{Path+Low}$, 5.4 [95% CI, 3.0–9.8] compared with HCM_{Path}, 2.0 [95% CI, 1.8 to 2.2] relative to $HCM_{SarcNeg}$). Multivariate analysis demonstrated that $HCM_{Path+Low}$ status remained an independent predictor of composite adverse events (other independent predictors included age of diagnosis and proband status; Table 3). Correspondingly, individuals with $HCM_{Path+Low}$ developed significantly more heart failure events (Figure S1B; Table S5). Composite arrhythmia adverse events were fewer in total number, and statistically significant differences were not proven among groups (Figure S1A; Table S5).

Assessment of *MYBPC3* c.442G>A as a LowSV Through Partial Splice Gain of Function

We next determined the effects of the LowSV *MYBPC3* c.442G>A on RNA splicing and encoded MyBP-C protein level. This variant has had conflicting interpretations

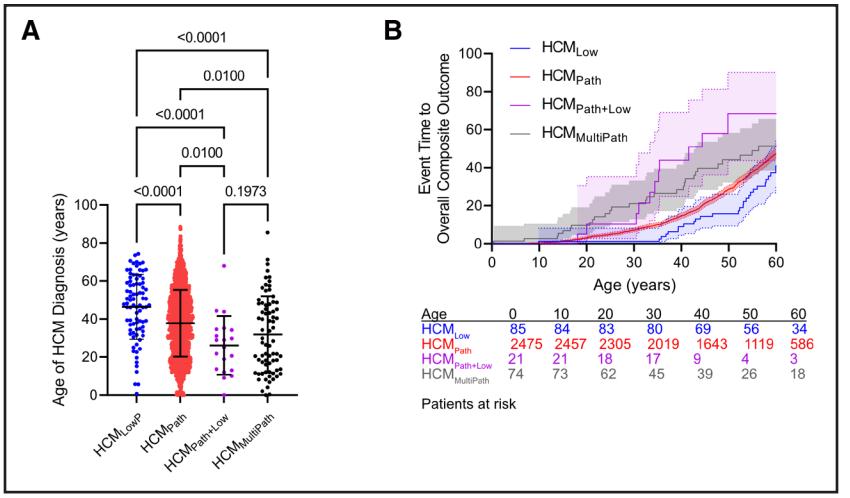

Figure 3. LowSV in combination with PathSV demonstrates markedly increased disease severity.
A, When compared with HCM cases with a single PathSV (HCM_{Path} red), HCM cases with LowSV combined with a PathSV ($HCM_{Path+Low}$, purple) demonstrate significantly early onset of HCM similar to that of patients with 2 or more PathSVs ($HCM_{MultiPath}$, gray) (*P* value by 1-way ANOVA, Bonferroni correction for multiple comparison). **B**, Similar to increased HCM severity as demonstrated by age of onset, patients with $HCM_{Path+Low}$ with combined LowSV and PathSV (purple) genotype have significantly worse outcomes than a single PathSV genotype by Kaplan-Meier time to event of overall composite outcome ($P<0.001$ by Breslow testing, HR by univariate Cox regression noted in Table S4). Patients with $HCM_{Path+Low}$ do statistically differ in outcomes compared with patients with $HCM_{MultiPath}$. Shaded area denotes 95% CI.

in the ClinVar database and was previously designated as a VUS after adjudication by SHaRe investigators.[10] We found *MYBPC3* c.442G>A to be enriched in SHaRe with an OR of 9.8 (95% CI, 5.6–24.6; Table 1). This exonic variant was predicted to cause a splice gain based on a spliceAI score of 0.94 (Table 1). Although c.442G>A is also a coding sequence variant, the amino acid change (p.G148R) was not strongly predicted to affect protein structure by AlphaMissense (0.44; Table 1). Among the SHaRe cohort, this variant occurred as an isolated sarcomere variant in n=12 (14% of single LowSV cases) and in combination with a pathogenic *MYBPC3* variant in 2 patients (Table S2). In both biallelic cases, the patients presented at young age (22 and 30 years) and developed heart failure over short-term follow-up (by age 25 and 35 years, respectively). One of the patients developed left ventricular wall thinning and systolic dysfunction over 5 years of follow-up (ejection fraction decline from normal to 35%), indicating progression to advanced disease,[40] but not as severe as patients presenting with homozygous PathSV variants in *MYBPC3*, which typically result in fatal infantile dilated cardiomyopathy.[41] Therefore, we hypothesized that the c.442G>A variant exerts an incomplete splice gain effect, thus behaving as a hypomorphic allele (ie, partial loss of function) that exerts an additive effect in combination with other sarcomere variants.

We generated an iPSC line from 1 of the biallelic patients to specifically test the hypothesis that the c.442G>A variant results in a partial splice gain effect with a consequent additive effect on encoded protein level. This patient harbored the *MYBPC3* c.442G>A LowSV with a definitively pathogenic *MYBPC3* splice variant (c.506-1G>A)[10] on the other allele. We differentiated these iPSCs into cardiomyocytes (iPSC-CMs) and quantified splicing of *MYBPC3* transcripts relative to control cardiomyocytes using RNA sequencing. We observed a partial alternative splice acceptor gain at the predicted location downstream of the c.442G>A variant (Figure 3A and 3B). The identified alternatively spliced transcript is frameshifted relative to the reference transcript by 37 nucleotides resulting in a premature stop codon. Premature stop codons render transcripts susceptible to nonsense mediated RNA decay, which is the predominant cause of haploinsufficiency in HCM caused by truncating *MYBPC3* variants (*MYBPC3*tv).[27,42] We calculated the percent of abnormally spliced *MYBPC3*

Table 3. Risk of Development of Overall Composite Outcome to Age 60 Years

Factor		Hazard ratio (95% CI)	*P* value
Sex	Female	Ref	
	Male	0.97 (0.88–1.08)	0.62
Age at diagnosis per 5 years		0.72 (0.71–0.73)	<0.001
Proband	No	Ref	
	Yes	1.27 (1.07–1.51)	0.006
Variant class	Negative	Ref	
	LowSV	1.22 (0.84–1.78)	0.30
	VUS	1.13 (0.95–1.35)	0.18
	PathSV	1.17 (1.05–1.31)	0.005
	Low+PathSV	2.62 (1.44–4.77)	0.002
	MultiPathSV	1.92 (1.32–2.79)	<0.001

Cox regression analysis of overall composite event.

transcripts from the c.442G>A allele by normalizing to adjacent, unaffected splice junctions and found that 41±13% of transcripts from this allele were alternatively spliced (Figure 4C). The pathogenic c.506-1G>A *MYBPC3* variant in this patient resulted in primarily in-frame alternatively spliced transcripts (Figure S2). Next, we performed mass spectroscopy to determine the cumulative impact of the compound heterozygous variants (c.442G>A combined with c.506-1G>A) on *MYBPC3*'s encoded protein (MyBP-C). We found that the patient-derived biallelic *MYBPC3* iPSC-CMs exhibited haploinsufficiency of MyBP-C compared with 2 control iPSC-CM lines (62±3% of control; $P<0.001$; Figure 4D) and also compared with our previously published heterozygous *MYBPC3*tv iPSC-CM lines ($P<0.001$), which we had shown to exhibit posttranslational compensation that

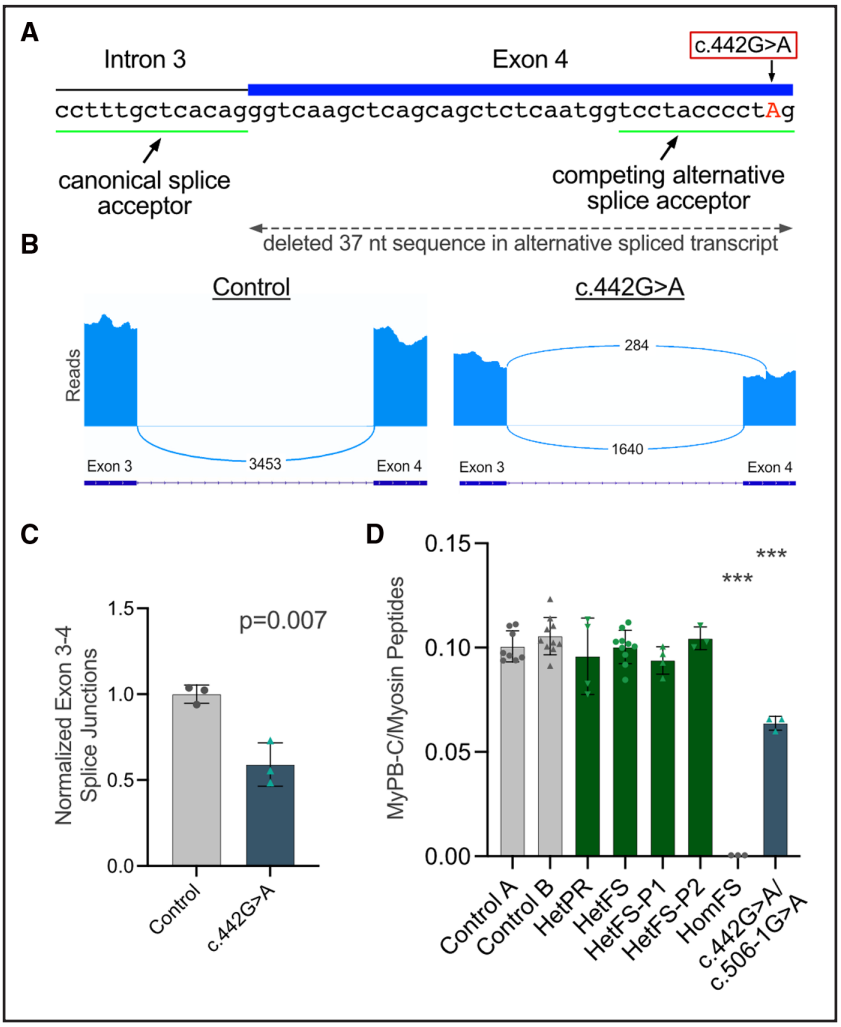

Figure 4. Functional testing of *MYBPC3* c.442G>A on splicing and protein stoichiometry.
A, The *MYBPC3* c.442G>A variant creates a novel splice acceptor site 27 nucleotides 3' of the canonical exon 4 splice acceptor site. **B**, iPSC-CMs were generated from a patient with the *MYBPC3* c.442G>A variant in trans with the pathogenic *MYBPC3* splice variant c.506-1G>A. The effect of each variant was quantified using RNA sequencing (n=3 separate differentiation batches for the biallelic *MYBPC3* line and for a control iPSC line). Reads aligning to splice junctions were quantified using the Integrative Genomics Viewer (IGV), shown here as Sashimi plots (1 example shown for control and biallelic *MYBPC3* lines). Lines connecting exons indicate the number of reads aligning with each junction. In the control iPSC-CMs (**left**), reads aligned only with the canonical splice junction. In the line containing the c.442G>A variant (**right**), reads also aligned to the predicted splice gain acceptor site. Exon 4-5 splice junction disruption by the c.506-1G>A variant is shown in Figure S2. **C**, Disruption of exon 3-4 splicing for the c.442G>A allele was calculated by normalizing normally spliced exon 3-4 junction reads to reads aligned to adjacent unaffected exons. This calculation could be performed because the c.506-1G>A variant results in primarily in-frame splice disruption not affecting transcript stability (Figure S2). **D**, The encoded protein (MyBP-C) was quantified relative to total myosin using mass spectroscopy for biallelic c.442G>A/c.506-1G>A iPSC-CMs compared with 2 controls and multiple previous *MYBPC3* model iPSC-CM lines. The control and other MYBPC3 variant lines' data were previously published but were obtained in the same mass spectroscopy experiments as the data for the c.442G>A/c.506-1G>A iPSC-CMs and are presented for comparison. The previous *MYBPC3* variant lines include HetPR (gene-edited heterozygous *MYBPC3* promoter deletion), HetFS (gene-edited heterozygous *MYBPC3* frameshift/truncating variant), HetFS-P1 (patient-derived heterozygous *MYBPC3* frameshift/truncating variant), HetFS-P2 (patient-derived heterozygous *MYBPC3* frameshift/truncating variant), and HomFS (gene-edited homozygous *MYBPC3* frameshift/truncating variant). Bars and error bars indicate mean and SD. ***$P<0.001$.

prevents haploinsufficiency.[27] Taken together, these analyses demonstrate that the *MYBPC3* c.442G>A LowSV yields an intermediate size effect on *MYBPC3* transcript level through a partial splice acceptor gain, and furthermore, this partial splicing effect results in an additive loss of total MyBP-C protein level when present together with a *MYBPC3* PathSV.

Assessment of *TNNT2* c.832C>T (p.R278C) as a LowSV Through Contractile Dysregulation

We next assessed the functional effects of the *TNNT2* c.832C>T (R278C) variant, which has been an unresolved variant with conflicting interpretations in the ClinVar database. This variant was previously designated as "likely benign" in the SHaRe cohort because of its high population prevalence (5×10^{-4}). However, in the present analysis, we found that this variant is clearly enriched in SHaRe with an OR of 6.5 (95% CI, 4.7–8.9; Table 1). In total, we observe that this variant is present in 43 patients in SHaRe, making it one of the single most common variants in SHaRe. This variant was predicted by AlphaMissense to affect protein structure/function (0.997; Table 1).

We hypothesized that the R278C variant has an intermediate effect on contractility between wild-type *TNNT2* and established missense variants in *TNNT2* that have been determined to be causative of HCM (ie, PathSV). To test for subtle functional effects between variants, we used the CMB approach,[28] which enables contractile studies of purified iPSC-CMs in highly aligned tissues with uniaxial contractions at a physiologic workload (~8 kPa). Moreover, because we previously have observed significant iPSC-CM batch variation in high-resolution contractile assays, even across experiments in the same iPSC-CM line,[28,29] we selected an approach that would control for both line and batch (ie, isogenic and isobatch) by using lentiviral expression of *TNNT2* in CMBs. The *TNNT2* R286C variant was used as a positive control because it is strongly enriched in SHaRe (OR, 55.6 [95% CI, 26.6–116.3]) and affects the same C-terminal TNNT2 tail domain as the R278C variant. We first optimized transduction conditions to attain near complete displacement with lentiviral-expressed *TNNT2* at the mRNA transcript level (Figure S4). We then showed that protein levels of total TNNT2 were unchanged between transduced and nontransduced iPSC-CMs using quantitative immunofluorescence (Figure 5A and 5B). This finding is consistent with the known precise stoichiometric regulation of TNNT2 in the sarcomere.[43] In addition, we verified that equivalent levels of transduced TNNT2 were expressed for each of the 3 constructs (Figure 5C). Having shown that this overexpression approach resulted in similar protein levels for each variant, we next performed contractility analyses in CMBs. Contractility was measured in CMBs, which individually contract at physiologic workload and allow for high precision of contractile mechanics, as previously described.[28] We found that both R278C and R286C CMBs exhibited increased maximal fractional shortening compared with wild type (control) at low media calcium concentration (7.3±2.6% and 7.6±2.3% versus 6.1±2.3%; P=0.005 and P=0.001, respectively; Figure 5D). However, these differences were not apparent at higher media calcium concentration (Figure 5E), consistent with an effect of the *TNNT2* variants on myofilament calcium sensitivity. Relaxation velocity at low calcium concentration was reduced in R286C CMBs compared with both R278C CMBs (3.8±0.8 µm/s versus 4.4±1.0 µm/s; P=0.001) and wild type CMBs (3.8±0.8 µm/s versus 4.4±0.8 µm/s; P=0.002; Figure 5F). At high calcium concentration, both R278C and R286C CMBs exhibited reduced relaxation velocity compared with wild type (4.6±1.0 µm/s and 4.6±1.2 µm/s versus 5.1±1.1 µm/s; P=0.01 and P=0.04, respectively Figure 5G). Taken together, these data demonstrate that the TNNT2 R278C variant dysregulates both contractile and relaxation function in cardiomyocytes, with a lesser effect on relaxation velocity compared with the R286C variant at lower calcium level.

LowSVs Are Associated With HCM-Adjacent Traits in the UK Biobank

We next hypothesized that HCM-associated LowSVs, being more common in the general population, may also be associated with cardiac morphologic traits along the HCM spectrum, even in the absence of a clinical diagnosis of HCM. To test this hypothesis, we analyzed whether LowSVs identified in SHaRe are associated with HCM-adjacent traits in the CMR subcohort of the UK Biobank without HCM (n=36 104). Specifically, we analyzed the association of individual LowSVs on left ventricular end diastolic volume, left ventricular end systolic volume, left ventricular ejection fraction, left ventricular mass, and maximum wall thickness. Five of the 12 LowSVs were associated with significant differences in these metrics (Table 4). All significant differences exhibited a direction of effect expected for HCM (ie, reduced left ventricular end diastolic volume or left ventricular end systolic volume; increased left ventricular ejection fraction and maximum wall thickness). For example, the *MYL3* c.170C>A variant was associated with decreased left ventricular end diastolic volume and left ventricular end systolic volume, metrics that have been associated with diastolic dysfunction.[44–47] *MYBPC3* variants c.3065G>C, 1828G>C, and 1224-52G>A demonstrated a significant association with a modestly increased average maximum wall thickness, as expected for a low penetrance variant. *TNNT2* variant c.832C>T was significant across multiple variables (left ventricular ejection fraction and left ventricular end systolic volume). The associations for all LowSVs are shown in Table S7 with similar concordant direction

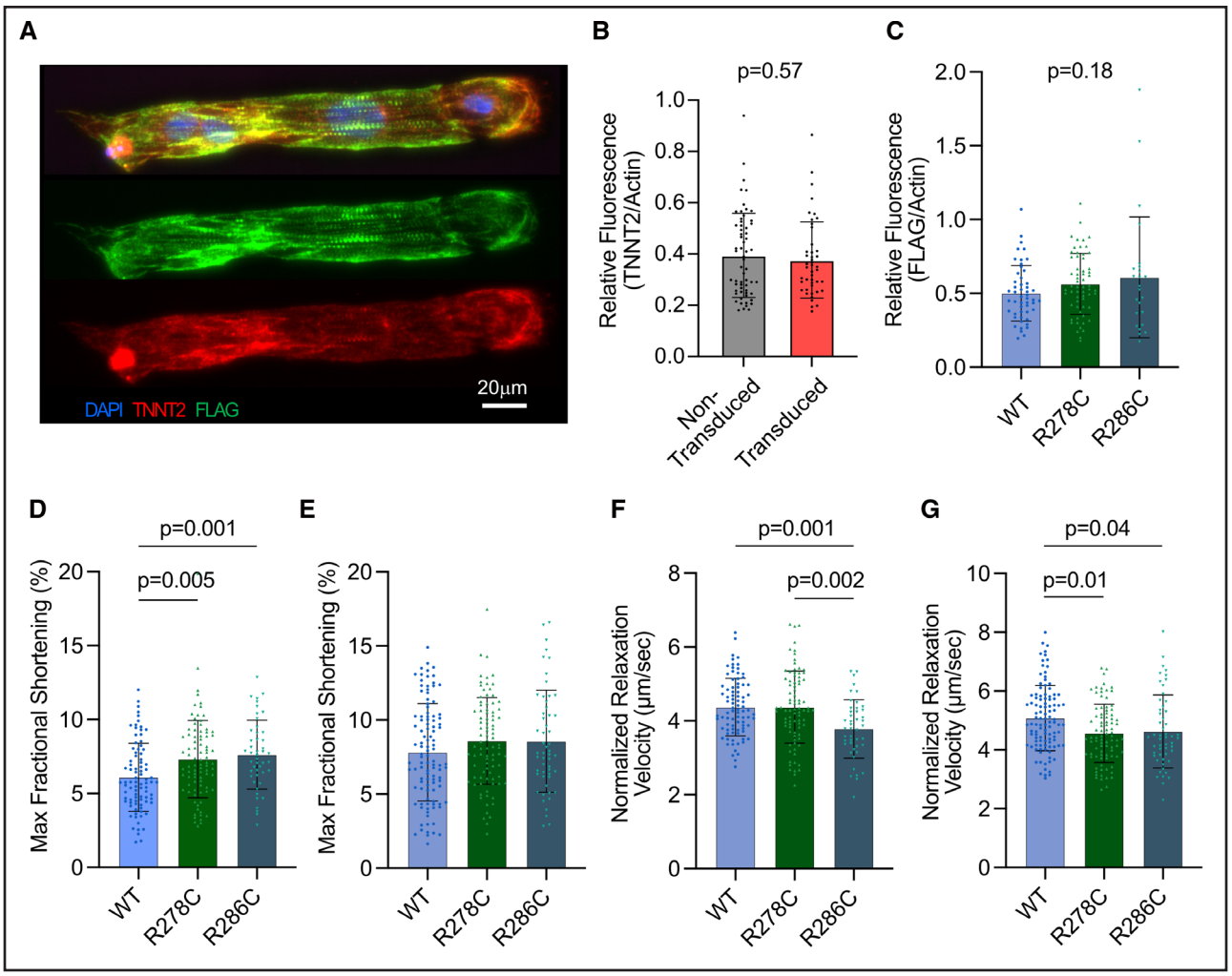

Figure 5. Functional testing of *TNNT2* c.832C>T (R278C) in cardiac muscle bundles.
A, iPSC-CMs were transduced with lentiviral expression vectors for wild-type (control) TNNT2, R278C TNNT2, or R286C TNNT2. All constructs were C-terminal epitope tagged with FLAG to enable immunofluorescence-based validation of transduction efficiency. A representative example of WT-TNNT2-FLAG is shown. Immunofluorescence imaging with an anti-FLAG antibody demonstrates high transduction efficiency as evidenced by colocalization with an anti-TNNT2 antibody (marking both endogenous and exogenous TNNT2). Examples for both R278C and R286C are shown in Figure S3. **B** and **C**, Total TNNT2 protein (**B**) and FLAG-tagged TNNT2 (**C**) were quantified by immunofluorescence relative to actin. Total TNNT2 protein was equivalent between individual iPSC-CMs that were transduced compared nontransduced CMs (**B**). Quantitative FLAG-tagged TNNT2 levels were equivalent across (control) TNNT2, R278C TNNT2, or R286C TNNT2 cardiac muscle bundles (**C**). **D** and **E**, Contractile function was measured in cardiac muscle bundles (CMBs) generated from transduced iPSC-CMs. CMBs were imaged under both low (0.75 mM) and high (1.5 mM) media calcium concentrations. Maximal fractional shortening was increased in both R278C and R286C CMBs at low calcium concentration (**D**) but was not significantly different at high calcium concentration (**E**). **F** and **G**, Relaxation velocity of CMBs was measured as a cell-based metric of diastolic function. Relaxation velocity was reduced in R286C CMBs at low calcium concentration (**F**) and was reduced in both R278C and R286C CMBs at high calcium concentration (**G**). Bars and error bars indicate mean and SD.

of effect, even in those not reaching statistical significance. Taken together, these data demonstrate that the common HCM-associated LowSVs are associated with cardiac morphologic changes consistent with adverse remodeling without meeting criteria for typical HCM.

DISCUSSION

Using the scope of genotypic and phenotypic data in SHaRe, population-level genetic data from gnomAD, functional validation in patient-derived iPSC lines, and the large-scale imaging and genetics in the UK Biobank, we have characterized a new class of LowSVs. LowSVs are relatively common in the general population with an overall prevalence of 1 of 350 individuals. When resulting in HCM (ie, penetrant), LowSVs typically cause milder HCM, based on later age of presentation and fewer adverse events. Further, our analyses support an additive effect model for understanding HCM disease severity. Although their effect size is moderate when isolated, LowSVs profoundly influence HCM severity when present along with PathSVs. In addition, establishing this set of LowSVs results in resolution of variant classification affecting 2.1% of patients in SHaRe, because

Table 4. Cardiac MRI Metrics of Carriers of LowSVs in UK Biobank

Cardiac MRI metric	Variant	Effect (95% CI)	*P* value
Left ventricular end diastolic volume (mL)	MYL3 c.170C>A	−28.7 (−51.6, −5.9)	0.01
Left ventricular ejection fraction (%)	TNNT2 c.832C>T	2.2 (0.6, 3.8)	0.01
Left ventricular end systolic volume (mL)	TNNT2 c.832C>T	−4.3 (−8.5, 0.0)	0.05
	MYL3 c.170C>A	−14.9 (−28.8, −1.1)	0.03
Maximum wall thickness (mm)	MYBPC3 c.3065G>C	2.2 (0.3, 4.0)	0.03
	MYBPC3 c.1828G>C	1.2 (0.1, 2.4)	0.04
	MYBPC3 c.1224-52G>A	1.3 (0.2, 2.5)	0.02

MRI indicates magnetic resonance imaging.

the LowSVs we identified all had previously disputed clinical significance. Last, using the UK Biobank CMR cohort, we show that LowSVs are associated with adverse cardiac remodeling even in the absence of a clinical diagnosis of HCM.

Clinical Implications of LowSVs

HCM has generally been considered a primarily autosomal dominant disease for which a single variant (ie, monogenic) is sufficient to cause HCM.[48] However, genetic testing has yielded diagnostic results in only ~40% of patients with HCM.[49] Our groups have previously shown that patients with negative genetic testing most often do not exhibit evidence of familial HCM, establishing the concept of more complicated non-Mendelian inheritance for many patients with HCM.[18,19] In contrast with classic monogenic causes of HCM, recent genome-wide association studies have clearly demonstrated that there exist common population variants in nonsarcomere genes that function as risk alleles for HCM.[11,12,15,24] Risk alleles do not cause HCM in isolation because of their small effect size but exert additive effects both with and without the presence of PathSVs with large influences on disease risk in combination.[17] Here, we have characterized an important group of lower penetrance sarcomere variants with intermediate effect sizes that function between PathSVs and risk alleles along a spectrum (Figure 1). Although the existence of lower penetrance sarcomere variants has been suggested previously (eg, *TNNI3* p.R79C, *TNNT2* p.R286H),[20–22] no previous study has systematically assessed low penetrance variants or determined how such variants can influence HCM. Our results are consistent with the paradigm recently proposed by the ClinGen Workgroup on low penetrance and risk alleles.[17] These analyses support an oligogenic disease model for HCM, wherein sarcomere variants contribute to disease severity correlated with their independent effect size and function additively when multiple variants are present.

The concept of lower penetrance variants has implications for clinicians performing cascade testing and screening evaluations in family members. The actual penetrance of individual sarcomere variants is strongly dependent on genetic background. As recently reviewed, average penetrance of PathSVs in non-proband family members of patients with HCM is 57%.[31] However, the penetrance is lower when PathSVs are discovered incidentally (eg, through whole genome sequencing for other indications), which is estimated at 11%.[20,31] We used a ratio of the ORs to estimate an average penetrance of LowSVs for non-proband family members of only ~10%. Correspondingly, we estimate incidentally discovered LowSVs likely have an even lower penetrance on the range of just 1% to 2%. Nevertheless, although HCM was milder on average in patients with isolated LowSVs, adverse events did occur, and family history of both HCM and SCD was positive in a significant proportion (33% and 4%, respectively). Therefore, clinical screening is still appropriate for family members of individuals with isolated LowSVs when a family history of HCM is present. Less frequent serial monitoring may be appropriate in such individuals.

Identification of LowSVs in patients also influences clinical assessment of longitudinal risks. Patients with an isolated LowSV typically develop mild HCM with later onset and few complications. In contrast, LowSVs profoundly alter disease course when present along with a single PathSV. These individuals presented, on average, 11 years earlier than those with a PathSV alone and experienced more than double the risk of adverse events. As risk models incorporating genetic variants continue to evolve, inclusion of low penetrance variants will be critical to maximize the potential for individualized risk prediction, follow-up, and management.

Adjudication of LowSVs Resolves a Substantial Proportion of VUSs

The majority of LowSVs adjudicated in this study were previously reported as VUSs or had disputed categorizations (Table 1). VUS interpretation has remained a major clinical challenge of genetic testing. In HCM, patients carrying sarcomere VUSs exhibit, on average, a milder clinical phenotype than patients with PathSVs[7] However, this observation could be a result of averaging of large (ie, PathSV), intermediate (ie, LowSV), and negligible/small

(ie, likely benign or risk allele) effect sizes of individual variants, limiting clinical actionability for the VUS category. Here, we show compelling evidence that some sarcomere variants previously classified as VUS are definitively intermediate effect size variants (ie, LowSVs). The low penetrance explains why these variants were previously unresolved with rules developed for PathSVs. For example, the low penetrance of the *TNNT2* c.832C>T variant is consistent with conflicting reports in ClinVar because of lack of clear variant cosegregation in families.

In contrast with PathSVs, which can be resolved from a small number of observations of variant-phenotype cosegregations within families, LowSVs require analyses of large numbers of individuals with HCM referenced to large populations. Because of the higher population frequency of the identified LowSVs, our study resolves previously disputed variants affecting 2.1% of all genetic test results for patients with HCM in SHaRe. Thus, our findings emphasize the need for large clinical-genetics registries like SHaRe to further improve genetic test interpretation. This approach may also be able to resolve substantial percentages of VUSs in other cardiac diseases, as has been done with long QT syndrome.[50]

Functional Variant Validation of Sarcomere LowSVs

A major goal across fields has been the development of cell-based assays to validate individual variants. In this work, we functionally validated effects of 2 specific individual sarcomere variants in iPSC-CMs using 2 different approaches. In the case of the *MYBPC3* c.442G>A variant, we used a patient iPSC line with biallelic variants to demonstrate that the *MYBPC3* c.442G>A variant causes only partial alternative splicing. When paired with a pathogenic *MYBPC3* splice variant, the combined result is severe protein level haploinsufficiency. This analysis shows that multiple variants affecting *MYBPC3* can exert additive effects on MyBP-C protein level, indicating 1 mechanism by which severe *MYBPC3*-associated HCM may develop. Additive dose reduction of *MYBPC3* transcription through unrecognized lower-penetrance variants or risk alleles that affect *MYBPC3* expression and the consequent additive effect on MyBP-C protein reduction may explain some of the clinical variability in patients with "isolated" *MYBPC3tvs*, the most common genetic form of HCM.

For the *TNNT2* c.832C>T variant, we used our recently developed iPSC-CM–derived CMB approach to analyze the effect of this variant on contractile function. The CMB approach was chosen to maximize the signal to noise of contractile function, which is an inherent limitation of standard iPSC-CM culture formats.[28,29] Moreover, we leveraged lentiviral expression, taking advantage of the tightly controlled stoichiometry of TNNT2 in the sarcomere,[43] to attain both isogenic and iso-batch control in these contractile analyses. The lentiviral expression approach has been effectively used by Pettinato et al[51] to test *TNNT2* variants. By removing line and batch variability,[28,29,52–54] we were able to detect relatively subtle differences between TNNT2 missense variants. We found that the *TNNT2* c.832C>T variant results in a similar contractile effect compared with the pathogenic *TNNT2* c.856C>T variant, with an increase in maximal fractional shortening but reduction in relaxation velocity, a cellular equivalent of diastolic dysfunction. However, the *TNNT2* c.856C>T variant demonstrated a greater effect on relaxation velocity at low calcium concentration, suggesting greater impairment of diastolic function. Given the large scope of unique sarcomere variants, a future challenge will be to increase the scalability of functional testing of sarcomere gene variants in iPSC-CMs while retaining a high degree of precision necessary to isolate moderate effects on contractile phenotypes.

Common Sarcomere Variants May Contribute to Age-Related Cardiac Remodeling

Given the high background frequency but lower penetrance of LowSVs, we hypothesized that LowSVs may contribute to adverse cardiac remodeling even in the absence of overt HCM. We based this hypothesis on the fact that the key cardiac morphologic contributor to diastolic heart failure is similar to the clinical phenotype of HCM—ie, an increased left ventricular mass to volume ratio.[44,55] In HCM, reduced left ventricular volume correlates with heart failure symptom class irrespective of obstruction.[55] Apart from HCM, the left ventricular volume to mass ratio gradually decreases with age and correlates with heart failure events.[45] Our analysis of the UK Biobank magnetic resonance imaging cohort supports the concept that LowSVs could accelerate this age-related process. In this orthogonal dataset, we found compelling evidence for an association of several LowSVs with reduced ventricular volume or increased wall thickness. Genetic variants that influence sarcomere function to more subtle degrees likely have additive effects with other diastolic heart failure risk factors, such as hypertension and obesity. This general concept is also consistent with recent genome-wide association studies that identified low effect size variants associated with later age onset nonsarcomere HCM and additive effects with hypertension.[14,56] These results suggest potential utility of subclassification of diastolic heart failure based on molecular pathogenesis and warrant further study. If verified in follow-up studies, an etiologic subclassification could better guide future treatment development for diastolic heart failure.

Limitations

SHaRe uses curated longitudinal data, and our observations are subject to the inherent limitations of registry-based studies. Our approach identified LowSVs

based on population prevalence. LowSVs that are not common in the population are not tractable for this approach. Genetic testing was performed at all sites as clinically indicated with the specific panels used being variable across over time. Variant identification and reporting have also varied over time. For example, in the past, deep intronic variants were not commonly recognized (eg, *MYBPC3* c.1224-52G>A). However, while influencing the precise OR measurements, underreporting of such variants in the SHaRe cohort would result in a lower statistical significance and not false positives. Similarly, our registry is not equipped at present to evaluate variants routinely designated as likely benign through clinical genetic testing. Variants currently classified as likely benign (eg, the LowSVs MYH7 c.3981C>A) are typically no longer included on genetic testing reports. However, underreporting of these variants in the SHaRe cohort would similarly result in a lower statistical significance and not false positives. Broader inclusion of all intermediate population frequency variants (eg, MAF <1%) on genetic testing reports would facilitate future studies of low penetrance and risk alleles. In addition, our cohort primarily consists of individuals with European ancestry, similar to the gnomAD population (83% and 77%, respectively; Table 2). The strong bias toward European ancestry in both registries resulted in increased statistical power for enrichment testing in this population but diminished statistical power for populations from non-European ancestries. For example, analysis of East Asian HCM cohorts identified 2 sarcomere variants that fit a low penetrance variant profile but were not identified in our study.[21,22] The *TNNI3* R79C variant had enrichment <2.0, and the *TNNT2* R286H variant was below our set MAF threshold. These variants highlight that incorporation of additional populations will expand the category of bona fide LowSVs. In addition, our analyses were limited to the 8 sarcomere genes most strongly associated with HCM (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, *TPM1*, *MLY2*, *MYL3*, *ACTC1*). Application of this approach to additional genes, including nonsarcomere genes associated with hypertrophy (eg, *RAF1*, *PTPN11*), would likely identify additional HCM-associated low penetrance variants. Last, we used threshold criteria to identify LowSVs based on established criteria[9,23,24] while recognizing that variants exist along a spectrum (Figure 1). We therefore may have excluded variants with biological impact that exist outside this predefined range. Thus, the present study serves as a starting point, with future work expected to iteratively add to a growing compendium of LowSVs that will increasingly enhance clinically actionable results from genetic testing for HCM.

CONCLUSIONS

This study establishes a novel category of low penetrance, intermediate effect size sarcomere variants that are common in the general population (1 of 350) and even more common in patients with HCM (1 of 50). LowSVs cause milder HCM when present alone but function additively when present with typical PathSVs. These findings support an oligogenic disease model with LowSVs explaining a portion of the marked variation in expressivity and penetrance in sarcomere-related HCM. Together, identification of this new class of LowSVs improves our understanding of sarcomere-based cardiac disease and will improve future efforts toward variant screening and risk stratification.

ARTICLE INFORMATION

Received February 23, 2024; accepted October 24, 2024.

Affiliations

Department of Pediatrics, Division of Pediatric Cardiology (J.K.M., M.W.R.), Department of Internal Medicine, Division of Cardiovascular Medicine (E.D.S., Y.-C.T., B.E., A.B., O.L.M., S.S., A.S.H.), Cellular and Molecular Biology Program, Medical School (A.R.), University of Michigan, Ann Arbor. National Heart and Lung Institute and Medical Research Council Laboratory of Medical Sciences, Imperial College London, United Kingdom (S.L.Z., J.S.W.). Cardiovascular Division, Brigham and Women's Hospital, Boston, MA (N.K.L., C.Y.H.). Penn Center for Inherited Cardiovascular Disease, Hospital of the University of Pennsylvania & Perelman School of Medicine at the University of Pennsylvania, Philadelphia (A.T.O., S.M.D.). Department of Pediatrics, University of Cincinnati College of Medicine, Heart Institute, Cincinnati Children's Hospital Medical Center, OH (T.D.R., E.M.M.). Department of Pediatrics, Children's Hospital of Philadelphia, PA (J.W.R., K.Y.L.). Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, MA (B.L.C.). Center for Inherited Cardiovascular Disease, Stanford Medicine, CA (E.A.A., V.N.P., M.T.W.). Department of Cardiology, Thoraxcenter, Erasmus Medical Center Rotterdam, The Netherlands (M.M.). Section of Cardiovascular Medicine, Yale School of Medicine, New Haven, CT (R.L., J.C.S.). Center for Cardiovascular Genetics, Boston Children's Hospital, MA (D.A.). Agnes Ginges Centre for Molecular Cardiology at Centenary Institute, Sydney Medical School Faculty of Medicine and Health, University of Sydney, Australia (C.S.). Department of Cardiology, Royal Prince Alfred Hospital, Sydney, Australia (C.S.). Genomics and Inherited Disease Program, Garvan Institute of Medical Research and University of New South Wales, Sydney, Australia (J.I.). Cardiomyopathy Unit, Careggi University Hospital, Florence, Italy (I.O.). Department of Molecular Physiology & Biophysics, University of Vermont, Burlington (M.P.).

Sources of Funding

This work was funded by the National Institutes of Health (K12 HD028820 - JKM, R03 HL148465 - ASH, T32 GM145470 – AR, T32 GM007863-43 – AR, T32 HL007853 - ES) and the National Science Foundation (EEC-1647837 - ASH), Sir Jules Thorn Charitable Trust (21JTA), Medical Research Council (UK), British Heart Foundation (RE/18/4/34215), and the National Institute for Health and Care Research Imperial College Biomedical Research Centre. SHaRe receives unrestricted research support from Bristol Myers Squib, Pfizer, and Cytokinetics; these companies were not involved in the project design, drafting, or editing of this article.

Disclosures

N.K.L. is a consultant for Bristol Myers Squibb, Pfizer, Cytokinetics, Tenaya, and Sarepta and receives research funding from Pfizer. J.W.R. is a consultant for AskBio, American Regent, Bayer, Merck, Enzvyant, and Bristol Myers Squibb. D.A. is a consultant for Rocket Pharmaceuticals. V.N.P. receives research funding from BioMarin and consults for Nuevocor and Viz.ai. S.M.D. is a consultant for Tenaya and Lexeo. S.S. is a consultant for Bristol Myers Squibb and Cytokinetics. M.M. is a consultant for Bristol Myers Squibb and Cytokinetics. I.O. is a consultant for Bristol Myers Squibb, Amicus, Sanofi, Cytokinetics, Bayer, Tenaya, Rocket Pharma, and Lexeo. M.P. is a consultant for and receives research funding from Tenaya. C.Y.H. is a consultant for or receives research funding from Bristol Myers Squib, Pfizer, Cytokinetics, Tenaya, Biomarin, viz.AI, and Lexicon. J.S.W. has consulted for MyoKardia (now Bristol Myers Squibb), Foresite Labs, HealthLumen, and Pfizer. A.S.H. is a consultant or receives research funding from Tenaya and Lexeo. The other authors report no conflicts.

Supplemental Material

Expanded Methods
Figures S1–S4
Tables S1–S7
Excel File All Tables
References 57–64

REFERENCES

1. Geisterfer-Lowrance AAT, Kass S, Tanigawa G, Vosberg H-P, McKenna W, Seidman CE, Seidman JG. A molecular basis for familial hypertrophic cardiomyopathy: a β cardiac myosin heavy chain gene missense mutation. *Cell*. 1990;62:999–1006. doi: 10.1016/0092-8674(90)90274-i
2. Watkins H, MacRae C, Thierfelder L, Chou YH, Frenneaux M, McKenna W, Seidman JG, Seidman CE. A disease locus for familial hypertrophic cardiomyopathy maps to chromosome 1q3. *Nat Genet*. 1993;3:333–337. doi: 10.1038/ng0493-333
3. Poetter K, Jiang H, Hassanzadeh S, Master SR, Chang A, Dalakas MC, Rayment I, Sellers JR, Fananapazir L, Epstein ND. Mutations in either the essential or regulatory light chains of myosin are associated with a rare myopathy in human heart and skeletal muscle. *Nat Genet*. 1996;13:63–69. doi: 10.1038/ng0596-63
4. Carrier L, Hengstenberg C, Beckmann JS, Guicheney P, Dufour C, Bercovici J, Dausse E, Berebbi-Bertrand I, Wisnewsky C, Pulvenis D. Mapping of a novel gene for familial hypertrophic cardiomyopathy to chromosome 11. *Nat Genet*. 1993;4:311–313. doi: 10.1038/ng0793-311
5. Thierfelder L, MacRae C, Watkins H, Tomfohrde J, Williams M, McKenna W, Bohm K, Noeske G, Schlepper M, Bowcock A. A familial hypertrophic cardiomyopathy locus maps to chromosome 15q2. *Proc Natl Acad Sci U S A*. 1993;90:6270–6274. doi: 10.1073/pnas.90.13.6270
6. Kimura A, Harada H, Park JE, Nishi H, Satoh M, Takahashi M, Hiroi S, Sasaoka T, Ohbuchi N, Nakamura T, et al. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat Genet*. 1997;16:379–382. doi: 10.1038/ng0897-379
7. Ho CY, Day SM, Ashley EA, Michels M, Pereira AC, Jacoby D, Cirino AL, Fox JC, Lakdawala NK, Ware JS, et al. Genotype and lifetime burden of disease in hypertrophic cardiomyopathy: insights from the Sarcomeric Human Cardiomyopathy Registry (SHaRe). *Circulation*. 2018;138:1387–1398. doi: 10.1161/CIRCULATIONAHA.117.033200
8. Richard P, Charron P, Carrier L, Ledeuil C, Cheav T, Pichereau C, Benaiche A, Isnard R, Dubourg O, Burban M, et al; EUROGENE Heart Failure Project. Hypertrophic cardiomyopathy: distribution of disease genes, spectrum of mutations, and implications for a molecular diagnosis strategy. *Circulation*. 2003;107:2227–2232. doi: 10.1161/01.CIR.0000066323.15244.54
9. Kelly MA, Caleshu C, Morales A, Buchan J, Wolf Z, Harrison SM, Cook S, Dillon MW, Garcia J, Haverfield E, et al. Adaptation and validation of the ACMG/AMP variant classification framework for MYH7-associated inherited cardiomyopathies: recommendations by ClinGen's Inherited Cardiomyopathy Expert Panel. *Genet Med*. 2018;20:351–359. doi: 10.1038/gim.2017.218
10. Helms AS, Thompson AD, Glazier AA, Hafeez N, Kabani S, Rodriguez J, Yob JM, Woolcock H, Mazzarotto F, Lakdawala NK, et al. Spatial and functional distribution of MYBPC3 pathogenic variants and clinical outcomes in patients with hypertrophic cardiomyopathy. *Circ Genom Precis Med*. 2020;13:396–405. doi: 10.1161/CIRCGEN.120.002929
11. Tadros R, Francis C, Xu X, Vermeer AMC, Harper AR, Huurman R, Kelu Bisabu K, Walsh R, Hoorntje ET, Te Rijdt WP, et al. Shared genetic pathways contribute to risk of hypertrophic and dilated cardiomyopathies with opposite directions of effect. *Nat Genet*. 2021;53:128–134. doi: 10.1038/s41588-020-00762-2
12. Tadros R, Zheng SL, Grace C, Jorda P, Francis C, Jurgens SJ, Thomson KL, Harper AR, Wilde AA, Olivotto I, et al. Large scale genome-wide association analyses identify novel genetic loci and mechanisms in hypertrophic cardiomyopathy. *Eur Heart J*. 2023;44: doi: 10.1093/eurheartj/ehad655.3197
13. Zheng SL, Jurgens SJ, McGurk KA, Xu X, Grace C, Theotokis PI, Buchan RJ, Francis C, Marvao AD, Curran L, et al. Evaluation of polygenic score for hypertrophic cardiomyopathy in the general population and across clinical settings. 2023;10:2023.2003.2014.23286621. doi: 10.1101/2023.03.14.23286621
14. Aung N, Lopes LR, van Duijvenboden S, Harper AR, Goel A, Grace C, Ho CY, Weintraub WS, Kramer CM, Neubauer S, et al. Genome-wide analysis of left ventricular maximum wall thickness in the UK Biobank cohort reveals a shared genetic background with hypertrophic cardiomyopathy. *Circ Genom Precis Med*. 2023;16:e003716. doi: 10.1161/CIRCGEN.122.003716
15. Harper AR, Goel A, Grace C, Thomson KL, Petersen SE, Xu X, Waring A, Ormondroyd E, Kramer CM, Ho CY, et al; HCMR Investigators. Common genetic variants and modifiable risk factors underpin hypertrophic cardiomyopathy susceptibility and expressivity. *Nat Genet*. 2021;53:135–142. doi: 10.1038/s41588-020-00764-0
16. Ning C, Fan L, Jin M, Wang W, Hu Z, Cai Y, Chen L, Lu Z, Zhang M, Chen C, et al. Genome-wide association analysis of left ventricular imaging-derived phenotypes identifies 72 risk loci and yields genetic insights into hypertrophic cardiomyopathy. *Nat Commun*. 2023;14:7900. doi: 10.1038/s41467-023-43771-5
17. Schmidt RJ, Steeves M, Bayrak-Toydemir P, Benson KA, Coe BP, Conlin LK, Ganapathi M, Garcia J, Gollob MH, Jobanputra V, et al; ClinGen Low Penetrance/Risk Allele Working Group. Recommendations for risk allele evidence curation, classification, and reporting from the ClinGen Low Penetrance/Risk Allele Working Group. *Genet Med*. 2024;26:101036. doi: 10.1016/j.gim.2023.101036
18. Ko C, Arscott P, Concannon M, Saberi S, Day SM, Yashar BM, Helms AS. Genetic testing impacts the utility of prospective familial screening in hypertrophic cardiomyopathy through identification of a nonfamilial subgroup. *Genet Med*. 2018;20:69–75. doi: 10.1038/gim.2017.79
19. Ingles J, Burns C, Bagnall RD, Lam L, Yeates L, Sarina T, Puranik R, Briffa T, Atherton JJ, Driscoll T, et al. Nonfamilial hypertrophic cardiomyopathy: prevalence, natural history, and clinical implications. *Circ Cardiovasc Genet*. 2017;10: doi: 10.1161/CIRCGENETICS.116.001620
20. McGurk KA, Zhang X, Theotokis P, Thomson K, Harper A, Buchan RJ, Mazaika E, Ormondroyd E, Wright WT, Macaya D, et al. The penetrance of rare variants in cardiomyopathy-associated genes: a cross-sectional approach to estimating penetrance for secondary findings. *Am J Hum Genet*. 2023;110:1482–1495. doi: 10.1016/j.ajhg.2023.08.003
21. Pua CJ, Tham N, Chin CWL, Walsh R, Khor CC, Toepfer CN, Repetti GG, Garfinkel AC, Ewoldt JF, Cloonan P, et al. Genetic studies of hypertrophic cardiomyopathy in Singaporeans identify variants in TNNI3 and TNNT2 that are common in Chinese patients. *Circ Genom Precis Med*. 2020;13:424–434. doi: 10.1161/CIRCGEN.119.002823
22. Wu G, Liu L, Zhou Z, Liu J, Wang B, Ruan J, Yang Q, Kanchwala M, Dai P, Zhang C, et al. East Asian-specific common variant in TNNI3 predisposes to hypertrophic cardiomyopathy. *Circulation*. 2020;142:2086–2089. doi: 10.1161/CIRCULATIONAHA.120.050384
23. Whiffin N, Walsh R, Govind R, Edwards M, Ahmad M, Zhang X, Tayal U, Buchan R, Midwinter W, Wilk AE, et al. CardioClassifier: disease- and gene-specific computational decision support for clinical genome interpretation. *Genet Med*. 2018;20:1246–1254. doi: 10.1038/gim.2017.258
24. Walsh R, Mazzarotto F, Whiffin N, Buchan R, Midwinter W, Wilk A, Li N, Felkin L, Ingold N, Govind R, et al. Quantitative approaches to variant classification increase the yield and precision of genetic testing in Mendelian diseases: the case of hypertrophic cardiomyopathy. *Genome Med*. 2019;11:5. doi: 10.1186/s13073-019-0616-z
25. Walsh R, Thomson KL, Ware JS, Funke BH, Woodley J, McGuire KJ, Mazzarotto F, Blair E, Seller A, Taylor JC, Minikel EV, MacArthur DG, Farrall M, Cook SA, Watkins H.; Exome Aggregation Consortium. Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples. *Genet Med*. 2017;19:192–203. doi: 10.1038/gim.2016.90
26. Saltzman AJ, Mancini-DiNardo D, Li C, Chung WK, Ho CY, Hurst S, Wynn J, Care M, Hamilton RM, Seidman GW, et al. Short communication: the cardiac myosin binding protein C Arg502Trp mutation: a common cause of hypertrophic cardiomyopathy. *Circ Res*. 2010;106:1549–1552. doi: 10.1161/CIRCRESAHA.109.216291
27. Helms AS, Tang VT, O'Leary TS, Friedline S, Wauchope M, Arora A, Wasserman AH, Smith ED, Lee LM, Wen XW, et al. Effects of MYBPC3 loss-of-function mutations preceding hypertrophic cardiomyopathy. *JCI Insight*. 2020;5:e133782. doi: 10.1172/jci.insight.133782
28. Tsan YC, DePalma SJ, Zhao YT, Capilnasiu A, Wu YW, Elder B, Panse I, Ufford K, Matera DL, Friedline S, et al. Physiologic biomechanics enhance reproducible contractile development in a stem cell derived cardiac muscle platform. *Nat Commun*. 2021;12:6167. doi: 10.1038/s41467-021-26496-1
29. Ufford K, Friedline S, Tong Z, Tang VT, Dobbs AS, Tsan YC, Bielas SL, Liu AP, Helms AS. Myofibrillar structural variability underlies contractile function in stem cell-derived cardiomyocytes. *Stem Cell Rep*. 2021;16:470–477. doi: 10.1016/j.stemcr.2021.01.007
30. Mbatchou J, Barnard L, Backman J, Marcketta A, Kosmicki JA, Ziyatdinov A, Benner C, O'Dushlaine C, Barber M, Boutkov B, et al. Computationally efficient whole-genome regression for quantitative and binary traits. *Nat Genet*. 2021;53:1097–1103. doi: 10.1038/s41588-021-00870-7
31. Topriceanu CC, Pereira AC, Moon JC, Captur G, Ho CY. Meta-analysis of penetrance and systematic review on transition to disease in genetic hypertrophic cardiomyopathy. *Circulation*. 2024;149:107–123. doi: 10.1161/CIRCULATIONAHA.123.065987
32. Cheng J, Novati G, Pan J, Bycroft C, Zemgulyte A, Applebaum T, Pritzel A, Wong LH, Zielinski M, Sargeant T, et al. Accurate proteome-wide missense

variant effect prediction with AlphaMissense. *Science*. 2023;381:eadg7492. doi: 10.1126/science.adg7492
33. Jaganathan K, Kyriazopoulou Panagiotopoulou S, McRae JF, Darbandi SF, Knowles D, Li YI, Kosmicki JA, Arbelaez J, Cui W, Schwartz GB, et al. Predicting splicing from primary sequence with deep learning. *Cell*. 2019;176:535–548.e24. doi: 10.1016/j.cell.2018.12.015
34. Bagnall RD, Ingles J, Dinger ME, Cowley MJ, Ross SB, Minoche AE, Lal S, Turner C, Colley A, Rajagopalan S, et al. Whole genome sequencing improves outcomes of genetic testing in patients with hypertrophic cardiomyopathy. *J Am Coll Cardiol*. 2018;72:419–429. doi: 10.1016/j.jacc.2018.04.078
35. Ito K, Patel PN, Gorham JM, McDonough B, DePalma SR, Adler EE, Lam L, MacRae CA, Mohiuddin SM, Fatkin D, et al. Identification of pathogenic gene mutations in LMNA and MYBPC3 that alter RNA splicing. *Proc Natl Acad Sci U S A*. 2017;114:7689–7694. doi: 10.1073/pnas.1707741114
36. Marston NA, Han L, Olivotto I, Day SM, Ashley EA, Michels M, Pereira AC, Ingles J, Semsarian C, Jacoby D, et al. Clinical characteristics and outcomes in childhood-onset hypertrophic cardiomyopathy. *Eur Heart J*. 2021;42:1988–1996. doi: 10.1093/eurheartj/ehab148
37. Lakdawala NK, Olivotto I, Day SM, Han L, Ashley EA, Michels M, Ingles J, Semsarian C, Jacoby D, Jefferies JL, et al. Associations between female sex, sarcomere variants, and clinical outcomes in hypertrophic cardiomyopathy. *Circ Genom Precis Med*. 2021;14:e003062. doi: 10.1161/CIRCGEN.120.003062
38. Miron A, Lafreniere-Roula M, Steve Fan CP, Armstrong KR, Dragulescu A, Papaz T, Manlhiot C, Kaufman B, Butts RJ, Gardin L, et al. A validated model for sudden cardiac death risk prediction in pediatric hypertrophic cardiomyopathy. *Circulation*. 2020;142:217–229. doi: 10.1161/CIRCULATIONAHA.120.047235
39. Lopes LR, Losi MA, Sheikh N, Laroche C, Charron P, Gimeno J, Kaski JP, Maggioni AP, Tavazzi L, Arbustini E, et al; Cardiomyopathy Registry Investigators Group. Association between common cardiovascular risk factors and clinical phenotype in patients with hypertrophic cardiomyopathy from the European Society of Cardiology (ESC) EurObservational Research Programme (EORP) Cardiomyopathy/Myocarditis registry. *Eur Heart J Qual Care Clin Outcomes*. 2022;9:42–53. doi: 10.1093/ehjqcco/qcac006
40. Marstrand P, Han L, Day SM, Olivotto I, Ashley EA, Michels M, Pereira AC, Wittekind SG, Helms A, Saberi S, et al; SHaRe Investigators. Hypertrophic cardiomyopathy with left ventricular systolic dysfunction: insights from the SHaRe registry. *Circulation*. 2020;141:1371–1383. doi: 10.1161/CIRCULATIONAHA.119.044366
41. Wessels MW, Herkert JC, Frohn-Mulder IM, Dalinghaus M, van den Wijngaard A, de Krijger RR, Michels M, de Coo IF, Hoedemaekers YM, Dooijes D. Compound heterozygous or homozygous truncating MYBPC3 mutations cause lethal cardiomyopathy with features of noncompaction and septal defects. *Eur J Hum Genet*. 2015;23:922–928. doi: 10.1038/ejhg.2014.211
42. O'Leary TS, Snyder J, Sadayappan S, Day SM, Previs MJ. MYBPC3 truncation mutations enhance actomyosin contractile mechanics in human hypertrophic cardiomyopathy. *J Mol Cell Cardiol*. 2019;127:165–173. doi: 10.1016/j.yjmcc.2018.12.003
43. Feng HZ, Hossain MM, Huang XP, Jin JP. Myofilament incorporation determines the stoichiometry of troponin I in transgenic expression and the rescue of a null mutation. *Arch Biochem Biophys*. 2009;487:36–41. doi: 10.1016/j.abb.2009.05.001
44. Kitzman DW, Little WC, Brubaker PH, Anderson RT, Hundley WG, Marburger CT, Brosnihan B, Morgan TM, Stewart KP. Pathophysiological characterization of isolated diastolic heart failure in comparison to systolic heart failure. *JAMA*. 2002;288:2144–2150. doi: 10.1001/jama.288.17.2144
45. Cheng S, Fernandes VR, Bluemke DA, McClelland RL, Kronmal RA, Lima JA. Age-related left ventricular remodeling and associated risk for cardiovascular outcomes: the Multi-Ethnic Study of Atherosclerosis. *Circ Cardiovasc Imaging*. 2009;2:191–198. doi: 10.1161/CIRCIMAGING.108.819938
46. Kuznetsova T, Herbots L, Lopez B, Jin Y, Richart T, Thijs L, Gonzalez A, Herregods MC, Fagard RH, Diez J, et al. Prevalence of left ventricular diastolic dysfunction in a general population. *Circ Heart Fail*. 2009;2:105–112. doi: 10.1161/CIRCHEARTFAILURE.108.822627
47. Borlaug BA. The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol*. 2014;11:507–515. doi: 10.1038/nrcardio.2014.83
48. Ommen SR, Ho CY, Asif IM, Balaji S, Burke MA, Day SM, Dearani JA, Epps KC, Evanovich L, Ferrari VA, et al; Writing Committee Members. 2024 AHA/ACC/AMSSM/HRS/PACES/SCMR guideline for the management of hypertrophic cardiomyopathy: a report of the American Heart Association/American College of Cardiology Joint Committee on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2024;83:2324–2405. doi: 10.1016/j.jacc.2024.02.014
49. Butters A, Bagnall RD, Ingles J. Revisiting the diagnostic yield of hypertrophic cardiomyopathy genetic testing. *Circ Genom Precis Med*. 2020;13:e002930. doi: 10.1161/CIRCGEN.120.002930
50. Schwartz PJ, Crotti L, George AL Jr. Modifier genes for sudden cardiac death. *Eur Heart J*. 2018;39:3925–3931. doi: 10.1093/eurheartj/ehy502
51. Pettinato AM, Ladha FA, Mellert DJ, Legere N, Cohn R, Romano R, Thakar K, Chen YS, Hinson JT. Development of a cardiac sarcomere functional genomics platform to enable scalable interrogation of human TNNT2 variants. *Circulation*. 2020;142:2262–2275. doi: 10.1161/CIRCULATIONAHA.120.047999
52. Laco F, Woo TL, Zhong Q, Szmyd R, Ting S, Khan FJ, Chai CLL, Reuveny S, Chen A, Oh S. Unraveling the inconsistencies of cardiac differentiation efficiency induced by the GSK3beta inhibitor CHIR99021 in human pluripotent stem cells. *Stem Cell Rep*. 2018;10:1851–1866. doi: 10.1016/j.stemcr.2018.03.023
53. Huo J, Kamalakar A, Yang X, Word B, Stockbridge N, Lyn-Cook B, Pang L. Evaluation of batch variations in induced pluripotent stem cell-derived human cardiomyocytes from 2 major suppliers. *Toxicol Sci*. 2017;156:25–38. doi: 10.1093/toxsci/kfw235
54. Mannhardt I, Saleem U, Mosqueira D, Loos MF, Ulmer BM, Lemoine MD, Larsson C, Ameen C, de Korte T, Vlaming MLH, et al. Comparison of 10 control hPSC lines for drug screening in an engineered heart tissue format. *Stem Cell Rep*. 2020;15:983–998. doi: 10.1016/j.stemcr.2020.09.002
55. Manganelli F, Betocchi S, Losi MA, Briguori C, Pace L, Ciampi Q, Perrone-Filardi P, Salvatore M, Finizio F, Pezzella E, et al. Influence of left ventricular cavity size on clinical presentation in hypertrophic cardiomyopathy. *Am J Cardiol*. 1999;83:547–552. doi: 10.1016/s0002-9149(98)00911-4
56. Khurshid S, Lazarte J, Pirruccello JP, Weng LC, Choi SH, Hall AW, Wang X, Friedman SF, Nauffal V, Biddinger KJ, et al. Clinical and genetic associations of deep learning-derived cardiac magnetic resonance-based left ventricular mass. *Nat Commun*. 2023;14:1558. doi: 10.1038/s41467-023-37173-w
57. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, et al; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405–424. doi: 10.1038/gim.2015.30
58. Whiffin N, Minikel E, Walsh R, O'Donnell-Luria AH, Karczewski K, Ing AY, Barton PJR, Funke B, Cook SA, MacArthur D, et al. Using high-resolution variant frequencies to empower clinical genome interpretation. *Genet Med*. 2017;19:1151–1158. doi: 10.1038/gim.2017.26
59. Altman DG. *Practical Statistics for Medical Research*. Chapman and Hall/CRC; 1990.
60. Hildebrandt M, Bender R, Gehrmann U, Blettner M. Calculating confidence intervals for impact numbers. *BMC Med Res Methodol*. 2006;6:32. doi: 10.1186/1471-2288-6-32
61. de Marvao A, McGurk KA, Zheng SL, Thanaj M, Bai W, Duan J, Biffi C, Mazzarotto F, Statton B, Dawes TJW, et al. Phenotypic expression and outcomes in individuals with rare genetic variants of hypertrophic cardiomyopathy. *J Am Coll Cardiol*. 2021;78:1097–1110. doi: 10.1016/j.jacc.2021.07.017
62. Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, et al. Chemically defined generation of human cardiomyocytes. *Nat Methods*. 2014;11:855–860. doi: 10.1038/nmeth.2999
63. Correia C, Koshkin A, Duarte P, Hu D, Teixeira A, Domian I, Serra M, Alves PM. Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Sci Rep*. 2017;7:8590. doi: 10.1038/s41598-017-08713-4
64. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15–21. doi: 10.1093/bioinformatics/bts635