

Targeted panel sequencing in pediatric primary cardiomyopathy supports a critical role of *TNNI3*

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Abstract

The underlying genetic mechanisms and early pathological events of children with primary cardiomyopathy (CMP) are insufficiently characterized. In this study, we aimed to

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characterize the mutational spectrum of primary CMP in a large cohort of patients ≤ 18 years referred to a tertiary center. Eighty unrelated index patients with pediatric primary CMP underwent genetic testing with a panel-based next-generation sequencing approach of 89 genes. At least one pathogenic or probably pathogenic variant was identified in 30/80 (38%) index patients. In all CMP subgroups, patients carried most frequently variants of interest in sarcomere genes suggesting them as a major contributor in pediatric primary CMP. In *MYH7*, *MYBPC3*, and *TNNI3*, we identified 18 pathogenic/probably pathogenic variants (*MYH7* $n = 7$, *MYBPC3* $n = 6$, *TNNI3* $n = 5$, including one homozygous (*TNNI3* c.24+2T>A) truncating variant. Protein and transcript level analysis on heart biopsies from individuals with homozygous mutation of *TNNI3* revealed that the *TNNI3* protein is absent and associated with upregulation of the fetal isoform *TNNI1*. The present study further supports the clinical importance of sarcomeric mutation—not only in adult—but also in pediatric primary CMP. *TNNI3* is the third most important disease gene in this cohort and complete loss of *TNNI3* leads to severe pediatric CMP.

KEYWORDS

cardiomyopathy, genetics, pediatrics, sarcomere, *TNNI3*

1 | INTRODUCTION

Primary cardiomyopathies (CMPs) are genetically heterogeneous disorders with a large number of disease-causing genes. The growing list of genes, reaching about 100 genes, and the overlap in genes involved in different CMP subtypes cause significant challenges to provide a genetic diagnosis for patients. CMP phenotypic subtypes include hypertrophic (HCM), dilated (DCM), restrictive (RCM), left ventricular non-compaction (LVNC), and arrhythmogenic right ventricular (ARVC) CMP. In adults, sarcomeric genes represent the major functional category, with the exception of ARVC, which is dominated by mutation of desmosomal genes. There are only few studies on current genetic testing in pediatric CMP. Depending on the phenotypic composition of the cohorts, also including secondary CMP, and different next-generation sequencing (NGS) strategies, recent studies lead to the identification of pathogenic variants in 26% to 39% of pediatric patients.^{1,2} These genetic defects exhibit variable expressivity and variable penetrance. Mutations in genes encoding components of the sarcomere and related binding proteins, Z-band, nuclear membrane, desmosomal, mitochondrial, and calcium handling proteins have all been found in children with CMP.³ Only now, NGS techniques and genetic variant interpretation guidelines enable to unravel the genetic background in a reasonable percentage of primary CMP in children more systematically. We assembled a cohort of pediatric primary CMP patients and present their clinical phenotypes as well as spectrum of genetic variants. We present the distribution of genetic variants in certain functional groups and highlight genetic variants which can lead to severe CMP. In clinical practice, pediatric patients with mutation of Troponin I require special attention.

2 | MATERIAL AND METHODS

2.1 | Clinical evaluation

Unrelated patients with CMP were recruited at the Charité – Universitätsmedizin Berlin and the German Heart Center Berlin, Berlin, Germany between November 2011 and February 2017 (study design: Figure S1). Written informed consent was obtained from each subject or their legal guardians according to the Declaration of Helsinki. The study was approved by the local institutional review board (ID EA2/083/13, EA2/131/10, Charité – Universitätsmedizin Berlin). Probands ≤ 18 years and available family members were evaluated by medical history, physical examination, 12-lead electrocardiography, and transthoracic echocardiography. For patients recruited after heart transplantation (HTX), the clinical and echocardiographic data were collected by retrospective review of patients' records. Patients with a primary diagnosis of HCM, DCM, RCM, LVNC, or ARVC were included in the study.^{4,5} Patients who had secondary CMP (syndromic, neuromuscular, metabolic, or myocarditis) or CMP in combination with a structural congenital heart defect were excluded. ARVC was not included in the analysis of subgroups.

2.2 | Targeted NGS

NGS was carried out in genomic DNA samples isolated from patient blood samples with the NucleoSpin Blood kit according to the manufacturer's protocol (Macherey-Nagel, Germany). DNA quantification occurred with a Qubit 3 fluorometer using appropriate Qubit dsDNA reagents (Thermo Fisher Scientific, Invitrogen). NGS was performed with a panel-based approach using the

Illumina TruSight Cardio Sequencing Kit (Illumina).⁶ With this approach, exonic regions of 174 cardiovascular disease genes specifically implicated in structural heart disease and arrhythmias were sequenced (https://support.illumina.com/sequencing/sequencing_kits/trusight-cardio-sequencing-kit.html). Library preparation and enrichment was performed according to the manufacturer's protocol. Quantification of DNA libraries was performed with a Bioanalyzer 2100 using the high sensitivity DNA kit or DNA 1000 kit (Agilent Technologies). Patient DNA libraries were sequenced on a NextSeq 500 platform with mid output cartridge v2 with paired end sequencing (150 cycles) and dual indexing. Sanger sequencing was applied to validate variants detected by NGS in index patients and in family members for segregation analysis. Sanger sequencing was performed according to standard laboratory protocols.⁷ The primer for genomic amplification of cardiac troponin I (*TNNI3*) polymerase chain reaction (PCR) products and Sanger sequencing is available in Table S1.

2.3 | Alignment of raw data and variant calling

The libraries were demultiplexed using *bcl2fastq* v2.17.1.14. The reads were then aligned using BWA-MEM v0.7.15⁸ to the reference GRCh37 (hs37d5.fa), separate read groups were assigned for all reads from one lane, and duplicates were masked using *Samblaster* v0.1.24.⁹ Standard quality control was performed using *FastQC*¹⁰ and further quality control was performed by considering the minimal coverage of all exons Illumina specifies as belonging to the target region for the panel. The variants were then called using *GATK UnifiedGenotyper* v3.7.¹¹

2.4 | Bioinformatic evaluation and variant classification

The called variants were evaluated with Variant Studio (Illumina) for their minor allele frequency (<0.001) and mutation specification. We used Genome Aggregation Database (gnomAD) as genetic reference database for unaffected individuals (<http://gnomad.broadinstitute.org/>).¹² We evaluated 89 CMP disease genes that have been ascertained in a series of patients, preferably including family segregation data; CMP genes suggested from single case reports were excluded (Table S2). Each detected variant was validated with databases displaying genetic variants for CMP: human gene mutation database (HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>) and atlas of cardiac genetic variation¹³ (ACGV, <https://cardiodb.org/ACGV/>). Variants of interest (VOI) were classified as pathogenic, probably pathogenic, or variant of uncertain significance (VUS) according to the guidelines of the American College of Medical Genetics and Genomics (ACMG)¹⁴ using the recommended frequency threshold in CMPs of <0.0001.¹⁵ For the purpose of this study, variant classification was reconfirmed by the classification on the National Center for Biotechnology (NCBI) database, ClinVar.¹⁶ The most recent variant classification was used in the final analysis (<https://www.ncbi.nlm.nih.gov/clinvar/>).¹⁷

For functional classification, the 89 genes evaluated in this study were arranged into functional groups (Table S2).¹⁸

2.5 | Protein and mRNA expression analysis

Age-matched human ventricular biopsies obtained at time of cardiac surgery (age range 5 months to 2.5 years) were subjected to protein and total RNA isolation after liquid nitrogen treatment and mechanical crushing. Powdered heart tissue was subjected to RNA isolation with Trizol reagent (Invitrogen, Carlsbad, CA) and protein lysates were obtained after incubation with RIPA buffer. Protein samples were analyzed by Western blot after sodium dodecylsulfate polyacrylamide gel electrophoresis and transfer onto polyvinylidene difluoride membrane (iBlot system; Invitrogen, Carlsbad, CA). For Western blot analysis, membranes were incubated with the following primary antibodies: anti-GAPDH (Life Technologies, AM4300), anti-HSC70/HSP70 (Enzo Life Science, ADI-SPA-820), anti-TNNI1 (Sigma, HPA028190), anti-TNNI3 (ThermoFisher, PA5-28964), anti-TNNT2 (ThermoFisher, MA5-12960), and anti-MYBPC3 (R&D systems, AF7439). Quantification of Western blots occurred with GelQuantNet (<http://biochemlabsolutions.com>). Normalization of target protein levels occurred with HSP70 expression.

For mRNA analysis, total RNA was isolated according to standard protocols and transcribed into cDNA with SuperscriptII (Invitrogen). Quantitative PCR (qPCR) was performed on a Taqman 7500 (Applied Biosystems) using *GAPDH* as endogenous control. The relative mRNA level was calculated according to the $\Delta\Delta CT$ method. The primers used for qPCR are listed in Table S1.

3 | RESULTS

3.1 | Patient characteristics

During the study period, 80 individuals were recruited at our tertiary center in the pediatric cardiology clinics (German Heart Center and Charité – Universitätsmedizin Berlin, both in Berlin) with a diagnosis of primary CMP at age ≤ 18 years. The cohort of unrelated Caucasian individuals (42 males and 38 females; mean age at diagnosis 4.8 years, range 1 day to 18 years) underwent genetic screening of 89 CMP-associated genes. The clinical characteristics of the entire cohort are summarized in Table 1. Our pediatric CMP cohort was composed of patients with DCM ($n = 34$), HCM ($n = 23$), LVNC ($n = 14$), RCM ($n = 7$), and ARVC ($n = 2$) (Figure 1A). The age at diagnosis was <10 years in 63 patients (79%) and <1 year in 34 patients (43%). Of note, the age of diagnosis did not affect the relative CMP subtype frequency (Figure 1A-C). Family members of 61 index patients (77%) underwent clinical evaluation. In 27 of these families, at least one additional affected family member was identified resulting in a 44% positive yield of family screening (Table S3). The largest group was composed of DCM patients (43%) of which 50% underwent HTX. In addition, the DCM group had the highest rate of implanted ventricular assist device systems (47%), extracorporeal membrane oxygenation systems (15%), and death

TABLE 1 Clinical characteristics of the pediatric cohort at initial diagnosis

	All (n = 80)	DCM (n = 34)	HCM (n = 23)	LVNC (n = 14)	RCM (n = 7)	ARVC (n = 2)
Sex						
Male, n (%)	42 (52.5)	11 (32.3)	18 (78.3)	7 (50)	4 (57.1)	2 (100)
Female, n (%)	38 (47.5)	23 (67.7)	5 (21.7)	7 (50)	3 (42.9)	0 (0)
Age at enrollment, mean in years	8.3	7.5	9.9	8.0	6.9	n.a.
Age at diagnosis, mean in years (IQR)	4.8 (8.8)	3.2 (4.7)	7.2 (12.1)	3.9 (7.8)	4.4 (3.0)	n.a.
Family screening performed, n (%)	61 (76.3)	23 (67.7)	19 (82.6)	12 (85.7)	5 (71.4)	n.a.
Positive result of family screening, n (%)	27 (33.8)	8 (23.5)	12 (52.2)	5 (35.7)	1 (14.3)	n.a.
IVSd, mean in mm (SD)	9.1 (\pm 7.6)	5.5 (\pm 1.8)	16.7 (\pm 9.6)	5.5 (\pm 1.3)	5.0 (\pm 1.3)	n.a.
IVSd, mean z-score (SD)	3.6 (\pm 6.8)	0.4 (\pm 1.6)	10.6 (\pm 8.4)	0.4 (\pm 0.6)	0.2 (\pm 0.8)	n.a.
LVEDD (m-mode), mean in mm (SD)	39.4 (\pm 13.0)	46.2 (\pm 13.7)	33.8 (\pm 11.1)	38.6 (\pm 7.1)	26.0 (\pm 4.4)	n.a.
LVEDD, mean z-score (SD)	2.8 (\pm 5.1)	7.0 (\pm 4.2)	-1.4 (\pm 2.8)	2.5 (\pm 3.0)	-2.0 (\pm 0.7)	n.a.
LV-EF (auto 4CH monoplan), mean in % (SD)	46.5 (\pm 22.4)	29.7 (\pm 15.4)	66.8 (\pm 14.7)	44.0 (\pm 19.5)	62.1 (\pm 12.6)	n.a.
FS, mean in % (SD)	27.6 (\pm 16.8)	15.1 (\pm 6.7)	44.7 (\pm 16.5)	26.2 (\pm 11.5)	31.5 (\pm 11.6)	n.a.
AICD, n (%)	8 (10)	1 (2.9)	7 (30.4)	0 (0)	0 (0)	n.a.
VAD, n (%)	22 (27.5)	16 (47.1)	1 (4.4)	2 (14.3)	3 (42.9)	n.a.
ECMO, n (%)	6 (7.5)	5 (14.7)	0 (0)	0 (0)	1 (14.3)	n.a.
HTX at enrollment, n (%)	17 (21.3)	13 (38.2)	0 (0)	3 (21.4)	1 (14.3)	n.a.
HTX during study, n (%)	9 (11.3)	4 (11.8)	1 (4.4)	0 (0)	4 (57.1)	n.a.
Deceased during study, n (%)	5 (6.3)	2 (5.9)	1 (4.4)	0 (0)	2 (28.6)	n.a.

Abbreviations: AICD, automatic implantable cardioverter defibrillator; ARVC, arrhythmogenic right ventricular; DCM, dilated cardiomyopathy; ECMO, extracorporeal membrane oxygenation; FS, fractional shortening; HCM, hypertrophic cardiomyopathy; HTX, heart transplantation; IQR, interquartile range; IVSd, interventricular septum thickness at end-diastole; LV-EF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic diameter (z-score, normal reference range between -2 standard deviation [SD] and +2 SD); LVNC, left ventricular non-compaction; n.a., not applicable; RCM, restrictive cardiomyopathy; VAD, long-term ventricular assist device.

(6%). Within the whole cohort, one-third of patients (n = 26; 33%) already had a HTX at the time of enrollment or underwent HTX during the study period (Figure 1D).

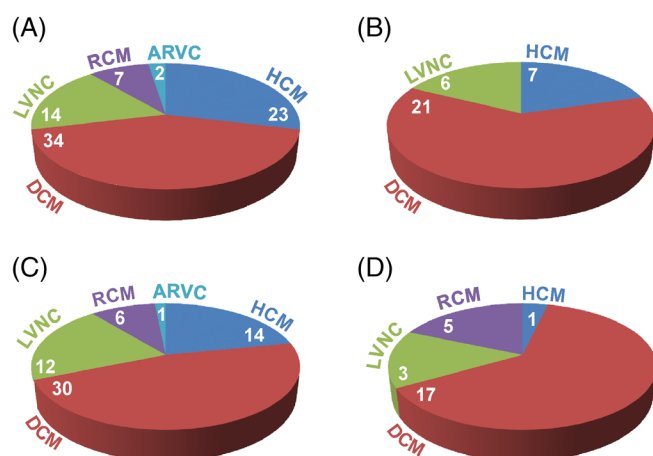


FIGURE 1 CMP phenotypes in the overall cohort of pediatric patients. A, Evaluation of the complete cohort of 80 pediatric patients with CMP according to clinical subtypes. B, CMP subtypes <1 year of age (age of diagnosis). C, CMP subtypes <10 years of age (age of diagnosis). D, CMP subtypes of patients that underwent HTX. CMP, cardiomyopathy; HTX, heart transplantation [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Spectrum of detected rare variants in pediatric primary CMP

To identify a potential genetic defect, we screened all index patients with a panel-based NGS approach detecting genetic variants in 89 CMP-associated genes. In 46 of these 89 genes, we identified at least one rare variant. In total, 126 detected variants were classified as pathogenic (n = 17, 14%), probably pathogenic (n = 15, 12%), or VUS (n = 94, 74%) (Table 2). Forty-two percentage (53/126) of all variants were novel and not annotated in disease reference databases; novel variants were mostly classified as VUS (42/53). One hundred three Missense variants were found in >80% of cases (103/126) and 37 of them were novel (37/103). In addition, 9 indel/frameshift, 3 stop gain, and 11 splice site rare variants were detected. From 75/126 rare variants, for which inheritance was tested in the parents, 7 (10%) arose de novo and 5/7 were classified as pathogenic and 2/7 were probably pathogenic. A detailed summary of all VOI is supplied in Table S4.

In 16 patients of our cohort, we could not detect any VOI (16/80). More than one VOI was detected in 36 out of 80 patients (1 VOI, n = 28; 2 VOI, n = 20; 3 VOI, n = 12; >3 VOI, n = 4). Each individual carried 1.58 VOI on average. Pathogenic variants were identified in 17 patients, probably pathogenic variants in 13 patients, and VUS in 55 patients. At least one pathogenic or probably

TABLE 2 Genetic variants of 80 index patients with pediatric cardiomyopathy

	Pathogenic ^a	Probably pathogenic ^a	Uncertain significance ^a	Total
Variants according to phenotype, n	17 (14%)	15 (12%)	94 (74%)	126 (100%)
DCM	4	4	44	52
HCM	11	2	26	39
LVNC	1	4	13	18
RCM	1	2	10	13
ARVC	0	3	1	4
Category of variants, n				
De novo	5	2	0	7
Not de novo (inherited)	4	9	55	68
Novel ^b	2	9	42	53
Not novel (known) ^c	15	6	52	73
Missense	11	9	83	103
Indel/frameshift	2	4	3	9
Stop gain	1	1	1	3
Splice site	3	1	7	11

Abbreviations: ARVC, arrhythmogenic right ventricular; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LVNC, left ventricular non-compaction; RCM, restrictive cardiomyopathy.

^aClassification according to Richards et al¹⁴ Genetics in Medicine 2015.

^bNovel indicates variants that have not been annotated in genetic disease reference databases ClinVar, ACGV, and HGMD.

^cNot novel indicates variants that are annotated in genetic disease reference databases ClinVar, ACGV, and HGMD.

pathogenic variant was identified in 30/80 (38%) index patients. Twenty-one patients (26%) with and 34 patients (43%) without a pathogenic/probably pathogenic variant had at least one additional VUS. None of the patients carried two different pathogenic variants. Two patients had a complex genotype with two probably pathogenic variants (Table S5).

3.3 | Rare variants indicate specific molecular functional groups and complex genotypes

VOI were most frequently detected in genes encoding for myosin heavy chain 7 (*MYH7*), cardiac myosin binding protein C (*MYBPC3*), *TNNI3*, desmoplakin (*DSP*), LIM domain binding 3 (*LDB3*), and myopalladin (*MYPN*) (Figure 2A). The VOI frequency per patient was highest for *MYH7* (16%) and *MYBPC3* (9%). For all CMP subgroups, except for RCM, *MYH7* yielded the highest number of patients with a VOI per CMP gene (Figure 2B-E). Of note, the specific VOI distribution per gene was distinct for each CMP subgroup.

Of the 80 patients, 39 had a VOI in a sarcomere gene (13 *MYH7*, 7 *MYBPC3*, 6 *TNNI3*, 4 troponin T2 cardiac type [*TNNT2*], 4 titin [*TTN*]), 16 in a desmosomal gene (6 *DSP*, 4 plakophilin 2 [*PKP2*], 3 desmocollin 2 [*DSC2*], 3 desmoglein 2 [*DSG2*]), 15 in a z-disc gene (5 *LDB3*, 5 *MYPN*, 4 nexilin [*NEXN*]), and 9 in a gene involved in transcription/splicing (4 PR domain-containing protein 16 [*PRDM16*]) (Figure S2A). The functional group "sarcomere" showed the highest frequency of genotype positive patients in all CMP subgroups (Figure S2B-E). Interestingly,

each CMP subgroup shows overrepresentation of distinct functional groups: protein quality control for DCM, dystrophin complex for HCM, mitochondria and nuclear envelope for LVNC, and transcription/splicing for RCM. Variants in desmosome genes are rarely detected in HCM patients. In children <1 year (n = 34), the relative VOI frequency for the functional groups sarcomere, desmosome, z-disc, and transcription/splicing was similar compared to the total cohort (n = 80) (Figure S2F). Note, functional groups comprising a large number of genes and/or exons are overrepresented in this illustration.

3.3.1 | TTN variants

VOI inducing truncations of the *TTN* protein (n = 4, 3% of all VOI) were rarely found and occurred in three RCM as well as in one DCM patient, two probably pathogenic, and two VUS (Table S5). In RCM patients, three *TTN* splice-site variants were identified in three individuals, respectively, two VUS and one probably pathogenic (Figure 2E).

3.3.2 | Homozygous/compound heterozygous VOI

The vast majority of detected VOI were heterozygous, nevertheless; we observed two homozygous (*NEXN*, *TNNI3*), and two compound heterozygous (*DSC2*, *MYBPC3*) VOI. In one case, we identified compound heterozygous mutation of *MYBPC3* p.S858R with complete deletion of the *MYBPC3* gene on the second allele.¹⁹

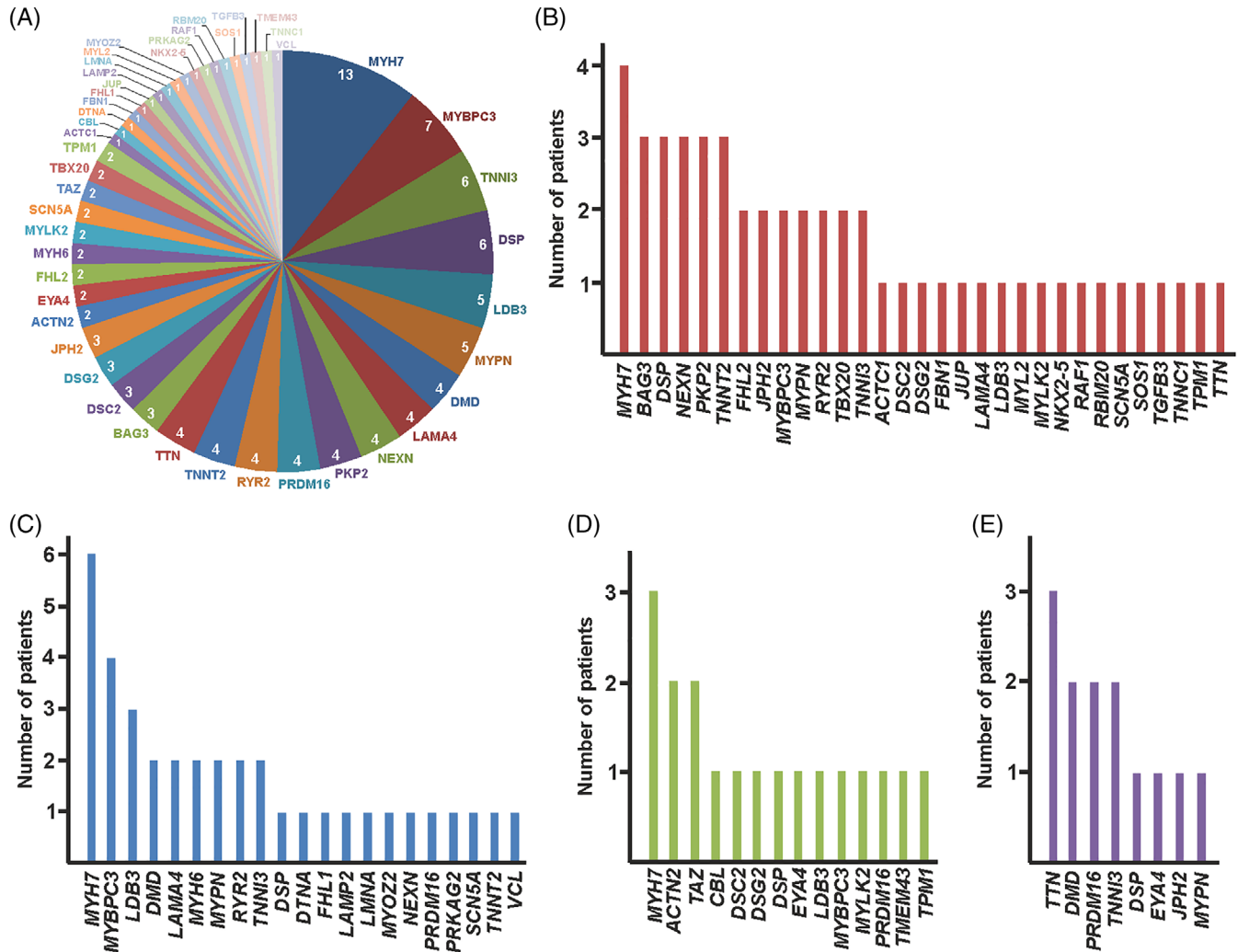


FIGURE 2 Distribution of variants in CMP genes. A, Number of VOI detected per gene, including pathogenic, probably pathogenic, and VUS. B, Number of DCM patients with VOI per gene. C, Number of HCM patients with VOI per gene. D, Number of LVNC patients with VOI per gene. E, Number of RCM patients with VOI per gene. Of note, *TTN* missense variants were not included. One patient may have several variants in one gene. VOI were considered for each patient. CMP, cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LVNC, left ventricular non-compaction; RCM, restrictive cardiomyopathy; VOI, variants of interest; VUS, variant of uncertain significance [Colour figure can be viewed at wileyonlinelibrary.com]

3.3.3 | Hemizygous VOIs

Four hemizygous dystrophin (*DMD*), 1 four and a half LIM domains 1 (*FHL1*), and two tafazzin (*TAZ*) variants (Table S5) were also observed.

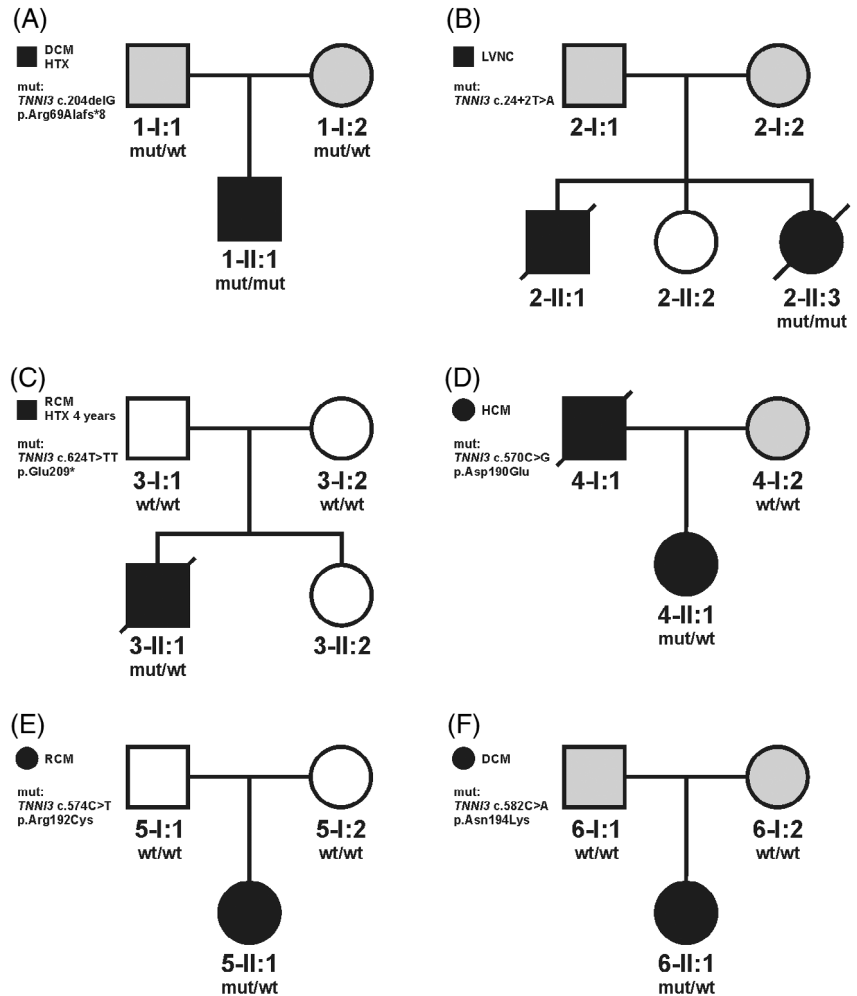
3.3.4 | VOI in HTX

Within this pediatric CMP cohort, one-third of patients ($n = 26$), mainly DCM and RCM, underwent HTX (Figure 1D). HTX patients most frequently carried variants in *DSP* (4 VUS), *DMD* (3 VUS), *PRDM16* (3 VUS), and *TTN* (2 probably pathogenic, 1 VUS). Of note, three *TTN* splice or truncating variants were found in RCM as well as in DCM patients with HTX (Table S4). Fourteen HTX CMP patients exhibited ≥ 2 VOI, six patients had one VOI, and no VOI was identified in six patients.

3.4 | Homozygous loss of *TNNI3* protein is compensated by upregulation of fetal *TNNI1*

Within the pediatric CMP cohort, VOI were most frequently detected in *MYH7*, *MYBPC3*, *TNNI3*, and *DSP* (Figure 2A). From this group of VOI, 18 pathogenic/probably pathogenic variants in sarcomere genes (*MYH7* $n = 7$, *MYBPC3* $n = 6$, *TNNI3* $n = 5$, *DSP* $n = 0$) were identified, including one homozygous (*TNNI3* c.24+2T>A) and one heterozygous *TNNI3* truncating variant (*TNNI3* c.624dupT, p. Glu209*). To further explore the impact of the five pathogenic/probably pathogenic detected *TNNI3* variants and another unrelated homozygous truncating *TNNI3* variant (c.240delG, p.Arg69Alafs*8), we assessed the associated families (family 1-6) for their cardiac phenotype and genotype (Figure 3A-F, Figure S3A-F and Table S6). Patients 1-II:1 and 2-II:3 (Figure S4) are affected by DCM and LVNC, respectively. Both patients were born to consanguineous

FIGURE 3 Families with CMP and mutation of *TNNI3*. A-F, Pedigrees of all families with rare genetic *TNNI3* variants. Open symbols depict unaffected individuals and filled symbols affected family members. The phenotype is indicated for each pedigree. The *TNNI3* genotype is presented for each pedigree, with the mutated allele marked as *mut* and the reference allele as *wt*. Individuals without genotype were not available for genetic testing. CMP, cardiomyopathy



parents and carry homozygous *TNNI3* variants that induce premature stop of protein translation. The splice site variant of 2-II:3 affects the donor splice site consensus sequence of intron_2-3 and results in the truncated *TNNI3* protein p.Ala8_Ala9insGluA-rgAlaAlaGly* (Figure S5A-C).

The *TNNI3* protein is composed of a cardiac specific region, the IT arm region, and the flexible C-terminal tail (Figure 4A).²⁰ All heterozygous *TNNI3* missense variants detected in this study cluster in exon 8 (Figure 4B). To further explore the molecular consequences of the truncating *TNNI3* variants (c.240delG, p.Arg69Alafs*8; c.24+2T>A; c.624dupT, p.Glu209*), we determined mRNA and protein levels in heart biopsies. The *TNNI3* protein is absent in patients 1-II:1 and 2-II:3 (Figure 4C,D). Of note, the protein level of *TNNI1*, the fetal *TNNI* variant, is markedly higher in patients 1-II:1 and 2-II:3. The *TNNI3* mRNA level is markedly reduced in patient 1-II:1, suggesting degradation by non-sense mediated decay, or not different from controls in patient 2-II:3 (Figure 4E). Levels of the sarcomeric proteins *TNNT2* and *MYBPC3* were not significantly affected. The *TNNI3* protein level in a heart biopsy of patient 3-II:1 (p.E209*) was normal (Figure S6A-C). In contrast, the *TNNI3* and *TNNI1* transcript levels were higher in heart tissue of patient 3-II:1.

4 | DISCUSSION

In this study, we report the genetic basis of a cohort of 80 pediatric patients with pediatric primary CMP referred to a tertiary center. VOI were most frequently detected in the *MYH7*, *MYBPC3*, *TNNI3*, and *DSP* genes. *TNNI3* is the third most important disease gene in this cohort and complete loss of myocardial *TNNI3* was associated with severe CMP and a compensatory increase in *TNNI1*.

Our study serves to assess the impact of genetic testing for counseling and management of pediatric primary CMP patients, and it shows wide genetic heterogeneity, depending on the stringency of phenotypes included in the cohort.² The strength of this pediatric study is that it included only patients with primary CMP.

4.1 | Spectrum of genetic variants in pediatric primary CMP

In the entire cohort, we mainly identified patients with DCM and HCM, which is consistent with the established incidence of pediatric CMP.⁵ Less frequently, we detected patients with LVNC and RCM.

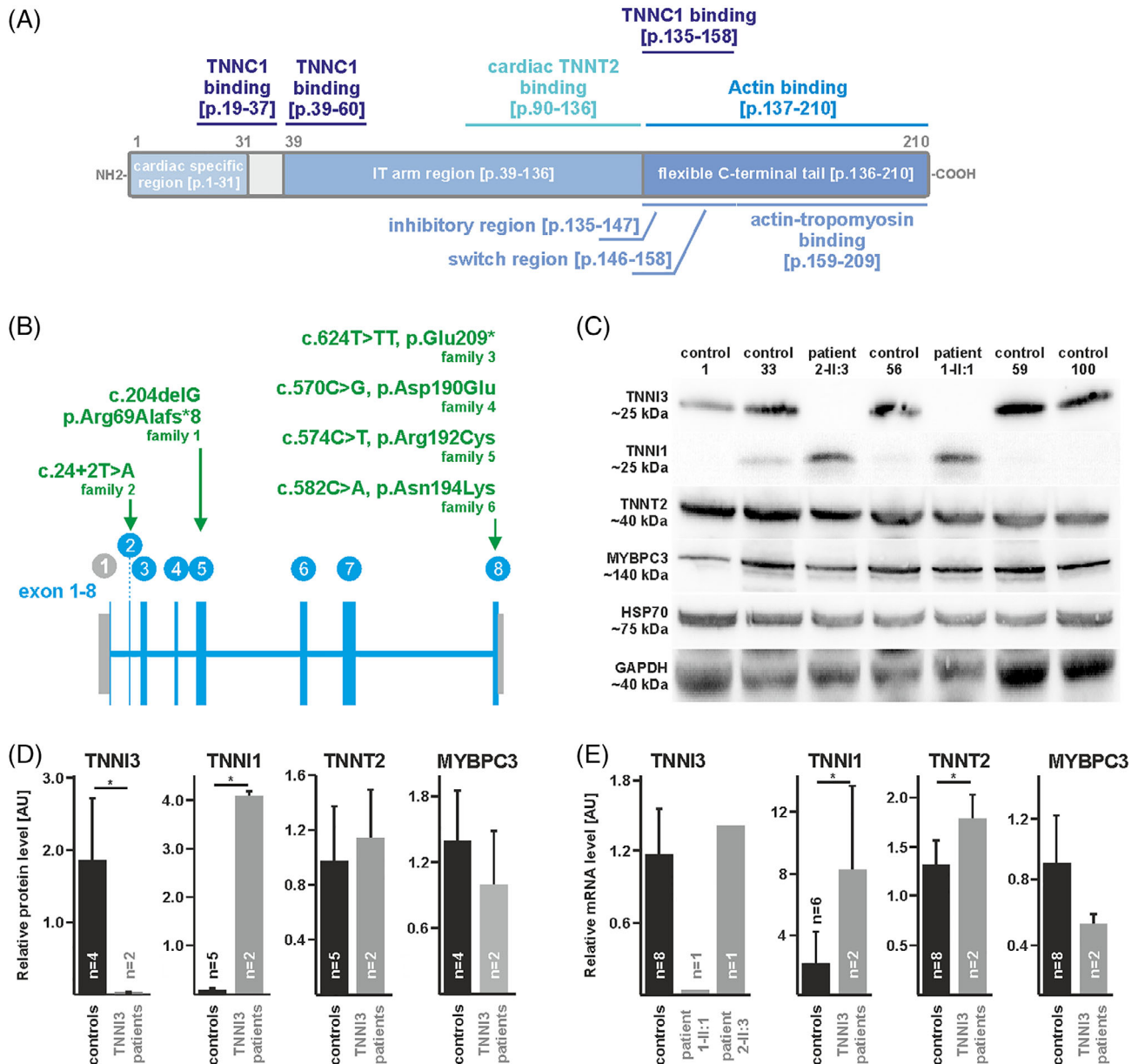


FIGURE 4 Localization of *TNNI3* variants and protein as well as mRNA expression. A, Structure of the TNNI3 protein with functional domains and interaction sites. B, Genomic structure of the *TNNI3* gene with localization of the detected genetic variants. C, Protein expression analysis of heart biopsies from two patients with homozygous *TNNI3* variants and controls. D, Quantification of protein levels occurred relative to HSP70 expression. E, Transcript levels of target genes were determined with quantitative polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase. Statistical analysis was performed with Student's *t* test, **P* < .05 [Colour figure can be viewed at wileyonlinelibrary.com]

Over all CMP subtypes, genetic variants were most frequently detected in sarcomeric genes (*MYH7*, *MYBPC3*, *TNNI3*) followed by desmosomal (*DSP*) and z-disc genes (*LDB3*, *MYPN*).

4.1.1 | Hypertrophic cardiomyopathy

Shared genetic causes for childhood-onset and adult CMP were first shown for HCM; in the study by Morita et al, mutations occurred predominantly (in >75% of the children) in *MYH7* and *MYBPC3*.²¹

4.1.2 | Dilated cardiomyopathy

The spectrum of contributing genes differs among the age groups, most prominently, when comparing the infant group with the pediatric and adult groups.²²

4.1.3 | Restrictive cardiomyopathy

A substantial proportion of pediatric RCM is caused by mutation of sarcomere genes.²³ In adults, genetic analysis of RCM is limited to individual consanguineous families and case reports.²⁴

4.1.4 | Left ventricular non-compaction

Targeted NGS approaches revealed a wide spectrum of genetic variations and a high incidence of pathogenic variants in pediatric LVNC patients, mostly sarcomere gene variants.^{25,26}

In adult cohorts, sarcomeric genes are the major disease genes for HCM (>70%),²⁷ DCM (14%, 35%, or 37%),^{22,28,29} and LVNC (26% or 29%).^{30,31} In all CMP subgroups, patients carried most frequently VOI in sarcomere genes suggesting them as major contributor in pediatric CMP as previously shown in meta-analyses for adult-onset CMP.^{32,33} A detailed genotype-phenotype correlation was not intended in this study of index-patients due to the composition and small size of the subgroups per CMP. The cohort is phenotypically heterogeneous which means that the five different CMPs under investigation have different clinical characteristics and severity cannot be accounted for by age of onset.

4.1.5 | Specific disease genes

MYH7 variants were most frequently associated with HCM, DCM, and LVNC in our cohort. Onset of disease in 12 individuals with *MYH7* variants ranges from infancy to adolescence. In the literature, 6/9 variants previously reported were found in adults as well as in children (Table S7). This suggests that *MYH7* variants found in children are not specific to individuals of a certain age group. Other, yet unknown genetic or exogenous factors contribute to the age of onset of disease. Seven *MYH7* missense variants were classified as pathogenic or probably pathogenic. All of these seven missense variants localize within the HCM cluster region *MYH7*_167-931 supporting their pathogenic character.¹⁵ Moreover, the high detection rate of already known missense variants (only three novel *MYH7* variants out of 12) solicits reliable *MYH7* disease variant identification as well as classification.^{14,15} Altogether, pathogenic assessment of *MYH7* variants is highly specific due to the availability of comprehensive genetic and functional data.

In this study, we excluded *TTN* missense variants, due to their currently uncertain interpretation. Nevertheless, we identified four heterozygous *TTN* variants inducing premature stop of translation (truncating variants) or potentially affecting splicing. One DCM and two RCM patients carrying truncation *TTN* variants underwent HTX. This suggests a link of *TTN* truncation with pediatric RCM, potentially in combination with a more complex genetic alteration, and a critical importance of both *TTN* alleles for postnatal development.²⁴ In line with our observations, other studies showed that in children with DCM, the frequency of truncating *TTN* variants is much lower compared to adults.^{22,34}

4.1.6 | Segregation analysis

In 77% of families, we tested the variant inheritance and clinical status in first degree members. This approach allowed us to identify seven de novo variants (10%). This is a lower ratio of de novo variants than previously reported.² Vasilescu et al reported 46% de novo variants in

a cohort of infant childhood-onset CMP (median age of diagnosis of 0.33 years) and 33% of individuals in this cohort presented with a systemic disorder. The interpretation of de novo variants has recently been challenged. Numerous de novo variants associated with cardiac disease were observed as standing variation in ExAC, thus these variants are less probably monogenic causes or major risk contributors for cardiac disease.³⁵

4.2 | Defective troponin I switch due to homozygous *TNNI3* mutation

This study identified two patients with either DCM (1-II:1, p.Arg69Alafs*) or LVNC (2-II:3, c.24+2T>A) carrying homozygous *TNNI3* loss-of-function variants. It is known that heterozygous *TNNI3* variants cause DCM, HCM, and RCM; however, homozygous mutation of *TNNI3* is a rare event,¹⁸ and to our knowledge so far, three homozygous *TNNI3* cases have been described: the missense variant p.Ala2Val (DCM), the synonymous variant c.G150A (p.Lys50Lys) causing aberrant splicing of *TNNI3* mRNA (DCM), and a genomic deletion comprising the *TNNI3* exon 8 as well as the entire *TNNT2* gene (DCM).³⁶⁻³⁸ Here, we showed absence of the *TNNI3* protein in heart tissue of patients with homozygous *TNNI3* mutation. Moreover, we suggest impaired *TNNI* isoform switching in the postnatal heart indicated by increased *TNNI1* (fetal isoform) protein and mRNA levels in both patients. The switch from fetal *TNNI1* to adult *TNNI3* monitors cardiomyocyte maturation in mammalian heart tissue and in human-induced pluripotent stem cell-derived cardiomyocytes.^{39,40} More importantly, *TNNI* switching critically determines Ca^{2+} sensitivity, resistance to hypoxia/acidosis, and responsiveness to adrenergic stimulation of the contractile system.⁴¹ Thus, defective *TNNI1* to *TNNI3* switching upon CMP associated *TNNI3* mutation may prevent adequate adaptation to postnatal heart physiology.

4.3 | Pediatric CMP: Implications for genetic testing?

It is a matter of ongoing debate whether whole genome sequencing (WGS) or whole exome sequencing (WES) in CMP would be necessary to provide more insight into the genetic disease mechanisms.⁴² The incremental yield of clinically actionable variants by WGS is limited by a paucity of genetic and functional evidence.⁴³ In a study by Herkert et al, combining copy number variant analysis with stepwise trio-based WES yielded a diagnosis in more than 50% of pediatric DCM patients.⁴⁴ They identified patients with familial and non-familial DCM or a mixed cardiac phenotype with age at onset of CMP <18 years. These included patients with extracardiac features or possible myocarditis. Other recent studies lead to the identification of pathogenic variants in 26% to 39% of pediatric patients.^{1,2} Our study suggests genetic testing for sarcomere gene variants in severely affected children as early as possible to support clinical decision making. Altogether, these studies support the use of genetic testing in clinical practice by NGS, to improve risk stratification for clinically affected patients in selected CMP, such as pediatric CMP.⁴⁵

In summary, with an NGS panel-based approach, we identified the genetic cause in 38% of pediatric patients with primary CMP. Most frequently, we detected pathogenic or probably pathogenic missense variants in sarcomere genes such as *MYH7*, *MYBPC3*, and *TNNI3*. Homozygous deactivation of *TNNI3* leads to severe CMP in pediatric patients and compensatory expression of *TNNI1*. We propose that this study advances the general genetic understanding of pediatric primary CMP and highlights certain genetic defects with severe clinical courses.

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


CONFLICT OF INTEREST

None of the authors declare any conflict of interest related to this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the ClinVar database at <https://www.ncbi.nlm.nih.gov/clinvar/>.¹⁷

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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