**VICTOR BABEȘ" UNIVERSITY OF MEDICINE AND PHARMACY
FROM TIMIȘOARA**

FACULTY OF MEDICINE

DEPARTMENT OF GENETICS

SAV OANA-RALUCA

PhD THESIS

GENETIC INSIGHTS INTO
PRIMARY CARDIOMYOPATHIES

– S U M M A R Y –

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**T i m i ș o a r a
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1. Introduction

Dilated cardiomyopathy (DCM) accounts for one third of all causes of heart failure, is associated with high morbidity and mortality, and is one of the common causes of sudden cardiac death (SCD). DCM has an estimated prevalence of 0.036–0.400% in the general population and is the main indication for cardiac transplantation.

Initially, primary DCM was considered an idiopathic disease, but access to genetic testing and advances in next-generation sequencing led to the identification of the genetic substrate and subsequent classification of DCM into genetic/inherited and acquired/secondary forms. Pathogenic/probably pathogenic genetic variants have been identified in more than 30 genes, most of which are involved in the coding of structural components of the myocardium, such as the sarcomere or the cardiac Z disc.

Next-Generation Sequencing (NGS) techniques play an important role in identifying the genetic substrate in DCM and have provided an approachable and applicable methodology in clinical practice. The identification of a disease-causing variant establishes the diagnosis of certainty and guides the targeted screening of the first-degree relatives of the proband, offering the chance of an early diagnosis, before the appearance of symptoms and prompt treatment.

The purpose of this research is to carry out the genetic testing by NGS of a cohort of adult patients, diagnosed with sporadic or familial DCM, but also to characterize the clinical and imaging phenotype and analyze the arrhythmic risk of these patients. This is the first study cohort consisting of 122 Romanian patients diagnosed with primary DCM who were followed in five University Hospitals in Romania. A subgroup analysis focused on electrocardiogram (ECG) changes and their correlation with patients' genotype.

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2. Specific objectives

The specific objectives of this thesis are:

1. Sequencing a cohort of patients with DCM through the new sequencing techniques to identify the causal genetic variants of the phenotype as well as investigating the yield of genetic diagnosis of these patients.;

2. Family screening of subjects with DCM who consented to participate in the study.

3. Analysis of the differences between patients with DCM and genetic variants causing the disease compared to those with negative results

4. Correlation of the genotype with the clinical phenotype of patients with DCM,

5. Analysis of the arrhythmic risk determined by the genetic variant.

6. A subgroup analysis focused on ECG changes and correlation with the identified genetic variant.

3. General research methodology

The current study was approved by the Ethics Committee of the Institute of Cardiovascular Diseases through decision no. 8152/25.09.2023 and received the approval of the Ethics Commission 60/15.12.2017 from the Victor Babes University of Medicine and Pharmacy Timisoara

The patients' participation in the study was freely consented, a fact certified by signing the informed consent form for genetic testing - Appendix 1. The samples that are the subject of this research were collected from 122 patients evaluated between September 2016 and December 2022 within the 5 University Hospitals - tertiary centers for diagnosis and treatment of cardiovascular diseases: Bucharest Emergency Clinical Hospital, Institute of Cardiovascular Diseases Timisoara, Cluj-Napoca, Iasi and Craiova.

The inclusion criteria were:

- Adult patients with a definite diagnosis of DCM in the absence of a secondary cause. DCM was defined according to the criteria of the current guidelines: end-diastolic volumes or diameters of the left ventricle above the established limit related to sex and indexed to the body surface area and the ejection fraction of the left ventricle <50%, in the absence of conditions of volume/pressure overloading.

- Patients who agreed to participate in the study and perform genetic testing after signing the informed consent.

Exclusion criteria:

- Patients who associate one or more of the following conditions: significant valvulopathies, congenital heart disease or significant coronary disease

- Patients with a history of cardiotoxic treatment,

- Patients diagnosed with DCM and chronic alcohol consumption

- Patients with clinically suspected myocarditis or myocarditis diagnosed on MRI imaging

- Patients with syndromic forms of DCM

Prior to enrollment, significant coronary artery disease was excluded by coronary angiography or multislice coronary computed tomography, depending on available local resources.

3.1 Evaluation methods

Participants selected and diagnosed with CMD were evaluated as follows:

• anamnesis and complete clinical examination;

• pedigree for 3 generations in which the family antecedents were synthesized;

- laboratory tests: NT pro BNP analysis

• standard electrocardiogram in 12 leads;

• holter ECG/ 24h;

• M-mode and two-dimensional transthoracic echocardiography

• cardiac magnetic resonance with contrast substance

• genetic testing by NGS

- implantation of a cardiac device as follows: pacemaker, implantable cardioverter-defibrillator, cardiac resynchronization therapy with defibrillator (CRTD), cardiac resynchronization therapy with pacemaker (CRTP);

- complications of the disease: significant ventricular arrhythmias (AV) (eg, sustained or unsustained ventricular tachycardia, history of resuscitated cardiac arrest), cardiac transplantation or death;

First and second degree relatives of the patients who presented cardiac changes supporting the diagnosis of DCM and consented to the inclusion in the study within the family screening, were evaluated using the same investigations.

3.1.2 Electrocardiogram in 12-leads

Each of the included subjects performed an electrocardiogram, where the following were observed:

• pathological Q waves (>0.04 s or >25% of the R wave in the same lead) or the presence of the QS complex

-repolarization anomalies: negative T waves in at least 2 contingent leads

- atrio-ventricular conduction disorders: degree I, II or complete AV block

-interventricular conduction abnormalities: RBBB, LBBB, fragmented QRS or non-specific interventricular conduction abnormality

-supraventricular arrhythmias such as: atrial flutter or atrial fibrillation

- Microvolted QRS was defined by QRS complexes less than 10 mm in limb leads and less than 5 mm in precordial leads

3.1.3 24h electrocardiogram monitoring

Each of the subjects with CMD included benefited at least once- at inclusion of 24h Holter monitoring. During the 24h Holter monitoring, the following were observed:

• supraventricular rhythm disorders (atrial fibrillation, atrial flutter);

• ventricular rhythm disturbances (sustained and/or unsustained ventricular tachycardia).

3.1.4 Echocardiography

All subjects were assessed by:

• 2D transthoracic cardiac ultrasound using a VIVID E9 ultrasound scanner, GE Healthcare Philips with the 3.5 MHz probe. All standard incidences of echocardiography: apical (long axis, four and two chambers), parasternal long and short axis, all were acquired at 50-70 frames per second;

• M mode ultrasound at the level of the tricuspid ring to evaluate the longitudinal systolic function of the right ventricle

Cardiac chamber dimensions were measured according to the American Society of Echocardiography Guidelines. Standard measurements of LV volume and LV FE were calculated using the modified Simpson formula. The cut-off values for LV dilatation were: an LV end-diastolic diameter >58 mm in men and >52 mm in women and an indexed LV volume of ≥75 mL/m2 in men and ≥62 mL/m2 in women and systolic dysfunction when LVEF<50%. RV systolic dysfunction was considered if TAPSE < 17 mm or/ and fractional RV area change < 35%, depending on available data.

Cardiac Magnetic Resonance (MR) Assessment

Cardiac MRI was performed using a 1.5 T scanner (Siemens). A standardized examination protocol was used for all subjects. LGE images were obtained 10 to 15 minutes after intravenous administration of a gadolinium contrast agent. The presence or absence of fibrosis was analyzed but not quantified. MRI was also used to analyze myocardial edema and rule out myocarditis.

Classification of dilated cardiomyopathy

1. Familial CMD has been diagnosed in the presence of more than one affected relative (deceased or living) with a definite diagnosis of DCM or MSC. All family members at risk of inheriting the disease underwent clinical investigations consisting of: a clinical evaluation, transthoracic echocardiography and ECG. Relatives who were identified with a positive clinical phenotype for DCM were included in the study.

2. Sporadic DCM was defined in the absence of a family history suggestive of DCM or MSC.

Genetic testing by new sequencing techniques

Subjects diagnosed with CMD were genetically tested using next-generation sequencing using a core panel that included 54 DCM-associated genes (Invitae Dilated Cardiomyopathy and Left Ventricular Noncompaction Panel). At the discretion of the prescribing physician. , an expanded panel (179 genes) was chosen that included a wider variety of cardiomyopathies and SCD genes: Invitae Arrhythmia Comprehensive Panel, Invitae Dilated Cardiomyopathy and Left Ventricular Noncompaction Panel, Invitae Cardiomyopathy Comprehensive Panel or Invitae Arrhythmia Panel Cardiohythomogenic Panel . First-degree relatives of a patient identified with a pathogenic or likely pathogenic genetic variant were included in the family screening and genetic testing for DCM was performed.

Another part of the CMD patients underwent genetic testing in the Genomic Center of the Victor Babeș Timișoara University of Medicine and Pharmacy. The TruSightCardio Illumina panel (San Diego, CA) was used, which provides a 174-gene sequencing panel using the TruSight Rapid Capture kit (Illumina). Sequencing was done 2 × 150 bp on a MiSeq platform (Illumina). Detected variants were annotated using the ANNOVAR system as previously reported.

DNA extraction was performed from a venous blood sample collected in an EDTA vacutainer.

Interpretation of genetic variants

The results were analyzed and interpreted using the following databases: Genome Aggregation Database (gnomAD version 3.1; http://gnomad.broadinstutite.org), VarSome (https://varsome.com/) and ClinVar (https: // www.ncbi.nlm.nih.gov/clinvar). The ClinGen platform, (https://www.clinicalgenome.org/) was used to analyze the correlation between identified gene variants and DCM, based on reports of clinical relevance, made by a team of experts. Genetic variant classification as benign (B), probably benign (LB), variant of uncertain significance (VUS), probably pathogenic (LP), and pathogenic (P) was performed based on the recommendations of the American College of Medical Genetics and Genomics and the

Association of Pathology Molecular (ACMG/AMP). Results were defined as positive (disease-causing) if they were P or LP and negative if B, LB or VUS, depending on the classification of the genetic variant detected and the mode of genetic transmission of the disease (autosomal dominant/recessive).

Statistical analysis

Continuous normally distributed variables were expressed as mean and standard deviation (SD), while nonparametric variables were expressed as median. Categorical variables were reported as proportions or numbers. Comparison between groups of continuous variables was performed using the t-test combined with Levene's test, and the Mann-Whitney U test was used to compare non-parametric variables. Categorical variables were compared using the chi-square test. Analysis of the survival rate without ICD intervention was performed using the Kaplan Meier curve. For the comparison of genetic positive vs. negative patients, male vs. female, TTN group vs. other genes group, and TTN group vs. LMNA group, P values were calculated using the Mantel-Cox test. Statistical significance was considered at a P value less than 0.05. SPSS 12.0 software (IBM SPSS Inc.) was used for data recording and statistical analysis.

4. Results

4.1. Clinical characteristics

• A total of 122 adult patients with DCM were included, predominantly men (66.6 %). The mean age at diagnosis was 41.4 ± 12.3 years. Heart failure symptoms led to the diagnosis in most cases (82.8%), while 2.4% of patients presented with resuscitated cardiac arrest as the first manifestation of the disease, 6.5% were diagnosed incidentally as following a routine assessment and 8.1% were diagnosed in the context of family screening.

• More than half of the participants (52.4%) had a positive family history of DCM or SCD. An LVEF <35% was observed in 48.4% of DCM patients, with a mean LVEF of 35.3±11.1% with no significant differences between those with the genetic variant positive versus those with the genetic test negative. Almost one-third (30.3%) of patients had a history of ventricular arrhythmia, and 18.8% had atrial fibrillation,

• One third (33.3%) of DCM patients had conduction abnormalities represented by any form of atrioventricular block (degree I, II, or total AV block) or intraventricular conduction disorders represented by LBBB, RBBB, or interventricular conduction disorder nonspecific

• 39.3% of the patients had an implanted device as follows: 6 (5%) had a pacemaker, 36 (29.3%) had a cardiac defibrillator (single-chamber ICD or triple-chamber - cardiac

resynchronization therapy CRTD) with a mean ICD implant age of 40.97±13.58 while 6 (5%) had CRT-P.

• 60% of participants were investigated by cardiac magnetic resonance identifying a high rate of late gadolinium uptake expressing myocardial fibrosis in three quarters of patients (74%).

• The main clinical characteristics and imaging changes of patients in whom a disease-causing genetic variant was identified versus patients with a negative genetic test, were not statistically different, except for atrial fibrillation, which was more frequent in patients with a genetic test positive.

4.2. Genetic characterization

• 122 adult patients with DCM were told about genetic testing. In 49.2% of the participants, genetic testing was done using panels with a variable number of genes from 54 to 179, chosen by the attending cardiologist or geneticist (Invitae) depending on local availability. 15/122 (12.3%) of the study population underwent familial screening based on sequencing of the target genes found in the affected first-degree relative (Invitae). The other patients with DCM, 47/122 (38.5%) underwent genetic testing using the Tru Sight Cardio Illumina kit, a panel of 174 genes, test performed in the Genomic Center of the University of Medicine and Pharmacy "Victor Babes" Timisoara .

• Genetic sequencing identified disease-causing, pathogenic or likely pathogenic variants in 62/122 (50.8%) participants, of whom 66.1% were male, while 30.3% were identified with variants of unknown significance (VUS)

• Disease-causing variants in 15 genes have been identified in patients with DCM, in the following genes: TTN, (MIM 118840), LMNA, (MIM 150330), DSP (MIM 125647), TNNT2 (MIM 191045), RBM20 (MIM 613171), PLN (MIM 172405), DMD (MIM 300377), ACTC1 (MIM 102540), TMEM43 (MIM 612048), MYO6 (MIM 600970), MYH7 (MIM 160760), MYBPC3 (MIM 600958), CRYAB (MIM 123590), and BAG3 (MIM 603883).

• Variants in TTN, LMNA and DSP accounted for 75% of genetic mutations for DCM. The characterization of the identified causative variants is as follows: 18 mutations were missense, 20 were nonsense, 2 were splice-site and 25 were frameshift indels.

• All 32 variants detected in the TTN gene were truncating variants: 18 variants were frame-shift, 13 were stop-gain/nonsense and 1 was splice-site. Regarding the LMNA gene, 4 of the identified variants were truncating (2 frameshift, 2 nonsense), while 5 variants were missense.

4.3. Genotype-phenotype correlation

• Patients with disease-causing variants in the TTN vs LMNA gene were compared. The group with variants in the LMNA gene was predominantly made up of female patients. They presented significantly more cardiac conduction anomalies, atrial fibrillation and more frequently required the implantation of a cardiac device. All patients (8/8) with variants in the LMNA gene who underwent CMR showed myocardial fibrosis, compared to 76.2% (16/21) of patients with a mutation in the TTN gene. However, these differences did not reach statistical significance.

• The level of BNP (brain-type natriuretic peptides) was significantly lower in patients with variants in the TTN gene, compared to other genes; However, the percentage of patients tested is too small to ensure a correct interpretation

• Comparison between the survival curves without cardiac device intervention was significantly lower in patients with disease-causing variants in the LMNA gene versus patients with pathogenic variants in the TTN gene.

4.4. ECG analysis

• ECG changes were analyzed in the group of patients with CMD, identifying the following aspects: 8.8% of patients presented pathological Q waves, 19% had microvolted QRS complexes, in 35% of cases a QRS complex>120 was identified, 43% presented an abnormal morphological QRS complex and approximately half of the patients (49.4%) had negative T waves.

• A comparison of ECG changes between the group of patients with disease-causing genetic variants versus the negative result on genetic testing was performed

• A QRS complex wider than 120 msec was more frequently identified in patients with a negative genetic test.

• abnormal morphology of the QRS complex which included: left bundle branch block, right bundle branch block, fragmented QRS or non-specific interventricular conduction abnormality was predominantly found in patients with negative genetic result compared to those with positive genetic variants (p=0.054) , a result at the limit of statistical significance.

• There were no significant differences in the presence of supraventricular or ventricular arrhythmias, first-degree AV block, pathological Q waves, microvolted QRS complex in limb leads or in precordial leads) or negative T waves between DCM patients with disease-causing genetic variants versus negative genetic test.

5. Personal Contributions and Insights

In the present study, a cohort of patients with primary dilated cardiomyopathy, familial or sporadic, was sequenced for the first time in Romania, through new generation sequencing techniques using a panel (54-179) of genes known in the literature to be associated with this pathology.

The diagnostic yield of genetic testing was 50.8%. Disease-causing variants (classified as pathogenic and probably pathogenic) were identified in the following 15 genes: TTN, LMNA, DSP, TNNT2, RBM20, PLN, DMD, ACTC1, TMEM43, MYO6, MYH7, MYBPC3, CRYAB and BAG3. 75% of the genetic causes for DCM have been identified in the TTN, LMNA and DSP genes. No significant statistical correlation could be identified between the genetic variant that determined the disease and the clinical-imaging phenotype of the patients. Potentially, a selection of genes with higher prevalence in the Romanian population could lead to more targeted and optimal genetic testing from the point of view of cost-effectiveness.

This study provides preliminary data for the genetic background of DCM in Southeast Europe, where there are few data on the prevalence of certain mutations compared to Western or Northern Europe, where most data on the incidence of mutations in DCM. In the future, the study group will be expanded by including a larger number of subjects, which will allow a possible differentiated analysis according to the genotype.

The group with variants in the LMNA gene presented significantly more atrio-ventricular conduction anomalies, device implantation and atrial fibrillation as well as myocardial fibrosis versus the group with variants in the TTN gene. Individuals with TTN variants had a lower rate of fibrosis on cardiac MRI assessment, lower BNP levels, and a higher survival rate without the need for cardiac device intervention compared to the LMNA group. However, none of these differences in clinical manifestation or phenotype reached statistical significance, arguing that we cannot predict the presence of a causative genetic variant based on the clinical-imaging picture of the patients alone.

We have described the most common ECG abnormalities that can be encountered in non-ischemic dilated cardiomyopathy. In addition, we aimed to analyze the gene-phenotype correlation in Romanian patients with DCM, to provide data for improving clinical management. We did not observe any significant relationship between electrocardiographic parameters and genotype. Instead, we observed a tendency towards a higher risk of ventricular arrhythmias in patients with a wide QRS, which along with LV dysfunction also associates right ventricular dysfunction, suggesting that these changes associated with a certain genotype could lead to the indication for the implantation of ICD in primary prevention of sudden cardiac death

Future studies including larger cohorts of patients with CMD should focus on the prognostic value of the ECG and its correlation with genotype and the course of dilated cardiomyopathy.