

ARTICLE

Genetic investigation of 100 heart genes in sudden unexplained death victims in a forensic setting

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In forensic medicine, one-third of the sudden deaths remain unexplained after medico-legal autopsy. A major proportion of these sudden unexplained deaths (SUD) are considered to be caused by inherited cardiac diseases. Sudden cardiac death (SCD) may be the first manifestation of these diseases. The purpose of this study was to explore the yield of next-generation sequencing of genes associated with SCD in a cohort of SUD victims. We investigated 100 genes associated with cardiac diseases in 61 young (1–50 years) SUD cases. DNA was captured with the Haloplex target enrichment system and sequenced using an Illumina MiSeq. The identified genetic variants were evaluated and classified as likely, unknown or unlikely to have a functional effect. The criteria for this classification were based on the literature, databases, conservation and prediction of the effect of the variant. We found that 21 (34%) individuals carried variants with a likely functional effect. Ten (40%) of these variants were located in genes associated with cardiomyopathies and 15 (60%) of the variants in genes associated with cardiac channelopathies. Nineteen individuals carried variants with unknown functional effect. Our findings indicate that broad genetic investigation of SUD victims increases the diagnostic outcome, and the investigation should comprise genes involved in both cardiomyopathies and cardiac channelopathies.

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INTRODUCTION

Sudden cardiac death (SCD) is responsible for a large proportion of sudden, unexpected deaths in young individuals (≤ 50 years). If the death is unexplained after thorough medico-legal investigation, including autopsy, histopathology and toxicology, the death is referred to as sudden unexplained death (SUD). In Denmark, it has been estimated that 7% of all deaths is due to SCD in the age group 1–35 years.¹ Of the SCDs that were autopsied in that study, 29% remained unexplained after medico-legal investigation.¹ In the age group 1–49 years, the fraction of SCD cases was 11% of all deaths in Denmark.² Generally, SUD is considered to be caused by inherited cardiac diseases, especially cardiac channelopathies such as long QT syndrome, Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia, short QT syndrome, progressive cardiac conduction disorder or early repolarization syndrome, that is, cardiac diseases without morphological abnormalities. Gene mutations associated with the development of cardiomyopathies have also been suspected to be involved in SUD, although the mechanisms are still unknown.^{3–6} In these cases, SUD may represent an early malignant arrhythmogenic presentation – preceding the development of the morphological cardiomyopathy phenotype. Many studies have been done to investigate the genetics in cases of SUD in the young. Most

studies have focused on the cardiac channelopathy-associated genes^{7–11} and only a few have investigated if cardiomyopathy-associated gene mutations might also be involved.^{12–14}

The purpose of this study was to explore the utility of next-generation sequencing (NGS) of a large number of genes associated with both the cardiac channelopathies and cardiomyopathies in a cohort of 61 SUD victims in a forensic setting in Denmark.

MATERIALS AND METHODS

Study population

Between 2009 and 2011, 348 young (1–50 years) deceased individuals, who were assumed to have died from natural causes, were autopsied at the Section of Forensic Pathology, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. Of these, 142 individuals were categorized as SCD, including cases with negative autopsy. In total, 61 individuals had died suddenly, unexpectedly and with no pathogenic findings after thorough macro- and histopathological investigations. Toxicology screening did not show any drug in a toxic level that could explain the death in any of these cases. The remaining 81 cases were examined in earlier studies due to other cardiac causes of death. Twenty individuals were classified as having cardiomyopathies (arrhythmogenic right ventricular cardiomyopathy (ARVC) = 14, hypertrophic cardiomyopathy (HCM) = 6), 52 individuals as

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having non-diagnostic structural abnormalities of the heart¹⁵ and 9 individuals as sudden unexpected death in infancy.¹⁶

Genetic investigation

We have previously described the methods for the genetic investigations in Hertz *et al.*¹⁵ In brief, DNA was extracted from whole blood in 57 of the individuals using the QIAamp DNA Mini Kit (Qiagen, Stockach, Germany). DNA was extracted from fresh frozen muscle from one individual and from fresh frozen spleen tissue from three individuals, using the EZ1 DNA Investigator Kit (Qiagen). The extracted DNA was quantified with the Quantifiler Human DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. DNA from two samples was whole-genome amplified using the REPLI-g Midi kit (Qiagen) following the manufacturer's protocol due to low DNA concentration.

NGS was used to investigate 100 genes previously reported to be associated with inherited cardiac diseases and SCD (Table 1). The genes were selected from the available literature in PubMed and Online Mendelian Inheritance in Man (<http://omim.org/>).

SureDesign (Agilent Technologies, Santa Clara, CA, USA) was used to design capture probes for the target regions of the exons and UTRs of the 100 genes for the Haloplex Target Enrichment system (Agilent Technologies) with 150 bp read lengths. The design included all coding exons, 25 bp of the adjacent introns and 5'- and 3'-UTR regions of the 100 genes of interest to a total of 2076 regions with a total size of 787 943 bp. Overall, 99.6% of the target was covered >50×. Two exons were not covered sufficiently by the sequencing and seven exons were not sequenced due to missing probe designs. Details of

the genomic regions not covered sufficiently is found in Supplementary Table S1. Median coverage for amplicons was 950 (range: 206–3065).

The Haloplex Target Enrichment protocol version D.5 was used according to the manufacturer's instruction. The protocol included the following steps: (1) digestion of 200 ng of genomic DNA with different restriction enzymes in eight tubes and analysis of the fragments using a 2100 Bioanalyzer (Agilent Technologies); (2) hybridization of the digested DNA to Haloplex probes with ends of the probes that were complementary to the fragments of the targets. During hybridization, the fragments were circularized and indexes, sequencing motifs and biotin were incorporated; (3) the target DNA was captured with Haloplex magnetic beads; (4) the nicks in the circularized fragments were closed by ligation; and (5) elution with NaOH, PCR amplification, purification and final elution with Tris-HCl buffer of the captured target DNA was performed. After library build, the amount of DNA was measured using a Qubit Fluorometer 2.0 with the dsDNA HS assay (Invitrogen, Life Technologies Europe BV, Nærum, Denmark). The size distribution of the DNA was analyzed using a 2100 Bioanalyzer and the High Sensitivity DNA kit (Agilent Technologies). All samples were sequenced on a MiSeq (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations with 150 bp paired-end sequencing using the MiSeq Reagent Kit V2 (300 cycles).

Data analysis and bioinformatics

We have previously described the data analysis in details in Hertz *et al.*¹⁵ In brief, fastq files generated by the MiSeq were used for further alignment and analysis. SureCall (Agilent Technologies) was used for post-base-calling analysis

Table 1 Investigated genes and diseases

Disease	Genes	Other associated diseases
ARVC	<i>DSC2</i> (NM_024422.3), <i>DSG2</i> ^a (NM_001943.3), <i>DSP</i> ^a (NM_004415.2), <i>JUP</i> (NM_021991.2), <i>PKP2</i> ^b (NM_004572.3), <i>RPSA</i> (NM_001012321.1), <i>TGFB3</i> (NM_003239.2), <i>TMEM43</i> (NM_024334.2)	^a DCM, ^b BrS
BrS	<i>CACNA1C</i> (NM_199460.3), <i>CACNB2</i> (NM_201596.2), <i>GPD1L</i> ^c (NM_015141.3), <i>HCN4</i> ^d (NM_005477.2), <i>KCND3</i> (NM_004980.4), <i>KCNE3</i> (NM_005472.4), <i>KCNJ8</i> ^e (NM_004982.3), <i>MOG1</i> (NM_016492.4), <i>SCN1B</i> ^{f,g} (NM_199037.3), <i>SCN3B</i> ^f (NM_018400.3), <i>SCN4B</i> ^h (NM_174934.3), <i>SCN5A</i> ^{a,d,e,f,g,h,i} (NM_001099404.1), <i>TRPM4</i> ^g (NM_017636.3)	^a DCM, ^b RBBB, ^c SSS, ^e SIDS, ^f FAF, ^g CCD, ^h LQTS, ⁱ PFVF
CPVT	<i>CASQ2</i> (NM_001232.3), <i>RYR2</i> (NM_001035.2)	ⁱ ARVC
CTD	<i>SLC22A5</i> (NM_003060.3)	
DCM	<i>ABCC9</i> (NM_005691.3), <i>ACTN2</i> ^k (NM_001103.3), <i>BAG3</i> (NM_004281.3), <i>CTF1</i> (NM_001330.3), <i>CRYAB</i> (NM_001289807.1), <i>DES</i> (NM_001927.3), <i>DMD</i> (NM_004006.2), <i>EYA4</i> (NM_172105.3), <i>FHL2</i> (NM_001450.3), <i>FKTN</i> (NM_006731.2), <i>GAA</i> (NM_000152.3), <i>ILK</i> (NM_001014794.2), <i>LAMA4</i> (NM_001105206.2), <i>LDB3</i> ^{k,l} (NM_001171610.1), <i>LMNA</i> ⁱ (NM_170707.2), <i>MYPM</i> ^k (NM_001256267.1), <i>NEBL</i> (NM_006393.2), <i>NEXN</i> ^k (NM_144573.3), <i>PLN</i> ^k (NM_002667.3), <i>PRDM16</i> ^l (NM_022114.3), <i>PSEN1</i> (NM_000021.3), <i>PSEN2</i> (NM_000447.2), <i>RBM20</i> (NM_001134363.2), <i>RPS7</i> (NM_001011.3), <i>SDHA</i> (NM_004168.2), <i>SGCD</i> (NM_000337.5), <i>STARD3</i> (NM_006804.3), <i>TAZ</i> ^l (NM_000116.3), <i>TCAP</i> (NM_003673.3), <i>TMPO</i> (NM_003276.2), <i>TNNC1</i> ^k (NM_003280.2), <i>TNNI3</i> ^k (NM_000363.4)	^f FAF, ⁱ ARVC, ^k HCM, ^l LVNC
FAF	<i>CACNA1D</i> (NM_000720.3), <i>GJA5</i> (NM_005266.6), <i>HCN1</i> ^d (NM_021072.3), <i>KCNA5</i> (NM_002234.3), <i>NPPA</i> (NM_006172.3), <i>RANGRF</i> (NM_016492.4), <i>SCN2B</i> (NM_004588.4)	^d SSS
FD	<i>GLA</i> (NM_000169.2)	
HCM	<i>ACTC1</i> ^{a,j} (NM_005159.4), <i>ANKRD1</i> (NM_014391.2), <i>CALR3</i> (NM_145046.4), <i>CAV3</i> ^m (NM_001234.4), <i>CSRP3</i> ⁿ (NM_003476.4), <i>JPH2</i> (NM_020433.4), <i>LAMP2</i> (NM_001122606.1), <i>MYBPC3</i> ^{a,j} (NM_000256.3), <i>MYH6</i> ^{a,d} (NM_002471.3), <i>MYH7</i> ^{a,j} (NM_000257.2), <i>MYL2</i> (NM_000432.3), <i>MYL3</i> (NM_000258.2), <i>MYLK2</i> (NM_033118.3), <i>MYOZ2</i> (NM_016599.4), <i>PRKAG2</i> ⁿ (NM_016203.3), <i>SLC25A4</i> (NM_001151.3), <i>TNNT2</i> ^{a,j} (NM_001276345.1), <i>TPM1</i> ^{a,j} (NM_000366.5), <i>TTM</i> ⁿ (NM_001267550.1), <i>VCL</i> ^a (NM_014000.2)	^a DCM, ^d SSS, ^l LVNC, ^m LQTS, ⁿ WPW
LQTS	<i>AKAP9</i> (NM_005751.4), <i>ANK2</i> (NM_001148.4), <i>CALM1</i> ^{e,p} (NM_006888.4), <i>CALM2</i> ^{e,p} (NM_001743.4), <i>CALM3</i> ^e (NM_005184.2), <i>KCNE1</i> (NM_000219.5), <i>KCNE1L</i> ^f (NM_012282.2), <i>KCNE2</i> ^f (NM_172201.1), <i>KCNE4</i> (NM_080671.3), <i>KCNH2</i> ^e (NM_000238.3), <i>KCNJ2</i> ^{e,o} (NM_000891.2), <i>KCNJ5</i> (NM_000890.3), <i>KCNQ1</i> ^{f,o} (NM_000218.2), <i>SNTA1</i> (NM_003098.2)	^e SIDS, ^f FAF, ^e SQTS, ^p CPVT
LVNC	<i>DTNA</i> (NM_001390.4)	
PFVF	<i>DPP6</i> (NM_130797.3)	

Abbreviations: ARVC, arrhythmogenic right ventricular cardiomyopathy; BrS, Brugada syndrome; CCD, cardiac conduction disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; CTD, carnitine transporter deficiency; DCM, dilated cardiomyopathy; ERS, early repolarization syndrome; FAF, familial atrial fibrillation; FD, Fabry disease; HCM, hypertrophic cardiomyopathy; LL, Lenegre-Lev syndrome; LQTS, long QT syndrome; LVNC, left ventricular non-compaction; PFVF, paroxysmal familial ventricular fibrillation; RBBB, right bundle branch block; SIDS, sudden infant death syndrome; SSS, sick sinus syndrome; SQTS, short QT syndrome; WPW, Wolff-Parkinson-White Syndrome.

using default settings and their standard algorithm. A complete list of identified variants was given in Variant Caller Format.

Alamut Batch v.1.2.0 and Alamut Visual v.2.5 (Interactive Biosoftware, Rouen, France) were used for the annotation and evaluation of variants as described by Hertz *et al.*¹¹ The variants were evaluated by (1) an in-house *in silico* analysis tool developed for determining the likelihood of variant pathogenicity and (2) frequencies of <1% in two reference population databases (the Exome Variant Server (ESP) (<http://evs.gs.washington.edu/EVS/>) and dbSNP (build 137; <http://www.ncbi.nlm.nih.gov/snp/>)) and associations with cardiac diseases reported in HGMD.¹⁷

The *in silico* analysis was based on the parameters of conservation (Grantham distance, AlignGVGD class, BLOSUM62 and orthologues), computational prediction (MAPP and SIFT), frequency in the relevant reference population (dbSNP (build 137) and ESP), the location in the genome (distance to nearest

splice site) and the coding effect. The available databases and literature were also reviewed for each variant to determine their possible effects.¹⁸ Evidence for pathogenicity included null variants (nonsense, frameshift, splice site and initiation codon), known disease-causing amino-acid changes or residues, functional studies, prevalence of the variants in affected individuals with the associated disease, location in exon and/or functional domain with known disease-causing variants, assumed *de novo*, co-segregation studies and a minor allele frequency below the disease prevalence.^{18–20} Given that rare variants are also subject to population stratification,²¹ we included a local Danish reference population ($n = 2000$) that was whole-exome sequenced, of which one half was comprised of metabolically healthy individuals and the other half was patients with type 2 diabetes.²² The variants were finally classified as likely, unknown or unlikely to have a functional effect by two medical doctors (CLH and MD).

A variant was classified as being of unknown significance, in case of frequency above the disease-prevalence according to available literature, non-conserved amino-acid substitution, multiple observations of the variant in the cohort or lack of significant association with any cardiac disease and nucleotides in the regions, in which the variant was found.

Genes and DNA variants were numbered according to the reference sequences applied in Table 1, using HGVS nomenclature (www.HGVS.org). Variants were submitted to the Leiden Open Variation Database (<http://databases.lovd.nl/shared/individuals>, individuals IDs: 00064702–00064741).

Coverage was calculated using a python script that calculates coverage for each amplicon using Bedtools coverageBed. The amplicons where all samples had <50× coverage were extracted to an excel file. The input was BAM files of each sample and a BED file containing all the amplicons from the SureDesign (Agilent Technologies) of the 100 genes.

The software R v.2.11.0²³ was used to perform the statistical analyses and the scoring of variants in the *in silico* analysis using a custom script.

Ethical standards

The study was approved by the Committees on Health Research Ethics in the Capital Region of Denmark (H-2-2012-017) and the Danish Data Protection Agency (2011-54-1262).

RESULTS

The study population

We included 61 individuals, who had died suddenly and unexpectedly with no pathogenic findings after thorough medico-legal investigations and were classified as SUD. The median age at the time of death was 39 years (range 1–50) and 43 (70%) were males (Table 2). Post-mortem toxicological screenings were performed in 54 (89%) of the cases of which 16 (30%) were negative. No drug was found in a toxic concentration that could explain the death in any of the cases.

Two deceased individuals (3%) had a family history of cardiac disease. The father of one individual died at the age of 56 years of unspecified cardiac cause. No variant with likely functional effect was found in this individual. Two close relatives of the other individual died during sleep at a young age with no recognized cause. In this individual, two variants with likely functional effects were found (Table 3, ID 7).

In our study, 16 (26%) of the SUD cases had psychiatric diseases according to the available medical records. Seven (13%) and eight (15%) of the deceased used antipsychotics or antidepressants, respectively. According to the available summary of product characteristics, the antipsychotics and antidepressants used by the deceased had a very low or unknown frequency of QT_c prolongation. Further, 14 (23%) of the SUD cases suffered from epilepsy and 10 (19%) had one or more antiepileptic drug(s) in the blood (Table 2). None of the drugs used to treat epilepsy had a known frequency of QT_c prolongation according to the available summary of product characteristics.

Table 2 Descriptive data of the cohort

Characteristics	Number	(N = 61)
Gender (male)	43	(70%)
Median age at time of death (years)	39	(Range 1–50)
Median BMI	25	(Range 17–45) ^a
Witnessed death	9	(15%)
Family history of SCD	2	(3%)
<i>Event at death</i>		
At rest	17	(28%)
Sleep	15	(25%)
Physical exercise	6	(10%)
Unknown	23	(38%)
<i>Symptoms immediately before death</i>		
Vomiting	1	(2%)
Fever	1	(2%)
Chest pain	2	(3%)
Dyspnea	1	(2%)
Palpitation	1	(2%)
<i>Post-mortem toxicology^b</i>		
Negative	16	(30%) ^c
Antiepileptic drugs	10	(19%) ^c
Anxiolytics/hypnotics	9	(17%) ^c
Antidepressant	8	(15%) ^c
Antipsychotics	7	(13%) ^c
Illegal drugs ^d	6	(11%) ^c
Strong analgesics	2	(4%) ^c
Other	27	(50%) ^c
<i>Medical history</i>		
Psychiatric disease ^e	16	(26%)
Epilepsy	14	(23%)
Diabetes mellitus	5	(8%)
Known cardiac problems ^f	4	(7%)
Hypertension	4	(7%)
Cerebral disorders	3	(5%)
Asthma	1	(2%)
Dyspnea on exertion	1	(2%)
Hepatitis C	1	(2%)

Abbreviations: BMI, body mass index; SCD, sudden cardiac death.

^aExcluding four children (ages 1 (2), 3 and 11 years).

^bNo drug was found in toxic level that could explain the cause of death.

^cPercentage of cases with post-mortem toxicology screening.

^dTetrahydrocannabinol (THC), cocaine, gamma-hydroxybutyrate (GHB), amphetamine or anabolic steroids.

^eSchizophrenia, psychosis, bipolar disorder or depression.

^fThird-degree AV block, tachycardia, cardiac arrest with seizures, syncope, ventricular extrasystole and unspecified cardiac diseases.

Table 3 Variants with likely functional effects

ID	Sex	Age	Gene	Function	Coding	Nuclotide	Evidence			
					effect	variant	AA variant	MAF	rsID	(primary literature)
1	F	1	<i>RYR2</i> (NM_001035.2)	Intracellular calcium homeostasis	Missense	c.1358G>C	p.(S453T)	0.03% ^a	rs761911597	44
2	M	3	<i>KCNH2</i> (NM_000238.3)	Potassium channel	Missense	c.442C>T	p.(R148W)	0.05% ^a	rs139544114	
3	F	11	<i>DSP</i> (NM_004415.2)	Desmosomal	Nonsense	c.2836G>T ^b	p.(E946*)	—	—	
4	M	15	<i>CACNB2</i> (NM_201596.2)	L-type calcium channel	Missense	c.641G>C	p.(S214T)	0.08% ^a	rs149253719	45
			<i>MYBPC3</i> (NM_000256.3)	Sarcomeric	Missense	c.649A>G	p.(S217G)	0.1% ^c	rs138753870	30
5	F	15	<i>KCNH2</i> (NM_000238.3)	Potassium channel	Missense	c.526C>T	p.(R176W)	—	rs36210422	46
6	M	22	<i>CACNB2</i> (NM_201596.2)	L-type calcium channel	Missense	c.1883A>C ^b	p.(H628P)	—	—	
7	F	24	<i>SCN5A</i> (NM_001099404.1)	Sodium channel	Missense	c.496G>A	p.(A166T)	0.01% ^a	rs201232332	
			<i>LMNA</i> (NM_170707.2)	Nuclear	Missense	c.1256G>A ^b	p.(R419H)	—	—	
8	M	26	<i>SCN5A</i> (NM_001099404.1)	Sodium channel	Missense	c.1127G>T ^b	p.(R376L)	—	—	
9	F	28	<i>MYBPC3</i> (NM_000256.3)	Sarcomeric	Missense	c.2992C>G	p.(Q998E)	—	rs11570112	47
10	M	35	<i>MYBPC3</i> (NM_000256.3)	Sarcomeric	Missense	c.3742G>A	p.(G1248R)	0.04% ^c	rs202147520	28
11	F	36	<i>KCNE2</i> (NM_172201.1)	Potassium channel	Missense	c.29C>T	p.(T10M)	0.03% ^a	rs199473648	48
12	M	37	<i>RYR2</i> (NM_001035.2)	Intracellular calcium homeostasis	Missense	c.12430C>A ^b	p.(R4144S)	—	—	
13	F	39	<i>KCNJ8</i> (NM_004982.3)	Potassium channel	Nonsense	c.1066C>T ^b	p.(R356*)	—	—	
14	M	41	<i>RYR2</i> (NM_001035.2)	Intracellular calcium homeostasis	Missense	c.13931A>G ^b	p.(Y4644C)	—	—	
15	M	42	<i>KCNQ1</i> (NM_000218.2)	Potassium channel	Missense	c.514G>A	p.(V172M)	—	rs199472694	49
			<i>TTN</i> (NM_001267550.1)	Sarcomeric	Nonsense	c.38356A>T ^b	p.(K12786*)	—	—	
16	F	45	<i>PKP2</i> (NM_004572.3)	Desmosomal	Missense	c.184C>A	p.(Q62K)	0.03% ^c	rs199601548	50
17	M	45	<i>SCN5A</i> (NM_001099404.1)	Sodium channel	Missense	c.1019G>A ^b	p.(R340Q)	—	—	29
18	F	46	<i>MYBPC3</i> (NM_000256.3)	Sarcomeric	Missense	c.442G>A	p.(G148R)	—	rs397516050	27
			<i>TNNC1</i> (NM_003280.2)	Sarcomeric	Missense	c.433G>A	p.(D145N)	0.01% ^c	rs142759728	
19	F	46	<i>SCN5A</i> (NM_001099404.1)	Sodium channel	Missense	c.6046G>A	p.(V2016M)	—	rs762981322	51
20 ^{d,e}	M	48	<i>DTNA</i> (NM_001390.4)	Dystrophin complex	Missense	c.257C>T ^b	p.(S86F)	—	—	
21 ^{d,e}	M	49	<i>KCNQ1</i> (NM_000218.2)	Potassium channel	Missense	c.592A>G	p.(I198V)	—	rs199472700	52

Abbreviations: AA, amino acid; F, female; M, male; MAF, minor allele frequency.

Clinical data and genetic variants with likely functional effects, including details of the functions of the gene, nucleotide- and amino-acid change and frequency in a healthy population.

^aMinor allele frequency in a Danish population study.²²

^bNovel variant.

^cMinor allele frequency in European-American populations in the NHLBI GO Exome Sequencing Project (ESP) (<http://evs.gs.washington.edu/EVS/>).

^dDNA purified from spleen samples.

^eDNA was whole genome amplified.

Variants with a likely and unknown functional effect

In total, 21 (34%) individuals had 25 genetic variants that were classified as having likely functional effects (Table 3). Ten of these variants were novel. The median age of individuals carrying variants with a likely functional effect was 36 years (range 1–49) and included 11 males (52%). Of the variants with a likely functional effect, 22 were missense mutations and 3 were nonsense mutations (Table 3). Ten (40%) and 15 (60%) variants were located in genes previously having been reported to be associated with cardiomyopathies or cardiac channelopathies, respectively. Seven of the individuals who had variants with likely functional effects also had variants of unknown functional effect. Further, 19 individuals only carried genetic variants of unknown functional effect (data on unknown variants are found in the Supplementary Table S2). In the remaining 21 (34%) individuals, no variant with likely or unknown functional effect was found.

Physical activity at the time of death

Six (10%) of the SUD cases died during the physical activity. Of these, four had a variant with a likely functional effect. A 39-year-old female collapsed while walking and had a variant with a likely functional effect in *KCNJ8* (c.1066C>T, p.(R356*)). She had no prior reported symptoms of cardiac disease. Two individuals died while diving: a 46-year-old female, who had one variant with a likely functional effect in *MYBPC3* (c.442G>A, p.(G148R)) and another in *TNNC1* (c.433G>A, p.(D145N)), and a 35-year-old male with a variant with

likely functional effect in *MYBPC3* (c.3742G>A, p.(G1248R)). The two divers had not reported any prior symptoms indicating cardiac disease. A 15-year-old male collapsed suddenly during soccer while running. He had one variant with a likely functional effect in *CACNB2* (c.641G>C, p.(S214T)) and another in *MYBPC3* (c.649A>G, p.(S217G)). He had previously experienced two syncope events during soccer and was under examination for cardiac disease when he died. The ECG showed ventricular extrasystoles, but no diagnosis had been established.

DISCUSSION

We sequenced 100 cardiac disease-associated genes in 61 Danish cases of SUD using NGS. In 21 (34%) of the individuals, one or more variants with a likely functional effect were identified. Of the 21 individuals, 12 had variants with likely functional effects exclusively in genes that have previously been associated with cardiac ion channel diseases. Six individuals had variants with likely functional effects exclusively in genes that have previously been associated with cardiomyopathic diseases. Three individuals had variants with likely functional effects in both channelopathy- and cardiomyopathy-associated genes.

The frequency of variants in cardiomyopathy-associated genes was higher than expected. Cardiomyopathies are often variable in expression and with incomplete penetrance, and the initial phenotypic alterations may not be visible at autopsy or may be considered

unspecific or within the normal range. However, variants previously reported to be associated with cardiomyopathies and structural alterations of the heart may give rise to arrhythmia – and in some cases mediated through cardiac channel dysfunctions. This is supported by functional studies of desmosomal genes, previously demonstrated to be associated with ARVC.^{3–6} The *PKP2* gene was first shown to be associated with ARVC, when Gerull *et al*²⁴ found 25 variants in *PKP2* in 32 ARVC patients. Variants in *PKP2* have been found in ~70% of all familial ARVC cases.^{4,25} Recently, *PKP2* was found to be associated with BrS with no structural abnormalities of the heart.^{3,4} Cerrone *et al*⁵ found *PKP2* variants in 2.5% of the investigated BrS patients. In that study, *PKP2* was therefore suggested to play a role in the sodium channel trafficking to the intercalated disk through an unknown mechanism.³ Zhang *et al*⁴ found equal numbers of *PKP2* variants in ARVC patients and SUD cases with negative autopsy, and concluded that sudden death could be due to arrhythmia via reduced localization of the Nav1.5 channel proteins to the intercalated disk and, hence, increase the distance between the plus end of the microtubules and the N-cadherins midline plaque. Variants in *DSP* and *DSG2*, which are also part of the cardiac desmosomal complex, have similarly been found to be associated with Nav1.5 dysfunction in mice and HL-1 cells, respectively.^{5,6} Also, sarcomeric gene mutations were present in sudden infant death syndrome (SIDS) cases, in which no structural change was seen. Brion *et al*²⁶ found multiple, previously described variants in sarcomeric genes in 286 SIDS victims. They suggested that variants in sarcomeric genes could be the cause of death in these infants even though the infants had no structural changes of the heart.

In the present study, we found two-thirds (40 individuals) of the SUD cases had variants with unknown functional effects or no variants with likely or unknown functional effects. These individuals' cause of death still remain unanswered and raises new questions; are there other genes or regulatory elements (methylation, miRNA, etc) that are only found in SUD cases, and never presents themselves in individuals that are living with a heart disease? It would be of interest to investigate these cases further with whole-genome sequencing and investigate epigenetic factors.

Physical activity at the time of death

Six (10%) of the SUD cases died during the physical activity, including walking, diving, biking or playing soccer. Four of these six individuals had variants with likely functional effects, whereof three were in *MYBPC3*. A 46-year-old female diver had a *MYBPC3* variant (c.442G>A) previously observed in an HCM patient.²⁷ A 35-year-old male diver had a *MYBPC3* variant (c.3742G>A) previously observed in another HCM patient²⁸ and classified as probably pathogenic.²⁹ A 15-year-old athlete football player had a *MYBPC3* variant (c.649A>G) previously identified in a study, where the variant was found in two SIDS cases and described as possibly pathogenic.²⁶ In another study, the variant was found in a 19-year-old female, who experienced cardiac arrest after running and was subsequently diagnosed with HCM.³⁰ Furthermore, the variant was described by the same study as above by Andreasen *et al*,²⁹ who found this variant to be of low frequency and possibly pathogenic. The three individuals with *MYBPC3* variants with a likely functional effect were all performing moderate to heavy exercise at the time of death.

Sudden unexpected death in psychiatric disease and epilepsy

In the present SUD cohort, 16 (26%) of the SUD cases had psychiatric diseases according to the available medical records (Table 2). One or more variants with a likely functional effect were found in five (31%)

of the individuals with a psychiatric disease. The variants were found in the *SCN5A*, *KCNQ1*, *TTN* and *LMNA* genes. The prevalence of SUD in the young with depression has not been widely studied. It is known that the life expectancy in Denmark in schizophrenic patients is 16–20 years shorter than that of the general population.³¹ In a recent study from Australia, Sweeting *et al*³² found that the most prevalent cause of death in schizophrenic patients was cardiovascular disease (23%) and that 11% of the deaths were unexplained after autopsy.

The underlying causes of SCD and SUD in psychiatric diseases are not yet established, but drug-induced arrhythmia has been proposed to be important in depression³³ and schizophrenia.^{34–37}

Furthermore, 14 (23%) of the SUD cases suffered from epilepsy and 10 (19%) had one or more antiepileptic drug(s) in the blood. Sudden unexpected death in epilepsy (SUDEP) is defined as sudden, unexpected, witnessed or unwitnessed, non-traumatic and non-drowning death in patients with epilepsy with or without evidence for seizure with exclusion of documented status epilepticus and when post-mortem examinations do not reveal any structural or toxicological causes of death.³⁸ In line with our findings, a Danish study by Holst *et al*³⁹ identified 166 SUD cases in the period 2000–2006. Among these, 26 cases were diagnosed as definite SUDEP giving a total percentage of epilepsy cases of 16% in their SUD cohort. SUDEP is suspected to be multifactorial and may relate to autonomic dysfunction, abnormalities in heart rate variability or catecholamine surge, antiepileptic medications and underlying cardiac arrhythmias.^{40,41} Risk factors for SUDEP in patients treated with antiepileptic medication include poor patient compliance, abrupt withdrawal, poly-pharmacy and specific antiepileptic drugs. Some antiepileptic drugs have been suspected to be associated with a negative effect on cardiac conduction and may induce sodium channel blockage or dose-dependently suppress the autonomic cardiac modulation.^{41,42} In our study, 3 of the 14 SUDEP cases (21%) had one or more variants with likely functional effects. These variants were identified in genes involved in the inwardly-rectifying potassium channel coded by the *KCNH2* and in a gene involved in the desmosomal complex (*DSP*) that is associated with the Nav1.5 channel function. In an Australian study, *KCNH2* and *SCN5A* mutations were found in 13% of the SUDEP cases.⁴³

There are some limitations to this study. First of all, this is a highly selected cohort potentially reducing the generalizability of our findings. To investigate the variants with a likely functional effect further, functional studies and co-segregation analyses in the families should be performed. The genetic findings in this study cannot stand alone to determine the cause of death, as many of the variants identified are novel and need to be investigated further.

In summary, we found that one-third of the deceased individuals with negative forensic autopsy had one or more variants with a likely functional effect in genes associated with cardiomyopathies and cardiac channelopathies. Furthermore, 19 and 21 individuals were found to have only variants of unknown significance or no variants of significance, respectively. Our findings suggest that broad genetic screening using NGS is a useful diagnostic part of the forensic investigation in SUD cases, in which the cause of death cannot be established after routine medico-legal autopsy. As a significant proportion of the variants with a likely functional effect were found in genes previously found to be associated with cardiomyopathies, we suggest that genetic investigations of SUD victims should comprise genes involved in both the structural and non-structural cardiac diseases. Furthermore, the two-thirds of individuals where no variant with likely functional effect was found make us wonder if there are other genes or regulatory elements that are yet to be discovered in these unexplained sudden death cases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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