Next-Generation Sequencing Gene Panels in Inheritable Cardiomyopathies and Channelopathies: Prevalence of Pathogenic Variants and Variants of Unknown Significance in Uncommon Genes

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1. Introduction

Inheritable cardiomyopathies and channelopathies are disorders with phenotypic and genetic heterogeneous features caused by the presence of structural or electrical heart abnormalities [1]. Variable penetrance and incomplete expression are also common and may be due to the interaction of the causal mutation with modifier genes, epigenetic changes, environmental factors, or individual factors such as age, gender, ethnicity, or physical activity [2]. According to their functional and morphological features, cardiomyopathies are commonly classified as hypertrophic cardiomyopathy (HCM), arrhythmogenic cardiomyopathy (ACM), dilated cardiomyopathy (DCM), or restrictive cardiomyopathy (RCM) [3].
Channelopathies are arrhythmic disorders in patients without structural heart abnormalities that are usually due to genetic alterations in genes coding for cardiac ion channels or their associated proteins. The main channelopathies are: Brugada syndrome (BrS), long QT syndrome (LQTS), short QT syndrome (SQTS), and catecholaminergic polymorphic ventricular tachycardia (CPVT) [4].

Both cardiomyopathies and channelopathies can cause sudden cardiac death (SCD), and this tragic event may represent the onset of an inherited heart disease in asymptomatic individuals, including subjects who practice intense physical activity, such as elite athletes [5–9].

Over the last two decades, the knowledge of the molecular bases of cardiomyopathies and channelopathies has gradually increased, and putatively associated variants have been identified in more than 200 genes [10–22]. Indeed, while some genes are strongly related to a clinical phenotype and are highly penetrant causative genes, others have only rarely been identified in affected subjects, and the association with the disease seems to be poor. Currently, mutations in about 100 different genes are associated to HCM [5,23].

Nevertheless, eight main genes, MYBPC3, MYH7, TNNT2, TNNI3, TPM1, ACTC1, MYL2, and MYL3, account for up to 65–70% of all HCM cases [24–26], while other uncommon genes seem to be globally involved in about 10% of HCM cases [12,27–30]. Similarly, 51 curated genes were associated with idiopathic DCM [31], explaining up to 40–50% of DCM cases [24]. Among these, mutations in TTN gene account for up to 20–25% of DCM cases, while mutations occurring in other genes have rarely been identified [32–42]. With regard to ACM, pathogenic variants in each of the main cardiac desmosome genes were identified in more than 50% of the affected subjects [24,43,44], a smaller fraction carrying pathogenic mutations in nondesmosomal genes [43,45,46]. Lastly, a combination of sarcomeric and cytoskeletal genes were detected in half of RCM patients [47].

Similar considerations also apply to channelopathies. About 20–25% of BrS patients carry a mutation in the SCN5A gene, while other disease-related genes explain an additional 5% of all BrS cases [5,48–50]. With regard to LQTS, mutations in the KCNQ1, KCNH2, and SCN5A genes are found in about 75% of patients, while 5–10% of cases are due to genetic mutations in uncommon genes. The main gene associated with SQTS is KCNH2, reported in about 15% of SQTS patients; other uncommon genes were also reported [51]. Lastly, genes mainly associated with CPVT are RYR2 (60%) and CASQ2 (5%); other genes are rarely reported (<1% each) [52]. Supplementary Table S1 shows the genes reported in the literature associated with the above mentioned cardiomyopathies and channelopathies [26].

Most previously reported genes are commonly included in diagnostic tests. Moreover, in recent years, advances in high-throughput sequencing strategies, such as next-generation sequencing (NGS), have revolutionized the diagnosis of inherited heart diseases in terms of expanding the number of involved genes and the discovery of new genes potentially associated with the diseases [5,53–57].

This wide genetic scenario highlights the need for a standardized method to estimate the genetic evidence and the clinical validity of gene–disease relationships. A proposed framework to evaluate relevant genetic and experimental evidence supporting or contradicting a gene–disease relationship is represented by the NIH-funded Clinical Genome Resource (ClinGen) (https://search.clinicalgenome.org/, accessed on 26 September 2022) [58,59], a standardized evidence-based framework, from expert panel curation. ClinGen is an authoritative open-access online resource that defines genes and variants on the basis of the level of evidence of disease association, which can be strong, definitive, or moderate. ClinGen also reports genes that have a gene–disease association in the literature data, but present limited or conflicting evidence to support a causal role in the disease since the time of the initial report. These genes are classified as limited, disputed, or refuted.

In this manuscript, we report the prevalence and type of genetic variants identified in patients with cardiomyopathies and channelopathies, focusing on genes with weaker evidence (limited, disputed, refuted) of disease association and genes not reported for the specific phenotype according to the ClinGen definition. Here, we define “uncommon
"genes" as all limited, disputed, or refuted genes, or those without a reported association with the clinical phenotype of the patients.

2. Materials and Methods

2.1. Patient Enrolment and DNA Extraction

In total, 133 unrelated patients with a clinical diagnosis or suspicion of an inherited cardiac disease (cardiomyopathy or channelopathy) were enrolled from June 2018 to date and followed up on an average period of 3 years at the Department of Cardiomyopathy and Inherited Heart Disease Clinic, UOC Cardiology, University of Campania ‘Luigi Vanvitelli’ of Naples, and at the Center for the Study of Hypertrophic Cardiomyopathies of the Cardiology Division of the Federico II University of Naples. Genetic testing was performed at the CEINGE diagnostic laboratory.

The reference clinicians of the patients required the molecular analysis to investigate the presence of a possible genetic disease. A blood sample was collected in EDTA from each study subject. Each patient gave their written informed consent to molecular analysis according to the tenets of the Helsinki Declaration [60] and the internal ethics committee (N. 77/21). Genomic DNA was extracted using a Maxwell 16 instrument (Promega, Madison, WI, USA). DNA quality was assessed with a Nanodrop (Thermo Fisher, Waltham, MA, USA) spectrophotometer and Tape Station (Agilent, Santa Clara, CA, USA) analyzers to verify their concentration, purity, and integrity before proceeding to library preparation.

2.2. Molecular Analysis

Molecular testing was carried out by analyzing a panel of target genes through an NGS-based procedure. In particular, 2 custom panels were used: (1) a first-level panel including 60 diagnostic genes associated to the different genetic cardiomyopathies, including HCM, DCM, ACM, and channelopathies, such as LQTS and BrS; and (2) an enlarged panel of 129 genes including rarer genes; this panel was used in cases of complex or unclear phenotypes and in the presence of a positive family history for SCD. In both cases, HaloPlex technology (Agilent, Santa Clara, CA, USA) was used for library preparation. In detail, each genomic DNA sample was fragmented using a pool of restriction enzymes. The obtained fragments were enriched with hybridization with the custom capture probes, and then purified and PCR-amplified to obtain a DNA library or sample. During this procedure, each genomic DNA sample was univocally tagged with a barcode sequence to allow for sample multiplexing during the subsequent sequencing step.

The obtained enriched and indexed libraries were sequenced using the MiSeq (Illumina, San Diego, CA, USA) instrument (2 × 250 PE). Alissa software (Agilent, Santa Clara, CA, USA) was used to perform sequence data analysis. This tool allows for the alignment of the sequences to the reference genome to obtain a list of genomic variants that can be prioritized using a customizable pipeline in order to highlight pathogenic mutations or potentially pathogenic variants. This pipeline allows for variant classification based on their position with regard to the gene structure (exonic or intronic), population frequency, coding effect, ClinVar classification, and functional predictions. In addition, we classified the variants according to American College of Medical Genetics and Genomics (ACMG) guidelines adapted to cardiomyopathy [61]. In detail, all variants were evaluated for their frequency in international population database gnomAD (http://gnomad.broadinstitute.org/, accessed on 26 September 2022): we excluded variants showing minor frequency alleles (MAF) below the calculated maximal tolerated allele frequency for the specific cardiomyopathy [62]. According to these criteria, the maximal credible allele frequency was 0.000084 for DCM, 0.00004 for HCM, 0.000092 for ACM, 0.00001 for BrS, and 0.0000082 for LQTS [62]. However, we kept 10 variants showing an MAF greater than the threshold because they are reported as pathogenetic in the HGMD database. The latter classifies variants on the basis of the interpretation at first reporting; thus, even if it is less valuable with respect to ACMG classification and more prone to subsequent modifications, it may provide additional data supporting the variants’ correct classification and subsequent interpretation. Moreover,
bioinformatic pathogenicity evaluation for missense variants was performed by using multiple in silico tools, including Grantham distance and an Align–GVGD matrix. The splicing module from Alamut visual v.2.7.0.0 software was used to test the pathogenicity of a possible splicing variant. In some cases, we had informative families, i.e., ones having at least 3 affected relatives in which we analyzed variant cosegregation. In families with one affected member being a noncarrier, the variant is considered to be non-disease-causing. Pathogenic or doubtful variants were confirmed with Sanger sequencing.

3. Results

In total, 133 independent subjects underwent genetic testing because of a clinical suspicion of inherited cardiomyopathy or channelopathy over a period of enrollment of 3 years (2018–2020). The average sequencing coverage of the target regions was in the range of 150–250X with a 50X minimal acceptable threshold. In detail, 60/133 patients showing a phenotype, and/or laboratory or instrumental data supporting a clinical diagnosis of a specific inherited cardiomyopathy according to international guidelines were analyzed using a first-level panel including 60 main diagnostic genes that had been associated with different cardiomyopathies/channelopathies. The remaining 73/133 subjects, showing unclear cardiological clinical signs and/or a positive family history for SCD, were analyzed using an enlarged 129 gene panel. In our cohort of 133 patients, we detected a total of 82 variants, excluding polymorphisms. The variants, shown in Table 1, were reported following Human Genome Variation Society (HGVS) nomenclature (http://www.HGVS.org/varnomen, accessed on 26 September 2022), and annotated according to the Human Gene Mutation Database (HGMD) Professional 2020.4, (http://www.hgmd.cf.ac.uk/, accessed on 26 September 2022), NCBI SNP Database (http://www.ncbi.nlm.nih.gov, accessed on 26 September 2022), and ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar, accessed on 26 September 2022).

Among the 82 detected variants, 27 were novel as they were missing in the reference SNP, ClinVar, and HGMD databases. The ClinVar database reported 39/82 variants. Of these variants, 20 were classified as pathogenic or likely pathogenic (P/LP), 13 as conflicting (CI), and 6 of uncertain or unknown significance (VUS). The remaining 43 variants were not reported (NR) in ClinVar. HGMD reported 38/82 variants as pathogenic or likely pathogenic, while the remaining 44 were not reported. According to ACMG interpretation criteria, 39/82 variants were classified as P/LP (48%), of which 6 were novel (without reference SNP ID number), and 41/82 as VUSs (50%), of which 26 were novel ones. Furthermore, two of the identified variants (2%) were classified by ACMG guideline as benign or likely benign (B/LB). According to ACMG criteria, we globally identified P/LP variants in 53 patients (40% of the analyzed population), VUSs in 38 patients (29%), and B/LB variants in 4 patients (3%). In addition, 11 patients carried multiple variants. We did not identify any pathogenic mutations or VUSs in the remaining 38 patients (29%), who showed only known polymorphisms and/or LB variants in the analyzed genes. If we separately analyzed the two cohorts of patients, among the 60 patients admitted to the first-level test, 24 carried a P/LP variant (41%, 7 channelopathy and 17 cardiomyopathy patients) and 24 carried a VUS (41%, 7 channelopathy and 17 cardiomyopathy patients). Similarly, among the 73 patients analyzed using the enlarged panel, 29 carried a P/LP variant (40%, 4 channelopathy and 25 cardiomyopathy patients) and 14 carried a VUS (19%, 6 channelopathy and 8 cardiomyopathy patients).
Table 1. List of genetic variants (n = 82) identified in the 133 patients of the study cohort.

| Gene     | Transcript | HGVS * Coding (cDNA) | HGVS * Protein Level | Patient's Phenotype | ClinGen ** | Reference SNP ID | ClinVar | HGMD $ | ACMG *** | ACMG Supporting Criteria $ | gnomAD Frequency |
|----------|------------|----------------------|----------------------|---------------------|------------|------------------|---------|--------|----------|----------------------------|------------------|
| ABC9     | NM_020297.4 | c.2937G>A             | p.Trp979Ter          | HCM                 | NR         | NR               | NR      | NR     | P        | PS1/PM2/PP3                | NF               |
| ANK2     | NM_00134273.1 | c.1873C>G             | -                    | HCM                 | NR         | NR               | NR      | NR     | P        | PS1/PM2/PP3                | 0.00000402       |
| ANK2     | NM_00134273.1 | c.1900C>A             | p.Gly64Arg           | BrS                 | DISPUTED   | rs1562805147    | NR      | VUS    | PM2/PP3               | NF               |
| CACNA1D  | NM_000720.4 | c.4187C>A             | p.Ala1396Asp         | BrS                 | DISPUTED   | rs745690505     | NR      | VUS    | PM2/PP3               | NF               |
| CACNA1D  | NM_000720.4 | c.1059C>G             | p.Ala353 Ala         | HCM                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CACNA1D  | NM_00136867.1 | c.1516-4C>A           | -                    | HCM                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CACNB2   | NM_201570.3 | c.645G>T              | p.Me215Ile           | ACM                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CACNB2   | NM_201570.3 | c.1652G>T             | p.Arg551Met          | ACM                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CASQ2    | NM_001232.4 | c.562C>T              | p.His188Tyr          | BrS                 | NR         | rs141314684     | CI      | P      | LB       | BS1/BP6                | 0.000001         |
| CASQ2    | NM_001232.4 | c.928G>A              | p.Asp310Asn          | BrS                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CAV3     | NM_033337.3 | c.233C>T              | p.Trh78Met           | HCM                 | NR         | rs72546668      | CI      | P      | B        | PP3/PP2/BS1/BS2         | 0.00263 vs. 0.0004 |
| CAV3     | NM_033337.3 | c.682delG             | -                    | HCM                 | NR         | NR               | NR      | NR     | VUS      | PM2/PP3                | NF               |
| CTNNB1   | NM_001354273.1 | c.1873C>G             | -                    | HCM                 | NR         | rs1485074194    | NR      | VUS    | PM2/PP3               | NF               |
| DSC2     | NM_024422.6 | c.726C>T              | p.Ser242Ser          | HCM                 | NR         | rs869025431     | NR      | VUS    | PM2/PP3               | NF               |
| DSP      | NM_000415.4 | c.8471_8483delGGTC    | p.Gly2824AlafsTer55  | ACM                 | DEFINITIVE | rs397516946     | LP      | P      | P        | PS1/PM2                | NF               |
| DSP      | NM_000415.4 | c.8171A>G             | p.Glu2724Arg         | ACM                 | DEFINITIVE | rs371666083     | NR      | VUS    | VUS      | PM2/PP3                | NF               |
| DSP      | NM_000415.4 | c.3284delA             | p.Ile950LeufsTer27   | ACM                 | DEFINITIVE | rs371666083     | NR      | VUS    | VUS      | PM2/PP3                | NF               |
| DSP      | NM_000415.4 | c.1748A>G             | p.Ala583Ser          | ACM                 | DEFINITIVE | rs1204195890    | NR      | VUS    | VUS      | PM2/PP3                | NF               |
| DSC2     | NM_024422.6 | c.1516-4C>A           | -                    | HCM                 | NR         | rs869025431     | NR      | VUS    | PM2/PP3               | NF               |
| DSC2     | NM_024422.6 | c.1059C>G             | p.Ala353 Ala         | HCM                 | NR         | rs1371737796    | NR      | VUS    | PM2/PP3               | NF               |
| DSC2     | NM_024422.6 | c.2785dupG            | p.Glu929GlyfsTer11   | ACM                 | DEFINITIVE | rs794728458     | CI      | P      | P        | PS1/PP5                | NF               |
| FHL1     | NM_001354273.1 | c.2764C>G             | p.Cys522Ser          | ACM                 | DEFINITIVE | rs761863251     | CI      | P      | P        | PS1/PP5                | NF               |
| KCNE1    | NM_005472.5 | c.41+1G>C             | -                    | ACM                 | DEFINITIVE | rs120074178     | P/LP    | P      | P        | PS3/PM1/PM2/PP3/PP5/BP1 | 0.0000401       |
| KCNE2    | NM_005472.5 | c.157C>T              | p.Arg53Cys           | ACM                 | DEFINITIVE | rs19947237     | CI      | P      | P        | PM1/PM5/PM2/PP3/PP5     | 0.000036         |
| KCNE2    | NM_005472.5 | c.41+1G>C             | -                    | ACM                 | DEFINITIVE | rs19947237     | CI      | P      | P        | PM1/PM5/PM2/PP3/PP5     | 0.000036         |
| KCNE3    | NM_005472.5 | c.41+1G>C             | -                    | ACM                 | DEFINITIVE | rs19947237     | CI      | P      | P        | PM1/PM5/PM2/PP3/PP5     | 0.000036         |
| LMNA     | NM_001282626.2 | c.9494G>C             | p.Ser3162Pro         | ACM                 | DISPUTED   | rs587775707    | P       | P      | P        | PS1/PS2/PS3/PS4/PS5     | 0.0000433        |
| MYBPC1   | NM_000256.3 | c.1591G>C             | p.Gly531Arg          | ACM                 | DEFINITIVE | rs39751912     | LP      | P      | P        | PM1/PM2/PP3/PP5/BS2     | 0.0000121        |
| MYBPC1   | NM_000256.3 | c.1790G>C             | p.Arg597Cln          | ACM                 | DEFINITIVE | rs727503195    | CI      | P      | P        | PP3/PM1/PM2/BS2        | 0.00003          |
| MYBPC1   | NM_000256.3 | c.1928-2A>G           | -                    | ACM                 | DEFINITIVE | rs39751937     | P       | P      | P        | PS1/PS2/PS3/PS4/PS5     | NF               |
| MYBPC1   | NM_000256.3 | c.2955G>T             | p.Lys958Asn          | ACM                 | DEFINITIVE | rs1299079662   | P       | P      | L        | PSV1/PM2                | NF               |
| MYBPC1   | NM_000256.3 | c.3627+2T>A           | -                    | ACM                 | DEFINITIVE | rs730880805    | P       | P      | P        | PS1/PM2/PP3/PP5         | NF               |
| MYH6     | NM_002347.1 | c.562C>G              | p.Val187Ile          | ACM                 | DEFINITIVE | rs1562805147   | NR      | VUS    | PM2/PP3               | NF               |

**Variant Classification**

- **ClinGen**: ClinGen Classification
- **Reference SNP ID**: Reference SNP ID
- **ClinVar**: ClinVar
- **HGMD**: HGMD
- **ACMG**: ACMG
- **ACMG Supporting Criteria**: ACMG Supporting Criteria
- **gnomAD Frequency**: gnomAD Frequency
| Gene | Transcript | HGVS * Coding (cDNA) | HGVS * Protein Level | Patient's Phenotype | ClinGen ** | Reference | SNP ID | ClinVar | HGMD $ | ACMG *** | ACMG Supporting Criteria # | gnomAD Frequency |
|------|-------------|---------------------|---------------------|--------------------|------------|-----------|--------|---------|--------|----------|-----------------------------|------------------|
| MYH7 | NM_000257.4  | c.1615A>C           | p.Met539Leu         | HCM                | DEFINITIVE  | rs73088930 | LP     | P       | LP     | PM1/PM2/PP2/PP5/PP3/PP5/BP1 | 0.0000657        |
| MYH7 | NM_000257.4  | c.2155C>T           | p.Arg719Trp         | HCM                | DEFINITIVE  | rs12191367 | P      | LP      | LP     | PM1/PM2/PP2/PP5/PP3/PP5/BP1 | 0.0000657        |
| MYH7 | NM_000257.4  | c.4066G>A           | p.Glu1356Lys        | HCM                | DEFINITIVE  | rs725703246 | LP     | LP      | LP     | PM1/PM2/PP2/PP3/PP5/BP1     | 0.0000398        |
| MYL2 | NM_000432.4  | c.2703G>T           | p.Glu893Val         | HCM                | DEFINITIVE  | rs397516201 | LP     | LP      | LP     | PM1/PM2/PP2/PP3/PP5/BP1     | 0.0000398        |
| OBPSCN| NM_00127223.2 | c.22911_22912delGT  | p.Ser7638MetfsTer30 | HCM                | NR          | rs3558418533 | NR     | NR      | LP     | PV51/PM2                  | 0.0000402        |
| PKP2 | NM_004572.4  | c.4130C>T           | p.Thr1377Met        | ACM                | DEFINITIVE  | rs397516994 | P/LP   | P       | P      | PV51/PM2/PP3/PP5            | 0.0000399        |
| PKP2 | NM_004572.4  | c.1378+1G>C         |                      | HCM                | DEFINITIVE  | rs397516784 | CI     | P       | LP     | PS3/PM2/PM4/PP5            | 0.0000398        |
| PLN# | NM_002667.5  | c.40_42delAGA       | p.Arg14del          | ACM/DCM            | MODERATE    | rs97516784 | CI     | P       | LP     | PS3/PM2/PM4/PP5            | 0.0000398        |
| POLG | NM_002693.3  | c.530G>A            | p.Arg177Gln         | ACM/DCM            | MODERATE    | rs113994094 | CI     | P       | LP     | PM2/PP5                  | 0.000155         |
| POLG | NM_002693.3  | c.752C>T            | p.Thr251Ile         | ACM                | MODERATE    | rs145843073 | CI     | P       | LP     | PM2/PP5/PP4               | 0.0000473 vs. 0.00001 |
| POLG | NM_002693.3  | c.1402A>G           | p.Asn468Asp         | BrS                | MODERATE    | rs113994096 | CI     | P       | P      | PP5/PM2/PP3               | 0.0000398        |
| PRDM16 | NM_0021114.4 | c.1400C>A          | p.Pro467His         | ACM                | MODERATE    | rs58777588 | P      | LP      | LP     | PM2/PM1/PM5/PP5            | 0.000159         |
| RAFL1| NM_001354689.3 | c.709G>A            | p.Ala237Thr         | HCM                | NR          | rs397516994 | P/LP   | P       | LP     | PM2/PP2/PP5/PP3            | 0.0000398        |
| RAFL1| NM_001354689.3 | c.945T>G            | p.Ala315Arg         | ACM                | MODERATE    | rs779308123 | CI     | P       | LP     | PM2/PP2/PP5               | 0.0000473 vs. 0.00001 |
| RYR2 | NM_0001033.3  | c.11467G>T          | p.Glu3829Stop       | ACM                | MODERATE    | rs728722059 | NR     | NR      | NR     | PM2                     | 0.0000409        |
| SCN10A| NM_005143.3  | c.3090C>T          | p.Glu1030Ter        | ACM                | MODERATE    | rs780649338 | NR     | NR      | NR     | PM2/PP3/PP5               | 0.0000159        |
| SCN3B | NM_001040151.2 | c.353C>T            | p.Tyr118Ter         | ACM                | MODERATE    | rs145843073 | CI     | P       | LP     | PM2/PP5/PP4               | 0.0000473 vs. 0.00001 |
| SCN5A | NM_198056.3   | c.4414_4416delAGA   | p.Asn1452del        | ACM/DCM            | MODERATE    | rs199473584 | CI     | P       | LP     | PS3/PM2/PM3/PP3/PP5        | 0.0000422 vs. 0.00001 |
| SCN5A | NM_198056.3   | c.4414_4416delAGA   | p.Asn1452del        | ACM/DCM            | MODERATE    | rs199473584 | CI     | P       | LP     | PM1/PM2/PM4/PP3/PP5        | 0.0000601 vs. 0.00001 |
| TNNI3 | NM_000363.5   | c.434G>A            | p.Arg145Lys         | HCM                | DEFINITIVE  | rs397516349 | P/LP   | P       | P      | PP5/PS3/PM1/PM5/PM2/PP3/PP3 | 0.0000161        |
| TNNI3 | NM_000363.5   | c.485G>A            | p.Asp162Glu         | HCM                | DEFINITIVE  | rs397516349 | P/LP   | P       | P      | PP5/PS3/PM1/PM5/PM2/PP3/PP3 | 0.0000402        |
| TNP1K | NM_0019793.3  | c.2187G>T           | p.Met729Ile         | HCM                | DEFINITIVE  | rs137281124 | NR     | NR      | NR     | PV51/PM2                  | 0.0000404        |
| TNP1K | NM_0019793.3  | c.2187G>T           | p.Met729Ile         | HCM                | DEFINITIVE  | rs397516463 | P       | P      | P      | PM1/PM2/PP2/PP3/PP5        | 0.0000401        |
| TTN# | NM_00126750.2 | c.50255C>G          | p.Pro1672Arg        | ACM/DCM            | MODERATE    | rs751095080 | NR     | NR      | NR     | LV51/PM2/PP4               | 0.0000401        |

**Table 1. Cont.**

- **HGVS**: Human Genome Variation Society mutation notation.
- **ClinGen**: Clinical Genomics.
- **ACMG**: American College of Medical Genetics and Genomics.
- **gnomAD**: Genome Aggregation Database.
- **Variant Classification**: Based on ACMG guidelines.
- **PM2/PM3/PM4/PP3/PP5**: Evidence supported by evidence levels specified by ACMG guidelines.
| Gene | Transcript | HGVS * Coding (cDNA) | HGVS * Protein Level | Patient's Phenotype | ClinGen ** | Reference SNP ID | ClinVar | HGMD § | ACMG *** | ACMG Supporting Criteria # | gnomAD Frequency |
|------|------------|----------------------|----------------------|-------------------|----------|----------------|---------|--------|---------|--------------------------|-----------------|
| TTN  | NM_001267550.2 | c.83378A>G           | p.Asp27793Gly        | DCM               | DEFINITIVE | NR              | NR      | NR     | NR      | VUS                      | NF              |
| TTN  | NM_001267550.2 | c.69500G>A           | p.Gly23167Asp        | HCM               | LIMITED   | rs747019187     | NR      | NR     | NR      | VUS                      | PM2             |
| TTN  | NM_001267550.2 | c.102565G>C          | p.Asp34189His        | BrS               | NR        | NR              | NR      | NR     | NR      | PM2                      | NF              |

For each genetic variant, the following information is shown: reference transcript, variant nomenclature at DNA and protein level according to Human Genome Variation Society (* HGVS) guidelines, clinical phenotype, gene classification according to the NIH-funded Clinical Genome Resource (** ClinGen) with respect to the clinical phenotype, reference single-nucleotide polymorphism (SNP) ID number (rs), clinical significance by the ClinVar database, HGMD database (§ Human Genetic Mutation Database (HGMD) and American College of Medical Genetics (*** ACMG) classification, including ACMG supporting criteria (#) according to [62]; variant allele frequency according to gnomAD exome database. §§ Mutations found in 2 patients; §§§ mutations found in 3 patients; º mutations found in 2 patients; ° GnomAD frequency versus the maximal tolerated allele frequency for the specific cardiomyopathy. NF: not found; NR: not reported; LP: likely pathogenic; P: pathogenic; LB: likely benign; VUS: variant of uncertain significance; CI: conflicting interpretation; ACM: arrhythmogenic cardiomyopathy; DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; BrS: Brugada syndrome; LQTS: long QT syndrome.
Figure 1 shows the distribution of P/LP and VUS variants among channelopathy (Figure 1A,C) and cardiomyopathy (Figure 1B,D) patients analyzed via the two genes panels. The percentage of patients with an unknown causal gene included subjects without pathogenic mutations or VUSs, carrying known polymorphisms and/or LB variants in the analyzed genes.

![Figure 1](image.png)

**Figure 1.** Percentage of channelopathy and cardiomyopathy patients carrying pathogenic/likely pathogenic (P/LP) and unknown significant (VUS) variants, as identified using (A,B) first-level and (C,D) extended panels. Variants were classified according to ACMG guideline classification with respect to patients without a known causative gene.

In total, 11 patients (8.3%) carried multiple variants: in particular, 3 patients carried two different P/LP mutations, 3 patients carried 1 P/LP mutation and a VUS, and 5 patients carried more than one VUS. We analyzed 5/11 patients with the first-level test, and 6/11 with the enlarged one. Furthermore, 7/11 patients showed double mutations in uncommon genes with respect to their phenotype, 2 patients were doubly mutated in main genes, and 2 patients showed one mutation in an uncommon and the other in a main gene.

Globally, among the 82 variants, 50 (61%) were localized in genes without reported evidence, or classified with limited or disputed evidence for a role in cardiomyopathies or channelopathies in ClinGen (uncommon genes), while 32 (39%) were had a definitive or moderate association with the disease.

Among the 50 identified variants in uncommon genes, 48 were P/LP or VUS (13 as P/LP and 35 as VUSs) and 2 as B/LB according to ACMG classification. In addition, 11/13 P/LP and 14/35 VUSs were identified among patients who had undergone extended molecular analysis.

In particular, 9 ACM patients carried variants in the CACNB2 (n = 2), KCNE3 (n = 1), LDB3 (n = 1), MYH6 (n = 1), PLN (n = 1), POLG (n = 1), and RYR2 (n = 1) genes. One ACM patient was a compound heterozygous (confirmed with segregation analysis) carrier of two missense variants (c.752C>T and c.1760C>T) in the POLG gene. Furthermore, c.40_42delAGA (p.Arg14del) in the PLN gene was present in both a patient with ACM and an independent DCM patient. ClinGen reports a definitive association between the PLN gene and the DCM but not ACM phenotype [63].

In addition, 17 independent HCM patients carried pathogenic variants or VUSs in the following uncommon genes: ABCC9, ANK2, CAV3, CTNNA3, DSP, KCNQ1, FHL1, OBSCN, PKP2, RYR2, TGFB3 (reported in one patient each); RAFI (n = 2), SCN10A (n = 2), and TTN (n = 2).
In total, 14 independent BrS patients carried P/LP or VUSs in the following uncommon genes: ANK2, CACNA1D, CACNA2D1, CASQ2 (n = 2), CAV3, DSC2, KCNE3, KRAS, MYBPC3, POLG, PRDM16, SGCD, and TGFB3.

In 3 DCM patients, we identified 3 VUSs in uncommon genes KCNQ1, TBX1, and TRPM4. Lastly, in LQTS patients, we identified 3 VUSs in uncommon genes KRT17, LAMA3, and POLG.

The detailed distribution of all identified variants divided by clinical phenotype is reported in Table 1.

Overall, in this study group, we detected 39 pathogenic or likely pathogenic mutations, of which 26 were identified in main genes (67%) and 13 (33%) in uncommon genes. Furthermore, we identified 41 VUSs, of which 35 (85%) in uncommon genes and 6 (15%) in main genes. Our findings indicate that P/LP variants are more likely to be identified in main genes, while VUSs are mostly identified in uncommon genes. When considering the two analyzed cohorts separately, we found that the number of variants (both P/LP and VUSs) in the main genes was almost the same within the two different panels; the number of P/LP variants identified in uncommon genes increased when the enlarged panel was used (Figure 2).

As shown in Figure 3A, P/LP mutations in both main and uncommon genes (accordingly with association with the reported cardiomyopathy in Clinical Genome Resource) were mainly detected in HCM patients (57% of the P/LP variants), followed by ACM (20%), LQTS, DCM, and BrS patients (12%, 6% and 6%, respectively). Among ACM, HCM, BrS, DCM, and LQTS patients carrying P or LP mutations, 4/10 (40%), 7/29 (24%), 2/3 (67%), 2/3 (67%), 1/6 (17%) were detected in uncommon genes, respectively (Figure 3B). Only 3 (19%) of these variants were identified in patients who had undergone the first-level test, while the remaining 13 (81%) were identified with the enlarged-gene panel.

Furthermore, among the 41 identified VUSs, 6 (15%) were detected in ACM, 6 (15%) in DCM, 13 (32%) in HCM, 13 (32%) in BrS, and 3 (7%) in LQTS patients (Figure 4A). Interestingly, 35/41 VUSs (85%) fell into the uncommon-gene group, with 22 (63%) identified in patients who had undergone the first-level test and 13 (37%) in those who performed the enlarged one. When we analyzed the distribution of the VUSs in uncommon and main genes per each disease, we found a higher percentage of VUSs in common genes in all the diseases with the exception of DCM (Figure 4B).

In the enrolled patients among 133 analyzed subjects, we detected 53 patients (40%) carrying at least one P or LP variants. In particular, 15 out of 53 patients (28%) carried P or LP variants in uncommon genes and 38 (72%) in main genes (Figure 5). Table 2 shows the demographic, clinical, and instrumental data of eight patients carrying pathogenic or likely pathogenic variants detected in uncommon genes with respect to the clinical diagnosis or suspicion for the inherited cardiac disease (cardiomyopathy or channelopathy). All except 1 patient (NA04) were analyzed with the enlarged panel of 129 genes, including rarer genes.

**Figure 2.** Number of pathogenic/likely pathogenic (P/LP) variants and VUSs according to ACMG guidelines, identified in the patients analyzed using (A) first-level and (B) enlarged panels and divided by main and uncommon genes.
These eight patients were recruited according to the physician’s clinical suspicion for an inherited cardiomyopathy or channelopathy. In detail, four HCM patients carried mutations in uncommon genes \( ABCC9, \ \text{KCNQ1}, \ \text{PKP2} \) and \( \text{RAFI} \), and two BrS patients in uncommon genes \( \text{MYBPC3} \) and \( \text{POLG} \). Furthermore, we identified one ACM patient carrying a mutation in uncommon gene \( \text{KCNE3} \) and one ACM patient \textit{in trans} carrying two variants in uncommon gene \( \text{POLG} \) (c.752C>T and c.1760C>T), classified as LP/P according to the novel ACMG criteria.

Furthermore, VUSs were identified in 38 out of 133 patients (29%), of which 32 (84%) showed VUSs in uncommon genes and 6 (16%) in main genes (Figure 5).
Table 2. Demographic, clinical, and instrumental data of patients carrying pathogenic or likely pathogenic variants in uncommon genes with respect to the clinical diagnosis or suspicion of an inherited cardiac disease (cardiomyopathy or channelopathy).

| ID  | Age at Diagnosis (Years) | Sex | Family History | Symptoms * | ECG * | Gene | Nucleotide Variant (Aminoacid Change) | Echocardiography/CMR ** | Definitive Diagnosis |
|-----|--------------------------|-----|----------------|------------|-------|------|----------------------------------------|------------------------|---------------------|
| NA01| 20                       | M   | Negative for SCD and HCM | Exertional dyspnoea (NYHA Class II) | Signs of LVH, diffuse repolarization abnormalities | ABCC9 | c.2937G>A (p.Trp979Ter) | Symmetric LVH with MWT (18 mm) at the level of the basal IVS and first-degree diastolic dysfunction | HCM |
| NA02| 47                       | M   | Negative for SCD, and ACM | Asymptomatic | Diffuse repolarization abnormalities | KCNE3 | c.-41+1G>C | Regional dyskinesia of right ventricle free wall. Identification of regional fibrosis with LGE | ACM |
| NA03| 44                       | M   | Negative for SCD and HCM | Asymptomatic | Right axis deviation; delayed intraventricular left conduction | KCNJ1 | c.877C>T (p.Arg293Cys) | Symmetric LVH with MWT (22 mm) at the level of the basal IVS | HCM |
| NA04| 51                       | F   | Negative for SCD and BrS | Syncope | Brugada type 1 pattern | MYBPC3 | c.906-1G>C | Normal wall thicknesses and chamber diameters | BrS |
| NA05| 79                       | M   | Negative for SCD and HCM | Asymptomatic | Diffuse repolarization abnormalities | PKP2 | c.1378+1G>C | Asymmetrical LVHMWT, 19 mm | HCM |
| NA06| 9                        | M   | Negative for SCD and BrS | Asymptomatic | Flecainide-induced Brugada type 1 pattern | POLG | c.1402A>G (p.Asn468Asp) | Normal wall thicknesses and chamber diameters | BrS |
| NA07| 20                       | M   | Positive for SCD and ACM | Syncope | Diffuse repolarization abnormalities | POLG | c.752C>T (p.Thr251Ile); c.1760C>T (p.Pro587Leu) | Normal wall thicknesses and chamber diameters. Presence of diffuse late gadolinium enhancement with subepicardial distribution in the basal segment of the inferior and lateral walls at CMR | ACM |
| NA08| 18                       | M   | Negative for SCD and HCM | Exertional dyspnoea (NYHA Class II) | Signs of LVH, negative T-waves in AVL, V5, V6 leads | RAF1 | c.709G>A (p.Ala237Thr) | Asymmetric obstructive LVH with MWT (15 mm) at the level of the basal IVS | HCM |

Abbreviations: ACM, arrhythmogenic cardiomyopathy; BrS, Brugada syndrome; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; LQTS, long QT syndrome; ECG, electrocardiogram; CMR, cardiac magnetic resonance; IVS, interventricular septum; MWT, maximal wall thickness; NYHA, New York Heart Association; SCD, sudden cardiac death; LGE, late gadolinium enhancement. * At first evaluation; ** at last follow-up.
4. Discussion

In this study, we report the results of a targeted-NGS-based genetic screening carried out on 133 unrelated patients from South Italy diagnosed with cardiomyopathy or channelopathy. We used a large panel of 129 cardiomyopathy-related genes to increase the yield of genetic testing considering the genetic heterogeneity and variable and overlapping phenotypes, which is a characteristic of hereditary structural and electric cardiac diseases. Using the clinical genome resource approach to assess the strength of gene–disease association, we focused on variants in genes without definitive or strong evidence for an association with cardiomyopathies or channelopathies, which we defined as uncommon causal genes.

Globally, our NGS panel test demonstrated a yield of 40% if we considered the genetic test to be positive in the presence of pathogenic or likely pathogenic variants in both main and uncommon genes. When we included the VUSs, the global yield of the test increased up to 69%.

We detected 39 pathogenic or likely pathogenic variants according to the ACMG classification in 53 patients (44 cardiomyopathies and 9 channelopathies), including 13 patients carrying P or LP variants in uncommon genes. The role of these uncommon genes as causal genes should be evaluated in the context of the clinical manifestations and the evidence of the functional effects of the variant on the encoded protein; moreover, when large pedigrees are available, the analysis of the segregation of the genetic variant with the phenotype allows for confirming the phenotype–genotype association. Furthermore, we verified whether similar gene–disease associations to those identified in our study were reported in the literature. For example, in our study cohort, we detected a mutation in sarcomeric gene MYBPC3 (c.906-1G>C; rs587776700) in a BrS patient. Experimental studies showed that this variant disrupts mRNA splicing, leading to a loss of protein function [64]. Some studies reported a MYBPC3 mutation in patients showing the Brugada phenotype [65,66]. In addition, the literature reported cases of Brugada phenocopies in patients carrying a pathogenic mutation in the MYBPC3 gene [67], speculating that Brugada type 1 ECG could be an early sign of an occult structural heart disease. Brugada syndrome is generally considered to be a disease of the ion channels. However, recent studies have also identified structural heart impairment, i.e., epicardial surface and interstitial fibrosis, increased collagen, and reduced contractility in BrS patients. These observations agree with the finding of causal mutations in genes that are molecular causes of cardiomyopathies, such as sarcomeric genes [66]. These data suggest that structural and electrical cardiac alterations may be present in BrS patients, which may develop from the same molecular alteration.

Similarly, although HCM is commonly considered to be a disease of the sarcomere, recent literature data reported an association of the HCM phenotype with mutations in the ABCC9 [48,68], PKP2 [69–71], and RAF1 [72] genes, and in other nonsarcomeric genes [73]. The pathogenic mechanisms leading to the development of the HCM phenotype need to be elucidated.

Furthermore, in our study, we identified (L)P variants in the POLG gene in two patients with BrS and ACM, respectively. Our data are in accordance with recently published data in which the POLG gene was associated with the ACM phenotype [74].

Overall, in our cohort, the prevalence of (L)P variants in uncommon genes was about 8% (3% in the first-level and 15% in the enlarged panels). This result supports the need for extended genetic testing including uncommon genes for such heterogeneous diseases as cardiomyopathies and channelopathies. Indeed, by limiting the genetic test to just phenotype-related (main) genes, we would have failed in identifying 13 mutations and the corresponding carrying patients (8% of the screened population). On the other hand, our approach was able to increase the diagnostic sensitivity of the test identifying these additional patients.

Biotechnological research is always looking for new diagnostic strategies [75]. As NGS-based applications are currently widely used in diagnostic laboratories, and their costs are decreasing progressively, their use is desirable to enlarge the spectrum of the genes
related to a specific disease. Indeed, accumulating data from different studies may allow for reconsidering the genotype–phenotype associations for some genes now considered to be uncommon.

In addition to pathogenic and likely pathogenic mutations, as expected in this kind of extended molecular screening, we identified many VUSs (41/82; 50% of the total number of identified variants) according to ACMG classification. Of the VUSs, 85% (35/41) were detected in uncommon genes. We detected variant c.764G>C in an HCM patient in the FHL1 gene, which is classifiable as a VUS according to ACMG criteria. However, it is reported as pathogenic by HGMD database, showing strong evidence for a primary pathogenic role in HCM [73,76]. The FHL1 gene was classified without evidence for a HCM association by ClinGen. The ACMG rule for classification is hypothesized to minimize the risk for false positive interpretations; however, it is also well-accepted that these rigorous criteria result in undercalling pathogenic variants in well-established cardiomyopathy genes.

Certainly, VUS interpretation and the consequent clinical management of their carriers is a key challenge following NGS-based molecular testing [77]. The possibility to study a specific VUS in the context of a large pedigree by allowing for the robust analysis of the variant’s segregation with the clinical phenotype may be decisive to establish its pathogenic role; however, this is often unfeasible [78]. To definitively assess the molecular consequence of an identified VUS and thus establish its functional effects, the most proper approach should include in vitro functional assays [79–82]. Nevertheless, these functional evaluations are often difficult to be realized, especially in a routine setting and on a large scale, since different cellular models and different assays need to be used on the basis of the gene involved and on the type of molecular defect. The more that we enlarge the analyzed genomic region, the more the number of VUSs per patient increases. Nevertheless, even if VUS communication may be challenging and frustrating for both patients and clinicians, it offers a great opportunity for the re-evaluation of cases over time. Indeed, monitoring these variants over time may allow for their reclassification, and hence provide novel clues for the management of carriers and their relatives. Moreover, some of the VUSs may act as modifier genes, influencing a patient’s clinical phenotype, and may contribute to also explaining the observed clinical heterogeneity within families.

5. Conclusions

Considering all the above, extensive genetic test approaches are required to unravel the molecular bases of such a complex group of diseases as cardiomyopathies and channelopathies. NGS-based approaches, by allowing for the simultaneous analysis of multiple genes, increase the detection rate of causal mutations in inherited structural and electric hereditary cardiac diseases, and provide epidemiological data regarding the prevalence of causative mutations in uncommon genes. In addition, several uncertain or unknown variants (VUSs) are being identified and require more efforts in the near future to assess their pathogenicity.

The use of an NGS-based approach in diagnostics also increases the yield of mutations carriers’ identification in the rarest and uncommon genes, as shown in our study. Indeed, our data are a parameter of the increased diagnostic yield of such extended molecular analyses that also enable screening for rare and uncommon genes in a simple, reliable, and cost-saving manner.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12101417/s1. Table S1: genes reported in the literature associated with cardiomyopathies and channelopathies.

Author Contributions: C.M., G.F. and V.D. conceived and designed the study; V.D., F.U. and M.V.E. performed experimental data; F.B., B.M., C.M., G.F. and V.D. analyzed the data; C.M., G.F., V.D. and R.L. wrote and revised the paper; G.L., M.A.L., R.L., M.C. and E.M. collected the clinical data. All authors have read and agreed to the published version of the manuscript.
Funding: This research was funded by the Italian Ministry of University and Research, grant numbers PON03PE_00060_7 and PRIN-20173ZWACS (R.L. and G.L.).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by an internal institutional review board (N. 77/21).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are reported within the text.

Acknowledgments: The authors thank Serena Conato for the technical support to the experimental work.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

NGS Next-generation sequencing
VUS Variants of unknown significance
SCD Sudden cardiac death
HCM Hypertrophic cardiomyopathy
ACM Arrhythmogenic cardiomyopathy
DCM Dilated cardiomyopathy
LQTS Long QT syndrome
BrS Brugada syndrome
SQTS Short QT syndrome
CPVT Catecholaminergic polymorphic ventricular tachycardia
MYBPC3 Cardiac myosin binding protein 3
MYH7 β-myosin heavy chain
TNNT2 Cardiac troponin T
TNNI3 Cardiac troponin I
TMPI α-tropomyosin
ACTC1 Cardiac α-actin
MYL2 Myosin regulatory light chain
MYL3 Myosin essential light chain
MYOZ2 Myozenin 2 gene
ACTN2 α-actinin-2
TTN Titin
MYOM1 Myomesin 1
ANKRD1 Cardiac ankyrin repeat protein 1
TCAP Telethonin
PKP2 Plakophilin
DSP Desmoplakin
DSC2 Desmocollin 2
DSG2 Desmoglein
TMEM43 Transmembrane protein 43
PLN Phospholamban
CDH2 Cadherin-2
CTNNAL3 Catenin alpha 3
FLNC Filamin C
TJP1 Tight junction protein 1
ANK2 Ankyrin 2
TP63 Tumor protein P63
SCN10A Sodium voltage-gated channel alpha subunit 10
CACNA1C Calcium voltage-gated channel subunit alpha 1 c
ABCC9 ATP binding cassette subfamily c member 9
SCN1B Sodium voltage-gated channel beta subunit 1
KCNH2 Potassium voltage-gated channel subfamily h member 2
CACNB2 Calcium voltage-gated channel auxiliary subunit beta 2
TRPM4 Transient receptor potential cation channel subfamily m member 4
ANK3 Ankyrin 3
CACNA2D1 Calcium voltage-gated channel auxiliary subunit alpha2delta 1
FGF12 Fibroblast growth factor 12
GPD1L Glycerol-3-phosphate dehydrogenase 1 like
HCN4 Hyperpolarization activated cyclic nucleotide gated potassium channel 4
KCNO1 Potassium voltage-gated channel subfamily q member 1
KCNJ2 Potassium voltage-gated channel subfamily j member 2
SCNSA Sodium voltage-gated channel alpha subunit 5
AKAP9 a-Kinase anchoring protein 9
RYR2 Ryanodine receptor 2
CASQ2 Calsequestrin 2
CALM1 Calmodulin 1
CALM2 Calmodulin 2
CALM3 Calmodulin 3
TRDN Triadin
ACMG American College of Medical Genetics and Genomics
RAF1 RAF-1 proto-oncogene, serine/threonine kinase
LDLR Low-density lipoprotein receptor
GAA Glucosidase alpha, acid
P Pathogenic
FHL1 Four and a half LIM domains 1
B Benign
LB Likely benign
ESC European Society of Cardiology
AHA American Heart Association
ACC American College of Cardiology

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