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Further exploration of cardiac channelopathy and cardiomyopathy genes in stillbirth

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Abstract

Objective: To explore genetic variation including whole genome copy number variation and sequence analysis of 98 genes associated with pediatric or adult cardiomyopathies, cardiac channelopathies, and sudden death in an unexplained intrauterine fetal death cohort.

Methods: The study population included 55 stillbirth cases that remained unexplained after thorough postmortem examination, excluding maternal, fetal, and placental causes of stillbirth. Molecular karyotyping was performed in 55 cases and the trio exome sequencing approach was applied in 19 cases.

Results: The analysis revealed six rare variants with predicted effects on protein function in six genes (*CASQ2*, *DSC2*, *KCNE1*, *LDB3*, *MYH6*, and *SCN5A*) previously reported in cases of stillbirth or severe early onset pediatric cardiac related phenotypes. When applying strict American College of Genetics and Genomics classification guidelines, these are still variants of uncertain significance.

Conclusions: Several potentially stillbirth-related genetic variants were detected in our cohort, adding to the growing literature on cardiac phenotype gene variation in stillbirth. However, the mechanisms of action, gene-gene interaction, and contribution of the uterine environment are still to be deciphered. In order to advance our knowledge of the genetics of unexplained fetal death, there is an evident need for international collaboration and field standardization.

Key points

What is already known about this topic?

- More than 60% of stillbirth cases remain unexplained even after comprehensive clinical evaluation.
- Cardiac channelopathies and cardiomyopathies are suggested as a contributing mechanism.

What does this study add?

- Several potentially stillbirth-related variants were identified in our cohort, two in previously reported stillbirth cases, four in severe early onset pediatric cardiac phenotype cases, and several others in genes related to cardiac arrhythmias.

1 | INTRODUCTION

Stillbirth is defined as fetal death after 22 weeks of gestation or having a birth weight of more than 500 g when the gestational age is unknown.[1] Common causes are classified into several groups, including obstetric complications, infections, placental insufficiency, placental abruption, umbilical cord complications, intrauterine growth restriction, and congenital abnormalities with or without a known genetic cause.[2] While several of the above mechanisms are considered to be purely environmental, genetic predisposition may be associated with some, including placental disease and umbilical cord anomalies, both as a part of a complex genetic phenotype or stand-alone complications.[3–5] However, even after a detailed evaluation of each case following recommended protocols, 25–60% of cases remain unexplained,[6,7] and are defined as a stillbirth with an unknown cause or an unexplained stillbirth.

Genetic syndromes have been acknowledged as an important contributor to fetal death and a substantial proportion of stillbirths may be attributed to various genetic causes, including chromosomal aberrations, copy number variations, and single gene disorders.[8] These genetic causes usually result in congenital malformations and/or specific dysmorphic signs. After performing a systematic review, our team learned that there is limited data regarding the monogenic causes of unexplained stillbirth, with some evidence suggesting a potential correlation with cardiomyopathies and cardiac channelopathies.[9,10]

Three cohort studies reported single nucleotide variants in selected gene panels comprising various numbers of genes (between 3 and 70 genes) related to long- and short- QT syndromes (LQTS and SQTS), cardiomyopathies, channelopathies, and sudden cardiac death. Overall, compared to variants present in the control database of unaffected individuals (ExAc),[11] the frequency of variants with evidence supporting pathogenicity is significantly higher in cohorts; however, these variants still meet the classification guidelines' definition of unknown significance in most cases. A single pathogenic finding was reported in the *KCNJ2* gene (p.Arg40Gln), and 3 putative pathogenic variants in the *KCNQ1* (2 variants) and *KCNH2*.[12] Only 1 study used a different approach by generating what they called an OMIM Morbid genes panel with the aim of expanding the genotype-phenotype correlation of known genes.[13] Among 246 cases, there were five pathogenic/likely pathogenic variants in cardiac genes, and 6 cases had a multisystem developmental disorder. In addition, several loss-of-function variants were discovered in genes that are currently not associated with a human disease but are crucial for in utero survival in mice.[13]

We identified two limitations of the aforementioned studies. Firstly, most of the studied cohorts included cases with underlying placental disease, umbilical cord anomalies, or fetal congenital anomalies as known or suspected causes of fetal death.[13,14] It is important to acknowledge that including those cases in unexplained stillbirth research may distort the research yield in both false positive and negative directions. Placental disease and umbilical cord anomalies present a common and known cause of stillbirth; therefore, including these cases in research on genetic variation in unexplained stillbirth cohorts results in a lower yield of fetal genetic contribution analysis. However, it has been shown that abnormal placental development affects the development of the fetal cardiovascular system [15] and may, if present, cause an environmental insult, affecting the related cardiac gene expression.[16] Furthermore, since a significant proportion of complex congenital anomalies have a genetic cause, including cases with known congenital anomalies in unexplained stillbirth genetic research leads to false positive results. In addition, previous studies did not include parental DNA and family history data, making it impossible to evaluate inheritance or de novo occurrence.[10,11] All this information might elucidate further details that would contribute to the variant classification. We addressed those limitations in this study.

Our research aimed to explore genetic variation in a cohort of unexplained stillbirth cases, including genome-wide copy number variation and comprehensive gene panel sequence analysis of 98 genes associated with cardiomyopathies, cardiac channelopathies, and sudden death.

2 | METHODS

2.1 | Study population

Our study population included unexplained stillbirth fetuses at the Department of Perinatology, Division of Gynecology and Obstetrics, University Medical Center Ljubljana, from January 2015 to July 2021. Unexplained stillbirth was defined as the death of a fetus occurring after the 22nd week of gestation. Our inclusion criteria were healthy women with normal singleton pregnancies, without known maternal, fetal, or placental causes for stillbirth, and without previously detected genetic abnormalities in the fetus. The fetal autopsy was performed and was normal in all cases. After a comprehensive postmortem examination, these stillbirth cases were defined as unexplained. Nineteen couples were included in the trio-based exome sequencing cohort and signed a written informed consent. Copy number variation syndromes were excluded using molecular karyotyping as previously described.[17] The samples underwent trio-based whole exome sequencing analysis with an in silico panel approach at the Clinical Institute for Genomic Medicine, University Medical Centre Ljubljana.

2.2 | Genetic testing

Molecular karyotyping was performed using an oligonucleotide microarray with approximately 55,000 probes distributed throughout the genome (Agilent, Human CGH microarray Kit 8 × 60k, hg19 UCSC, NCBI Build 37, February 2009), which provides an average resolution of approximately 100 kb. Each sample was compared with a commercial reference sample of DNA (Agilent). The results were

analyzed by CytoGenomics 5.3.0 software (Agilent). The library was enriched using in-solution capture with Illumina Nextera Coding Exome probes for trio exome sequencing. Whole exome sequencing of the shotgun library was completed using the Novaseq 6000 platform in the 2 × 101 reads paired-end sequencing mode. Median coverage of more than 90 × was established, and more than 98% of regions were covered at 20 ×. Sequencing results were evaluated using an in-house analysis pipeline as described in previous publications by our group.[18,19] Homozygosity mapping analyses were performed using an in-house algorithm followed by the validation of the findings using Homozygosity mapper software.[20] A digital panel of 98 genes associated with cardiac arrhythmias, cardiomyopathies, and sudden unexplained death in young people extracted from PanelApp[21] was used (Table S1). De novo variants were identified by filtering the parental variants.

2.3 | Variant filtering and classification

Called CNVs were aligned with known aberrations in publicly available databases ClinGen (http://dbsearch.clinicalgenome.org/search/), DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (https://decipher.sanger.ac.uk/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), and the Database of Genomic Variants—DGV (http://dgv.tcag.ca/dgv/app/home), as well as with the in-house database of detected variants and their clinical significance ascertained by trained analysts according to the American College of Genetics and Genomics (ACMG) Standards and Guidelines.[22] The variants in the 98 genes in the above panels that were present in <500 heterozygotes and ≤2 homozygotes in gnomAD v3.1.2 and in <100 heterozygotes and ≤2 homozygotes from our internal database of Slovenian individuals were further analyzed. We included only those having a moderate (missense, in frame deletion/duplication) and high (loss of function) effect on protein function. Synonymous and intronic variants were considered if computer algorithms confidently predicted the splicing effect of the variant. In addition, the variants classified as pathogenic, likely pathogenic, or conflicting between pathogenic, likely pathogenic, risk factor, and/or variant of uncertain significance in ClinVar were also included in the consideration, even if their frequency in the population bases was greater than the above cutoffs. The variants described above that met both the loss frequency and the moderate or high protein effect criteria were named rare functional variants. We classified the rare functional variants based on the ACMG Criteria.[23] All research articles on unexplained stillbirth were scanned for other variants in the genes identified in this study.

2.4 | Ethics

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (consent number 0120-03/2020/7).

3 | RESULTS

3.1 | Maternal and fetal baseline characteristics

Our study population included unexplained stillbirth fetuses at the Department of Perinatology, Division of Gynecology and Obstetrics, University Medical Centre Ljubljana, from January 2015 to July 2021. In this period, there were 37.020 births and 472 stillbirths (1.27%), defined as the death of a fetus occurring after the 22nd week of gestation. More than half (246) were excluded due to causal congenital anomalies and an additional 89 due to an underlying placental cause, significant intrauterine growth restriction, and/or infection. An additional 23 cases were excluded due to multiple pregnancies and 41 due to maternal pre-existing chronic conditions, maternal age, or non-existing DNA material. Fetal autopsies were performed and were normal in all cases. Fifty-five cases were screened for copy number variation, with 19 couples signing informed consent to participate in exome sequencing.

Individual maternal and fetal characteristics of each case were collected. The following parameters are shown in Table 1: maternal age, parity, previous miscarriages, gestation age at stillbirth, fetal sex, weight and length. The median maternal age was 35 (22–47) years at the time of delivery, 10 of them were nulliparous and nine multiparous. Five women had previous miscarriages (one had two and four had one miscarriage). Genetic testing was not utilized in any of the previous miscarriage cases.

The study cohort consisted of 13 male and 6 female fetuses. The median fetal gestational age at stillbirth was 32 (25–39) weeks. The median weight of stillborn fetuses was 1800 (410–3710) g with a median length of 42 (28 – 54) cm (Table 2).

3.2 | Genetic variants

Molecular karyotyping only showed common benign variation in the tested cohort. In a panel of 98 genes, 56 (54 different) rare functional variants were detected. After a rigorous manual classification, 31 (29 different) variants in 23 genes, associated with autosomal dominant diseases, were classified as variants of uncertain significance.

Despite a small cohort, 2 VUS variants were previously already reported in stillbirth cases: variant *CASQ2*:c.1186G>A[11] and common variant *KCNE1*:c.253G>A; the latter was reported in several other cardio related phenotypes, has a population frequency of 0.9% (gnomAD v2.1.1), and was present in two cases from our cohort (Table 3).[14] Additional 5 VUS variants in 4 genes from our panel (*DSC2*, *LDB3*, *MYH6*, and *SCN5A*) were previously reported in at least 7 cases of severe early onset cardiac-related phenotypes, including 4 cases of rhythm disturbances and 3 cases of cardiomyopathy (Table 3, marked in bold). Further, 9 VUS variants in 7 genes from the panel (*CACNB2*, *DSP*, *HCN4*, *KCNQ1*, *PRDM16*, *RBM20*, and *TRPM4*) have been previously identified in cases with other cardiac phenotypes, as detailed further in Table 3. According to the current guidelines, we reclassified

TABLE 1 Characteristics of the study population.

Characteristic	*N*	%
Maternal age (years)		
<20	0	0
20–34	12	63.2
35–39	4	21.0
≥40	3	15.8
Parity		
nulliparous	10	52.6
multiparous	9	47.4
Previous miscarriages		
None	14	73.7
One	4	21.0
Two	1	5.3
Gestational age at stillbirth (weeks of gestation)		
22–27	6	31.6
28–31	4	21.0
32–36	4	21.0
≥37	5	26.3
Fetal sex		
Female	6	31.6
Male	13	68.4
Weight (g)		
500–1500	8	42.1
1501–2500	6	31.6
>2501	5	26.3
Length (cm)		
<30	2	10.5
31–49	12	63.2
≥50	5	26.3

1 variant identified in our cohort and in a previous case of sudden death (*DSP*:c.1696G>A) from likely pathogenic to VUS variant due to its uncertain functional effect and relatively high population prevalence.[24,25] Further literature and database searches of the above-mentioned 13 genes with recurrent variants revealed 73 additional variants previously reported in cohorts of stillbirths and other cardiac-related and sudden death phenotypes. These are presented in the Supplement (Table S2).

Additional 5 variants were previously unreported in relevant scientific literature but were present in genes, where a different variant has been previously reported in stillbirth (genes *CACNA1C*, *DMD*, *TMEM43*, Table 4). Nine variants were detected in 7 genes (*FLII*, *MYLK3*, *NKX2-5*, *RPL3*, *TNNI3K*, *TRDN*, and *TRIM63*) which have not been linked to stillbirth cases so far (Table S3). Last but not least, the analysis revealed a compound heterozygous state of *TNNI3K* variants (c.1995C>A and c.512T>C) in a single case (Table S4). This gene has previously been associated with a recessive form of cardiac-conduction disease.[26] The variants were previously unreported and classified as VUS (PM3_SUP).

Altogether, 31 variants (18 maternally inherited, 13 paternally inherited) were present in 17 cases from our cohort, meaning that 89.5% of our stillbirth population are carriers of a VUS variant in a gene linked to stillbirth, sudden (infant) death, cardiomyopathy, and/or arrhythmogenic heart disease. The distribution of variants according to the pathogenicity, mode of inheritance, and phenotype correlation is presented in Figure 1. None of the detected variants appeared de novo in our study.

4 | DISCUSSION

Presented study aimed to identify monogenic cardio related causes of unexplained stillbirth. The analysis of an in silico panel of 98 genes related to stillbirth, sudden death, cardiomyopathy, and/or arrhythmogenic heart disease revealed that as many as 89.5% of cases carry a rare functional variant in a gene that might contribute to stillbirth; however, no definite genetic cause has been detected.

Fifteen of the identified variants have already been published in sudden (infant) death, cardiomyopathy, and/or arrhythmogenic heart disease cases and are discussed below. Despite being classified as variants of uncertain significance, they represent a potential contributing genetic factor in stillbirth, making them attractive candidates for further analysis. The classification of a variant as a VUS is a consequence of insufficient data favoring the variant's benign or pathogenic nature.[27] This is especially challenging in cardiac-related genes with known variable expression and incomplete penetrance, where the presence of the variant in a control population does not automatically assume its benign nature. Experimental data regarding the variant's effect featuring in vitro or animal models are scarce in rare disease research; additionally, existing databases are lacking fetal phenotypes,[28] and the expansion of prenatal phenotypes would support phenotype-driven prenatal genetic diagnosis.[29] Experimental studies confirming the discovered variant's impact on the protein function are needed to establish its pathogenic effect. Our study provides further exploration of cardiogenic gene sequence variation within a stillbirth population by reporting several interesting variants of uncertain significance in our cohort.

Most importantly, two of the 15 variants have already been identified in stillbirth cases. The variant *CASQ2*: c.1186G>A results in an amino acid change from aspartate to asparagine at position 396 in the protein. It has been reported in a stillbirth male fetus at 35 + 4 weeks of gestation, where an autopsy also revealed a hypolobation of the right lung.[11] The *CASQ2* gene has been associated with a recessive form of catecholaminergic polymorphic ventricular tachycardia[30] with a dominant inheritance model proposed recently.[31,32] Our variant is located in the C-terminal end of the *CASQ2* protein. Even though this variant has not been functionally

TABLE 2 Characteristics of the stillbirth cases.

Case	Gestational age at stillbirth (weeks of gestation)	Fetal sex	Weight (g)	Length (cm)	Gene transcript position	Variant classification
1	39	Male	2930	50	*ATAD3A* c.229C>G	Variant in AR gene
					TNNI3K c.1995C>A	Compound heterozygous variants in *TNNI3K*
					TNNI3K c.512T>C	
2	39	Female	3230	54	*HCN4* c.3577G>C	VUS
3	30	Female	1360	38	*RBM20* c.1244G>A	VUS
					TTN c.48320A>C	Missense VUS in TTN
4	28	Male	1505	40	*KCNE1* 253G>A	VUS
					TTN c.64811G>A	Likely benign
					TULP3 c.1093delG	Variant in AR gene
5	38	Male	3710	53	*CASQ2* c.1186G>A	VUS
					RPL3 c.970G>A	VUS
					TCAP c.37_39delGAG	Likely benign
					TMEM43 c.413A>G	VUS
					TNNI3K c.2499dupA	VUS
6	32	Male	1670	43	*HCN4* c.3577G>C	VUS
					KCNQ1 c.590C>T	VUS
					NKX2-5 c.767A>T	VUS
					TTN c.46G>A	Missense VUS in TTN
7	29	Female	1420	40	*ALPK3* c.862G>C	Variant in AR gene
					MYH6 c.679G>A	VUS
8	25	Male	410	28	*DOLK* c.703G>A	Variant in AR gene
					LDB3 c.664G>A	VUS
					MYLK3 c.2204C>T	VUS
					TTN c.85309G>C	Missense VUS in TTN
					TTN c.34474C>A	Likely benign
9	38	Male	2880	50	*DSP* c.1696G>A	VUS
					TTN c.37421T>C	Likely benign
					TTN c.102877A>G	Missense VUS in TTN
10	26	Male	690	33	*FKTN* c.929A>G	Variant in AR gene
					FLII c.253G>A	VUS
					MYLK3 c.1453G>A	VUS
					NRAP c.4504C>T	Variant in AR gene
					TRPM4 c.2561A>G	VUS
					TTN c.89G>C	Likely benign
11	27	Male	1160	42	*TRIM63* c.1012G>T	VUS
					TTN c.70231G>A	Missense VUS in TTN
12	25	Male	265	26	*PRDM16* c.2666C>T	VUS
					TTN c.71300G>A	Missense VUS in TTN
					TTN c.54818C>T	Missense VUS in TTN

TABLE 2 (Continued)

Case	Gestational age at stillbirth (weeks of gestation)	Fetal sex	Weight (g)	Length (cm)	Gene transcript position	Variant classification
13	36	Female	2090	48	*LDB3* c.2092G>A	VUS
					SCN5A c.998+5G>A	VUS
14	36	Female	2740	51	*CACNA1C* c.4841G>A	VUS
					CACNB2 c.209G>A	VUS
					KCNE1 253G>A	VUS
15	37	Male	2260	48	*DMD* c.11G>C	VUS
					TMEM43 c.351dupG	VUS
16	27	Male	710	36	*DMD* c.7093G>A	VUS
					RBM20 c.773C>T	VUS
					TRDN c.17C>T	VUS
					TTN c.93250G>C	Likely benign
					TTN c.71584C>G	Missense VUS in TTN
					TTN c.54532C>T	Likely benign
17	27	Female	1780	45	No results	No results
18	36	Male	2640	49	No results	No results
19	39	Female	2940	51	*DSC2* c.2194T>G	VUS
					NKX2-5 c.639_641dupGCC	VUS
					SPEG c.1622C>T	Variant in AR gene
					TTN c.99922G>A	Likely benign

Abbreviation: AR, autosomal recessive.

assessed previously, the C-terminus of the *CASQ2* has been described as highly conserved among species and involved in protein phosphorylation.[33] The second variant previously reported in stillbirth is a *KCNE1*:c.253G>A variant that was detected in 2 cases in our cohort. It represents a known *KCNE1*-D85N variant, which has previously been reported to be significantly more frequent in LQTS probands[34] despite also being present in 0.94% of the gnomAD population. However, we have discovered this variant in two of our 19 stillbirth patients, making it tenfold more frequent in our cohort of unexplained stillbirth cases than in the general population and might contribute to the phenotype. Moreover, it has been previously published in an unexplained intrauterine death with normal autopsy and placental histology,[14] in two sudden unexpected infant death cases,[35] as well as in several studies focused on Long QT syndrome.[12,14,36] The *KCNE1* gene encodes the beta-subunit of cardiac voltage-gated K channels, and this polymorphism is known to be associated with drug-induced LQTS.[37,38] In a large cohort of LQTS patients, its frequency was significantly higher in LQTS patients compared with healthy controls (3.9% vs. 0.81%).[34] This variant is likely a disease-causing genetic variant acting by modifying KCNQ1-and KCNH2-coded channel function and reducing its ion currents.[39] Neither of the 2 cases in our cohort carried any additional putative pathogenic variants in the KCNQ1 or KCNH2 genes, where it has been demonstrated that the risk factor significantly affects the phenotype.[34] Nevertheless, it is known that fetal progesterone levels rise and QT intervals become longer during the late stages of pregnancy[40–42] which indirectly implies that the intrauterine fetal death-related LQTS channelopathy might occur more frequently.[12] The risk of lethal ventricular arrhythmias during this gestational period could be increased with the presence of a pathogenic LQTS-related variant or a rare variant with an abnormal electrophysiological phenotype.[12] Further studies are needed to answer the question of whether and how this polymorphism acts on QT duration and its potential link to stillbirth.

Four identified variants have been previously reported in an early onset severe phenotype, including heart rhythm disturbances and early cardiomyopathy that might be of importance in the prenatal period. One was classified as (likely) pathogenic, and the remaining three as a VUS. Variant *DSC2*:c.2194T>G has been reported as pathogenic in the HGMD database in a case of clinically definite ARVD/C,[43] and possible disease causing variant in a cohort of arrhythmogenic right ventricular cardiomyopathy (ARVC).[44] Lacking additional convincing criteria, such as functional studies, we have classified it as a VUS in our cohort. The *SCN5A* gene, one of the most commonly mutated genes in cohorts of LQTS and Brugada syndrome patients, is also a promising candidate. Several variants have been reported in stillbirth, some of them as putative/likely

TABLE 3 Cohort variants that have been previously reported in stillbirth cases or cases with cardiac-related phenotypes.

Gene (transcript): Variant (protein change)	Related phenotypes (OMIM), mode of inheritance	Classification (ACMG criteria)	Previous reports of this variant (classification of the variant in previous study) [PMID reference]
CACNB2 (NM_201596.3): c.209G>A (p.Arg70His)	Brugada syndrome 4 (611876), AD	VUS (PS4_MOD)	Unexplained death of a 42-year- old male during exercise, negative autopsy (PPV) [27930701, 28255936]
CASQ2 (NM_001232.4): c.1186G>A (p.Asp396Asn)	Ventricular tachycardia, catecholaminergic polymorphic 2 (611938), AR	VUS (PS4_SUP, BP4)	Stillbirth at 35 + 4 gestational weeks, male fetus with hypolobation of the right lung [30615648]
DSC2 (NM_024422.6): c.2194T>G (p.Leu732Val)	Arrhythmogenic right ventricular dysplasia 11 (610476), AD, AR	VUS (PS4_MOD, BP4)	15-year-old boy suffered sudden death during football, negative autopsy (VUS) [28255936] 3-month-old male sudden death during sleep (pathogenic in HGMD for ARVC CM0910201) [25447171]
DSP (NM_004415.4): c.1696G>A (p.Ala566Thr)	Arrhythmogenic right ventricular dysplasia 8 (607450), AD	VUS (PS4_MOD, BP4)	Sudden death with cardiomyopathy (ARVC) in a patient and a clinically positive family member (likely pathogenic) [27000522] Patient with ARVC (pathogenic) [16774985]
HCN4 (NM_005477.3): c.3577G>C[a] (p.Glu1193Gln)	Brugada syndrome 8 (613123); Sick sinus syndrome (163800), AD	VUS (PS4_MOD, BP4)	70-year-old man with Brugada syndrome (VUS) [26230511] 21-year-old female suffered sudden cardiac death (VUS) [30327538] Sudden unexpected death in epilepsy (SUDEP) (VUS) [26704558]
KCNE1 (NM_001127670.3): c.253G>A[a] (p.Asp85Asn)	Long QT syndrome 5 (613695), AD	RF (PS3, PS4, BS1)	Female fetus, unexplained intrauterine death at 40 gestational weeks (PPV) [29874177] Six patients carried a genetic risk factor in KCNE1 (with unspecified arrhythmia, CPVT, sudden cardiac arrest) [30847666]
KCNQ1 (NM_000218.3): c.590C>T (p.Pro197Leu)	Atrial fibrillation, familial, 3 (607554), AD, long QT syndrome 1 (192500), AD, short QT syndrome 2 (609621), AD	VUS (PS4_SUP, PP3)	Sudden unexplained death during daily activities with negative autopsy in the Chinese Han population (VUS) [25639344]
LDB3 (NM_007078.3): c.664G>A (p.Ala222Thr)	Cardiomyopathy, dilated, 1C, with or without LVNC (601493), AD, cardiomyopathy hypertrophic, 24 (601693), AD	VUS (PS4_SUP, BP4)	Identified in one patient with myofibrillar myopathy (VUS) [33802723]
LDB3 (NM_007078.3): c.2092G>A (p.Ala698Thr)	Cardiomyopathy, dilated, 1C, with or without LVNC (601493), AD, cardiomyopathy hypertrophic, 24 (601693), AD	VUS (PS4_MOD)	Case with dilated cardiomyopathy (VUS) [27532257] A child with dilated cardiomyopathy (VUS) [36178741] 2-year-old with aborted sudden cardiac death and a severe form of hypertrophic cardiomyopathy (VUS) [37092670] Dilated cardiomyopathy (VUS) [30847666]
MYH6 (NM_002471.4): c.679G>A (p.Ala227Thr)	Cardiomyopathy, dilated, 1EE (613252), AD, cardiomyopathy, hypertrophic, 14 (613252), AD	VUS (PM2, PS4_SUP)	6-year-old male patient with progeroid phenotype, severe developmental delay, and no cardiac anomaly (VUS) [34805759] Pediatric dilated cardiomyopathy (VUS) [35026164]
PRDM16 (NM_022114.4): c.2666C>T (p.Pro889Leu)	Cardiomyopathy, dilated, 1LL (615373), AD	VUS (PS4_SUP)	Wolff-Parkinson-White arrythmia and supraventricular tachycardia (VUS) [32233023]

TABLE 3 (Continued)

Gene (transcript): Variant (protein change)	Related phenotypes (OMIM), mode of inheritance	Classification (ACMG criteria)	Previous reports of this variant (classification of the variant in previous study) [PMID reference]
RBM20 (NM_001134363.3): c.773C>T (p.Ser258Leu)	Cardiomyopathy, dilated, 1DD (613172), AD	VUS (PM2, PS4_SUP, BP4)	Hypertrophic cardiomyopathy (VUS) [30847666] Unexplained SCD case with nondiagnostic structural abnormalities of the heart (VUS) [26383259]
RBM20 (NM_001134363.3): c.1244G>A (p.Ser415Asn)		VUS (PM2, BP4, PS4_SUP, PS3_SUP)	Woman with early onset DCM and heart failure (VUS) [ClinVar accession: VCV000548140.4]
SCN5A (NM_001099404.2): c.998+5G>A	Atrial fibrillation, familial, 10 (614022), AD, Brugada syndrome 1 (601144), AD, cardiomyopathy, dilated, 1E (610154), AD, heart block, nonprogressive (113900), AD, long QT syndrome 3 (603830), AD, sudden infant death syndrome (272120), AR	VUS (PP3)	Two female children with LQTS (VUS) [32383558] 29-year-old male with LQT syndrome, positive family anamnesis, and no history of cardiac arrest (VUS) [23631430]
TRPM4 (NM_017636.4): c.2561A>G (p.Gln854Arg)	Progressive familial heart block, type IB (604559), AD	VUS (PS4_SUP, BP4)	53-year-old female with a complete heart block and syncopa (VUS) [29568272]

Abbreviations: AD, autosomal dominant; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilatative cardiomyopathy; HGMD, the human gene mutation database; MOD, moderate; SCD, sudden cardiac death; SUP, supporting.

[a]Present in two cases in our cohort.

TABLE 4 Cohort variants appearing in genes that have been previously reported in stillbirth cases.

Gene (transcript): Variant (protein change)	Related phenotypes (OMIM), mode of inheritance	Classification (ACMG criteria)
CACNA1C (NM_000719.7): c.4790G>A (p.Ser1597Asn)	Brugada syndrome 3 (611875), AD; long QT syndrome 8 (618447), AD	VUS (PM2)
DMD (NM_004006.3): c.11G>C (p.Trp4Ser)	DMD (310200); cardiomyopathy, dilated 3B (611938), X-linked	VUS (PM2, BP1)
DMD (NM_004006.3): c.7093G>A (p.Val2365Ile)	DMD (310200); cardiomyopathy, dilated 3B (611938), X-linked	VUS (PM2, BP1, BP4)
TMEM43 (NM_024334.3): c.413A>G (p.Gln138Arg)	Arrhythmogenic right ventricular dysplasia 5 (604400), AD, Auditory neuropathy, autosomal dominant 3 (619832), AD, Emery-Dreifuss muscular dystrophy 7 (614302), AD	VUS (PM2, BP4)
TMEM43 (NM_024334.3): c.351dupG (p.His118Alafs)	Arrhythmogenic right ventricular dysplasia 5 (604400), AD, Auditory neuropathy, autosomal dominant 3 (619832), AD, Emery-Dreifuss muscular dystrophy 7 (614302), AD	VUS (PM2)

Abbreviation: AD, autosomal dominant.

pathogenic as well (Table S2).[12] The variant identified in our cohort is one with a possible effect on splicing. It has been identified in cases with ARVD, dilatative cardiomyopathy, and arrhythmia, but despite its frequency, its functional impact has not been tested or confirmed. The variant c.2092G>A in *LDB3* has been reported in four different cardiomyopathy patients in previous studies: in a 2-year-old with aborted sudden cardiac death and a severe form of hypertrophic cardiomyopathy,[45] a child with dilated cardiomyopathy,[46] and two other cases with dilated cardiomyopathy.[33,47] Our stillbirth cases all had normal autopsy results; however, this does not exclude the possibility of dilated/hypertrophic cardiomyopathy becoming detectable in the first years of life. Even though these reports do not provide any final conclusions about the variants' pathogenicity, we believe that their repeated occurrence in cardiac disease patients and their relatively low prevalence in the control database point towards their possible clinical significance.

Two additional detected variants have been previously classified as likely or putatively pathogenic in a cardiac-disease related context. The variant *DSP*:c.1696G>A has been considered (likely) pathogenic in two patients clinically and histologically diagnosed with ARVC.[48,49] The variant is located in a mutational hot spot but it has been recently reclassified to VUS due to its population prevalence and in silico predictions,[24] which is in line with our classification. Its functional consequence is unclear, with a functional study reporting no difference with end-binding one protein association compared to a wild type protein but concluding that other adverse effects might contribute to variant expression.[25] A possible explanation for the variant's pathogenicity might be its co-appearance with another

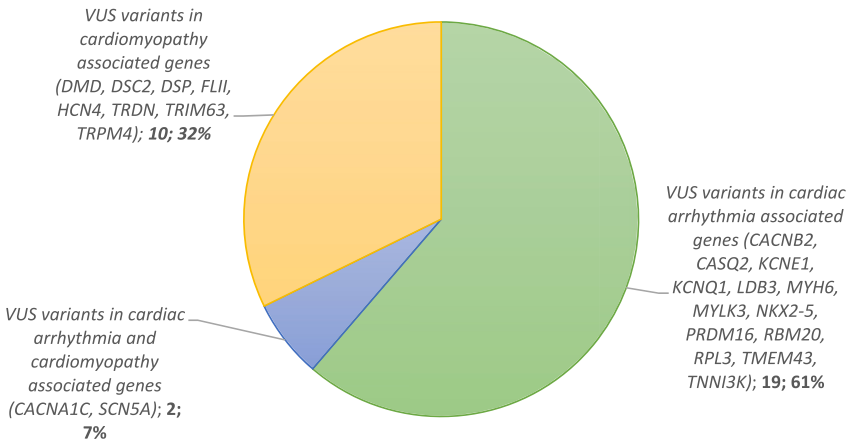

FIGURE 1 Distribution of variants of uncertain significance related to disease group. VUS variants in autosomal dominant genes were grouped according to the disease they were associated with. Most variants (19 variants, 61%) were found in genes associated with cardiac arrhythmia, and 10 variants (32%) were found in genes associated with cardiomyopathy. Two variants were found in genes associated with both of those disease groups (CACNA1C and SCN5A). [Colour figure can be viewed at wileyonlinelibrary.com]

variant in a compound heterozygous state: a detected variant has been reported with another *DSP* variant in an ARVD patient.[49] It is present in the GnomAD database at a frequency of 0.02%, meaning that one in approximately 5.000 people is a carrier of this variant. Since ARVC is a progressive and non-fully penetrant disease with age-dependent clinical expression, it is expected that clinically significant variants might exist in the general population. Nevertheless, future studies might reveal whether this is a rare but benign polymorphism. The stillbirth case in our cohort lacked an additional DSP variant to support this double hit model. The variant c.209G>A in *CACNB2* gene has been previously reported as a likely pathogenic variant in a case of a 42-year- old male who died of sudden death during exercise and had an otherwise normal heart.[49] *CACNB2* gene is linked to Brugada syndrome, a syndrome with a high incidence of sudden death in patients with structurally normal hearts, including infancy and childhood. Importantly, a different missense variant in this gene, c.1439C>T, has been previously reported as a putative pathogenic variant, and an additional one (c.1598C>T) as a VUS in two stillbirth cases.[11]

To conclude, we identified several potentially stillbirth-related variants in our cohort, two of them already previously reported in stillbirth cases, four in severe early onset cardiac phenotype cases, and several others in genes related to cardiac arrhythmias. Studies focused on monogenic causes of unexplained stillbirth are accumulating and international collaboration would significantly enhance data curation and clinical applicability of such analyses.

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CONFLICT OF INTEREST STATEMENT

We have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available in the article and its supplementary materials.

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