Human Genomics

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Whole-exome sequencing to identify causative variants in juvenile sudden cardiac death

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Abstract

Background Juvenile sudden cardiac death (SCD) remains unexplained in approximately 40% of cases, leading to a significant emotional burden for the victims' families and society. Comprehensive investigations are essential to uncover its elusive causes and enable cascade family screening. This study aimed to enhance the identification of likely causative variants in juvenile SCD cases (age ≤ 50 years), particularly when autopsy findings are inconclusive.

Results Autopsy revealed diagnostic structural abnormalities in 46%, non-diagnostic findings in 23%, and structurally normal hearts in 31% of cases. Whole-exome sequencing (WES), refined through a customized virtual gene panel was used to identify variants. These variants were then evaluated using a multidisciplinary approach and a structured variant prioritization scheme. Our extended approach identified likely causative variants in 69% of cases, outperforming the diagnostic yields of both the cardio panel and standard susceptibility gene analysis (50% and 16%, respectively). The extended cardio panel achieved an 80% diagnostic yield in cases with structurally normal hearts, demonstrating its efficacy in challenging scenarios. Notably, half of the positive cases harboured a single variant, while the remainder had two or more variants.

Conclusion This study highlights the efficacy of a multidisciplinary approach employing WES and a tailored virtual gene panel to elucidate the aetiology of juvenile SCD. The findings support the expansion of genetic testing using tailored gene panels and prioritization schemes as part of routine autopsy evaluations to improve the identification of causative variants and potentially facilitate early diagnosis in first-degree relatives.

Keywords Sudden cardiac death, Genetic, Molecular autopsy, NGS, Cardiomyopathy, Arrhythmia

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Background

Sudden cardiac death (SCD) represents a dramatic event and a significant public health concern accounting for 15–20% of all deaths in the general population, with an estimated incidence of 1.3–2.8 per 100,000 person-years in individuals under the age of 50 [1, 2]. By definition, sudden natural death is presumed to be of cardiac cause when it occurs within 1 h from the onset of symptoms in witnessed cases, and within 24 h from the last time the subject was seen alive when it is unwitnessed [1, 2].

The aetiology of SCD is still largely unknown and varies with age. In individuals below the age of 50, SCD often stems from inherited disorders that induce structural and/or functional abnormalities triggering fatal arrhythmias [3, 4]. Such disorders encompass cardiomyopathies (CMP), heart structural abnormalities like hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and arrhythmogenic cardiomyopathy (AC), and primary electrical disorders including Brugada syndrome (BrS), long QT syndrome (LQTS), short QT syndrome (SQTS), and catecholaminergic polymorphic ventricular tachyarrhythmias (CPVT) [4–6]. These conditions involve various genetic and clinical characteristics. HCM is characterized by unexplained left ventricular (LV) hypertrophy, myocyte disarray, and fibrosis. Hypertrophic cardiomyopathy (HCM) is typically associated with mutations in sarcomere and sarcomere-associated genes (e.g., Myosin Binding Protein C3 - MYBPC3 and Myosin Heavy Chain 7 - MYH7). Notably, non-sarcomere HCM and phenocopies are also reported, as well as digenic and polygenic risk factors [4, 5]. DCM features LV enlargement and fibrotic substitution, leading to systolic dysfunction and increased arrhythmic risk; common associated genes coding for cytoskeletal proteins among which Lamin A/C (LMNA) and Desmin (DES) are associated with a particularly arrhythmogenic phenotype [4, 5]. ACM is characterized by fibro-fatty replacement of the myocardium, usually due to genetic defects affecting cardiac desmosomes (e.g., Plakophilin-2 - PKP2 and Desmoplakin - DSP). Primary electrical diseases increase the risk of ventricular arrhythmias and SCD without apparent structural abnormalities. These include LQTS (often due to mutations in Potassium Voltage-Gated Channel Subfamily Q Member 1 - KCNQ1, Potassium Voltage-Gated Channel Subfamily H Member 2 - KCNH2, or Sodium Voltage-Gated Channel Alpha Subunit 5 - SCN5A), SQTS (associated with potassium channel gene mutations), BrS (often linked to SCN5A mutations), and CPVT (commonly caused by Ryanodine Receptor 2 - RYR2 and Calsequestrin 2 - CASQ2 mutations) [1, 3, 4]. Notably, all these cardiac disorders exhibit autosomal dominant pattern of inheritance with incomplete penetrance and variable expressivity making genetic assessment even more complex [1, 3–5, 7]. Other involved disorders are represented by myocarditis, congenital heart defects, including coronary artery anomalies and valve diseases, and storage cardiomyopathies. In the older individuals, coronary artery diseases and valve diseases represents the main cause of SCD [7, 8].

Autopsy plays a crucial role in identifying the cause of death, which is particularly relevant for risk prediction in family members. However, establishing a post mortem diagnosis remains challenging despite standardized autopsy guidelines [9, 10]. In particular, differentiating non-diagnostic findings from pathological abnormalities can be difficult, with up to 40% of SCD victims below the age of 50 remaining undiagnosed after a comprehensive autopsy [7, 8, 11]. Cases are classified as unexplained when autopsy reveals either non-diagnostic structural findings or no cardiac abnormalities, a scenario named sudden arrhythmic death (SAD) [1, 11, 12]. Notably, in 88% of autopsied SCD cases, the fatal event represents the first manifestation of an underlying, often asymptomatic and undiagnosed, life-threatening cardiac condition [3, 4]. Moreover, the concept of "concealed cardiomyopathy" has recently emerged, describing potentially fatal arrhythmias in inherited heart disorders that occur before visible structural changes. This highlights the complex interplay between genetic predisposition and phenotypic expression in SCD cases [11, 13, 14].

Consequently, a thorough investigation of young SCD victims is essential and post-mortem genetic testing may prove beneficial [12, 13, 15]. However, genetic testing has been limited for decades to four major susceptibility genes (*KCNQ1, KCNH2, SCN5A*, and *RYR2*), typically sequenced with the Sanger method [15, 16]. The advent of Next Generation Sequencing (NGS) has slightly enhanced diagnostic accuracy. The diagnostic yield increases by 25–40% with NGS, when a wider spectrum of genes linked to cardiomyopathies or channelopathies are included, analysing 40–200 genes depending on the method and panel used for sequencing. For instance, the TruSight Cardio Panel (Illumina, San Diego, California, U.S.) include 174 genes currently analysed for inherited heart disease (https://emea.illumina.com/products/by-type/clinical-research-products/trusight-cardio.html) [17, 18]. Current guidelines advocate for genetic testing in SCD cases with a probable genetic origin [1] but caution is advised against examining genes without a definitive link to the clinical phenotype [1]. This broader approach often results in the more frequent identification of variants of unknown significance (VUS), whit subsequent problems in interpreting the results [19–21].

American College of Medical Genetics (ACMG) guidelines for variant interpretation state that VUS are not clinically actionable but stress the importance of make an effort to reclassify VUS as either "pathogenic" or "benign [22]. In addition to globally standardized guidelines, diagnostic genetics laboratories often adopt supplementary practical guidelines, especially for managing VUS (https://www.acgs.uk.com/; https://sigu.net/). These laboratory-specific protocols guide practice and provide clarifications based on user experiences.

In light of these challenges and evolving practices, our study hypothesizes that employing whole exome sequencing (WES), refined through a bespoke virtual gene panel and a structured scheme for prioritizing variants (particularly VUS), coupled with a meticulous, case-specific variant evaluation using a multidisciplinary approach, could substantially increase the diagnostic performance of post-mortem genetic testing in SCD cases.

Methods

Study cohort

Our study is part of the "JUvenile Sudden cardiac deaTh: JUST know and treat" (JUST) project, which started in 2016 and involved both retrospective evaluation of old cases and prospective evaluations of new cases of juvenile SCD (age ≤ 50 years). In the former, young individuals were scrutinized from the Forensic Medicine Department of University Hospital of Pisa (1995–2016), while in the latter also cases from the Forensic Medicine Department of Lucca were included (2017–2023). In both cases, exclusion criteria were: non-cardiac death causes; ischemic heart and/or coronary disease; positive toxicology tests. According to forensic reports, all individuals were either completely asymptomatic or exhibited only nonspecific symptoms. Report of previously cardiological investigations with inconclusive results was reported for two subjects. None of the individuals had been diagnosed with a specific cardiac disorder prior to death (Supplemental Table 1). We secured consent for genetic testing and research use of data from the relatives of the deceased. This study was conducted in agreement with the Helsinki Declaration and received approval from the Ethic Committee of the Tuscany Region, Area Vasta Nord-Ovest (no. 14870).

Autopsy examinations

Autopsy examinations were conducted according to the latest guidelines [10]. The heart and lungs were removed "en bloc" and the pulmonary vessels were explored. Subsequently, an analysis of the right and left sections of the heart (atrial and ventricular) and the valve planes was carried out. Multiple transverse cuts at 3-mm intervals along the course of the main epicardial arteries, including branches, such as the diagonal and obtuse marginal, were performed to check patency. Finally, cuts were made along the short axis of the heart to obtain slices about 1 cm thick. Wall thickness was then verified: the endocardium was carefully inspected, and the thickness of the mid-cavity free wall of the left ventricle (LV), right ventricle (RV), and interventricular septum (IVS) (excluding trabeculae) was measured. At the end, we compared the measurements against tables of normal thickness by age, gender, and body weight [23]. The forensic experts (M.D.P., D.B.) retrospectively reviewed all the reports of macroscopic and histopathology examinations, and classified the cases into three categories: "diagnostic structural abnormalities", if the macroscopic and/or histopathological alterations fell within the diagnostic criteria for a specific cardiomyopathy; "non-diagnostic autopsy findings", if the macroscopic and/or histopathological alterations were subtler, such as non-diagnostic small areas of fibrosis, inflammation or fatty replacement; "structurally normal heart", if no relevant cardiac alterations were found.

DNA extraction and whole exome sequencing

For retrospective cases, formalin-fixed, paraffin-embedded (FFPE) samples of the heart, kidney, or spleen were used. Conversely, for prospective cases, 5 to 10 mL of whole blood using a hypodermic syringe from either the femoral or inferior vena cava were collected, subsequently storing it in ethylenediaminetetraacetic acid (EDTA). The blood was refrigerated at 2 to 8 °C for analyses within 4 weeks, or frozen at − 20 °C or − 80 °C for later examinations.

DNA extraction from FFPE samples was performed using Promega's Maxwell 16 LEV DNA FFPE Purification Kit (Promega, Madison, Wisconsin U.S.). In prospective cases, DNA extraction from whole blood was done using the Maxwell® 16 LEV Blood DNA Kit (Promega, Madison, Wisconsin U.S.). Whole exome library preparation followed the manufacturer's guidelines for the Illumina DNA Prep with Enrichment Kit (Illumina, San Diego, California, U.S.). Sequencing was conducted using Illumina's NextSeq™ 500 instrument (Illumina, San Diego, California, U.S.).

Bioinformatic analysis and extended cardio panel

Primary exome data analysis was executed using the SeqMule pipeline [24]. FastQC and FastqScreen Quality Control (QC) systems identified and rectified errors, trimmed low-quality reads, and removed adaptors [25]. The reads were aligned to the reference human genome (GRCh37/hg19) using BWA-MEM software (https://github.com/MGPC-Nantes/MEM). Variant calling was conducted using the Genome Analysis Toolkit (GATK)

[26], annotating only variants in genetic regions with a quality score ≥ 30 and a read depth ≥ 20 at the altered position [27]. VarAFT [28] and BaseSpace Variant Interpreter software (Illumina Inc., San Diego, CA, USA) were used to filter annotated variants, excluding those with a minor allele frequency (MAF) > 0.01 (1%) in GnomAD (http://gnomad.broad institute.org) and including only missense, nonsense, frameshift, and splice site variants. WES data were filtered to create a virtual panel specifically constructed from selected genes associated with cardiac diseases to expand the possible associations with SCD. Genes were first selected exploiting two databases:

1. Human Phenotype Ontology (HPO): this provides a standardized vocabulary of phenotypic abnormalities encountered in human disease. We searched for "Abnormal Myocardium Morphology" (HP:0001637), identifying 689 diseases and 565 associated genes, and "Abnormality of Cardiovascular System Electrophysiology" (HP:0030956), identifying 750 diseases and 524 associated genes.
2. Human Protein Atlas: this integrates various omics technologies to map all the human proteins in cells, tissues and organs. We searched for "heart-specific proteome" and selected the 419 genes with elevated expression in the heart compared to other tissue types. We have also checked genes used in previous NGS studies (13–36), such as those included in TruSight Cardio Panel (Illumina) (Supplemental Table 1). The final virtual panel used to filter the annotated variants thus included 1304 genes (Supplemental Table 2).

Variant interpretation

We prioritized filtered variants based on pathogenicity through VarSome (https://varsome.com) and Franklin by Genoox (https://franklin.genoox.com), which automatically classify variants according to the ACMG guidelines [22]. We also reconsidered the PM2 criterion, fulfilled for ACMG if the MAF was ≤ 0.01% (ultra-rare variants): after looking for a possible pathogenic variant among the rare variants (MAF < 1% during filtration), we evaluated the variants considering, together with the other ACMG criteria, the frequency of the different pathologies underlying SCD (e.g., HCM 1:625–1:344, MAF ~0.2%; DCM 1:250–400, MAF ~0.2 up to 1:2000, MAF ~0.005%; AC and channelopathies ~1/2000, MAF 0.005%). Splicing and frameshift variants' functional impacts were further examined using Human Splicing Finder (https://hsf.genomnis.com) and Regulation Spotter (https://www.regulationspotter.org/) [29, 30]. We have sub-classified the VUS identified from VCF after excluding pathogenic (P) or likely pathogenic (LP). VUS that met specific criteria were classified as highly suspicious of pathogenicity and defined as VUS/LP:

1. Each ACMG criterion describing the variant falls within the pathogenic criteria set. This ensures that we do not include variants classified as VUS by ACMG solely due to conflicting evidence, even if some criteria suggest them as benign.
2. Franklin's aggregated prediction, which combines results from various prediction tools, based on latest recommendations for PP3/BP4 rules [25], indicates a high likelihood of pathogenicity. If this criterion was not fully met, we considered whether the variants were in a recognised hot spot and/or in a critical functional domain (PM1).
3. The variant resides in a gene whose functional pathway aligns with the structural and/or functional cardiac alterations probably responsible for the SCD in the cases examined.

Using these prioritization schemes, variant reported as VUS based only on the PM2 criterion, when all other evidence suggests that it is benign, were considered VUS/likely to be benign and were not reported, in line with recent practical guidelines (https://www.acgs.uk.com/; https://sigu.net/). Variants reported underwent further scrutiny for clinically relevant information via ClinVar (https://www.ncbi.nlm.nih.gov/clinv), OMIM (https://www.omim.org/), ClinGen (https://clinicalgenome.org/), and PubMed (https://pubmed.ncbi.nlm.nih.gov), along with an evaluation of the variants' presence in pertinent regions (e.g., protein functional domains, binding sites) via UniProt (https://www.uniprot.org/). Genotype associations were determined based on OMIM and ClinGen for genes already recognized in association with cardiomyopathy/channelopathies. For genes not previously linked, we considered whether the gene was reported in ClinGen as "challenged" or "limited" in association with the cardiac phenotype, previously published studies on cardiac involvement, the reported phenotype, and/or the pathway involved in cardiac function found on Human Phenotype Ontology and GeneCard (https://genecards.org/). The phenotype and/or pathway were retrieved from the Human Phenotype Ontology (HPO) and GeneCard (https://genecards.org/) databases. Globally, all information on identified variants and genes were interpreted by a multidisciplinary team, including geneticists, bioinformaticians, cardiologists and forensic medicine specialists.

We refer to the 4 main susceptibility genes as "Core genes", the 174 genes from the TruSight Cardio Panel (Illumina, San Diego, California, USA) as "Cardio panel", and the 1304 genes examined in this study as "Extended panel".

Table 1 Characteristics of sudden cardiac death cases

Sample ID	Gender	Age	Event at death	Symptoms at death	Weight (kg) × height (cm)	Heart weight (g)	Autoptic diagnosis	Sample type
SCD01	M	26	Sport	Syncope	85 × 175	700	HCM	FFPET
SCD02	M	29	Sleep	Dyspnoea	71 × 174	n/a	SAD	n/a
SCD03	M	22	Sport	Syncope	87 × 171	365	HCM	FFPET
SCD04	M	36	Light activity	n/a	n/a	n/a	SAD	n/a
SCD05	M	29	Sleep	None	70 × 177	340	SAD	FFPET
SCD06	M	37	Rest	None	63 × 172	350	SAD	FFPET
SCD07	M	20	Sport	Syncope	n/a × 175	500	AC (RV, LV)	FFPET
SCD08	M	25	Sleep	None	77 × n/a	370	SAD	FFPET
SCD09	F	25	n/a	n/a	n/a	n/a	Non-diagnostic autopsy findings (MVP)	n/a
SCD10	M	21	Sport	Syncope	90 × 187	520	DCM	FFPET
SCD11	M	37	Sleep	None	n/a × 168	360	AC (RV)	FFPET
SCD12	M	25	Rest	Dyspnoea	n/a	360	AC (RV)	FFPET
SCD13	F	14	Sleep	None	n/a	260	AC (RV, LV)	n/a
SCD14	F	21	Light activity	none	n/a × 160	310	HCM	FFPET
SCD15	F	22	Sleep	n/a	n/a	220	HCM	n/a
SCD16	F	35	Rest	n/a	87 × 171	365	HCM	n/a
SCD17	F	45	Rest	Dyspnoea	n/a	290	AC (RV)	FFPET
SCD18	M	20	Rest	None	n/a × 178	360	HCM	FFPET
SCD19	M	40	n/a	n/a	n/a × 180	530	Non-diagnostic autopsy findings (foci of fatty replacement of the myocardium, RV)	Autoptic blood
SCD20	F	50	Rest	n/a	n/a × 164	300	AC (RV)	Autoptic blood
SCD21	F	29	Light activity	Syncope	n/a	n/a	MVP (Myxomatous degeneration of the mitral valve leaflets)	Autoptic blood
SCD22	M	42	Sleep	None	n/a	n/a	SAD	Autoptic blood
SCD23	M	34	Light activity	Syncope	n/a	n/a	Non-diagnostic autopsy findings (small foci of fibrosis)	Autoptic blood
SCD24	M	31	Rest	Fever	n/a × 188	415	Non-diagnostic autopsy findings (foci of fatty replacement of the myocardium, LV)	Autoptic blood
SCD25	F	37	Light activity	n/a	n/a	530	SAD	Autoptic blood
SCD26	M	23	Rest	n/a	n/a × 190	565	Non-diagnostic autopsy findings (mild fatty replacement, RV, LV)	Autoptic blood
SCD27	M	50	Light activity	None	n/a	630	HCM	Autoptic blood
SCD28	M	29	Sport	Syncope	n/a	380	SAD	Autoptic blood
SCD29	M	42	Light activity	None	n/a	540	Non-diagnostic autopsy findings (slightly dilated chambers and mild fatty replacement of the myocardium)	Autoptic blood
SCD30	M	40	Rest	Chest pain	n/a	450	Non-diagnostic autopsy findings (small foci of fibrosis)	Autoptic blood
SCD31	F	45	Rest	Chest pain	n/a	286	SAD	Autoptic blood
SCD32	M	29	Sport	n/a	n/a × 184	315	AC (RV, LV)	Autoptic blood
SCD33	M	50	Sport	n/a	n/a	510	Non-diagnostic autopsy findings (mild fatty replacement, RV, LV)	Autoptic blood
SCD34	M	45	n/a	n/a	n/a	n/a	SAD	Autoptic blood
SCD35	M	39	Light activity	Palpitations	175 × n/a	575	HCM	Autoptic blood
SCD36	F	50	Sport	n/a	173 × 70	300	HCM	Autoptic blood
SCD37	M	42	Sleep	None	185 × 90	610	HCM	Autoptic blood
SCD38	F	50	Rest	None	160 × 80	400	SAD	Autoptic blood
SCD39	F	45	Rest	None	160 × 60	200	SAD	Autoptic blood

AC arrhythmogenic cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *SAD* sudden arrhythmic death, *MVP* mitral valve prolapse, *LV* left ventricle, *RV* right ventricle, *FFPET* formalin-fixed and paraffin-embedded tissue, *n/a* not available

Results

The final study cohort included 39 subjects (18 from the retrospective cohort and 21 from the prospective cohort; Table 1). Subjects were mainly male (n = 26, 67%), with an age of 33 ± 10 years. Autopsy results revealed a diagnostic structural abnormality in 18 cases (46%), which could be classified as HCM in 10 (56%), AC in 7 (39%), and DCM in 1 (6%). Additionally, 9 cases (23%) displayed non-diagnostic autopsy findings including fatty replacement (n = 4), mitral valve prolapse (n = 2), myocardial fibrosis (n = 2), and mild left ventricular dilation (n = 1). The remaining 12 cases (31%) displayed structurally normal hearts. Overall, 21 cases (54%) displayed non-diagnostic autopsy findings or structurally normal hearts.

WES was performed in 32 cases (7 HCM, 6 AC, 1 DCM, 10 SAD, 8 with non-diagnostic autopsy findings), due to FFPE unavailability in 6 samples and bacterial DNA contamination in 1 FFPE sample, precluding the completion of the remaining retrospective cases. Likely causative variants were found in 22 cases, translating to a detection rate of 69%, outperforming both the Cardio panel and standard susceptibility gene analysis (50% and 16%, respectively, as shown in Fig. 1).

Detailed variant descriptions are provided in Tables 2, 3 and 4. All identified variants were heterozygous. Half of positive cases (n = 11) harboured a single variant, while the remainder had ≥ 2 variants. P/LP and VUS/LP variants were distributed in the different autopsy groups as follows: SCD with diagnostic structural abnormalities, 8 out of 14 (57%; 6/7 in HCM, 2/6 in AC, 0/1 DCM); non-diagnostic autopsy findings, 6 out of 8 (75%); structurally normal heart, 8 out of 10 (80%; Fig. 2). Overall, 78% of cases with non-diagnostic autopsy findings or structurally normal heart had a positive genetic test with the extended cardio panel.

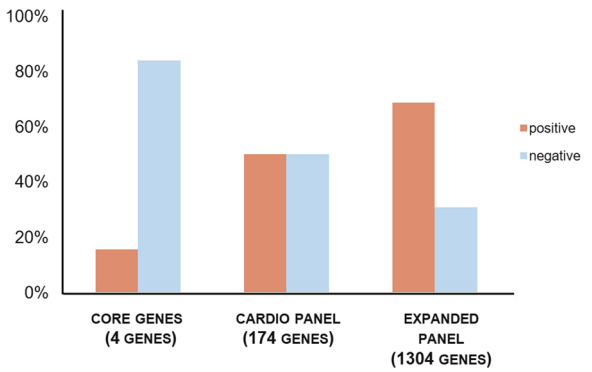

Fig. 1 Added diagnostic value of the extended cardio panel. The variant detection rate (%) found using different virtual panels in all autopsy cases: core genes 16%, cardio panel 50%, extended cardio panel 69%. "Core genes" represent the 4 main susceptibility genes, "Cardio panel" includes the 174 genes from the TruSight Cardio Panel (Illumina, San Diego, California, USA) and the "extended panel" includes the 1304 genes selected in this study

P/LP variants were found in 12 cases (38%): 7 in genes affecting myocardial structure/morphology and 4 in genes related to cardiac electrical function. VUS/LP variants were identified in 10 cases (31%): 6 in structural protein genes and 10 in ion channel-related genes. Fifty percent of sudden cardiac death (SCD) cases, whether they exhibit diagnostic structural abnormalities or have non-diagnostic autopsy findings, showed variants in cardiomyopathy genes. In contrast, 80% of cases with structurally normal hearts (SAD) presented variants in channelopathy genes (Fig. 3). Fifteen genes recognized for their association with cardiac disease were identified: 3 (*RYR2*, *SCN5A*, *KCNH2*) from the primary susceptibility panel and 12 (*TTN, MYH7*, *MYBPC3, TGFB2*, *CACNA2D1*, *CALM1*, *LAMA4*, *DSP, CACNA1C, FBN1, SNTA1 BAG3*) from the Cardio panel. Three cases with variants in Cardio panel genes had causative variants exclusively identified in the extended panel. Nine genes uniquely included in our extensive panel (*HCN1, KCNJ14, SCN9A, SLC4A3, PSEN2, SCN10A, KCNMA1, ATP1A2, CDH2*) were found to have a disease association labeled as "disputed" or "limited" (ClinGen) and/or cardiac-related pathways (GeneCards). No significant differences were found in the diagnostic yield across the different age groups (p = 1 for each age class; Fig. 4).

Discussion

Juvenile SCD remains unexplained in approximately 40% of cases, despite forensic and molecular autopsy. This highlights the need for a more in-depth search for gene variants. Our study employed a multidisciplinary approach involving geneticists, cardiologists, bioinformaticians, and forensic medicine specialists. The expertise of this team was exploited in a stepwise manner: (1) review of macroscopic and microscopic findings from SCD cases, (2) comprehensive assessment of available guidelines and relevant literature to develop a robust variant prioritization scheme, (3) analysis of variants identified through WES. A multidisciplinary discussion was crucial to sub-classify VUS and evaluate prioritized variants in the context of individual case characteristics. This integrated approach enabled a thorough and nuanced interpretation of genetic findings in relation to the clinical and pathological features of each SCD case, identifying likely causative variants in 22 out of 32 cases (69%), compared to 16 cases (50%) when using the standard cardiac panel.

Our diagnostic yield is higher than previous studies. Among 26 other NGS studies that adhered to ACMG guidelines and with a sample size comparable to ours, reported diagnostic yields ranged from 6 to 44% [15, 17,

Table 2 Variants assessed as P/LP or VUS/LP in SCD cases with diagnostic structural abnormalities

Sample ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Variant type	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/ cardiac association	ClinGen cardiac association	HP no. phenotype association/gene pathway from GeneCards	Protein
HCM											
SCD01	26	n/a	/	/	/	/	/	/	/	/	/
SCD03	22	MYH7 (NM_000257.4): c.4258C > T; p.Arg1420Trp	missense	LP/P	PS4 PM1 PM2 PP3 PM5	P-HCM	(1–6)	192600/HCM	HCM/definitive	0001645-SCD/ Cytoskeleton remodeling: regulation of actin cytoskeleton	Myosin Heavy Chain 7
SCD14	21	negative	/	/	/	/	/	/	/	/	/
SCD15	22	n/a	/	/	/	/	/	/	/	/	/
SCD16	35	n/a	/	/	/	/	/	/	/	/	/
SCD18	20	KCNH2 (NM_000238.4): c.1010C > G; p.Thr337Ser	missense	VUS/LP	PM2 PP3 PP2	u.s.-LQTS	n/a	613695/LQTS	LQTS/SQTS/definitive BrS/disputed	0001645-SCD/ Cardiac conduction: phase3-rapid repolarisation	Potassium Voltage-Gated Channel (Subfamily H Member 2)
		KCNE1 (NM_000219.6): c.95G > A; p.Arg32His	missense	VUS	PM2 PM5	u.s.-LQTS	(7,8)	176261/LQTS	LQTS/limited	0001645-SCD/ Cardiac conduction: phase2-plateau phase; phase3-rapid repolarisation	Potassium Voltage-Gated Channel (Subfamily E Regulatory Subunit 1)
SCD27	50	FBN1 (NM_000138.4): c.979A > G; p.Arg327Gly	missense	LP/P	PP3 PP2 PS4 PM2	LP-Marfan	(9)	134797/Marfan Syndrome	Marfan syndrome/ definitive	0001640-Cardiomegaly; 0001635-Congestive heart failure/ ERK Signaling	Fibrillin 1
SCD35	39	SLC4A3 (NM_001326559.2): c.1318G > T; p.Asp440Tyr	missense	VUS/LP	PM2 PP3	n/a	n/a	106195/SQT	SQTS/moderate	0001645-SCD 0001962-Palpitations/ Transport of inorganic cations/anions and amino acids/oligopeptides	Solute Carrier Family 4 Member 3 (Sodium-independent anion exchanger)
		PSEN2 (NM_000447.3): c.506A > G; p.His169Arg	missense	VUS/LP	PM2 PP3 PP2	u.s	n/a	600759/DCM	DCM/limited	0005110-Atrial fibrillation/ 0001279-Syncope ERK Signaling	Presenilin 2
SCD36	50	SCN10A (NM_001293307.2): c.916G > C; p.Asp306His	missense	VUS/LP	PP3 PM2	n/a	n/a	604427/episodic pain/BrS/VT	BrS/disputed	0001645-SCD/ Cardiac conduction: phase0-rapid depolarisation	Sodium Voltage-Gated Channel (Alpha Subunit 10)
		KCNMA1 (NM_001161352.2): c.3457A > C; p.Lys1153Gln	missense	VUS/LP	PP3 PP2 PM2	u.s.-epilepsy	n/a	600150/seizure	n/a	0030680-Abnormality of cardiovascular system morphology/ Ca2 + activated K+ channels	Potassium Calcium-Activated Channel (Subfamily M Alpha 1)

Table 2 (continued)

Sample ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Variant type	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/ cardiac association	ClinGen cardiac association	HP no. phenotype association/gene pathway from GeneCards	Protein
SCD37	50	BAG3 (NM_004281.3): c.67C > T; p.Arg203Trp	missense	VUS/LP	PM2 PP3 PM1	u.s-DCM/ CMP	n/a	603883/DCM	DCM/definitive	0030872-Abnormal cardiac ventricular function/ Cellular responses to stimuli and heat stress	BAG Cochaperone 3
AC											
SCD07	20	negative	/	/	/	/	/	/	/	/	/
SCD11	37	RYR2 (NM_001035.3): c.8779C > T; p.Gln2927Ter	nonsense	LP/P	PVS1 PM2	n/a	n/a	180902/VA/CPVT	CPVT/definitive HCM/limited	0001645-SCD/ Cardiac conduction: ion homeostasis	Ryanodine Receptor 2 (Calcium channel on sarcoplasmic reticulum)
SCD12	25	negative	/	/	/	/	/	/	/	/	/
SCD13	14	n/a	/	/	/	/	/	/	/	/	/
SCD17	45	negative	/	/	/	/	/	/	/	/	/
SCD20	50	negative	/	/	/	/	/	/	/	/	/
SCD32	29	DSP (NM_004415.4): c.7000C > T; p.Arg2334Ter	Nonsense	LP/P	PVS1 PP5 PM2	P-AC u.s.-CMP	n/a	125647/AC	AC/definitive HCM/disputed	0001645-SCD/ Signaling by Rho GTPases;Apoptosis	Desmoplakin
DCM											
SCD10	21	Negative	/	/	/	/	/	/	/	/	/

AC arrhythmogenic cardiomyopathy, *ACMG* American College of Medical Genetics, *BrS* Brugada syndrome, *CMP* cardiomyopathy, *CPVT* catecholaminergic polymorphic ventricular tachyarrhythmias, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LP* likely pathogenic, *LQTS* long QT syndrome, *n/a* not available, *P* pathogenic, *SCD* sudden cardiac death, *SQTS* short QT syndrome, *u.s.* uncertain_significance on ClinVar, *VUS/LP* variant with unknown significance/likely pathogenic, *PVS1* Very Strong—Null variants (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single exon or multiexon deletion) in a gene where loss of function is a known mechanism of disease, *PM1* Mutational hot spot and/or critical and well-established functional domain, *PM2* Absent from controls or at extremely low frequency in Exome Sequencing Project, 1000 Genomes or ExAC, *PM5* Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before, *PP5* Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation, *PP2* Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease, *PP3* Multiple lines of computational evidence support a deleterious effect on the gene or gene product

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Table 3 Variants assessed as P/LP or VUS/LP in SCD cases with non-diagnostic autopsy findings

ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Variant type	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/ cardiac association	ClinGen cardiac association	HP no. phenotype association/gene pathway from GeneCards	Protein
Mild fatty replacement											
SCD19	40	CACNA1C (NM_000719.7): c.2558 T > C; p.Met853Thr	missense	VUS/LP	PM2 PM1 PP3 PP2	u.s.-LQTS	n/a	114205/BRs/LQTS	LQTS/moderate BrS/SQTS/disputed	0001645-SCD/ Cardiac conduction: phase 2-plateau phase	Calcium Voltage-Gated Channel (Subunit Alpha1 C)
SCD24	31	negative	/	/	/	/	/	/	/	/	/
SCD26	23	ATP1A2 (NM_000702.4): c.1352A > C; p.Glu451Ala	missense	VUS/LP	PP3 PP2 PM2	n/a	n/a	182340/n/a	n/a	0011675-Arrhythmia/ Cardiac conduction: ion homeostasis; ion transport by P-type ATPases	ATPase Na+/K+ Transporting Subunit Alpha 2
SCD33	50	SNTA1 (NM_003098.3): c.583delC; p.Leu195Phefs*23	frameshift	LP	PVS1 PM2	n/a	n/a	612955/LQTS	LQTS/disputed	0001645-SCD/ NGF Pathway	Syntrophin Alpha 1
Mitral valve prolapse											
SCD09	25	n/a	/	/	/	/	/	/	/	/	/
SCD21	29	TGFB2 (NM_001135599.3): c.1312 T > C; p.Ser410Pro	missense	LP	PP3 PM2	n/a	n/a	190220/Loeys-Dietz syndrome	Loeys-Dietz syndrome/definitive	0001654-Abnormal heart valve morphology/ ERK Signaling	Transforming growth factor beta 2
		SCN5A (NM_001160161.2): c.1820G > A; p.Gly607Asp	missense	VUS	PM2 PP2	n/a	n/a	600163/BrS/LQTS	Brs/LQTS/DCM/ definitive AC/limited CPVT/SQTS/ disputed	0001645-SCD/ Cardiac conduction: phase0-rapid depolarisation	Sodium Voltage-Gated Channel (Alpha Subunit 5)
Small foci of fibrosis											
SCD23	34	TTN (NM_001267550.2): c.69936C > G; p.Tyr23312Ter	nonsense	P	PVS1 PM2	n/a	n/a	188840/DCM/HCM	DCM/definitive HCM/AC/limited	0001645-SCD/ Striated muscle contraction; cardiac conduction	Titin
SCD30	40	negative	/	/	/	/	/	/	/	/	/
Slightly dilated chambers											
SCD29	42	LAMA4 (NM_002290.4): c.719-2A > G;	splicing	LP	PVS1 PM2	u.s-DCM	(1)	600133/DCM	DCM/limited	0001644-DCM; 0012664-Reduced left ventricular ejection fraction/ ERK Signaling	Laminin (Subunit Alpha 4)

Table 3 (continued)

ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Variant type	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/ cardiac association	ClinGen cardiac association	HP no. phenotype association/gene pathway from GeneCards	Protein
		CDH2 (NM_001792.4): c.1729G > C; p.Ala577Pro	missense	VUS/LP	PM2 PP3	u.s	n/a	114020/AC	AC/limited	0004756-Ventricular tachycardia; 0011675-Arrhythmia/ Cell junction organization; ERK Signaling	Cadherin 2

AC arrhythmogenic cardiomyopathy, *ACMG* American College of Medical Genetics, *BrS* Brugada syndrome, *CMP* cardiomyopathy, *CPVT* catecholaminergic polymorphic ventricular tachyarrhythmias, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LP* likely pathogenic, *LQTS* long QT syndrome, *n/a* not available, *P* pathogenic, *SCD* sudden cardiac death, *SQTS* short QT syndrome, *u.s.* uncertain_significance on ClinVar, *VUS/LP* variant with unknown significance/likely pathogenic, *PVS1* Very Strong—Null variants (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single exon or multiexon deletion) in a gene where loss of function is a known mechanism of disease, *PM1* Mutational hot spot and/or critical and well-established functional domain, *PM2* Absent from controls or at extremely low frequency in Exome Sequencing Project, 1000 Genomes or ExAC, *PP3* Multiple lines of computational evidence support a deleterious effect on the gene or gene product, *PP2* Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease

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Table 4 Variants assessed as P/LP or VUS/LP in SCD cases with structurally normal heart: SAD (sudden arrhythmic death)

Sample ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Protein impact	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/Cardiac association	ClinGen cardiac association	HP no. phenotype association/ gene pathway from GeneCards	Protein
SCD02	29	n/a	/	/	/	/	/	/	/	/	/
SCD04	36	n/a	/	/	/	/	/	/	/	/	/
SCD05	29	HCN1 (NM_021072.3): c.2560C > T; p.Arg854Ter	nonsense	P/LP	PVS1 PM2	n/a	n/a	602780/seizure	seizure/definitive	n/a Contributes to the native pacemaker currents in heart/ Potassium Channels	Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 1
		KCND3 (NM_004980.5): c.416G > A; p.Arg139His	missense	VUS	PM2 PP2	n/a	n/a	616399/BrS	BrS/disputed	0001695-Cardiac arrest/ Cardiac conduction: phase1-inactivation of fast Na + channels	Potassium Voltage-Gated Channel (Subfamily D Member 3)
SCD06	37	MYBPC3 (NM_000256.3): c.1255C > T; p.Arg419Cys	missense	VUS/LP	PM2 PM1 PP3 PP2	us-HCM us-CMP	(1,2)	600958/HCM/DCM/ LVNC	HCM/definitive DCM/AC/limited	0001695-Cardiac arrest/ Striated muscle contraction pathway; Cardiac conduction	Myosin Binding Protein C3
		RYR2 (NM_001035.2): c.12168G > T;p. Lys4056Asn	missense	VUS/LP	PM2 PM1 PP2	us-CMP	n/a	180902/VA/CPVT	CPVT/definitive HCM/limited	0001645-SCD/ Cardiac conduction: ion homeostasis	Ryanodine Receptor 2 (Calcium channel on sarcoplasmic reticulum)
SCD08	25	CALM1 (NM_006888.4): c.10C > T; p.Gln4Ter	nonsense	P/LP	PVS1 PM2	n/a	n/a	614916/CPVT/LQT	LQTS/definitive CPVT/moderate	0001645-SCD/ Cardiac conduction:ion homeostasis	Calmodulin 1 (calcium signal transduction pathway)
		RYR2 (NM_001035.2): c.7034C > T; p.Ala2345Val	missense	VUS/LP	PM2 PM1 PP2	n/a	n/a	180902/VA/CPVT	CPVT/definitive HCM/limited	0001645-SCD/ Cardiac conduction:Ion homeostasis	Ryanodine Receptor 2 (Calcium channel on sarcoplasmic reticulum)
SCD22	42	negative	/	/	/	/	/	/	/	/	/
SCD25	37	negative	/	/	/	/	/	/	/	/	/

Table 4 (continued)

Sample ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Protein impact	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/Cardiac association	ClinGen cardiac association	HP no. phenotype association/ gene pathway from GeneCards	Protein
SCD28	29	RYR2 (NM_001035.2): c.4972 C > G; p.Leu1658Val	missense	VUS/LP	PM2 PP3 PP2	n/a	n/a	180902/VA/CPVT	CPVT/definitive HCM/limited	0001645-SCD/ Cardiac conduction:Ion homeostasis	Ryanodine Receptor 2 (Calcium channel on sarcoplasmic reticulum)
		CACNA2D1 (NM_000722.4): c.1393C > A;p.Pro465Thr	missense	VUS/LP	PP3 PP2 PM2	n/a	n/a	114204/–	BrS/SQTS/disputed	0001645-SCD/ CREB Pathway: intracellular calcium signaling	Calcium Voltage-Gated Channel (Auxiliary Subunit Alpha2delta 1)
		LMNA (NM_170707.3): c.1122 C > G; p.His374Gln	missense	VUS/LP	PM2 PM1 PP2	n/a	n/a	150330/DCM	DCM/definitive AC/limited	0001645-SCD/ Apoptosis and survival FAS signaling cascades	Lamin A/C
SCD31	45	TTN (NM_001267550.2): c.97556_97557insA; p.Trp32520LeufsTer7	frameshift	P/LP	PVS1 PM2	n/a	n/a	188840/DCM	DCM/definitive	0001645-SCD/ Striated muscle contraction;Cardiac conduction	Titin
SCD34	45	KCNJ14 (NM_013348.4): c.599 T > C; p.Phe200Ser	missense	VUS/LP	PM2 PP3	n/a	n/a	603953/–	n/a	n/a Cardiac conduction: classical Kir channels; phase4-resting membrane potential	Potassium Inwardly Rectifying Channel (Subfamily J Member 14)
		SCN5A (NM_001160161.2): c.1820G > A; p.Gly607Asp	missense	VUS	PM2 PP2	us-BrS	n/a	600163/BrS/LQTS/ AF/VF	BrS/LQTS/DCM/ definitive AC/limited CPVT/SQTS/disputed	0001645-SCD/ Cardiac conduction: phase0-rapid depolarisation	Sodium Voltage-Gated Channel (Alpha Subunit 5)
SCD38	50	KCND3 (NM_004980.5): c.905G > A; p.Arg302His	missense	P/LP	PM1 PM5 PP3 PM2	n/a	n/a	605411/BRS	BrS/disputed	0001695-Cardiac arrest/ Cardiac conduction: phase1-inactivation of fast Na+ channels	Potassium Voltage-Gated Channel (Subfamily D Member 3)
		MYH7 (NM_000257.4): c.5606A > G; p.Asp1869Gly	missense	VUS/LP	PP3 PM2	us	(3,4)	192600/HCM	HCM/definitive	0001645-SCD/ Cytoskeleton remodeling: regulation of actin cytoskeleton	Myosin Heavy Chain 7

Table 4 (continued)

Sample ID	Age	Genetic alteration (gene, nucleotide and aminoacidic change)	Protein impact	ACMG	ACMG criteria	ClinVar	Variant literature (PMID)	Gene MIM number/Cardiac association	ClinGen cardiac association	HP no. phenotype association/ gene pathway from GeneCards	Protein
SCD39	45	SCN9A (NM_002977.3): c.2633G > C; p.Gly878Ala	missense	VUS/LP	PP3 PM2	n/a	n/a	603415/–	n/a	0011675-Arrhythmia/ Cardiac conduction: phase0-rapid depolarisation	Sodium Voltage-Gated Channel (Alpha Subunit 9)

AC arrhythmogenic cardiomyopathy, *ACMG* American College of Medical Genetics, *BrS* Brugada syndrome, *CMP* cardiomyopathy, *CPVT* catecholaminergic polymorphic ventricular tachyarrhythmias, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LP* likely pathogenic, *LQTS* long QT syndrome, *n/a* not available, *P* pathogenic, *SCD* sudden cardiac death, *SQTS* short QT syndrome, *u.s.* uncertain_significance on ClinVar, *VUS/LP* variant with unknown significance/likely pathogenic, *PVS1* Very Strong—Null variants (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single exon or multiexon deletion) in a gene where loss of function is a known mechanism of disease, *PM5* Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before, *PM1* Mutational hot spot and/or critical and well-established functional domain, *PM2* Absent from controls or at extremely low frequency in Exome Sequencing Project, 1000 Genomes or ExAC, *PP3* Multiple lines of computational evidence support a deleterious effect on the gene or gene product, *PP2* Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease

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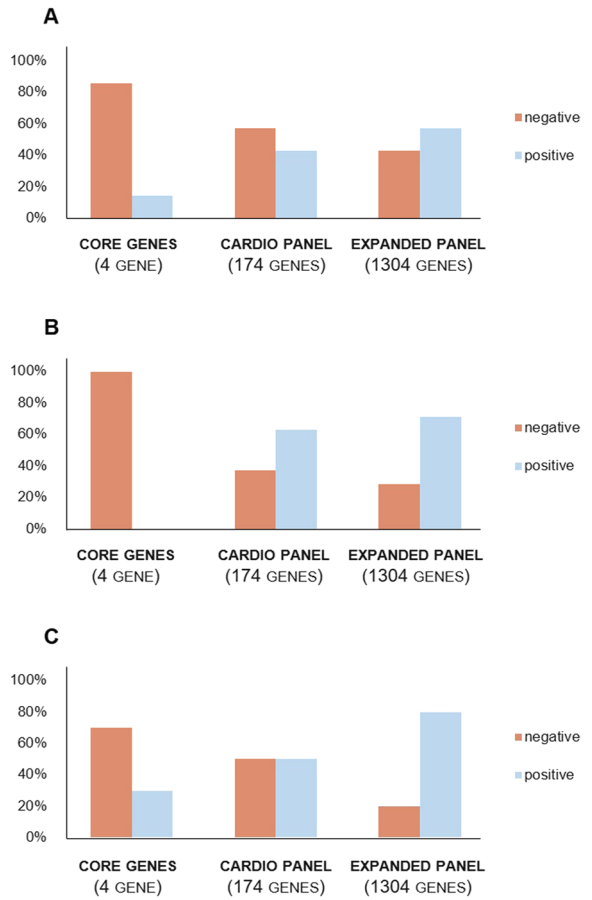

Fig. 2 Variants diagnostic detection rate (%) in each autopsy group. The cohort of "SCD with diagnostic structural abnormalities" (**A**) (macroscopic and/or histopathological alterations fell within the diagnostic criteria for a specific cardiomyopathy) shows a detection rate of 14% (2/14) with the major susceptibility genes (core genes), 43% (6/14) with the cardio panel and 57% (8/14) with the extended cardio panel; the core genes have a detection rate of 0% in the group of "SCD with non-diagnostic autopsy findings" (**B**) (macroscopic and/or histopathological alterations were subtler) (0/8) and of 30% in the group of "SCD with structurally normal heart (SAD)" (3/10); the increase in the detection rate of likely causative variants ranges from 63% (5/8) with the cardio panel to 75% (6/8) with the extended panel for "SCD cases with inconclusive autopsy findings" (**C**) and from 60% (6/10) to 80% (8/10) for the SAD group

21, 31–33], as detailed in Supplemental Table 1. Several studies do not include VUS in the diagnostic yield reporting only their detection percentage [17, 21, 34–36]. Conversely, other studies have found that including VUSs in genetic evaluation increases the variant detection rates to approximately 50–80% [18, 31, 37]. However, these studies did not further categorize VUSs based on their probabilities of being benign or pathogenic, unlike our study. Some variants were classified as VUS based solely on the PM2 criterion, even when other criteria suggested benignity, or when the PP3 criterion was not fully satisfied. In our prioritization scheme, such VUS are considered 'cold,' which excludes variants that are more likely to be reclassified as benign over time. VUS are considered "not clinically actionable" and cannot be used directly for diagnostic purposes [22]. However, recent publications [38, 39] and practice guidelines (https://www.acgs.uk.com/; https://sigu.net/) advocate for a more nuanced approach to manage VUS, rather than simply categorizing them as uncertain. Multidisciplinary discussions to evaluate VUS in specific cases can help to determine which VUS should be reported to clinicians, particularly when there is high level of evidence supporting pathogenicity and potential for obtaining additional evidence [40]. Further studies are necessary to validate variants we have classified as VUS/LP and achieve final reclassification. However, our results underscore the importance of expanding genetic testing through tailored gene panels and specific prioritization, with variant assessment conducted in a multidisciplinary setting that considers the case context. Integrating this approach into laboratory practice facilitates comprehensive reporting to clinicians and enables further variant studies and patient follow-up. The data collected may contribute to future reclassification of the VUS/LP identified in this study. Furthermore, the stored raw exome data can be re-evaluated in future trio-family analyses.

All cases with positive genetic results exhibited heterozygous variants, and 27% had 2 or more variants, highlighting the complexity of the SCD phenotype. These observations support the theory that SCD might result from interactions among common variants with moderate impacts or clusters of rarer variants with more pronounced effects. Distinguishing pathogenic from benign variants is particularly challenging, given the potential for incomplete penetrance, variable expressivity, and phenotypic overlap in channelopathies and cardiomyopathies, which typically follow an autosomal dominant inheritance pattern. Combinations of known and unknown genetic and environmental factors may contribute to incomplete penetrance and variable expressivity [41–44].

In our study, 9 altered genes would not have been identified in the standard cardiac panel. Some of these genes— *Solute Carrier Family 4 Member 3 - SLC4A3, Presenilin 2 - PSEN2, Sodium Voltage-Gated Channel Alpha Subunit 10 - SCN10A,* and Cadherin 2 - *CDH2*— are currently under investigation, but are likely to be associated with SQTS, DCM, BrS and AC, respectively. The remaining genes are implicated in ion homeostasis and cardiac conditions. For instance, *Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel 1 (HCN1)*, highly expressed in human sinoatrial node, could have a key role in pacemaker current [45–47]. *ATPase Na+/K+ Transporting Subunit Alpha 2 (ATP1A2)* had "cardiac conduction" among its related pathways, with a role

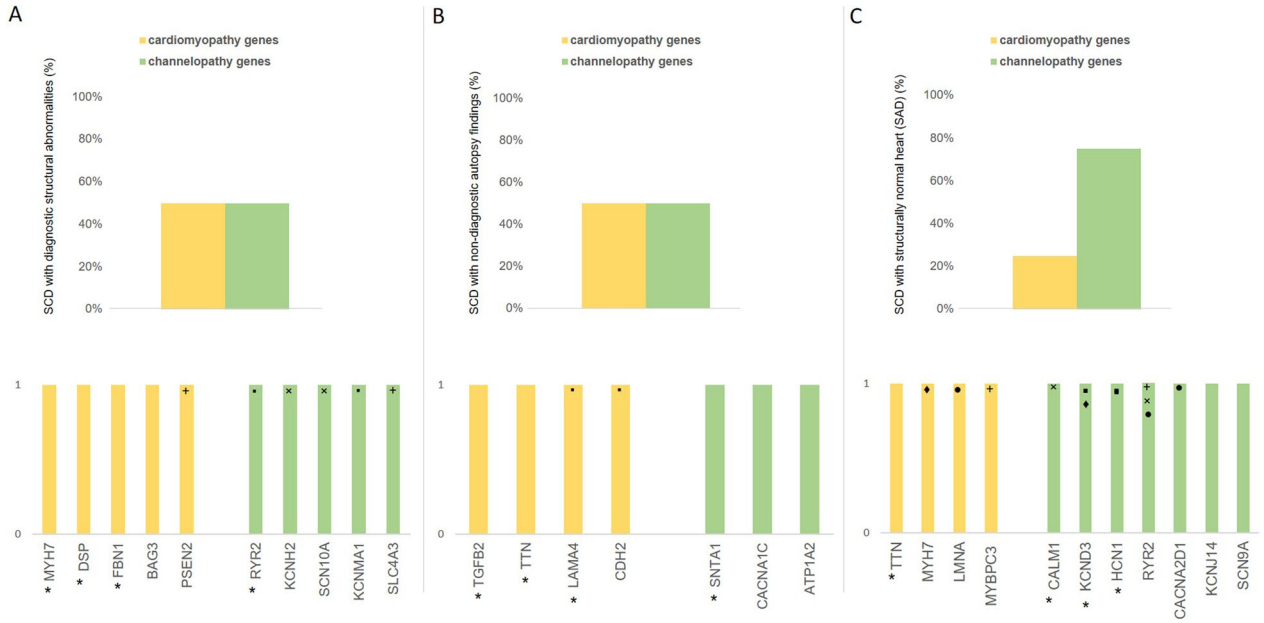

Fig. 3 Cardiomyopathy versus channelopathy genes in different autopsy groups. Probable causative variants in genes associated with cardiomyopathy (orange) are observed more frequently in cases of SCD with diagnostic structural abnormalities (50%) (**A**) and with non-diagnostic autopsy findings (50%) (**B**), compared to those with structurally normal hearts (SAD) (25%) (**C**). Bars are marked with the same symbol when represent the same subject; genes marked with an asterisk present (P/LP) variant

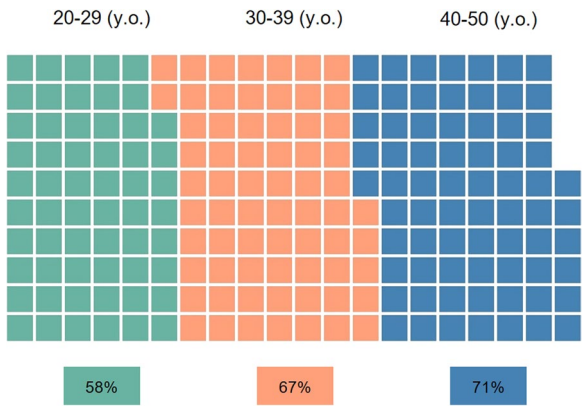

Fig. 4 Diagnostic yield percentages across age groups. Waffle charts depicting diagnostic yield percentages for three age groups: 20–29 years (58%), 30–39 years (67%), and 40–50 years (71%). Each square represents 1% of the total sample. Despite apparent differences, statistical analysis revealed no significant variation in diagnostic yield across age groups (p = 1 for each age class)

in ion homeostasis and ion transport by P-type ATPases and recently, Staehr et al., suggesting its potential role in cardiac function and metabolism through the Src/Ras/Erk1/2 pathway [48]. Most variants in these genes are classified as VUS/LP, given the absence of definitive gene/disease associations. Current guidelines advocate genetic testing in SCD cases with a probable genetic basis, but also advice caution against analysing genes not definitively associated with the clinical phenotype [1]. This is a significant issue, particularly in cases with non-diagnostic autopsy findings or structurally normal hearts, when clinical and family histories are unavailable to guide post-mortem genetic testing. We propose a combined approach extending the search for genetic variants beyond specific panels, while limiting the likelihood of casual results from broad screenings.

The diagnostic yield found in SCD with diagnostic structural abnormalities (57%) aligns with results from earlier studies on inherited cardiomyopathies [49]. Ion channel-related genes were found in 4 cases revealed a potential overlapping phenotype. This result is consistent with previous studies that have detected variants in cardiac conduction-related genes in cases of SCD with autopsy diagnosis of cardiomyopathy [50, 51]. This could derive from the use of a broader gene panel including classical "channelopathies" genes to test subjects with structural alteration. On the other hand, structural and conduction alterations might coexist, and CPVT, LQTS, and SQTS can present as asymptomatic, but still lead to cardiac arrest as its first manifestation, as in our cohort.

Our results underscore the efficacy of a multidisciplinary approach to elucidate the aetiology of juvenile SCD, particularly in unexplained cases. We identified probable causative variants in 14 cases with either non-diagnostic autopsy findings or structurally normal hearts, achieving a yield of 78%. This approach reduced

the proportion of unexplained cases from conventional autopsy to molecular autopsy. Variants in genes associated with channelopathies were found in 50% of cases, supporting previous findings [18, 44]. Notably, RYR2 VUS/LP variants were identified in 9% of SAD cases, aligning with previous reports that observed a prevalence of 5–10% [52, 53]. However, all three cases in our cohort also harboured additional probable causative variants, underscoring the importance of comprehensive genetic analysis. Conversely, variants in cardiomyopathy-associated genes were found in 28% of cases, supporting the hypothesis that variants in structural proteins can trigger functional abnormalities in cardiomyocytes before any macroscopic or histopathological changes are evident [11, 14, 44]. Sudden death in these individuals, who carry a likely causative variant in a cardiomyopathy-associated gene, could be attributed to a "concealed cardiomyopathy," where malignant arrhythmias occur in the absence of overt clinical disease. As instance, case [SCD29] had slightly dilated chambers found at autopsy and carried the splicing LP LAMA4:c.719-2A > G already found in a DCM patient [54], while case [SCD31] with structurally normal heart carried a frameshift LP variant in TTN. Therefore, these results support recent recommendations suggesting that genes linked to cardiomyopathy should be included in the molecular autopsy [1].

The co-segregation of the variant with the disease in family members is a powerful tool for identify the causative variant as well as for reclassification of VUSs. Functional validation studies of genetic variants represent another effective approach, though their expense and time-consuming nature limit their routine use, especially considering the vast number of variants uncovered by NGS. We did not perform either co-segregation or functional validation studies, and the small sample size and the limited information about previously clinical data and family history did not allow searching for associations between specific variants with clinical data. These limitations have led to a lack of definitive confirmation of the pathogenicity of the variants considered VUS/LP. However, we have tried to address this limitation through the specific VUS prioritization scheme and careful case-specific variant assessments by multidisciplinary team.

In conclusion, WES optimized with a customized virtual gene panel, a structured variant prioritization scheme and a multidisciplinary approach for case-specific variant evaluation can significantly improve the identification of likely causative variants in juvenile SCD cases, particularly when autopsy findings are inconclusive. This approach should be considered as a routine basis in this setting for achieving a thorough autopsy diagnosis and potentially facilitating early diagnosis in first-degree relatives.

Abbreviations

AC	Arrhythmogenic cardiomyopathy
ATP1A2	ATPase Na+/K+ Transporting Subunit Alpha 2
ACMG	American College of Medical Genetics
BrS	Brugada syndrome
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CASQ2	Calsequestrin 2
DES	Desmin
DSP	Desmoplakin
FFPE	Formalin-fixed: paraffin-embedded
HCN1	Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel 1
IVS	Interventricular septum
LMNA	Lamin A/C
LQTS	Long QT syndrome
LV	Left ventricle
MAF	Minor allele frequency
MYBPC3	Myosin Binding Protein C3
MYH7	Myosin Heavy Chain 7
PKP2	Plakophilin-2
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1
KCNH2	Potassium Voltage-Gated Channel Subfamily H Member 2
RYR2	Ryanodine Receptor 2
RV	Right ventricle
SAD	Sudden arrhythmic death
SCD	Sudden cardiac death
SCN5A	Sodium Voltage-Gated Channel Alpha Subunit 5
SQTS	Short QT syndrome
VUS	Variant of unknown significance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40246-024-00657-x.

Additional file 1

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Author contributions
MM conceived the work, carried out the experimental analysis of the samples, performed the bioinformatic analysis and variant interpretation and drafted the manuscript. AG and AA conceived the work, interpreted the results, critically reviewed and edited the manuscript. ME and MDP conceived the work, supervised the study and critically revised the manuscript. PA performed the bioinformatics analysis. NB and SM critically reviewed the variant evaluation. MDP, DB and AS contributed to the autopsy diagnosis and critically reviewed the cases for classification and autopsy diagnosis. All authors contributed in a multidisciplinary team to the final variant evaluation on a case-by-case basis.

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Availability of data and materials
All data described in this study are provided within the article and Supplemental Material. The raw DNA sequencing data for the cases are not available due to privacy and ethics restrictions.

Declarations

Ethical approval
We secured consent for genetic testing and research use of data from the relatives of the deceased. This study was conducted in agreement with the

Helsinki Declaration and received approval from the Ethic Committee of the Tuscany Region, Area Vasta Nord-Ovest (no. 14870).

Consent to participate
We secured consent for genetic testing and research use of data from the relatives of the deceased.

Competing interests
The authors declare that they have no competing interests.

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