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The impact of the European Society of Cardiology guidelines and whole exome sequencing on genetic testing in hereditary cardiac diseases

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Abstract

In the last decade, an incredible improvement has been made in elucidating the genetic bases of cardiomyopathies. Here we report the impact of either the European Society of Cardiology (ESC) guidelines or the use of whole exome sequencing (WES) in terms of a number of variants of uncertain significance (VUS) and missed diagnoses in a series of 260 patients affected by inherited cardiac disorders. Samples were analyzed using a targeted gene panel of 128 cardiac-related genes and/or WES in a subset of patients, with a three-tier approach. Analyzing (i) only a subset of genes related to the clinical presentation, strictly following the ESC guidelines, 20.77% positive test were assessed. The incremental diagnostic rate for (ii) the whole gene panel, and (iii) the WES was 4.71% and 11.67%, respectively. The diverse analytical approaches increased the number of VUSs and incidental findings. Indeed, the use of WES highlights that there is a small percentage of syndromic conditions that standard analysis would not have detected. Moreover, the use of targeted sequencing coupled with "narrow" analytical approach prevents the detection of variants in actionable genes that could allow for preventive treatment. Our data suggest that genetic testing might aid clinicians in the diagnosis of inheritable cardiac disorders.

KEYWORDS

exome sequencing, expert consensus statement, genetic predisposition to disease, heart diseases, high-throughput nucleotide sequencing, targeted sequencing

1 | INTRODUCTION

Inheritable cardiac disorders (i.e., cardiomyopathies and channelopathies) is an umbrella term that covers a wide range of diseases with phenotypic and genetic heterogeneous features caused by the presence of structural or electrical heart abnormalities.[1] Cardiomyopathies are a significant public health issue as they are highly prevalent (1:200 to 1:2500 in the general population). Hypertrophic cardiomyopathy is considered the most common inherited cardiovascular disease, in which a genetic cause is estimated to be present in 1:500 individuals.[2] According to their functional and morphological features, inheritable cardiac disorders can be divided in four subgroups: (1) arrhythmia syndromes; (2) cardiomyopathies; (3) sudden arrhythmic death syndrome (SADS) or unexplained cardiac arrest, and (4) congenital heart

diseases. Moreover, the cardiomyopathy subtypes include hypertrophic (HCM), dilated (DCM), restrictive (RCM), non-dilated left ventricular cardiomyopathy (NDLVC), and arrhythmogenic right ventricular (ARVC) cardiomyopathies.[3,4]

Inheritable cardiac disorders are mainly characterized by an autosomal-dominant mode of inheritance, but incomplete penetrance and variable expression are also common. Notwithstanding, some specific forms of mitochondrial, autosomal recessive, and X-linked recessive inheritance have also been reported.[5]

Due to the progress and democratization of next-generation sequencing (NGS) approaches, clinical testing capacity in familial cardiomyopathy has been revolutionized.[6] Indeed, targeted sequencing investigating known cardiac-associated genes is a highly accurate and customizable approach. The size of gene panels is still a matter of debate, and there is no clear consensus on whether wider cardiac gene panels are more useful than narrower, cardiomyopathy-specific ones or whether they simply increase the number of unclear genetic results.[7] Moreover, targeted panels do not allow novel cardiac-related gene discovery. Whole-exome sequencing (WES) is a technique in which the protein-coding regions of almost 25 000 genes are sequenced, allowing investigation of the most frequent genes, the ones most rarely associated with the disease and those not previously associated with cardiac disorders. While capture techniques have now achieved such a high degree of specificity allowing the assessment of both single nucleotide variants (SNVs) and copy number variations (CNVs), on the other hand this technology allows for the analysis of only a small portion of the human genome (around 1%).[8] To this aim, whole-genome sequencing (WGS) enables unbiased sequencing of all genes and regulatory regions, including variation in intronic and intergenic regions. To date, few studies on cardiomyopathies using WGS are available, mostly due to the cost of this approach and the data storage burden associated with data analysis.[3,6,9]

Over the last two decades, applying the above-described technologies, the knowledge of the genetic bases of inheritable cardiac diseases has gradually increased, and putatively associated variants have been identified in more than 100 genes.[10] Notwithstanding, depending on the sequencing approach used and the type of cardiomyopathy analyzed, between 10% and 60% of patients may be expected to be identified as carriers of a pathogenic variant.[11,12]

Considering both the rapid improvement of genetics achieved in the last years and the emerging problem of handling the increasing amount of variants of unknown significance (VUS) related to the broader number of genes tested, there is a deep debate about the analysis to choose for the genetic diagnosis of inherited cardiac disorders.

In 2022, the European Society of Cardiology (ESC) published an International Expert Consensus Statement concerning the genetic testing for cardiac diseases, upgrading recommendations for genetic counseling and testing in the context of cardiac pathologies.[13] With genetic testing having the ultimate goal of determining the cause, ESC provides strict criteria on who should be assessed and recommend which gene list should be examined for the diverse heart conditions. Once a genetic cause is identified in the patient, cascade testing in family members should be performed. Moreover, in 2023 ESC released updated guidelines for the management of cardiomyopathies.[4] According to this, only the subset of genes strongly related to the hypothesized diagnosis should be tested but more extended sequencing or analysis may be indicated when suspicion of a monogenic cause remains high.

In this manuscript, we report the impact of three different strategies using: (i) the 2023 guidelines for cardiomyopathies and the 2022 International Expert Consensus Statement for the other cardiac diseases, (ii) a large panel of 128 cardiac-related genes or (iii) the WES analysis in terms of number of VUS and missed diagnosis in a series of 260 patients affected by inherited cardiac disorders.

2 | MATERIALS AND METHODS

2.1 | Patient enrolment and DNA extraction

This study uses clinical information and biological samples from 260 individuals with a clinical diagnosis or suspicion of an inherited cardiac disease enrolled from January 2019 to December 2023 and followed by the Cardiothoracic Department of the Azienda Sanitaria Universitaria Friuli Centrale (ASUFC) in Udine. Genetic testing was performed at the Medical Genetics Institute of ASUFC. Informed consent to molecular analysis according to the Helsinki Declaration was obtained from all patients. This study was approved by the Institutional Review Board of the Department of Medicine (IRB-DMED, RIF. Prot IRB: 269/2023).

Cardiological assessments were made considering both personal/familial history and clinical information were retrieved. A full physical evaluation was performed, not only assessing cardiac-related symptoms, but also multisystemic ones, since cardiomyopathies could be associated with complex/syndromic features. All patients underwent ECG-based evaluations as it may be the only phenotypic manifestation of disease but, more frequently, directs the diagnosis together with multimodal imaging. First-line imaging was based on echocardiography, while cardiac resonance was crucial to obtain an accurate assessment of tissue characterization. In doubtful cases, endomyocardial biopsy was performed which remains a gold standard for the diagnosis of specific disorders.

Genomic DNA was extracted from peripheral blood samples collected into 10 mL EDTA K_2 blood collection tubes using the QIAsymphony® SP/AS instrument (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA concentration was estimated using the Qubit™ dsDNA HS Assay Kit on a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 | Next-generation sequencing and data analysis

For targeted sequencing, barcoded libraries were generated from 200 ng of DNA per sample and the exonic regions and flanking splice

junctions of 128 coding genes were captured using Sophia Extended Cardio Solution (Sophia Genetics SA, Rolle, Switzerland). Sequencing was performed in paired-end 2 × 251 bp on a MiSeq system (Illumina Inc, San Diego, CA, USA). Reads were aligned to human genome build GRCh37/hg19. Variant calling and annotation were performed with the Sophia DDM platform (Sophia Genetics SA).

For WES, barcoded libraries were generated from 50 ng of DNA per sample and the exonic regions and flanking splice junctions (±25 bp flanking each exon) of about 22 000 coding genes were captured using the WholEX pro sequencing kit (4bases SA, Manno, Switzerland). Sequencing was performed in paired-end 2 × 150 bp on a NextSeq system (Illumina Inc). Reads were aligned to human genome build GRCh38/hg38. Variant calling and annotation were performed with the Varsome Clinical platform (Saphetor SA, Lausanne, Switzerland).

For both analytical workflows, a minimum depth coverage of 20X and a minimum alternate allele frequency of 20% (VAF≥0.2) were considered suitable for analysis. Variants with frequency <0.01% in population-based databases (i.e., gnomAD), exonic missense, splicing, stop-gain, stop-loss, and frameshift insertion and deletion variants were retained for further evaluation. The following public databases were used for the interpretation of the variants: HGMD Professional (https://my.qiagendigitalinsights.com/bbp), LOVD (https://databases.lovd.nl/shared/genes), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Varsome Premium (https://varsome.com/), Franklin by Genoox (https://franklin.genoox.com/clinical-db/home), Cardioclassifier v0.2.0 (https://www.cardioclassifier.org/), the Atlas of Cardiac Genetic Variation (https://www.cardiodb.org/acgv/), CardioBoost (https://www.cardiodb.org/cardioboost/) and the database of Titin Variants in Dilated Cardiomyopathy (https://www.cardiodb.org/titin/). Variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines.[14] The diagnostic yield, that is, the detection rate, was computed as the percentage of sample bearing at least a pathogenic/likely pathogenic variant associated with the cardiac phenotype over the whole number of samples analyzed in each analytical setting.

2.3 | Sanger sequencing

Amplification was performed using 50 ng of DNA and GoTaq® Colorless Master Mix (Promega, Madison, WI, USA) as previously described.[15] PCR primer sequences are available on demand. The amplified products were analyzed by direct sequencing using the Big Dye Terminator Cycle Sequencing Kit v3.1 and capillary electrophoresis on the 3500 Dx Series Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

2.4 | Quantitative PCR

Quantitative PCR (qPCR) was performed using the PowerUp SYBR Green master mix (ThermoFisher Scientific) in a QuantStudio 3™ PCR System (Applied BioSystems, Foster City, CA, USA). The ΔΔCT method was performed by the QuantStudio Design & Analysis software v1.4 (Applied BioSystems). qPCR primer sequences are available upon request.

2.5 | Statistical analysis

Statistical analysis was performed using GraphPad Prism Software v.5 (GraphPad Software Inc, Boston, MA, USA). Categorical data were expressed as frequency and percentage. D'Agostino-Pearson normality test was first performed to test the shape of data distribution. Mann–Whitney *U* test was used for group comparison. *p*-value <0.05 was considered statistically significant.

3 | RESULTS

Overall, 260 patients underwent genetic testing because of a clinical suspicion of inherited cardiac disorder in a 5-year period (2019–2023). In details, 219/260 (84.23%) were diagnosed with a cardiomyopathy, 32/260 (12.31%) with an arrhythmic syndrome, 7/260 (2.69%) with a positive familiarity for sudden arrhythmic death syndrome/unexplained cardiac arrest and 2/260 (0.77%) with a congenital heart disease. Patients' clinical characteristics are summarized in Table 1.

In a first analytical setting, probands' DNA was extracted and the coding region of 128 cardiovascular disease-associated genes was analyzed by NGS. Initially, data were analyzed strictly following ESC guidelines.[4,13] In silico gene panels used for analysis are included in Table S1. After filtering benign and likely benign variants, 100 variants were found in 90 patients. A positive genetic test was assessed in 20.77% cases (54/260) while an inconclusive one in 13.85%

TABLE 1 Clinical characteristics of the cohort.

Cardiac disease diagnosis (*n*)	Phenotype (*n*)
Cardiomyopathies (219)	Dilated (133)
	Hypertrophic (52)
	Arrhythmogenic (18)
	Unclassified (9)
	Restrictive (7)
Arrhythmia syndromes (32)	Long QT (11)
	Brugada syndrome (10)
	Unclassified arrhythmia (4)
	Ventricular tachycardia (3)
	Early repolarization syndrome (2)
	Short QT (2)
Sudden arrhythmic death syndrome (7)	Unexplained cardiac arrest (7)
Congenital Heart Disease (2)	

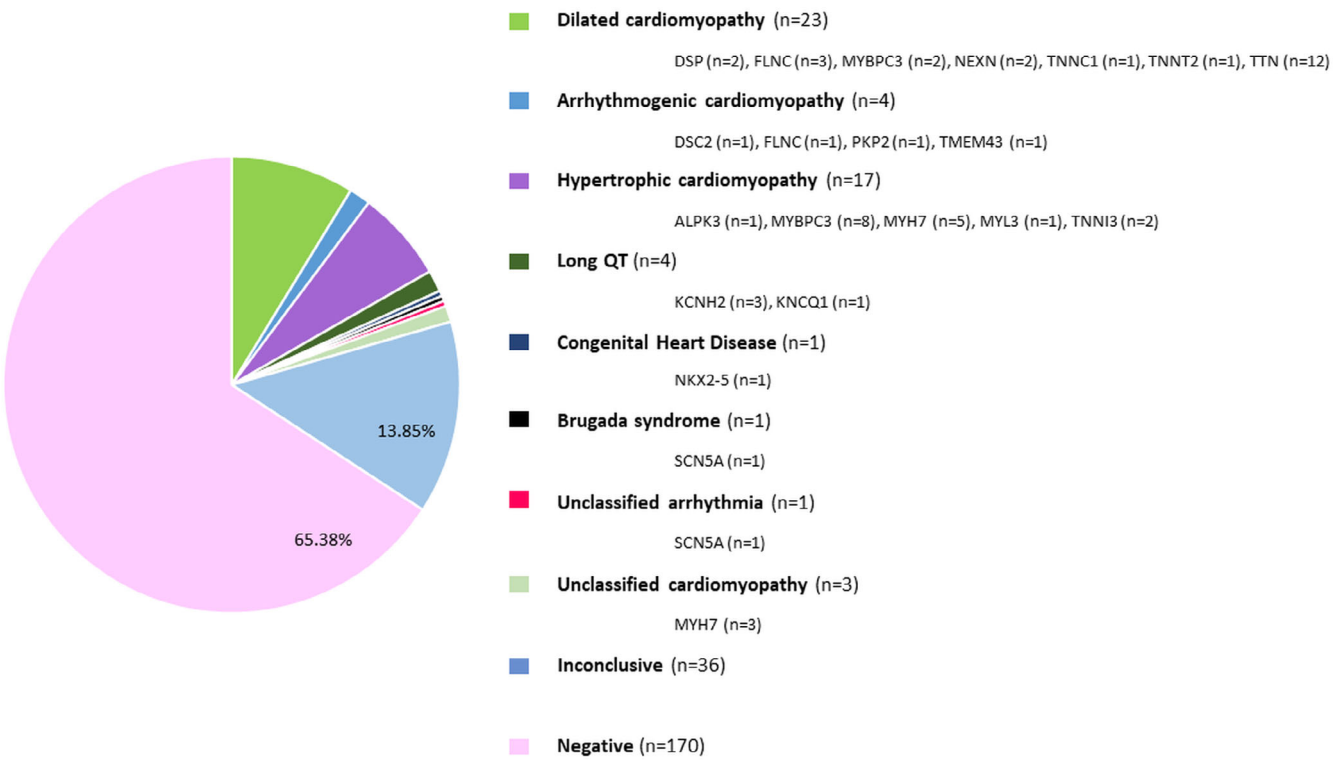

FIGURE 1 Pie chart showing the results of the ESC guidelines-based analysis. Percentages indicate the number of patients in whom an inconclusive (pale blue) or negative test (pink) was assessed. The diagnostic yield (20.77%) is distributed according to patients' phenotype. [Colour figure can be viewed at wileyonlinelibrary.com]

($n = 36/260$). Specifically, 30 pathogenic (P), 26 likely pathogenic (LP) variants and 44 VUS were assessed. Among all P/LP variants, *TTN*, *MYBPC3*, and *MYH7* represented the most prevalent altered genes with 12/56 (21.43%), 11/56 (19.64%), and 8/56 (14.29%) variants, respectively. Besides, a heterozygous LP variant was found in the recessive *ALPK3* gene, thus leading to an inconclusive test. These data are summarized in Figure 1.

The percentage of positivity among the various categories of heart disease reflects their distribution within the cohort ($p = 0.86$). Molecular data are summarized in Table S2.

Subsequently, negative samples ($n = 170$) were re-analyzed using data coming from the whole 128 cardiovascular disease-associated genes included in the targeted panel. The complete list of genes assessed in this analysis are included in Table S3. After filtering benign and likely benign variants, 53 variants were found in 45 patients. A positive genetic test was assessed in 4.71% cases ($n = 8/170$) while an inconclusive one in 21.76% ($n = 37/170$). Specifically, 7 pathogenic (P), 1 likely pathogenic (LP) variants and 45 VUS were assessed.

Table 2 summarizes P/LP findings assessed with the second analytical approach.

A nonsense *TTN* variant was identified in a patient affected by recurrent ventricular tachycardia. A phenotypical re-evaluation was performed and a diagnosis of idiopathic DCM with ventricular tachycardia and paroxysmal atrial fibrillation was made. The result of genetic testing is consistent with the updated phenotype.

A missense *LMNA* variant was assessed in a patient diagnosed with arrhythmogenic cardiomyopathy. A phenotypical update highlighted a mild dilated cardiomyopathy with reduced left ventricle ejection fraction. Family history was positive for DCM. Phenotype is consistent with genetic findings.

A missense *TTR* variant was identified in a 56-year-old patient affected by DCM with a mild left ventricular dysfunction. To date, no signs of cardiac amyloidosis were detected. Notwithstanding, previously published papers on the same variants suggested an age of onset of about 60–65 years old.[16,17]

A missense *GLA* variant was assessed in a patient diagnosed with ischemic and hypertrophic cardiomyopathy. The N215S variant is a common cause of Fabry, occurring in 4.8% of people in a large cohort of primarily North American and European descent.[20] To date, phenotypes with diverse degrees of severity have been associated with this variant (i.e., classical, cardiac variant or "mild" Fabry), with some patients presenting solely a sarcomeric HCM.

A nonsense *TTN* variant was identified in a patient affected by unexplained cardiac arrest. A phenotypical update highlighted a mild DCM with reduced left ventricle ejection fraction. The result of genetic testing is consistent with the updated phenotype.

A nonsense *TTN* variant was identified in a patient diagnosed with arrhythmogenic cardiomyopathy. A phenotypical re-evaluation was performed and a diagnosis of DCM with atrioventricular node dysfunction was made. The result of genetic testing is consistent with the updated phenotype.

A nonsense *LMNA* variant was assessed in a patient diagnosed with ARVC. *LMNA* variants have been already associated with this phenotype[19] but this association has not been reviewed by ESC or the ClinGen consortium.

A deep intronic variant in *ABCC9* was evaluated in a patient affected by ventricular tachycardia and right bundle branch block in differential diagnosis for Brugada syndrome. This variant has been already described in literature but reported in a different transcript (ABCC9(NM_005691.4):c.4570_4572delinsAAAT;p.-Leu1524Lysfs*5).[21,22] Notwithstanding, *ABCC9* gain of function variants have been associated with early repolarization syndrome with or without right bundle branch block and to ventricular tachycardia.[23] Nonetheless, although not conclusive, the result of genetic testing seems to be consistent with patients' phenotype.

The complete set of molecular data obtained from this analysis are summarized in Table S4.

TABLE 2 Pathogenic and likely pathogenic variants identified analyzing the 128-gene panel.

Cardiac phenotype	Genetic alteration	ACMG classification
Dilated cardiomyopathy	*TTR*(NM_000371.4):c.293A>T;p.Tyr98Phe[a]	Pathogenic PS4, PS3, PM1, PP2, PM2, PP3
Arrhythmogenic cardiomyopathy	*LMNA*(NM_170707.4):c.736C>T;p.Gln246*[b]	Pathogenic PS4, PP1, PVS1, PM2
	LMNA(NM_170707.4):c.949G>A;p.Glu317Lys[c]	Pathogenic PS4, PM1, PP2, PM2, PP3
	TTN(NM_001267550.2):c.60931C>T;p.Arg20311*	Pathogenic PVS1, PM2, PS4, PP5
Hypertrophic cardiomyopathy	*GLA*(NM_000169.3):c.644A>G;p.Asn215Ser[d]	Pathogenic PM3, PM2, PM1, PP3, PP2,PS3, PP1, PP5
Unexplained cardiac arrest	*TTN*(NM_001267550.2):c.26351G>A;p.Trp8784*	Likely pathogenic PVS1, PM2
Ventricular tachycardia	*TTN*(NM_001267550.2):c.2347A>T;p.Lys783*	Likely pathogenic PVS1, PM2
Brugada syndrome	*ABCC9*(NM_020297.4):c.4512 + 744_4512 + 746delTTAinsAAAT[e]	Likely pathogenic PM2, PP5

[a]Reported in References 16,17.
[b]Reported in Reference 18.
[c]Reported in Reference 19.
[d]Reported in Reference 20.
[e]Reported as *ABCC9*(NM_005691.4):c.4570_4572delinsAAAT;p.Leu1524Lysfs*5 in References 21–23.

TABLE 3 Pathogenic and likely pathogenic variants identified by WES and related to cardiac diseases.

Cardiac phenotype	Genetic alteration	ACMG classification
Dilated cardiomyopathy and bicuspid aortic valve (BAV)	FBN1(NM_000138.5):c.7732C>A;p.Gln2578Lys[a]	Likely Pathogenic PM2, PM1, PP3, PP2, PP5
Dilated cardiomyopathy	NRAP(NM_198060.4):c.619delG;p.Val207Trpfs*20	Likely Pathogenic PVS1, PM2
Dilated cardiomyopathy	POLR2A(NM_000937.5):c.2528dupG;p.Arg844Serfs*2	Likely Pathogenic PVS1, PM2
Dilated cardiomyopathy	chr18-31 082 235-31 093 643-DEL[b]	Likely Pathogenic 2E
Hypertrophic cardiomyopathy	SOS1(NM_005633.4):c.1660C>T;p.Leu554Phe	Likely Pathogenic PM1, PM2, PP3
	LZTR1(NM_006767.4):c.353G>A;p.Arg118His[c]	Likely Pathogenic PM2, PP3, PM1, PP2, PP5
Unexplained cardiac arrest	SCN4A(NM_000334.4):c.4609G>A;p.Gly1537Ser	Likely Pathogenic PP3, PM2, PP2

[a]Reported in Reference 24.
[b]Spanning from exon 2 to exon 9 of DSC2 (NM_024422).
[c]Reported to be associated to hypertrophic cardiomyopathy in the European Network on Noonan syndrome (https://nseuronet.com/php/index.php).

Finally, 60 negative samples, selected by considering the proportion of different inherited heart diseases in the main cohort, were subjected to WES to assess a further increase in the diagnostic yield. After filtering benign and likely benign variants, 33 variants were found in 25 patients. A positive genetic test was assessed in 11.67% cases ($n = 7/60$), an inconclusive one in 10% ($n = 6/60$) while the detection of solely an incidental finding was highlighted in 20% patients ($n = 12/60$). Specifically, 11 pathogenic (P), 16 likely pathogenic (LP) variants and 6 VUS were assessed. Table 3 summarizes P/LP findings related to cardiac phenotype assessed with the third analytical approach.

A homozygous *NRAP* frameshift variant was assessed in a patient affected with DCM. NRAP is a multi-domain cytoskeletal protein specifically expressed in heart and skeletal muscle.[25] The variant introduces a premature stop codon resulting in a truncation that leads to missing more than two-thirds of the protein. Recent studies reported the association of loss-of-function *NRAP* variants with autosomal recessive DCM.[26,27] Indeed, in vivo experiments in Zebrafish highlighted that *NRAP* knockouts showed a decreased expression of genes related to heart development, resulting in disordered arrangement of cardiomyocytes, enlarged intercellular space, and loose muscle fibers.[25] Moreover, cohort analysis demonstrated that *NRAP* seems to be associated with a more severe phenotype similar to *LMNA*-related cardiac laminopathy.[26] Whether preliminary, these data make *NRAP* a novel gene associated with DCM. Thus, patient' phenotype is consistent with genetic findings.

A missense variant in *SCN4A* was identified in a patient affected with cardiac arrest. *SCN4A* encodes the pore-forming alpha subunit of the voltage-gated sodium channel $Na_V1.4$, highly expressed in skeletal muscle.[28] Mutations in this gene are responsible for muscular sodium channelopathies. Cardiac arrhythmias were described in *SCN4A*-mutated patients,[29,30] suggesting a possible role of *SCN4A* in cardiac arrhythmogenesis.[31,32] Indeed, a phenotypical re-evaluation of this patient was performed after the genetic test and a diagnosis of ventricular arrhythmia was made.

A heterozygous frameshift *POLR2A* variant was assessed in a patient diagnosed with DCM. This variant is located in the bridging helix of the pol II enzyme and is thought to promote polymerase translocation by acting as a molecular ratchet, bending to maintain contact with the coding base during forward translocation, and then snapping back to engage with the next base for another round of the process.[33] The one-bp insertion creates a frameshift of two amino acids before the introduction of a premature stop codon. This result in a truncation that leads to missing more than half of the protein, which is unable to form a stable multimeric complex since lacks binding sites for several other pol II subunits. Indeed, pathogenic variants in *POLR2A* have been recently associated with an emerging syndromic neurodevelopmental condition.[34,35] Functional data highlighted that missense variants led to a malfunctioning pol II enzyme, thereby inducing a dominant-negative effect on gene transcription, exerting a severe phenotype dominated by profound infantile-onset hypotonia and developmental delay. Conversely, loss-of-function variants (i.e., nonsense, frameshifts and splice site variants) reduce levels of functional pol II enzyme, triggering the mildest phenotypes.[34] Moreover, it has been reported that 25% *POLR2A*-mutated patients present cardiac abnormalities (i.e., dilated cardiomyopathy, bicuspid aortic valve, or atrial septal defects). A clinical re-evaluation of this patient, including medical history and other clinical and instrumental

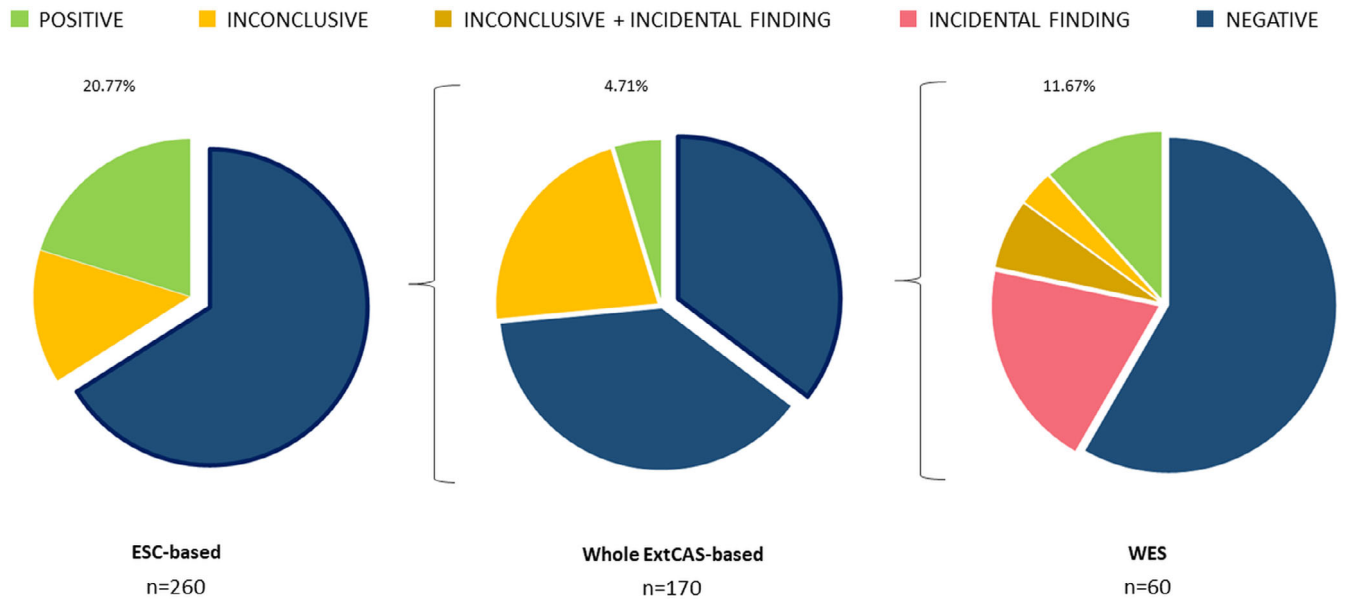

FIGURE 2 Pie chart showing the results of the three analytical approaches used in this study. Percentages indicate the number of patients in whom a positive test was assessed. Positive test after each analytical setting are depicted in green while inconclusive tests are represented in light yellow. Negative tests are represented in blue. The concurrent occurrence of VUSs and incidental findings is depicted in ochre and the solely presence of incidental findings is represented in pink. Brackets indicate the group of negative patients whose DNA was analyzed using the subsequent approaches. [Colour figure can be viewed at wileyonlinelibrary.com]

investigations other than cardiological ones, demonstrated a complex phenotype characterized by speech delay, ataxic gait, gastroesophageal reflux and vision problems. These findings are consistent with the identified hypomorphic variant affecting the *POLR2A* gene.

A missense *FBN1* variant was assessed in a patient affected with DCM. Variants in the *FBN1* gene, encoding fibrillin-1, are primarily linked to connective tissue disorders (i.e., Marfan syndrome). Several studies hypothesize that *FBN1* gene mutations can lead to DCM since this gene has a major role in the regulation of the myocardial extracellular matrix during cardiac development.[36–38] Concurrently, cardiomyopathy has been reported in a subset of patients with Marfan syndrome.[36,38] Indeed, reassessment of patient' phenotype confirmed the diagnosis of DCM with a moderate left ventricular dysfunction and bicuspid aortic valve. The result of genetic testing is consistent with the updated phenotype.

Two patients diagnosed with hypertrophic cardiomyopathy bore a missense variant in *SOS1* and *LZTR1*, respectively. Heterozygous mutations in genes encoding RAS proteins upregulating the RAS-MAPK signaling cascade are associated with Noonan syndrome, a clinically variable disorder characterized by a wide spectrum of congenital heart disease, HCM, reduced postnatal growth, facial dysmorphism, variable cognitive defects and predisposition to certain cancers.[39] The clinical re-assessment of the patient carrying the *SOS1* variant highlighted the presence of facial dysmorphisms and typical ectodermal features while the patient with the *LZTR1* variant showed a short stature with short neck, hypertelorism and downslanted palpebral fissures. Patients' phenotype is consistent with genetic findings.

Lastly, a homozygous deletion involving the *DSC2* gene was assessed in a patient diagnosed with DCM. *DSC2* variants are associated with a cardiac-restricted phenotype of an early-onset biventricular arrhythmogenic cardiomyopathy. Indeed, disease presentation is highly variable and it might initially mimic a dilated cardiomyopathy. Reassessment of patient' phenotype demonstrated the presence of an arrhythmogenic cardiomyopathy that lead the patient to an Automatic Implantable Cardioverter-Defibrillator (AICD) implantation.

The complete set of molecular data obtained from this analysis are summarized in Table S5.

Indeed, the increased diagnostic yield using WES results from the detection of variants associated with congenital diseases that exhibit cardiac involvement. Moreover, the vast majority of P/LP detected with this approach is related to the assessment of incidental findings, that is, deleterious variants in genes associated diverse genetic conditions other than inherited cardiac disorders ($n = 20/27$). A summary of the data obtained using the three different analytical approaches is represented in Figure 2.

4 | DISCUSSION

Hereditary structural and electric cardiac diseases are characterized by genetic heterogeneity and overlapping phenotypes. In the last decade, an incredible improvement has been made in elucidating the genetic bases of cardiomyopathy,[40] mostly due to the tremendous advance in sequencing techniques. The main outcome of this progress was certainly the identification of a substantial genetic overlap between cardiomyopathies. This notion suggest that a better understanding of cardiac pathophysiology related to specific gene variants is mandatory and may lead to the identification of new therapeutic targets and herald the dawn of precision medicine.

In this context, evidence-based guidelines meant to answer the question of what tests to perform and when to perform them has been proposed. In 2022, ESC provided a collection of recommendations with the ultimate purpose of suggesting the analysis of only genes with strong scientific evidence supporting their disease association, considering the somewhat complex issue of variant interpretation. Moreover, in 2023 ESC released update guidelines for the management of cardiomyopathies. Genes that do not have sufficient evidence to date as single-gene causes for disease should not receive variant interpretations.[13] Accordingly, only the subset of genes strongly related to the hypothesized diagnosis should be tested but more extended sequencing or analysis may be indicated when suspicion of a monogenic cause remains high.[4]

This concept contrasts with the overall trend in genetic testing for rare diseases (i.e., multisystemic disorders) in which the use of comprehensive techniques such as WES, coupled to comprehensive phenotyping mandatory for variant interpretation, has been routinely implemented in clinical practice.

In this scenario, we report the result of a NGS-based genetic test performed in a cohort of 260 patients diagnosed with inherited cardiac diseases. To account for the well-known genetic heterogeneity of inherited cardiac diseases, we used a commercial panel including 128 known cardiomyopathy-related genes. We analyzed data following three analytical approaches: (i) strictly following the updated guidelines published by the ESC on genetic testing for cardiac diseases; (ii) assessing variants from the whole targeted gene panel used in our laboratory; (iii) assessing variants from WES in a subset of patients.

Compared to the use of the ESC recommendations, the analysis of the whole gene panel results in an increased diagnostic yield of 4.71% while the use of WES of 11.67%. Besides an obvious increase in the number of VUSs, the use of WES highlights a percentage of syndromic conditions that previous analyses would not have detected. 2/60 patients carried germline pathogenic variants in genes associated with the RASopathies, a group of developmental syndromes sharing similar features and caused by pathogenic variants in components of the RAS/mitogen-activated protein kinase (RAS/MAPK) pathway.[39] A frequent manifestation of this spectrum of diseases is the cardiomyopathy. The most common cardiovascular anomalies are congenital heart disease (CHD) involving pulmonary valve stenosis (50%–60%) and hypertrophic cardiomyopathy (20%).[41] Notwithstanding, the disease is clinically heterogeneous and can manifest at any age, leading to a substantial rate of underdiagnoses.[42] It is worth to remark that WES analysis uncovered a discrete number of incidental findings, 74.07% of all deleterious variants found exclusively using this method ($n = 20/27$). Indeed, the detection of incidental findings by WES or WGS always aroused a vast debate in the literature.[43] The

concept of incidental finding has been gradually amended since its introduction in 2011 and the term has been divided into two categories: unsolicited findings are P/LP variants not related to the initial clinical question but that may nonetheless be of medical relevance for the patient; secondary findings are deleterious variants not related to the initial clinical question but that are actively looked for.[44] Indeed, the ACMG created a secondary findings minimum list (ACMG SF v3.2) that include 81 medically actionable disease genes, that is, for which preventive measures and/or treatment are available, which should be reported to patients.[45] Clearly, the use of targeted sequencing made the detection of unsolicited findings very unlikely; but in an era where the WES has been gradually implemented as a first-tier test, the probability of detecting these findings has increased. To date, no consensus has yet been reached.

Bringing these concepts back to our cohort and considering all deleterious variants outside the ESC-guidelines as incidental findings, 33 P/LP variants were detected. Only 36.4% variants ($n = 12/33$) are located in genes that could be classified as secondary findings as they are enlisted as actionable genes by ACMG.[45] As expected, WES generated a great number of unsolicited findings, ranging from susceptibility to late-onset genetic diseases or cancer predisposition syndromes to multisystem congenital defects (such as the aforementioned RASopathies).

Overall, we are aware that our results are flawed by the reduced number of WES analyses. Although it is possible that the increased diagnostic yield by WES could be less than that reported here, our data show that including genes involved in syndromic congenital defects that may involve the cardiovascular system in the first-tier analysis should be preferred for the proper management of these patients. Moreover, these results hint that genetic testing might aid clinicians in the diagnosis of inheritable cardiac disorders.

In conclusion, extended genetic testing dissolves the border between diagnostics and research and is required to unravel the molecular bases of such a complex group of diseases as the inherited cardiac diseases, increasing the detection rate and providing epidemiological data regarding the prevalence of causative mutations in uncommon genes. Notwithstanding, a great number of VUSs are identified, burdening geneticists with a periodical reassessment of their pathogenicity. The use of targeted sequencing coupled to "narrow" analytical approach (i.e., strictly based on ESC-guidelines) prevents the detection of variants in actionable genes whose assessment when patients are still asymptomatic allows for preventive treatment, which is pivotal to the theme of personalized medicine that permeates modern medicine.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data that support the findings of this study are included in the article or uploaded as supplemental information.

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