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ORIGINAL RESEARCH ARTICLE

Molecular Diagnosis of Inherited Cardiac Diseases in the Era of Next-Generation Sequencing: A Single Center's Experience Over 5 Years

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Abstract

Background and objective Molecular diagnosis in inherited cardiac diseases is challenging because of the significant genetic and clinical heterogeneity. We present a detailed molecular investigation of a cohort of 4185 patients with referrals for inherited cardiac diseases.

Methods Patients suffering from cardiomyopathies (3235 probands), arrhythmia syndromes (760 probands), or unexplained sudden cardiac arrest (190 cases) were analyzed using a next-generation sequencing (NGS) workflow based on a panel of 105 genes involved in sudden cardiac death.

Results (Likely) pathogenic variations were identified for approximately 30% of the cohort. Pathogenic copy number variations (CNVs) were detected in approximately 3.1% of patients for whom a (likely) pathogenic variation were identified. A (likely) pathogenic variation was also detected for 21.1% of patients who died from sudden cardiac death. Unexpected variants, including incidental findings, were present for 28 cases. Pathogenic variations were mainly observed in genes with definitive evidence of disease causation.

Conclusions Our study, which comprises over than 4000 probands, is one of most important cohorts reported in inherited cardiac diseases. The global mutation detection rate would be significantly increased by determining the putative pathogenicity of the large number of variants of uncertain significance. Identification of "unexpected" variants also showed the clinical utility of genetic testing in inherited cardiac diseases as they can redirect clinical management and medical resources toward a meaningful precision medicine. In cases with negative result, a WGS approach could be considered, but would probably have a limited impact on mutation detection rate as (likely) pathogenic variations were essentially clustered in genes with strong evidence of disease causation.

Key Points

(Likely) pathogenic variations were identified for approximately 30% of the cohort of 4185 patients.

Pathogenic CNVs was detected in approximately 3.1% of patients for whom a (likely) pathogenic variation were identified.

Identification of "unexpected" variants showed the clinical utility of genetic testing in inherited cardiac diseases as they can redirect clinical management including therapeutic approaches.

Pathogenic variations were mainly observed in genes with definitive evidence of disease causation.

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1 Introduction

Sudden cardiac death (SCD) is defined as a "natural death due to cardiac causes, heralded by abrupt loss of consciousness within 1 h of the onset of acute symptoms" [1, 2]. It represents a major public health issue in industrial countries, with an incidence rate ranging from 18.6 to 128 cases/100,000 inhabitants/year, and can occur at any age [3]. In patients aged 35 years and more, coronary artery disease and ischemic cardiomyopathy are the most frequent causes [4]. In younger patients, however, a significant number of SCD cases may be attributed to inherited cardiac diseases, such as cardiomyopathies and arrhythmia syndromes [1].

Cardiomyopathies are a large group of diseases that could be divided into five different subgroups, according to the World Health Organization: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), left ventricular non-compaction (LVNC), and a large group of unclassified cardiomyopathies [5]. Cardiomyopathies are a significant public health issue as they are highly prevalent. The prevalence has been estimated to be between 1:500 for HCM and 1:2500 for DCM, but this is likely underestimated [6, 7]. More recently, using different approaches, the prevalence has been reevaluated and estimated to be in the range of 1:250 for DCM and 1:200 for HCM [8, 9]. They are mainly characterized by an autosomal-dominant mode of inheritance. Over the past decade, molecular genetic diagnosis of cardiomyopathies have identified mutations in more than 50 different genes encoding proteins involved mainly in the sarcomere, but also in the cytoskeleton, in mitochondrial function, and in calcium handling [10–12].

In about 5% of SCD cases, at autopsy, the heart is apparently healthy. More than half of these cases can be explained by channelopathies, which are genetic disorders involving genes encoding either for ion channels or for proteins involved in the formation of action potential [13, 14]. Arrhythmia syndromes are less prevalent than cardiomyopathies. They are divided into four main groups according to electrocardiographic criteria: long QT syndrome (LQTS, prevalence of 1:2000), Brugada syndrome (BrS, prevalence of 1:5000), short QT syndrome (SQTS), and catecholaminergic polymorphic ventricular tachycardia (CPVT). As cardiomyopathies, they are mostly characterized by an autosomal-dominant mode of inheritance and show an important phenotypic, genetic, and allelic heterogeneity [14, 15].

With the progress and the democratization of next-generation sequencing (NGS) approaches, molecular diagnosis is now increasingly requested for these highly prevalent diseases. In this study, based on a previously reported NGS mutation detection strategy, we report our personal 5-year experience of molecular diagnosis performed on a cohort of 4185 patients affected with cardiomyopathies and/or arrhythmia syndromes but also from patients who died from unexplained sudden cardiac arrest [16].

2 Methods

2.1 Patients

The study, which relied on clinical data provided by ordering cardiologists and/or geneticists at the time of testing, was performed on a cohort of 4185 unrelated patients containing either probands suffering from cardiomyopathies and/or arrhythmia syndromes or probands who developed a sudden cardiac arrest and died as resuscitation was ineffective. The clinical diagnostic criteria were established according to international criteria (http://www.escardio.org/Guidelines-&-Education).

Inclusion of some patients was made in slightly permissive conditions as a precise description of the clinical phenotype including classification of the disease (sporadic or familial form), ECG, cardiac imaging (TTE and/or MRI), or familial data was not obtained systematically for each proband. The study was conducted in accordance with the principles of the Declaration of Helsinki and informed consent was obtained for all cases.

2.2 Molecular Study

Genomic DNAs, extracted from a peripheral blood sample, were tested by NGS as previously described [16]. The panel was designed to identify disease-causing variations in 105 SCD-causing genes (Table S1, Online Supplemental Material (OSM)). These genes were initially selected, in 2015, according to the Online Mendelian Inheritance in Man (OMIM) database and/or publications evoking either a robust or a putative implication of a given gene in inherited cardiac diseases. Target regions included coding exons (with a 30 pb padding), 5′- and 3′-UTR regions. Each run allowed the targeted resequencing of 72 patients simultaneously. Pathogenic or likely pathogenic variations were further verified using either conventional dideoxy sequencing using BigDye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) for point mutations and short indels, or quantitative PCR for copy number variation (CNV).

2.3 Bioinformatic Pipeline

Bioinformatics analyses were further performed using a private pipeline developed by Sophia Genetics (Sophia Genetics, Lausanne, Switzerland) [16].

2.4 Variant Classification

Detected variants were classified using VarSome's implementation of ACMG/AMP guidelines [17, 18]. All variations were described with respect to the transcript IDs in Table S1 (OSM). A large number of them were easily classified as previously reported and characterized. For novel null variants (nonsense, frameshift, canonical splice sites, initiation codon, single or multiexon deletion), classification as likely pathogenic was proposed if PVS1 (null variant in a gene where LOF is a known mechanism of disease variants) and PM2 (absent/extremely rare (< 0.004% in the Genome Aggregation Database (gnomAD) criteria could be applied. If only one of these two criteria could be applied, the null variant was classified as a variant of uncertain significance (VUS). For novel missense variants, classification as likely pathogenic was proposed if the following criteria could be applied : PM2 (absent/extremely rare (< 0.004% in gnomAD), and PP1 (cosegregation with disease in more than two affected family members in a gene definitively known to cause the disease), and PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease) and PP3 (multiple lines of computational evidence (PolyPhen-2, SIFT, Mutation Taster, and CADD) support a deleterious effect on the gene). If only three of the four criteria could be applied, the missense variant was classified as a VUS.

3 Results

Genetic testing was performed using a NGS workflow based on a panel of 105 genes known to be involved in sudden cardiac death (Table S1 (OSM)). Coverage analysis showed us that 100% of targeted regions were covered with a threshold limit of 30X coverage for each proband. This cohort, constituted between 2015 and 2020, included 4185 cases (Table 1). Referrals were mostly for carriers of cardiomyopathies: 3235 cases with a cardiomyopathy (77.3%) versus 760 cases with an arrhythmia syndrome (18.2%). Molecular testing was also performed for 190 patients with unexplained death and for whom a cardiac disease was suspected but for whom no autopsy reports were performed (4.5%) (Table 1). Due to the large number of patients included in this study, more precise information about a given patient or a given genetic variation can be obtained upon request (Tables S2–S13 (OSM)).

3.1 Hypertrophic Cardiomyopathy (HCM) Cohort

Our molecular approach allowed us to investigate 1,622 HCM patients. Using a virtual panel including 57 cardiomyopathy-causing genes, 534 cases (32.9%) were genotyped as a carrier of a pathogenic or likely pathogenic gene variant and 495 cases (30.5%) were carriers a VUS.

Among all (likely) pathogenic variants on cardiomyopathy-causing genes, *MYBPC3* variants (295/534; i.e. 55.2%) represent the most prevalent cause of HCM, whereas *MYH7* ones (115/534; i.e. 20.9%) rank second in the pathogenesis (Table 2). As expected, most *MYBPC3* (likely) pathogenic variations were truncating variants (223/295; i.e. 75.6%). For other known prevalent HCM-causing genes, identified (likely) pathogenic variations were almost exclusively missense. Recurrent variants were detected in this cohort, the most frequent being *MYBPC3*-c.1928-2A>G (30 cases) and *MYH7*-p.Thr1377Met (15 cases) (Table 3).

Whereas the initial indication provided by ordering physicians (cardiologists and/or geneticists) was "Hypertrophic Cardiomyopathy," 29 patients (1.8 % of the HCM cohort) were carriers of (likely) pathogenic variants of genes (*GLA, LAMP2, PTPN11, PRKAG2* and *TTR*) associated with HCM phenocopies (Table 4) [19]. Inclusion of these genes in diagnostic panels for HCM is advantageous given their pronounced phenotypic similarity with the classic sarcomeric form and the importance of a prompt differential diagnosis, enabling correct treatment decisions and optimal patient

Table 1 Description of pathologies carried by probands included in the cohort

Pathologies	Cases genotyped
HCM	1622
DCM	1361
LQTS	331
BrS	273
Sudden cardiac death	190
LVNC	130
CCD	75
Other arrhythmia syndromes	72
ARVC	68
RCM	52
CPVT	9
Barth syndrome	2
	4185

ARVC arrhythmogenic right ventricular cardiomyopathy, *BrS* Brugada syndrome, *CCD* cardiac conduction disorder, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LQTS* long QT syndrome, *LVNC* left ventricular non-compaction, *RCM* restrictive cardiomyopathy

Table 2 Distribution of (likely) pathogenic variations among hypertrophic cardiomyopathy patients

	Pathogenic (P) + likely pathogenic (LP)		P	LP
MYBPC3	295	53.73%	235	60
MYH7	115	20.95%	40	75
TNNI3	24	4.37%	12	12
FLNC	20	3.64%	1	19
TNNT2	12	2.19%	5	7
TPM1	6	1.09%	3	3
MYL2	6	1.09%	3	3
MYL3	4	0.73%	2	2
Other CM-causing genes	67	12.20%	37	30

CM cardiomyopathy

management. Most of them were *TTR* variants involved in Transthyretin cardiac amyloidosis (16 cases; 1% of the HCM cohort) and *GLA* involved in Fabry disease (six cases; 0.37% of the HCM cohort).

Other cardiomyopathy-causing genes with a limited or no evidence of HCM association were also analyzed [20]. This led us to highlight patients with variants usually associated with other cardiomyopathies (see OSM). This could be illustrated by detection of likely pathogenic *TTN* truncating variants (main molecular explanation of familial DCM) and pathogenic *PKP2* truncating variants (mostly expected in ARVC patients) in 12 HCM patients [21, 22]. Of note, some cases with HCM referral, with any putatively pathogenic variant in known validated HCM-causing genes, were carriers of missense variants with PM2 and PP3 ACMG criteria. However, as no clear association could be determined between these variants and HCM, they remained classified as VUS.

3.2 Dilated Cardiomyopathy (DCM) cohort

Comprehensive genetic testing of 1361 DCM patients allowed us to report 374 pathogenic or likely pathogenic variants (27.5% of DCM cohort). More than 80% of them were concentrated in eight genes (*TTN, FLNC, LMNA, DSP, MYH7, TNNT2, BAG3, MYBPC3*) (Table 5). As expected, truncating *TTN* variants (TTNtv) were the most prevalent (n = 132/1361; 9.7%). Due to the presence of approximately 0.5–0.7% of TTNtv on healthy populations, only TTNtv found in the regions of the gene included in Cronos titin were considered to be likely pathogenic [23]. With regard to the 18 DCM referrals with TTNtv (1.3% of DCM cohort) located upstream, the internal start site encoding for the isoform Cronos, they remained classified as VUS. Further segregation data are ongoing to better evaluate their pathogenicity. As previously reported, pathogenic and likely pathogenic variants were mostly truncating variants located in *FLNC, DSP, LMNA*, and *BAG3* (n = 88/1361; 6.5%) [24]. Except for variants *TTN*-p.Arg17983* (five cases), *TTN*-p.Arg14454* (four cases), *FLNC*-p.Ser792Ile (three cases), and *FLNC*-p.Arg1354* (three cases), no pathogenic variant was detected more than twice in this cohort. Known DCM variants affecting the RS-domain of RBM20 (five cases),

Table 3 Recurrent pathogenic hypertrophic cardiomyopathy (HCM) variations (identified in more than two HCM cases)

Gene	Nucleotide change	Effect on protein	No. of cases
MYBPC3	c.1928-2A>G	p.?	30
MYH7	c.4130C>T	p.Thr1377Met	15
MYBPC3	c.3732C>A	p.Cys1244*	12
MYBPC3	c.3065G>C	p.Arg1022Pro	11
MYBPC3	c.1624G>C	p.Glu542Gln	7
MYBPC3	c.226C>T	p.Gln76*	7
MYBPC3	c.772G>A	p.Glu258Lys	6
MYBPC3	c.913_914del	p.Phe305Profs*27	6
MYBPC3	c.2441_2443del	p.Lys814del	5
MYBPC3	c.26-2A>G	p.?	5
MYBPC3	c.2258dupT	p.Lys754Glufs*79	5
MYBPC3	c.2827C>T	p.Arg943*	5
MYBPC3	c.2905-280_*485del	deletions exons 27_35	5
MYBPC3	c.655G>T	p.Val219Phe	5
MYBPC3	c.821+5G>A	p.?	5
MYBPC3	c.927-9G>A	p.?	5
MYH7	c.1063G>A	p.Ala355Thr	5
MYH7	c.2389G>A	p.Ala797Thr	5
MYBPC3	c.1483C>G	p.Arg495Gly	4
MYBPC3	c.2308G>A	p.Asp770Asn	4
MYBPC3	c.2373dupG	p.Trp792Valfs*41	4
MYBPC3	c.2670dupG	p.Arg891Alafs*160	4
MYH7	c.2602G>C	p.Ala868Pro	4
TNNI3	c.497C>T	p.Ser166Phe	4
TNNT2	c.388C>T	p.Arg130Cys	4
MYBPC3	c.1227-13G>A	p.?	3
MYBPC3	c.1625-1G>A	p.?	3
MYBPC3	c.2149-2delA	p.?	3
MYBPC3	c.2221dupG	p.Ala741Glyfs*6	3
MYBPC3	c.2308+1G>A	p.?	3
MYBPC3	c.237C>A	p.Tyr79*	3
MYBPC3	c.2905+5G>A	p.?	3
MYBPC3	c.3697C>T	p.Gln1233*	3
MYBPC3	c.821+1G>A	p.?	3
MYH7	c.2156G>A	p.Arg719Gln	3
MYH7	c.3346G>A	p.Glu1116Lys	3
TPM1	c.644C>T	p.Ser215Leu	3

Table 4 Pathogenic (P) or likely pathogenic (LP) variations identified on genes involved in hypertrophic cardiomyopathy phenocopies

Gene	Nucleotide change	Effect on protein	Status	Pathogenicity	No. of cases
GLA	c.644A>G	p.Asn215Ser	Hemizygous	P	2
GLA	c.713G>A	p.Ser238Asn	Hemizygous	P	1
GLA	c.899T>C	p.Leu300Pro	Hemizygous	P	1
GLA	c.901C>T	p.Arg301*	Hemizygous	P	1
GLA	c.317T>C	p.Leu106Pro	Hemizygous	LP	1
LAMP2	c.118_124del	p.Thr40Phefs*7	Hemizygous	P	1
PRKAG2	c.905G>A	p.Arg302Gln	Heterozygous	P	1
PTPN11	c.1507G>A	p.Gly503Arg	Heterozygous	P	1
PTPN11	c.1528C>G	p.Gln510Glu	Heterozygous	P	1
PTPN11	c.1529A>G	p.Gln510Arg	Heterozygous	P	1
PTPN11	c.836A>G	p.Tyr279Cys	Heterozygous	P	1
PTPN11	c.846C>G	p.Ile282Met	Heterozygous	P	1
TTR	c.148G>A	p.Val50Met	Heterozygous	P	2
TTR	c.262A>T	p.Ile88Leu	Heterozygous	P	1
TTR	c.379A>G	p.Ile127Val	Heterozygous	P	1
TTR	c.424G>A	p.Val142Ile	Heterozygous	P	10
TTR	c.191T>A	p.Phe64Tyr	Heterozygous	LP	1
TTR	c.206C>T	p.Thr69Ile	Heterozygous	LP	1

Table 5 Distribution of (likely) pathogenic variations among dilated cardiomyopathy patients

	Pathogenic (P) + likely pathogenic (LP)		P	LP
TTN	132	35.87%	0	132
FLNC	39	10.60%	31	8
LMNA	35	9.51%	24	11
DSP	32	8.70%	27	5
MYH7	25	6.79%	6	19
TNNT2	15	4.08%	10	5
BAG3	13	3.53%	13	0
MYBPC3	13	3.53%	7	6
RBM20	7	1.90%	5	2
Other CM- causing genes	57	15.49%	14	43

or the *SCN5A*-p.Arg222Gln (two cases) were also detected [25, 26].

An overview of other cardiomyopathy-causing genes led us to identify definitive pathogenic variations usually observed either in sarcomeric HCM (truncating *MYBPC3* variants (seven cases); *MYL2*-p.Arg58Gln (one case)) or in HCM phenocopies (*GLA*-p.Ser238Asn involved in Fabry-disease (one case); *TTR*-p.Val142Ile known as the most common pathogenic variant involved in cardiac amyloidosis (one case), *PTPN11*-p.Thr468Met associated with LEOPARD syndrome (one case), or *DES*-p.Glu413Lys associated with myofibrillar myopathy (one case)) [27–31]. In addition, two DCM patients were carriers of hemizygous *EMD* variations for whom pathogenicity was supported by additional clinical and/or functional evidence.

3.3 Other Cardiomyopathies

Although cases with DCM or HCM referrals represented the vast majority, some cases were clinically diagnosed as carriers of other types of cardiomyopathies such as LVNC, RCM, or ARVC.

The spectrum of 45 (likely) pathologic variations identified in the cohort of 130 cases with LVNC referrals is close to the one observed in cases with DCM referrals but with specific genes overrepresented, such as *HCN4* [32, 33].

Among them, *MYH7* missense variants (8.5% of LVNC cohort) represent the most prevalent cause of LVNC, whereas *TTN*tv (6.9% of LVNC cohort) and *HCN4* missense variants (6.2% of LVNC cohort) rank second and third, respectively, in the pathogenesis. Similar to DCM patients, *TTN* tv classified as pathogenic variations were only those identified in the distal I-band and in the A-band regions of titin, which are also the regions included in Cronos titin. As previously reported, *HCN4* variants were mostly identified in patients with a combined clinical presentation of bradycardia and LVNC [34, 35]. More surprisingly, three patients (two children aged 8 and 14 years, respectively, and a woman aged 50 years with a congenital AV block) were genotyped with *NKX2-5* truncating variants, a gene known to be associated with congenital heart disease (CHD) and/or

conduction disorders but not with LVNC [36]. In the same way, two patients (one child aged 10 years and a woman aged 50 years with a congenital AV block) were genotyped with two novel *TBX20* missense variants close to each other: p.Val148Asp and p.Ile152Met. The p.Ile152Met variation was previously reported as associated with a family history of CHD and a complex spectrum of developmental anomalies [37]. These observations strengthen the hypothesis that LVNC could be associated with mutations in various genes involved in myocardial development [38].

Comprehensive genetic testing of 52 RCM patients allowed us to report 20 (likely) pathogenic variants on cardiomyopathy-causing genes (38.5% of RCM cohort). Although relatively small, distribution of genes with (likely) pathogenic variants involved in our RCM cohort were quite similar to other cardiomyopathies except an expected enrichment of *FLNC* (eight cases) variations [39–41]. All *FLNC* variations were missense variations except one truncating variant that was identified in a woman aged 40 years also carrier of a *RBM20* likely pathogenic variation. As previously reported, *FLNC* pathogenic variants (p.Ala1186Val, p.Arg2133Cys) were identified in three pediatric RCM cases [42].

Finally, molecular diagnosis was performed on 68 patients for whom a clinical diagnosis of ARVC was indicated. Using a virtual panel of genes associated with arrhythmogenic cardiomyopathy (ACM), pathogenic or likely pathogenic variations were detected for 14 probands (20.6% of ARVC cohort) [43]. As expected, all mutations, expect one, were detected on desmosomal genes, *PKP2* being the most commonly affected one (ten cases). In a woman aged 25 years a homozygous *PLN* truncating variant (p.Leu39*) was also detected. ARVC diagnosis was based on the 2010 task force criteria [44]. Because of a frank decreased left ventricular ejection fraction, she underwent an implantable cardioverter defibrillator procedure. A similar case was previously reported [45].

3.4 Long QT Syndrome

Three hundred and thirty-five probands with a referral of long QT syndrome (LQTS) were molecularly evaluated. Ninety-six cases (28.7% of LQTS cohort) were carriers of (likely) pathogenic variations on one of the 17 genes reported as being causative for LQTS [46]. As expected, most of them were clustered on three genes: *KCNQ1* (44 cases), *KCNH2* (38 cases) and *SCN5A* (seven cases) (Table 6). Some of them were recurrent as identified in more than two LQTS cases (*KCNQ1*-p.Val254Met; *KCNH2*-p.Ala561Val). The seven remaining cases were carriers of variations affecting either *CACNA1C* (four cases), *KCNJ2* (two cases), or *KCNE1* (one case). The rate of genotype-positive probands was lower than expected as approximately 75% of patients with a clinically certain LQTS diagnosis would have pathogenic variations in one of three major LQTS-causing genes [46]. This difference could be explained by inclusion of patients who were not clinically certain LQTS cases but rather cases with a "suspected" LQTS or carriers of an arrhythmia syndrome; LQTS was the most common inherited arrhythmia. These clinical misdiagnoses could be explained by identification of (likely) pathogenic variations in *KCNJ2* (usually linked to Andersen-Tawil syndrome) or *RYR2* (usually linked to CPVT) but also in genes predisposing to arrhythmogenic cardiomyopathy such as *DSP, HCN4,* or *TTN* [47, 48].

3.5 Brugada Syndrome

Genetic testing of 273 probands with a referral of Brugada syndrome (BrS) was performed. Thirty-eight cases (13.9% of BrS cohort) were carriers of a pathogenic or likely pathogenic variation on *SCN5A*, which is the only gene classified as definitive BrS-causative [49]. Truncating *SCN5A* variants were observed for 16 cases with BrS referrals. Among 40 minor genes associated with BrS, ten additional putative pathogenic variations were identified including two *TTN* variants and one *DSP* truncating variant more commonly detected in cases with DCM referrals. Other putative pathogenic variants will necessitate further segregation and/or in vitro analysis to definitely validate their pathogenicity.

3.6 Other Arrhythmia Syndromes

Although LQTS or BrS represented the vast majority of referrals, some cases were also carriers of other types of arrhythmia syndromes caused by abnormalities in the generation or conduction of electrical impulses or both.

An initial sub-group of 75 patients with cardiac conduction disorder was analyzed. Six pathogenic variations were identified. All were truncating variants affecting progressive cardiac conduction disease susceptibility genes (*LMNA, SCN5A, NKX2-5*) [50]. A novel putative pathogenic missense variation (p.Asp155Tyr) was identified in *SCN2B*. *SCN2B* is not known to be implicated in cardiac conduction disorder but it could be considered as a valuable gene

Table 6 Distribution of (likely) pathogenic variations among long QT syndrome (LQTS) patients

	Pathogenic (P) + likely pathogenic (LP)		P	LP
KCNQ1	44	45.83%	34	10
KCNH2	38	39.58%	34	4
SCN5A	7	7.29%	4	3
Other LQTS-causing genes	7	7.29%	4	3

candidate as pathogenic variations have previously been reported in other genes (*SCN5A, SCN1B*) encoding subunits of cardiac sodium voltage-gated ionic channels. Validation of the pathogenicity for this *SCN2B* variant will necessitate further segregation and/or in vitro analysis. The presence of *TTN* and *PKP2* truncating variants, more commonly detected on DCM and ARVC cases, respectively, were also observed in this sub-group.

The second sub-group consisted of 72 cases with arrhythmia syndrome without an accurate clinical diagnosis. Using a virtual panel including 48 arrhythmia-causing genes, 13 cases (23.6%) were genotyped as a carrier of a (likely) pathogenic variant among which there were four pathogenic *KCNJ2* variations.

3.7 Sudden Cardiac Death

A molecular study was performed on a cohort of 190 patients who died from SCD without clinical data. This approach led us to identify (likely) pathogenic variants for 40 patients (21.1% of SCD cases): 27 on cardiomyopathy-causing genes and 13 on arrhythmia-causing genes. Of these, 30 were in the following genes: *MYBPC3* (six cases), *RYR2* (five cases), *PKP2* (four cases), *FLNC* (three cases), *KCNH2* (three cases), *LMNA* (three cases), *SCN5A* (three cases), and *TTN* (three cases). These data confirm than an extended molecular analysis with multi-phenotype genetic testing can identify a concealed cardiomyopathy or arrhythmia syndrome, and increase the diagnosis rate for clinically idiopathic SCA survivors [51].

3.8 Copy Number Variations (CNVs)

Before the NGS era, methods to detect CNV were rarely performed. Now, routine use of NGS methods in diagnosis, allowing simultaneous detection of CNV, single-nucleotide variations, and short indels is a real improvement in medical care. Global CNV analysis allowed the detection of 25 pathogenic or likely pathogenic CNVs (Table 7). Their identification allowed us to provide a genotype-positive status for 35 patients: 21 with cardiomyopathies, 12 with arrhythmia, and two with sudden cardiac death. A (likely) pathogenic CNV was identified in approximately 0.9% of patients included in our cohort, which is equivalent to 3.1% of patients for whom a (likely) pathogenic variation were identified. The frequency of CNVs among cases of sudden unexplained death, patients with a cardiomyopathy, or patients with an arrhythmia syndrome was 1.1% (2/190), 0.7% (22/3233), and 1.6% (12/755), respectively. Detection rates were lower than previously reported [52].

The most recurrent pathogenic CNV was a *MYBPC3* large deletion (g.47309385_47312889del) identified in five cases with HCM referrals [53]. A total heterozygous *PKP2* gene deletion was also identified for four patients with cardiomyopathies. A total homozygous *PKP2* gene deletion, associated with a neonatal severe cardiomyopathy with a left ventricular non compaction phenotype, was also detected [54].

3.9 Unexpected Identified Pathogenic Variations

To promote standardized reporting of actionable information from clinical genomic sequencing, the American College of Medical Genetics and Genomics published a minimum list of 59 genes to be reported as incidental or secondary findings [55]. Twenty of them are associated with inherited cardiac diseases. As an adaptation of these guidelines, individuals with cardiomyopathies were screened for secondary findings in the arrhythmia and vice versa. This screening allowed us to complete our molecular diagnosis for 28 patients (0.7% of the cohort) (Table 8). *PKP2* truncating variants were detected in 12 patients with non-ARVC referrals: six HCM, two DCM, two LVNC, one RCM, and one CCD. Conversely, (likely) pathogenic variations affecting prevalent LQTS genes (*KCNQ1, KCNH2, and SCN5A*) were identified in five patients with cardiomyopathies. Pathologic truncating variants in genes associated with dilated cardiomyopathy (*TTN, BAG3,* and *DSP*) were identified in seven patients with arrhythmia syndromes including three cases with LQTS referrals. Only three probands were carriers of a (likely) pathogenic variant that could explain the phenotype related to the referral (Table 8).

3.10 Homozygous Variations

Hereditary forms of cardiomyopathies or arrhythmia syndromes are mostly associated with an autosomal dominant form of inheritance. However, X-linked and autosomal recessive forms can also occur. In some cases, homozygous mutations may be present in genes typically associated with autosomal dominant inheritance, often leading to a more severe phenotype. Our cohort led us to detect 14 cases with homozygous mutations (Table 9). Homozygous *CSRP3* variants were identified on three cases with HCM referrals, reinforcing the hypothesis that *CSRP3* variants could result in HCM with an autosomal recessive inheritance rather than with an autosomal dominant transmission [56]. A similar hypothesis could be evoked for *MYLK2*, a gene categorized as having limited evidence of HCM causation as our molecular study also led to detection of, for the first time, a homozygous *MYLK2* variant (p.Pro57Thr) for one of our HCM referrals [20]. However, this novel missense variation remains a VUS as prediction software classified it as benign. In the context of cardiomyopathies, two homozygous *MYH7* missense variations were also identified:

Table 7 Pathogenic copy number variations (CNVs) identified in the cohort

Gene	CNV	Pathology	No. of cases
BAG3	g.121422264_121431805del	DCM	1
DSP	total gene deletion	DCM	2
FHL1	exons 3_8 deletion	RCM	1
FLNC	total gene deletion	HCM	1
KCND3	total gene duplication	Arrhythmia syndrome	1
KCNH2	exons 5_11 deletion	LQTS	1
KCNH2	exons 5_15 deletion	LQTS	1
KCNH2	total gene deletion	LQTS	2
KCNQ1	exon 2 deletion	LQTS	1
KCNQ1	exon 7 deletion	LQTS	2
KCNQ1	exons 7_ 10 deletion	LQTS	1
LMNA	exons 1_2 deletion	DCM	1
LMNA	total gene deletion	DCM	1
LMNA	g.156102440_156108545del	DCM	1
MYBPC3	total gene deletion	Sudden cardiac death	1
MYBPC3	g.47309385_47312889del	HCM	5
NKX2-5	g.172660719_172662184del	CCD	1
PKP2	exon 11 deletion	Arrhythmia syndrome	1
PKP2	exons 13_14 deletion	Sudden cardiac death	1
PKP2	exon 8 deletion	HCM	1
PKP2	total gene deletion	HCM	5[a]
TTN	exons 192_245 deletion	BrS	1
TTN	exons 272_313 deletion	DCM	1

BrS Brugada syndrome, *CCD* cardiac conduction disorder, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LQTS* long QT syndrome, *RCM* restrictive cardiomyopathy

[a]Including one case with a total homozygous *PKP2* gene deletion (LVNC with neonatal severe cardiomyopathy)

p.Arg807His detected in a 49-year-old woman with DCM and p.Arg1050Gln detected in 7-year-old boy with LVNC.

4 Discussion

Based on an NGS approach, a cohort of 4185 patients affected with either cardiomyopathies or arrhythmia syndromes was tested. This NGS workflow allowed us to detect simultaneously point mutations such as single nucleotide variations (SNVs), short indels, but also large rearrangements such as CNV. Using this strategy, SCD-causing genes of 72 cases can be explored in approximately 2 weeks and clearly represents a sensitive, specific, and high-capacity low-cost mutation detection method (approximately 130 €/patient).

A vast majority of our genetic testing requests were for cardiomyopathies (77.3%) rather than for arrhythmia syndromes (18.2%). This disequilibrium is not surprising as prevalence of HCM or DCM are higher than prevalence of LQTS or BrS. Moreover, 190 genetic testing were also performed for patients who died suddenly due to a suspected cardiac disease but for whom no autopsy was performed (4.6%). Although always requested, a precise description of the clinical phenotype including classification of the disease (sporadic or familial form), ECG, cardiac imaging (TTE and/or MRI), or familial data was not obtained systematically for each proband. Consequently, we could not exclude the possibility that some patients were either misdiagnosed or have benefited from a molecular diagnosis when it was not justified (over-referral). Overall, even in these slightly permissive conditions, our custom panel allowed us to identify a (likely) pathogenic variation for approximately one-third of the cohort (28.3%).

More interestingly, it also allowed us to identify unexpected variants such as (i) pathogenic variations in arrhythmia-causing genes for patients with cardiomyopathies, (ii) pathogenic variations in cardiomyopathy-causing genes for patients with arrhythmia syndromes, and (iii) pathogenic variations in HCM-mimicking genes for patients initially only diagnosed as suffering from HCM. These results showed once again the clinical utility of genetic testing in CV diseases, as identification of these "unexpected" variants can redirect clinical management and diagnostic and

Table 8 Unexpected pathogenic variations identified in our cohort

Unexpected pathogenic variation			Referral	Other detected (likely) pathogenic variant that could explain the phenotype
Gene	Nucleotide change	Effect on protein		
TTN	c.90085C>T	p.Arg30029*	AS	No
MYH7	c.2609G>A	p.Arg870His	ARVC	No
MYH7	c.2782G>A	p.Asp928Asn	ARVC	No
TTN	c.93376delA	p.Arg31126Glyfs*26	BrS	No
TTN	exons 192_245 deletion	CNV	BrS	No
PKP2	c.1643del	p.Gly548Valfs*15	CCD	No
TTN	c.57154_57157del	p.Asp19052Metfs*62	CCD	No
DSG2	c.630delG	p.Phe211Serfs*3	DCM	No
KCNQ1	c.569G>A	p.Arg190Gln	DCM	No
KCNQ1	c.1031C>T	p.Ala344Val	DCM	*TTN*_p.Trp21446*
PKP2	c.2146-1G>C	p.?	DCM	*MYBPC3*_p.Leu66Pro
PKP2	c.2553_2562delinsA	p.Glu852_Leu879delins27*	DCM	No
KCNE1	c.292C>T	p.Arg98Trp	HCM	No
KCNH2	c.2768del	p.Pro923Argfs*51	HCM	No
PKP2	c.2148dupG	p.Pro717Alafs*26	HCM	No
PKP2	c.219_223+5delCAACGGTGAG	p.?	HCM	No
PKP2	c.2489+1G>A	p.?	HCM	No
PKP2	c.2578-2A>C	p.?	HCM	No
PKP2	exon 8 deletion	CNV	HCM	No
PKP2	total gene deletion	CNV	HCM	No
BAG3	c.1636delC	p.His546Thrfs*20	LQTS	*KCNQ1*_del exon 7
DSP	c.5779C>T	p.Gln1927*	LQTS	No
MYBPC3	c.1928-2A>G	p.?	LQTS	No
TTN	c.69070_69071del	p.Thr23024Valfs*8	LQTS	No
PKP2	c.2146-1_2146del	p.?	LVNC	No
PKP2	c.2198_2202delACACC	p.His733Profs*8	LVNC	No
SCN5A	c.1797_1798delGG	p.Val600Glyfs*120	LVNC	*TBX20*_p.Ile152Met
PKP2	total gene deletion	CNV	RCM	No

ARVC arrhythmogenic right ventricular cardiomyopathy, *BrS* Brugada syndrome, *CCD* cardiac conduction disorder, *DCM* dilated cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LVNC* left ventricular non-compaction, *RCM* restrictive cardiomyopathy

medical resources toward a meaningful precision medicine. These unexpected variants have to be further systematically discussed with the prescribing physician in order to avoid an over-interpretation of variants not validated for the clinical presentation provided in the initial referral. Indeed, a different hypothesis could explain such results. Identification of an unexpected variant could either be due to a misdiagnosis or indicate that probands have two unrelated pathogenic variants that could cause different phenotypes or, finally, correspond to a novel gene-disease association and the detected variant is causative for the phenotype. On the other hand, this variant could also simply represent a background variation, as observed for TTNtv.

Although SNPs and short indels are the most often identified mutations in probands, it is noteworthy that pathogenic CNVs was also detected in approximately 3.1% of patients for whom a (likely) pathogenic variation were identified. As expected, for each pathology, pathogenic variations were mainly observed in genes having definitive evidence of disease causation. This is illustrated by the large number of *MYBPC3*tv in HCM referrals or *TTN*tv for DCM referrals. Globally, the mutation detection rate was higher for patients with cardiomyopathies (30.7%) than for patients for arrhythmia syndromes (20.3%). More interestingly, a (likely) pathogenic variation was also detected for 21.1% of patients who died from SCD.

The mutation detection rate would be significantly increased by testing only familiar forms. It could also be increased thanks to more detailed clinical description, familial segregation, or functional studies of some variants of interest [57–61]. These additional data would allowed us to better define the putative pathogenicity of

Table 9 Pathogenic or likely pathogenic variations identified with a homozygous status

Pathogenic variation			Pathology of the proband
Gene	Nucleotide change	Effect on protein	
CSRP3	c.168C>G	p.Ile56Met	HCM
CSRP3	c.369T>A	p.Cys123*	HCM
CSRP3	c.483dupC	p.Lys162Glnfs*	HCM
MYBPC3	c.659A>G	p.Tyr220Cys	HCM
MYBPC3	c.1684G>A	p.Ala562Thr	HCM
MYH7	c.2420G>A	p.Arg807His	DCM
MYLK2	c.169C>A	p.Pro57Thr	DCM
HCN4	c.1501G>A	p.Val501Met	DCM
MYH7	c.3149G>A	p.Arg1050Gln	LVNC
PKP2	total gene deletion	CNV	LVNC
DSC2	c.354+3A>C	p. ?	ARVC
PLN	c.116T>G	p.Leu39*	ARVC
KCNH2	c.1807G>A	p.Gly603Ser	LQTS
TNNI3	c.204delG	p.Arg69Alafs*8	Sudden cardiac death

ARVC arrhythmogenic right ventricular cardiomyopathy, *HCM* hypertrophic cardiomyopathy, *LQTS* long QT syndrome, *LVNC* left ventricular non-compaction

the large number of VUS and, putatively, reclassify some of them as likely pathogenic variations. It would also allow us to detect the coexistence of digenic mutations for some cases. Although more expensive, an alternative approach would be to use a WGS approach instead of NGS targeted sequencing. This strategy would allow the detection of new causative genes, but would, probably, have a limited impact on the mutation detection rate. Whether for cardiomyopathies or arrhythmia syndromes, our study highlights that pathogenic or likely pathogenic variations were essentially detected in genes having strong evidence of disease causation and rarely in genes having a limited evidence. One benefit of a WGS approach would be to obtain exhaustive information about intronic sequences of genes having strong evidence of disease causation. As previously shown for *MYBPC3* in HCM referrals, deep splice mutations would be detected for a significant proportion of cases [62].

In conclusion, our study, which includes over than 4,000 probands, is one of most important cohorts reported in inherited cardiac diseases. To our knowledge, only two similar large cohorts were previously reported: one containing 2500 unrelated cases referred for the FAMILION LQTS clinical genetic test [63] and one containing a retrospective review of 1376 patients with a suspected clinical diagnosis of HCM [64]. Although this study showed some limitations such as lack of detailed clinical description and/or familial segregation data for some probands, (likely) pathogenic variants were detected in about 30% of the referrals, including unexpecting findings and results associated with opportunities for therapy. Identification of such novel (likely) pathogenic variations is still necessary for a better elucidation of the molecular basis of cardiac diseases. It provides new insights into genotype/phenotype relationships and, consequently, an accurate knowledge of the physiopathology of these inherited cardiac diseases characterized by incomplete penetrance, variable expressivity, and phenotypic overlap.

Our study suggests that a NGS approach based on a targeted panel of genes provided a rapid, low-cost, and highly efficient workflow for identification of genomic variants in the most prevalent genes involved in sudden cardiac death. In cases of negative results, and after discussion with the clinician and the geneticist, strategies based either on WES or even WGS could also be considered. Globally, NGS approaches are definitely necessary for patients with cardiomyopathies and/or arrhythmia syndromes but also for victims of sudden unexplained death syndrome. These approaches now have to be combined with high throughput in vivo or in vitro model systems for functional interrogation of the hundreds of identified VUS [57, 60, 61].

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Declarations

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Conflicts of interest AJ, LJ, CC, AD, PC, and GM are employees of Hospices Civils de Lyon (France). None of the authors have potential conflicts of interest to declare.

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Author contributions AJ, LJ, CC, and GM are molecular biologists involved in all molecular diagnosis steps (from blood sample to clinical reports). AD and PC are cardiologists and coordinators of the National Reference Center of inherited cardiac diseases in Lyon.

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