

Next-generation sequencing of 34 genes in sudden unexplained death victims in forensics and in patients with channelopathic cardiac diseases

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Abstract Sudden cardiac death (SCD) is responsible for a large proportion of sudden deaths in young individuals. In forensic medicine, many cases remain unexplained after routine postmortem autopsy and conventional investigations. These cases are called sudden unexplained deaths (SUD). Genetic testing has been suggested useful in forensic medicine, although in general with a significantly lower success rate compared to the clinical setting. The purpose of the study was to estimate the frequency of pathogenic variants in the genes most frequently associated with SCD in SUD cases and compare the frequency to that in patients with inherited cardiac channelopathies. Fifteen forensic SUD cases and 29 patients with channelopathies were investigated. DNA from 34 of the genes most frequently associated with SCD were

captured using NimbleGen SeqCap EZ library build and were sequenced with next-generation sequencing (NGS) on an Illumina MiSeq. Likely pathogenic variants were identified in three out of 15 (20 %) forensic SUD cases compared to 12 out of 29 (41 %) patients with channelopathies. The difference was not statistically significant ($p=0.1$). Additionally, two larger deletions of entire exons were identified in two of the patients (7 %). The frequency of likely pathogenic variants was >2-fold higher in the clinical setting as compared to SUD cases. However, the demonstration of likely pathogenic variants in three out of 15 forensic SUD cases indicates that NGS investigations will contribute to the clinical investigations. Hence, this has the potential to increase the diagnostic rate significantly in the forensic as well as in the clinical setting.

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Introduction

Sudden cardiac death (SCD) is a major cause of unexpected, non-traumatic deaths in young and apparently healthy individuals. The major aetiologies of SCD include cardiomyopathies, channelopathies and ischaemic heart diseases. In about two thirds of SCD cases, structural abnormalities are evident at autopsy [1–3], whereas no structural pathogenic variation of the heart is found in the remaining cases. These cases are called sudden unexplained deaths (SUD). Many of the SUD cases are suspected to be caused by inherited cardiac channelopathies [4] among which the most common ones are long QT syndrome (LQTS), Brugada syndrome (BS), catecholaminergic polymorphic ventricular tachycardia (CPVT) and short QT syndrome (SQTS). More than 100 genes have been

shown to be associated with inherited SCD [5–8]. With modern DNA technology like next-generation sequencing (NGS), it is possible to investigate large numbers of genes and thus support the clinical investigation and increase the possibility to establish a likely cause of death. This may be of importance for legal reasons but may also be valuable information when considering screening of family members for the same disease.

In the forensic setting, SUD is a major challenge. Investigation of pathogenic variants in genes associated with cardiac diseases is possible, but the diagnostic yield is not well known. The aim of this study was to estimate the frequency of pathogenic variants in 34 of the genes most frequently associated with channelopathic diseases, comparing SUD cases and patients with clinically diagnosed inherited cardiac channelopathies.

Material and methods

Study population

A total of 15 SUD cases from the Section of Forensic Pathology, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, were investigated. The individuals were <50 years of age and randomly selected if (i) deceased within a time period of 1 year (2009–2010), (ii) they had died suddenly, unexpectedly and with no diagnosis after thorough macro- and microscopic investigations by the forensic pathologists and (iii) the toxicology screenings were negative. The SUD cases included 11 males with a median age of 38 years (range 3–49) and four females with a median age of 19 years (range 1–42). The majority, 12 (80 %), was last seen alive and healthy less than 24 h prior to the death and none had a known family history of SCD.

A total of 29 clinically affected patients from the Department of Cardiology, National University Hospital, Rigshospitalet, Copenhagen, Denmark, were included. Twelve (41 %) patients had LQTS, nine (31 %) Brugada syndrome, one (3 %) SQTS and seven (24 %) non-diagnostic phenotypes with symptoms and findings suggestive of channelopathic diseases. The median age at time of diagnosis was 36 years (8–64) with 22 (79 %) under the age of 50 years. A total of 17 (59 %) were males. Family history of SCD was reported in 10 (34 %) patients (Table 1).

Genetic investigation of single nucleotide variants and large insertion-deletions

DNA was extracted from anti-coagulated whole blood using the QIAamp DNA Mini Kit (Qiagen, Germany) and quantified with the Quantifiler Human DNA Quantification Kit

(Thermo Fisher, USA) following the manufacturer's protocol. The 15 SUD samples were investigated with the AmpF/STR Identifiler PCR Amplification Kit (Thermo Fisher, USA) to measure the degree of DNA degradation. Full STR profiles were found, thus no SUD sample was degraded. Two patient samples were whole genome amplified due to low DNA concentrations, using the REPLI-g Midi kit (Qiagen, Germany) following the manufacturer's protocol.

A total of 34 genes (*ABCC9*, *AKAP9*, *ANK2*, *CACNA1C*, *CACNB2*, *CASQ2*, *CAV3*, *DPP6*, *GJA5*, *GPD1L*, *HCN4*, *KCNA5*, *KCNE1*, *KCNE1L*, *KCNE2*, *KCNE3*, *KCNE4*, *KCNH2*, *KCNJ2*, *KCNJ5*, *KCNJ8*, *KCNQ1*, *MYH6*, *MYH7*, *NPPA*, *PRKAG2*, *RYR2*, *SCN1B*, *SCN2B*, *SCN3B*, *SCN4B*, *SCN5A*, *SNTA1* and *TRPM4*) were investigated using NGS. All genes have been reported to be associated with inherited cardiac diseases often presented with arrhythmia. The genes were selected from the literature and Online Mendelian Inheritance in Man (OMIM) [9]. The gene *MYH7*, which is primarily associated with structural cardiac diseases, was included as a result of the similarity to *MYH6* and the capture design. The total length of the target regions was 387,395 bp and involved 639 target regions. Target regions included exons, 250 bp of adjacent introns and 5'- and 3'-UTR regions. Targeted sequencing capture probes were custom designed by Roche NimbleGen (USA). Library preparation was performed by using the TruSeq LT DNA Sample Preparation Guide (Illumina, USA). This protocol involved the steps "Fragment Genomic DNA" to "Ligate Indexed Paired-End Adapters". Following this, the preparation of the libraries were continued using the NimbleGen SeqCap EZ Library SR User's Guide v4.0 (Roche, Germany) according to the manufacturer's recommendations. After library build, the samples were analysed on Agilent's 2100 Bioanalyzer and the DNA concentrations were measured using the Qubit 2.0 Fluorometer (Thermo Fisher, Germany). Libraries were sequenced on an Illumina MiSeq using the MiSeq Reagent kit v2 (500 cycles) with 250-bp paired-end sequencing.

Genetic investigations of large insertion-deletions (indels) in *KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2* and *SCN5A* were performed with multiplex ligation-dependent probe amplification (MLPA) [10] using pre-designed kits (SALSA MLPA P114 Long-QT probe mix, SALSA MLPA P108 SCN5A probe mix (MRC-Holland, Holland). The analyses were performed according to the protocol provided by the supplier. In brief, the procedure involved denaturation of the DNA, hybridisation, ligation reaction, PCR and fragment separation by means of electrophoresis. Each sample was investigated in duplicate. If a rearrangement was observed, a third investigation was performed. Only concordant results were reported.

Data analysis and annotation

MiSeq Reporter (Illumina, USA) was used for the analysis of fastq-files generated by the MiSeq with default settings for

Table 1 Characteristics of cardiac patients

		All	LQTS/SQTS	BS	Unknown phenotype
<i>N</i>		29	13	9	7
Sex	Male	17 (59 %)	4 (31 %)	7 (78 %)	6 (86 %)
	Female	12 (41 %)	9 (69 %)	2 (22 %)	1 (14 %)
Age of diagnosis	Median	37	31	41	45
	Range	8–64	8–64	17–61	33–59
	<50 years	21 (72 %)	10 (77 %)	6 (67 %)	3 (43 %)
Symptoms at onset	Chest pain	11 (38 %)	3 (23 %)	7 (78 %)	1 (14 %)
	Dyspnoea	2 (7 %)	1 (8 %)	1 (11 %)	0
	Dizziness	5 (17 %)	3 (23 %)	1 (11 %)	1 (14 %)
	Palpitation	5 (17 %)	4 (31 %)	1 (11 %)	0
	Syncope	14 (48 %)	8 (61 %)	4 (44 %)	2 (29 %)
	Other arrhythmias	5 (17 %)	0	3 (33 %)	2 (29 %)
	VF/TdP	11 (38 %)	5 (38 %)	2 (22 %)	4 (57 %)
	Cardiac arrest	9 (31 %)	3 (23 %)	1 (11 %)	5 (71 %)
Family history of SD ^a		10 (34 %)	7 (54 %)	1 (11 %)	2 (29 %)
ICD treatment		17 (59 %)	8 (61 %)	6 (67 %)	4 (57 %)

LQTS long QT syndrome, SQTS short QT syndrome, BS Brugada syndrome, VT ventricular fibrillation, TdP Torsades de Pointes, SD sudden death, ICD implantable cardioverter-defibrillator

^aBased on anamnestic information from the patients

PCR amplicon analysis. The human genome sequence UCSC/hg19 [11] was used as reference sequence. The following steps were performed using the MiSeq Reporter: Reads were aligned to the reference genome using BWA [12]. Variant calling was carried out using The Genome Analysis Toolkit (GATK) [13]. Aligned reads were provided in BAM-files (Binary compressed SAM format) and lists of variants in variant caller format (VCF). A total of 98.4 % of the target regions were covered more than 10 times. The median coverage for all regions and samples was 1216 (range 619–3191). In total, all exons in the investigated 34 genes were sequenced except for exon 1 and the 5'-UTR region in *SCN1B*. Moreover, the first exons and 5'-UTR regions of *GPD1L*, *KCNH2*, *SCN5A* and *SNTA1* were not sufficiently covered (maximum coverage <50). Variants were included according to the following criteria: (1) passing the MiSeq Reporter quality filter on default settings based on coverage depth, variant score, mapping quality and quality score and (2) the coverage was above 50.

Alamut-HT v. 1.1.8 (Interactive Biosoftware, France) and Alamut v.2.2 (Interactive Biosoftware, France) was used for the annotation and evaluation of variants. In brief (Fig. 1), variants were selected by (1) frequency in reference populations [14, 15] and associations to cardiac diseases in HGMD [16], (2) in silico analysis of the likelihood of pathogenicity (Tables 2 and 3) and (3) evaluation and classification of each variant by two medical doctors. Variants were classified as likely pathogenic or of unknown significance based on the allele frequency of the variant, segregation evidence, in vitro

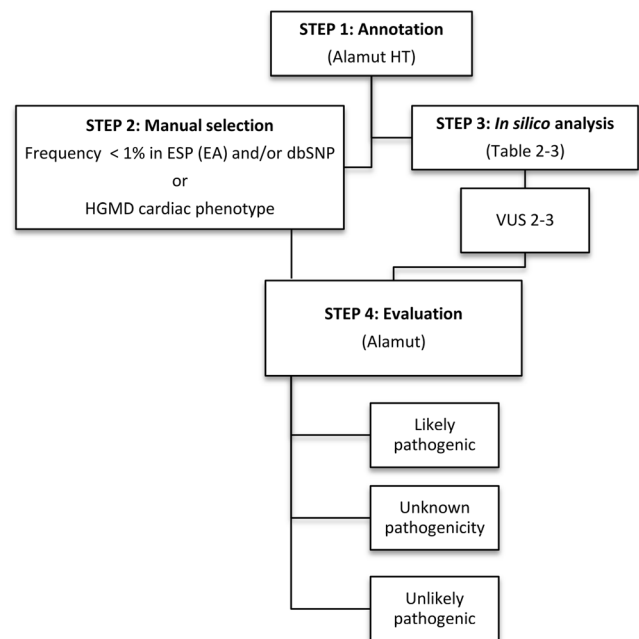


Fig. 1 Selection and evaluation of variants. After annotation (step 1), variants were selected manually (step 2) or through the in silico analysis (step 3). Using Alamut (Version 2.2, Interactive Biosoftware, LLC, France), variants were evaluated in terms of clinical relevance (step 4) and categorised as “likely pathogenic”, “unknown pathogenicity” or “unlikely pathogenic”. *ESP* (EA) minor-allele frequency in European American population in NHLBI GO Exome Sequencing Project (ESP) [14]; *dbSNP* variation global minor allele in the single nucleotide polymorphism database [15], *HGMD* Human Gene Mutation Database [16], *VUS* variant of unknown significance

Table 2 In silico analysis scoring scheme for missense and nonsense variants

Parameter		Score
1. MAPP prediction	Bad	1
	Good	0
2. SIFT prediction	Deleterious	1
	Tolerated	0
3. Grantham distance	>140	2
	71–140	1
	≤70	0
4. AlignGVGD class	C65	1.25
	C55	1
	C45	0.75
	C35	0.5
	C21/C25	0.25
	C0	0
5. BLOSUM62	≤−2	1
	−1	0.5
	≥0	0
6. Conservation between species (calculated percentage between conserved residues in alignment and number of orthologous in alignment)	100 %	1
	90 %	0.75
	50 %	0.5
	<50 %	0
7. Frequency in dbSNP	0 < freq ≤ 0.02	1.5
	0.02 < freq ≤ 0.05	1
	0.05 < freq ≤ 0.1	0.5
	>0.1	0
8. Frequency in ESP (EA)	0 < freq ≤ 0.02	1.5
	0.02 < freq ≤ 0.05	1
	0.05 < freq ≤ 0.1	0.5
	>0.1	0
9. Distance to nearest splice site (bp)	−2 ≥ SS ≤ 2	1
	−5 ≥ SS ≤ −3	0.5
	5 ≤ SS ≤ 3	0.5
	−5 < SS > 5	0
10. Coding effect	Frameshift or nonsense	4

The table shows the parameters of the in silico analysis and the scoring scheme. The parameters were based on conservation and the biophysical alterations of the amino acid substitutions, frequencies of the variants in a control population and splice site predictions. Parameters 1–9 were used for missense and in-frame variants and parameters 7–10 were used for nonsense and frameshift variants

MAPP prediction multivariate analysis of protein polymorphism prediction, *SIFT prediction* sorting intolerant from tolerant prediction, *BLOSUM62* Blosum62 score, *Frequency in dbSNP* variation global minor allele in the single nucleotide polymorphism database [15], *Frequency in ESP (EA)* minor-allele frequency in European American population in NHLBI GO Exome Sequencing Project (ESP) [14]

functional studies, number of affected individuals found with the variant, computational prediction of the functional effect of amino acid substitutions and status as a de novo mutation.

Table 3 Sub-classifications of variants based on *in silico* analysis

VUS classification	Percentage of maximum possible score
VUS3	≥70
VUS2	45–69
VUS1	25–44
VUS0	<25

Based on the scores given in the in silico analysis, all variants were sub-classified as VUS 0, 1, 2 or 3 with an increasing prognostic severity
VUS variant of unknown significance

The algorithm used for in silico analysis was inspired by van Spenonck-Zwarts et al. [17].

The fragment lengths of the MLPA investigations were analysed with GeneMapper v. 4.0 (Thermo Fisher, USA) and visual inspection of the electropherograms. Probe ratios below 0.7 or above 1.3 were regarded as heterozygous deletions or duplications, respectively.

The software, R v. 2.11.0 [18] was used to perform the statistical analysis of medians, means and standard deviations. The genetic results of the two investigated groups were compared with Fisher's exact test.

Ethical standards

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

Results

Possibly pathogenic variants in the SUD victims and cardiac patients

Among the 44 investigated samples, the mean number of variants per sample that passed the MiSeq Reporter quality filter was 528 (s.d. 124). After selection of candidate variants by the process described in Fig. 1 and Tables 2 and 3, a mean of 40 (s.d. 8) possibly pathogenic variants per sample were further evaluated and classified.

Among the 15 SUD victims, three likely pathogenic variants including one novel, were identified in three (20 %) cases (Table 4). No larger rearrangement (indels) was found. In 12 (41 %) of the 29 patients, 18 likely pathogenic variants including three novel, were identified. Large deletions were found in a Brugada patient (*SCN5A* deletion in exon 23) and a LQTS patient (*KCNQ1* deletion in exon 7, 8 and 9). The coverage in the deleted areas was approximately 50 % of the average coverage. Both deletions were verified with MLPA.

Table 4 Specifications of likely pathogenic and novel variants identified in the study

Case	Disease	Gender	Age at diagnosis/SUD	Symptoms at onset	Gene	Nucleotide change	Amino acid change	Coding effect	In silico analysis	MAPP	SIFT
2	BS	M	61	Cardiac arrest	SCN5A	1403C>T	P468L	Missense	VUS3	Bad	Deleterious
5	BS	M	37	Chest pain during physical exercise	SCN5A	5228G>A	G1743E	Missense	VUS3	Bad	Deleterious
7	BS	M	37	Palpitations, ventricular extrasystoles	MYH6	4136C>T	T1379M	Missense	VUS3	Bad	Deleterious
8	BS	M	27	Syncope	TRPM4	2740A>T	K914*	Nonsense	VUS3	NA	NA
9	BS	M	17	Syncope	SCN5A	Exon 23	Deletion	–	–	–	–
10	LOTIS	F	64	Dyspnoea, chest pain	SCN5A	361C>T	R121W	Missense	VUS3	Bad	Deleterious
12	LQTS	M	47	VF, cardiac arrest	GJA5	286G>T	A96S	Missense	VUS3	Bad	Deleterious
13	UP	F	41	Chest pain, syncope (QTc 460–500 ms)	NPPA	190A>G	S64R	Missense	VUS1	Good	Tolerated
16	LQTS	F	17	Syncope (QTc 480 ms)	KCNQ1	Exon 7,8,9	Deletion	–	–	–	–
17	LQTS	F	32	Chest pain (QTc 460–480 ms)	AKAP9	6176A>G	E2059G	Missense	VUS3	Bad	Deleterious
18	LQTS	M	8	Syncope (QTc 460 ms)	KCNH2	1286del	A429Vfs*5	Frameshift	VUS3	NA	NA
19	LQTS	F	33	Syncope in association with audio triggers (QTc 540–600 ms)	HCM4	2275G>A	V759I	Missense	VUS2	Bad	Deleterious
21	LQTS	F	19	Syncope in association with audio triggers (QTc 550 ms)	KCNH2	326T>C	L109P	Missense	VUS2	Bad	Deleterious
22	LQTS	F	20	Syncope, chest pain, palpitations (QTc 520 ms)	KCNH2	526C>T	R176W	Missense	VUS2	UK	Deleterious
30	SUD	M	3	CP, epilepsy, died during sleep	KCNQ1	1096C>T	R366W	Missense	VUS3	Bad	Deleterious
37	SUD	M	48	Unwitnessed (<24 h)	KCNH2	301A>G	K101E	Missense	VUS1	Good	Deleterious
38	SUD	M	37	Unwitnessed (<24 h)	KCNH2	1286del	A429Vfs*5	Frameshift	VUS3	NA	NA
22	LQTS	F	20	Syncope, chest pain, palpitations (QTc 520 ms)	RYR2	8162T>C	I2721T	Missense	VUS3	Bad	Deleterious
30	SUD	M	3	CP, epilepsy, died during sleep	KCNH2	1640A>G	N547S	Missense	VUS2	Bad	Deleterious
37	SUD	M	48	Unwitnessed (<24 h)	KCNH2	442C>T	R148W	Missense	VUS2	UK	Deleterious
38	SUD	M	37	Unwitnessed (<24 h)	SNYAI	770C>G	A257G	Missense	VUS2	Bad	Deleterious
21	LQTS	F	19	Syncope in association with audio triggers (QTc 540–600 ms)	RYR2	12430C>A	R4144S	Missense	VUS3	Bad	Deleterious

Case	Grantham distance	AGVGD class	BLOSUM62	Conservation	Frequency in ESP (EA)	Frequency in dbSNP	Distance to splice site (bp)	dbSNP ID	HGMID ID	Classification
2	98	C15	-3	90	NA	NA	65	–	–	Likely pathogenic
5	98	C65	-2	100	NA	NA	415	rs199473629	CM023682	Likely pathogenic
81	81	C65	-1	100	0.001	NA	-40	rs145611185	–	Likely pathogenic
7	NA	NA	NA	NA	0.001	0.002	-39	rs140799936	CM131518	Likely pathogenic
8	–	–	–	–	–	–	–	–	Likely pathogenic	Likely pathogenic
9	101	C65	-3	100	NA	NA	-32	rs199473556	CM095355	Likely pathogenic
99	99	C65	1	100	NA	0.001	319	rs121434557	CM068637	Likely pathogenic
110	110	C0	-1	75	0.002	0.001	67	rs61757261	CM109498	Likely pathogenic
10	–	–	–	–	–	–	–	–	Likely pathogenic	Likely pathogenic
12	98	C65	-2	100	0.0008	0	-35	–	–	Novel
13	NA	NA	NA	NA	NA	NA	158	–	CD097837	Likely pathogenic
29	29	C25	3	88	0.002	0.003	132	rs62641689	CM134354	Likely pathogenic
98	98	C0	-3	86	NA	NA	19	–	–	Likely pathogenic
101	101	C0	-3	71	NA	NA	54	rs36210422	CM001198	Likely pathogenic

Table 4 (continued)

Case	Grantham distance	AGVGD class	BLOSUM62	Conservation	Frequency in ESP (EA)	Frequency in dbSNP	Distance to splice site (bp)	dbSNP ID	HGMD ID	Classification
18	101	C65	-3	100	NA	NA	-33	rs199473411	CM981130	Likely pathogenic
19	56	C0	1	79	NA	NA	-7	rs199472856	CM013272	Likely pathogenic
21	NA	NA	NA	NA	NA	NA	158	-	CD097837	Likely pathogenic
89	64	C65	-1	100	0.0005	NA	33	rs201500134	-	Novel
22	64	C0	0	93	NA	NA	-105	rs199472944	CM970803	Likely pathogenic
46	46	C45	1	100	NA	NA	28	-	-	Novel
30	101	C0	-3	71	0.002	NA	-31	rs139544114	CM096514	Likely pathogenic
37	60	C0	0	91	0.003	0.001	69	rs56157422	CM087843	Likely pathogenic
38	110	C65	-1	100	NA	NA	468	-	(CM087241)	Novel

The table shows detailed information of the likely pathogenic and novel variants found in the study

In silico analysis sub-classification based on *in silico analysis*, *MAPP* multivariate analysis of protein polymorphism prediction, *SIFT* sorting intolerant from tolerant prediction, *Grantham* Grantham distance, *AGVGD class* AlignGVGD class, *BLOSUM62* Blosu62 score, *Conservation* calculated percentage between conserved residues in alignment and number of orthologous in alignment, *Frequency in ESP (EA)* minor-allele frequency in European American population in NHLBI GO Exome Sequencing Project (ESP) [14], *Frequency in dbSNP* variation global minor allele in the single nucleotide polymorphism database [15], *Distance to splice site (bp)* distance to nearest splice site in bp, *dbSNP ID* reference SNP id number in dbSNP, *HGMD ID* Human Genome Mutation Database ID [16], *Classification* classification after evaluation based on *in silico analysis*, available databases and literature, *LQTS* long QT syndrome, *UP* unknown phenotype, *SQTS* short QT syndrome, *BS* Brugada syndrome, *SUD* sudden unexplained death, *VUS* variant of unknown significance, *VF* ventricular fibrillation, *QTc* corrected QT interval, *CP* cerebral palsy, *UK* unknown

No other insertion or deletion of large gene segments was observed. Details of all possibly pathogenic variants are described in Table 4. The difference in the number of observed likely pathogenic variants in the SUD cases and the patients was not statistically significant ($p=0.1$).

Variants of unknown significance

In all of the SUD victims and in 26 (90 %) of the patients, variants previously associated with cardiac diseases but with an unknown significance, were identified (Supplementary material online, Table S1). Two previously identified variants (K1459N and A1777T in MYH7) in patients diagnosed with hypertrophic cardiomyopathy (HCM) were found in two of our BS patients. Both variants were categorised as being of unknown significance because of the phenotypic variation between the patients.

Discussion

With NGS, we identified likely pathogenic variants in three of the 15 SUD victims (20 %). In the patients, the corresponding frequency was 41 %. In addition, two larger deletions of whole exons were identified in the patient cohort, increasing the diagnostic rate of the genetic investigation to 48 %. The frequency of identified likely pathogenic variants in the clinically diagnosed patients was >2-fold compared with that of the SUD victims, although not statistically significant ($p=0.1$). The difference is most likely due to the reduced possibility of phenotypic characterisation of the forensic victims [19].

Previous genetic studies of SUD cases showed pathogenic variants in 11–17 % of the deceased [20–22] when investigating up to five genes associated with channelopathies (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*). However, Tester et al. [4] found putative pathogenic variants in 26 % of the individuals in the same five genes when investigating a large cohort comprising 173 SUD victims. In the 26 % with a pathogenic variant, the median age was 16 years. The corresponding number in our study was 37 years. Moreover, Tester et al. [4] found a higher frequency of pathogenic variants in the female SUD cases compared to that of the male SUDs (39 and 18 %, respectively). In our study, the males constituted 11 of the 15 SUD cases. Disease-causing variants in patients diagnosed with LQTS, BS and CPVT were previously identified in 19–50 % of the investigated cases [23–27]. None of the studies were performed with NGS and the number of investigated genes was smaller.

Compared to previous studies with similar cohorts, we found higher frequencies of likely pathogenic variants in both deceased and living patients when investigating 34 genes associated with channelopathies. This suggests that the gene

panel used in this study is relevant in both clinical and forensic investigations. Due to the wide screening, we found that five of the cardiac patients carried several likely pathogenic variants in two or more genes. This may result in a compound effect, worsening the phenotypic trait of the disease. We also observed an overlap in the established phenotypes and the identified genotypes, specifically two BS diagnosed patients were identified with two variants previously found in patients diagnosed with hypertrophic cardiomyopathy [28, 29]. This could suggest that the diseases are expressed differently among individuals or not related to the described phenotypes and low frequent variants. In both the clinical and forensic settings, many cases have unspecific symptoms or presentation that makes it impossible to establish a clear phenotypic diagnosis. Hence, a wider genetic screening would be preferable in these cases and could contribute to the clinical investigation. Papadakis et al. [30] evaluated family members of victims of either sudden arrhythmic death syndrome (SADS) ($n=163$) or with unspecific abnormalities of the heart ($n=41$). They found a similar proportion (47 and 51 %, respectively) of channelopathic diseases in the relatives of the two groups. The predominant diagnoses were LQTS and BS in both cohorts. This demonstrates that there is diversity in both phenotypic and genotypic expression and that we should also include genes associated with structural cardiac diseases when investigating arrhythmogenic diseases.

We found a high frequency of variants associated with a cardiac disease with unknown significance. The variants were present in ESP and dbSNP in higher frequencies than the expected phenotype prevalence or present in several individuals in our study cohort. Similarly, Andreasen et al. [31] recently investigated the frequency of previously cardiomyopathy-associated variants in population-based exome data. They found a significant overrepresentation of variants previously thought to be pathogenic in the control population which indicated that the genetic variants may not be disease-causing but may have a modifying effect or may even be benign.

To establish the real pathogenicity of the described variants in this study, family co-segregation studies and functional testing should be performed. Non-coding and regulatory regions were not analysed unless they had previously been found to be associated with a cardiac disease or symptom. A large amount of genetic information that may be of importance has not yet been published. For future work, focus ought to be extended to the intronic and UTR regions in order to fully understand the genetics behind the diseases causing SCD. Also, epigenetic factors may have a possible influence on the pathogenesis and the medical relevance of epigenetic regulation remains largely unexplored in cardiac pathology [32].

This study shows that NGS is a valuable tool in a postmortem forensic setting with enhanced results to previous first-

generation sequencing studies. Additionally, it is possible to use NGS to detect large deletions of entire exons. As described above, we found inconsistencies between phenotypes and genotypes, suggesting that there is an overlap among the channelopathic and cardiomyopathic diseases. For future studies, wide genetic screening of both channelopathic and cardiomyopathic associated genes should be performed in suspected SCD victims to fully understand the phenotype-genotype correlation.

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