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ORIGINAL RESEARCH ARTICLE

Redefining the Genetic Architecture of Hypertrophic Cardiomyopathy: Role of Intermediate-Effect Variants

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BACKGROUND: Hypertrophic cardiomyopathy (HCM) is a genetically heterogeneous disorder linked primarily to rare variants in sarcomeric genes, although recently certain nonsarcomeric genes have emerged as important contributors. Nonmendelian genetic variants with reproducible moderate-effect sizes and low penetrance, intermediate-effect variants (IEVs), can play a crucial role in modulating disease expression. Understanding the clinical impact of IEVs is crucial to unravel the complex genetic architecture of HCM.

METHODS: We conducted an ancestry-based enrichment analysis of 14 validated HCM genes, including the 9 core sarcomeric and 5 nonsarcomeric genes *(ALPK3*, *CSRP3*, *FHOD3*, *FLNC*, and *TRIM63)*. Enrichment of intermediate frequency missense variants was evaluated in 10 981 patients with HCM, 4030 internal controls of European-ancestry, and 590 000 external controls from gnomAD non-Finnish Europeans. The population-attributable fraction was calculated to assess contribution of IEVs to HCM. Age-related disease penetrance, phenotypic severity (left ventricular maximum wall thickness), and major adverse cardiac events were analyzed in 11 991 HCM cases of the whole cohort according to 5 genetic groups: genotype negative, isolated IEV, monogenic, monogenic+IEV, and double monogenic.

RESULTS: Fourteen IEVs in 8 genes were identified in 731 individuals (6.1% of the cohort), of whom 570 patients (4.8%) had IEVs in isolation: 198 (34.7%) in sarcomeric genes and 372 (65.3%) in nonsarcomeric genes. The contribution of IEVs to HCM genetics according to population-attributable fraction was estimated to be 4.9% (95% CI, 3.2–6.7). A significant gradient in penetrance, phenotypic severity, and major adverse cardiac events was observed across genetic groups. Compared with genotype-negative patients, IEV carriers displayed a younger median age at diagnosis (59 years of age [95% CI, 46–69] versus 61 years [95% CI, 49–70]; P=0.0073) and a higher mean left ventricular maximum wall thickness (18.1±3.7 versus 19.0±4.3; P=0.0043). IEVs also modified disease expression in individuals with monogenic variants, causing a more aggressive phenotype than in individuals from the monogenic-only group with HCM onset at younger age and a higher left ventricular maximum wall thickness (all P<0.0001), with major adverse cardiac event–free survival being significantly lower (93.3% versus 69.3% at 70 years of age; P<0.0001).

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CONCLUSIONS: IEVs are present in 6.1% of HCM cases and account for 4.8% of HCM genetic burden. IEVs also influence disease severity and outcomes, particularly when combined with monogenic disease-causing variants. Evaluation of IEVs should be considered when HCM genetic testing is performed.

Key Words: cardiomyopathy, hypertrophic ■ genetic predisposition to disease ■ genetic testing ■ genetic variation ■ inheritance patterns ■ penetrence ■ risk factors

Clinical Perspective

What Is New?

- This study identifies and quantifies the contribution of intermediate-effect variants (IEVs) to hypertrophic cardiomyopathy in a large, ancestrally homogeneous cohort.
- IEVs are associated with earlier age at diagnosis, greater left ventricular hypertrophy, and a higher risk of major adverse cardiac events compared with genotype-negative cases.
- The clinical impact of IEVs is more pronounced when co-occurring with monogenic variants, modifying disease course and contributing to greater severity through a cumulative effect.

What Are the Clinical Implications?

- IEVs should be recognized as relevant genetic contributors in hypertrophic cardiomyopathy, especially in the interpretation of genetic test results that do not meet classic Mendelian thresholds.
- Comprehensive variant assessments, including IEVs, may enhance risk stratification and clinical decision-making.
- Genetic counseling and cascade screening protocols may benefit from incorporating the potential modifying role of IEVs in affected families in the future.

Nonstandard Abbreviations and Acronyms

FAF	filtered allele frequency
HCM	hypertrophic cardiomyopathy
IEV	intermediate-effect variant
LVMWT	left ventricular maximum wall thickness
MACE	major adverse cardiac event
NFE	non-Finish European
OR	odds ratio
P/LP	pathogenic/likely pathogenic
PCA	principal component analysis

Hypertrophic cardiomyopathy (HCM) is characterized by significant genetic and clinical heterogeneity. The diagnostic yield of genetic testing varies depending on the cohort studied. In most cases, the frequency of actionable positive findings does not exceed 50% and is <30% in more recent cohorts.[1–3] The accuracy of interpretation of the variant and the comprehensiveness of the genetic study are critical factors to maximize the potential of genetic testing in inherited heart conditions.[4–6]

Although HCM is caused predominantly by rare pathogenic/likely pathogenic (P/LP) variants (monogenic variants) in core sarcomeric genes, structural and regulatory genes that have been recently identified also contribute to the genetic spectrum of HCM. The updated ClinGen gene-disease curation for HCM has classified several nonsarcomere genes with moderate to definitive evidence, including *ALPK3*, *CSRP3*, *FHOD3*, and *TRIM63*.[7] Overall, variants in these nonsarcomeric genes are estimated nowadays to account for 5% to 10% of HCM cases based on their reported prevalence in several studies.[8–11]

Despite these advances, a substantial proportion of the genetic basis of HCM remains unexplained. Determining whether a variant is associated with HCM is frequently challenging because of incomplete penetrance and variable expressivity, suggesting that environmental, epigenetic, and additional genetic factors play a significant role in disease expression. Recently, it has been suggested that nonrare genetic variants can modify the penetrance and phenotypic severity of HCM cases caused by rare monogenic variants.[12,13] These nonrare genetic variants could also potentially contribute to phenotype in HCM cases without monogenic variants, commonly referred as genotype negative.[14] Therefore, the genetic architecture of HCM can be conceptualized as a continuum. At one end, there are common polymorphisms with minimal individual impact; their collective contribution to disease risk is beginning to be explored through polygenic risk scores.[15] At the other end, there are rare monogenic variants with high clinical impact, characterized by high penetrance and familial aggregation. Between these extremes lies a spectrum of variants with nonnegligible allele frequencies in controls, above the maximum credible frequency for the disorder, that would be enriched in HCM cases. These variants, with an intermediate-effect size, can be broadly classified as intermediate-effect variants (IEVs).

Here, we identify and characterize the effect and contribution of IEVs in a large single-center sequenced cohort

of HCM probands, focusing on 14 validated HCM disease-causing genes and using a principal component analysis (PCA) with ancestry-matched internal and external controls. We explore the potential role of IEVs as contributors to the genetic burden of HCM and as phenotypic modifiers in the presence of monogenic variants in primary HCM genes.

METHODS

Data Availability

Data and materials that support the findings of this study are available from the corresponding author on reasonable request.

Study Population and Phenotypic Characterization

This report adheres to the Strengthening the Reporting of Observational Studies in Epidemiology reporting guideline, which is given in the Supplemental Material.

Between May 2014 and June 2024, >35 000 consecutive unrelated probands with different inherited cardiac conditions were sequenced in Health in Code S.L. by next-generation sequencing. Patients with HCM and individuals with other cardiac conditions, excluding cardiomyopathies and overlapping phenotypes, were selected. The HCM cohort consisted of 14 113 probands; the control cohort comprised 8144 probands. Phenotypes were determined by the respective referring centers before genetic testing.

Phenotypic data were collected retrospectively from clinical records from those patients that agreed to participate and gave informed consent; Institutional Review Board approval was obtained from of A Coruña/Ferrol Ethics Committee (registry COV27-061). This study adhered to the ethical principles outlined in the Declaration of Helsinki and was conducted in compliance with international ethical standards to ensure the protection, rights, and well-being of participants.

Variant Genotyping and Classification

All probands were sequenced by customized next-generation sequencing libraries. The number of genes varied through time, ranging from 242 genes in the first library in 2014 to 368 genes in the latest one; libraries were updated regularly to include genes with new evidence of association with inherited cardiac diseases (Table S1).

For the variant enrichment analysis, all cases and controls in whom the evaluated genes were sequenced were used. For the phenotypic characterization, only 11 991 cases sequenced after 2017 were included to ensure the homogeneity of the cohort because previous libraries did not include *ALPK3*, *CSRP3*, and *TRIM63*, facilitating consistent sequencing depth, gene coverage, and confounder detection.

Each genetic variant was classified according to tailored American College of Medical Genetics criteria (Supplemental Material). The pathogenicity assessment of the 1189 variants identified in the cohort can be found in Table S2.

HCM primary genes were defined as those with definitive, strong, or moderate associations with HCM per current ClinGen curation, encompassing 9 sarcomeric (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, *TNNC1*, *ACTC1*, *TPM1*, *MYL3*, and *MYL2*) and 5 nonsarcomeric (*ALPK3*, *CSRP3*, *FLNC*, *FHOD3*, and *TRIM63*) genes. HCM genocopy genes such as *TTR*, *GLA*, *PTPN11*, *LAMP2*, *PRKAG2*, and *RAF1* and mitochondrial genes were not considered.

Enrichment Analysis and IEV Selection

Enrichment analysis was performed comparing frequencies of the variants in HCM cases with both internal controls (non-cardiomyopathy cases) and external controls extracted from gnomAD (https://gnomad.broadinstitute.org/, version V4.1.0) as described before.[16] Enrichment was measured by 2-sided odds ratios (ORs) with 95% CIs, with statistical significance determined by the Fisher exact test.

A PCA was performed with common variants present in the sequencing library to select European ancestry probands for both cases and internal controls to mitigate potential biases associated with the asymmetric variant frequencies across different ancestries. The non-Finnish European (NFE) subpopulation of gnomAD was used as the external control. The estimated penetrance for each variant was calculated by comparing the allele frequency of individual variants in our HCM cohort (after PCA-based ancestry adjustment) with the background frequency of the same variants in the gnomAD-NFE population (Supplemental Material and Figure S1 provide details on the detailed methods).[17]

Only missense variants in HCM primary genes were evaluated. Variants affecting splicing by previous functional studies obtained from the literature (Table S3) and probands with P/LP variants in genes considered genocopies (metabolic disorders, RASopathies, glycogen storage diseases, mitochondrial diseases, and cardiac amyloidosis) were excluded from enrichment and phenotypic analysis.

The criteria for defining IEVs were as follows:

- Intermediate range of filtered allele frequency (FAF), the maximum credible genetic ancestry group allele frequency in nonbottlenecked ancestry groups in gnomAD V4.1.0:
  - Upper limit: 0.01 (threshold for considering a variant a polymorphism)
  - Lower limit: 0.00004 (maximum credible frequency to classify a variant as monogenic for HCM primary genes)[18]
- Significant enrichment in cases/controls of broad European ancestry based on PCA:
  - Case count ≥5
  - Derivation external control cohort (gnomAD-NFE): OR ≥2 and $P<0.05$
  - Replication internal control cohort (noncardiomyopathy cases): OR ≥2 and $P<0.1$
  - Estimated penetrance <15%, OR <15 (against internal controls), or both for excluding possible monogenic variants. This value was used as established in the specific ClinGen recommendations for evaluating risk alleles to define a variant as monogenic.[19]

The population-attributable fraction associated with IEVs was assessed through an adjusted etiologic fraction analysis (Supplemental Material).[20]

Phenotypic Analysis According to Genetic Findings

The clinical variables evaluated were age-related disease penetrance (age at diagnosis), left ventricular maximum wall

thickness (LVMWT), and a composite end point of major adverse cardiac events (MACEs) that included major arrhythmic events (sudden cardiac death, aborted sudden cardiac death, and appropriate implantable cardioverter defibrillator shock) and heart failure death, which includes heart failure death and cardiac transplantation.

We categorized the HCM cohort into 5 distinct genetic groups for phenotypic analysis:

- Negative: cases without candidate genetic variants that could explain the disease, including monogenic variants, variants of uncertain significance in HCM primary genes, and IEVs. Cases harboring LP/P variants in simple heterozygosis in genes with exclusively recessive inheritance (*TRIM63, KLHL24,* and recessive genocopy genes) were also included in this group.
- IEV: probands harboring an IEV in HCM primary genes in isolation.
- Monogenic: probands harboring a P/LP monogenic variant in HCM primary genes in isolation.
- Monogenic-IEV: probands carrying a P/LP monogenic variant in HCM primary genes and at least 1 IEV.
- Double monogenic: Probands harboring ≥2 P/LP monogenic variants in HCM primary genes.

Patients carrying a variant of uncertain significance, whether in isolation or in combination with other variant classes, were excluded from phenotypic and intersection analyses. Variants of uncertain significance include both potentially pathogenic and likely benign variants, and they might introduce analytical noise and compromise interpretability.

Statistical Analysis

Continuous variables were expressed as mean±SD (normal distribution) or median and 25th to 75th percentiles (nonnormal); categorical data were expressed as frequencies (percent). Continuous variables were compared with the Student *t* test (2 groups, normally distributed) or ANOVA (multiple groups, normally distributed) with *P* values adjusted with the Benjamini-Hochberg method. For nonparametric data, the Mann-Whitney *U* test (2 groups) or Kruskal-Wallis test (multiple groups) followed, when significant, by the Dunn post hoc test with Bonferroni correction was applied. For categorical variables, χ^2 tests were used for overall group comparisons, and when significant, pairwise comparisons of proportions with Bonferroni-adjusted *P* values were performed.

Kaplan-Meier curves and log-rank tests assessed age-related penetrance and survival; post hoc pairwise comparisons and Cox regression (univariable and age adjusted) were performed to address survival curve biases. The proportional hazards assumption was tested with Schoenfeld residuals. All tests were 2 tailed; $P<0.05$ was considered significant. Analyses were performed in R Studio 4.3.2.

RESULTS

IEVs Selection

We identified 108 candidate missense variants that showed an FAF between 0.004% and 1% and were individually present in >5 HCM cases in the cohort. Using a PCA-adjusted strategy focused on European ancestry cases and controls, we found 66 of these variants to be enriched in HCM cases compared with gnomAD-NFE external controls, of which 17 were also validated through comparison with internal controls. Without restriction on European ancestry cases, 127 candidate variants would have been selected, of which 69 were enriched compared with external controls and 19 compared with internal controls (Figure 1).

Three of the 17 variants initially identified were classified as monogenic variants: *TPM1*:p.Met281Val and *MYL3*:pAla143Thr had an estimated penetrance above the established threshold of 15%, and *MYBPC3*:p.Arg502Trp had an OR ≥15 compared with internal controls (Figure 1; Table S4). Figure 2 displays the architecture of the genetic variants identified in HCM cases, showing the correlation between the ORs (HCM cases versus internal controls) and the FAF of each variant.

Last, 14 variants were classified as IEVs (Figure 3; Table 1; Table S5). Of these, 10 were present in sarcomeric genes and 4 in nonsarcomeric genes. IEVs in sarcomeric genes included the following: 4 in *MYBPC3* (p.Arg1022Pro, p.Arg1036His, p.Arg1226Cys, and p.Glu441Lys), 2 each in *MYH7* (p.Asp1652Tyr and p.Ile1927Phe) and *TNNT2* (p.Arg278Cys and p.Arg286His), and one in *MYL3* (p.Ala57Asp) and in *TNNI3* (p.Arg162). The 4 IEVs present in nonsarcomeric genes were as follows: 2 in *FHOD3* (p.Arg637Gln and p.Arg638Trp) and one each in *FLNC* (p.Ala2430Val) and *TRIM63* (p.Cys23Tyr). Of note, 65.3% of the probands with isolated IEV had a nonsarcomeric IEV, with the most frequent variant being *FHOD3* p.Arg637Gln, present in 49.3% of probands carrying IEVs (Table S6).

The comparison of ORs calculated with internal versus external (gnomAD-NFE) controls showed similar enrichment patterns for most of the selected variants. However, variants such as *TNNI3*:p.Arg162Trp, *TNNT2*:p.Arg286His, *MYH7*:p.Asp1652Tyr, *MYBPC3*:p.Arg1226Cys, and *TRIM63*:p.Cys23Tyr exhibited greater enrichment compared with gnomAD, potentially highlighting genetic differences between our cohort and the NFE population in gnomAD, even after PCA-based selection of European ancestry individuals (Figure S2). Some of the variants excluded in the filtering process exhibited IEV-like enrichment in cases in our cohort (OR ≥2) but were not statistically significant (Tables S7 and S8).

The identification of the previously published recessive variant in *TRIM63*:p.Cys23Tyr as an IEV[11] and the results of a new study suggesting that null variants in heterozygosis might be associated with HCM[21] prompted us to investigate whether these type of variants could also represent an intermediate-effect genetic substrate. Enrichment analysis of these null variants in heterozygosity against gnomAD-NFE

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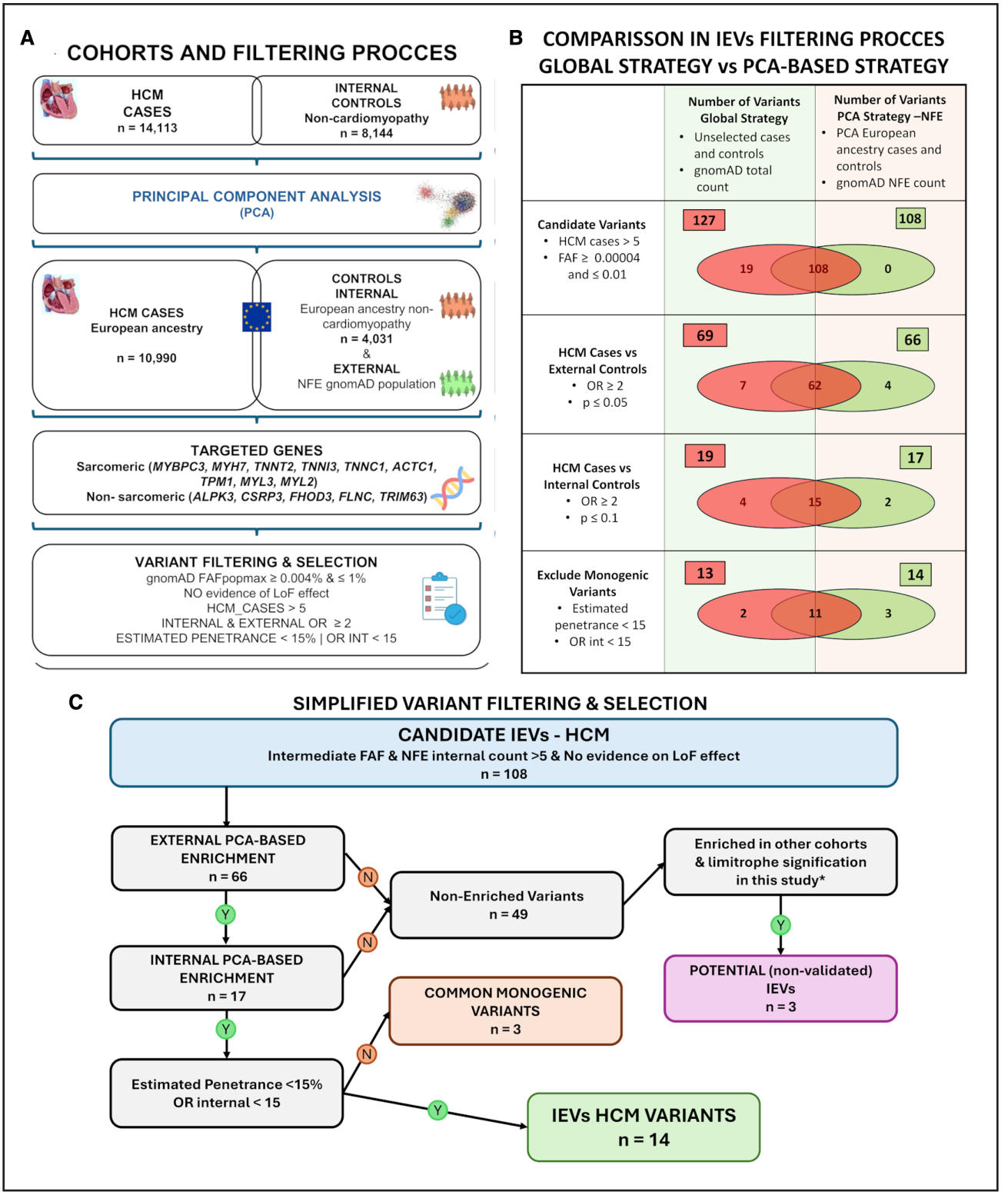

Figure 1. Study IEVs selection flow chart and methodology for filtering and selection of IEVs.
A, Methodology for variant selection and cohort composition. Global numbers for cases and controls, both before and after selection of the non-Finnish European (NFE) with principal component analysis (PCA), the genes targeted for exploration, and the criteria applied for variant filtering. **B**, Selection and validation of intermediate-effect variants (IEVs). Comparison of strategies and enrichment analysis with internal and external controls, global and PCA analysis. Venn diagram illustrates the overlap in variants identified by the global and PCA-adjusted strategies. Numbers represent the count of variants meeting specified criteria for each strategy and their overlap. **C**, Simplified IEV filtering and selection. FAF indicates filtered allele frequency; HCM, hypertrophic cardiomyopathy; and OR, odds ratio.

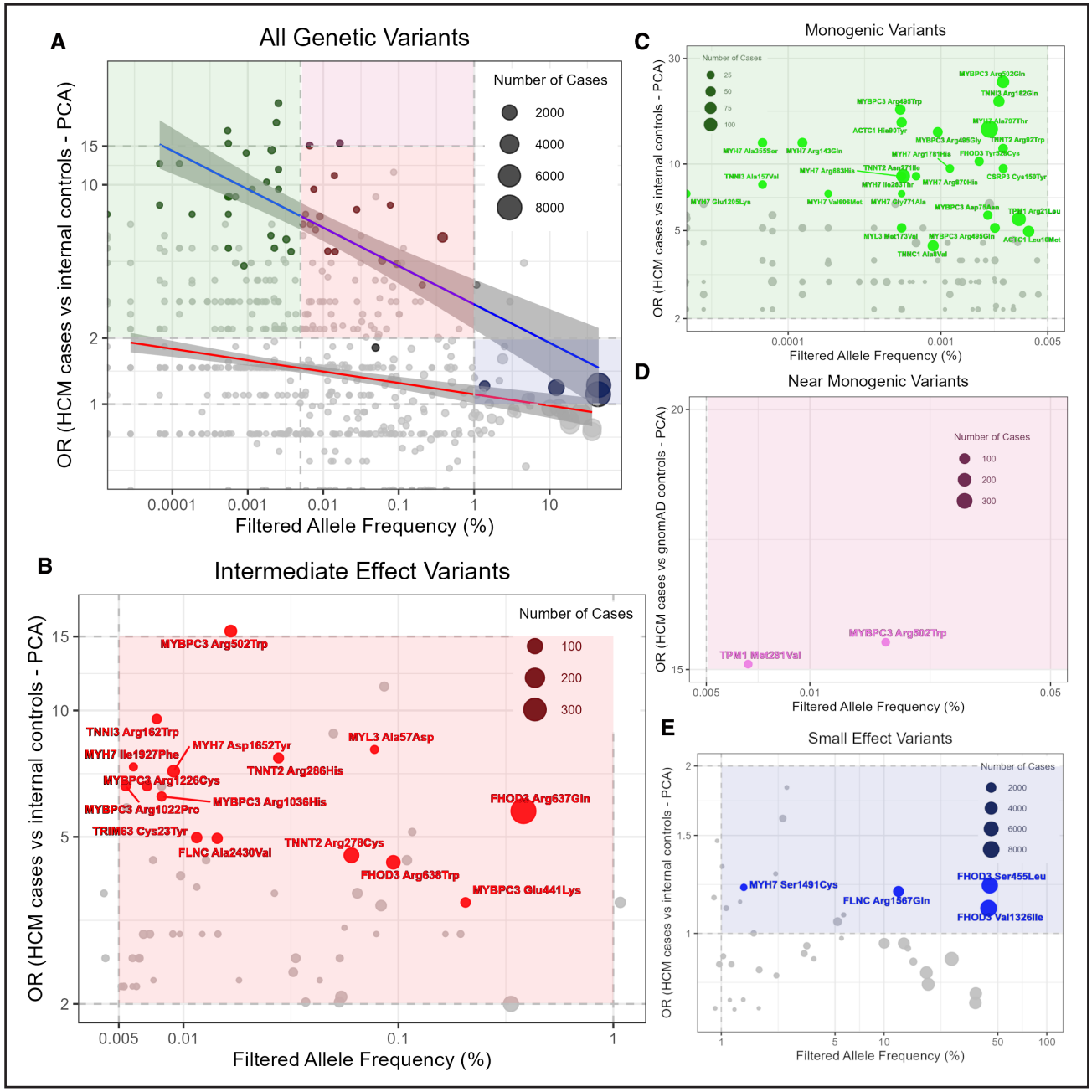

Figure 2. Architecture of variants identified in the HCM cohort.

A, Genetic variants identified in the study, with the filtered allele frequency in gnomAD v4.1 version on the *x* axis, and the enrichment (odds ratio [OR]) of the variants in hypertrophic cardiomyopathy (HCM) cases compared with internal controls (using principal component analysis [PCA] analysis) on the *y* axis. Black dots correspond to the variants that were significantly enriched (OR ≥1) after PCA validation, selecting individuals of European ethnicity in HCM cases and internal controls, and using gnomAD non-Finnish European (NFE) data. Blue line represents the linear regression line for the model of the variants enriched in our study ($OR=10^{-0.386}.FAF^{-0.465}$; $R^2=0.725$, $P<0.001$), and red line represents the variants not significantly enriched ($OR=10^{-1.080}.FAF^{-0.417}$; $R^2=0.629$, $P<0.001$). **B**, Intermediate-effect variants (IEVs), defined as those significantly enriched in HCM cases compared with internal and external controls, with an OR ≥2. Red dots represent variants validated in both global and PCA (NFE) analysis, and gray dots represent variants not significantly enriched in this last analysis. **C**, Monogenic variants, defined as those with an filtered allele frequency (FAF) $<5\times10^{-5}$ (0.005%) and significantly enriched in HCM cases compared with internal and external controls, with an OR ≥2. Green dots represent variants validated in both global and PCA (NFE) analysis. **D**, Near-monogenic variants (green dots), defined as those with an FAF between FAF $>5\times10^{-5}$ (0.005%) and 0.01 (1%), enriched in HCM cases with an OR ≥2, but an estimated penetrance >15% or an internal OR ≥15 (with a high penetrance to be considered IEV). **E**, Small-effect variants, defined as those with an FAF >0.01 (1%) and significantly enriched in HCM cases with an OR ≥1. Blue dots represent variants that were validated in both global and PCA (NFE) analysis, and gray dots represent variants not significantly enriched in internal PCA analysis. Tables S4, S5, S7, and S8 provide full details.

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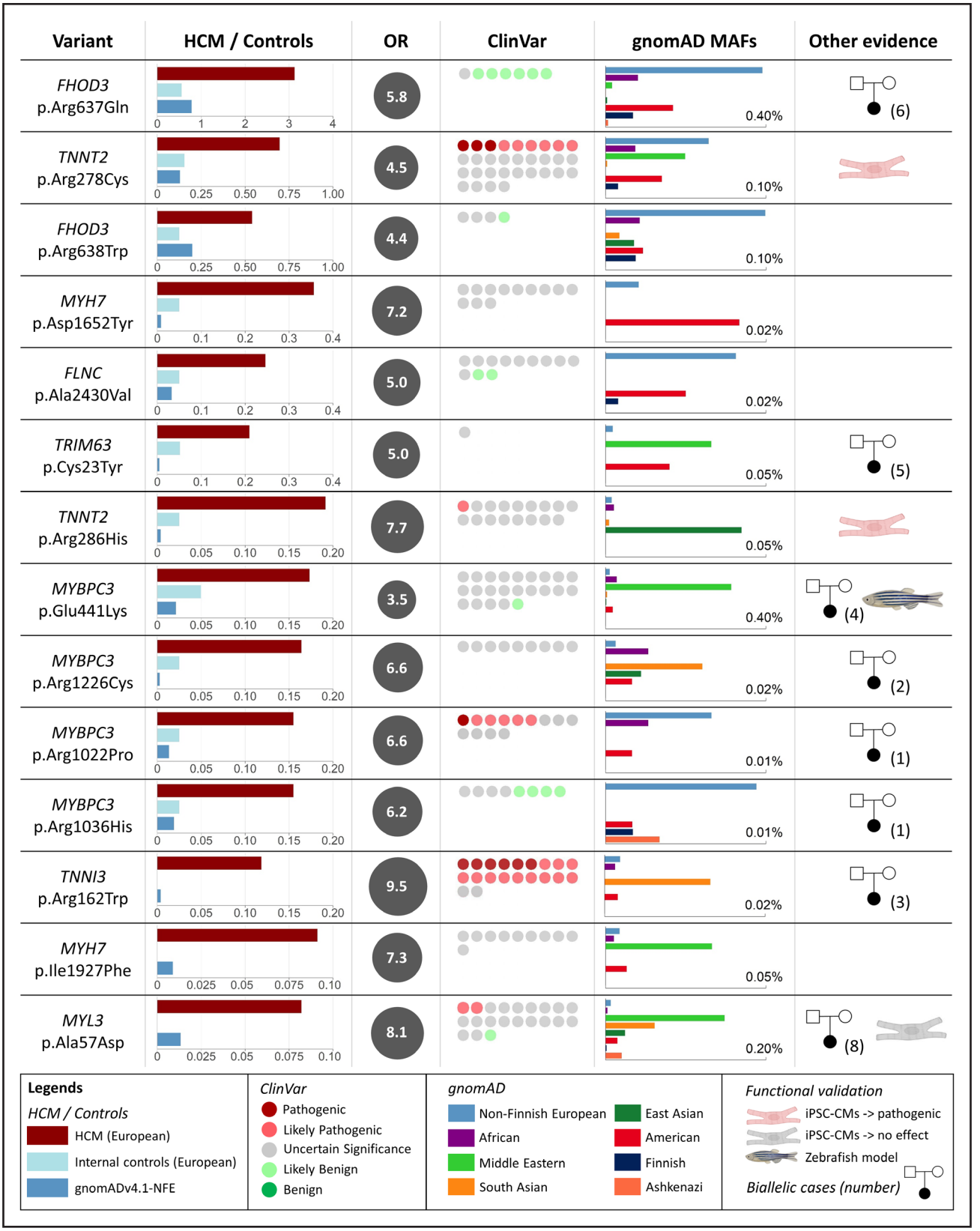

Figure 3. Selected IEVs.
Selected intermediate-effect variants (IEVs) with enrichment in hypertrophic cardiomyopathy (HCM) cases (European ancestry) vs internal controls and gnomADv4.1 non-Finnish European (NFE) individuals. Odds ratios (ORs) of the HCM cohort vs internal controls; variant classifications in ClinVar (Feb 2025); minor allele frequencies (MAF) in gnomADv4.1 populations; and additional evidence of pathogenicity. Numbers of biallelic cases and previous functional validation studies in human-induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) or animal models (Tables S10 and S11 provide details).

Table 1. IEVs: Variant Enrichment, ORs, and Estimated Penetrance Compared With Internal and External Control Groups

IEV	gnomAD FAF, population	Variant cases (PCA)	Variant internal controls (PCA)	gnomAD NFE AC/AN	OR internal controls (PCA; 95% CI), *P* value	OR gnomAD NFE (95% CI), *P* value	Estimated penetrance NFE (%)	ClinVar classification	Functional evidence	Enriched in other cohort	ClinGen risk allele curated evidence
MYBPC3 p.Arg1022Pro	0.000054 NFE	17/10 973	1/4029	78/1 179 572	6.61 (1.04–275.14) *P*=0.036	11.71 (6.49–19.98) *P*=1.88E-12	2.34	Conflicting	No	Yes	Very strong
MYBPC3 p.Arg1036His	0.000079 NFE	17/10 974	1/4028	111/1 179 840	6.24 (0.98–260.64) *P*=0.058	8.23 (4.63–13.79) *P*=2.54E-10	1.64	Conflicting	No	No	Strong
MYBPC. p.Arg1226Cys	0.000068 SAS	18/ 10,973	1/ 4029	15/1 179 896	6.61 (1.04–275.14) P=0.036	64.51 (30.72–137.73) *P*=4.13E-23	12.88	Uncertain significance	No	No	Strong
MYBPC3. p.Glu441Lys	0.002053 MID	19/ 10,972	2/ 4027	124/1 179 668	3.49 (0.84–30.89) *P*=0.085	8.24 (4.79–13.42) *P*=2.29E-11	1.64	Conflicting	Yes, supp	Yes	Very strong
MYH7 p,Asp1652Tyr	0.000090 AMR	39/ 10,952	2/ 4028	49/1 180 058	7.17 (1.86–61.37) *P*=5.82E-04	42.88 (27.40–66.65) *P*=9.73E-44	8.55	Uncertain significance	No	No	Strong
MYH7 p.Ile1927Phe	0.000058 MID	10/ 10,981	0/ 4030	52/1 180 052	7.34 (0.52–159.21) *P*=0.071	10.33 (4.68–20.56) *P*=1.88E-07	2.06	Uncertain significance	No	No	Strong
MYL3 p.Ala57Asp	0.000774 MID	11/ 10 980	0/ 4030	78/1 180 010	8.07 (0.46–144.74) *P*=0.044	6.20 (2.73–12.39) *P*=3.23E-05	1.51	Conflicting	No	Yes	Very strong
TNNI3 p.Arg162Trp	0.000075 SAS	13/ 10 978	0/ 4030	23/1 179 988	9.54 (0.72–202.70) *P*=0.026	30.38 (14.13–62.51) *P*=3.97E-14	6.07	Conflicting	Yes, supp	Yes	Very strong
TNNT2 p.Arg278Cys	0.000604 NFE	76/ 10 915	6/ 3893	758/1 179 234	4.52 (1.98–12.71) *P*=2.23E-05	5.41 (4.21–6.86) *P*=1.21E-29	1.08	Conflicting	Yes, mod	Yes	Very strong
TNNT2 p.Arg286His	0.000277 EAS	21/ 10 970	1/ 4027	23/1 178 912	7.71 (1.24–318.48) *P*=0.015	49.06 (25.81–92.99) *P*=4.30E-25	9.79	Uncertain significance	Yes, supp	Yes	Very strong
FHOD3 p.Arg637Gln	0.003818 NFE	333/ 10 649	22/ 4007	4616/1 179 896	5.76 (3.74–9.33) *P*=2.64E-24	3.98 (3.54–4.45) *P*=1.51E-89	0.78	Conflicting	No	No	Strong
FHOD3 p.Arg638Trp	0.000947 NFE	59/10 927	5/4024	1174/1 179 828	4.35 (1.76–13.89) *P*=1.94E-04	2.71 (2.05–3.52) *P*=6.59E-11	0.54	Conflicting	No	No	Strong
FLNC p.Ala2430Val	0.000144 NFE	27/10 963	2/4028	192/1 180 024	4.96 (1.25–43.02) *P*=0.011	7.57 (4.86–11.36) *P*=1.04E-14	1.51	Conflicting	No	No	Strong
TRIM63. p.Cys23Tyr	0.000115 AMR	23/10 963	2/3899	27/1 180 042	4.98 (1.25–43.11) *P*=0.012	45.84 (25.09–83.19) *P*=6.95E-27	9.15	NA	No	No	Strong

AC indicates Allele Count; AMR, Latino/Admixed American; AN, Allele Number; EAS, East Asian; FAF, filtered allele frequency; IEV, intermediate-effect variant; MACE, major adverse cardiac event; MID, Middle Eastern; mod, moderate; NFE, non-Finnish European; OR, odds ratio; PCA, principal component analysis; SAS, South Asian; and supp, supportive.

Negative (identified variants), IEV (IEVs in isolation), monogenic (single pathogenic variant), monogenic+IEV (both a monogenic and an IEV variant), and double monogenic (s monogenic variants). MACEs are a combined end point of sudden cardiac death, aborted arrest, appropriate implantable cardioverter defibrillator shock, heart failure death, cardiac transplantation, and death related to a cardiovascular procedure.

revealed a marginally significant enrichment (OR, 2.39 [95% CI, 1.65–3.47], *P*<0.0001) that was not validated against internal controls (OR 1.34 95% CI, 0.74–2.42], *P*=0.337; Table S9), reinforcing our approach of treating cases with heterozygous null variants in *TRIM63* as negative.

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Yield of Genetic Testing and Genetic Composition of the Cohort

Overall results of genetic testing in the 11 991 HCM probands with complete genetic evaluation of the 14 HCM primary genes are shown in Figure 4. A P/LP monogenic variant in an HCM disease-causing gene was identified in 2767 individuals (23.1%), and 731 (6.1%) had IEVs. Among those individuals with monogenic variants, 81.6% had a variant in a sarcomeric gene, with the 2 most frequently affected genes being *MYBPC3* (49.6%) and *MYH7* (23.5%). The relative contribution of each of the remaining sarcomeric genes was <6%. In addition, 230 cases (8.3% of the positive cases) had a single HCM disease-causing variant in a nonsarcomeric gene, with *ALPK3tv* and *FHOD3* being the most prevalent (4.3% and 2.0%, respectively). The remaining positive cases were explained by genocopies (4.4%, n=123) or complex genotypes of multiple P/LP variants or combination of a P/LP variant with IEV (5.8%, n=156). In addition, 557 subjects (4.7%) had a relevant variant of uncertain significance in isolation.

IEVs Contribute to HCM

A total of 570 subjects carried an IEV in isolation, representing 4.76% of the whole cohort. Using the approximation of the population-attributable fraction showed that the proportion of HCM cases in the entire cohort attributable to IEVs was estimated to be 4.91% (95% CI, 3.22%–6.66%).

The presence of a P/LP monogenic HCM variants in individuals with IEVs was significantly lower than in the overall cohort (13.1% versus 23.1%; $P<0.0001$), providing additional evidence that IEVs contribute to HCM phenotype because otherwise the proportion of individuals with monogenic variants should have been similar among individuals with IEVs and the overall cohort.

Furthermore, we analyzed the presence of IEVs across some well-known specific substrates for HCM such as *MYBPC3* truncating variants (*MYBPC3tv*), *MYH7* P/LP missense variants, and *ALPK3tv* (considered low-penetrance monogenic variants). Among *ALPK3tv* variant carriers, 5.2% also had an IEV. In contrast, the co-occurrence of IEVs was lower in *MYBPC3*tv (3.6%) and *MYH7* P/LP (3.0%) variant carriers yet higher than the co-occurrence of other P/LP HCM variants in these groups (double-monogenic cases: 1.3% and 1.1%, respectively). A gradient in the frequency of co-occurrence of IEVs was observed according to genetic substrates, increasing from highly penetrant monogenic substrates to those with lower penetrance and reaching the higher value in cases without monogenic variants (Figure 4C).

IEVs Influence HCM Phenotype

Patients with HCM were stratified into 5 distinct groups for phenotypic comparative analysis: negative (n=8091), IEV (n=570), monogenic (n=2434), monogenic+IEV (n=90), and double-monogenic (n=46). Probands with at least 1 relevant variant of uncertain significance in a primary HCM gene or a disease-causing variant in genocopies or secondary genes were not considered (n=770).

The phenotypic results across groups are presented in Table 2 and Figure 5. A progressive increase in age-related penetrance was observed across the different groups, with the lowest penetrance observed in negative

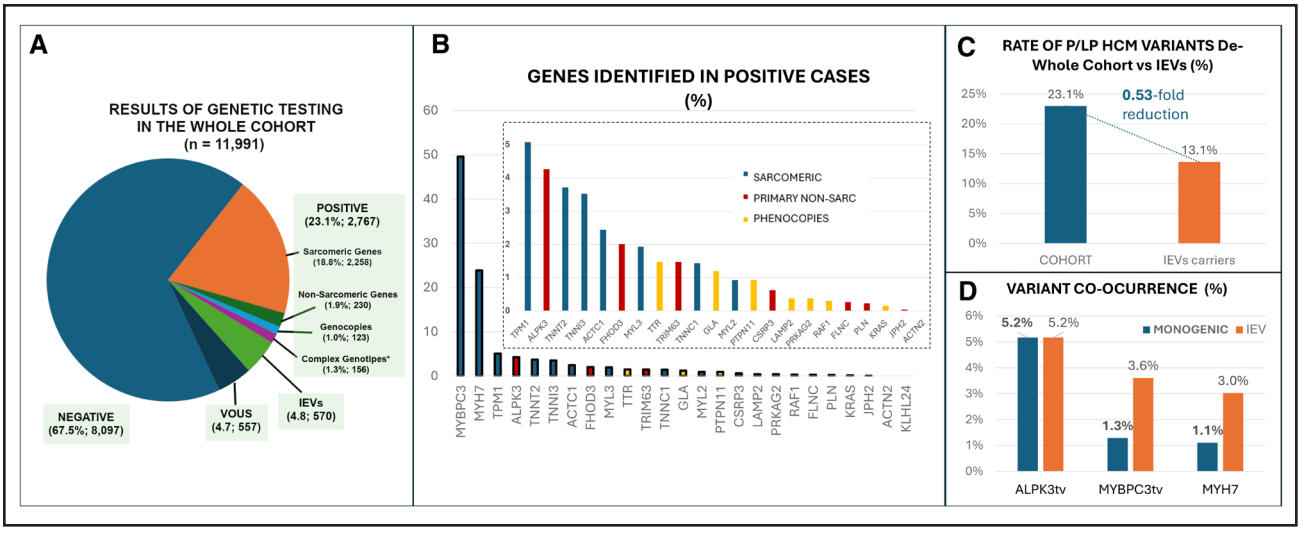

Figure 4. Results of genetic testing.
A, Genetic testing results in the whole cohort. **B**, Contribution of each gene among cases with positive genetic results: sarcomeric (blue bars), primary nonsarcomeric (red bars), and phenocopy genes (yellow bars). **C**, Proportion of pathogenic/likely pathogenic (P/LP) variants (diagnostic yield) in the whole cohort compared with carriers of intermediate-effect variants (IEVs). **D**, Co-occurrence rate of a second monogenic variant and IEVs in different genetic subpopulations. HCM indicates hypertrophic cardiomyopathy; and VOUS, variant of uncertain significance.

Table 2. Clinical Characteristics According to Genetic Findings

	Negative (n=8091)	IEV (n=570)	Monogenic (n=2,434)	Monogenic+IEV (n=90)	Double monogenic (n=46)	*P* value	Total cohort (n=11 231)
Sex (male), %	66.34 (5311/8006)	63.56 (361/568)	63.76 (1534/2406)	56.18 (50/89)	53.49 (23/43)	0.12	65.51 (7279/11 112)
Age of diagnosis (25th–75th percentile), y	61 (49–70)	59 (46–69)	50 (38–62)	46 (31–58)	37 (21–56)	<0.0001	58 [46-69]
Follow-up, y	6.96±7.42	7.69±7.66	9.82±9.02	14.20±9.02	11.50±15.20	<0.0001	7.91±8.15
LVMWT, mm	18.10±3.74	19.00±4.31	20.70±4.99	22.20±5.23	25.00±6.91	<0.0001	18.86±4.31
LVMWT >25 mm, %	5.87 (196/3339)	11.11 (35/315)	18.81 (218/1159)	31.91 (15/47)	45.83 (11/24)	<0.0001	9.73% (475/4884)
LVMWT >30 mm, %	1.17 (39/3339)	3.81 (12/315)	7.16 (83/1159)	8.51 (4/47)	37.50 (9/24)	<0.0001	3.01% (147/4884)
MACEs, %	2.43 (184/7562)	3.48 (19/545)	3.52 (78/2212)	12.19 (10/82)	10 (4/40)	<0.0001	2.83% (295/10 441)
MAEs	2.26 (171/7562)	2.38 (13/545)	2.93 (65/2212)	12.19 (10/82)	5 (2/40)	<0.0001	2.49% (261/10 441)
HFD	0.17 (13/7562)	0.73 (4/545)	0.45 (10/2212)	0.00 (0/40)	5.00 (2/40)	<0.0001	

HFD indicates heart failure death; IEV, intermediate-effect variant; LVMWT, left ventricular maximal wall thickness; MACE, major adverse cardiac event; and MAE, major arrhythmic event.

Negative (no identified variants), IEV (IEVs in isolation), monogenic (single pathogenic variant), monogenic+IEV (both a monogenic and an IEV variant), and double monogenic (2 monogenic variants).

MACEs are a combined end point of MAEs (combined end point of sudden cardiac death, aborted arrest, and appropriate implantable cardioverter defibrillator shock) and HFD (combined end point of HFD and cardiac transplantation).

individuals (median, 61 years of age; 25th–75th percentile, 49–70 years of age), followed by IEV (59 years of age; 25th–75th percentile, 46–69 years of age), monogenic (50 years of age; 25th–75th percentile, 38–62 years of age), and monogenic+IEV (46 years of age; 25th–75th percentile, 31–58 years of age), with the highest penetrance in the double monogenic (37 years of age; 25th–75th percentile, 21–56 years of age). These differences were statistically significant ($P<0.0001$). Pairwise comparisons between the groups demonstrated that significant differences were maintained for all comparisons except for monogenic+IEV versus the double-monogenic group ($P=0.43$), which is probably explained by the lower number of observations in these 2 groups.

Because the *FHOD3*:p.Arg637Gln variant was identified in 50% of probands carrying IEVs, we compared its effects with the other IEVs. No significant differences were observed in age at diagnosis, MACE-free survival, or LVMWT distribution between *FHOD3*:p.Arg637Gln carriers and those with other IEVs, suggesting no distinct phenotypic impact for this variant within the cohort (Figure S3). A sensitivity analysis excluding *FHOD3*:p.Arg637Gln showed consistent differences in age at diagnosis, MACE-free survival, and LVMWT across genetic groups (all $P<0.0001$). Monogenic+IEV carriers still exhibited more severe phenotypes than monogenic-only cases, demonstrating that the additive effect of IEVs is not driven solely by *FHOD3*:p.Arg637Gln (Figure S4).

In addition, we included the *TNNI3*:p.Arg162Gln variant as a separate group, given that some previous reports have considered it monogenic, and the *MYBPC3*:p.Arg502Trp variant, which exhibited several characteristics of an IEV but was ultimately excluded and classified as monogenic in the final step of the analysis. There were no differences in age-related penetrance between the IEVs, *FHOD3*:p.Arg637Gln, and *TNNI3*:p.Arg162Gln, whereas *MYBPC3*:p.Arg502Trp was significantly higher, supporting its inclusion in the monogenic group (Figure S5).

The survival analysis for MACEs (Figure 6) also revealed significant differences across groups ($P<0.0001$). MACEs were significantly higher in the monogenic+IEV group compared with the monogenic group ($P<0.0001$) and similar to the double-monogenic group ($P=0.67$). Because the divergence in MACE-free survival curves beginning at ≈40 years of age may be influenced by a decreasing number at risk and the possibility of survival bias, we performed a Cox regression analysis, univariable analysis, and an analysis adjusted by age of diagnosis (Figures S6 and S7). The results were consistent, with the monogenic+IEV group being the only category with a significant increase in MACE risk after adjustment, confirming that its effect is not driven solely by differences in age at presentation.

LVMWT also exhibited a statistically significant gradient of severity across groups ($P<0.0001$), from negative to double monogenic (Figure 6C and 6D). In analyses of the proportion of cases with severe (LVMWT >25 mm) and massive (LVMWT >30 mm) thickness, the differences between groups were both statistically significant

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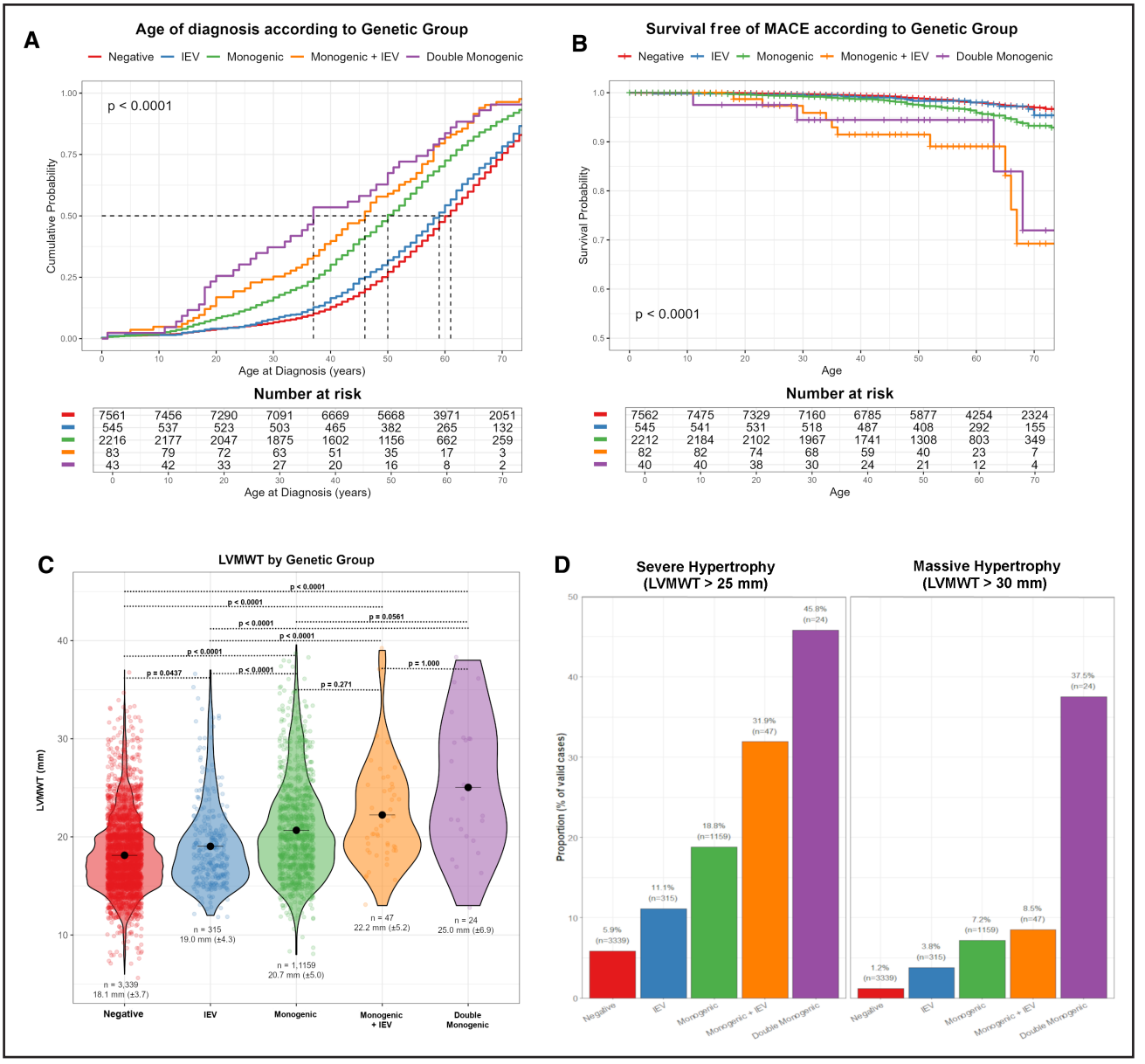

Figure 5. Age at diagnosis, survival free of MACEs, and LVMWT according to genetic groups.
A, Kaplan-Meier curves showing the age-related penetrance. **B**, Kaplan-Meier major adverse cardiac event (MACE)–free survival. **C**, Violin plots of left ventricular maximal wall thickness (LVMWT); black dots represent mean LVMWT. **D**, Proportion of individuals with severe left ventricular hypertrophy (LVMWT >25; **left**) and massive left ventricular hypertrophy (LVMWT >30; **right**). IEV indicates intermediate-effect variant.

(*P*<0.0001), with the same gradient of severity observed for the 5 genetic categories.

An analysis of age-related penetrance, MACEs, and LVMWT stratified by sex resulted in similar findings across genetic groups. In both sexes, adding an IEV to a monogenic variant was associated with earlier diagnosis, increased wall thickness, and higher event rates; these differences were more pronounced in women (Figures S8 through S10). We observed that age-related penetrance of HCM was higher in men than in women, but this sex effect decreased toward higher-burden subgroups and disappeared in the monogenic+IEV and double-monogenic groups (Figure S7B).

We next assessed whether the combination of a sarcomeric or nonsarcomeric IEV in carriers of only monogenic sarcomeric variants modifies disease expression (Figure S11). No statistically significant differences were observed, although a nonsignificant trend toward greater LVMWT (*P*=0.063) and higher MACE incidence (*P*=0.141) was noted in carriers of sarcomeric IEVs.

A spectrum of effect by zygosity was observed for 9 of the 14 IEVs identified, with a total of 28 homozygous or compound heterozygous cases described in this cohort (10 cases) or in the literature (18 cases; Figure 6; Table S10). The age at disease onset in patients with biallelic IEVs was significantly lower than in heterozygous

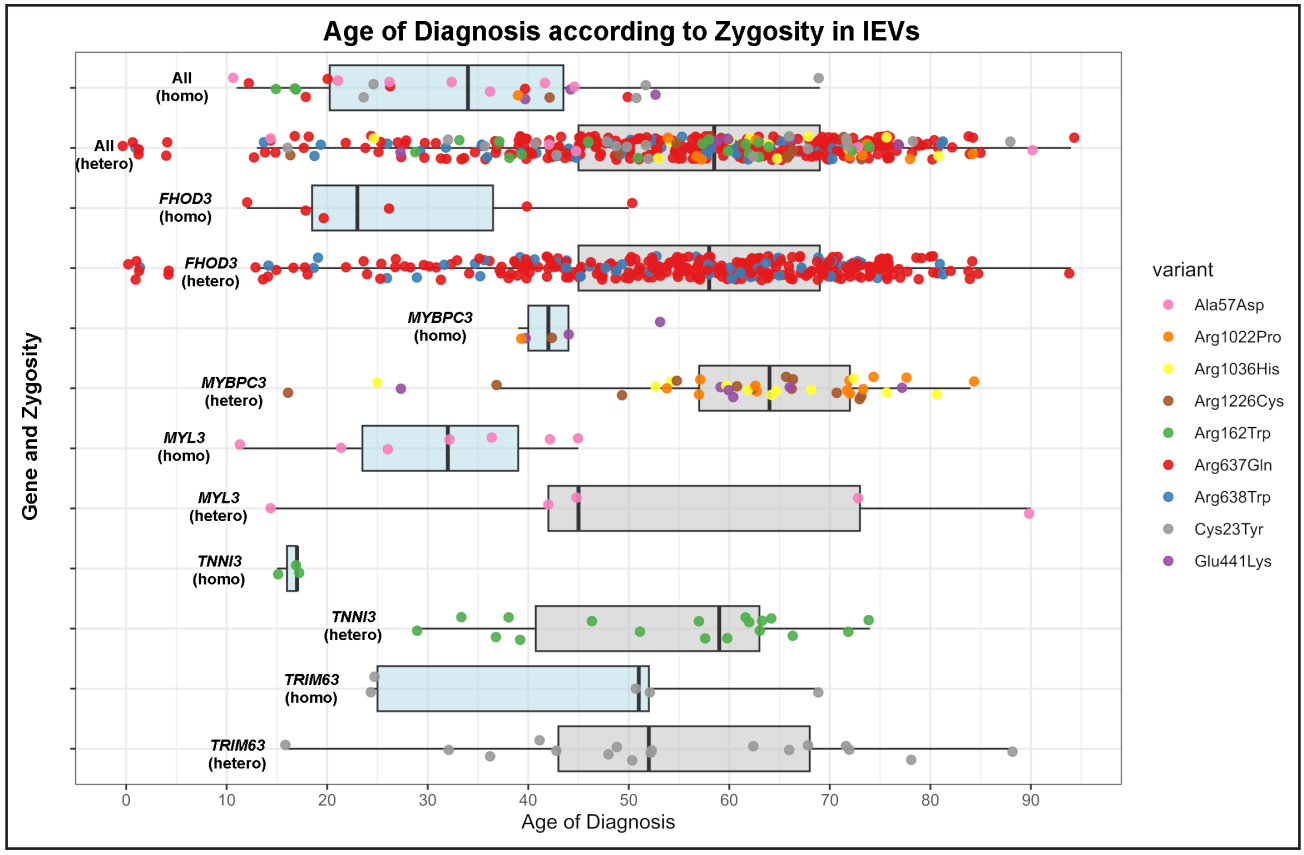

Figure 6. Age at diagnosis according to IEV zygosity (biallelic vs heterozygous carriers).
Age at diagnosis in 9 intermediate-effect variants (IEVs) as described in the literature or in our cohort in homozygous/compound-heterozygous carriers compared with heterozygous carriers. Light blue bars represent homozygous/compound heterozygous carriers; gray bars represent heterozygous carriers (Table S10 provides details).

cases in our cohort: 33.34±15.20 years of age versus 55.73±17.41 years ($P<0.0001$). In addition to further evidence for their pathogenic role, these findings reinforce the low penetrance of these variants in heterozygosis and a more severe phenotype under biallelic involvement.

DISCUSSION

Our study, focused on exploring the contribution and impact of IEVs in HCM, includes the largest cohort of patients described to date analyzing this type of variant and is built on robust methodological grounds, providing valuable insights into the HCM genetic landscape. We included both sarcomeric and nonsarcomeric genes with a validated association with primary HCM, and our selection of IEVs was conducted using PCA-based ancestry and incorporating a dual validation approach with both internal noncardiomyopathy controls (with a uniform sequencing approach) and large population datasets. Our study describes 14 IEVs across 9 genes that were present in 6.1% of HCM cases and shows that IEVs account for 4.8% of HCM of the overall genetic risk attributable to IEVs. We also demonstrated that IEVs influence disease severity and outcomes, particularly when combined with monogenic disease-causing variants. Both sarcomeric and nonsarcomeric IEVs modulate age-related penetrance in monogenic sarcomeric HCM, with sarcomeric IEVs exerting a more pronounced impact on hypertrophy and clinical outcomes. These contrasting effects may reflect differential synergistic interactions between each type of IEV and the monogenic sarcomeric background.

These results highlight the importance of IEVs in HCM and suggest that evaluation of IEVs should be routinely considered when HCM genetic testing is undertaken.

Description of IEVs in HCM is challenging because identifying these variants is highly dependent on the patient and control cohorts used and confirming its biological influence is difficult. Our study used cases and controls with the same broad ancestral background, reducing the potential for bias in observed associations between genetic variants and disease traits attributable to differences in allele frequencies across diverse populations. Although this method does not achieve the level of precision offered by genome-wide profiling in tightly matched genome-wide association studies, it enhances the accuracy of variant identification and strengthens genotype-phenotype correlations. The co-occurrence of monogenic and IEVs in the same gene was rare in our

cohort and, when observed, typically involved variants on separate alleles (Table S11), arguing against linkage disequilibrium as a major contributor to enrichment.

A subset of the IEVs identified in our study have also been functionally characterized through experimental studies, providing supportive to moderate evidence for their potential pathogenicity, including *MYBPC3*:p.Glu441Lys,[22,23] *TNNI3*:p.Arg162Trp,[24–26] *TNNT2*:p.Arg278Cys,[13,27–30] and *TNNT2*:p.Arg286His.[31] However, the challenges in functionally validating variants of modest effect sizes are highlighted by the *MYL3*:p.Ala57Asp variant identified in our study for which no pathogenic effect was observed in heterozygous or homozygous CRISPR-Cas9–edited cardiomyocytes despite compelling human genetic evidence of its pathogenicity.[32] Moreover, some of the identified IEVs are in mutational hot spots or affect the same residue of one definitive disease-causing variants, further underlining their biological relevance (Table S12). Last, the analysis of biallelic cases with IEVs performed also demonstrated a clear dose-gradient effect, with homozygous or compound heterozygous carriers exhibiting significantly earlier disease onset and associated with a more severe phenotype and worse prognosis compared with heterozygous carriers, which also supports the pathological role of these variants.

Most of the 14 selected IEVs were homogeneously enriched when we compared external and internal controls in our cohort. However, some IEVs such as *TNNI3*:p.Arg162Trp, *TNNT2*:p.Arg286His, *MYH7*:p.Asp1652Tyr, *MYBPC3*:p.Arg1226Cys, and *TRIM63*:p.Cys23Tyr demonstrated an increased external OR (effect sizes) compared with the internal OR. Although PCA adjustment mitigates some ancestry-related biases, the internal OR serves as our primary metric to accurately assess the strength of effect when analyzing variants. Ancestry in the HCM cohort was defined with PCA, a statistical technique that identifies major patterns of genetic variation across individuals. This is particularly important in multicenter cohorts with diverse backgrounds, improving downstream association analyses. Complementarily, the use of internal controls reduces the influence of population stratification and technical heterogeneity while also accounting for cohort-specific confounders. Because the internal control cohort is smaller than public reference datasets, it remains possible that some variants were conservatively filtered out and could meet IEV criteria in the future.

A further advantage of these large-scale cohorts is the identification of IEVs at low frequencies in Europeans but that are likely much more prevalent in other ancestries based on gnomAD data (eg, *TNNT2*:p.Arg286His in East Asian people and *MYBPC3*:p.Glu441Lys in Middle Eastern people).

The fact that genetic findings affect phenotypic expression in HCM is widely recognized. Patients with HCM with monogenic sarcomeric variants have been reported to present a higher risk of complications than sarcomere-negative patients.[33] In addition, the presence of multiple sarcomere mutations has been linked with a poorer prognosis.[34] However, the variability clinical course observe within and between sarcomeric genes and the possible modifying effects of additional genetic and nongenetic factors have complicated genetic risk stratification.[35,36] Furthermore, nonsarcomeric genes contribute to HCM clinical heterogeneity, often showing a less penetrant or milder phenotype than the majority of sarcomeric genetic substrates.[10]

Here, we have described the contribution of IEVs to HCM phenotypic expression, showing a robust gradient of severity in LVMWT, MACEs, and median age at diagnosis according to genetic findings. Gene-elusive HCM was associated with a milder disease phenotype, characterized by smaller LVMWT, lower age-related penetrance, and a more favorable prognosis compared with cases with known sarcomeric monogenic variants, whereas carriers of IEVs in isolation exhibited an intermediate phenotype compared with the other groups. Furthermore, the presence of an IEV in combination with a known monogenic variant significantly modified the clinical course and expression, underscoring the crucial role of IEVs as key modifiers, with potential implications for risk stratification and clinical management in affected individuals and their families.

The modifying effect of IEVs appeared more pronounced in women, potentially reflecting greater sensitivity to genetic burden and a corresponding attenuation of baseline sex-related differences in age at diagnosis, severity of hypertrophy, and MACE risk. The additive effect of multiple variants may, in part, override or mask the modulatory effect of sex on disease expression. One possible explanation is that in individuals carrying both monogenic and IEVs, the genetic burden may be sufficiently high to dominate the phenotypic outcome, thereby reducing the relative influence of sex-dependent modifiers such as hormonal or epigenetic factors.

Using the population-attributable fraction weighing the frequency of each variant by its estimated penetrance, we estimated that IEVs collectively could contribute to nearly 5% of the HCM burden in our cohort. Despite limitations inherent in accurately determining the penetrance of IEVs, this approximation provides a valuable and reasonably valid estimate of the overall contribution of IEVs to HCM and shows how routine examination of IEVs can improve current HCM genetic testing. However, it should be noted that these variants probably do not drive disease in most individuals on their own but rather contribute to HCM expression within a liability threshold model, acting as necessary factors for disease manifestation through interactions with other genetic and environmental factors.

A recent study from SHaRe (Sarcomeric Human Cardiomyopathy Registry) has also evaluated the role of low-penetrant variants in modulating HCM phenotype.[13]

Although the approach used for variant selection in that study has some similarities with ours, particularly in terms of the intermediate frequency range and significance thresholds used, there were a number of key methodological differences in our study. We used the FAF instead of minor allele frequencies to accurately identify variants present at low frequency across all ancestries. We also performed an ancestry-based analysis and validated findings with both gnomAD external controls and ancestry-matched internal controls, which is critical for accurate variant selection. Last, we included nonsarcomeric genes.

As a result, only 4 IEVs were shared by both studies: *TNNT2*:p.Arg278Cys, *MYL3*:p.Ala57Asp, *MYBPC3*:p.Arg1022Pro, and *MYBPC3*:p.Glu441Lys. In our opinion, the relatively low concordance between our study and SHaRe is related to the methodological differences across both studies (Table S13). First, our approaches for assessing variant frequency in control populations differed. For instance, the variants *MYH7*:p.Asp1652Tyr, *TNNI3*:p.Arg162Trp, and *TNNT2*:p.Arg286His selected in our study were enriched in the SHaRe study but excluded because of rarity based on overall gnomAD minor allele frequencies, although these IEVs were in the intermediate frequency range in non-NFE FAFs. Conversely, the *MYBPC3*:p.Asn1327Lys variant, selected in the SHaRe work, was excluded in our analysis because of a low FAF (and a lack of significant enrichment compared with our internal controls). Notably, this variant is highly enriched in individuals of Ashkenazi descent (this bottleneck population is not included in FAF calculations), suggesting that a population-specific analysis may be required for validation of this variant.

Three of the variants identified in the SHaRe study (*MYBPC3*:p.Arg810His, p.Asp610His, and p.Asp605Asn) showed borderline significance when we compared the HCM cohort with our internal controls. This discrepancy might arise from random stochastic effects attributable to minor ancestry differences between the cohorts or, more likely, from limitations in statistical power. The point estimates of ORs for these variants were broadly similar between analyses, suggesting that our internal controls do not demonstrate a lack of enrichment but rather an underpowered ability to detect it. Establishing significance and effect size thresholds is essential for the inclusion and validation of IEVs, but we cannot rule out that some variants, particularly those with borderline significance, could qualify as IEVs in future studies with larger datasets.

However, the main reason for discrepancy in the number of individuals with IEVs emerges from the larger number of genes analyzed in our study. The inclusion of nonsarcomeric genes increased the proportion of cases with IEVs from 2.1% to 2.5% (for sarcomeric IEVs in both studies) to 6.1%. The importance of including nonsarcomeric genes in the analysis of IEVs is highlighted by the fact that although nonsarcomeric IEVs accounted for one-third of the identified IEVs, they were present in two-thirds of probands carrying IEVs. Nonsarcomeric genes may play a more significant role in this nonmendelian inheritance pattern than sarcomeric genes, despite explaining a relatively small percentage of mendelian monogenic cases.

Although some of these genes such as *TRIM63*, *ALPK3*, *CSRP3*, and *FHOD3* have been associated with differing inheritance patterns (autosomal dominant, recessive, or semidominant), we applied a uniform, threshold-based framework to assess phenotypic impact across all genes. This strategy avoids binary assumptions about inheritance, aligning with recent insights that penetrance and expressivity lie along a spectrum and may be more variant specific than gene specific, especially for nontruncating variants.

Last, although our study focused on individuals of NFE ancestry to ensure unbiased results through ancestry homogeneity, the contribution and distribution of IEVs may differ across ancestral backgrounds. This highlights that genetic architecture is not uniform across populations and that generalization of these findings should be made with consideration of ancestral context.

Limitations

For IEV analysis, we focused on 14 primary genes with strong or moderate evidence according to recent ClinGen curation. Although *ACTN2* and *JPH2* meet the criteria for moderate evidence, they were excluded because of their negligible representation in our cohort (0% and 0.02%, respectively), in line with their low diagnostic yield in other large studies. We acknowledge this as a limitation of our gene selection approach.

The deliberate exclusion of HCM genocopy genes from enrichment and phenotypic analyses may have resulted in the omission of cases in which these genes contributed to phenotype or MACEs, although their distinct clinical behavior justified this decision.

Our analysis focused on genes with established HCM associations, which may have limited the detection of IEVs in emerging candidate genes or genes not yet linked to HCM. Although this conservative approach enhanced interpretability, it may have missed additional contributors to disease risk. Broader genomic strategies will be needed to fully define the IEV landscape in HCM.

We did not perform functional studies to confirm the functional effect of the IEVs identified in our study. However, we believe this limitation is mitigated by the large number of patients studied and the robust methodology used in our study. In addition, we did not incorporate polygenic risk scores into our analysis, which are increasingly recognized to influence phenotypic expression in HCM. Future studies should integrate polygenic risk scores,

IEVs and monogenic variants to fully assess the contribution of genetics to HCM expression.

Conclusions

IEVs constitute a key component of the spectrum of HCM genetic architecture, accounting for nearly 5% of the overall disease burden based on population attributable fraction. IEVs influence disease severity and outcomes, particularly when combined with monogenic disease-causing variants. Evaluation of IEVs should be considered when HCM genetic testing is performed.

ARTICLE INFORMATION

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Supplemental Material

Expanded Methods
Figures S1–S11
STROBE Checklist for Observational Studies
Excel File S1 (Tables S1–S13)
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