European Journal of Human Genetics (2017) 25:1313–1323
https://doi.org/10.1038/s41431-017-0004-3

ARTICLE

Repeat genetic testing with targeted capture sequencing in primary arrhythmia syndrome and cardiomyopathy

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Received: 10 February 2017 / Revised: 1 August 2017 / Accepted: 4 August 2017 / Published online: 10 October 2017

Abstract

In inherited primary arrhythmia syndromes (PAS) and cardiomyopathies (CMP), the yield of genetic testing varies between 20 and 75% in different diseases according to studies performed in the pre next-generation sequencing (NGS) era. It is unknown whether retesting historical negative samples with NGS techniques is worthwhile. Therefore, we assessed the value of NGS-based panel testing in previously genotype negative–phenotype positive probands. We selected 107 patients (47 PAS and 60 CMP) with a clear phenotype who remained genotype negative after genetic analysis of the main genes implicated in their specific phenotype. Targeted sequencing of the coding regions of 71 PAS- and CMP-related genes was performed. Variant interpretation and classification was done according to a cardiology-specific scoring algorithm ('Amsterdam criteria') and the ACMG-AMP criteria. Co-segregation analysis was performed when DNA and clinical data of family members were available. Finally, a genetic diagnosis could be established in 21 patients (20%), 5 PAS (11%) and 16 CMP (27%) patients, respectively. The increased detection rate was due to sequencing of novel genes in 52% of the cases and due to technical failures with the historical analysis in 48%. A total of 118 individuals were informed about their carrier state and either reassured or scheduled for proper follow-up. To conclude, genetic retesting in clinically overt PAS and CMP cases, who were genotype negative with older techniques, resulted in an additional genetic diagnosis in up to 20% of the cases. This clearly supports a policy for genetic retesting with NGS-based panels.

Introduction

At the end of last century and the beginning of the 2000s, a candidate gene approach was used to identify novel primary arrhythmia syndrome (PAS) and cardiomyopathy (CMP) genes. A multitude of studies have been published in that era illustrating the yield of genetic testing for specific genes in specific disease entities. In the following years, small panels of genes were analysed in specific phenotypes using variant scanning techniques such as denaturing high-pressure liquid chromatography (DHPLC) and subsequent DNA sequencing of abnormal results using the Sanger sequencing technique. These small panels of genes included the so-called 'core genes' or main genes and the diagnostic yield of this approach varies between 20 and 75% in the different phenotypes according to literature data (Table 1) [1]. Overviews of disease-causing variants in these core genes per specific phenotype were published, like for example in Brugada syndrome (BrS) [2], long QT syndrome (LQTS) [3] and hypertrophic cardiomyopathy (HCM) [4]. Despite these great advances, many patients with a clear phenotype remained genetically unresolved. Original ideas to tackle this quest for the genetic cause of Mendelian traits were published in the recent years. These include evaluation of larger genomic deletions or duplications that are missed by conventional variant scanning techniques [5–8]. Another drawback of these techniques is that they are time consuming and costly. However, identifying the underlying genetic defect is of importance, since it

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41431-017-0004-3) contains supplementary material, which is available to authorised users.

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Table 1 Core genes and estimated yield per phenotype

		Core genes (+ estimated yield in literature)	Yield at our institution
PAS	LQTS	KCNQ1, KCNH2, SCN5A (70%)	60%
	BrS	SCN5A (25%)	19%
	CPVT	RYR2, CASQ2 (65%)	80%
	iVF/SD	KCNQ1, KCNH2, SCN5A (25%)	
CMP	HCM	MYBPC3, MYH7, TNNT2 (50%)	32%
	DCM	MYBPC3, MYH7, TNNT2 (15%)	7.5%

Yield at our institution indicates the diagnostic yield of a gene-by-gene approach of the core genes using DHPLC or direct Sanger sequencing

PAS primary arrhythmia syndrome, *CMP* cardiomyopathy, *LQTS* long QT syndrome, *BrS* Brugada syndrome, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *iVF* idiopathic ventricular fibrillation, *SD* sudden death, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy

allows for predictive genetic testing in asymptomatic family members, genetic counselling regarding reproductive risks and even genotype-specific treatment in some diseases [1]. It is unknown whether retesting historical samples with next-generation sequencing (NGS) techniques is worthwhile. Therefore, we assessed the value of NGS-based testing of a comprehensive panel of genes in patients with a clear phenotype that had previously tested negative for the main genes using traditional techniques in the setting of a diagnostic testing laboratory. This allowed us to evaluate the additional genetic yield of NGS-based panel testing in PAS and CMP.

Subjects and methods

Patient selection

The database of our hereditary heart disease clinic was evaluated to select patients that fulfilled the following criteria:

- Clear phenotype of PAS (LQTS, BrS, catecholaminergic polymorphic ventricular tachycardia (CPVT) or idiopathic ventricular fibrillation (iVF)) or CMP (HCM or familial dilated or left ventricular non-compaction cardiomyopathy (DCM or LVNC)) according to the latest guidelines [9–11]; and
- Genetic testing performed for at least the major implicated genes as defined by the EHRA/HRS expert consensus statement published in 2011 (Table 1) [1]. The exception was that patients with HCM were tested for variants in *MYBPC3*, *MYH7* and *TNNT2* only, and not for *TNNI3* and *TPM1*.

There was no selection based on number of affected family members, so both simplex and familial cases were included. The study was approved by an institutional review committee and the subjects gave informed consent for further genetic testing at the time of their initial counselling.

NGS-based panel testing

A panel containing 71 genes implicated in PAS and CMP (Supplementary Table 1) was designed using an in-solution NimbleGen SeqCap EZ capture array (Roche Nimblegen, Madison, Wisconsin, USA) in May 2013. The genes were selected based on careful evaluation of reports in the literature. The test was developed and validated to detect single nucleotide variations and small insertions and deletions in the captured regions (exons and intron/exon boundary (±20 bp) of targeted genes). The probe design covered ~850 kb, corresponding to 95% of the targeted region. Genomic DNA (1 μg) was fragmented by sonication and libraries were prepared by use of the TruSeq DNA Sample Preparation Kit (Illumina, San Diego, California, USA). Six libraries were pooled for each capture reaction. Sequencing was performed on an Illumina HiSeq 2500 (San Diego, California, USA) as 150 bp paired-end reads. Mapping of the reads was performed against the reference genome build 19. Further bio-informatics analysis was done with an in-house pipeline (base calling, alignment and variant calling were performed using the GATK package). Sequencing coverage was around 99.5% and uncovered regions were covered by Sanger sequencing. Variant annotation and classification of variants was performed within the BENCHlab NGS® module (Cartagenia, Leuven, Belgium). Genetic testing in these diseases was phenotype directed [1]. Therefore, subpanels covering the different disease entities were defined as follows: LQTS, BrS, CPVT, iVF, HCM and DCM (Supplementary Table 1). We made a distinction between variants found in the predefined phenotype-related subpanel of a specific patient and variants found in other phenotype-unrelated subpanels, so-called genes of unknown significance (GUS). Sarcomere genes in a patient with LQTS are exemplary for this, since the

identification of a disease-causing variant in this gene subset for the given phenotype is very unlikely. Nevertheless, variants in GUS were reported to envisage the background genetic noise and the presence of rare variation in these genes. Background genetic noise was defined as the chance of identifying a class 3 or above variant according to any of the two classification schemes in a gene linked to a certain phenotype. Finally, copy number variation (CNV) was assessed. Reads per kilobase per million mapped reads (RPKM) values were computed for all samples using the python script of CoNIFER [12]. RPKM values were visualised by chromosome after normalisation. Abnormalities were confirmed with multiplex ligation-dependent probe amplification (MLPA).

Variant interpretation and classification

Variants underwent thorough analysis of their disease-causing potential by two distinct variant interpretation scoring systems. For both algorithms, variants were classified in 5 different classes (1–5); not pathogenic or benign (1), unlikely to be pathogenic or likely benign (2), variant of unknown significance (3), likely pathogenic (4) and pathogenic (5). All variants in the phenotype-related subpanel that were classified as class 3, 4 or 5 were confirmed using Sanger sequencing using primers designed to specifically amplify the corresponding exon (available upon request). BENCHlab NGS® (Cartagenia, Leuven, Belgium) and Alamut® (Interactive Biosoftware, Rouen, France) software tools were used for the analysis. The first algorithm was proposed by the Amsterdam group ('Amsterdam criteria') and was specifically designed for evaluation of variants in CMP [13] and PAS [14] genes. A detailed description of this method has been published previously [13]. In short, first, the frequency of a variant in the human genome was checked in publicly available databases like the exome sequencing project (ESP version ESP6500SI-V2) [15], the 1000 genomes project (1000G; Phase 3) [16], Exome Aggregation Consortium (ExAC, version 0.3.1) [17], but also in our local database. If the minor allele frequency exceeded 1%, the variant was considered to be a class 1 variant. Subsequently, the location (intronic versus exonic; functional domain), type of variation (deletion, insertion, nucleotide substitution) and its effect (frameshift, introduction of a stop codon, nonsynonymous variant, possible splicing effect) were evaluated. The variants were then subjected to in silico prediction tools that are based on conservation among species (phylogenetic analysis) and alteration in biophysical characteristics of the amino acid substitution (physicochemical analysis). The tools that were used were Grantham difference, A-GVHD, BLOSUM62, polyphen-2, SIFT, orthologous annotation and conservation among protein families (paralogous annotation). The final classification using the 'Amsterdam criteria' was based on two additional assessments. First, an extensive search in the literature, including Medline, HGMD, locus specific and ClinVar [18] databases, was performed to evaluate prior reports linking the specific variant with a phenotype. In these reports, we evaluated critically whether co-segregation was described and whether functional studies compatible with the specific phenotype were done. Finally, co-segregation analysis was performed if DNA and clinical data from at least one family member was available.

The second algorithm was the non-cardiology-specific guidelines proposed by the American College of Medical Genetics and Genomics (ACMG) and Association of Molecular Pathology (AMP), hereinafter referred to as ACMG guidelines [19]. Some issues have been reported since the publication of these guidelines, including the lack of definition of interpretation of co-segregation data and in silico tools, resulting in discordance of interpretation between laboratories [20, 21]. Therefore, we used the recently proposed rules for co-segregation by Jarvik and Browning, and considering co-segregation in clearly affected individuals only [22]. Variants that were not identified in an affected family member were classified as likely benign. For interpretation of in silico tools, we used the score obtained by the 'Amsterdam criteria' before adding any extra information but the in silico tools: supporting benign factor if compound score <25%, supporting pathogenic factor if compound score >70%. Finally, only variants that were classified as likely pathogenic or pathogenic by both scoring algorithms were retained as (likely) pathogenic. All variants reported in this paper have been submitted to ClinVar database with the submission name 'Leuven Retrospective Study Cardiogenetics' (www.ncbi.nlm.nih.gov/clinvar/) [18].

Results

Patient selection

In total, out of 384 index patients lacking a genetic diagnosis in the database of our hereditary heart disease clinic, 107 patients fulfilled inclusion criteria. Patients were excluded from the study for different reasons: 76 patients due to lack of DNA to perform the analysis, 87 due to lack of clinical data or unfulfilled diagnostic criteria, 22 because not all core genes had been sequenced for the specific phenotype and finally 92 patients because they were lost to follow-up at our institution. The selected patients consisted of 47 patients with PAS (25 LQTS, 11 BrS, 8 iVF and 3

CPVT) and 60 with CMP (52 HCM, 7 DCM and 1 LVNC). All patients were from Caucasian ancestry, apart from seven in the HCM group of whom four were from African and three from Middle Eastern descent. Different testing techniques had been used for evaluation of the core genes in the past: 52 DHPLC scanning and Sanger sequencing of abnormal results, 51 direct Sanger sequencing and 4 had a custom DNA resequencing array in an external lab [23].

Variant interpretation

Pathogenic and probably pathogenic variants are listed in Table 2, while variants of unknown significance are listed in Table 3. All variants were single nucleotide variations or small insertions and deletions apart from one variant, where we observed a reduction of the RPKM value for exon 3 of the *RYR2* gene (patient 21). This reduction was further confirmed with MLPA (P168-C2, MRC Holland).

First, we analysed all variants with the 'Amsterdam criteria' (Fig. 1). Before entering co-segregation and literature data into the algorithm, 35 variants of class 3, 4 or 5 were retained in 31 patients. DNA and clinical data of other family members were available for 31 variants allowing co-segregation analysis. Co-segregation analysis aided in variant classification for 24 variants, and remained inconclusive for another 4 variants. Three variants were classified as (likely) benign because they were not detected in one or more affected family members, and thus displaying non-fitting co-segregation (Supplementary Table 2).

Second, we analysed all variants using the ACMG criteria (Fig. 1). All variants classified as (likely) pathogenic by the 'Amsterdam criteria' were also classified as (likely) pathogenic applying these ACMG criteria. A reclassification from a class 4 or 5 variant to a class 3 variant or vice versa influences medical decision making. Such a transition was only observed for one variant of unknown significance using the 'Amsterdam criteria' that was reclassified as likely pathogenic by the ACMG criteria (*TNNC1* c.430A>G or p.(Asn144Asp); family 29 and 30; Table 3). Thus far, this specific variant was found in 13 unrelated HCM probands in our diagnostics lab. Further workup with evaluation of a possible founder effect is planned. No variants that were classified as class 3 according to the 'Amsterdam criteria' were downgraded to a (likely) benign variant by the ACMG criteria. On the other hand, six variants that were classified as (likely) benign by the 'Amsterdam criteria' were classified as variant of unknown significance using the ACMG criteria.

Finally, our analysis resulted in a genetic diagnosis (class 4 or 5 variants) in 21 patients, i.e., an additional yield of 20% (11% in PAS and 27% in CMP). The genetic yield per phenotype is shown in Fig. 2. Detailed variant interpretation and description of the phenotypes of families with a class 3 or above variant is available in the Supplementary Data.

Reasons for initial detection failures

In 11 of these 21 patients, the (likely) disease-causing variant was located in a gene that was not part of the core genes and therefore had not been evaluated in the past. This concerned five pathogenic and six likely pathogenic variants according the ACMG criteria. One of these variants was an exon 3 deletion of the *RYR2* gene detected in proband 21 causing an overlap between LVNC and CPVT. This deletion would not have been picked up with DHPLC that was historically performed. Surprisingly, in the other 10 patients, 11 disease-causing variants were found in a gene that had been scanned or sequenced before. According the ACMG criteria, six of these variants were considered as pathogenic and five variants as likely pathogenic (of which one was detected in the historical analysis in a patient with compound heterozygous variants in *CASQ2*-related autosomal recessive CPVT). These detection failures included cases of (1) variants not detected by the laboratory, where the original investigation was performed (N=4), (2) functional reclassification (N=3), (3) allelic dropout with DHPLC (N=2) and (4) failed heterozygous calling with sequencing (N=1). Disease-causing variants were identified in 8 out of 51 (16%) patients for whom Sanger sequencing was performed as their historical analysis technique, 11 out of 53 (21%) for whom DHPLC was performed and 2 out of 4 for whom a custom DNA resequencing array was performed.

Variants in genes of unknown significance

Analysis of genetic variation in genes of unknown significance resulted in 31 additional variants in 30 patients (Table 4). Ten of these variants were detected in a patient that also carried a (likely) pathogenic variant. Variants in a gene of unknown significance were more prevalent in patients with CMP (42%) compared to PAS (13%, P=0.001). All these variants were classified as VUS according to the ACMG criteria. Evaluation of these variants using the Amsterdam criteria resulted in 6 class 1 variants (19%), 7 class 2 variants (23%), 14 class 3 variants (45%) and 4 class 4 variants (13%). Since most of these variants are novel, classification using the Amsterdam criteria was mainly based on in silico prediction tools. We thus identified a VUS in a GUS in 28% of the included probands. The background genetic noise varied depending on the phenotype from 1% in CPVT genes to 19% in iVF genes (Table 5) and was generally higher using an extended panel of genes compared to the core genes only (P=0.001).

Table 2 Overview of (likely) pathogenic variants

Family	Pheno	Gene	RefSeq	cDNA	Protein	AC	ACMG	ExAC	Clinvar	Novel	Co-segregation	Reason initial detection miss
1	LQTS	KCNH2	NM_000238.3	c.2038del	p.(Val680Cysfs*34)	5	P	0/121080	–	No	11 G+Ph+; 6G-Ph−	Allelic dropout
2	LQTS	KCNH2	NM_000238.3	c.1952T>G	p.(Met651Arg)	5	LP	0/119932	–	Yes	De novo	Allelic dropout
3	LQTS	KCNQ1	NM_000218.2	c.1032G>A	p.(Ala344Ala)	5	P	0/120972	2 Cl5	No	4G+Ph+	Functional reclassification
4	CPVT	CASQ2	NM_001232.3	c.164A>G	p.(Tyr55Cys)	4	LP	0/121408	–	No	Brother same G and SD	Detected
4	CPVT	CASQ2	NM_001232.3	c.783G>A	p.(Trp261*)	4	LP	1/121216	–	Yes	Brother same G and SD	Not detected by external lab
5	CPVT	RYR2	NM_001035.2	c.11996T>C	p.(Met3999Thr)	5	LP	0/99964	–	Yes	De novo	Not detected by external lab
6	HCM	MYBPC3	NM_000256.3[c]	c.505+5G>C		5	LP	0/23738	1 CL5	No	1G+Ph+, 2G+Ph−; 2G-Ph−	Functional reclassification
7[a]	HCM	MYBPC3	NM_000256.3	c.1790G>A	p.(Arg597Gln)	4	P	0/25908	1 Cl4; 1 Cl3	No	2G+Ph+	Functional reclassification
8	HCM	MYBPC3	NM_000256.3	c.2373dup	p.(Trp792Valfs*41)	5	P	1/26444	5 Cl5	No	2G+Ph+; 1G+Ph −;2G-Ph−	Not detected by external lab
9	HCM	MYH7	NM_000257.2	c.1436A>G	p.(Asn479Ser)	5	P	0/121380	1 Cl3	No	5G+Ph+; 3G+Ph−; 4G-Ph−	Heterozygous calling with sequencing failed
10[a]	HCM	MYH7	NM_000257.2	c.2334C>G	p.(Asp778Glu)	5	P	0/121342	–	No	3G+Ph+; 2G+Ph−; 1G-Ph−	Not detected by external lab
11[a]	HCM	MYL3	NM_000258.2	c.170C>G	p.(Ala57Gly)	5	P	11/121338	2 Cl5; 1 Cl4	No	2G+Ph+	Not sequenced before
12	HCM	PRKAG2	NM_016203.3	c.879C>A	p.(Phe293Leu)	5	LP	0/114896	1 Cl4	Yes	7G+Ph+; 4G+Ph−; 7G-Ph−	Not sequenced before
13	HCM	TNNI3	NM_000363.4	c.433C>T	p.(Arg145Trp)	5	P	1/120492	1 Cl4	No	3G+Ph+	Not sequenced before
14[a]	HCM	TNNI3	NM_000363.4	c.433C>T	p.(Arg145Trp)	5	P	1/120492	1 Cl4	No	2G+Ph+	Not sequenced before
15	HCM	TNNI3	NM_000363.4	c.434G>A	p.(Arg145Gln)	4	LP	3/120502	1 Cl5; 3 Cl4	No	3G+Ph+; 1G+Ph−; 1G-Ph−	Not sequenced before
16	HCM	TNNI3	NM_000363.4	c.508C>T	p.(Arg170Trp)	5	LP	0/119486	1 Cl5; 1 Cl4; 1 Cl3	Yes	De novo	Not sequenced before
17	HCM	TNNI3	NM_000363.4	c.611G>A	p.(Arg204His)	5	LP	0/120734	1 Cl5, 1 Cl4	No	De novo	Not sequenced before
18	HCM	TPM1	NM_001018005.1	c.640_645del	p.(Tyr214_Ser215del)	5	LP	0/121278	–	Yes	De novo	Not sequenced before
19[a,b]	DCM	DES	NM_001927.3	c.1360C>T	p.(Arg454Trp)	5	P	0/61410	2 Cl4	No	1G+Ph+; 3G-Ph−	Not sequenced before
20	DCM	LMNA	NM_005572.3	c.1622G>A	p.(Arg541His)	5	LP	2/21390	–	No	De novo	Not sequenced before
21	LVNC + CPVT	RYR2	NM_001035.2	c.(168+1_169-1)_(273+1_274-1)del [d]	p.(Asn57_Gly91)del	5	P	0/120742	–	No	2G+Ph+	Not sequenced before, large deletion

In the clinvar database, evaluations based on literature reports were ignored for this assessment

G indicates positive (+) or negative (−) genotype; Ph indicates positive (+) or negative (−) phenotype

Pheno phenotype, *LQTS* long QT syndrome, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *LVNC* left ventricular non-compaction, *RefSeq* reference sequence, *AC* classification according to 'Amsterdam criteria', *ACMG* classification according to the American college of medical genetics and genomics, *ExAC* Exome Aggregation Consortium, *LP* likely pathogenic variant, *P* pathogenic variant, *Cl* class

[a]Detected in compound with variant in gene of unknown significance (Table 4)

[b]Detected in compound with variant of unknown significance in panel of interest (Table 3)

[c]NG_007667.1:g.7694G>C

[d]Chr1(GRCh38):g.(237270617_237355964)del

Table 3 Overview of class 3 variants

Family	Pheno	Gene	RefSeq	cDNA	Protein	AC	ACMG	ExAC	Clinvar	Novel	Co-segregation	Reason initial detection miss
22	BrS	SCN5A	NM_198056.2	c.5540G>A	p.(Arg1847His)	3	VUS	0/120772	1 Cl3	No	ND	Detected
22	BrS	KCNH2	NM_000238.3	c.2606C>T	p.(Pro869Leu)	2	VUS	1/120148	1 Cl3	Yes	ND	Not sequenced before
23	iVF	HCN4	NM_005477.2	c.1928T>G	p.(Leu643Arg)	3	VUS	0/121358	–	Yes	1G+Ph+; 1G+Ph−; 1G-Ph−	Not sequenced before
24	iVF	RYR2	NM_001035.2	c.14650A>G	p.(Met4884Val)	3	VUS	0/120724	–	Yes	3G+Ph+, 2G+Ph−; 6G-Ph−	Not sequenced before
25	HCM	MYBPC3	NM_000256.3	c.223G>A	p.(Asp75Asn)	3	VUS	3/32862	1 Cl4; 2 Cl3	No	1G+[c]Ph+; 2G+Ph−	Detected; homozygous
26	HCM	MYH6	NM_002471.3	c.3220G>A	p.(Asp1074Asn)	3	VUS	5/121412	1 Cl3	Yes	ND	Not sequenced before
27	HCM	MYH7	NM_000257.2	c.3169G>A	p.(Gly1057Ser)	3	VUS	1/121398	1 Cl3	No	2G+Ph+; 1G+Ph−; 3G-Ph−	Detected
28[a]	HCM	MYLK2	NM_033118.3	c.1189A>G	p.(Met397Val)	1	VUS	0/121220	–	Yes	ND	Not sequenced before
28[a]	HCM	RYR2	NM_001035.2	c.1392C>A	p.(His464Gln)	1	VUS	6/120284	–	Yes	ND	Not sequenced before
29[a]	HCM	RYR2	NM_001035.2	c.4186G>A	p.(Asp1396Asn)	2	VUS	0/106780	–	Yes	ND	Not sequenced before
30	HCM	TNNC1	NM_003280.2	c.430A>G	p.(Asn144Asp)	3	LP	0/120590	1 Cl4	Yes	3G+Ph+	Not sequenced before
31	HCM	TNNC1	NM_003280.2	c.430A>G	p.(Asn144Asp)	3	LP	0/120590	1 Cl4	Yes	ND	Not sequenced before
32	HCM	TNNI3	NM_000363.4	c.298C>T	p.(Leu100Phe)	3	VUS	1/119904	–	Yes	1G+Ph+; 1G+Ph−	Not sequenced before
33	HCM	TPM1	NM_001018005.1	c.851T>C	p.(Ile284Thr)	3	VUS	1/108270	1 Cl3	Yes	ND	Not sequenced before
34[a]	HCM	VCL	NM_014000.2	c.2046A>T	p.(Leu682Phe)	2	VUS	8/121406	2 Cl3	Yes	ND	Not sequenced before
35	DCM	MYH7	NM_000257.2	c.1128C>A	p.(Asp376Glu)	2	VUS	1/121388	1 Cl2	Yes	ND	Detected
19[a,b]	DCM	TMPO	NM_003276.2	c.370C>T	p.(Leu124Phe)	3	VUS	3/121398	–	Yes	ND	Not sequenced before

In the clinvar database evaluation based on literature reports were ignored for this evaluation

G indicates positive (+) or negative (−) genotype; Ph indicates positive (+) or negative (−) phenotype

Pheno phenotype, *BrS* Brugada syndrome, *iVF* idiopathic ventricular fibrillation, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *RefSeq* reference sequence, *ACMG* classification according to the American college of medical genetics and genomics, *ExAC* Exome Aggregation Consortium, *VUS* variant of unknown significance, *Cl* class, *ND* not done

[a]Detected in compound with variant in gene of unknown significance (Table 4)

[b]Detected in compound with (likely) pathogenic variant (Table 2)

[c]Homozygous

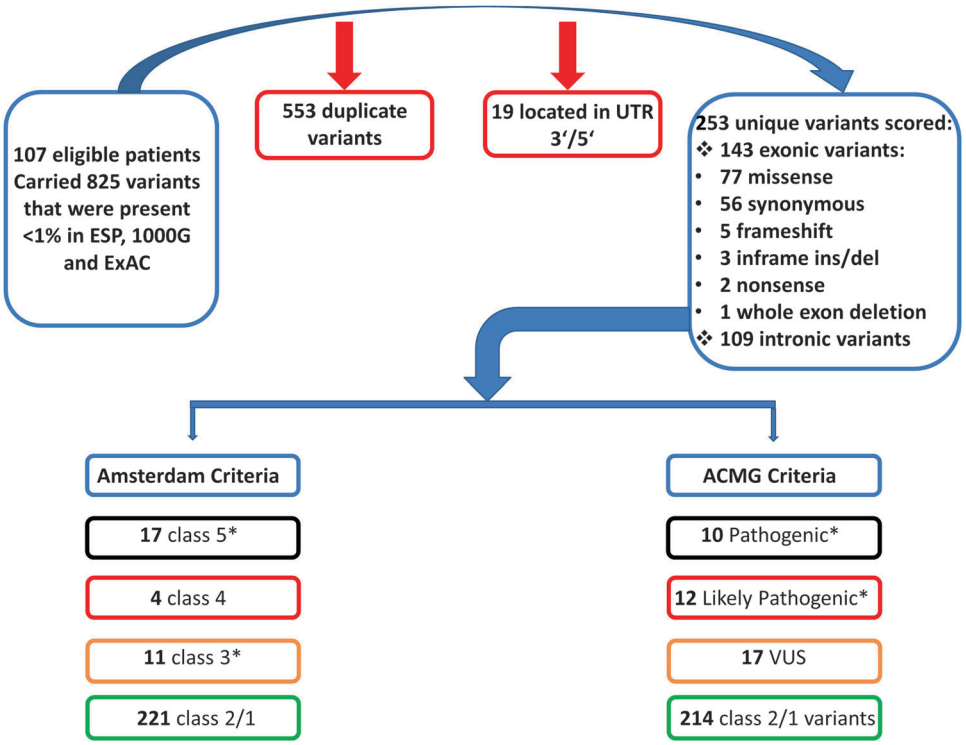

Fig. 1 Summary of the results of variant interpretation and classification according to the 'Amsterdam criteria' and ACMG criteria. *One variant was identified in 2 index cases; *ESP* exome sequencing project, *1000G* 1000 genomes project, *ExAC* exome aggregation consortium; duplicate variants indicates the total number any particular variant was observed in more than one patient; *UTR* untranslated region, *ins* insertion, *del* deletion

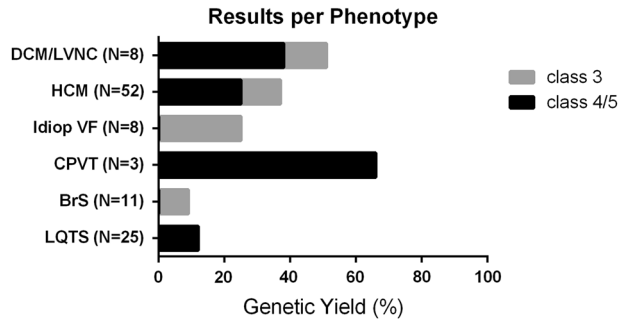

Fig. 2 Results per phenotype. The percentage of patients carrying a class 3 (variants of unknown significance), class 4 (probably pathogenic variant) or class 5 (definite pathogenic variant) variant are illustrated. The number on the *Y*-axis indicates the number of patients that were included per phenotype

Discussion

We here demonstrate that retesting with NGS-based extended gene panels of CMP or PAS patients that tested negative for the core genes using traditional techniques results in an additional 20% of genetic diagnoses. This indicates that genetic retesting is indicated.

We performed genetic retesting of clear phenotype-positive individuals that tested negative with previous variant scanning techniques or direct DNA sequencing. Such an approach was already described in a cohort of 44 LQTS patients, where direct DNA sequencing with the Sanger technique of the three LQTS core genes identified a disease-causing variant in 16% of patients who had tested negative with DHPLC in research context [24]. However, our study was performed in multiple PAS and CMP phenotypes and with a broader panel of genes, not only including the core genes, but also genes that have been implicated in only a minority of patients with the specific phenotype (minor disease-associated genes).

The genetic yield of retesting differed per phenotype. In HCM and DCM, the yield was 27%. This was due to sequencing of novel genes in two-thirds of the patients. In PAS, the global yield of retesting was lower (11%) and all of the identified disease-causing variants were located in genes that had been evaluated in the previous analysis. Therefore, technical issues with the historical genetic analyses were the only reason for detection failure in PAS. This also highlights the limited additional value of analysing the minor disease-associated genes in these diseases. For example, in 11 probands with BrS genetic retesting by NGS engendered just one previously reported variant of unknown significance in *SCN5A*. Firm conclusions in such a small cohort may be premature, but our results support the previous conclusion that in BrS the clinical value of genetic testing beyond *SCN5A* is questionable [25]. In LQTS, we identified a disease-causing variant in 12% of probands. Two misses were due to allelic dropout caused by the reverse primer of exon 8 of *KCNH2* residing over a SNP

Table 4 Overview variants in genes of unknown significance (GUS)

Family	Pheno	Gene	RefSeq	cDNA	Protein	AC	ACMG	ExAC	Clinvar	Novel
36	LQTS	ABCC9	NM_005691.2	c.2878G>C	p.(Glu960Gln)	2	VUS	0/119964	–	Yes
37	LQTS	CSRP3	NM_003476.4	c.437G>A	p.(Arg146His)	3	VUS	3/121330	1 Cl3	Yes
38	LQTS	MYBPC3	NM_000256.3	c.2783C>T	p.(Ser928Leu)	3	VUS	1/119088	–	No
39	LQTS	MYH7	NM_000257.2	c.5471A>G	p.(Asn1824Ser)	1	VUS	3/121362	–	Yes
40	CPVT	MYBPC3	NM_000256.3	c.841C>T	p.(Arg281Trp)	3	VUS	0/28648	1 Cl3	Yes
41	BrS	KCNQ1	NM_000218.2	c.1885G>A	p.(Gly629Ser)	1	VUS	5/26340	–	Yes
42	HCM	AKAP9	NM_005751.4	c.11682G>T	p.(Gln3894His)	2	VUS	1/120586	–	Yes
43	HCM	ANK2	NM_001148.4	c.3652C>T	p.(Pro1218Ser)	3	VUS	2/121332	–	Yes
44	HCM	ANK2	NM_001148.4	c.6078A>T	p.(Lys2026Asn)	2	VUS	1/119198	–	Yes
34[a]	HCM	DSC2	NM_024422.3	c.2603C>T	p.(Ser868Phe)	4	VUS	7/121334	–	No
45	HCM	DSP	NM_004415.2	c.4166G>A	p.(Ser1389Asn)	1	VUS	0/120960	–	Yes
46	HCM	DSP	NM_004415.2	c.5570A>C	p.(Lys1857Thr)	3	VUS	0/121396	–	Yes
47	HCM	DSP	NM_004415.2	c.8014C>G	p.(Gln2672Glu)	2	VUS	0/120764	–	Yes
28[a]	HCM	GJA5	NM_005266.6	c.586T>G	p.(Cys196Gly)	4	VUS	0/121088	–	Yes
48	HCM	HCN4	NM_005477.2	c.520C>T	p.(Pro174Ser)	2	VUS	5/39178	1 Cl3	Yes
49	HCM	HCN4	NM_005477.2	c.724C>T	p.(Arg242Cys)	3	VUS	4/120588	1 Cl3	Yes
50	HCM	KCNE1	NM_000219.4	c.273C>G	p.(Asp91Glu)	1	VUS	0/121148	–	Yes
7[a]	HCM	KCNH2	NM_000238.3	c.778G>C	p.(Ala260Pro)	2	VUS	0/2166	–	Yes
10[a]	HCM	KCNH2	NM_000238.3	c.3124C>G	p.(Leu1024Val)	2	VUS	0/12034	–	Yes
14[a]	HCM	KCNJ5	NM_000890.3	c.616G>A	p.(Ala206Thr)	3	VUS	2/120176	–	Yes
14[a]	HCM	LDB3	NM_001080116.1	c.91C>T	p.(Arg31Trp)	4	VUS	7/118678	1 Cl3	Yes
51	HCM	LMNA	NM_005572.3	c.1517A>C	p.(His506Pro)	3	VUS	0/84000	2 Cl3	Yes
29[a]	HCM	SCN5A	NM_198056.2	c.3795G>T	p.(Lys1265Asn)	3	VUS	0/121368	–	Yes
11[a]	HCM	SCN5A	NM_198056.2	c.5849A>G	p.(Tyr1950Cys)	3	VUS	0/116260	1 Cl3	Yes
52	HCM	TGFB3	NM_003239.2	c.787G>C	p.(Asp263His)	3	VUS	0/121406	–	No
53	HCM	TRPM4	NM_017636.3	c.739A>T	p.(Asn247Tyr)	3	VUS	0/91590	–	Yes
54	HCM	TRPM4	NM_017636.3	c.1603G>A	p.(Glu535Lys)	1	VUS	0/90200	–	Yes
55	DCM	AKAP9	NM_005751.4	c.2478A>G	p.(Ile826Met)	1	VUS	1/120582	–	Yes
56	DCM	CACNB2	NM_201590.2	c.1114G>A	p.(Ala372Thr)	3	VUS	0/120880	–	Yes
19[a,b]	DCM	KCNE2	NM_172201.1	c.285G>T	p.(Lys95Asn)	3	VUS	0/120226	–	Yes
57	DCM	SCN2B	NM_004588.4	c.332C>T	p.(Ser111Leu)	4	VUS	0/121232	–	Yes

In the clinvar database, evaluations based on literature reports were ignored for this assessment

Pheno phenotype, *LQTS* long QT syndrome, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *BrS* Brugada syndrome, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *RefSeq* reference sequence, *AC* classification according to 'Amsterdam criteria', *ACMG* classification according to the American college of medical genetics and genomics, *VUS* variant of unknown significance, *ExAC* Exome Aggregation Consortium, *Cl* class

[a]Detected in compound with (likely) pathogenic variant (Table 2)

[b]Detected in compound with variant of unknown significance (Table 3)

[26]. This is a known issue of DHPLC, since it was shown in an American cohort that 2–5% of presumed genotype negative LQTS patients harboured a disease-causing variant that was missed due to allelic dropout [24].

Functional reclassification of genetic variants was the reason for detection of a disease-causing variant in three patients. One synonymous variant in *KCNQ1* (c.1032G>A; family 3) located at the last nucleotide of exon 7 had been classified as likely benign back in 2004 based on absence of amino acid change or convincing literature reports. However, in the meantime, evidence has emerged indicating exon skipping caused by this variant [27]. Similarly, in the HCM subgroup, two variants in *MYBPC3* were reclassified based on novel literature reports, indicating activation of a cryptic donor splice site and subsequent introduction of a frameshift and premature stop codon for the splicing variant c.505+5G>C (family 6) [28] and skipping of exon 18 for missense variant c.1790G>A (family 7) [29]. Therefore, periodic reassessment of genetic test results and variants is appropriate and recommended [30]. Similar findings on

Table 5 Background genetic noise according to phenotype

Phenotype	N patients	N variants	N genes	Genetic noise (%)	
				Extended panel	Core genes
LQTS	82	12	16	14.6	6.1
BrS	96	8	15	8.3	2.1
CPVT	104	1	4	1.0	0
iVF	99	19	32	19.2	5.1
HCM	55	5	27	9.1	5.5
DCM	100	11	27	11	3
LVNC	106	6	11	5.7	2.8
ARVC	107	5	10	4.7	3.7

N patients indicates the number of patients that did not have the specific phenotype; N variants indicates the number of variants that were identified in the specific disease panel; N genes indicates the number of genes that were included in the extended panel of the specific disease

periodic reassessment have been recently reported in an Australian HCM cohort [31].

In the total cohort, only one clinically relevant CNV was identified, illustrating the rarity of CNVs in cardiogenetic diseases. A deletion of exon 3 of *RYR2* was identified in a family with left ventricular non-compaction (family 21). Initially, this family was regarded as a case of isolated LVNC, but recently the daughter of the index patient developed exercise-induced syncope and showed bidirectional ventricular tachycardia on an exercise stress test compatible with the diagnosis of CPVT. The daughter is also carrier of the exon 3 deletion. The particular combination of LVNC and CPVT has been previously described in several families [32, 33].

We identified a pathogenic or probably pathogenic variant in 21 out of 107 probands. In these 21 families, co-segregation analysis was performed if DNA, clinical data and consent of the individual family member were available. This allowed 98 relatives to be informed about their carrier state and counselled appropriately. A total of 46 individuals could be reassured since they were found not to be carrier of the familial disease-causing variant. Counselling regarding reproductive risks is an additional benefit of identifying a genetic cause. For severe, highly penetrant phenotypes, in vitro fertilisation with preimplantation genetic diagnosis can be offered. Until now, one patient with severe LQT2 in this cohort gave birth to a child after preimplantation genetic diagnosis.

The biggest challenge in genetic diagnosis remains variant interpretation and determining pathogenicity. This is especially so in the minor disease-associated genes since the pre-test probability of a disease-causing variant in these genes is much lower compared to the core genes. NGS produces an enormous amount of genetic variants that need interpretation and classification. In the so-called core genes, based on a greater experience of interpreting variants in these specific genes, efforts have been made to help variant interpretation. For LQTS, core genes for example, algorithms to score the probable pathogenicity of a variant have been developed based on a combination of the type of variation and its effect (missense, frameshift, introduction of a stop codon), their topological location and a combination of in silico prediction tools [34, 35]. Based on observations like these, it seems reasonable that algorithms implementing multiple variables enhance variant classification. One of these algorithms is the PES- and CMP-specific scoring system reported by the Amsterdam group [13, 14]. In 2015, the ACMG published a consensus document describing their standards and guidelines for variant interpretation [19]. Compared to the 'Amsterdam criteria', this guideline is not developed specifically for interpretation of variants in cardiac genes. It does not use a quantitative scoring system and therefore could be more dependent upon the operator's interpretation. Comparison of the two scoring systems in our data set produced a very similar outcome with regard to variant classification of (likely) disease-causing variants (class 4 or 5 and (likely) pathogenic variants, respectively). However, the ACMG criteria resulted in more variants of unknown significance that were classified as (likely) benign by the 'Amsterdam criteria' (17 versus 11). However, a limitation to this comparison of the two interpretation algorithms is that the interpretation was performed by a single rater (T.R.), who was of course not blinded for the result of the interpretation using the other scoring algorithm, possibly resulting in some interpretation bias.

Further limitations of the study are inherent to the selection of the core genes. We acknowledge that nowadays *TNNI3* and *TPM1* in HCM and *TTN* and *LMNA* in DCM should be part of so-called core genes. Most of the patients that were included in this study had their historical DNA analysis before the publication of the EHRA/HRS expert consensus statement in 2011, partly explaining why it was decided not to include these genes in routine screening prior to 2011 [1]. This is especially relevant in HCM, since the yield of genetic retesting would have been 14% instead of 27% using our definition of core genes. In DCM on the other hand, we identified a disease-causing variant in *LMNA* in one patient that underwent heart transplantation for end-stage dilated cardiomyopathy without conduction defect (family 20). This case was not especially suggestive for a laminopathy at that time. However, in the meantime, he developed a mild peripheral myopathy compatible with a laminopathy. *TTN* was not included in our panel because it was not established as a definite disease-causing mutation at the time the panel was developed (2013). Furthermore, interpretation poses many difficulties due to the magnitude of the gene, the multiple transcripts and the extensive

genetic variation [36]. However, with today's knowledge, we do agree to include *TTN* in a diagnostic workup of familial DCM, with the above-mentioned shortcomings. The same is true for several other genes that are not included in our panel, but that have been established as definite disease-causing genes in the meantime, like for example, *RBM20*, in DCM [37]. Finally, three variants had also been detected in the historical analysis, but reappraisal of the pathogenicity resulted in their reclassification towards a (likely) disease-causing variant. Excluding these variants from the additional diagnostic yield still resulted in 17% of patients with a genetic diagnosis in this cohort.

Conclusions

In the setting of a genetic diagnostic laboratory, genetic retesting identified a disease-causing variant in a substantial amount of historical samples of clear phenotype-positive cases of PAS or CMP that were previously evaluated using a gene-by-gene DHPLC or Sanger sequencing approach of core disease-related genes. This was partly due to evaluation of minor disease-associated genes and partly due to technical issues with these older techniques. These results definitely support genetic retesting with NGS-based panels of historical samples that were negative after the former analyses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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