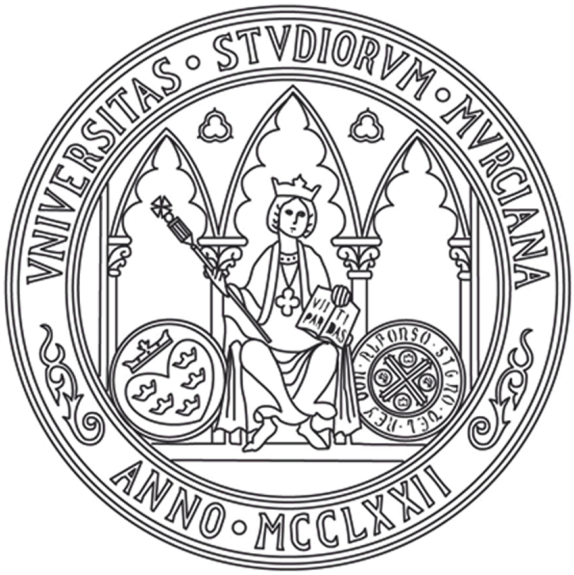

UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA

Clinical Characterization of the Arrhythmogenic Cardiomyopathy in the Region of Murcia: Genotype-Phenotype Correlation and Identification of Signs of Early Disease

Caracterización de la Miocardiopatía Arritmogénica en la Región de Murcia, Correlación Genotipo-Fenotipo e Identificación de Marcadores Precoces de Enfermedad

D. José María López-Ayala

2015

“CLINICAL CHARACTERIZATION OF THE ARRHYTHMOGENIC CARDIOMYOPATHY IN THE REGION OF MURCIA: GENOTYPE-PHENOTYPE CORRELATION AND IDENTIFICATION OF SIGNS OF EARLY DISEASE”

“CARACTERIZACIÓN DE LA MIOCARDIOPATÍA ARRITMOGÉNICA EN LA REGIÓN DE MURCIA, CORRELACIÓN GENOTIPO-FENOTIPO E IDENTIFICACIÓN DE MARCADORES PRECOCES DE ENFERMEDAD”.

DOCTORANDO: ***JOSE MARÍA LÓPEZ -AYALA***

DIRECTORES:

DR. JUAN RAMÓN GIMENO-BLANES

DR. JUAN JOSÉ SÁNCHEZ- MUÑOZ

DRA.MARIA JOSÉ OLIVA- SANDOVAL

PROGRAMA DE DOCTORADO: CIENCIAS CLÍNICAS EN MEDICINA

FACULTAD DE MEDICINA

UNIVERSIDAD DE MURCIA

INDEX

To my grandfather

To the contributors of this project

"Understanding the mechanisms of ARVC is not a goal but a vehicle for future advancements beyond the spectrum of this disease."

Dr Angeliki Asimaki

Harvard Medical School

Boston, Massachussetts

"True science teaches, above all, to doubt and to be ignorant"

Miquel de Unamuno

Beth Israel Deaconess Medical Center

Harvard Medical School

Chairman, Department of Pathology

Jeffrey E. Saffitz, M.D., Ph.D.

Mallinckrodt Professor of Pathology

Beth Israel Deaconess Medical Center
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617 667-4343 • Fax 617 667-2943
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October 18, 2012

To whom it may concern:

This letter certifies that Jose Maria Lopez-Ayala (ID 23049732Y), fellow of Cardiology at the University Hospital Virgen de la Arrixaca (Murcia, Spain), worked in my laboratory at Beth Israel Deaconess Medical Center/Harvard Medical School from September 13 to October 19, 2012.

During this period, he acquired skills in the histological and immunohistochemical analysis of arrhythmogenic right ventricular cardiomyopathy and in basic science techniques to define mechanisms of disease. He also participated in weekly laboratory meetings, seminars, grand rounds and other academic activities at our Medical Center.

Sincerely,

Jeffrey E. Saffitz, MD, PhD

ROYAL FREE AND UNIVERSITY COLLEGE MEDICAL SCHOOL
UCL DIVISION OF POPULATION HEALTH SCIENCES
CARDIOLOGY IN THE YOUNG
Professor William J. McKenna BA, MD, DSc, FRCP, FMEDSCI, FESC, FACC
Director, Inherited Cardiovascular Disease
Program Director - Cardiovascular, UCL Partners

WJM/sd/

20 December 2013

To Whom It May Concern

Re: Dr Jose María López Ayala

Jose is a cardiology fellow from Murcia who was as an academic visitor at The Heart Hospital from August 25th to December 23rd, 2013. During this period he attended cardiomyopathy clinics and post clinic meetings, in-patient ward rounds, and he observed non-invasive evaluations in the echocardiography, magnetic resonance and metabolic exercise laboratories. This involved seeing and presenting patients to the consultants, and presenting at the cardiomyopathy weekly meeting.

Despite the short stay, Jose collected data for a project on left ventricular function in hypertrophic cardiomyopathy and also helped with the ARVC database. He has a strong scientific and clinical base, integrated well into a community of more senior research fellows and overall it was a pleasure to have him with us as an academic visitor. Hopefully there will be opportunities for him to return for a longer period in the future.

Yours sincerely

William J McKenna MD FRCP
Professor of Cardiology

William J. McKenna MD, DSc, FRCP
UCL Division of Population Health Sciences Centre for Cardiology In The Young
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Beth Israel Deaconess Medical Center
Harvard Medical School
330 Brookline Avenue
Boston, MA 02215
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September 29th, 2015

INFORME DE VALORACION DE TESIS DOCTORAL

"CARACTERIZACIÓN DE LA MIOCARDIOPATÍA ARRITMOGÉNICA EN LA REGIÓN DE MURCIA, CORRELACIÓN GENOTIPO-FENOTIPO E IDENTIFICACIÓN DE MARCADORES PRECOCES DE ENFERMEDAD".

Departamento de Medicina Interna. Universidad de Murcia.

Doctorando: D. José María López Ayala
Directores: Dr., Juan Ramón Gimeno Blanes
Dr. Juan José Sánchez Muñoz
Dra. Maria Jose Oliva Sandoval

01/10/2015

La miocardiopatía arritmogénica es una causa frecuente de arritmias ventriculares y muerte súbita. Presenta una prevalencia de 1.3000-1:5000 habitantes y afecta fundamentalmente a pacientes jóvenes. Se encuentra producida por una sustitución de las fibras miocárdicas por tejido fibroadiposo. Su diagnóstico es a menudo complejo debido al solapamiento con otras enfermedades como la miocardiopatía dilatada o la miocarditis. Esta tesis doctoral es de gran relevancia por dicho motivo.

El trabajo realizado por *D. Jose Maria Lopez Ayala*, licenciado en Medicina, analiza diferentes aspectos de la miocardiopatía arritmogénica:

1. Diferencias epidemiológicas, fenotípicas, genéticas y clínicas entre la miocardiopatía arritmogénica derecha e izquerda.

2. Diferencias anatomopatológicas e inmunohistoquímicas entre ambos fenotipos.

3. Identificación de signos electrocárdiográficos de enfermedad precoz en portadores sanos de mutaciones asociadas a esta miocardiopatía.

4. Correlación genotipo-fenotipo e identificación de nuevos genes asociados a la enfermedad.

5. Estudio de las bases genéticas de la miocarditis en miocardiopatía arritmogénica y su importancia diagnóstica y pronóstica.

En conclusión, el trabajo de *D. Jose Maria Lopez Ayala* es de importante relevancia clínica y es de interés para el diagnóstico y manejo clínico de esta compleja enfermedad. El trabajo es metodológicamente correcto y responde adecuadamente a los objetivos planteados. Los resultados son fácilmente transferibles a la práctica clínica.

Yours faithfully,

Angeliki Asimaki, PhD
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Beth Israel Deaconess Medical Center and Harvard Medical School
Email: aasimaki@bidmc.harvard.edu
Tel: 001 617 642 9543

October 7th, 2015

INFORME DE VALORACION DE TESIS DOCTORAL

"CARACTERIZACIÓN DE LA MIOCARDIOPATÍA ARRITMOGÉNICA EN LA REGIÓN DE MURCIA, CORRELACIÓN GENOTIPO-FENOTIPO E IDENTIFICACIÓN DE MARCADORES PRECOCES DE ENFERMEDAD".

Departamento de Medicina Interna. Universidad de Murcia.

Doctorando: D. José María López Ayala
Directores: Dr. Juan Ramón Gimeno Blanes
Dr. Juan José Sánchez Muñoz
Dra. Maria Jose Oliva Sandoval

La miocardiopatía arritmogénica es una causa frecuente de arritmias ventriculares y muerte súbita en jóvenes y deportistas en España y en Italia. Su diagnóstico es a menudo complejo debido al solapamiento con otras enfermedades como la miocardiopatía dilatada o la miocarditis. Esta tesis doctoral es de gran relevancia por dicho motivo.

El trabajo realizado por D. José Maria López Ayala, licenciado en Medicina, analiza diferentes aspectos de la miocardiopatía arritmogénica:

1. Realiza una descripción detallada desde el punto de vista de la imagen de la enfermedad en un grupo amplio de pacientes.

2. Explora las diferencias clínicas y genéticas entre la miocardiopatía arritmogénica derecha e izquierda. Lo cual es uno de los aspectos mas originales de la tesis.

3. Analiza desde el punto de vista histológico diferentes formas de la enfermedad con técnicas poco frecuentes y novedosas, que requieren destreza técnica y experiencia.

4. Desde el punto de vista formal, la tesis está bien diseñada y los métodos empleados son acordes con los objetivos. La presentación es adecuada.

5. El trabajo de investigación viene avalado por la publicación de los resultados en revistas internacionales.

En conclusión, el trabajo de D. José Maria López Ayala es de relevancia y aplicabilidad clínica.

Atentamente,

Fdo: Dr. Giuseppe Limongelli

INTRODUCTION

A BRIEF HISTORICAL REVIEW

Sudden death has been a matter of interest for the medical community for centuries.

The diagnosis of families affected with ARVC goes back to the XVIII century, when it was reported by the Italian physician Giovanni Maria Lancisi it in his book "The Motu Cordis et aneurysmatubus opus postubum" **(Figure 1)**.

The modern approach to this disease started in 1961 and it was made by Professor Dr. Sergio Dalla Volta. He reported on a 35 year old patient than underwent cardiac transplantation due to a massive right ventricular enlargement and right heart failure.[1] More than two decades thereafter, the association of ventricular tachycardia of left bundle left block morphology associated with patients with right ventricular dilatation was discovered.[2]

A defect in the muscle development was the first hypothesis of the underlying mechanism. This prompted Prof. Frank Marcus and Prof. Guy Fontaine to propose the term dysplasia in 1982.[3] It was described as a cause of palpitations, ventricular arrhythmias, sudden death and heart failure.

At that time, it was recognized as an important cause of sudden death in the Veneto region. Particularly concerning was the fact that sudden death was frequently its first clinical presentation. The first histologic studies published by Prof. Gaetano Thiene depicted two patterns of disease (lipomatous and fibrolipomatous transformation) associated with occasional mononuclear infiltrates[4]**(Figure 2)**.Despite its initial description as a disease circumscribed to the right ventricle, the involvement of the interventricular septum and the left ventricle was reported by Prof. Domenico Corrado.[5]

Despite ARVC being inherited in a dominant trait, a minority of patients follows a recessive trait associated with cutaneous disorders. Dr. Nikos Protonotarios noticed that a subset of patients presented the triad arrhythmogenic right ventricular cardiomyopathy, wolly hair and non-epidermolitic palmoplantar queratodermia in the Greek Island of Naxos (1986) which prompted the term of Naxos syndrome [6] **(Figure**

3). A few years later, a similar triad was observed in Guayaquil by Dr. Carvajal-Huerta (1998). However, contrary to the cardiomyopathy found in Naxos syndrome, these patients associated left ventricular cardiomyopathy, which was named as Carvajal Syndrome.[7]

Further pathological research identified cases of predominant left ventricular involvement in postmortem.[8] These left-dominant and biventricular forms were also recognized in the clinical scenario by Professor Mckenna´s group.[9, 10]

The molecular basis of Naxos syndrome was reported in 2000: a homozygous mutation in the plakoglobin gene that caused a two-pair delection in the protein.[11] This discovery laid the foundations of the molecular basis of the disease: the desmosomal disruption.The identification of the mutations in desmosomal genes, the lymphocytic infiltrates in histology as well as controversial results in virus detection in cardiac samples led to the proposal of the inflammatory theory by Prof. Cristina Basso.[12]

New insights in the molecular basis of the disease were the remodelling in plakoglobin and GAP junctions reported by Prof. JE Saffitz and Dr. Angeliki Asimaki.[13]

The diagnostic hallmark of this disease is still the lack of a gold standard. Two different diagnostic criteria have led the diagnostic work-up of the disease (1994 criteria[14] and Task Force Criteria 2010)[15] being the integration of electrocardiographic, imaging and arrhythmic findings alongside pathology, genetics and family history the way to reach an usually difficult diagnosis **(Figure 4)**.

An International Task Force Consensus Statement on treatment, life style recommendations and ICD indications have been recently issued.[16]

FIGURES

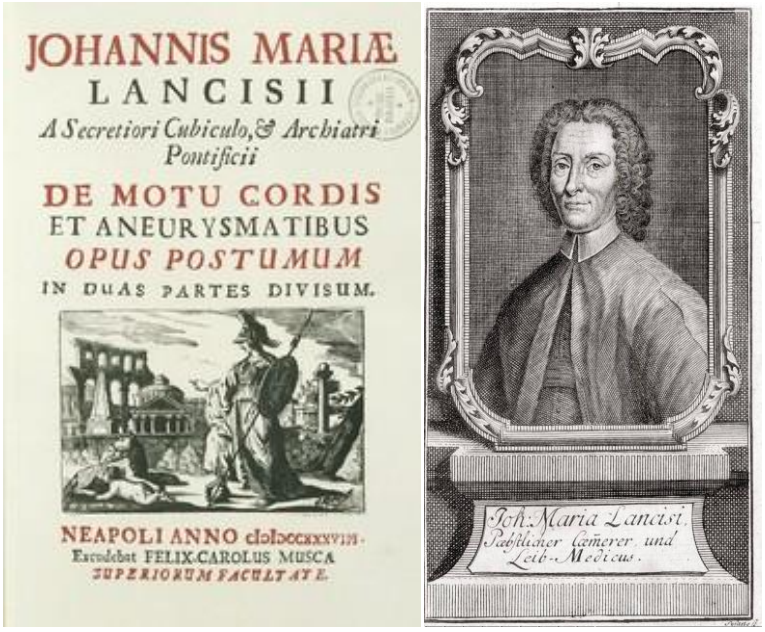

Figure 1: "The Motu Cordis et aneurysmatubus opus postubum", XVIII century. Physician to Pope Clement XI, undertook this study of sudden deaths under papal directive after an unusually large number of such deaths had occurred in Rome.

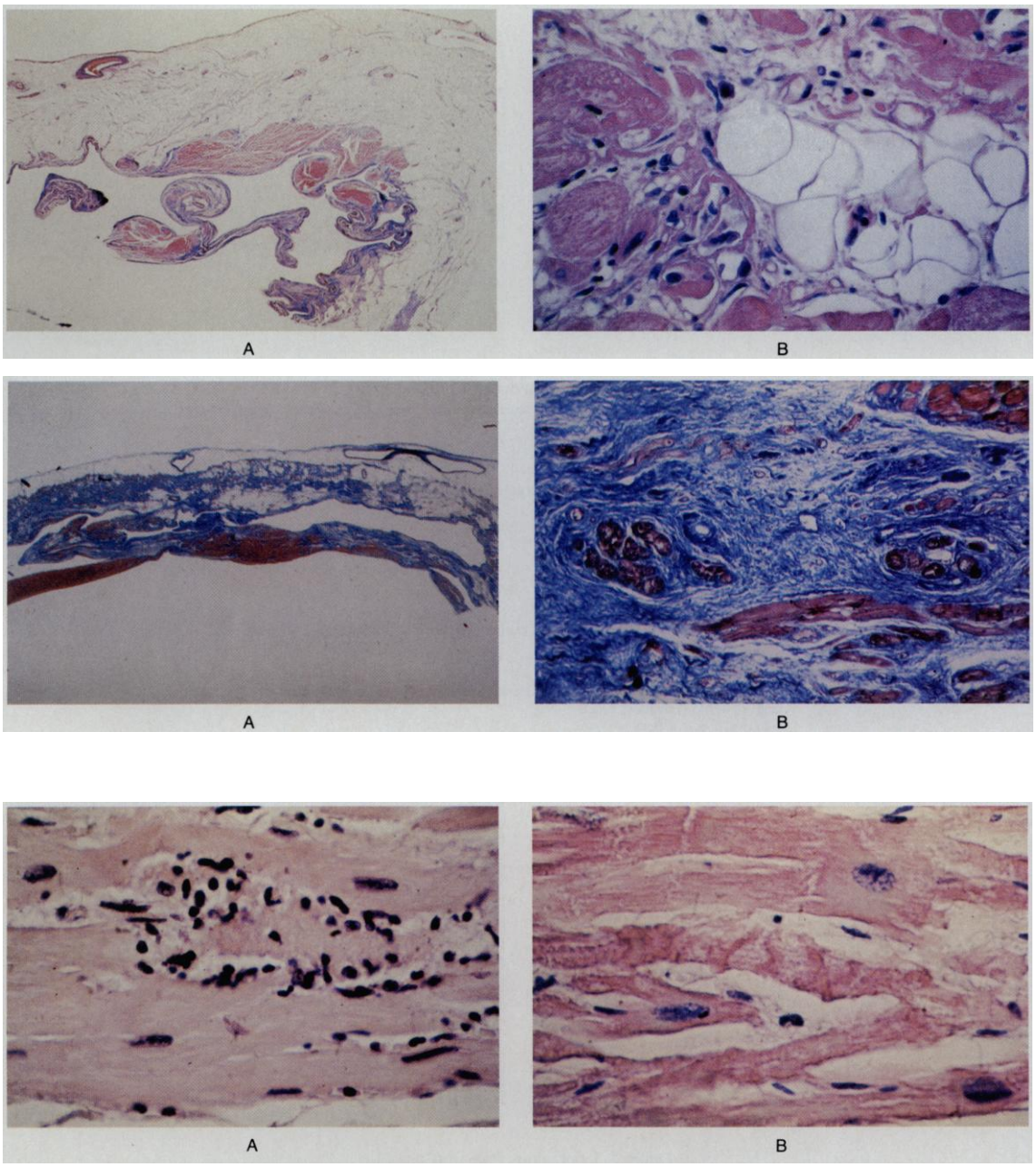

Figure 2: Initial histological patterns described by Thiene et al. Lipomatous (top) and fibrotic patterns (middle). Mononuclear infiltrates (left bottom) and contraction-band necrosis (right bottom).

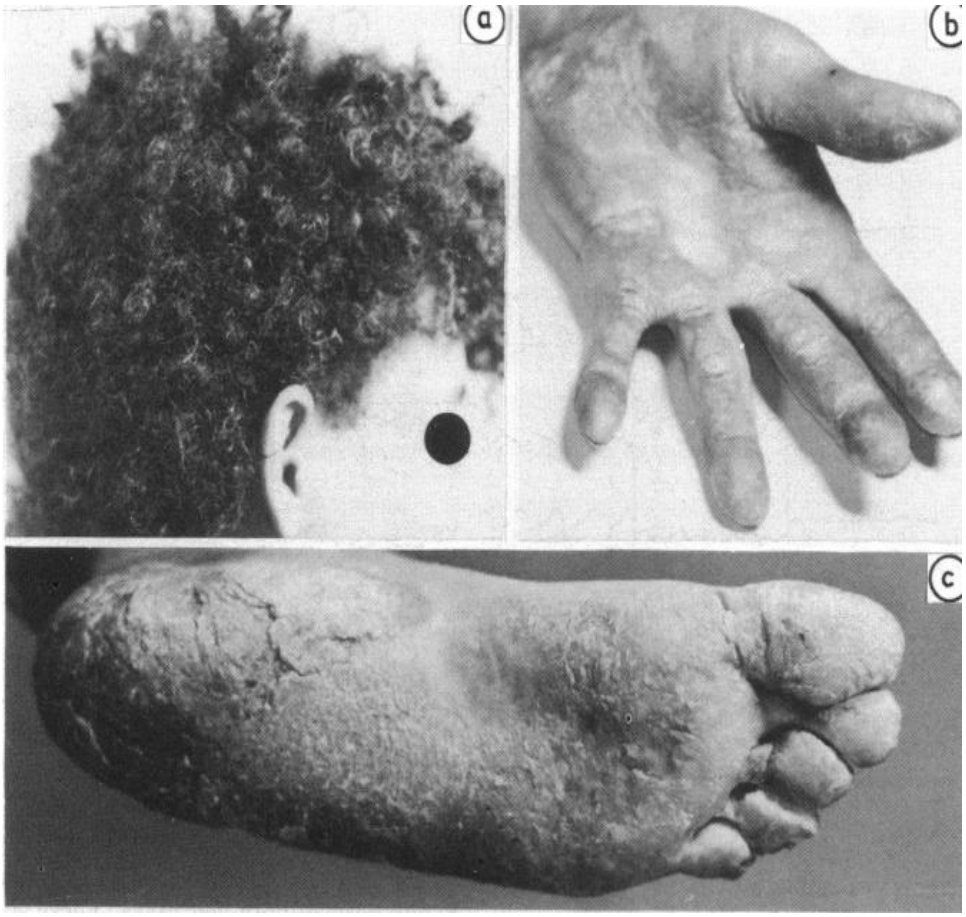

Figure 3: The triad ARVC, wolly hair and palmoplantar keratodermia known as Naxos syndrome.

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2010 Task Force Criteria for the Diagnosis of Arrhythmogenic Right Ventricular Cardiomyopathy

I. Global and/or regional dysfunction and structural alterations

Major

- *By 2-dimensional echocardiogram*:

Regional RV akinesia, dyskinesia, or aneurysm and 1 of the following (end diastole):

-PLAX RVOT ≥32 mm (corrected for body size [PLAX/BSA] ≥19 mm/m^2)

-PSAX RVOT ≥36 mm (corrected for body size [PSAX/BSA] ≥21 mm/m^2)

-Fractional area change ≤ 33%

- *By MRI*:

Regional RV akinesia or dyskinesia or dyssynchronous RV contraction and 1 of the following:

-Ratio of RV end-diastolic volume to BSA ≥110 mL/m^2 (male) or ≤100 mL/m^2 (female)

-RV ejection fraction ≤40%

- *By RV angiography*: regional RV akinesia, dyskinesia, or aneurysm

Minor

- *By 2-dimensional echocardiogram*:

Regional RV akinesia or dyskinesia and 1 of the following (end diastole):

- PLAX RVOT ≥29 to <32 mm (corrected for body size [PLAX/BSA] ≥16 to <19 mm/m2)

-PSAX RVOT ≥32 to <36 mm (corrected for body size [PSAX/BSA] ≥18 to <21 mm/m2)

-Fractional area change >33% to 40%

- *By MRI*:

Regional RV akinesia or dyskinesia or dyssynchronous RV contraction and 1 of the following:

-Ratio of RV end-diastolic volume to BSA ≥100 to < 110 mL/m^2 (male) or ≥90 to <100 mL/m^2 (female)

-RV ejection fraction >40% to ≤45%

II. Tissue characterisation

Major

Residual myocytes <60% by morphometric analysis (or <50% if estimated) with fibrous replacement of the RV free wall myocardium in ≥1 sample, with or without fatty replacement of tissue on endomyocardial biopsy.

Minor

Residual myocytes 60% to 75% by morphometric analysis (or 50% to 65% if estimated), with fibrous replacement of the RV free wall myocardium in ≥1 sample, with or without fatty replacement of tissue on endomyocardial biopsy.

III. Repolarisation abnormalities

Major

Inverted T waves in right precordial leads (V1, V2, and V3) or beyond in individuals >14 years of age (in the absence of complete RBBB QRS ≥120 ms).

Minor

-Inverted T waves in leads V1 and V2 in individuals>14 years of age (in the absence of complete RBBB) or in V4, V5, or V6

-Inverted T waves in leads V1, V2, V3, and V4 in individuals>14 years of age in the presence of complete RBBB.

IV. Depolarisation/conduction abnormalities

Major

Epsilon wave (reproducible low-amplitude signals between the end of the QRS complex and the onset of the T wave) in the right precordial leads (V1 to V3).

Minor

- Late potentials by SAECG in ≥1 of 3 parameters in the absence of a QRS duration of ≥110 ms on the standard ECG:

  1.Filtered QRS duration (fQRS)≥114 ms.

  2.Duration of terminal QRS<40 mV (low-amplitude signal duration) ≥38 ms

  3.Root-mean-square voltage of terminal 40 ms ≤20 mV

- Terminal activation duration of QRS ≥55 ms measured from the nadir of the S wave to the end of the QRS, including R', in V1, V2, or V3, in the absence of complete RBBB.

V. Arrhythmias

Major

Nonsustained or sustained ventricular tachycardia of left bundle-branch morphology with superior axis (negative or indeterminate QRS in leads II, III, and aVF and positive in lead aVL)

Minor

- Nonsustained or sustained ventricular tachycardia of RVOT configuration, LBBB morphology with inferior axis (positive QRS in leads II, III and aVF and negative in lead aVL) or of unknown axis
- >500 ventricular extrasystoles per 24 hours (Holter)

VI. Family history

Major

- ARVC/D confirmed in a first-degree relative who meets current Task Force criteria.
- ARVC/D confirmed pathologically at autopsy or surgery in a first-degree relative.

- Identification of a pathogenic mutation* categorized as associated or probably associated with ARVC/D in the patient under evaluation.

Minor

- History of ARVC/D in a first-degree relative in whom it is not possible or practical to determine whether the family member meets current Task Force criteria.
- Premature sudden death (<35 years of age) due to suspected ARVC/D in a first-degree relative.
- ARVC/D confirmed pathologically or by current Task Force criteria in second-degree relative.

Abbreviations

ARVC/D: Arrhythmogenic right ventricular cardiomyopathy/dysplasia; **BSA**: Body surface area; **LBBB**: Left bundle branch block; **MRI**: Magnetic resonance imaging; **PLAX**: Parasternal long-axis view; **PSAX**: Parasternal short-axis view; **RBBB**: Right bundle branch block; **RV**: Right ventricle; **RVOT:** Right ventricular outflow tract; **SAECG**: signal-averaged ECG.

* A pathogenic mutation is a DNA alteration associated with ARVC/D that alters or is expected to alter the encoded protein, is unobserved or rare in a large non-ARVC/D control population, and either alters or is predicted to alter the structure or function of the protein or has demonstrated linkage to the disease phenotype in a conclusive pedigree.

RECOMMENDATIONS FOR ICD IMPLANTATION

FLOW CHART FOR ICD IMPLANTATION

HIGH RISK (≥10%/YEAR)	INTERMEDIATE RISK (1-10%/YEAR)		LOW RISK (≤1%/YEAR)
1. ABORTED SD 2. SUSTAINED VT 3.SEVERE DISFUNCTION OF RV (EF ≤35%,FAC≤17%),LV≤35%OR BIVENTRICULAR	≥1 MAJOR RISK FACTORS: 1. SYNCOPE 2. NS-VT 3. MODERATE DYSFUNCTION OF RV, LV OR BIVENTRICULAR	≥1 MINOR RISK FACTORS* TEXT*	1.NO RISK FACTORS 2.HEALTHY GENE CARRIERS
ICD INDICATED (CLASS I)	ICD SHOULD BE CONSIDERED (CLASS IIA)	ICD MAY BE CONSIDERED (CLASS IIB)	ICD IS NOT INDICATED (CLASS III)

* MINOR RISK FACTORS: PROBAND STATUS;YOUNG AGE; COMPOUND MUTATIONS; MALE GENDER; INDUCIBLE VT/VF; EXTENSIVE ELECTROANATOMIC SCAR IN THE RV; ITW INFERIORLY; ITW IN ≥3 PRECORDIAL LEADS; FRAGMENTED QRS; SUM OF QRS VOLTAGES V1-V2-V3/ SUM OF QRS VOLTAGES V4-V5-V6 <0.48

Modified from: Corrado D, Wichter T, Link MS, et al. Treatment of Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia: An International Task Force Consensus Statement. Circulation 2015; 132:441-53.

ABBREVIATIONS

-ACM: Arrhythmogenic cardiomyopathy

-AF: Atrial fibrillation

-AFl: Atrial flutter

-ARVC: Arrhythmogenic right ventricular cardiomyopathy

-ALVC: Arrhythmogenic left ventricular cardiomyopathy

-BSA: Body surface area

-CMR: Cardiac magnetic resonance

-Cx43: Connexin 43

-DCM: Dilated cardiomyopathy

-DSC-2: Desmocollin-2

-DSG-2: Desmoglein-2

-DSP: Desmoplakin

-ECG: Electrocardiogram

-EDD: End-diastolic diameter

-ESD: End-systolic diameter

-EMB: Endomyocardial biopsy

-EPS: Eletrophysiological study

-EVM: Endocardial voltage mapping

-HCM: Hypertrophic cardiomyopathy

-ICD: Implantable cardioverter defibrillator

-ITW: Inverted T waves

-LBBB: Left bundle branch block

-LDB3: Limb domain binding protein 3

-LGE: Late gadolinium enhancement

-LV: Left ventricle

-LVEF: Left ventricular ejection fraction

-LVNC: Left ventricular non compaction

-MRI: Magnetic resonance imaging

-NGS: Next Generation Sequencing

-PKP-2: Plakokilin-2

-PG: Plakoglobin

-PLN: Phospholamban

-PLAX: Parasternal long axis

-PSAX: Parasternal short axis

-RBBB: Right bundle branch block

-RV: Right ventricle

-RVOT: Right ventricle outflow tract

-SA-ECG: Signal averaged ECG

-SD: Sudden death

-S-VT: Sustained ventricular tachycardia

-VF: Ventricular fibrillation

AIMS

The aims of this thesis are:

1. To highlight the importance of the family screening for the diagnosis of arrhythmogenic cardiomyopathy.
2. To identify clinical, genetic and molecular differences between right and left dominant arrhythmogenic cardiomyopathy.
3. To analyze differences in the arrhythmic outcome between right-dominant and left-dominant arrhythmogenic cardiomyopathy.
4. To identify early signs of electrical involvement on surface ECG in genotype positive/phenotype negative individuals.
5. To carry out a genotype/phenotype correlation in left-dominante arrhythmogenic cardiomyopathy caused by truncating mutations in desmoplakin.
6. To analyze the clinical phenotype of carriers of the European mutation *PLN R.14del.*
7. Identification of novel genes responsible for arrhythmogenic cardiomyopathy.
8. To study the clinical implications of myocarditis in patients affected with arrhythmogenic cardiomyopathy..

METHODS

A detailed explanation of the methods followed for each aim is provided in each chapter. Briefly, the clinical evaluation consisted of a 12- lead digital ECG, signal averaged-ECG, 2D-doppler echocardiogram, cardiac magnetic resonance, exercise test and 24-hour Holter monitoring. The genetic analysis was performed by dideoxy Sanger sequencing of all exons and flanking intron-exon boundaries in *DSP*, *DSC, DSG2, PKP2, JUP, TMEM43, LMNA A/C and PLN in patients recruited before 2012* and by Next Generation Sequencing onwards.

Myocardial samples from RV free wall, septum and LV (two samples from each location) were analyzed. Sections were stained with hematoxilin-eosin and Masson trichrome. The following lesions were evaluated: fibrofatty evidence, inflammation, necrosis and apoptosis.

Plakoglobin, Connexin-43 and desmoplakin patterns in the intercellular junctions were analyzed by immunohistochemistry and confocal microscopy.

CHAPTER 1: ARRHYTHMOGENIC CARDIOMYOPATHY: A DISEASE WITH A CHALLENGING DIAGNOSIS

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Lancet. 2015 ;385:662

ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY

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MANUSCRIPT

A 21-year-old Caucasian semiprofessional footballer presented in the Emergency Department in April 2012 complaining of recurrent palpitations and chest pain. Previous medical and family history was unremarkable. The ECG showed ventricular trigemini with left bundle branch block morphology, inferior axis and late transition, which suggested a right ventricular outflow tract origin. Normal repolarisation at admission **(Figure 1A)** alternated with transient flat and notched T waves in left precordial leads some days thereafter **(Figure 1B).** Blood tests (including troponin), echocardiogram and cardiac magnetic resonance (CMR) were normal. The signal averaged-ECG showed late potentials (1/3). Ventricular ectopics (VE) disappeared

during the exercise test and a non-sustained ventricular tachycardia (NS-VT) associated with chest pain and light headedness was documented.

Despite the diagnosis of idiopathic right outflow ventricular tachycardia (ROVT) (a benign condition) being the most likely, we had to rule out arrhythmogenic right ventricular cardiomyopathy (ARVC) (an important cause of malignant ventricular arrhythmias and sudden death (SD)) [1] and others such as myocarditis.[2] ARVC diagnosis is based upon imaging, ECG, arrhythmia, histology, genetics and family history criteria.[3] Next available tests were endomyocardial biopsy (an invasive procedure with a modest diagnostic yielding) and genetic testing (positive in 50% of cases).[4] A family screening was also carried out. The ECG of his asymptomatic 50-year-old father was highly suspicious of ARVC **(Figure 2A)**. His echocardiogram **(Figure 2B)** and CMR depicted a severely dilated (124 ml/m^2 , RV outflow tract of 45mm and 46 mm in parasternal long (PLAX) and short axis (PSAX) respectively) and impaired right ventricle (ejection fraction of 30% and fractional area change (FAC) of 32%) and he was diagnosed with ARVC. The proband was started on sotalol and he had a defibrillator (ICD) implanted because of newly documented sustained-VT. He gave up playing football and was discouraged from strenuous exercise. His father was started on betablockers. In May 2013, the proband was found to have inverted T waves in V1-3 on the ECG **(Figure 1C)** and a non-dilated (PSAX 25mm , PLAX 28 mm) but impaired RV (FAC of 35%). The genetic testing at that time failed to demonstrate a causative mutation. The proband met 2 major (ECG, family history of definitive ARVC in a first degree relative) and 3 minor criteria (>500 VE in 24 hours, late potentials and NS-VT of inferior axis) for the diagnosis of ARVC. During a 2-year follow-up both patients have been stable. The proband remains on sotalol and no ICD therapies have been required. His father remains asymptomatic on betablockers.

ARVC ranges from overt to mild phenotypes (concealed stage) and SD may occur at any stage (sometimes in subtle forms that mimic idiopathic ROVT). Conversely, severely dilated RV is not a strong predictor of ventricular arrhythmias. [5] Family screening is essential for the diagnosis of relatives. A positive family history increases the probability of the diagnosis sharply from 1:5000 to 1:2 [3] and allows diagnosis in mild phenotypes. ARVC diagnosis is problematic amongst athletes in whom RV dilatation and T wave inversion in right precordial leads (the latter particularly in

African-Caribbean athletes) may be present as physiologic adaptation to exercise (although these findings tend to normalize following sport deconditioning). Since SD is frequently the first presentation, early diagnosis in asymptomatic patients is essential. ECG being widely available among the medical community means it is a valuable diagnostic tool. Special attention should be paid to T wave inversion in V1-3 or V5-6, a finding which should raise the suspicion of ARVC.

CONTRIBUTORS

JM Lopez-Ayala, MJ Oliva-Sandoval, JJ Sanchez-Muñoz and JR Gimeno contributed equally to the clinical management and writing of the manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

FIGURES

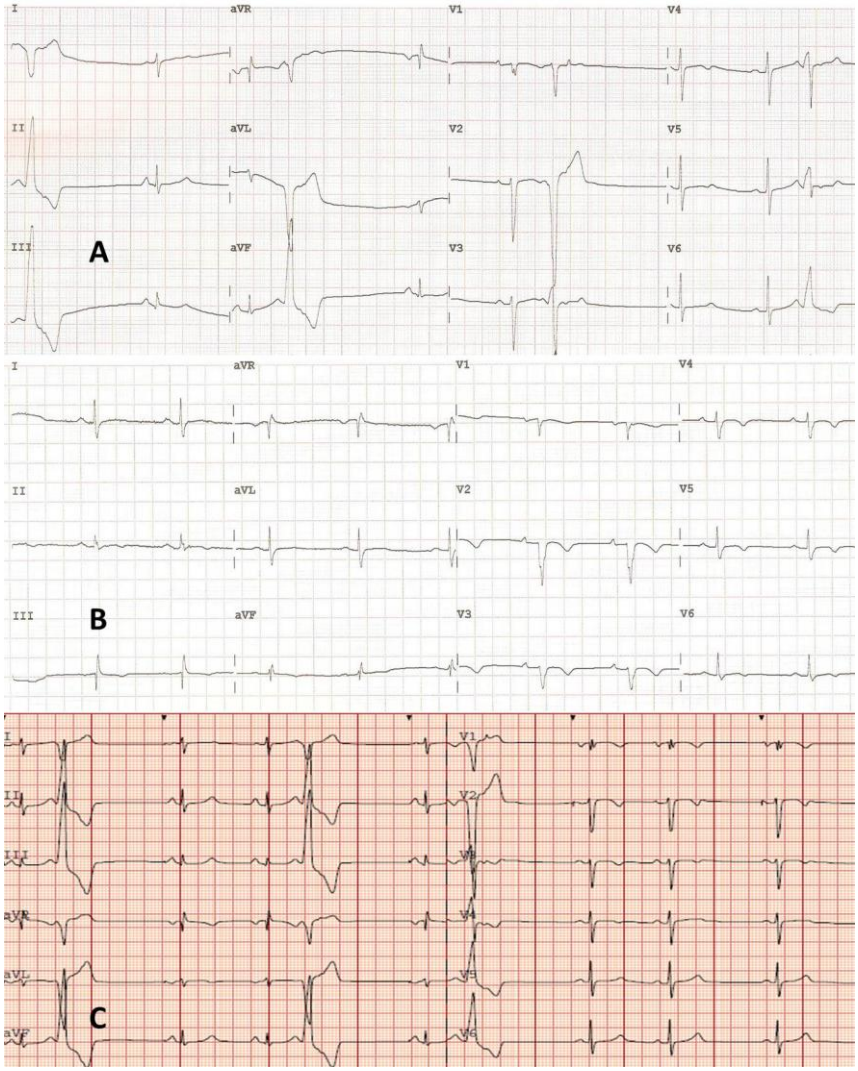

Figure 1.**A**:Proband´s ECG. A: Low ECG voltage in limb leads with normal repolarisation. **B:** Notched T waves in V1-3 with frequent VE**. C:** New T wave inversion in V1-4 discovered one year later.

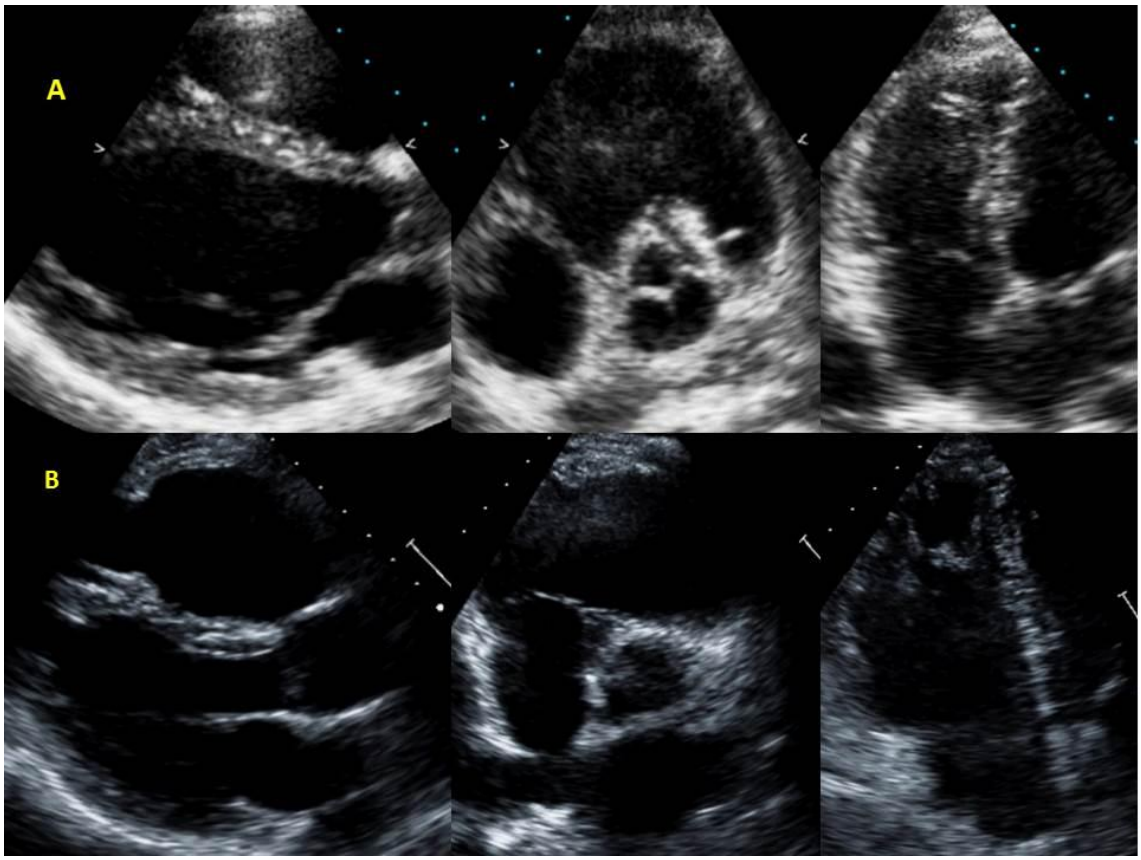

Figure 2. **A**: Father´s ECG depicts fragmented QRS (II, V1-3) and inverted T waves in inferior and precordial leads. **B:** Echocardiogram shows an enlarged and severely impaired RV with dyskynetic areas (arrows) in the apex and outflow tract.

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CHAPTER 2: CLINICAL, GENETIC AND HISTOLOGIC CHARACTERIZATION OF ARRHYTHMOGENIC CARDIOMYOPATHY

CLINICAL, GENETIC AND PATHOLOGICAL DIFFERENCES BETWEEN RIGHT AND LEFT-DOMINANT ARRHYTHMOGENIC CARDIOMYOPATHY

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ABSTRACT

Background

We lack data on the clinical differences between right dominant (RDACM) and left dominant (LDACM) arrhythmogenic cardiomyopathy. A better knowledge of the clinical profile of LDACM patients will facilitate their diagnosis and arrhythmic risk assessment.

Objectives

9. To identify clinical differences between RDACM and LDACM.
10. To analyze differences arrhythmic outcomes in carriers of implantable defibrillator (ICD).

Methods

Clinical work-up comprised ECG (71), signal- averaged ECG (50), 24 hour Holter (61) 2D-echocardiogram (69), cardiac magnetic resonance (43), electrophysiological study (5), endomyocardial biopsy or postmortem (17) and genetic testing (79). Patients were consecutively recruited between the years 2003-2014.

Results

38 LDACM and 68 RDACM patients were diagnosed. The mean follow-up was 45.8±29.6 months. Survival free from sustained-VT/SD was similar between RDACM and LDACM (log rank, *p: 0.270*). Patients that suffered S-VT/VF during the follow-up had previously suffered S-VT/VF (OR: 4.67 CI 95% [0.87-21.13], *p: 0.059* or syncope (OR: 3.47, CI 95% [1.05-11.37], *p: 0.033*). Annual rates of S-VT/VF were similar in primary and secondary prevention in both phenotypes: 8.4%/ year, CI 95% (1.0-32.2%) versus 9.2%/year, CI 95% (3.0-21.3%). NS-VT was not associated with higher risk of appropriate therapies (OR 0.56, 95% CI [0.13-2.52], *p: 0.56*). Plakoglobin and connexin 43 patterns were consistently normal in myocardial samples in LDACM contrary to the remodelling frequently observed in RDACM (66.7% and 38.5% respectively)

Conclusion

The incidence of ventricular arrhythmias, sudden death and annual rate of appropriate therapies is similar in both phenotypes. Differences in the composition of the intercalated disks are observed in RDACM and LDACM

KEY WORDS:

RDACM; LDACM; implantable defibrillator; sudden death; immunohistochemistry; cardiomyopathies; genetics.

ABBREVIATIONS

ACM= Arrhythmogenic cardiomyopathy; **RDACM=** Right-dominant arrhythmogenic cardiomyopathy; **LDACM=**Left-dominant arrhythmogenic cardiomyopathy; **DCM=** Dilated cardiomyopathy; **HCM=** Hypertrophic cardiomyopathy; **ICD=** Implantable cardioverter defibrillator; **LV=** Left ventricle; **LGE=** Late gadolinium enhancement; **LVNC=** Left ventricular non-compaction; **RV =** Right ventricle;

MANUSCRIPT

INTRODUCTION

RDACM is an important cause of sudden death (SD) and ventricular arrhythmias[1] particularly in young patients and athletes with an estimated prevalence of 1/3,000 to 1/5,000.[2] It is usually inherited as a dominant trait, being the underlying mechanism of disease a genetically determined trouble of the development.[3] Although it may be initially circumscribed to the RV, biventricular forms are commonly found. Some phenotypes also affect the LV (LDACM) at an initial stage. [4] The goals of this study are:

1) To characterize LDACM from a clinical perspective and to remark the differences with RDACM.
2) To propose diagnostic criteria for the clinical diagnosis of LDACM.
3) Indentify predictors of ventricular arrhythmias in the highest risk patients who had an ICD implanted.
4) To perform a detailed digital ECG analysis that allows the identification of early electrocardiographic changes in their genotype-positive / phenotype-negative relatives.

METHODS

1. Definitions

(1) The ACM group was made up of patients affected by either right or left dominant phenotypes:

A) Classical RDACM: diagnosis made according to the current diagnostic criteria.[5]

B) LDACM: In the absence of formal diagnostic criteria for this phenotype, the proposed criteria were (a or b):

a. Clinical criteria

- Identification of a RDACM-causing mutation alongside LV dilatation (left ventricular end-diastolic diameter >117% of predicted by age and body surface) and/or systolic dysfunction (LVEF < 45%).
- Ratio end diastolic RV/LV volume <1 on CMR.

b. Pathological criteria

Histopathological findings suggestive of ACM (fibrofatty replacement+ myocardial apoptosis and/or necrosis± lymphocytic patches) predominantly involving the LV.

(2) Genotype positive/ phenotype negative relatives were defined as disease causing mutation carriers in genes previously associated with ACM with no relevant cardiac history, non pathological ECG and Holter and no signs suggestive of ACM in cardiac imaging.

2. Clinical work-up

The study was approved by the Ethics Committee of our hospital (Virgen de la Arrixaca University Hospital, Spain). It complied with the ethical principles of the Declaration of Helsinki. All patients were assessed in a dedicated inherited cardiovascular disease clinic from 2003 to 2014 and attend the clinic on a 1-2 year basis. This clinic is the only referral unit in the county of Murcia (a region containing 1.4 million population). Reasons for referrals were clinical suspicion of a cardiomyopathy (HCM, DCM, LVNC, ACM) or family screening in relatives of probands affected by cardiomyopathy (including family history of sudden death). Consecutiveness was assured for the inclusion of all ACM patients. Referral centers included the 7 local hospitals of the county as well as the Legal and Forensic Medicine Institute of Murcia.

The diagnostic protocol was based on the recommended work-up for the diagnosis of cardiomyopathies and channelopathies.[6] If RDACM or LDACM was considered in the differential diagnosis, the clinical work-up included: 12- lead digital ECG, a signal averaged ECG (SA-ECG), a 2D-doppler echocardiogram, cardiac magnetic resonance (CMR), exercise test and 24-hour Holter. Electrophysiological study (EPS) was performed in patients with recurrent ventricular arrhythmias despite medical treatment. Coronary artery disease was ruled out by angiography or CT angiogram in all patients presenting with aborted sudden death, ventricular tachycardia, systolic dysfunction or chest pain and raised troponin level.

Appropriate ICD therapy was an anti tachycardia pacing or shock delivered to terminate a sustained ventricular tachycardia (S-VT) or ventricular fibrillation (VF). Non sustained ventricular tachycardia (NS-VT) was defined as three or more consecutive

beats arising below the AV node with an RR interval of less than 600 ms (> 100 bpm) and lasting less than 30 seconds. Sustained tachycardia is defined as tachycardia that lasts for more than 30 seconds unless requiring termination because of hemodynamic collapse.

Athletes were individuals of young and adult age, either amateur or professional enrolled in exercise training on a regular basis and who participates in official sports.[7]

The diagnosis of left ventricular non-compaction (LVNC) was made according to the Jenni´s[8] or Petersen´s criteria (when CMR data were available).[9]

In order to identify ECG findings suggestive of concealed stage, genotype-positive and phenotype- negative relatives underwent digital ECG analysis. Results were compared with a matched control group (genotype/negative relatives from LQTS and HCM families). Control individuals belonged to families where a disease causing mutation was harbored in all cases.

3. Genetic testing

Genetic testing was carried out by Sanger sequencing of 5 desmosomal genes, *TMEM43* and *PLN*. A panel of 123 candidate genes was available for screening by next generation sequencing (NGS) from 2012 onwards. All the relatives were screened for the mutation identified in the proband.

33 DCM probands were tested for mutations in *DSP*. Mutations in *PLN* were excluded in 71 probands.

Mutations were classified into 3 categories according to the predicted effect on the encoded protein: missense mutations, truncating mutations and mutations that affected the splicing. Truncating mutations and mutations that affected the splicing were considered by default pathogenic unless previous publications considered them otherwise. Missense mutations were considered pathogenic should they met any of the following criteria: A desmonstrated family segregation; a frequency in the NHLBI 6500 Exome date sets ≤ 0.05% and an *in silico* prediction to affect the protein function (tolerance index score of 0.02 (SIFT) and a PolyPhen-2 score of 0.900).

4. Microsatellite analysis and founder mutations

Although these families were not known to be related, the microsatellite analysis carried out in 3 of them suggested the presence of a common ancestor. 3 families harbored the previously reported *DPS p.Q447** [10] and 2 bore *PLN p.R14del.* [11]

PLN published primer sequences were used for amplification: Ex2 *PLN* forward: TCAGACTTCCTGTCCTGCTG. Ex2*PLN* reverse, TAAGCTGATGTGGCAAGCTG). Haplotype analyses for the region surrounding *PLN* have been described. [6, 7] Four published markers that flank the *DSP* gene were used to investigate the presence of a potential founder effect in carriers of *DSP p.Q447**. All of them are located within the *DSP* gene. Microsatellite 1 (Des.mic.1) is located in intron 1 and Microsatellite 2 (Des.mic.23) is located in intron 23 of the *DSP* gene. Both of them, were indentified in a sequence of a BAC clone maping to 6p23-p24 (GeneBank accession no. AL031058). The primer sequences for Des.mic 1 and 3 are as follows:

Des.mic.1 forward, 5´-CCCATCTATGCATAATGCAACC-3´; reverse, 5´-GTCCTCACGGATGTGCTACAAG-3´

Des.mic.23 forward, 5´-CGCTTTTGATCATGGCCCTAGTG-3´; reverse, 5´-CTCACCTGTTACAGCTAGATG-3´"

5. Histology and immunohistochemistry

Myocardial samples from RV free wall, septum and LV (two samples from each location) were analyzed. They were formalin fixed, paraffin embedded, cut at a thickness of 5µm and mounted on slides. Sections were stained with hematoxilin-eosin and Masson trichrome. The presence of fibrofatty tissue, inflammatory infiltrates and necrotic and apoptotic myocites was evaluated. We incubated the slides with a primary antibody (monoclonal mouse anti-N-cadherin (1:400) (Sigma); monoclonal mouse anti- plakoglobin (1:1000) (Sigma); Monoclonal rabbit anti-connexin 43 (1:400) (Sigma); and Monoclonal rabbit anti-desmin (1:200)(Abcam). The slides were incubated with secondary goat anti-mouse or goat anti-rabbit (1:400) (Jackson ImmunoResearch). Stained slides were checked using a confocal microscope (LSM510, Meta Zeiss). Findings were compared with a control sample from a patient deceased for no cardiovascular reason in which necropsy did not show evidence of cardiac

disease. Case and control samples were fixed and embedded according to the same protocol and stained under similar conditions.

6. Electrocardiogram and ECG monitoring tests

All digital ECG were recorded at 25 mm/s and 10 mm/mV. Intraventricular conduction delays, QRS fragmentation and TWI were evaluated by 2 independent cardiologists blinded to the clinical diagnosis.

The fiducial points for all ECG parameters were carefully set by trained clinicians using an ad-hoc tailored tool that enables the amplification of the signal. ECGs were all shuffled and anonymized for minimizing human bias. All electrocardiographic intervals were analyzed with this tool.

PR, QRS, TAD (measured from the S-nadir to the end of the end of the QRS) and QRS amplitude were automatically recorded. Parietal block was evaluated by the ratio QRS duration in V1-3/QRS duration in V4-6. QRS amplitude (mV) was measured from top to bottom peak. Terminal activation delay (TAD) was defined as the QRS duration from S-nadir to the last potential of the QRS. Early repolarisation was considered if a ST elevation of 1mm or more was observed in two or more contiguous leads.

ST morphology was assessed by the slope from the baseline. ST slope was evaluated using the analytically developed first order polynomial curve for the segments: a) first half of the ST segment, b) full ST segment, and c) second half of the ST segment.

Epsilon waves were defined as an electrical potential detected after the end of the QRS in right precordial leads. Fragmented QRS included notched complexes in the R, top portion or S wave. Inverted T waves (ITW) in anterior leads were included in the analysis if they extended beyond V1 in patients older than 14 years. TW amplitude was estimated as watios $^{-1/2}*10^{-9}$ and negative waves were represented by negative values.

The presence of one or more abnormal vectors in SA-ECG (MAC 5500 ECG Diagnosis System - GE Healthcare, United Kingdom) was considered as pathological according the diagnostic criteria for RDACM. Patients with right bundle branch block (RBBB), left bundle branch block (LBBB), paced rhythm or frequent ventricular ectopics were excluded from the SA-ECG analysis. The noise level was < 0.5 μV in all cases.

Tracings from ambulatory 24-hour Holter monitoring (Marquette Electronics, Milwaukee, USA and Synetec, ELA Medical, Sorin, USA) and treadmill exercise tests

(Marquette Electronics Inc., Milwaukee, USA and General Electric T-2100, Milwaukee,USA) were reviewed.

7. Echocardiography

A *Philips iE33* was used for the studies. They comprised the assessment of the end systolic and end diastolic diameters and volumes of the left ventricle (LV), ejection fraction, wall motion abnormalities, and pathological valvular flows. The right ventricle (RV) analysis included the basal and outflow tract dimensions (measured on both the long and short axis), TAPSE, tricuspid doppler tissue imaging (DTI) and the fractional shortening area to assess the RV systolic function.

8. Cardiac Magnetic Resonance (CMR)

A 1.5T magnet (Achieva CV, Philips Medical Systems, Best, Netherlands) was used for CMR studies. Epicardial and endocardial borders were manually contoured on the cine short-axis images, encompassing the LV and RV from base to apex (8–12 contiguous slices) to obtain a volumetric evaluation at end-diastole and end-systole. LV end systolic and end diastolic volumes were used to calculate the ejection fraction. SSFP end-expiratory breath-hold cine imaging was acquired. T1 measurement was calculated using standard late gadolinium enhancement (LGE) sequences after a bolus injection of Gadobutrol (0.1 mmol/kg, Gadovist; Bayer Shering Pharma, Berlin, Germany). Images were evaluated qualitatively for the presence of LGE and its distribution was classified into subendocardial, midwall, subepicardial and transmural pattern. T2-weighted STIR images were obtained in basal, mid, and apical SAX and 3 long axis planes. The analysis was performed using a personal computer and semi-automated software (Philips, Software work space 2.6.3.2).

9. Statistical analysis

Continuous variables are reported as mean± SD (or median [interquartile range] if they did not follow a normal distribution. These variables were compared with *T-Student* test (or *U Kruskall-Wallis* when appropriate). Frequencies are reported as (%). Categorical variables were compared by *Pearson´s Chi* test (*Fisher* test was used for comparison of rare events). Cumulative survival was calculated with the *Kaplan-Meier*

curves and compared with the log rank test. Sensitivity and specificity were obtained receiver operator curves (ROC). Annual rates were calculated by dividing the number of patients with the event during the follow-up by the number of person-year. SPSS (version 15.0; SPSS Inc, Chicago, IL) was used for the statistical analysis.

RESULTS

1. A cause of sudden death put into perspective

ACM patients presented the poorest survival free from SD amongst all cardiomyopathies (log rank, *p <0.001*) **(Figure 1)**. The risk of SD in ACM was significantly increased compared with that found in other cardiomyopathies (HCM (OR 2.01 95% CI [2.01-5.63], p<0.001) or DCM (OR 7.63 [3.16-18.56], p<0.001) **Table 1**). Survival free from SD/S-VT was similar in RDACM and LDACM phenotypes (log rank, *p: 0.270*) (**Figure 2A).** The majority of SD cases in ACM occurred between the third and sixth decade of life.

A total of 169 patients with a diagnosis of cardiomyopathy or channelopathy suffered a SD (either resuscitated or not) during the 12 year-follow-up. 18 (10.7%) were athletes and ACM was the second more frequent diagnosis in this subgroup (10 (55.6%) HCM patients, 7 (38.9%) ACM patients and 1 (5.6%) LQTS patients)**.**

2. Clinical presentation

The cohort was made up of 106 patients (17 cases diagnosed at postmortem) from 66 unrelated families. The clinical profile and family history of 49 RDACM families (12 (24.5%) of which had ≥ 2 affected members) was analyzed and compared with that from 16 LDACM families (9 (56.3%) of which had ≥2 affected relatives). 68 RDACM and 38 LDACM patients were included in the study. No DNA was available for analysis in 3 LDACM patients diagnosed at postmortem. 38 healthy carriers were identified during the family screening, 13 (34.2%) males. Baseline characteristics are showed in **Table 2.**

Age at the time of diagnosis was similar in both phenotypes, 43.3±15.7 years (range 12-90) in RDACM and 47.0±21.0 years (range 12-90) in LDACM group **(***p: 0.769***)**. The median age of the healthy carriers was 36.3±21.8 years (range 6-85). 3 of them were athletes. Reasons for diagnosis are shown in **Figure 3.**

The proportion of probands was significantly higher in the RDACM group, 46 (67.7%), compared with LDACM, 17 (44.7%), *p: 0.025*. Family history of cardiomyopathy was significantly lower in RDACM patients, 32 (47.1%) versus 29 (79.3%), *p: 0.013*. Family history of SD, was similar 21 (30.9%) RDACM versus 18 (47.4%) LDACM (*p: 0.145*). However, history of multiple SD was higher in LDACM (10 (26.3%) versus 6 (8.8%) *p: 0.016*.)

Probands of ACM were at a higher risk for the combined outcome SD/S-VT than their relatives (RR: 4.70 CI 95% [1.98-11.18], *p<0.001*). However this risk was higher in RDACM (RR: 7.00 CI 95% [2.04-24.06], *p: 0.02*) than in LDACM probands (RR: 2.81 CI 95% [0.73-10.77] *p: 0.126)*.

3. Epidemiology of sudden death

This diagnosis of ACM was made after SD in 25 (23.6%) patients (15 (60.0%) males and 10 (40.0%) females). 8 patients in this group were resuscitated and were clinically diagnosed with RDACM or LDACM.17 patients were diagnosed on postmortem. The median age was 35.5 [29.5-45.5] years (range 14-66). SD cases occurred significantly later in life in females (47.0±11.7 versus 35.5± 11.1 years, *p < 0.001*). 17 (16.0%) patients were diagnosed with RDACM and 8 (7.5%) with LDACM (*p: 0.189*). 18 (17.0 %) were probands and 7 (6.6%) had previous family history of cardiomyopathy or SD.

Amongst the deceased patients, 7 patients were athletes (6 amateurs and 1 professional) and 4 of whom died suddenly on exertion (2 during a football match, 1 while running and 1 while jogging). 5 were diagnosed with RDACM and 2 with LDACM. 2 patients had suffered alarming symptoms before: 1 suffered palpitations and breathlessness on exertion days before collapsing and 1 had suffered palpitations previously.

4. Clinical profile

4.1 Sex differences

Despite a majority of males 60 (56.6%) being affected with ACM, this proportion differed in the two phenotypes: 45 (62.5%) in RDACM versus 15 (40.5%) in LDACM (*p:*

0.014). Males were significantly younger than females at the time of diagnosis (39.8±17.5 versus 48.0±17.9 years, *p: 0.019*). However, this difference did not reach statistical significance within groups (RDACM: 40.2±16.7 (males) versus 45.4±16.6 years (females), *p: 0.218*); LDACM: 38.6±20.1 (males) versus 50.9 ±19.1 years (females), *p: 0.068*). There was a majority of males amongst RDACM probands (7 out of 10, 41.2%) compared to the probands of the LDACM group (34 out of 46, 73.9%), *P: 0.016.*

The survival free of the combined outcome SD/ S-VT was poorer in males ACM patients compared to females (log rank, *p: 0.010*). This difference remained significant in the RDACM group (log rank, *p: 0.045*) although it did not in the LDACM group (log rank, *p: 0.277*). First cases of SD occurred after the third decade of life reaching a plateau at the age of 50 years in males and a decade later in females **(Figures 2B-2D).**

4.2 Comorbidities and medication

Atrial fibrillation

10 (9.4%) patients had a past medical history of atrial fibrillation (AF) or atrial flutter (Aflu). 3 (7.9%) LDACM patients: 1 permanent AF; 1 persistent AF and 1 paroxismal AF. 7 (10.3%) RDACM had AF/Aflu: 2 permanent AF, 2 persistent AF, 2 paroxismal AF and 1 AFlu. The median age at diagnosis was 59.5 [50.3-73.0] years. Median LA diameter in this subgroup was 47 [40.3-49.3] mm.

Stroke/transient ischemic accident/pulmonary embolism

3 (4.4%) RDACM patients (48, 50 and 90 years old) had a previous stroke/TIA (2 males). 2 (5.3%) LDACM patients had previous stroke: 46 year-old male and 84- year-old female. 2 embolic strokes were secondary to AF. Pulmonary embolism was diagnosed in one oncologic RDACM patient.

Hypertension and diabetes mellitus

11 (16.2%) RDACM patients were hypertensive and 2 patients were type 2 diabetics. In LDACM group, 10 (26.3%) patients were hypertensive and 4 patients were type 2 diabetics.

Cancer

5 patients (4 females and 1 male) were diagnosed with cancer over the fifth decade of life. In all cases were solid tumors (breast (2), uterus (2), renal (1)). 1 family showed of multiple relatives affected (digestive and lung cancer). 4 patients were diagnosed of cardiomyopathy before and 1 was diagnosed after the diagnosis of cancer. 2 patients whom received anthracycline suffered an ongoing LV dilatation and worsening in the LVEF.

Medications

13 (19.1%) RDACM and 12 (31.6%) LDACM were on ACEI/ARB-2; 1 (1.5%) RDACM and 8 (21.1%) LDACM on Spironolactone; 15 (22.1%) RDACM and 19 (50.0%) LDACM were on betablockers; 13 (19.1%) RDACM and 1 (2.6%) LDACM patients were on sotalol; 1(1.5%) RDACM patient and 5 (13.2%) LDACM were on amiodarone.

4.3 Symptoms

Syncope of a cardiogenic profile was reported in 11 (16.2%) RDACM and 3 (7.9%) LDACM patients, *p: 0.240.* It was the first symptom of the disease in all cases. All patients passed out at rest. 2 (2.9%) RDACM patients presented with heart failure (NYHA III-IV) versus 7 (10.4%) LDACM patients (*p: 0.018*). Common symptoms in both groups were chest pain (25 (36.8%) RDACM and 15 (39.5%) LDACM patients) and palpitations (27 (39.7%) RDACM and 13 (34.2%) LDACM patients).

4.4 Sport

20 patients (18.9%) were engaged in sports: 15 (22.1%) RDACM and 5 (13.2%) LDACM patients (*p: 0.190*). 5 (4.7%) patients had been professional athletes, 12 (11.3%) amateurs and 3 (2.8%) had a physically demanding job.

Athletes were diagnosed with ACM at a significantly earlier age than non-athletes: 33.0±11.8 vs 46.1±18.7 years, *p: 0.004*. This difference remained significant in each group: 26.3±13.0 vs 47.8±20.3 years (*p: 0.024*) in LDACM; 35.2±10.8 vs 45.0±10.6 years (*p: 0.048*) in RDACM. Athletes presented a similar risk of SD/S-VT than their non-athletes counterparts (OR: 1.61 CI 95% [0.61-4.39], *p: 0.321*).

4.5 Diagnostic criteria for RDACM

Results are summarized in **Figure 4.** The most sensitive diagnostic criteria were those belonging to the arrhythmic category (major and minor criteria were met by 72.7% and 50.1% of patients respectively) followed by electrocardiographic criteria (major criteria in 41.8% and minor criteria in 58.2% of patients). The sensitivity of the remaining categories was similar (family history and genetics: major criteria in 49.1% and minor 14.5% in patients; imaging: major criteria in 43.6% and minor criteria in 23.6% patients).

4.6 Prevention of sudden death: indications for implantable defibrillator

23 (33.8%) RDACM patients had an ICD implanted, 9 (13.2%) for primary prevention (7 males) 14 (20.6%) for secondary prevention (10 males).

Amongst LDACM patients, 19 (50.0%) had an ICD, 10 (26.3%) for primary prevention (6 males), and 9 (23.7%) for secondary prevention (4 males).

5. Imaging characterization

A comparison of the imaging findings in RDACM and LDACM is displayed in **Table 3**. Global LV hypokynesia alongside regional wall motion abnormalities (RWMA) were frequently observed in the **LDACM group** (13 (34.2%) patients) being RV abnormalities rarely detected by echocardiography. 8 (22.2%) patients with RWMA had a preserved LVEF. Mean LV ejection fraction was moderately impaired (42.4±10.0%) and the diameters were in the upper limit of normality (LVED 56.8±5.0 mm; LVES diameter 45.1±8.4 mm).

RWMA and RV aneurysms were detected in less than one third of the **RDACM group**, being the RVOT the location most frequently affected (22 (32.4%)). Global LV hypokynesia was detected in 5 (7.2%) patients. LV RWMA were present in a minority of RDACM patients, being the septum 8 (11.5%) and apex 7 (10.1%) the locations more frequently affected. Mean LV diameters (LVED 47.9 ±5.4 mm and LVES 32.3±6.6 mm) and LVEF (56.9±10.6%) were within normal limits.

LGE was more commonly found in LDACM (20 (54.1%)) than in RDACM patients (7 out of 48 (15.6%)), *p: 0.006*. Septum and lateral wall were the locations more commonly

affected (with a lineal midwall/epicardic distribution). 1 LDACM presented focal transmural distribution. No subendocardial pattern was seen in any patient.

LVNC was significantly more prevalent in LDACM: 16 (42.1%) versus 4 (5.9%), *p<0.001*. Those patients showed a lower LVEF 45.0±11.0% vs 54.0±13.0% (*p: 0.035)* although the prevalence of heart failure symptoms was similar (*p: 0.640*). Patients with LVNC presented similar end diastolic volumes (LVED) than patients without LVNC: 173±31 ml versus 168±47 ml, *p: 0.753).*

Biventricular involvement and overlapping phenotypes were frequently found in both groups: 26 (38.2%) RDACM patients versus 16 (42.1%) in LDACM (*p: 0.937*) as it was the presence of biventricular systolic dysfunction: 18 (26.5%) in RDACM group versus 13 (34.2%) in the LDACM one, *p: 0.786*.

Athletes presented significant greater ventricular volumes than non-athletes: EDLV 201.7±41.6 vs 162.8±40.5 ml, *p: 0.018*; end diastolic right ventricular (RVED) 217.8±99.9ml vs 164.7±62.1 ml, *p:0.05*. LVEF (50.0±8.0% vs 52.0±13.0%, *p: 0.668*) and RVEF (43.0±16.0% vs 45.0±12.0%) were similar in athletes and non-athletes.

Other findings such as the presence of LGE (55.6% vs 38.1%, *p: 0.443*), LVNC (18.8% vs 24.6% *p: 0.693*), biventricular involvement (47.4% vs 41.9%, *p: 0.667*) or biventricular dysfunction (33.3% vs 33.3%, *p: 1.000*) were similar.

Cardiac imaging of the 38 genotype positive/phenotype negative did not demonstrate signs of ACM in neither serial echocardiograms (all individuals had at least 1 echocardiogram) or CMR (available in 14).

6. Clinical events

6.1 Phenotypic progression

The mean follow-up was 45.8±29.6 months (range 1-107 months). 1 RDACM patient that presented with syncope died suddenly while playing football (he had previously rejected the ICD implantation). 1 RDACM patient with severe RV dilatation suffered recurrent presyncopes and was also fitted with an ICD. 5 patients presented recurrent chest pain and troponin elevation during the follow-up (associated with increase in the LGE pattern and drop in the ejection fraction in 3 LDACM, S-VT in 1 RDACM and new TWI in 1 RDACM). 1 RDACM presented symptomatic NS-VT on Holter and had an ICD fitted. 1 LDACM with a longstanding history of heart failure died of cardiogenic shock

in her eighties. All patients were on betablockers and 1 on sotalol since the time of diagnosis. None of the RDACM developed isolated right-sided heart failure.

Amongst the genotype positive/phenotype negative carriers, 1 patient developed anterior TWI. None of the carriers suffered any significant cardiac symptoms or echocardiographic changes compared to the baseline scan.

6.2 Sustained ventricular arrhythmias

17 (25.0%) RDACM and 10 (26.3%) LDACM patients suffered at least one episode of S-VT /VF (*p: 0.854)*. All patients that suffered S-VT/VF during the follow-up had suffered S-VT (either hemodinamically stable or not) or VF previously as first sign of the disease (OR: 4.67 CI 95% [0.87-21.13], *p: 0.059* or syncope (OR: 3.47, CI 95% [1.05-11.37], *p: 0.033*). The median age when the first S-VT/VF occurred was similar in RDACM and LDACM: 45.0 [26.0-55.0] years versus 46.0 [24.5-59.8] years respectively, *p: 0.841*).

10 (37.0%) out of 27 events were triggered by exertion and 17 (63.0%) happened at rest or during mild daily life activities. Amongst the patients that suffered a S-VT/VF/SD on exertion, there were 8(11.7%) RDACM and 2 (5.3%) LDACM patients.

5 patients suffered recurrent S-VT despite medical treatment and underwent an EPS and VT ablation **(Figure 5).** 1 LDACM patient had arrhythmias of a RV origin, and another LV origin. 5 patients presented VT with RBBB morphology (which may suggest a LV origin). 2 patients had documented VF (the ICD recoding demonstrated direct VF in one case). In 1 RDACM patient the VT originated in the LV. In 2 RDACM patients, a RV origin was documented (in one case it also associated polymorphic VT). 13 patients had VT with LBBB morphology (suggestive of a RV origin).

Possible risk factors for ventricular arrhythmias such as biventricular disease (OR: 1.30 CI 95% [0.57-2.94]), *p: 0.531*; LGE (OR: 0.57 CI 95% [0.14-2.34], *p: 0.501*) or LVNC (OR: 0.41, CI 95% [0.10-1.71], *p: 0.288)* were not associated with an increased risk S-VT/VF. Moderate LV dysfunction (LVEF<45%) was associated with a higher risk for SD-S-VT (OR: 5.67, CI 95% [1.37-23.46], *p: 0.013*) in LDACM patients although it showed poor sensitivity and specificity (77% and 50% respectively) (AUROC 0.57, *p 0.524*) **(Figure 6)**.

The sensitivity and specificity of LV and RV systolic dysfunction for the identification of individuals at risk for ventricular arrhythmias was evaluated in ICD carriers. Reduced LVEF (AUROC 0.64, *p: 0.227*), RVEF (AUROC 0.34, *p: 0.389*) or TAPSE (AUROC 0.56, *p: 0.647)* did not predict ventricular arrhythmias during the follow-up. However, the

presence of a reduced tricuspid DTI showed high sensitivity and specificity (AUROC 0.77, *p: 0.021*) **(Figure 7).**

6.3 Ventricular arrhythmias after ICD implantation

Reasons for ICD implantation are summarized in **Table 2**. Patients were followed after the device implantation during a median follow-up of 24.2 [10.8-51.0] months. Survival free from S-VT/VF/appropriate therapy after ICD implantation was similar in primary and secondary prevention subgroups (log rank, *p: 0.133*). No difference was observed between primary and secondary prevention within LDACM patients (log rank, *p: 0.264).* This difference was marginal in the RDACM subgroup (log rank, *p: 0.059)*. ICD therapies (shock and/or ATP) in all subgroups clustered in the first 2 years after implantation (median follow-up until ICD therapy 24 [11-51] months **(Figure 8)**.

Annual rates of S-VT/VF were similar in primary and secondary prevention: 8.4%/ year, CI 95% (1.0-32.2%) versus 9.2%/year, CI 95% (3.0-21.3%). None of the patients that received the ICD for primary prevention for an indication different from syncope or hemodinamically stable S-VT received an appropriate therapy.

The presence of NS-VT on Holter was more frequently detected amongst patients affected with more severe phenotypes in whom the initial presentation of the disease was S-VT/VF (OR 4.76, 95% CI [1.58-14.3], *p: 0.004*. However, its presence in ICD recording was not associated with further appropriate therapies (OR 0.56, 95% CI [0.13-2.52], *p: 0.560*).

6.4 ICD complications

9 (20.9%) patients (2 LDACM and 7 RDACM) had a complication related to the device or inappropriate therapy (1 phrenic paralysis, 1 death for anaesthetic complication, 2 pneumothorax, 1 lead dislodgement, 1 lead thrombosis, 1 sensing problems, 1 inappropriate shock that triggered VF and 1 reactive depression).

7. Digital ECG in RDACM, LDACM and healthy mutation carriers

Main electrocardiographic findings of ACM patients and their healthy genotype positive/phenotype negative relatives are displayed in **Table 4**. First degree AV block was a rare finding more frequently observed in RDACM, 6 (10.7%) versus 1 (4.5%) patient. Complete right bundle branch block (RBBB) was more common in RDACM, 11

(21.6%) patients than in LDACM, 1 (4.5%) patient. Incomplete RBBB was present in 22 (43.1%) RDACM patients versus 15 (68.1%) LDACM, *p: 0.677*) and left anterior hemiblock in 3 (5.8%) RDACM versus 0 (0%) LDACM patients, *p: 0.257*. LBBB was observed in 1 LDACM patient.

Mean durations of PR, QRS and the ratio of the duration of the right precordial leads /left precordial leads were similar in both groups. The TAD in V1-3 was slightly longer in LDACM although this difference was non-significant (54.5±48.7 vs 42.3+14.2 ms, *p: 0.409*). Fragmented QRS were observed in half the population (28 (50%) RDACM versus (18 (51.4%) LDACM, *p: 0.420*). No difference was observed in the number of fragmented leads (1.2±1.4 in RDACM and 1.1±1.8 in LDACM, *p: 0.979*).

Final notched QRS in inferolateral leads was commonly found in both groups 13 (23.2%) RDACM versus 7 (20.0%) LDACM, *p: 0.420*. The morphology of the ST-segment after the notched QRS was horizontal or slightly descendant in the vast majority of cases. Since this morphology has been associated with a higher risk of ventricular arrhythmias in early repolarisation syndrome,[(12)] we carried out a better characterization of the ST slope. Mean slope in inferolateral leads was similar in RDACM and LDACM (11.78 º±30.74º versus 9.94º±24.67º, *p:0.792*). Postdespolarizations potentials were observed in 5 (7.4%) RDACM and 3 LDACM (7.9%) patients. They were found in anterior (3), inferior (2) and lateral (3) leads. Repolarisation abnormalities were significantly more extensive (median number of affected leads) in RDACM: 2.4±2.3 versus 1.5±1.6, *p: 0.009*). T wave area was significantly smaller in LDACM compared to that of the RDACM (8.0±15.1 versus 16.8±11.6 $W^{-1/2}$*10^{-9}, *p: 0.019*). Leads more frequently affected were anterior and inferior in RDACM and inferior and lateral leads in LDACM.

Electrocardiographic characterization of genotype-positive (38 cases) and phenotype-negative controls (113 cases) was carried out. There was no difference in the length of any ECG intervals (PR, QRS, QT) compared to control. The median number of fragmented leads whether affecting the beginning, top or final QRS were also similar. Mean inferolateral ST slope demonstrated a flat ST slope morphology amongst mutation carriers (9.6º±8.5º vs 13.2º±11.2º inferolaterally (*p: 0.04*) and 9.0º±9.1º vs 13.4±13.4º inferiorly (*p: 0.024*) (**Figure 9)**. This sign showed low sensitivity specificity

for the identification of healthy mutation carriers AUROC was 0.58 in both cases *(p: 0.122)*.

8. Genetic background

79 patients underwent genetic testing (44 in RDACM and 35 in LDACM) **(Table 5)**. At least one pathogenic mutation was found in 14 out of 44 screened RDACM patients (31.8%). Mutations of unknown significance and benign variants were present in another 15 (21.7%) patients. Amongst the carriers of pathogenic mutations, the penetrance reached the 66% at the age of 70 years **(Figure 10).**

LDACM patients were carriers of at least one disease causing mutation as per clinical definition criteria. In 3 patients diagnosed at postmortem DNA was not available.

4 patients had double hit mutations (3 in LDACM and 1 in RDACM). 1 RDACM patient was homozygous for a mutation in *DSC-2*. Only one family was diagnosed with cardiocutaneous syndrome: LDACM associated with palmar keratodermia and woolly hair (heterozygous, *DSP p.R425**).

The distribution of the involved genes differed between LDACM (mostly *DSP, DSG-2* and *PLN*) compared with those involved in RDACM (a heterogeneous group made up of a majority of *LDB3* and *PKP-2* mutations). Mutations predicted to truncate the protein were more frequently detected in LDACM (20 (57.1%) versus 8 (6.8%), *p: 0.031* **(Figure 11)**. Affected carriers of *PLN p.R14del* were overwhelmingly diagnosed with LDACM. Only one carrier was diagnosed with RDACM. One RDACM family (carrying *LDB3 p.T351A*) presented one case of biventricular disease.

Carriers of truncating mutations were not at a significant higher risk for SD/S-VT (OR: 1.05 CI 95% [0.43-2.55], *p: 0.918*) compared with carriers of other mutations. Survival free from S-VT/SD was similar in both groups (RDACM group, log rank, *p: 0.125*; LDACM log rank, *p: 0.550*) **(Figure 12).**

2 genotype positive/phenotype negative individuals carried a double hit mutation. The genetic background of this group is summarized in **Figure 13**.

9. Pathologic characterization

The postmortem of 15 patients (12 RDACM, 3 LDACM, 10 males) was available for macroscopic and histologic review. Macroscopically, the RV presented pathological

fibrofatty tissue in all cases. Septal and LV fibrofatty tissue was observed in 5 patients. Microscopically, the predominant lesion was fibrofatty tissue in the RV and patchy or diffuse fibrosis in the LV (present in 11 patients).When the interventricular septum was affected, the left side was relatively preserved in contrast to the commonly involved right side. Lymphohistiocytic infiltrates were observed in the RV ventricle in 12 cases and in the LV in 6 cases.

Immunohistochemical study of the intercellular junctions of the above mention cases and another 2 endomyocardial biopsies (1 RDACM and 1 LDACM) was performed. N-cadherin (N-Cad) signal was normal in all cases and control samples which reflect the integrity of the intercellular junctions. Plakoglobin (PG) signal were severely reduced or absent in 8/17 (47.1%) cases. It was extensive in all cases and affected both ventricles. Connexin-43 (Cx43) showed a heterogeneous pattern in 5/17 (29.4%). There was no correlation between the abnormal expression of these two proteins: Cx43 expression was normal in 4 out of 8 patients with abnormal PG. Likewise, PG signal was normal in 2 out of 5 patients with normal Cx43. Desmoplakin expression was normal in all cases.

All cases with an abnormal PG or Cx43 pattern were diagnosed with RDACM (some of them showing focal abnormalities suggestive of RDACM). The sensitivity of PG and Cx43 remodelling for the diagnosis of RDACM was 66.7% and 38.5% respectively. Interestingly, none of the cases diagnosed with RDACM with severe LV involvement or LDACM showed an abnormal protein pattern **(Figures 14 and 15)**.

DISCUSSION

The extension of RDACM to the LV was described years ago [13, 14] leading to the description of dominant left-sided forms thereafter.[15, 16] However, this distinction between right and left phenotypes is usually difficult in clinical practice due to the commonly found biventricular forms and overlapping phenotypes.[11] The terms RDACM and LDACM highlights the continuum spectrum of disease **(Figure 16)**. Despite its increased recognition, LDACM is an entity still under-diagnosed, a fact that bears prognostic implications due to the clinical impact of diagnostic delays.[10] In the absence of formal diagnostic criteria for the diagnosis of LDACM, the ones herein proposed integrated clinical, genetic and pathological criteria

Mutations found in LDACM families usually led to a truncation in desmoplakin *(DSP p.R425*, DSP p.Q447*)* or were European founder mutations which segregates with a high penetrance *(PLN p.R14del)*[17] . On the contrary, a high proportion of variants of unknown significance were found in RDACM families. Many of these variants play a role as modulators but they are not disease-causing.

Histologic differences between phenotypes were found in the intercalated disks. PG expression [18] was severely depressed or absent in 66.7% of RDACM patients. Cx43 displayed a heterogeneous pattern in 38.5% of RDACM cases, being this remodelling recognized as a potential arrhythmic substrate.[19] Interestingly, none of the samples from LDACM or biventricular cases showed an abnormal protein pattern. These differences may suggest a different underlying mechanism of disease leading to different phenotypes.

Despite RDACM being an important cause of SD amongst hitherto healthy patients <35 years old (particularly in males) [20] , the diagnosis of ACM should be also considered in older patients with cardiovascular comorbidities and risks factors. LDACM was diagnosed significantly later in females (mean age 50 years) and it usually coexisted with other co-morbidities or cardiovascular risks factors**.** Some families presented a high prevalence of cancer, which initially led to the diagnosis of anthracycline cardiomyopathy in some patients. The question whether anthracycline exposure may accelerate the development of the cardiomyopathy or modulate its expression resulting in a more severe phenotype will require further research.

RDACM is a common cause of morbidity in athletes and physical activity is known to be a modulator of the disease. [21] Athletes affected with ACM presented higher ventricular volumes than their counterparts, although there were no differences in the systolic function, presence of LVNC or LGE. Arrhythmic risk/SD was not increased in this group, although this finding may be underestimated by the sample size. As previously described, SD and ventricular arrhythmias showed a lower prevalence amongst relatives of both groups compared with other abnormalities such as ventricular dilatation, systolic dysfunction and ECG abnormalities. [22] Despite RDACM relatives being at a lower risk for SD/S-VT compared with probands, this difference was not present in LDACM .The hypothesis of a more aggressive phenotype found amongst LDACM relatives may be considered cautiously due to the small sample size.

From an imaging perspective, a RV/LV volume ratio <1 in CMR [12] (inclusion criteria) , a mildly dilated LV, a higher prevalence of LGE and LVNC were distinctive features in LDACM compared to RDACM. Superimposed LVNC was present in up to 30% of the patients, a proportion similar to that previously reported in DCM. [23] Neither LGE nor LVNC were associated with a higher risk of SD/S-VT or syncope. However, LVNC was associated with a poorer LVEF. A LVEF< 45% was found to be a sensitive cut-off value for the identification of subjects at risk of ventricular arrhythmias in LDACM (similar to that previously proposed for *PLN* mutation carriers [24] and LMNA[25]) although it shows a low sensitivity and specificity for the prediction of future events.

Annual rates of S-VT/VF were higher in RDACM patients with previous syncope, LDACM with a LVEF<45% or patients with a previous cardiac arrest. LDACM recipients of an ICD for secondary prevention showed the poorest survival free of appropriate therapy, although the difference did not reach the statistical significance (log rank, *p: 0.059)*. Reduced tricuspid DTI in RDACM might be a marker of severity and hence a predictor or arrhythmias. The explanation of the association between tricuspid DTI and arrhythmias in LDACM needs further analysis and should be assessed in future studies. RDACM patients with tricuspid DTI values < 10 cm/s showed typically biventricular dysfunction and overt disease (LVEF 58.1% ± 14.6% versus 45.3 ± 26.1%, *p: 0.041)*. The identification of NS-VT on ICD checks was more frequent in patients presenting with S-VT/VF but it did not associate more sustained arrhythmias/appropriate therapies during the follow-up.

Device-related complications reached 20%, a proportion in keeping with similar publications.[26]

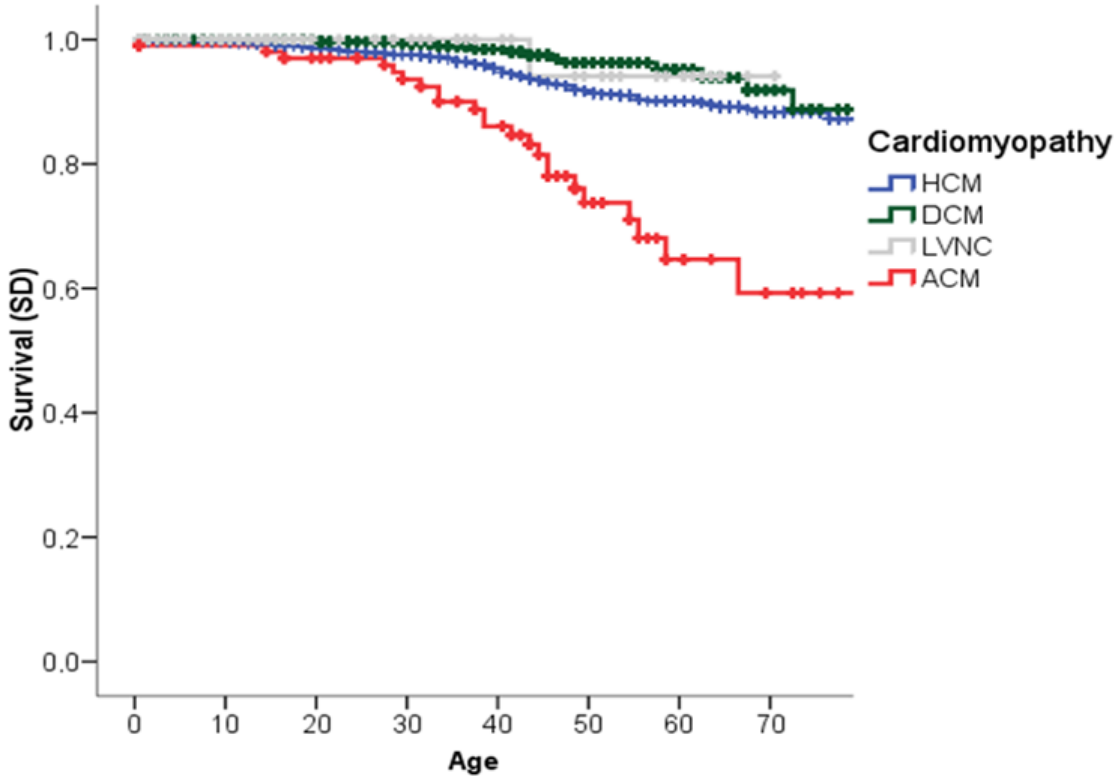
We also describe an electrocardiographic pattern of early disease in healthy mutation carrier: final QRS fragmentation associated with flat ST slope which was observed in the absence of other repolarisation abnormalities or intraventricular conduction delays. However, this sigh showed a low diagnostic yield.

Finally, it is worth mentioning the high prevalence of ICD complications found (20%, a proportion in keeping with similar publications[22]) a fact highlight the necessity of an optimal selection of the candidates in order to minimize this burden.

LIMITATIONS

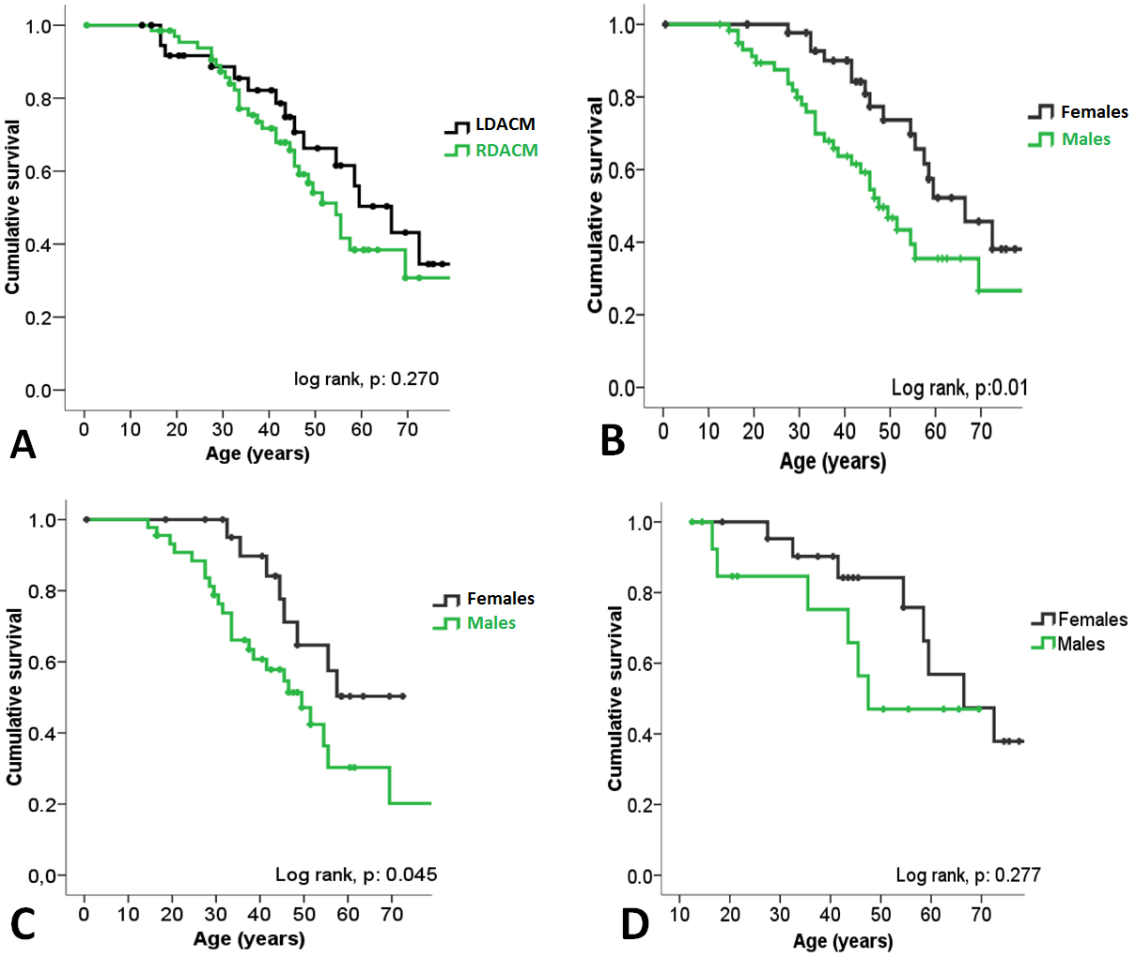
1) The small size of the population required further validation of these findings in other populations, particularly the findings derived from athletes. Longer follow-up of carriers of ICD will be also required. 2) Categorical classification into RDACM or LDACM is sometimes difficult and may result arbitrary in some cases. 3) The clinical relevance of electrical signs of early disease regarding the arrhythmic risk will required further investigations. This is an important issue since SD may occur in patients with non- specific ECG abnormalities in the absence of overt disease.[27] Basic research will be required to shed light on different molecular pathways leading to these different phenotypes.[3] 4) NGS was available for genetic diagnosis from 2012 onwards. Sanger sequencing of desmosomal genes, *PLN* and *TMEM43* was carried out before that year.

FIGURES



Individuals at risk of SD (Age, years)	0	20	40	60
HCM	1032	922	693	343
DCM	394	301	214	77
ACM	106	90	64	16
LVNC	96	29	19	9

Figure 1: Survival of different cardiomyopathies. ACM showed the poorest survival. The table shows the individuals at risk of SD at the beginning of each age interval.



ACM (age, years)	0	20	40	60
LDACM	38	32	24	9
RDACM	68	61	39	9
LDACM				
Males	16	11	8	3
Females	22	21	16	6
RDACM				
Males	44	39	22	5
Females	24	22	17	4

Figure 2: A: Survival free from the combined SD/S-VT in RDACM and LDACM**.** Gender and **s**urvival of SD/S-VT in ACM **(B)** RDACM (C) and LDACM **(D)**. The table shows the individuals at risk of SD/S-VT at the beginning of each age interval.

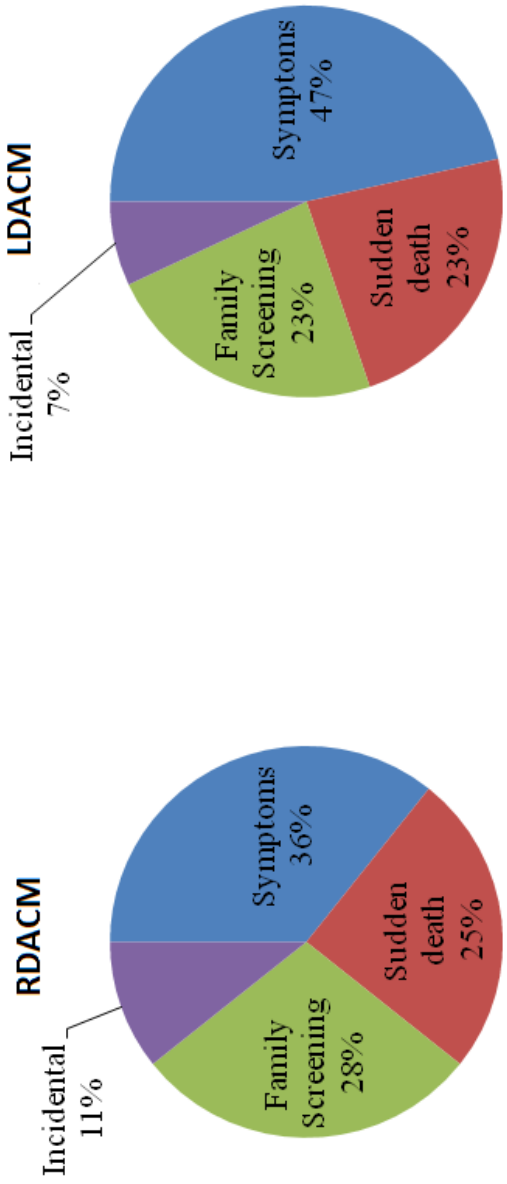


Figure 3: Reasons for diagnosis in both phenotypes.

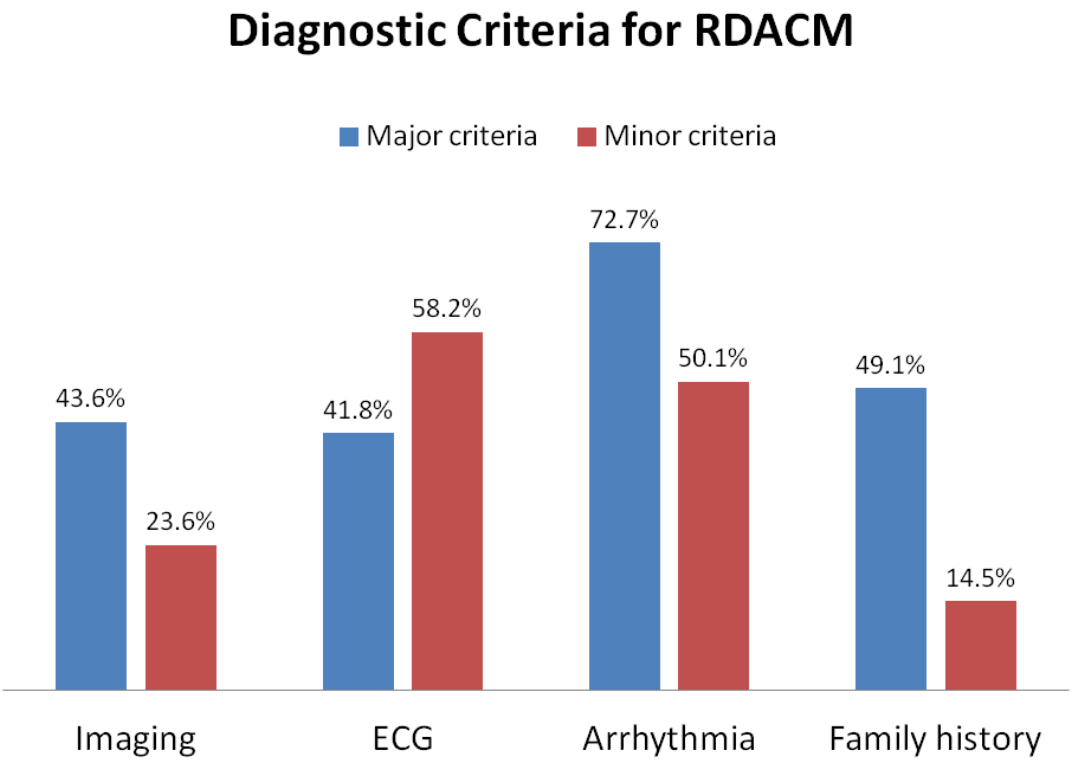


Figure 4: Yield of the different categories in the diagnostic criteria in RDACM.

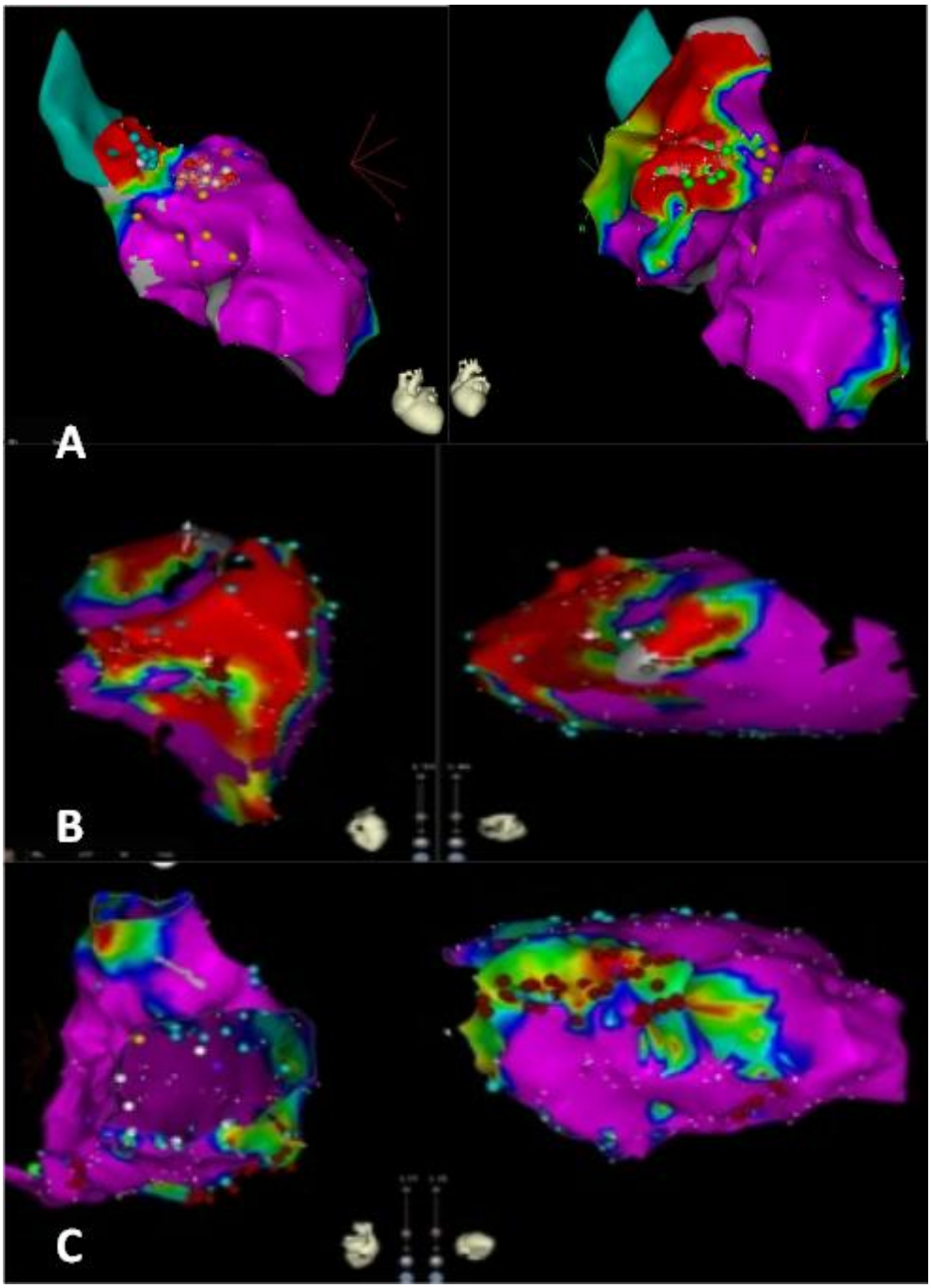


Figure 5: Five patients (3 RDACM and 2 LDACM) underwent EPS and ablation of ventricular tachycardia because recurrence of S-VT despite medical treatment.

Patient 1 (A): 33 year old patient diagnosed with DCM after recurrent syncope and S-VT. Her ECG showed remarkable low QRS voltages and ITW from V1-V3. She had severe LV dysfunction (with normal volumes) and mild impairment of the RV (with normal volumes). She carried a European founder mutation in *PLN* (p.R14del) and

after completing the family cascade screening her initial diagnosis was reclassified to LDACM. She was started on betablockers and ACEI and had an ICD implanted. She suffered 2 S-VT (one of them required a shock delivery) The EPS triggered a S-VT originated from the RV. The endocardic monopolar EVM suggested epicardial scar in the RVOT. Successful ablation guided by pacemapping was carried out. No low voltages areas in the LV were detected.

Patient 2 (B): a 48- year-old RDACM patient with recurrent syncope. His ECG showed widespread TWI and low voltages. His CMR showed a non-dilated RV with a normal ejection fraction. The LV was normal. No LGE was observed. He was started on sotalol and he had an ICD implanted for primary prevention. Despite sport discouragement, he suffered recurrent S-VT on exertion. He underwent an EPS. The endocardic EVM failed to demonstrate low voltage areas in RV. An epicardial approach revealed extensive low voltage areas which were ablationated. No arrhythmias have been documented arrhythmias in a 2-year follow-up.

Patient 3 (C): A 27-year-old amateur footballer suffered an aborted SD during a football match. His ECG showed RBBB and ITW in V1-3. The CMR showed a severely dilated and impaired RV (EDV 290 ml, RVEF 35%). The LV was normal and no LGE was observed. He was started on sotalol and he had an ICD implanted for secondary prevention. One year later, he suffered another S-VT that required ATP for cessation. The EVM showed inferobasal and apical low voltage areas.

Patient 4: He presented with well tolerated S-VT at the age of 51. His ECG showed sinus bradycardia, first degree AV block and giant epsilon waves. The CMR showed biventricular dilatation and dysfunction and a LGE spot in the lateral wall of the LV. He was finally diagnosed with RDACM with LV involvement. An EPS was performed after an S-VT storm and recurrent ICD shocks. Bipolar EVM revealed a low voltage area in the LV that corresponded to the LGE spot was found in the CMR. A successful endocardial ablation was performed. No other ventricular arrhythmias have been documented during 2-year follow-up.

Patient 5: 42 year old woman with 2 episodes of S-VT, global LV dysfunction and family history of LDACM. She carried a truncating mutation in *DSP (p.Q447**). The EVM did not demonstrate low voltage areas.

Low voltage areas were present in the RV outflow tract in 2 (1 RDACM and 1 LDACM) patients; there was a widespread epicardial low voltage area in the RV in 1 RDACM patient and it was localized in the LV lateral wall (1 RDACM with LV involvement). All the patients with RV low voltage areas were found to have ITW in right precordial leads despite their function and volume.

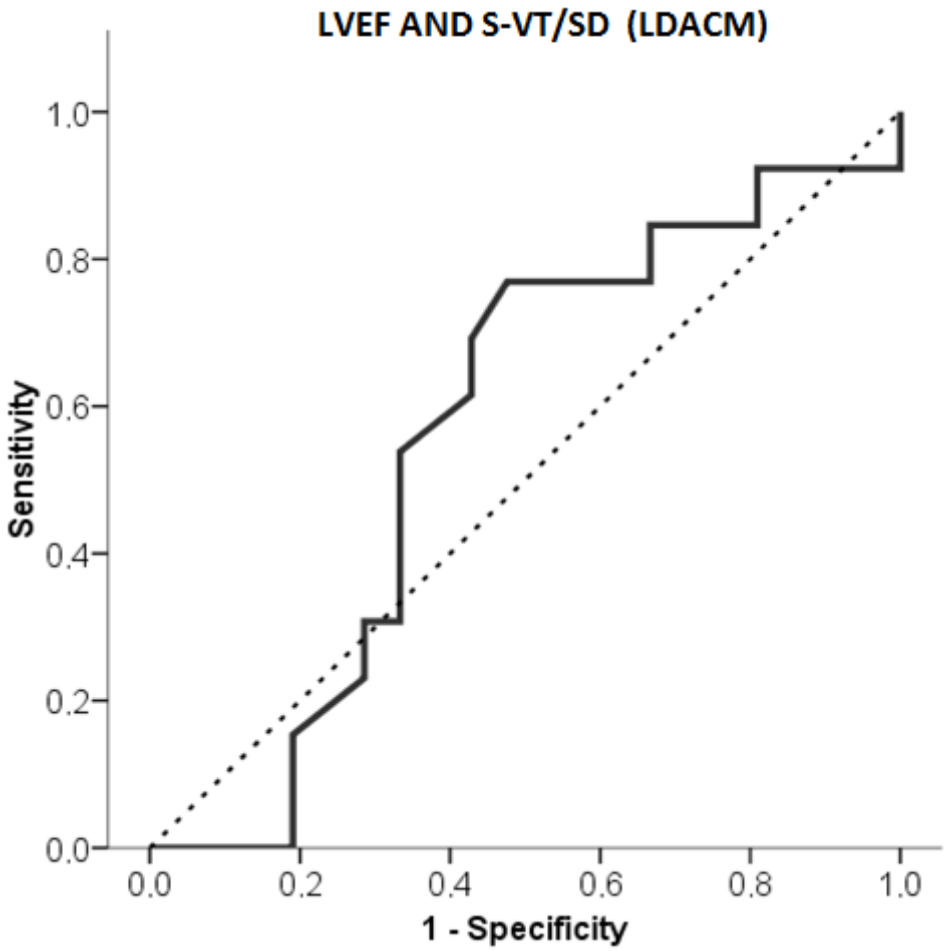


Figure 6: ROC curve of LVEF and S-VT/SD in LDACM. AUROC 0.57. LVEF of 45% yields sensitivity of 77% and specificity of 50%.

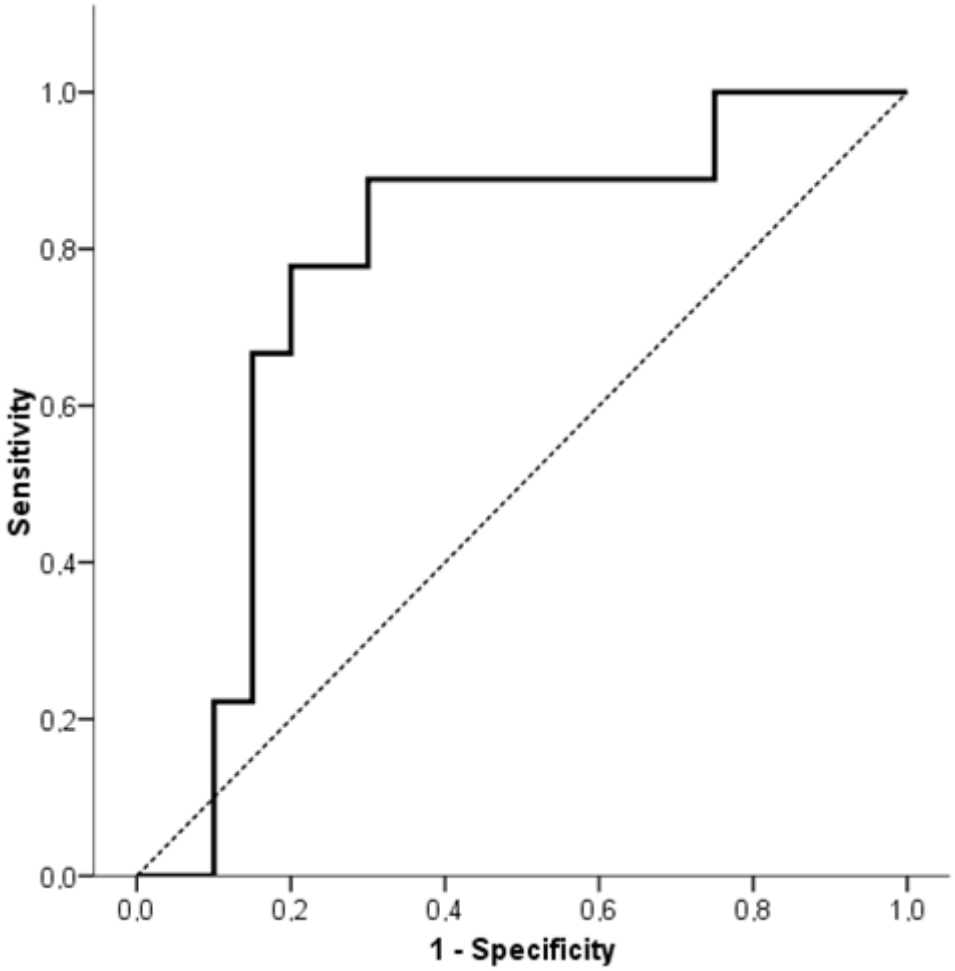
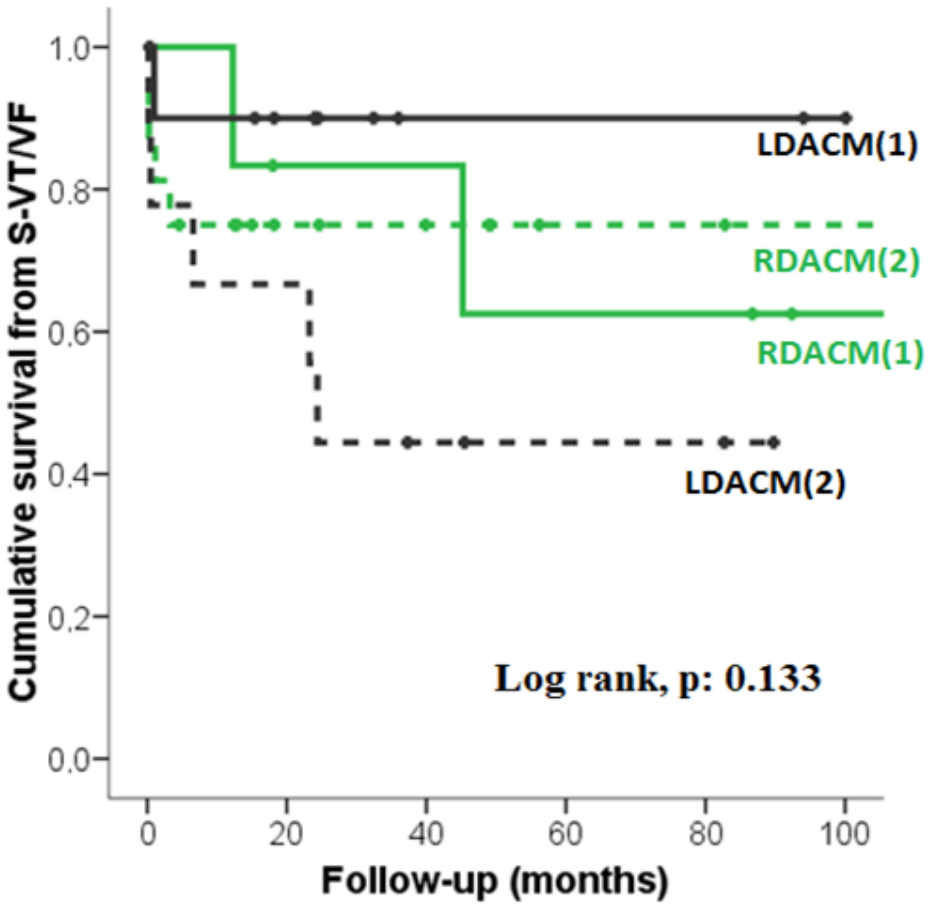


Figure 7: ROC curve of tricuspid DTI and S-VT/VF during the follow-up of ACM patients with ICD. (AUROC 0.77, *p: 0.021)*. Values below 10 cm/s shows a sensitivity of 78% and specificity of 80%.



Individuals at risk (S-VT/VF)	0-11 months	12-23 months	24-35 months	36-47 months
RDACM (Primary prevention)	9	7	5	4
RDACM (Secondary prevention)	14	10	6	6
LDACM (Primary prevention)	10	9	7	3
LDACM (Secondary prevention)	9	6	4	4

Figure 8: Survival free from appropriate ICD therapy after the implant in primary and secondary prevention. (1 and 2 stand for primary and secondary prevention respectively). The table shows the number of individuals at risk of S-VT/VF at the beginning of each interval.

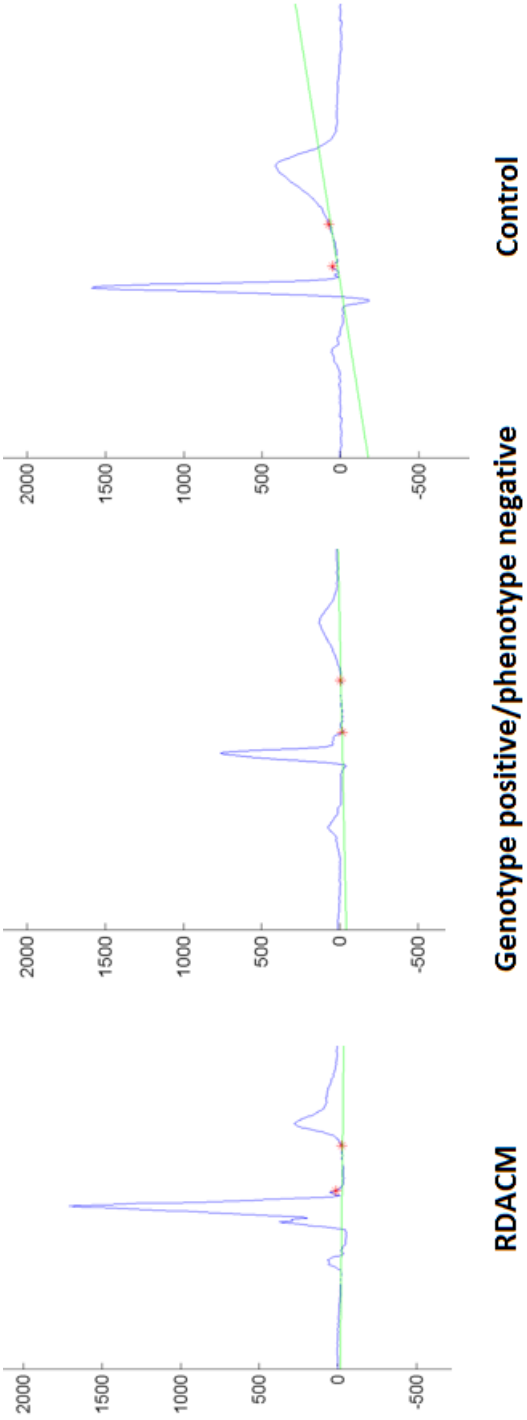


Figure 9: QRS of a RDACM patient (A), healthy mutation carrier (B) and control (C). Notice the fragmented QRS associated with flat ST in A (-0.4º) and B (1.9º). The ST slope is 9.25º in the control (C).

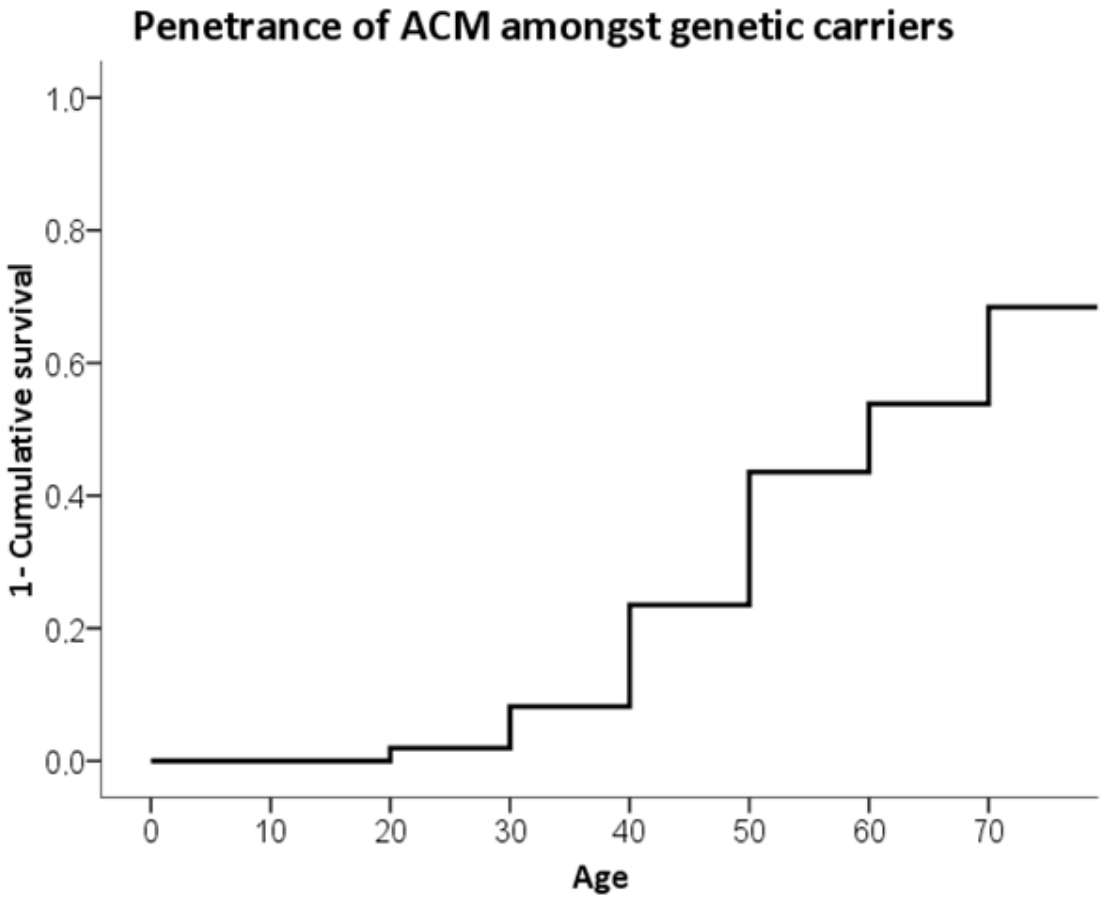


Figure 10: Age and cumulative penetrance in genetic carriers.

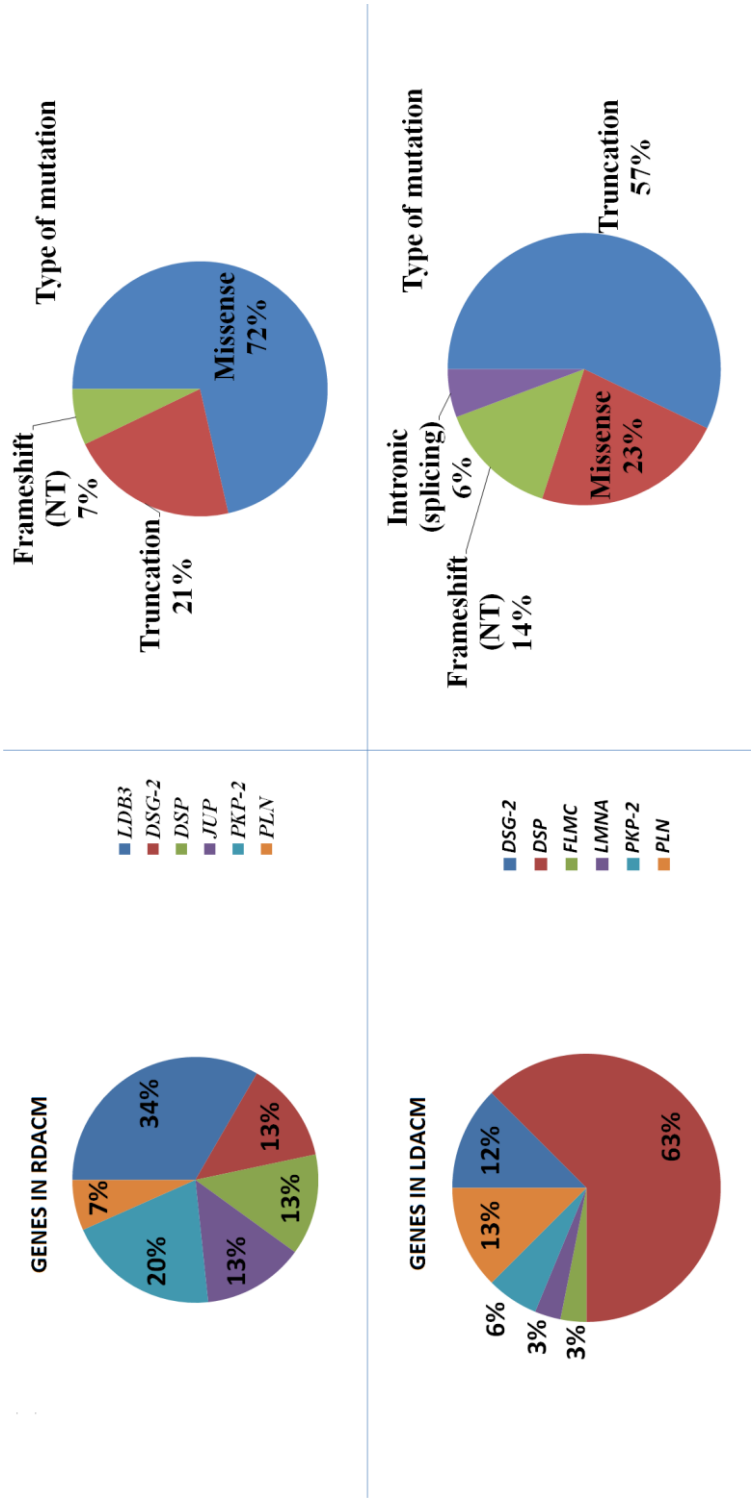
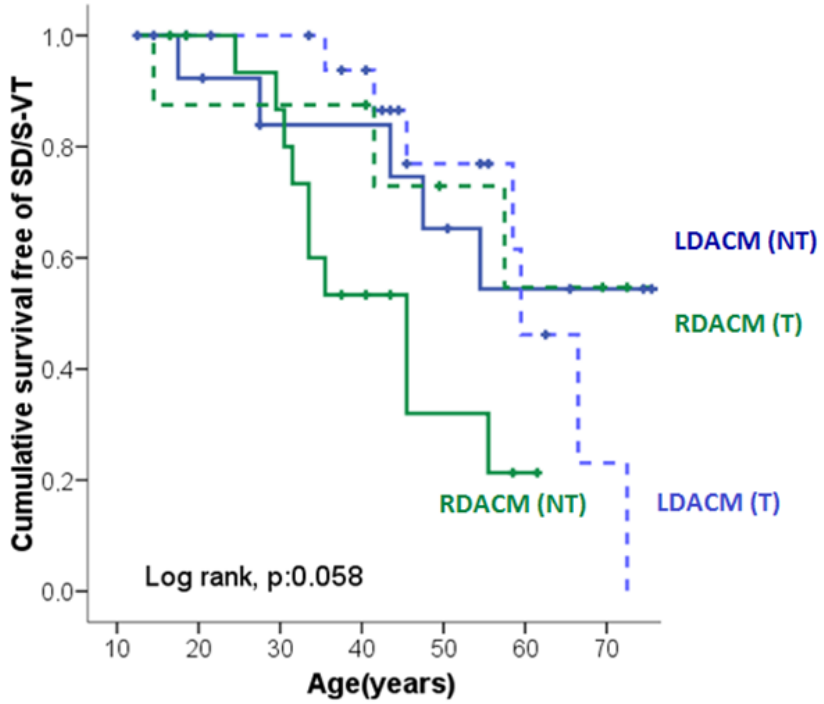


Figure 11: Distribution of the mutations found in RDACM and LDACM and mechanism.



Age (number at risk of S-VT/SD)	0	20	40	60
LDACM (Truncation)	20	18	14	3
LDACM (No truncation)	15	12	9	5
RDACM (Truncation)	8	7	7	3
RDACM (No truncation)	17	15	7	1

Figure 12: Survival free from S-VT/SD in RDACM and LDACM (truncating mutations versus non truncating mutations). Variants of unknown significance were not included in the analysis.

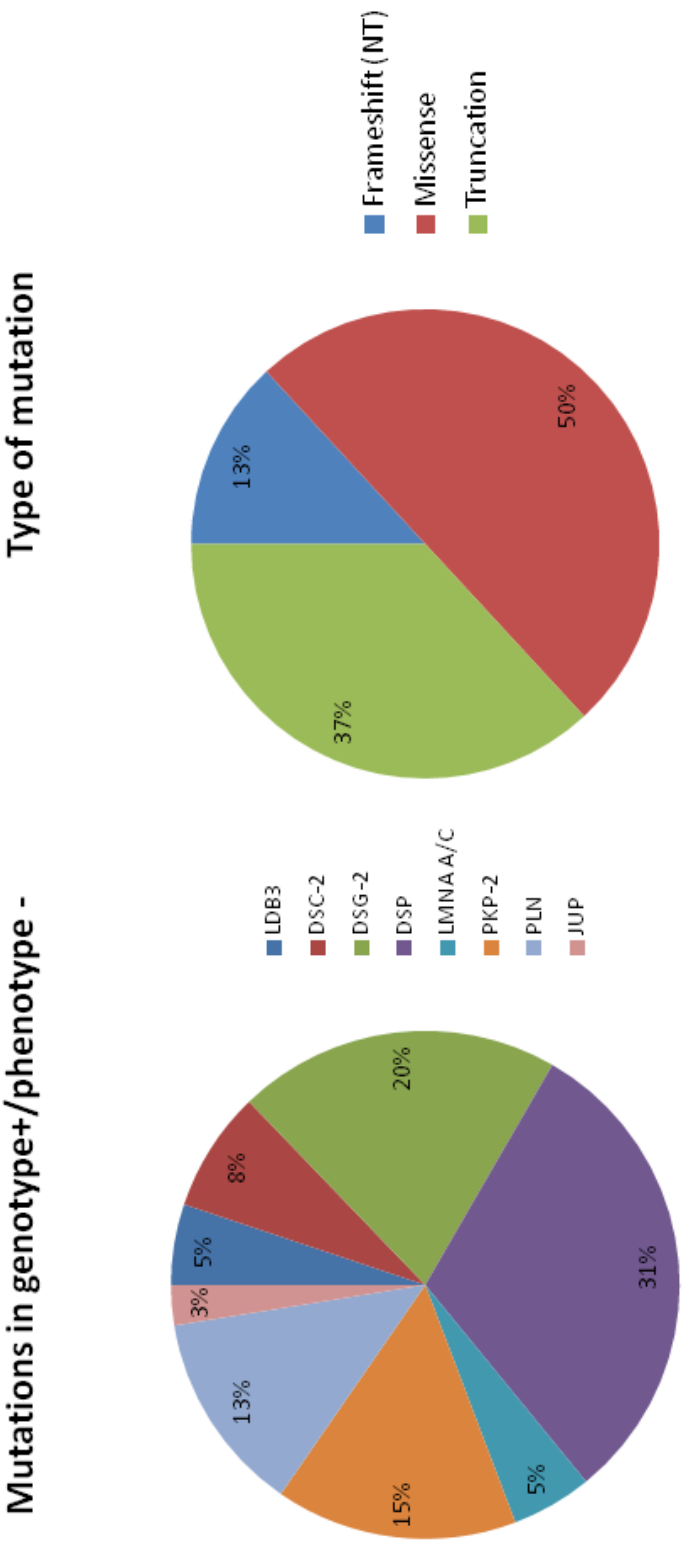


Figure 13: Genetic background in healthy mutation carriers.

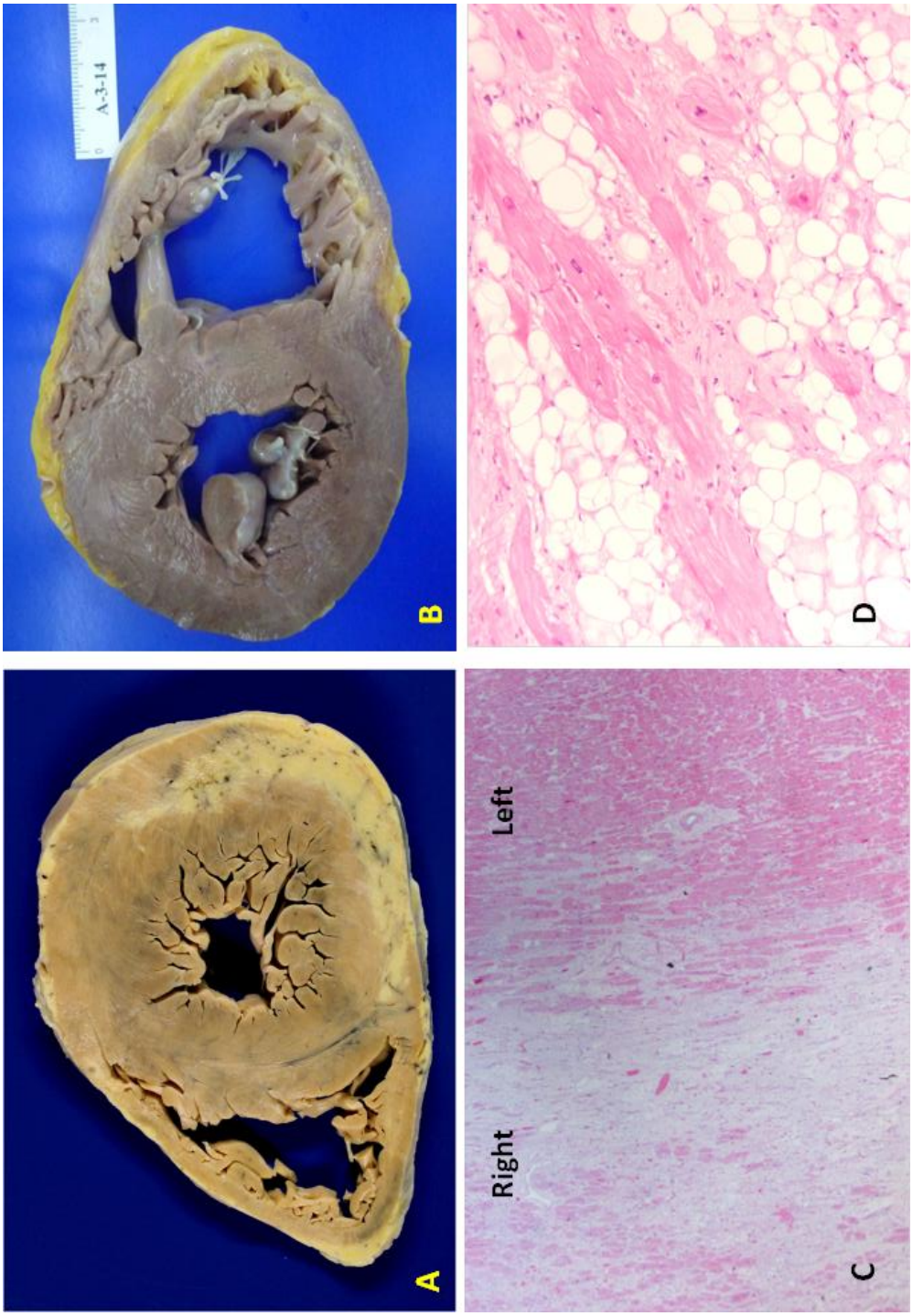


Figure 14: Macroscopic findings in LDACM **(A)** and RDACM **(B)**. Hematoxilin-eosin (H-E) of interventricular septum in RDACM (10X). Extensive fibrosis is observed on the right side in a RDACM case. The epicardial circumferential fibrotic pattern of the LV extends towards the right side of the interventricular septum in LDACM**(C).** Fibrofatty tissue in mid lateral wall of the RV in RDACM (H-E, 20X) **(D).**

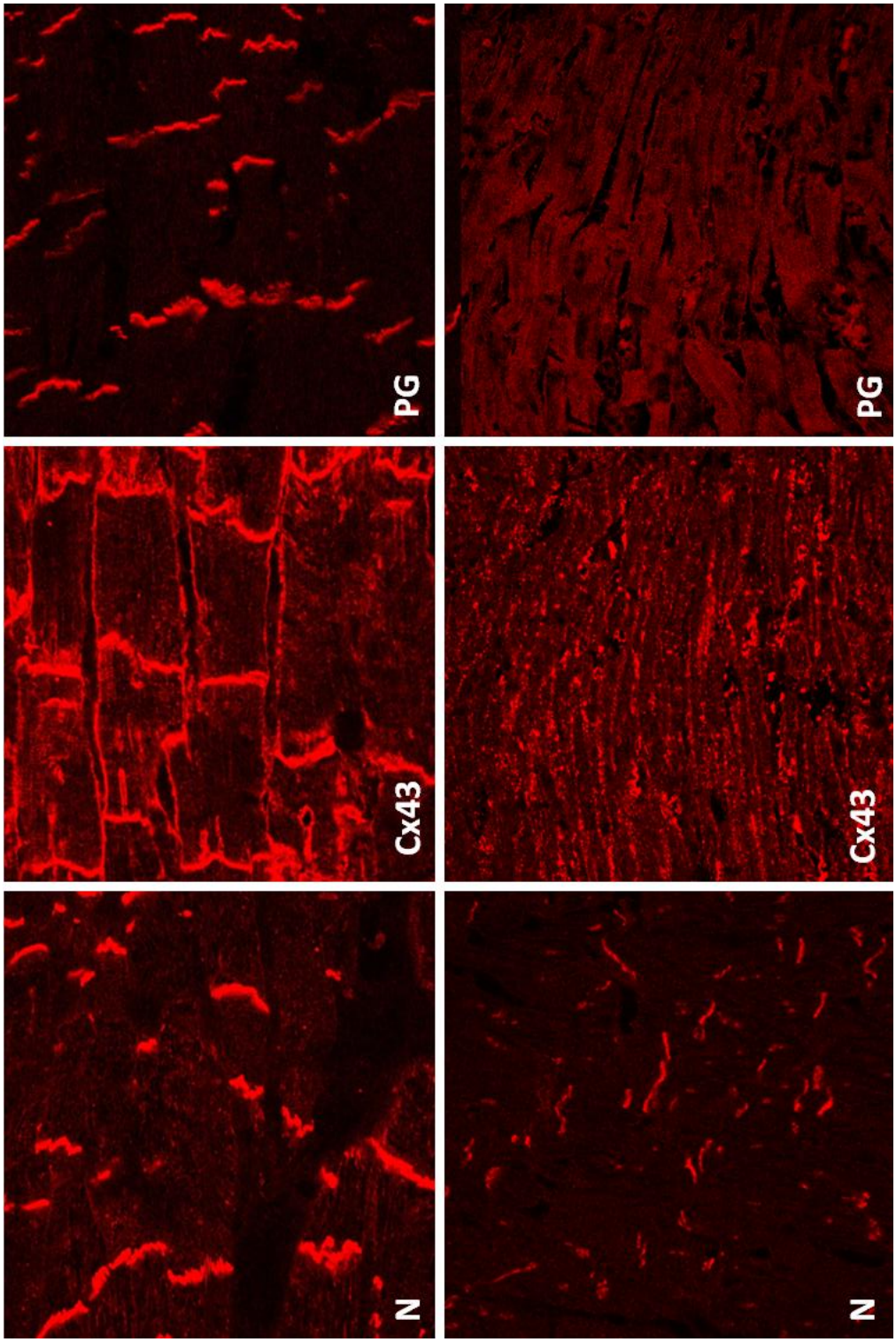


Figure 15: Immunohistochemistry. N: N-cadherin; Cx43: Connexin-43; PG: Plakoglobin. LDACM (top) and RDACM (bottom).

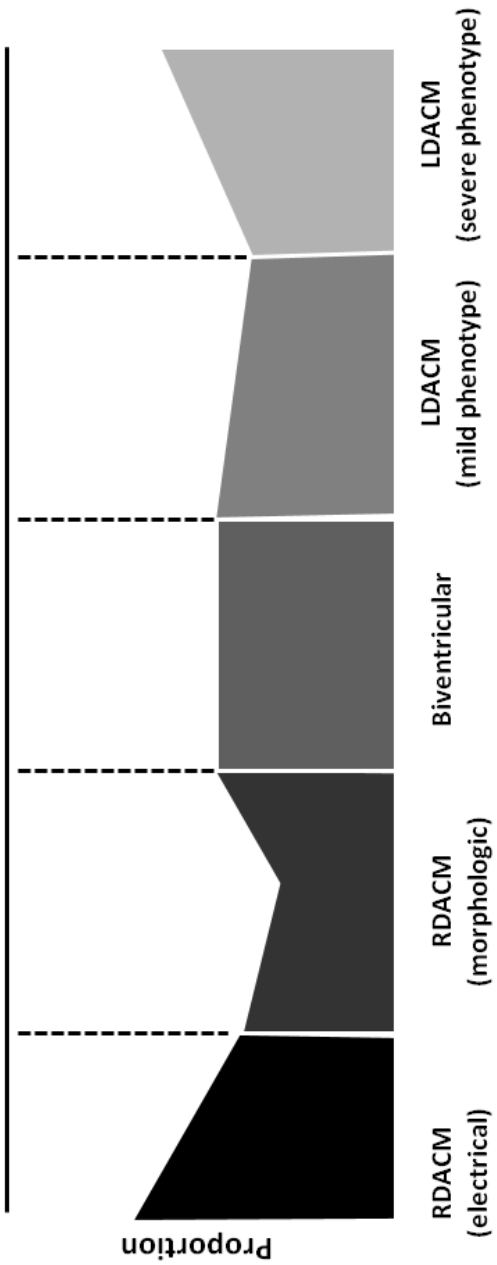


Figure 16: Spectrum of ACM and proportion of patients in each category.

TABLES

		HCM	DCM	LVNC	ACM
Global	Males (%)	639(61.7)	270(67.3)	64(66.7)	65 (61.3)
	Median age	49 [34-64]	41[20-56]	10[0.1-31.8]	43 [35.5-54.0]
	Ratio Proband: Affected relative	1:1.85	1:1.53	1:2.18	1:1.68
Total		1035	401	96	106
SD subgroup	Males (%)	63(80.8)	7(53.8)	1(100)	15(60)
	Age	39[24.5-47.3]	44.5[34.0-60.8]	43	38 [29.0-48.0]
Total		78	13	1	25

Table 1: Demographics of ACM, HCM, DCM and LVNC.

	RDACM (N=68)	LDACM (N=38)	P	Genotype +/ Phenotype – (N=38)
Sex (M)	45(62.5%)	15 (40.5%)	0.014	13 (34.2%)
Age at diagnosis	43.3±15.7	47±20.1	0.769	36.3±21.8
SD	17 (25.0%)	8 (21.1%)	0.189	0(0%)
Family history of cardiomyopathy	32 (47.1%)	29 (79.3%)	0.013	38 (100%)
Probands	46 (67.7%)	17 (44.7%)	0.025	-
Ratio proband: affected relative	1:0.48	1:1.24	-	-
Athletes	15 (21.5%)	5 (13.2%)	0.190	3(7.9%)
ICD	23 (33.8%) Biventricular dysfunction (1) Syncope (4) Stable S-VT (1) Others (NS-VT, presyncope)(3) Secondary prevention (14)	19 (50.0%) Severe LV systolic dysfunction±S-VT (5) Moderate systolic dysfunction±S-VT (5) Secondary prevention (9)	-	-
Comorbidities				
1. Atrial fibrillation/flutter	7(10.3%)	3 (8.3%)	-	1 (2.6%)
2. Stroke/TIA	3(4.4%)	2 (5.3%)	-	1 (2.6%)
3. Hypertension	11 (16.2%)	10 (26.3%)	-	2 (5.3%)
4. Diabetes	2(2.9%)	2(5.3)	-	2 (5.3%)
5. Cancer	1(1.5%)	4(10.5%)	-	0 (0%)
Medications				
Betablockers	15 (22.1%)	13 (19.1%)	-	1 (2.6%)
Sotalol	13 (19.1%)	1 (2.6%)	-	0 (0%)
Amiodarone	1 (1.5%)	5 (13.2%)	-	0 (0%)
Investigations				
ECG ITW (V1-3) ITW (V1-2; V5-6) ITW V1-4 (RBBB) Final QRS duration (V1-3)	**51 (75%)** 20 (39.2%) 14(27.5%) 11(21.6%) 45.3±14.21	**22 (57.9%)** 2 (9.1%) 9 (41.0%) 2 (9.1%) 54.5±48.7	 0.409	**38 (100%)** 0 (0%) 0 (0%) 0 (0%)
SA-ECG 0/3 1/3 2/3 3/3	**36 (52.9%)** 13 (36.1%) 7 (19.4%) 5 (13.8%) 11 (30.6%)	**14 (36.8%)** 8 (57.1%) 0 (0%) 2 (7%) 4 (28.6%)	 0.432 	**27 (71.1%)** 17 (63.0%) 3 (11.1%) 3 (11.1%) 4 (14.8%)

Holter	**40 (58.8%)**	**21 (55.3%)**		**16 (42.1%)**
Normal	19 (47.5%)	8 (38.1%)		16(100%)
Bigeminy, trigeminy or couplets	10 (25%)	5(23.8%)		0(0%)
NS-VT	11 (27.5%)	8 (38.1%)		0(0%)
Ventricular ectopics (>500/24h)	19 (47.5%)	10 (47.6%)		0(0%)
Ventricular ectopics (>1000/24h)	17 (42.5%)	10 (47.6%)		0(0%)
Exercise test	**25 (36.8%)**	**10 (26.3%)**		**18 (47.4%)**
Normal	12 (48%)	6 (60%)		16(88.9%)
NS-VT	8 (32%)	2 (20%)		0 (0%)
ABPR	5 (20%)	2 (20%)		2 (11.1%)

Table 2: Clinical summary of the ACM population and healthy genetic carriers. ABPR: Abnormal blood pressure response.

	LDACM (38) ***Echocardiogram (32)*** ***CMR (18)***	**RDACM (68)** ***Echocardiogram (42)*** ***CMR (42)***	**p**
EDV (LV) (ml) **Index (ml/m2)**	192.9±38.2 109.8±19.3	156.2±40.0 110.4±89.1	<0.001 0.98
ESV (LV) (ml) **Index (ml/m2)**	112.3±32.2 64.7±19.4	71.4±29.9 44.9±22.7	<0.001 0.019
LVEF (%)	42.4±10.0	56.9±10.6	<0.001
EDV (RV) (ml) **Index (ml/m2)**	146.6±33.6 80.9±16.6	197.5±79.3 140.5±116.0	0.004 0.003
ESV (RV) (ml) **Index (ml/m2)**	73.2±21.6 37.4±21.6	118.1±70.7 70.9±70.7	0.004 0.003
RVEF (%)	48.5±9.8	42.8±14.2	0.122
TAPSE (mm)	19.5±8.3	18.3±9.0	0.588
Tricuspid DTI (cm/s)	10.4±4.0	9.9±5.1	0.702
Ratio EDV RV/EDV LV **Index**	0.75±0.13 0.73±0.14	1.32±0.32 1.26±0.32	<0.001 <0.001
LGE	20 (54.1%)	7(15.6%)	0.006
Biventricular disease	16 (42.1%)	26 (38.2%)	0.937
LVNC (Ratio NCL/CL>2.3)	16 (42.1%)	4 (5.9%)	<0.001

Table 3: Imaging characterization of the ACM population. Volumes and RV and LV ejection fraction were calculated with CMR. PLAX: Parasternal long axis. PSAX: Parasternal short axis. EDV: End systolic volumes. ESV: End systolic volume. NCL: Non compacted layer. CL: Compacted layer.

	LDACM (22)	RDACM (51)	p	Genotype+/Phenotype- (38)	Control (113)	P
FIRST DEGREE AV BLOCK	1 (4.5)	6 (11.8)	0.231	0(0)	1 (0.9)	-
MEAN PR (ms)	174.6±31.2	182.7±35.0	0.209	179.9±18.5	187±13.1	0.140
MEAN QRS DURATION (ms)	95.1±28.9	103.0±22.8	0.247	85.7±13.1	87.5±19.6	0.419
DURATION V1-V3 (ms)	99.3±27.82	104.0±21.4	0.468	89.3±12.3	93.1±29.3	0.379
DURATION V4-V6 (ms)	97.6±49.9	104.1±31.4	0.479	84.9±16.0	86.7±29.6	0.556
V1+2+V3/V4+V5+V6	1.21±0.80	1.03±0.14	0.165	1.06±11.67	1.08±22.6	0.539
QRS DISPERSION (ms)	92.3±14.0	48.2±50.4	0.174	31.9±19.0	50.0±70.4	0.013
TERMINAL ACTIVATION DELAY (V1-3) (ms)	54.5±48.7	45.3±14.2	0.409	40.2±13.0	34.0±34.5	0.475
TERMINAL ACTIVATION DELAY (PRECORDIALS)	76.5±49.8	76.0±20.9	0.957	62.7±12.6	65.4±26.0	0.468
MEAN VOLTAGE (FRONTAL) (mV)	1.1±0.6	1.1±0.4	0.873	1.4±0.6	1.5±0.4	0.036
LOW VOLTAGE CRITERIA (FRONTAL)	15(68.2)	33(43.1)	0.098	27 (71.2)	63 (55.8)	0.201
MEAN VOLTAGE (PRECORDIAL)(mV)	1.2±0.6	1.2±0.4	0.944	1.4±0.6	1.5±0.5	0.036
LOW VOLTAGE CRITERIA (PRECORDIAL)	13(59.1)	22(43.1)	0.829	17 (44.7)	34 (30.1)	0.014
FRAGMENTED QRS	18 (81.9)	28 (54.9)	0.42	21 (55.3)	46 (40.7)	0.413
FRAGMENTED DERIVATIONS	1.1±1.8	1.2±1.4	0.566	0.7±0.9	0.8±1.1	0.575
FINAL NOTCHED QRS	7 (31.8)	13 (25.4)	0.893	11 (28.9)	37 (32.7)	0.503
NOTCHED DERIVATIONS	1.2±0.2	1.3±0.2	0.550	0.3±0.5	0.6±1.2	0.490
EPSILON WAVES AND OTHER POSTDESPOLARIZATIONS	3 (13.6)	5 (9.8)	0.894	0(0)	0(0)	-
EARLY REPOLARISATION	2 (9.1)	4 (7.8)	0.436	7 (18.4)	26 (23)	0.308
MEDIAN INFEROLATERAL ST SLOPE	9.94±24.67	11.78±30.74	0.792	9.6±8.5	13.2±11.2	0.040
T WAVE INVERSION (EXCLUDING AVR)	16(72.7)	26 (51.0)	0.274	18 (47.4)	38(33.6)	0.176
MEAN NUMBER OF INVERTED T WAVES	1.5±1.6	2.4±2.3	0.009	1.6±1.3	1.2±0.8	0.032
MEDIAN TW AREA ($W^{1/2}$*10^{-9})	8.0±15.1	16.8±11.6	0.019	19.6±0.8	23.1±12.2	0.139

Table 4: Characterization and comparison of electrocardiographic findings in RDACM and LDACM (left) and genotype positive/phenotype negative relatives and controls (right).

LDACM			RDACM			HEALTHY CARRIERS		
GENE	MUTATION	Total carriers (families)	GENE	MUTATION	Total carriers (families)	GENE	MUTATION	Total carriers (families)
DSG-2	p.V920G	3(1)	*DES*	p.G75E	1(1)	*PLN*	p.R14del	5(1)
DSG-2	p.G638R	1(1)	*DSG-2*	p.A969V	1(1)	*PKP-2*	p.V837fs*930	3(1)
DSP	p.R425*	1(1)	*DSG-2*	p.S303F	1(1)	*PKP-2*	p.S140F	5(1)
DSP	p.Q447*	18(4)	*DSC-2*	p.E896fs*900	3(3)	*PKP-2*	p.R79Q	2(1)
DSP	p.L1773Yfs*1781	1(1)	*DSP*	p.Q1925*	1(1)	*PKP-2*	p.D26N	1(1)
DSP	p.D2070N	1(1)	*DSP*	p.A1074Sfs*1087	1(1)	*PKP-2*	p.A418D	1(1)
FLNC	p.G1800*	1(1)	*DSP*	p.R1537C	1(1)	*LMNA*	p.R545H	2(1)
JUP	IVS5+6C>T	1(1)	*GJA1*	p.A6T	1(1)	*LDB3*	p.T351A	2(1)
JUP	p.T19I	1(1)	*JUP*	p.T19I	1(1)	*JUP*	p.T19I	1(1)
LMNA	p.R545H	1(1)	*JUP*	p.P35T	1(1)	*GJA1*	p.A6T	1(1)
PKP-2	p.R79Q	2(1)	*JUP*	p.A521A	1(1)	*DSP*	p.R425*	1(1)
PLN	p.R14del	4(2)	*LDB3*	p.T351A	5(1)	*DSP*	p.Q447*	4(3)
			PKP-2	p.V837fs*930	1(1)	*DSP*	p.Q1925*	2(1)
			PKP-2	p.S140F	2(1)	*DSP*	IVS8-1G>A	1(1)
			PKP-2	p.R79*	1(1)	*DSP*	p.D2070N	2(1)
			PKP-2	Exon8del	1(1)	*DSP*	p.A1074Sfs*1087	4(1)
			PKP-2	p.D26N	1(1)	*DSG-2*	p.V920G	8(2)
			PKP-2	p.A418D	1(1)	*DSC-2*	p.A133T	4(1)
			PKP-2	p.R355*	1(1)	*DSC-2*	p.R450R	2(1)
			PLN	p.R14del	1(1)	*DSC-2*	p.E896fs*900	4(3)
			RyR2	p.S376F	1(1)			
			TMEM43	IVS4+16G>A	1(1)			
Total		**35(16)**			**29 (24)**			**55(25)**

Table 5: List of mutations and variants of unknown significance identified in patients and their genotype positive/phenotype negative relatives.

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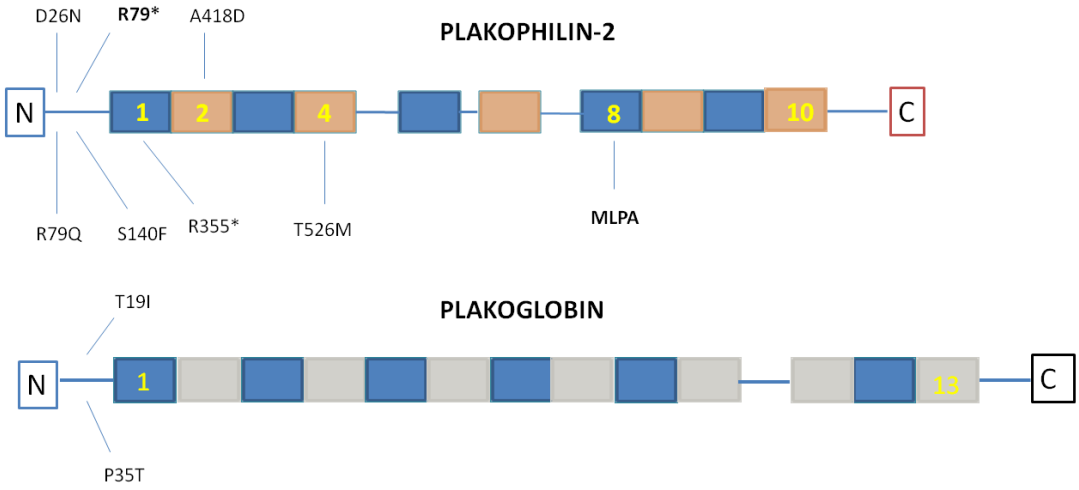
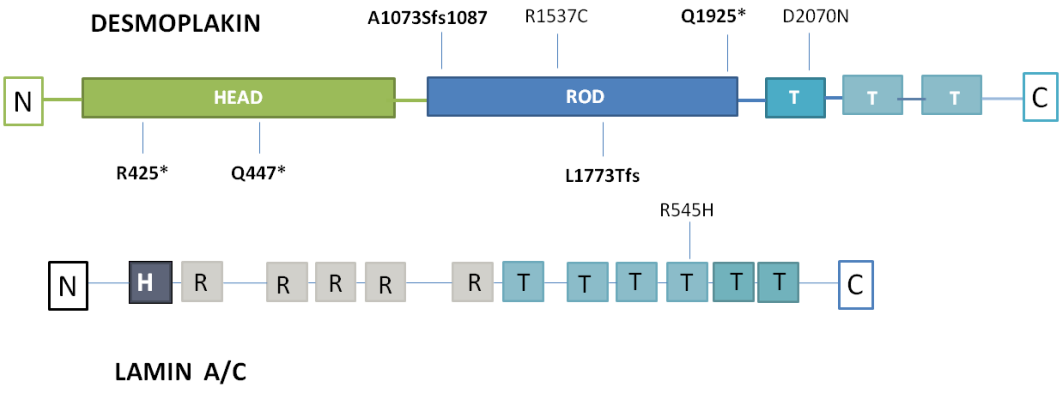
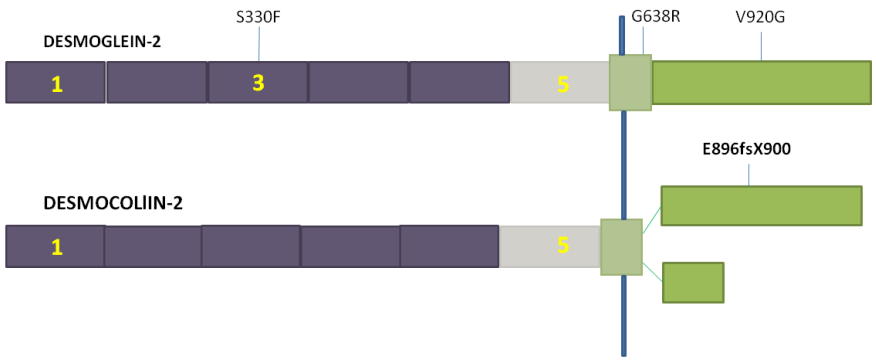
SUPPLEMENTARY MATERIAL

DISTRIBUTION OF MUTATIONS



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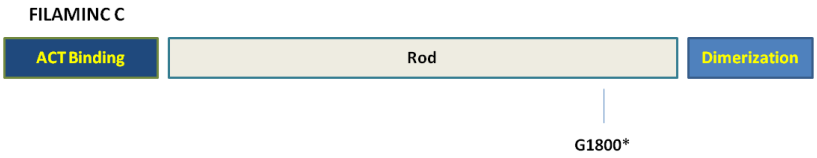
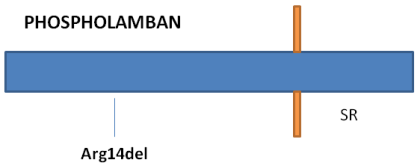

FILAMINC C
ACT Binding
Rod
Dimerization
G1800*


PHOSPHOLAMBAN
SR
Arg14del


CYPHER/ZASP
N
PDZ domain
ZM motifs
LIM domain
C
T351A

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Josefa Gonzalez; Esperanza Garcia-Molina; Ivan Gomez; Diana Domingo (Department of Cardiology Virgen de la Arrixaca University Hospital) Luis Polo (Department of Pathology, Virgen de la Arrixaca University Hospital).

CHAPTER 3: GENOTYPE-PHENOTYPE CORRELATION BEYOND THE DESMOSOME: LEFT DOMINANT PHENOTYPES AND DESMOPLAKIN, PHOSPHOLAMBAN CARDIOMYOPATHY AND Z BAND MUTATIONS

Aims

1. Clinical characterization of families harbouring the novel *DSP p.Q447** and to delineate the phenotype of all DSP truncating mutations reported to date
2. To characterize the clinical profile of the first known RDACM family with a mutation in the Z band gene *LDB3 p.A351T.*
3. Clinical and historical study of the European founder mutation *PLN p.R14del.*

DESMOPLAKIN TRUNCATIONS AND ARRHYTHMOGENIC LEFT VENTRICULAR CARDIOMYOPATHY: CHARACTERIZING A PHENOTYPE

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Running head: Desmoplakin Mutations and Arrhythmogenic Cardiomyopathy.

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ABSTRACT

Aims

Risk stratification for sudden death in arrhythmogenic right ventricular cardiomyopathy is challenging in clinical practice. We lack recommendations for the risk stratification of exclusive left- sided phenotypes. The aim of this study was to investigate genotype-phenotype correlations in patients carrying a novel *DSP p.Q447**, and to review the literature on the clinical expression and the outcomes in patients with *DSP* truncating mutations.

Methods

Genetic screening of the *DSP* gene was performed in 47 consecutive patients with a phenotype of either an arrhythmogenic right ventricular cardiomyopathy (ARVC) (n=24), or an idiopathic dilated cardiomyopathy (DCM), who presented with ventricular arrhythmias or a family history of sudden death (n=23) (aged 40±19 years, 62% males). Three unrelated probands with DCM were found to be carriers of a novel mutation (*DSP p.Q447**). Cascade family screening led to the identification of 15 relatives who are carriers.

Results

Penetrance in *DSP p.Q447** carriers was 83%. Sustained ventricular tachycardia was the first clinical manifestation in six patients and nine patients were diagnosed with left ventricular impairment (two had overt severe disease and seven had a mild dysfunction).Cardiac magnetic resonance revealed left ventricular involvement in nine cases and biventricular disease in three patients. Extensive fibrotic pattern in six and non-compaction phenotype in five patients was the hallmark in imaging.

Conclusion

*DSP p.Q447** is associated with an aggressive clinical phenotype of left-dominant arrhythmogenic cardiomyopathy and left ventricular noncompaction. Truncating mutations in desmoplakin are consistently associated with aggressive phenotypes and must be considered as a risk factor of sudden death. Since ventricular tachycardia

occurs even in the absence of severe systolic dysfunction, an implantable cardioverter-defibrillator should be indicated promptly.

Selected abbreviations and acronyms: ARVC= Arrhythmogenic right ventricular cardiomyopathy; SCD= Sudden cardiac death; DCM= Dilated cardiomyopathy; ACM= Arrhythmogenic cardiomyopathy; LVNC= Left ventricular noncompaction; TFC= Task Force Criteria; SA-ECG= Signal averaged; CMR=Cardiac magnetic resonance; RBBB= right bundle branch block; LBBB= left bundle branch block; ITW= Inverted T wave; FTW=Flat T wave; LGE= late gadolinium enhancement; ICD= Implantable cardioverter defibrillator; S-VT= Sustained ventricular tachycardia; EPS= Electrophysiological study; EMB= Endomyocardial biopsy

Key words: Arrhythmogenic cardiomyopathy; dilated cardiomyopathy; sudden death; implantable cardioverter defibrillator; desmoplakin

CONDENSED ABSTRACT

We indentified a novel desmoplakin truncating mutation in 3 families of arrhythmogenic left ventricular cardiomyopathy. This mutation is associated with a particularly aggressive phenotype and a high prevalence of ventricular arrhythmias. This phenotype is in keeping with all desmoplakin truncating mutations reported in literature.

MANUSCRIPT

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary myocardial disorder clinically characterized by ventricular arrhythmias, sudden cardiac death (SCD) and end stage heart failure. It is a common cause of SCD amongst young people and athletes[1], and fatal arrhythmia may even occur in the absence of significant ventricular remodelling.

ARVC was thought to primarily affect the right ventricle (RV) although in a proportion of cases, the disease has been shown to affect the left ventricle (LV) not only at end stage but also as a primary target. In this scenario, isolated arrhythmogenic left ventricular cardiomyopathy (ALVC) is frequently misdiagnosed as dilated cardiomyopathy (DCM) or myocarditis.[2, 3]

Mutations in genes encoding desmosomal proteins (desmoplakin, plakophilin-2, plakoglobin, desmocollin-2 and desmoglein-2)[4], were first identified in classical ARVC and were later found in left dominant forms. Other non desmosomal genes have recently been associated with arrhythmogenic cardiomyopathy (ACM).[5]

The aim of this study was to investigate genotype-phenotype correlations in patients carrying a novel *DSP p.Q447**, and to review the literature on the clinical expression and the outcomes in patients with *DSP* truncating mutations.

METHODS

The study was approved by the Ethics Committee of our hospital (Virgen de la Arrixaca University Hospital, Murcia) and it complied with the ethical principles of the Declaration of Helsinki. All participants gave their written consent. A genealogical pedigree of each family was obtained. Clinical assessment consisted of a 12- lead digital ECG, a signal averaged ECG (SA-ECG), a 2D-doppler echocardiogram, cardiac magnetic resonance (CMR), an exercise test and 24-hour Holter monitoring. In addition, an electrophysiological study (EPS) was performed in three patients. Patients were followed up annually (median 26[13-87] months).

Study population

The population studied included 47 unrelated patients (29 (62%) males, aged 40 ± 19 years). 23 (49%) met the criteria for ARVC and 24 for DCM showing either sustained ventricular tachycardia (S-VT) or a history of SCD in a first or second degree relative under 35 years of age. We chose a DCM population with such a particular aggressive arrhythmic burden that rose the suspicion of arrhythmogenic left ventricular cardiomyopathy as the first diagnosis to be considered.

Genetic study of the 5 desmosomal genes (*DSP*, *DSG2, DSC2, PKP2, PKG), PLN* and *LDB3* was performed in the 47 patients. A novel *DSP p.Q447** mutation was identified in 3 unrelated probands. First degree relatives from these 3 families were invited to participate in a clinical and genetic investigation. 15 relatives were found to carry *DSP p.Q447**. 2 additional patients previously diagnosed with DCM, mild systolic dysfunction and ventricular arrhythmia died before the study started and in light of their family history, they were considered to be obligatory carriers.

The presence of a LV end-diastolic diameter (LVEDd) of >117% from normal and/or LV systolic impairment (<45%) were used for the diagnosis of DCM in probands. Familial criteria were applied in the remaining carriers.[6]ARVC diagnosis was made according to the revised Task Force Criteria (TFC).[7] Jenni et al criteria were employed for the definition of left ventricular non-compaction.[8]

Genetic analysis

DNA was extracted from peripheral blood samples using the Maxwell 16 Blood Purification Kit. Previously published *DSP*, *DSC2, DSG2, PKP2, PKG, PLN* primer sequences were used for amplification. Results were compared with the sequences available in Gen Bank (RefSeq: NC_000006.11) and analyzed by SeqScape software (AppliedBiosystem).[9] MYBPC3 and MYH7 were studied in 2 selected individuals from families with *DSP p.Q447** with left ventricular non-compaction phenotype. *DSP p.Q447** variant was absent in 500 Spanish control samples and was not present in the 5000 exome project (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [1/12/2013].) Four published markers that flank the *DSP* gene were used to investigate the presence of a

potential founder effect in carriers of *DSP p.Q447**. All of them are located within the DSP gene. Microsatellite 1 (Des.mic.1) is located in intron 1 and Microsatellite 2 (Des.mic.23) is located in intron 23 of the *DSP* gene. Both of them, were indentified in a sequence of a BAC clone maping to 6p23-p24 (GeneBank accession no. AL031058). The primer sequences for Des.mic 1 and 3 are as follows:

Des.mic.1 forward, 5´-CCCATCTATGCATAATGCAACC-3´; reverse, 5´-GTCCTCACGGATGTGCTACAAG-3´
Des.mic.23 forward, 5´-CGCTTTTGATCATGGCCCTAGTG-3´; reverse, 5´-CTCACCTGTTACAGCTAGATG-3´"

Immunohistochemistry

We analyzed endomyocardial biopsy (EMB) samples from RV septum in one case (C.III.12). They were formalin fixed, paraffin embedded, cut at a thickness of 4 micrometres and mounted on slides. We incubated the slides with a primary antibody (monoclonal mouse anti-N-cadherin (1:400) (Sigma); monoclonal mouse anti-plakoglobin (1:1000) (Sigma); Monoclonal rabbit anti-connexin 43 (1:400) (Sigma); and Monoclonal rabbit anti-desmin (1:200)(Abcam). The slides were incubated with secondary goat anti-mouse or goat anti-rabbit (1:400) (Jackson ImmunoResearch). Stained slides were checked using a confocal microscope (LSM510,Meta Zeiss). Findings were compared with a control sample from a patient deceased for no cardiovascular reason in which necropsy did not show evidence of cardiac disease. Case and control samples were fixed and embedded according to the same protocol and stained under similar conditions.

Electrocardiogram and ECG monitoring tests

One or more abnormal parameters in SA-ECG were considered as pathological (MAC 5500 ECG Diagnosis System - GE Healthcare, United Kingdom). Patients with right bundle branch block (RBBB) or left bundle branch block (LBBB) were excluded from the SA-ECG analysis. Tracings from ambulatory 24-hour Holter monitoring (Marquette Electronics, Milwaukee, USA and Synetec, ELA Medical, Sorin, USA) and treadmill exercise tests (Marquette Electronics Inc., Milwaukee, USA and General Electric T-

2100, Milwaukee,USA) were reviewed. Non sustained ventricular tachycardia (NS-VT) was defined as the presence of ≥ 3 ventricular beats at a rate >100 bpm. Sustained ventricular tachycardia (S-VT) was defined as VT lasting >30 seconds or lasting less if it was poorly tolerated.

Echocardiogram

All participants underwent 2D-doppler echocardiogram (Hewlett-Packard Sonos 7500, Hewlett-Packard, Andover, MA, USA). The standard analysis of the left ventricle (LV) included the measurement of end systolic and end diastolic diameters and volumes, ejection fraction, wall motion abnormalities, and pathological valvular flows. Right ventricle (RV) analysis consisted of outflow tract diameter calculations (measured on both the long and short axis) and in order to assess RV performance, TAPSE, doppler tissue imaging (DTI) and the fractional shortening ratio were both measured.

Cardiac Magnetic Resonance (CMR)

CMR was performed on 10 *DSP p.Q447** carriers using a 1.5T magnet (Achieva CV, Philips Medical Systems, Best, Netherlands). SSFP end-expiratory breath-hold cine imaging was acquired. After a bolus injection of Gadobutrol (0.1 mmol/kg, Gadovist; Bayer Shering Pharma, Berlin, Germany) the T1 measurement was calculated using standard late gadolinium enhancement (LGE) sequences. The analysis was performed using a personal computer and semi-automated software (Philips, Software work space 2.6.3.2). 5 patients were excluded from this analysis (3 already carried an implantable cardioverter-defibrilator (ICD) and 2 rejected the test).

RESULTS

A novel heterozygous *DSP* variant (*p.Q447**) inherited in an autosomal dominant manner with high penetrance (83%) was identified in 3 patients. The mutation was a C to T transition at exon 11 in the *DSP* gene leading to a premature stop codon. This change occurred at the N-terminal region, and it is thought to generate a truncated peptide (85% in length). The affected amino-acid at position *p.Q447** is located in one of the globular head domains of desmoplakin. This domain is shown to mediate the interaction of desmoplakin with two catenin proteins: plakoglobin and plakophilin-

2(10) **(Figure 1)**. Microsatellite study suggested a founder effect in the 3 families and the presence of a common ancestor.

Family A

The 37-year-old proband (A.III.5) **(Figure 2)** was diagnosed after an episode of sustained ventricular tachycardia (S-VT) at rest. An echocardiogram demonstrated severe LV dilatation and systolic impairment. The patient had a history of longstanding alcohol drinking, smoking and occasional cocaine use. The angiogram excluded coronary lesions and an ICD was implanted.

His mother (A.II.3) had died suddenly at 67 years of age. She had been diagnosed with DCM after ruling out coronary disease. The maternal grandfather suffered from heart failure and died in his sleep at the age of 37 years (A.I.1). A maternal aunt had previously died of heart failure (A.II.2).

Prior to the proband's admission, a cousin aged 55 years (A.III.1) who presented with recurrent S-VT and a structurally normal heart underwent ICD implantation. Familial cascade screening led to a new diagnosis of 4 asymptomatic, though affected, individuals (A.III.4, A.III.6, A.IV.6 and A.IV.7). It should be noted that the diagnosis of A.III.4 and A.III.6 were left ventricular noncompaction (LVNC). A severely increased late gadolinium enhancement (LGE) pattern was observed in A.IV.6 and A.IV.7 alongside mild systolic dysfunction. During the follow-up period both siblings have suffered multiple episodes of chest pains with troponin rise. Coronary artery disease was ruled out is both cases. They had an ICD implanted for primary prevention in the view of progressive systolic dysfunction.

Family B

The proband (B.II.1), a 42-year-old woman, was admitted after a presyncopal S-VT episode. Echocardiography revealed global left ventricular hypokinesia and a moderately impaired systolic function.

The mother (B.I.1) aged 72 years, had a history of presyncopal episodes associated with S-VT. An echocardiogram showed severe systolic impairment and angiography ruled out coronary disease. She underwent an EPS where poorly tolerated S-VT of a different morphology was induced. Both patients received an ICD.

A maternal aunt had previously died suddenly at the age of 40 years. Family genetic screening led to diagnosis of an asymptomatic non-affected carrier: a sister (B.II.2) aged 50 years. The echocardiogram was normal and the patient rejected CMR due to claustrophobia. Four maternal aunts, one maternal uncle and a nephew refused a cardiac and genetic study.

Family C

The 62-year-old female proband was admitted to hospital after a first episode of acute heart failure (C.II.1). Echocardiography demonstrated moderately impaired systolic function and MRI results led to the diagnosis of LVNC.

His daughter (C.III.1) had suffered an episode of acute myocarditis at the age of 32 years.

Six years after the diagnosis of the proband, a 45-year-old nephew (C.III.5) presented with cardiac arrest while walking. He was diagnosed with LVNC and moderate systolic dysfunction. One month after having an ICD implanted he received four shocks due to S-VT during mild exertion **(Figure 3)**. Both his 13 year old son (C.IV.1) and his 17 year old daughter (C.IV.2) were asymptomatic and they were diagnosed with LVNC in familial screening. The latter was found to have frequent ventricular ectopics and couplets on holter monitoring.

Clinical presentation and therapy

The first clinical presentation was presyncopal S-VT in five patients, resuscitated SCD in 1 and heart failure in 2 patients. Cardiac and genetic work-up led to the identification of 7 asymptomatic patients, as well as 3 non-affected asymptomatic carriers. 8 carriers met the criteria for DCM, 4 for LVNC and seven carriers also fulfilled the diagnostic criteria for the definitive diagnosis of ARVC.

Beta-blockers were started in all patients. 9 patients with systolic impairment were on ACE inhibitors or ARB-II blockers. Spironolactone was started in four patients. 5 patients received an ICD for secondary prevention of sudden death (A.III.1; A.III.5; B I.1; B II.1; and C.III.5) and 4 for its primary prevention (A III.4; AIII.6; A.IV.6 and A.IV.7). Primary prevention was considered in the presence of any of the following events: moderate systolic impairment; frequent ventricular ectopic beats; and/or couplets on

Holter monitoring despite betablocker administration. 2 siblings (A.IV.6 and A.IV.7) had the ICD implanted for recurrent chest pains and troponin rise anlongside with worsening of the ejection fraction. During follow-up, two patients, B III.1 and C.III.5, had an appropriate ICD shock due to S-VT.

12-lead ECG and SA-ECG

ECGs from 16 mutation carriers were available for analysis. 9 (56%) showed T wave inversion in the precordial and/or frontal leads. Intraventricular conduction abnormalities were present in 4 patients (LBBB in 2 patients, bi-fascicular block in 1 and non-specific conduction abnormalities in another case). A SA-ECG was performed on 14 patients (2 had died before the study and 2 were excluded due to the existence of baseline wide QRS). 6 showed late potentials (43%) **(Figure 4).**

24-hour ECG-Holter monitoring, an exercise test and ICD recordings

NS-VT was evidenced in 4 patients using 24-hour Holter monitoring. 2 additional patients had a NS-VT episode on ICD recordings, one of which had an episode of NS-VT while undergoing an exercise treadmill test. 1 patient suffered S-VT one month after ICD implantation requiring four shocks to restore sinus rhythm. Frequent ventricular ectopics occurred during the treadmill test in 2 cases. Another young carrier without ECG or MRI abnormalities presented frequent ventricular ectopics and couplets.

Electrophysiological Study (EPS)

An EPS was performed on 3 patients, 2 of which (B.I.1 and B.II.1) were prior to ICD implantation. During the procedure the proband (B.I.1) developed S-VT different from the one previously recorded during hospitalization. Her daughter (B.II.1) developed S-VT during the EPS similar to the one originally recorded. Successful ablation was achieved. However, she did suffer a relapse 4 years later and further VT ablation was required. Endocardial voltage mapping using the CARTO navigator system in this later case showed no low voltage areas.

Echocardiogram

An echocardiographic study showed global or inferoposterior hypokinesia in 11 of 18 (61%) carriers. 2 patients had severe systolic dysfunction and 5 had moderate systolic impairment. In 3 cases, LV apical trabeculae fulfilling diagnostic criteria for LVNC were present. Neither RV enlargement nor dysfunction was detected in any case.

Cardiac Magnetic Resonance

CMR was performed in 10 patients. Pathological findings were found in 8 of them. Biventricular disease was observed in 3 patients and isolated LV involvement was present in 6. Sub-epicardial myocardial fibrosis at the LV was the hallmark and it was detected in 6 patients. Fibrosis was distributed in the lateral wall in 3 cases, the inferoposterior wall in 2 and it was globally distributed 2 more patients. 5 patients were diagnosed with LVNC **(Figure 5)**.

Immunohistochemistry

An EMB was analyzed from C.III.5 during ICD implantation after cardiac arrest. Histology showed normal cardiomyocytes and no trace of either fibrofatty replacement, disarray, granuloma, inflammatory infiltrates or apoptosis. Staining for N-Cadherin, desmoplakin, plakoglobin, Connexin 43 and desmin were normal and no differences when comparing the case with a control was observed **(Figure 6)**.

DISCUSSION

To date, 42 *DSP* mutations leading to premature termination of translation have been reported. There is clinical information available on 120 carriers from 44 families. Of these, 86 (72%) are affected, with 18 (15%) SCD cases reported in carriers of one of these mutations. In the 14 families where clinical cascade screening was possible, disease penetrance was > 50% in ten families (71%) and >90% in seven (50%) **(Figure 7)**.

Herein we present the largest series of carriers of the same *DSP* truncating mutation reported to date. Microsatellite study suggests *DSP p.Q447** to have a founder effect. Several ARVC and/or ALVC families bearing truncating *DSP* mutations have been published, some of them, suffering SCD as a first clinical manifestation.[11-13] These

studies have reported highly-penetrant disease phenotypes, with predominant LV involvement, a high incidence of malignant arrhythmias and a poor prognosis. In keeping with these studies, the novel mutation herein presented is also associated with high disease penetrance (83%), LV dysfunction, extensive fibrosis, mild/no LV enlargement, left/anterior precordial lead repolarisation abnormalities and a high prevalence of ventricular arrhythmias. It is notable that myocardial fibrosis was present even in young carriers (14 and 19 years old). Such extensive fibrosis at an early age is an uncommon finding in cardiomyopathies and particularly rare in heterozygous carriers of autosomal dominant mutations underlying ACM.

Characterizing a phenotype using an ECG

60% of the patients showed ECG abnormalities, even in the absence of overt structural disease. New diagnostic criteria for the diagnosis of ARVC include T wave inversion in V4-V6 as a marker of LV involvement. In the light of our results and those previously reported in other series [(11)] we would like to point out that T-wave inversion in inferior leads may be sensitive markers of ALVC even in the absence of ventricular remodelling. SA-ECG findings failed to correlate with ECG or imaging abnormalities.

Characterizing a phenotype with CMR

Heterogeneous myocardial fibrosis pattern evidenced by means of gadolinium on CMR has been associated with progression to heart failure in patients with hypertrophic cardiomyopathy, and, additionally, ventricular arrhythmias have been reported to be more frequent in patients with LGE in ischemic and idiopathic DCM.[(14)] The vast majority of the *DSP p.Q447** carriers showed extensive fibrosis in the LV, particularly at the sub-epicardium, similar to that seen in localized myocarditis. During a "hot phase", ACM may mimic myocarditis in its clinical presentation or CMR appearance.[(2, 15)] In our series, CMR was performed on clinically stable patients. Despite morphological findings being compatible with the diagnosis of LVNC in 5 patients, the presence of a truncating mutation in *DSP*, arrhythmic burden being out of proportion with the degree of systolic dysfunction and the family history led us to the diagnosis of arrhythmogenic cardiomyopathy.

Implications for prevention of malignant arrhythmias and sudden death

Identifying individuals at high risk of SCD is of undisputed importance. Whether ALVC is associated with a poorer prognosis than ARVC or DCM remains a matter of debate. In terms of risk stratification in ARVC, several factors have been proposed over the past few years, the presence of which would prompt ICD implantation. These include resuscitated cardiac arrest, syncope, recurrent or syncopal S-VT, and severe RV or LV involvement.[16, 17] Other markers such as family history of sudden death, induction of VT upon EPS, extensive bipolar low voltage area in RV [18], fragmented potentials within the scar[19], T wave inversion in ≥3 leads [20] and dispersion of depolarization and repolarisation have been also reported.[21, 22] On the other hand, primary ICD implantation in DCM is based almost exclusively on the severity of impairment of LV ejection fraction and functional status.[23] The decision on ICD implantation in ALVC poses a real challenge. To date, there is no general consensus in support of the use of genetic studies for ICD implantation. There is, however, no doubt that some genetic conditions such as laminopathies are associated with a high incidence of arrhythmic events and SCD, which may precede severe myocardial remodelling and dysfunction.

In recent years, the classic concept or ARVC has been broadened to the general concept of ACM. We have found a high prevalence of a LVNC-like phenotype in our families, leading us to recommend screening for *DSP* mutations in LVNC patients affected by a high burden of personal and familial arrhythmia. In this particular scenario, LVNC may be another phenotype to be considered in the broad spectrum of ACM.

Desmoplakin *p.Q447: an unexpected immunohistochemistry pattern**

Through its N-terminus, desmoplakin associates with armadillo proteins at the cell membrane. Through its C-terminus, it anchors intermediate filaments tethering them to junctional sites. Herein, we describe a novel truncating mutation in the N-terminal domain, anticipated to disrupt the synthesis of desmoplakin

Confocal microscopy revealed no difference in the expression of desmoplakin, plakoglobin, connexine-43 and desmin compared to the control. It is notable that the

hallmark in ARVC immunohistochemistry; plakoglobin shifting from junctions [24] was not present. This finding is in keeping with a series of phospholamban mutation carriers where the plakogobin distribution significantly differed in the DCM phenotype compared to the ARVC phenotype.[5]

This difference in plakoglobin remodelling leads us to raise the question whether the signalling pathways differs in ALVC and ARVC. Further studies will be required to shed light on the pathophysiology of this particular phenotype.

CONCLUSION

We have reported on a novel mutation in desmoplakin *(DSP p.Q447**) associated with a phenotype of ALVC and LVNC. Truncating mutations in desmoplakin seem to consistently cause extensive LV fibrosis with near normal RV performance. Ventricular arrhythmias and SCD occur in the absence of overt LV dysfunction or dilatation, and as a consequence, ICD implantation must be considered promptly. Genetic information seems to be of paramount prognostic value in this setting.

ACKNOWLEDGEMENTS

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FIGURES

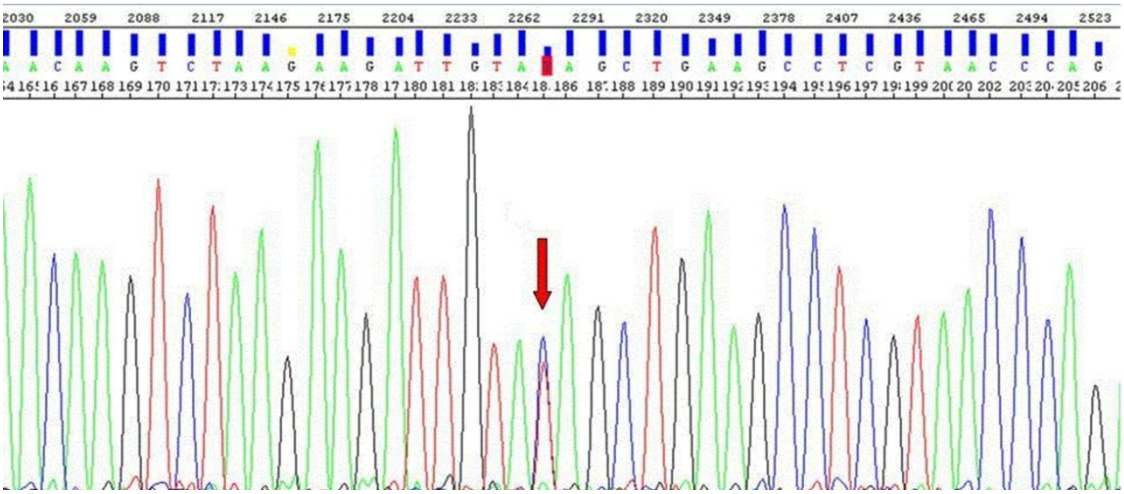

Figure 1. Electropherogram. The mutation was a C -> T transition at exon 11 in the *DSP* gene leading to a premature stop codon.

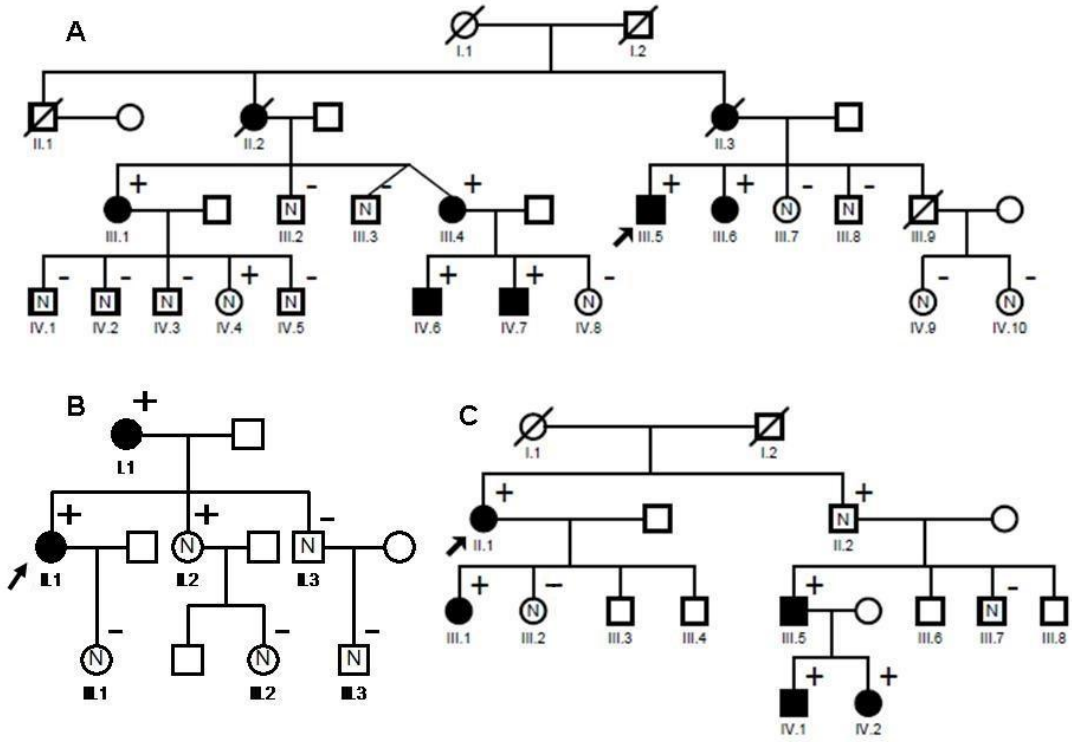

Figure 2. Family tree of families A, B and C. Arrow points to probands. Black filled symbols stand for affected carriers.

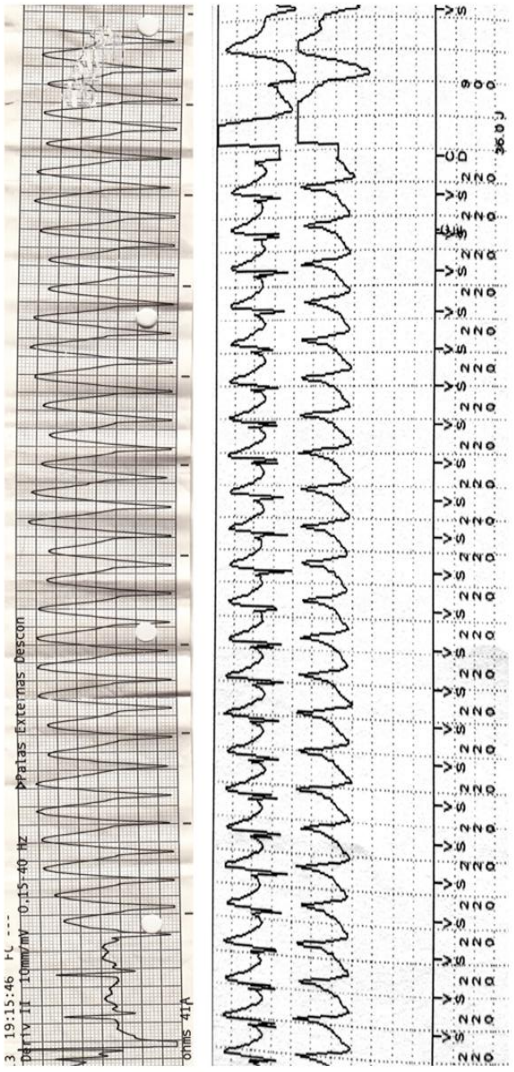

Figure 3. S-VT recorded at the emergency department during resuscitation maneuvers (top). ICD recordings showing S-VT and the beginning of another S-VT of a different morphology after shock delivery (bottom). Four shocks were necessary to restore sinus rhythm (C.III.5).

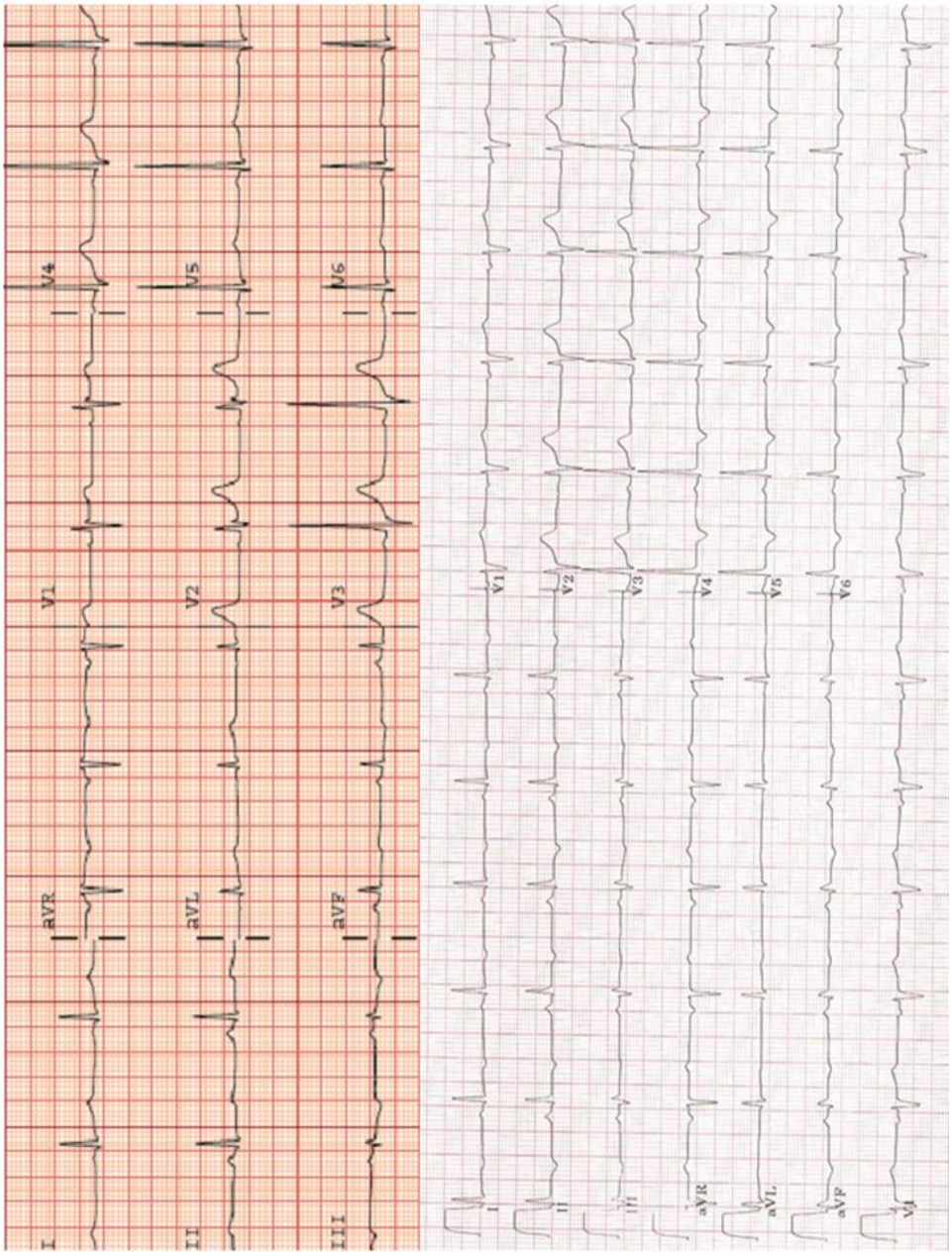

Figure 4.ECG from a LVNC patient (Top)(C.III.5) and a DCM patient (Bottom) (A.IV.7) T wave inversion in inferolateral leads was the most common abnormality in ECG.

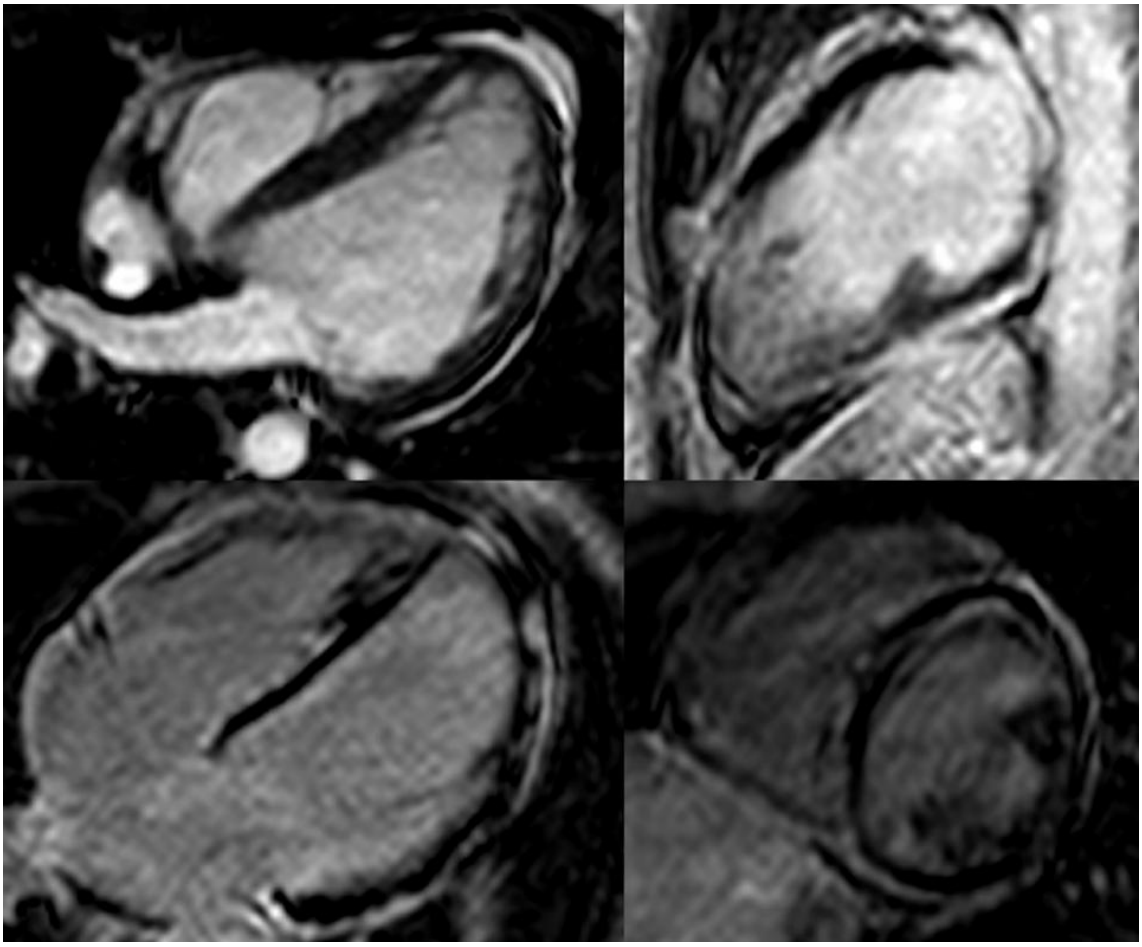

Figure 5. CMR from LVNC (C.III.5) (top) and DCM (A.IV.7) (bottom). Extensive LGE is observed.

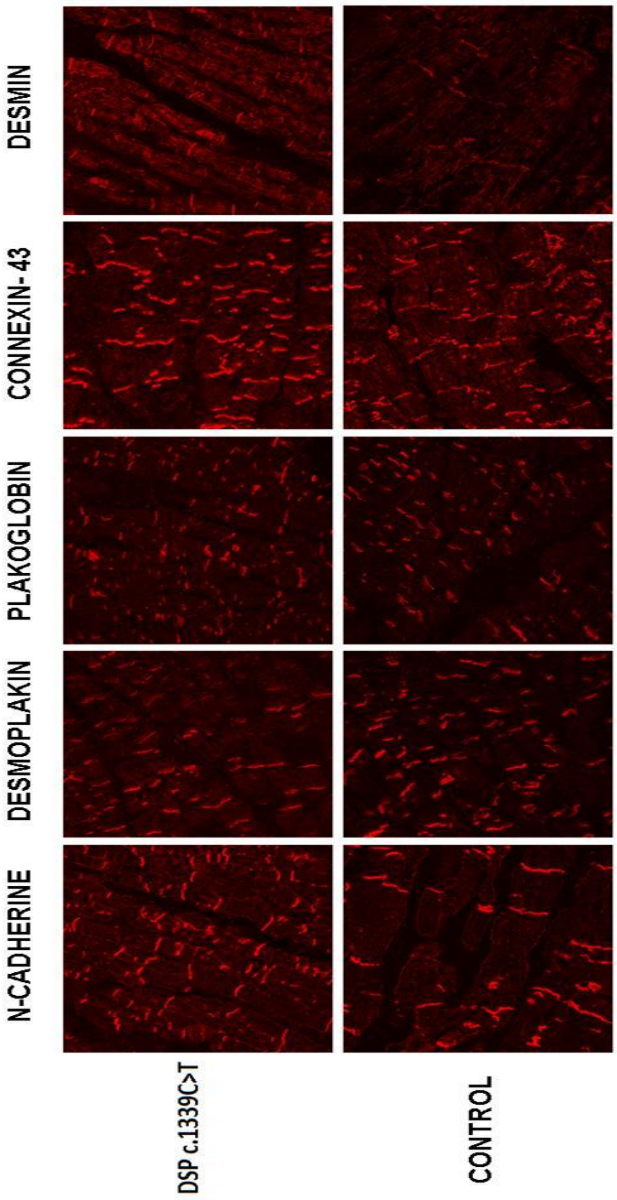

Figure 6. Immunohistochemistry from an EMB. No difference in the distribution of desmoplakin present in *DSP p.Q447** compared to the control. Plakoglobin, connexin-43 and desmin also shows an identical distribution

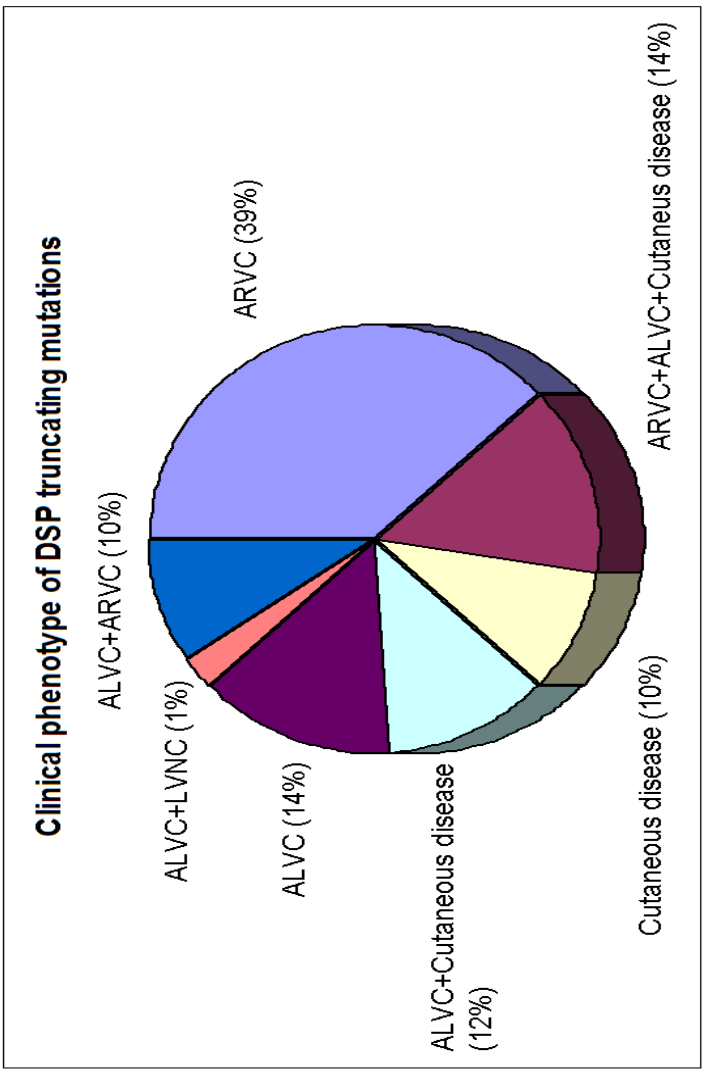

Figure 7. Clinical phenotypes of the 42 *DSP* truncating mutations reported to date. + indicates overlap syndrome.

TABLE

Case	Family	Pedigree	Age/ Sex	Syncope	Sustained VT	Morphology VT	CL (ms)	Non sustained VT	NYHA	ARVC diagnosis	LVEF	LGE distribution	Hypertrabeculation (CMR)
1	A	II.2	59F	Yes	Yes	RBBB	330	Yes	I	Borderline	40	NA	NA
2	A	II.3	65F	No	No	NA	No	No	II	Possible	40	NA	NA
3	A	III.1	55F	No	Yes	NA	NA	No	I	Definitive	64	NA	NA
4	A	III.6	45F	No	No	No	No	No	I	Definitive	40	Inferoposterior and lateral	Yes
5	A	III.4	44F	No	No	NA	NA	Yes	I	Borderline	50	Global	Yes
6	**A**	**III.5**	**36M**	**No**	**Yes**	**NA**	**NA**	**Yes**	**I**	**Definitive**	**30**	**NA**	**NA**
7	A	IV.4	38F	No	No	No	No	No	I	Possible	60	No	NA
8	A	IV.6	14M	No	No	No	No	No	I	Possible	45	Lateral	No
9	A	IV.7	19M	No	No	No	No	No	I	Possible	55	Lateral	No
10	B	I.1	72F	No	Yes	LBBB. Axis −30°	320	Yes	II	Definitive	30	NA	NA
11	B	II.2	49F	No	No	No	No	No	I	Possible	58	NA	NA
12	**B**	**II.1**	**42F**	**No**	**Yes**	**NA**	**NA**	**No**	**I**	**Definitive**	**40**	**NA**	**NA**
13	**C**	**II.1**	**62F**	**No**	**Yes**	**NA**	**NA**	**Yes**	**II**	**Definitive**	**43**	**Inferoposterior and lateral**	**No**
14	C	III.5	45M	No	Yes	NA	220	No	I	Definitive	42	Global	Yes
15	C	II.2	73M	No	No	No	No	No	I	Borderline	60	No	No
16	C	III.1	38F	No	No	No	No	No	I	Borderline	60	No	No
17	C	IV.1	13M	No	No	No	No	No	I	Borderline	66	No	Yes
18	C	IV.2	17F	No	No	No	No	No	I	Borderline	60	No	Yes

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A MUTATION IN THE Z-LINE CYPHER/ZASP PROTEIN IS ASSOCIATED WITH ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY

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ABSTRACT

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an important cause of malignant arrhythmia and sudden death particularly in young people. Although it is considered a desmosomal disease, mutations in non-desmosomal genes have also been identified. We report on a family where a mutation in *LDB3* is associated with this condition. The index case and first and second degree relatives underwent a complete clinical evaluation: physical examination, ECG, signal-averaged ECG, 2D echocardiogram, cardiac magnetic resonance and 24- hour monitoring. After ruling out mutations in the 5 desmosomal genes, genetic testing by means of Next Generation Sequencing was carried out on the proband. A heterozygous missense mutation in *LDB3 (p.T351A)* was identified. This result was confirmed by subsequent Sanger sequencing. Another 6 carriers were identified amongst her relatives. Three subjects fulfilled the criteria for a definitive diagnosis of ARVC and one reached a borderline diagnosis. In conclusion, we report on the first family with ARVC where a mutation in LDB3 is associated with ARVC. Due to the modest yielding through conventional genetic testing in this cardiomyopathy, the use of next generation sequencing has arisen as a particularly useful tool to point to new causative genes.

Key words: Arrhythmogenic right ventricular cardiomyopathy; Cypher/ZASP; LDB3; Next Generation Sequencing

MANUSCRIPT

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a familiar cardiomyopathy inherited in a dominant trait which is characterized by a high incidence of ventricular arrhythmia and sudden death, particularly amongst young patients and athletes[1]. In some cases, sudden death is the first clinical presentation and it is usually diagnosed in the post-mortem study. ARVC not only affects the right ventricle (RV) but also the left ventricle (LV) even as a primary target. Despite being initially considered as a merely desmosomal disease caused by mutations in any of the five desmosomal genes, recent investigations have reported that other non-desmosomal genes (such as *TMEM43*[2], *PLN*[3], *DES*[4], *LMNA A/C*[5], *TTN*[6] and *CTNNA3*[7], *TGF-β3*[8] and *RyR2*[9]) are also involved in the development of ARVC. Identification of a causative heterozigous mutation is only reached in the order of 50% of the cases [10, 11].

Herein we report on a family where a mutation in *LDB3* (Lim-domain binding protein 3) is linked to the condition.

MATERIAL AND METHODS

The proband and the first and second degree relatives keen to be screened underwent a complete clinical evaluation which consisted of a 12- lead digital ECG, a signal averaged ECG (SA-ECG), a 2D-doppler echocardiogram, cardiac magnetic resonance (CMR), an exercise test and 24-hour Holter. Genetic testing was approved by the Ethics Committee of our hospital. All participants gave their written consent. DNA was extracted from peripheral blood samples using the Maxwell 16 Blood Purification Kit. Sanger DNA sequencing failed to find a disease-causing mutation in any of the 5 desmosomal genes in the proband (*DSP, JUP, DSC-2, DSG-2, PKP-2*). We performed Next Generation Sequencing (NGS) with a selection of 134 related and non-related genes in order to discover another hypothetical disease-causing gene **(Supplemental material**). These genes are included in a commercial Inherited Cardiac Disease library. Pathogenicity of the identified variants was assessed by means of three softwares (Polyphen-2, Polymorphism Phenotyping and Sorting Intolerant From Tolerant) and genotype-phenotype correlation.

RESULTS

A missense heterozygous mutation (*c.1051A>G*) was identified in *LDB3*. Sanger DNA sequencing was used to confirm the presence of this variant in the proband. Bidirectional sequencing was performed using BigDye v1.1 chemistry in an ABI3130 analyzer (Applied Biosistems, Foster City, CA). The sequence of the patient was compared with the *LDB3* sequence in the National Center for Biotechnology Information (NCBI) sequence database (NM_001080114.1). We aimed to assess its segregation within the family as well as its genotype-phenotype correlation.

The proband was a caucasian 45-year-old female with a long-standing medical history of presyncope. She required medical attention after suffering a syncopal sustained ventricular tachycardia that required electrical cardioversion to restore sinus rhythm. Her ECG showed complete right bundle branch block (RBBB) and inverted and flat T waves in precordial leads. An echocardiogram and CMR showed biventricular dilatation as well as severe biventricular systolic dysfunction. Midwall late gadolinium enhancement (LGE) affecting the LV was also identified. In the view of these findings, a definitive diagnosis of ARVC was made. An angiogram ruled out coronary artery disease and an implantable cardioverter defibrillator (ICD) was recommended. Tragically, she died in the operating theatre during the surgical intervention as a result of an anaesthetic complication. The post mortem examination revealed extensive fibro-fatty replacement in the RV, extensive fibrosis in the LV and limited inflammatory patches. There were neither signs of superimposed acute myocarditis in keeping with a "hot phase".

Cascade screening identified another six carriers (four women; median age 47 years) of *LDB3 p.T351A*. Having not considered the carrier status as a major criterion for the diagnosis of ARVC, the clinical work-up led to a definitive diagnosis in two carriers (brother and sister) and a borderline diagnosis in one niece who also carried the mutation **(Figure 1 and Table 1).**

A 43-year old sister (II.6) complained of frequent palpitations. Her ECG showed inverted T waves in right precordial and inferior leads and the SA- ECG showed late potentials. Several 24-hour holter monitoring showed a run of idioventricular rhythm and 100 ventricular ectopic beats per day. The CMR revealed a dilated RV with severe

systolic dysfunction and normal LV. No LGE was observed. She was started on betablockers.

The 57-year-old brother (II.3) was asymptomatic. His ECG did not show repolarisation abnormalities and his SA-ECG showed late potentials. The CMR revealed a hypokinetic RV free-wall and moderate systolic dysfunction. The LV was normal and there was no LGE.

A 17-year-old niece (III.11) was also found to be a carrier. Although in the first evaluation she was asymptomatic and her ECG, SA-ECG, echocardiogram, CMR and holter failed to show any sign of the condition, she developed inverted T waves in precordial leads 2 years later. Therefore a borderline diagnosis of ARVC was accomplished.

The 59 years-old sister (II.5) was asymptomatic. Cardiac evaluation was normal although she developed late potentials in the SA-ECG during the follow up.

The 16-year old proband´s daughter (III.2) and the 54 year-old brother (II.2) were asymptomatic and all tests were normal. None of the wild-type relatives presented signs in suggestive of ARVC.

DISCUSION

Despite the fact that ARVC is a disease caused by disruptions to the desmosomes and that mutations in desmosomal genes are identified in 50% of patients, the physiopathology of this condition remains unclear.

Recent studies support a more holistic approach to the condition in which not only desmosomes but GAP junctions, ion channels, ion currents and area composita proteins have been found to be involved in the development of ARVC and arrhythmogenesis. [7, 12]

Although a particular type of ARVC associated with myofibrillar myopathy and conduction disturbances is linked to the chromosome 10q (the locus where *LDB3* is located), no mutation in any of the candidate genes proposed was identified.[13]

For the first time we identified a mutation in *LDB3* in a family suffering ARVC.

LDB3 encodes the ZASP protein in humans (homologous of the Cypher protein in mice). It is a cardiac and skeletal protein expressed in the cytoplasm and it plays an essential role stabilizing the Z-line. Alternative splicing of the protein leads to multiple

isoforms, with the PDZ domain being common to all of them (it interacts with the last 150 amino acids of α-actinin), whereas the ZM domain and the C-terminal LIM are isoform-specific.[14-16] Previous studies have shown that the suppression of Cypher in mice is associated with DCM alongside with death due to heart failure and sudden arrhythmic death. Notably, in this knockout model, there was an overt cardiac dilatation of the RV. [17]Sudden death may occur in the setting of missense mutation in *LDB3* even in the absence of apparent cardiomyopathy.[18] Other study recapitulated a DCM phenotype in a transgenic mouse model (*S196L*). Not only ECG abnormalities may be present at an early stage but disturbances in calcium and sodium currents and electrical abnormalities are also present (14). Although the evidence supported by genotype-phenotype studies is very limited, there is some data on mutations in exon 4 causing left ventricular non-compaction (LVNC) and mutations in exon 6 causing mixed phenotypes (LVNC and DCM).[18] Other mutations in LDB3 have been also associated with autosomal dominant dystrophy [19] and hypertrophic cardiomyopathy.[20]

LDB3 p.T351A is localized in the Exon 7. The frequency of this genetic variant could be very low in general population (less than 0.5%) according to the Exome Variant Server, were *c.1051A>G* was identified to date in 5 out of 8595 alleles from European-American individuals and in none of 4406 alleles from Afro-American subjects (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/).

LDB3 p.T351A affects a moderately conserved residue and the change of a polar amino acid (T) for a polar amino acid (A) is predicted to modify the polarity, mass and hydrophobicity of the protein, although Polyphen-2, PMut and SIFT predicts a tolerate protein function.

This mutation has been previously identified in patients with DCM and LVNC which led to its inclusion in a DCM genetic testing panel. [21, 22]

Amongst the carriers reported in the family herein described, 3 fulfilled criteria for a definitive diagnosis of ARVC and 1 reached a borderline diagnosis. The penetrance of the condition was 4/7 carriers. The phenotype ranged from severe biventricular systolic dysfunction with RV aneurysms in the proband to repolarisation abnormalities with normal CMR in the youngest case. There was no evidence of skeletal muscular disease in any carrier and there was no identifiable “red flags” in any patient as

previously reported in ARVC associated to other non desmosomal genes (eg: atrial arrhythmias and conduction disturbances in lamin A/C mutation carriers.[5] CK levels were normal in all cases. Serial ECG and holter monitoring did not now show AV block or pathological pauses in any carrier. [13]

The present findings highlight the complexity of the genetic basis of the disease since a mutation in a new non-desmosomal gene emerges as a cause of ARVC. Further studies will be required to clarify the causative relationship between *LDB3* mutations and ARVC.

In view of the discrete yield of genetic testing in ARVC, the implementation of

NGS not only in research but in clinical practice has arisen as a new tool for the identification of candidate genes associated with the disease. Having said that, and in order to avoid any confusion due to "genetic noise", unnecessary medical tests, and emotional stress in patients and relatives, these results should ideally be validated by functional studies, animal models and a detailed family cascade screening to assess its co- segregation.

In conclusion, for the first time we have reported on a mutation in *LDB3* which causes ARVC. This finding highlights the blurry boundary that exists not only in the phenotype but also in the genetic basis of different cardiomyopathies.

FIGURES

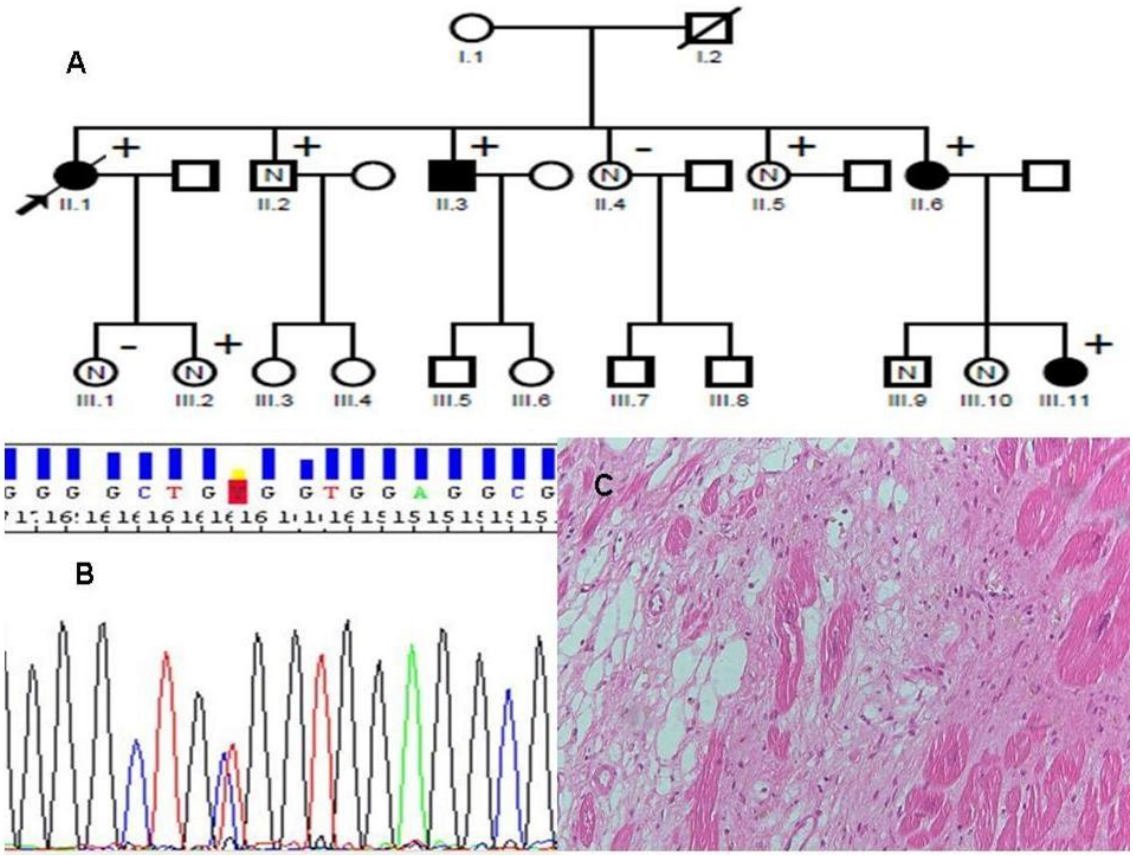

Figure 1. A: Family tree. B: Electropherogram: double peak at the site of the missense mutation. C: Hematoxilin-eosin (20X) from the RV (post-mortem of the proband, which demonstrated widespread fibrofatty replacement.

TABLES

Number	Pedigree ID	Sex	Age at diagnosis	ARVC criteria (TFC 2010)	Symptoms	ECG	SA-ECG	CMR	VT	Ventricular ecopics
1	II.1	Female	45	Definitive	Syncope	RBBB+ITW	NA	Biventricular dilatation and failure	Yes	>500
2	II.6	Female	43	Definitive	Palpitations	ITW inferior and RPL	3/3	RV dilatation and failure	No	<200
3	II.3	Male	57	Definitive	No	Incomplete RBBB	3/3	RV dilatation and failure	No	<200
4	III.11	Female	17	Definitive	No	IWT in RPL	0/3	Normal	No	<200
5	II.2	Male	54	Posible	No	Normal	0/3	Normal	No	<200
6	II.5	Female	59	Borderline	No	Normal	2/3	Normal	No	<200
7	III.2	Female	16	Posible	No	Normal	0/3	Normal	No	>200

Table 1: Clinical features of *LDB3 p.T351A* carriers. NA: not available. ITW: inverted T waves.

Cromosome	Gene	Cromosome	Gene	Cromosome	Gene	Cromosome	Gene
chr12	ABCC9	chr6	EYA4	chr11	KCNQ1	chr3	RAF1
chr10	ACTA2	chr15	FBN1	chr20	KCNQ2	chr17	RANGRF
chr15	ACTC1	chr5	FBN2	chr12	KRAS	chr10	RBM20
chr1	ACTN2	chrX	FHL1	chr6	LAMA4	chr1	RYR2
chr10	ADRB1	chr2	FHL2	chrX	LAMP2	chr19	SCN1B
chr5	ADRB2	chr9	FKTN	chr10	LDB3	chr11	SCN2B
chr8	ADRB3	chr7	FLNC	chr19	LDLR	chr11	SCN3B
chr1	AGL	chr9	FXN	chr1	LMNA	chr11	SCN4B
chr7	AKAP9	chr17	GAA	chr12	LRP6	chr3	SCN5A
chr4	ANK2	chr8	GATA4	chr15	MAP2K1	chr16	SCNN1B
chr10	ANKRD1	chr6	GJA1	chr19	MAP2K2	chr16	SCNN1G
chr2	APOB	chr1	GJA5	chr11	MYBPC3	chr5	SGCD
chr10	BAG3	chrX	GLA	chr14	MYH6	chr10	SHOC2
chr2	BMPR2	chr3	GPD1L	chr14	MYH7	chr4	SLC25A4
chr7	BRAF	chr5	HCN1	chr12	MYL2	chr20	SNTA1
chr9	CACNA1B	chr15	HCN4	chr3	MYL3	chr2	SOS1
chr12	CACNA1C	chr11	HRAS	chr20	MYLK2	chrX	TAZ
chr3	CACNA1D	chr20	JAG1	chr5	MYOT	chr7	TBX20
chr7	CACNA2D1	chr20	JPH2	chr4	MYOZ2	chr17	TCAP
chr10	CACNB2	chr17	JUP	chr10	MYPN	chr14	TGFB3
chr19	CALR3	chr12	KCNA5	chr1	NEXN	chr9	TGFBR1
chr1	CASQ2	chr1	KCND3	chr5	NKX2-5	chr3	TGFBR2
chr3	CAV3	chr21	KCNE1	chr1	NPPA	chr1	TGFBR3
chr11	CRYAB	chrX	KCNE1L	chr1	NRAS	chr3	TMEM43
chr11	CSRP3	chr21	KCNE2	chr4	PDLIM3	chr12	TMPO
chr16	CTF1	chr11	KCNE3	chr12	PKP2	chr3	TNNC1
chr2	DES	chr2	KCNE4	chr2	PKP4	chr19	TNNI3
chrX	DMD	chr7	KCNH2	chr8	PLEC	chr1	TNNT2
chr18	DSC2	chr11	KCNJ11	chr6	PLN	chr15	TPM1
chr18	DSG2	chr17	KCNJ12	chr14	PNN	chr2	TTN
chr6	DSP	chr17	KCNJ2	chr7	PRKAG2	chr18	TTR
chr18	DTNA	chr2	KCNJ3	chr14	PSEN1	chr10	VCL
chr7	ELN	chr11	KCNJ5	chr1	PSEN2		
chrX	EMD	chr12	KCNJ8	chr12	PTPN11		

Table 1. List of 134 candidate genes included in NGS.

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PHOSPHOLAMBAN P.ARG14DEL MUTATION IN A SPANISH FAMILY WITH ARRHYTHMOGENIC CARDIOMYOPATHY: EVIDENCE FOR A EUROPEAN FOUNDER MUTATION

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Abbreviations:

ARVC: Arrhythmogenic right ventricular cardiomyopathy

ACM: Arrhythmogenic cardiomyopathy

CMR: Cardiac magnetic resonance

DCM: Dilated cardiomyopathy

ICD: Implantable defibrillator

LGE: late gadolinium enhancement

ABSTRACT

INTRODUCTION AND AIMS

The mutation phospholamban *(PLN) p.R14del* causes arrhythmogenic cardiomyopathy as well as dilated cardiomyopathy, being this mutation endemic in The Netherlands. The aim of this study was two-fold: 1) to analyse the phenotype of a Spanish family which carries this mutation, 2) to study the founder effect of the mutation.

METHODS

12 probands of arrhythmogenic cardiomyopathy families with no causal mutation identified in any of the 5 desmosomal genes, *TMEM43* and *LMNA* underwent *PLN* sequencing.

Clinical work-up consisted of physical examination, ECG, signal averaged-ECG, 2D echocardiomgram, exercise test, 24-hour Holter and cardiac magnetic resonance. Haplotype analyses for the region surrounding *PLN* was carried out to evaluate a founder effect of the mutation.

RESULTS

PLN p.R14del was identified in a 27 years old lady and in 7 relatives (6 females, 2 males median 45 [23-55] years). The penetrance was 6/8 (75%) and a variable phenotype (ranging from aggressive forms: severe biventricular impairment and arrhythmias, arrhythmogenic right ventricular cardiomyopathy with ventricular tachycardia, to mild phenotypes: poor R wave progression). Poor R wave progression and low voltages were a “red flag” (6/8). Spanish patients shared 4 out of 5 markers from the shared Dutch haplotype suggesting a common founder ancestor.

CONCLUSION

PLN p.R14del is a European founder mutation which causes both arrhythmogenic cardiomyopathy and biventricular dysfunction. It shows a typical electrocardiographic pattern.

Key words: Phospholamban; Arrhythmogenic cardiomyopathy; Dilated cardiomyopathy, ECG, Founder effect.

MANUSCRIPT

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a common cause of sudden death particularly in young people and athletes.[1] This condition poses a diagnostic challenge for the clinician, especially in the setting of mild phenotypes and in the absence of a family history. Moreover, it usually overlaps with other cardiomyopathies (dilated cardiomyopathy (DCM), cardiac sarcoid, myocarditis, etc) and channelopathies, particularly with Brugada syndrome. [2]

But the overlap between ARVC and DCM exists not only in clinical practice but also in their underlying genetic basis.[3] Mutations in the nuclear protein *LMNA A/C* are an example of this overlap, since it causes DCM (typically associated with conduction disease and arrhythmias) [4] as well as ARVC. [5]

Phospholamban is an inhibitor of the sarcoplasmic calcium pump (SERCA2a). On phosphorylation, it separates from SERCA2a activating the calcium pump, which regulates contractility and relaxation. Mutations in its gene, *PLN,* have been hitherto associated with aggressive phenotypes of both DCM and ARVC. [6, 7]

Herein we report on a family diagnosed with arrhythmogenic cardiomyopathy (ACM) with some peculiar features, carrying a Dutch founder mutation in phospholamban (*PLN* c.*40_42delAGA; p.R14del*). The genotype-phenotype correlation allowed the identification of some red flags which should draw attention in the clinical work-up.

METHODS

Patients and clinical work-up

The study was approved by the Ethics Committee of our hospital (Virgen de la Arrixaca University Hospital, Murcia) and it complied with the ethical principles of the Declaration of Helsinki. All participants gave their written consent. A genealogical pedigree of the family was obtained. Clinical assessment consisted of a 12- lead digital ECG and signal averaged ECG (SA-ECG), a 2D-doppler echocardiogram, exercise test and 24-hour Holter monitoring. Cardiac magnetic resonance (CMR) was performed in seven mutation carriers. SSFP end-expiratory breath-hold cine imaging was acquired. After a bolus injection of Gadobutrol (0.1 mmol/kg, Gadovist; Bayer Shering Pharma,

Berlin, Germany) the T1 measurement was calculated using standard late gadolinium enhancement (LGE) sequences. The analysis was performed using a personal computer and semi-automated software (Philips, Software work space 2.6.3.2)

Since mutations *PLN* are associated with DCM and ARVC, we aimed to evaluate both diagnosis within the family.

1.The presence of a LV end-diastolic diameter (LVEDd) of >117% from normal and/or LV systolic impairment (<45%) were used for the diagnosis of DCM in the proband and familial criteria were applied in the carriers.[8] RV diameters were evaluated in short and long axis views in echocardiogram. RV performance was assessed by TAPSE, tricuspid annulus DTI as well as fractional area change.

2. ARVC diagnosis was made on the grounds of the revised Task Force Criteria (TFC 2010).[9]

Patients were followed up annually (median 72 [67-77] months).

Genetics

DNA was extracted from peripheral blood samples using the Maxwell 16 Blood Purification Kit. 12 unrelated probands with ACM with negative genetic study of the desmosomal genes (*PKP2*, *DSG2*, *DSC2*, *DSP*, *JUP*) and *TMEM43* and *LMNA*, were sequenced for *PLN (*published primer sequences were used for amplification: Ex2 *PLN* forward, TCAGACTTCCTGTCCTGCTG;Ex2 *PLN* reverse , TAAGCTGATGTGGCAAGCTG).

Haplotype analyses for the region surrounding *PLN* have been described elsewhere. [7,10]

RESULTS

Genetic testing led to the identification of eight *PLN p.R14del* carriers from one family:

1. The proband (III.2, **Figure 1**) was a 28 years old lady with a past medical history of vasovagal presyncopes. She was admitted in emergency department after collapsing while standing up. The clinical examination was unremarkable but her ECG showed QS inferiorly-and striking low voltages throughout all leads **(Figure 2A and 2B)**.

Echocardiogram showed a non-dilated left ventricle (LVEDd 48mm, 108% of predicted) with global systolic impairment and a calculated ejection fraction of 40%. Cardiac magnetic resonance (CMR) revealed global left ventricular hypokinesia (LVEF 35%),

more prominent at the apex. Right ventricle was not dilated and had global normal systolic (RVEF 51%) function although the apex was remarkably hypokinetic. Gadolinium sequences did not show late enhancement (LGE) **(Figure 3)**.

Blood tests were unremarkable (including ferritin, CK, troponin and thyroid hormones). Coronary CT scan showed normal coronaries. Tilt test was positive and reproduced the symptoms that led to consultation. 24 hours ECG-Holter monitor was normal. She was discharged on betablockers ACEI and spironolactone.

She was re-admitted for a new collapse three months thereafter. During that admission a sustained ventricular tachycardia (S-VT) **(Figure 2B)** was documented which prompted to the implantation of an implantable defibrillator (ICD). Two years later, she presented a S-VT that required ICD therapy (ATP). Sotalol was started at that point but few months thereafter, she collapsed due to a S-VT that required an ICD shock. Echocardiogram showed a severe biventricular systolic dysfunction (extensive apical akynesia, LVEF: 35%, tricuspid DTI 7 cm/s, TAPSE 15 mm and fractional shortening area 14%). She remained in NYHA I functional class.

Genetic analysis ruled out pathogenic mutations in the five desmosomal genes, *LMNA A/C* and *TMEM43*. However, a pathogenic mutation in *PLN*, *p.R14del* was identified.

Cascade family screening led to the identification of 7 additional carriers of the *PLN p.R14del* mutation in the maternal side **(Figure 1)**. There was no other relevant family history and no evidence of a sudden death cases in the family.

2. An asymptomatic 25 years old brother (III.1, Figure 1) had underwent sternal surgery for pectum excavatum. From a cardiovascular standpoint he was asymptomatic although he presented frequent (>1000/24 hours) ventricular ectopics which persisted throughout the exercise test. His ECG showed late R wave transition in precordial leads. Although the echocardiogram was normal, CMR revealed a hypokinetic RV apex and a subepicardic LGE patch in the lateral wall of left ventricle **(Figure 4)**. Biventricular systolic function was normal. One year later, he was admitted because of palpitations. Recurrent episodes of non -sustained VT (15-20 beats) were recorded. Betablocker was started and he had an ICD implanted for primary prevention.

3. Her 52 years old mother (II.1, Figure 1) was asymptomatic. Her ECG showed late R transition in the precordial leads. Echocardiogram and CMR were normal.

4. The maternal grandmother (I.1, Figure 1) was diagnosed incidentally at the age of 74 in a preoperative cardiac evaluation. Her ECG demonstrated negative T waves in inferior and lateral leads. The echocardiogram showed moderate LV systolic impairment (LVEF 45%) with normal size LV diameters (EDD 49 mm, 109% of predicted). Exercise echocardiogram was negative for ischaemia and Holter monitoring failed to demonstrate any arrhythmia. She had a good response to betablockers and ACEI with LVEF normalization.

5. Three maternal aunts (II.2, II.3, II.4, Figure 1) all in their fifties were found to carry *PLN R.14del*. Their ECG showed poor R waves progression and flat T waves throughout. Echocardiograms as well as CMR were normal in two. The other aunt was diagnosed with rheumatic mitral stenosis.

6. A 16 years old cousin (III.3, Figure 1) was asymptomatic and the clinical work-up did not show any abnormality.

None of the Holter performed showed ventricular arrhythmias in the relatives (apart from the proband and her brother). Exercise test failed to trigger ventricular arrhythmia is all cases. LGE was identified only in one case. SA-ECG was performed in 6 patients and it failed to show late potentials (0/3) in all cases. None of the patients had ventricular dilatation. Due to the status of being a carrier, the morphology of ventricular tachycardia and ECG abnormalities, 2 patients met criteria for definitive ARVC whilst 3 reached a borderline diagnosis.

Haplotype analyses of markers around *PLN*, in 2 affected Spanish *PLN* mutation carriers were compared to the Dutch series. [7,10] Interestingly, Spanish patients shared 4 out of 5 markers from the shared Dutch haplotype suggesting a common founder ancestor **(Table 1)**.

DISCUSSION

Mutations in *PLN* (particularly *p.R14del*) have been identified in severe forms of DCM, leading to severe dysfunction requiring heart transplantation and a high incidence of sudden death [11], although less aggressive and late onset forms have been also reported. [12]

PLN p.R14del accounts for 15% of the cases of DCM and 12% of the cases of ARVC in the Netherlands. [7] Interestingly, there is a remarkable overlap between phenotypes, being ventricular arrhythmias and sudden death commonplace in the DCM group and being left ventricle involvement frequently found in the ARVC group. This mutation arose 575-825 years ago, likely in the northern of the Netherlands and the emigration in the 19th and early 20th century is thought to be the reason for its identification in Germany, and the United States and Canada. [10] Because we identified a similar haplotype, a common founder may underlie the presence of this specific mutation both in Spain and the Netherlands. From a historic point of view, two possibilities for the introduction of this mutation from Spain to the Netherlands or vice versa are: the 80-years Spanish siege of the Netherlands leading to a Dutch revolt (AD 1568-1648) when thousands of Spanish soldiers resided in the low countries including the Northern parts where the mutation is believed to originate from, or the movement of several hundred Dutch male volunteers during the Spanish civil war in the thirties of last century. This latter possibility is however unlikely because the year of birth of the eldest Spanish mutation carrier **(I.1, Figure 1)** was before the start of the civil war. Interestingly, an identical *MYL2* mutation with a shared haplotype was recently identified in both Spanish and Dutch hypertrophic cardiomyopathy patients underscoring the "genetic" link between Spain and the Netherlands (personal communication, Arthur van den Wijngaard).

Herein we report on a family affected with arrhythmogenic cardiomyopathy where *PLN p.R14del* was identified as the cause of the disease. The penetrance of the disease is 6/8 (75%). Being *PLN p.R14del* a pathogenic mutation, this family is a clear example of the variability of the clinical phenotype of ACM in relatives. The clinical expression of the disease ranges from overt phenotypes, consisting of biventricular disease, systolic dysfunction and high arrhythmic burden to asymptomatic relatives where only minor ECG changes are present and no documented arrhythmia throughout the follow-up **(Table 2)**.Of note, recent evidence shows a trend for female carriers to suffer milder phenotypes when comparing to males yet malignant ventricular arrhythmias did not occur more often in the latter. [13]

Affected carriers of *PLN p.R14del* may present with an ARVC, DCM or mixed phenotype. Of note, the two siblings presenting with ventricular arrhythmias had biventricular disease without ventricular dilatation, not fulfilling criteria for the diagnosis of DCM consequently. Since Task Force Criteria for the diagnosis of ARVC includes T wave inversion in V5-6 as a minor criterion and considering the status condition and VT morphology, two patients met criteria for a definitive diagnosis and three fulfilled a borderline diagnosis.

It is notable the wide variety of ECG abnormalities found in the family: widespread low QRS voltage (even reminiscent of restrictive cardiomyopathy) in the proband; inverted T waves in inferolateral leads (typical of arrhythmogenic left ventricular cardiomyopathy); and poor R wave progression in all but one carriers **(Figure 5)**.

Despite mutations in *PLN* being associated with a widespread myocardial fibrosis in animal models and humans, CMR only identified a small area of LGE in one patient. Consequently it is worth mentioning that fibrosis in CMR does not correlate with low voltages in ECG in this family.

CMR protocols for identification homogeneous increase in interstitium might lead to identification of diffuse fibrosis in these *PLN* cases. [14]

Low voltage and poor R wave progression in ECG is the red flag that should raise suspicion on a *PLN* mutation which is paramount particularly when assessing the timing for ICD implantation which in the view of current data, should be indicated before entering a severely depressed systolic dysfunction.

CONCLUSION

PLN R.14del is a European founder mutation that causes arrhythmogenic right ventricle cardiomyopathy, dilated cardiomyopathy and or biventricular impairment. Poor R wave progression and low voltages are a hallmark which should lead to the screening of *PLN* mutations.

Acknowledgments:

We thank David Lopez Cuenca for his help in the recollection of blood samples and the pedigree design.

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FIGURES

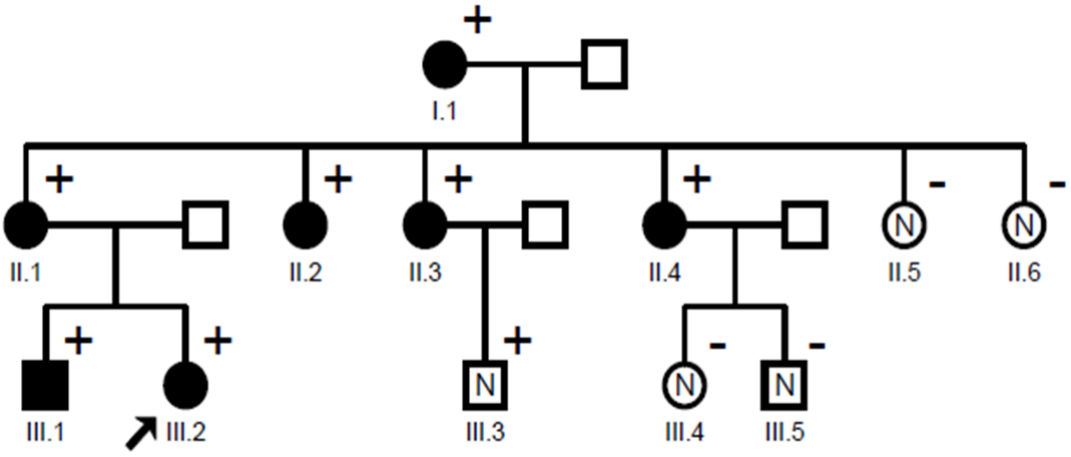

Figure 1: Family tree. The black arrow points to the proband (III.2). + stands for carriers. Filled black figures stand for affected carriers.

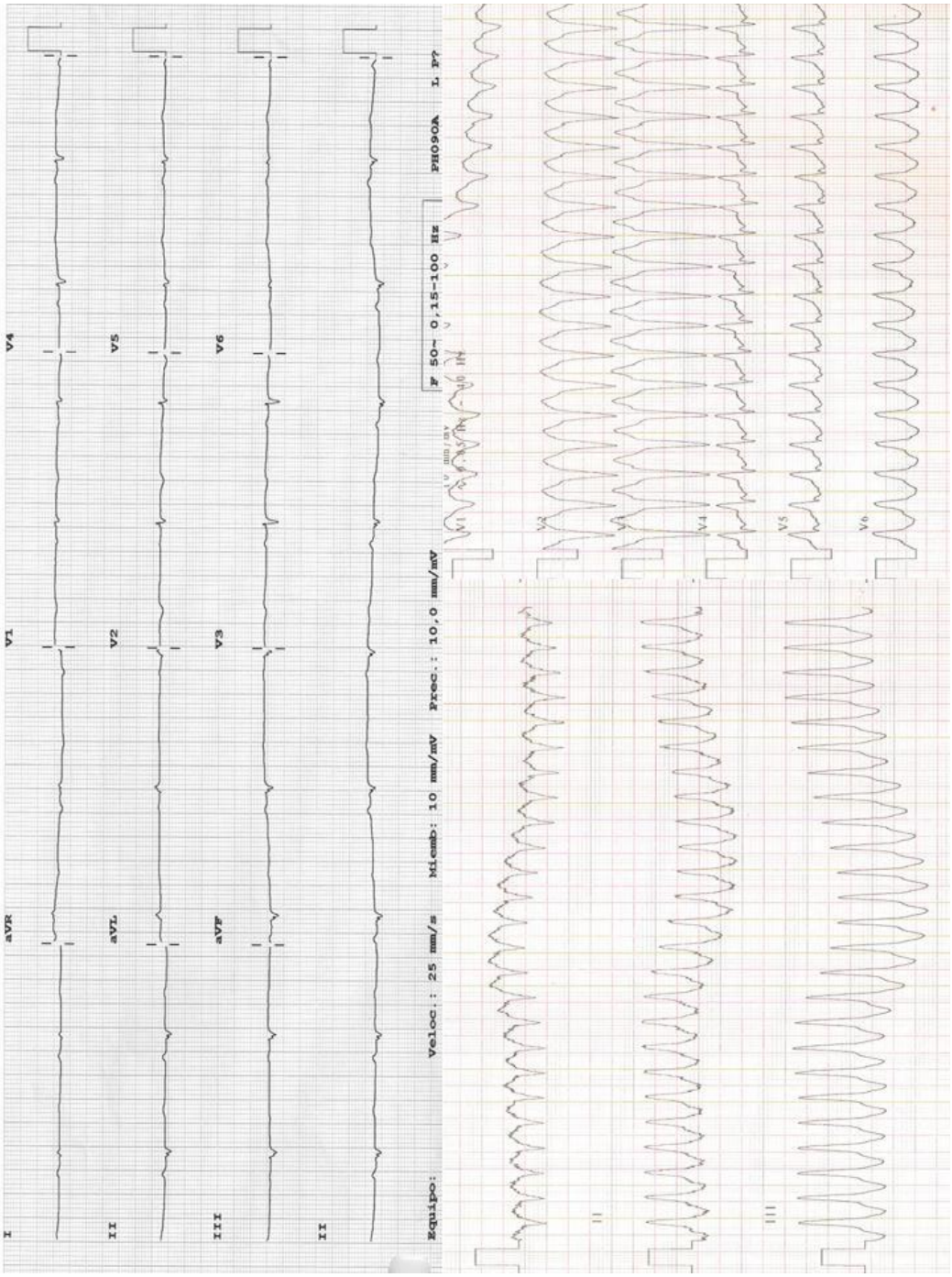

Figure 2: (A, top): Proband´s ECG. Notice the striking low voltages throughout and inverted T waves in right precordial leads. (B, bottom): S-VT suggestive of a RV outflow tract origin.

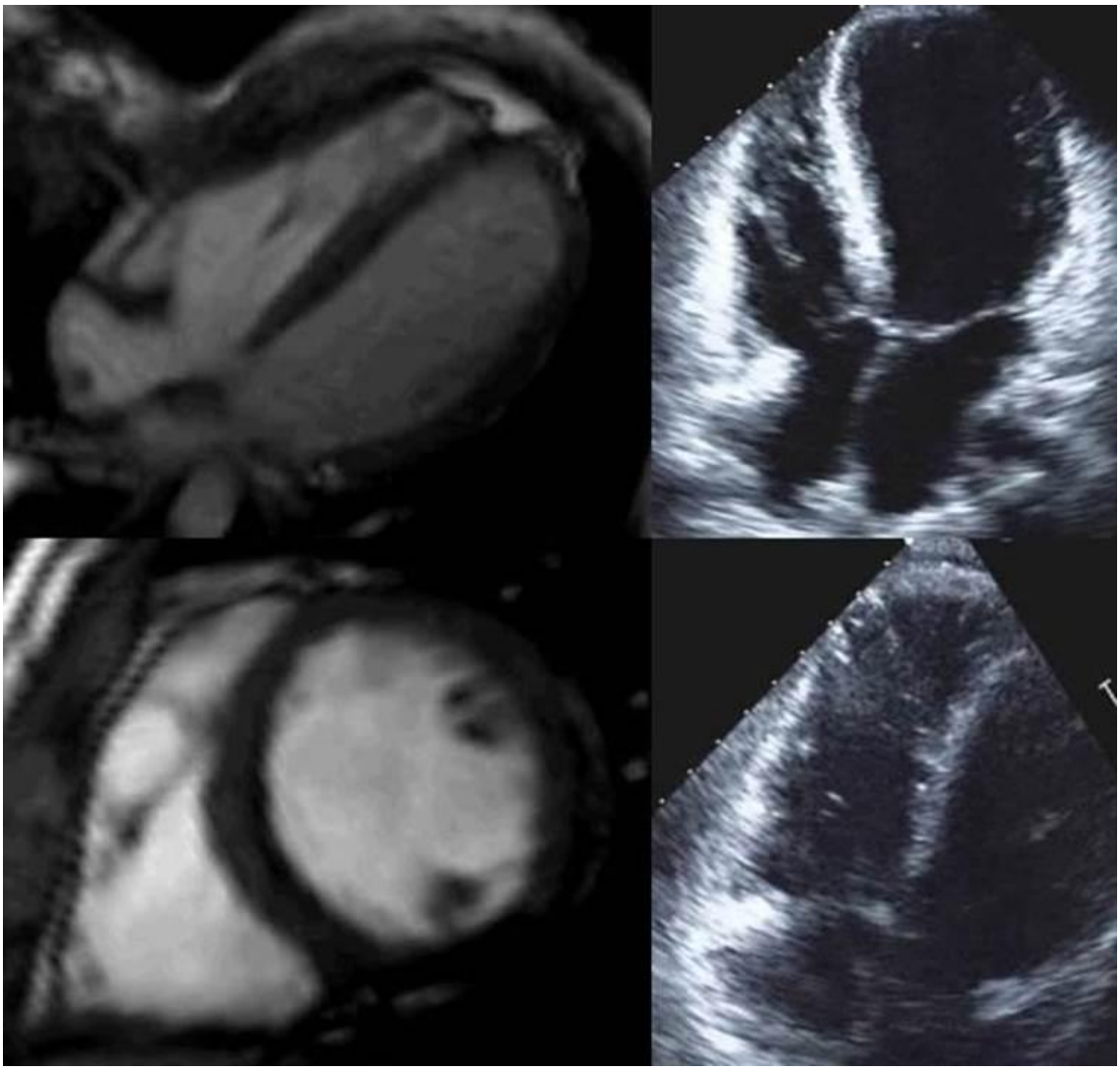

Figure 3: Proband´s echocardiogram and CMR revealed normal biventricular volumes, severe biventricular dysfunction and absence of LGE.

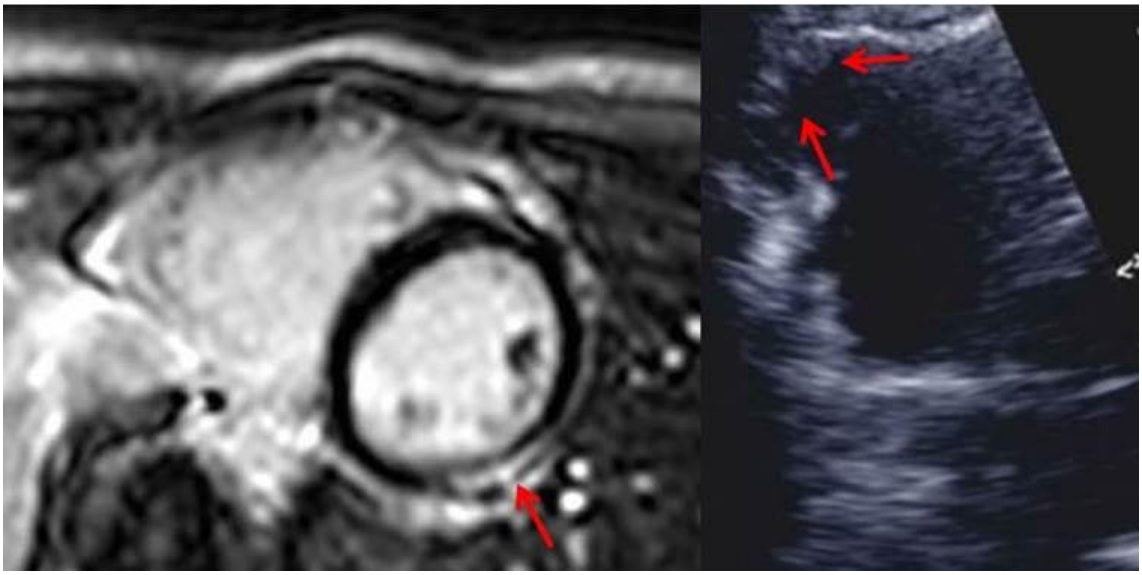

Figure 4: Cardiac imaging findings of III.1 On the left, red arrows point to LV lateral LGE patch. On the right, a RV aneurysm of the free wall is observed.

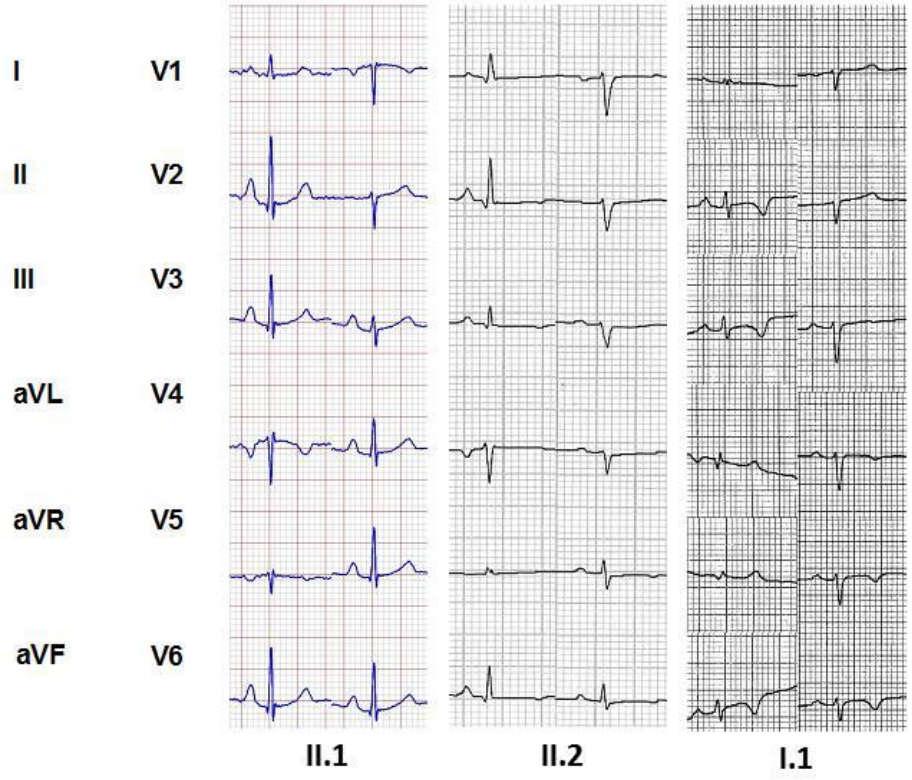

Figure 5: ECG of other carriers. Low voltages and poor R wave progression were the hallmark. Notice the inverted T waves in left precordial leads.

TABLES

Marker		Position	III.2		I.1		D07		D08	
			SPAIN		SPAIN		20473	NL	21163	NL
PLN-650K	B_FAM	118.250K	394	396	394	392	394	390	394	396
PLN-200K	C_FAM	118.700K	441	441	441	443	439	443	439	439
PLN-50K	B_HEX	118.850K	288	294	288	294	288	292	288	284
PLN+200K	D_FAM	119.100K	339	335	339	355	339	355	339	357
D6S304	D_HEX	119.450K	237	249	237	249	237	237	237	237

Table 1: Haplotypes of patients from the Spanish family and 2 Dutch patients carrying the *PLN p.R14del* mutation. The shared haplotype, with the previously described Dutch haplotype as a reference, is shown in bold and grey. Haplotype analysis shows that Spanish and Dutch patients share 4 out of 5 markers identified previously in the Dutch mutation carriers suggesting a common founder.

Case	Pedigree	Age at diagnosis	LVEF	LGE	RV involvement	LV dilatation	ARVC diagnosis	ECG	SA-ECG	VT
1	I.1	77	45%	NA	No	No	Borderline	Poor R wave transition	NA	No
2	II.1	52	Normal	No	No	No	Borderline	Lox voltages. Inverted T waves in lateral leads	0/3	No
3	II.2	56	Normal	No	No	No	Borderline	Low voltages. Inverted T waves in lateral leads	0/3	No
4	II.3	46	Normal	No	No	No	Possible	Low voltages	0/3	No
5	II.4	44	Normal	No	No	No	Possible	Normal	0/3	No
6	III.1	22	55%	Lateral wall	Yes	No	Definitive	Poor R wave transition	0/3	Yes
7	III.2	27	35%	No	Yes	No	Definitive	Low voltages. Inverted T waves precordial in precordial leads	NA	Yes
8	III.3	14	Normal	No	No	No	Possible	Normal	0/3	No

Table 2: Clinical findings of *PLN p.R14del* mutation carriers

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CHAPTER 4: INFLAMMATION IN ARRHYTHMOGENIC CARDIOMYOPATHY

GENETICS OF MYOCARDITIS IN ARRHYTHMOGENIC RIGHT VENTRICULAR "DYSPLASIA"

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ABSTRACT

BACKGROUND

Myocarditis is sometimes related to arrhythmogenic right ventricular dysplasia (ARVD), overlapping sometimes at early stages, which may lead to misdiagnosis. Acute myocarditis may reflect an active phase of ARVD.

OBJECTIVE

To evaluate the genetic basis of myocarditis in ARVD and investigate the association with a poorer prognosis and a higher risk of ventricular arrhythmias.

METHODS

Two groups were analysed : A) 131 affected patients, 84 ARVD cases (62% males, age 45[33-55]) and 47 left-sided forms (ALVD) patients (47 cases, 47% males, age 45[25-61] years old); B) 64 non affected mutation-carrying relatives (36% males, age 42[22-56]; 23 from classical ARVD families and 41 from ALVD families).

RESULTS:

7 (3.5%) patients presented with a clinical diagnosis of acute myocarditis over a median follow up of 34 months. It was the first clinical presentation in 6/7 cases. In 2 patients, acute myocarditis preceded a worsening of left ventricle systolic function. In 1 case, it was associated with an increase in the gadolinium pattern in CMR. Two patients presented with changes in the ECG weeks following the myocarditis resolution. It preceded the development of VT in other 2 patients. Myocarditis clustered in families bearing *DSP p.Q447** and *LDB3 p.A351T.*

CONCLUSION

Acute myocarditis reflects an active phase in ARVD, leading to changes in the phenotype and abrupt progression of the disease. An active phase should be suspected in the event of myocarditis associated with a family history of ARVD. Certain mutations may increase the susceptibility to superimposed myocarditis in ARVD.

Key words: ARVD; Myocarditis; Genetics, Desmoplakin, *LDB3*.

LIST OF ABBREVIATIONS

ARVD=Arrhythmogenic right ventricular dysplasia; ALVD= Arrhythmogenic left ventricular dysplasia; CMR=Cardiac magnetic resonance; CRP=C reactive protein; EMB=Endomyocardial biopsy; EPS= Electrophysiological study; LGE=Late gadolinium enhancement; LV=left ventricle; RV=Right ventricle; SA-ECG=Signal averaged ECG; SD= Sudden death; S-VT=Sustained ventricular tachycardia; TrI=troponin I.

MANUSCRIPT

INTRODUCTION

Arrhythmogenic right ventricular dysplasia (ARVD) is a frequently inherited myocardial disease whose clinical spectrum includes palpitations, syncope, ventricular arrhythmias, heart failure and sudden death.[1] The initial hypothesis of a "trouble of development" has been recently confirmed in vitro as the underlying mechanism of the disease.[2] The estimated prevalence of ARVD in the general population ranges from 1:1,000-1:5,000 [3] this condition being an important cause of sudden death, particularly in young people and athletes. [4] It may also extend to the left ventricle at the late stage[5] or affect the left ventricle as an initial target (ALVD).[6]

Since the initial description of a recessive mutation in plakoglobin as a cause of ARVD[7], the widespread implementation of genetic testing has led to the identification of desmosomal gene mutations in over 50% of the ARVD patients.[8] Moreover, mutations in other non-desmosomal genes has been associated with ARVD and ARVD-like phenotypes (*PLN*[9], *TMEM43*[10], *LMNA A/C*[11], *DES*[12], *CTNNA*[13] , *TTN* [14]).

Other factors such as viral infections have been reported as a co-factor of morbidity.[15] However, not only is myocarditis known to be related to ARVD but possible myocarditis (chest pain and troponin rises) also seem to be part of the clinical presentation as a superimposed phenomenon in the natural history of the disease.[16]

We aimed to evaluate the clinical features of these so-called active or “hot phases” and to investigate whether they are associated with poor prognosis and a higher risk of ventricular arrhythmias. For that purpose, we included two groups of patients: ARVD patients and their gene positive but phenotype negative relatives. The rationale for the inclusion of the second group was to assess whether the carrier status (in otherwise asymptomatic relatives with a vulnerable myocardium) is associated with a higher prevalence of myocarditis.

METHODS

We evaluated the medical history of a cohort of ARVD patients and their relatives. All patients provided written consent. The study was approved by the Ethical Committee of our hospital.

Patients were classified into 2 groups: Group A) Definitive diagnosis of ARVD (Task Force Criteria 2010[17]) or ALVD patients, defined as ARVD- mutation carriers alongside a phenotype of dilated cardiomyopathy (DCM), systolic dysfunction and a past medical history of ventricular arrhythmias and/or a family history of sudden death. Group B) Consisted of non-affected mutation-carriers, all of them relatives of patients diagnosed with ARVD or ALVD.

Patients with ischemic heart disease were excluded from the final analysis.

Clinical evaluation

This consisted of a 12- lead digital ECG, SA-ECG, 2D-doppler echocardiogram, exercise test and 24-hour Holter monitoring. CMR with LGE was performed in 5/7 patients with an probable myocarditis.

Genetic analysis

Mutation analysis was performed in all patients by dideoxy Sanger sequencing of all exons and flanking intron-exon boundaries in *DSP*, *DSC, DSG2, PKP2, JUP, TMEM43, LMNA A/C and PLN.* In cases where no conclusive mutation was found the study was expanded thereafter with the inclusion of a panel of 124 inherited cardiac genes which were analysed with Next Generation Sequence (NGS) **(Table 1, supplemental material).**

Inflammatory and myocardial damage biomarkers

Blood tests analyzed consisted of blood count, inflammation markers including C-reactive protein (CRP), CK, CK-MB and troponin I (TrI).

Histology and immunohistochemistry

Myocardial samples from RV free wall, septum and LV (two samples from each location) were analyzed. They were formalin fixed, paraffin embedded, cut at a thickness of 5 µm, and mounted on slides. Sections were stained with hematoxilin-eosin and Masson trichrome. The following lesions were addressed: fibrofatty evidence, inflammation, necrosis and apoptosis. Lymphocytic population was characterized by immunohistochemistry.

In order to make a certain diagnosis of ARVD in a case with a massive lymphocytic myocarditis and subtle fibrofatty evidence, patterns of plakoglobin, connexin-43, and desmoplakin was analysed. Slides with a primary antibody [monoclonal mouse anti-Ncadherin (1:400)(Sigma); monoclonal mouse anti-plakoglobin (1:1000)(Sigma); monoclonal rabbit anti-connexin 43 (1:400)(Sigma) and monoclonal mouse anti-desmoplakin (1:10)(Fitzgerald)]. The slides were incubated with secondary goat anti-mouse or goat anti-rabbit (1:400) (Jackson ImmunoResearch).Stained slides were checked using a confocal microscope (LSM510, Meta Zeiss). The results were compared with a control sample from a patient deceased for no cardiovascular disease.

Apoptosis was analysed by TUNEL assay and caspase-3 activity.

RESULTS

131 patients affected with ARVD (definitive or borderline diagnosis) or ALVD were included in group A which comprised classical ARVD (84 patients, 62% males, age 45[33-55] years old) and ALVD (47 patients, 47% males, age 45[25-61] years old). Group B, consisted of 64 non-affected mutation-carrying relatives, 36% males, age 42[22-56]; 23 from classical ARVD families and 41 from predominant ALVD families.

7 (3.5%) patients (2 ARVD patients, 4 predominant ALVD patients and 1 gene-positive and phenotype- negative relative) presented with a clinical diagnosis of acute myocarditis over a median follow up of 34 months. 6 (4.5%) cases occurred in the group of affected ARVD/ALVD patients and 1(1.5%) case in the group of non-affected mutation carriers.

Patient 1 (*DSP p.L1773Yfs*1781*)

A 20 year-old male patient was diagnosed with probable acute myocarditis after an episode of chest pain and TrI, CK and MB-CK rise. The ECG at the time did not show repolarisation abnormalities. The echocardiogram revealed mild left ventricular (LV) dilatation and dysfunction (LVEF= 45%). An angiogram ruled out coronary artery disease. Since this initial episode, he presented with recurrent episodes of chest pain and troponin elevation, progressive worsening of the LV function, developing a biventricular disease **(Figure 1, supplementary material)**. Genetic testing identified a

nonsense mutation in desmoplakin (*DSP p.L1773Yfs*1781*), predicted to truncate desmoplakin in the middle of the C-terminal domain of the protein.

Patients 2 and 3 *(DSP p.Q447*)*

Two siblings aged 14 and 19 years **(Figure 2, supplementary material)** were diagnosed during family screening with ALVD. *DSP p.Q447**, a dominant mutation leading to a stop codon, was identified in both. The ECG showed dynamic T wave inversion in inferolateral leads in the youngest **(Figure 1)** and flat T waves in the eldest sibling.

The echocardiogram revealed mild systolic dysfunction in the younger and normal in the elder sibling. In both cases the CMR showed a similar pattern consisting of a widespread LGE pattern **(Figure 3, supplementary material)**. Both siblings were admitted due to recurrent episodes of chest pain and TrI rise after the initial diagnosis. They suffered a progressive LV impairment documented during the follow-up.

Patient 4 *(DSP p.Q447*)*

A maternal aunt of the previous cases had been diagnosed with myocardial infarction with normal coronary arteries at the age of 48. The ECG showed inverted T waves in the inferolateral leads. Yet the inferior wall was hypokinetic, the volumes and LVEF were normal. Six years after, she was admitted with chest pain and palpitations. S-VT of left bundle branch block and axis +30º was recorded and electrical cardioversion successfully restored sinus rhythm. The angiogram was normal. She underwent an electro-physiological study that failed to induce VT. Moreover, the voltage mapping did not reveal low voltage areas. She was discharged on sotalol. One year later, she presented with a new episode of chest pain on exertion and syncopal S-VT. She had an ICD implanted for secondary prevention. No CMR was performed at that time. Years later she was found to be a carrier of *DSP p.Q447**. Following this, she developed new motion abnormalities, affecting the interventricular septum.

Patient 5 (*DSP p.Q447*)*

A 38-year old woman was admitted suffering from acute chest pain and MB-CK and a TrI rise two weeks after giving birth. Her past medical history had been unremarkable. Her mother had been previously diagnosed with ARVD. Both patients were found to

carry *DSP p.Q447**. Apparently she was not related to patients 2, 3 and 4. Her ECG, echocardiogram and CMR were normal.

Patient 6 and 7 (*LIM-domain Binding Protein-3, LDB3 p.T351A*)

A 45 year-old woman was admitted for recurrent chest pain and presyncope. Blood tests showed a rise in CK-MB and TrI. On monitoring, she suffered a poorly tolerated S-VT, successfully restored into sinus rhythm. The angiogram was normal and the echocardiogram and CMR showed a severe biventricular dilatation and dysfunction with multiple dyskynetic areas in the RV and extensive LGE. An ICD was indicated but unfortunately she died in the operating theatre due to a neurological complication related to the anaesthetic procedure. Postmortem showed a severe RV fibrofatty replacement, myocytolisis and scattered lymphocytic patches **(Figures 2 and 3)**.

Her 40 year- old sister reported a longstanding history of palpitations. Her ECG showed a narrow QRS and inverted T waves in right precordial and inferior leads as well as Q waves in inferior leads **(Figure 4)**. The CMR showed a dilated and impaired RV with multiple hypokinetic areas. Three years after the initial diagnosis, she presented with an episode of chest pain and ST elevation in lateral leads as well as a TrI rise. An angiogram ruled out coronary lesions and the diagnosis of acute myo-pericarditis was made. Another CMR performed four weeks after the acute episode, showed no change in RV volume or function. Neither oedema or LGE were observed **(Figure 3 supplementary material).**

After this episode, the ECG showed a pseudonormalization of T waves in right precordial leads and Q wave disappearance that remained for 6 months. Subsequently, a new T wave inversion occurred **(Figure 4)**

NGS identified a missense mutation in *LDB3 (LIM-domain Binding Protein-3)* (*p.T351A)* which was found to segregate in this family.

In no case there was a history of fever or infection in the weeks prior to the acute myocarditis. Notably, the blood tests carried out at the time of the episodes did not show an elevation in inflammatory markers such as CRP or leucocytosis. On imaging, none of the afore-mentioned patients developed pericardial effusion or myocardial oedema on CMR in the days after the acute episode.

DISCUSSION

The link between ARVD and inflammation is well known. Inflammatory infiltrates are present in 60%-88% ARVD samples and there is a higher prevalence of inflammation in postmortem of sudden death cases compared to ARVD patients who had died due to heart failure and amongst patients with left ventricular involvement [5,18-20] Theories that merged viral infections and a specific genetic background as a cofactor in ARVD have been proposed[19]. More recently, plakoglobin remodelling at cardiac myocyte junctions in granulomatous myocarditis (cardiac sarcoidosis and giant cell myocarditis) has been also described. On the contrary, plakoglobin signal is not depressed in viral lymphocytic myocarditis.[20]

We identified 7 patients that presented with acute myocarditis (4 patients with ALVD (out of 47 patients), 2 cases of classical ARVD (out of 84) and 1 non-affected ALVD mutation carrier (out of 23 genotype positive phenotype negative carriers). These episodes of acute myocarditis were recurrent in 4 cases **(Table 1)**. 5 patients carried *DSP p.Q447** and *p.L1773Yfs*1781* and 2 carried *LDB3 p.A351T*. All these patients were relatives or stem from a common ancestor, which points to a familiar distribution of myocarditis.

Chest pain was the first clinical presentation in 5 cases. 2 patients were diagnosed with acute chest pain with normal angiogram and only a detailed clinical work up alongside the family history led to a correct diagnosis.

In 3 patients (patients 1, 2 and 3) probable myocarditis did precede a worsening in LV systolic function. In one case, they were associated with an increase in the LGE (patient 3) **(Figure 5)**. It is worth mentioning the absence of signs which is in keeping with acute myocarditis in CMR, like myocardial oedema. In 2 patients the chest pain did precede the development of S-VT (Patients 4 and 6).

Since myocarditis were the frequently observed first clinical manifestation in ARVD/ALVD patients in this study, we also aimed at evaluating the prevalence of myocarditis in a subset of non-affected gene positive carriers so as to evaluate whether they may uncover an initial ARVD/ALVD phenotype. Only one in this group (patient 5) suffered an episode of chest pain and troponin rise which was not associated with a later change in ECG, holter or imaging. The small sample comprising the genotype positive and phenotype negative relatives prevent us to draw

conclusions on the susceptibility of healthy carriers to suffer myocarditis. Therefore, further investigations are required to shed light on this point.

Dynamic changes in repolarisation have been previously described as a sign of early disease in ARVD[21], with these changes being postulated as a sign of gap junction remodelling. In this series, 2 ARVD patients with severe RV dysfunction and 1 with ALVD presented dynamic changes (including T wave pseudonormalization in one case) a few weeks after the acute myocarditis (patients 2, 3 and 7).

Although useful for an accurate diagnosis, there was no consensus on performing an endomyocardial biopsy in all patients since all of them responded properly to medical treatment and were clinically stable. However, myocardial sample from postmorten was available in one case (patient 6). Hematoxilin-eosin showed fibro- fatty replacement, myocyte necrosis and scattered apoptotic myocytes **(Figure 4, Supplementary material)**. The extensive hyaline fibrosis found in this case alongside lymphocytic clusters inside the fibrotic areas suggests recurrent superimposed myocarditis throughout the natural history of the disease **(Figure 3)**.

Despite chronic myocarditis being considered the cause of some cases of DCM such a dilemma remains yet to be clarified in ARVD. Despite the prevalence of viral genome in ARVD patients being variable[22, 23] lymphocytic infiltrates in both ventricles are a common postmortem finding in ARVD.[5] Interestingly, we have documented that it may be the predominant histologic pattern at initial stages of disease, where fibrofatty tissue is scarce or even absent and a complete plakoglobin shifting has already occurred at the intercellular junctions **(Figures 6 and 7).**

In our series, 5/7 cases presented with signs of acute myocarditis in several members of a family at different times. From a clinical perspective, this superimposed phenomenon may occur during the natural history of the disease in the course of the same disease which reinforces the concept of a superimposed phenomenon on the genetically demonstrated ARVD pattern. These episodes may mirror a histologic active phase and progression of the myocardial disease.

Although the evidence is based on a small series of cases, there seem to be a higher incidence of myocarditis in *DSP* mutation carriers affected by ALVD[16] which should prompt the clinician to look for mutations in *DSP* in this clinical scenario.

This study raises awareness of an under-recognized clinical presentation of ARVD, involving chest pain and a rise in myocardial biomarkers either as the first sign of the disease or as a marker of progression.

Although rare in the whole ARVD population, signs of acute myocarditis should draw the attention of the clinicians on the possibility of ARVD especially when this occurs in other relatives. This clinical presentation is especially suggestive when it occurs in the absence of specific causes, infection, fever, pericardial effusion or a rise in blood inflammation biomarkers.

CONCLUSION

This is the first work to establish a straight link between genetics, myocarditis and ARVD. Relatives bearing the same disease-causing genes show a particular susceptibility to suffer myocarditis, a concept that is well known in other cardiomyopathies like DCM. Moreover, this concept can be further extended to other forms of cardiomyopathies.

Myocarditis reflects an active phase in ARVD and may be the first clinical presentation. Changes in the phenotype could occur during these phases, ranging from isolated ECG changes to worsening in the systolic function or ventricular arrhythmia. An active phase should be suspected in the event of myocarditis (especially if recurrent) associated with a family history of ARVD.

LIMITATIONS

This is a retrospective study based upon a small series of cases. Due to the small sample of healthy relative carriers, results in this subgroup should be interpreted cautiously. The extension of this concept to other ARVD mutations and other cardiomyopathies remains to be demonstrated yet. Another limitation was the absence of virus in myocardial samples, a technique difficult to perform which may explain the failure in the viral identification reported by some groups.

FIGURES

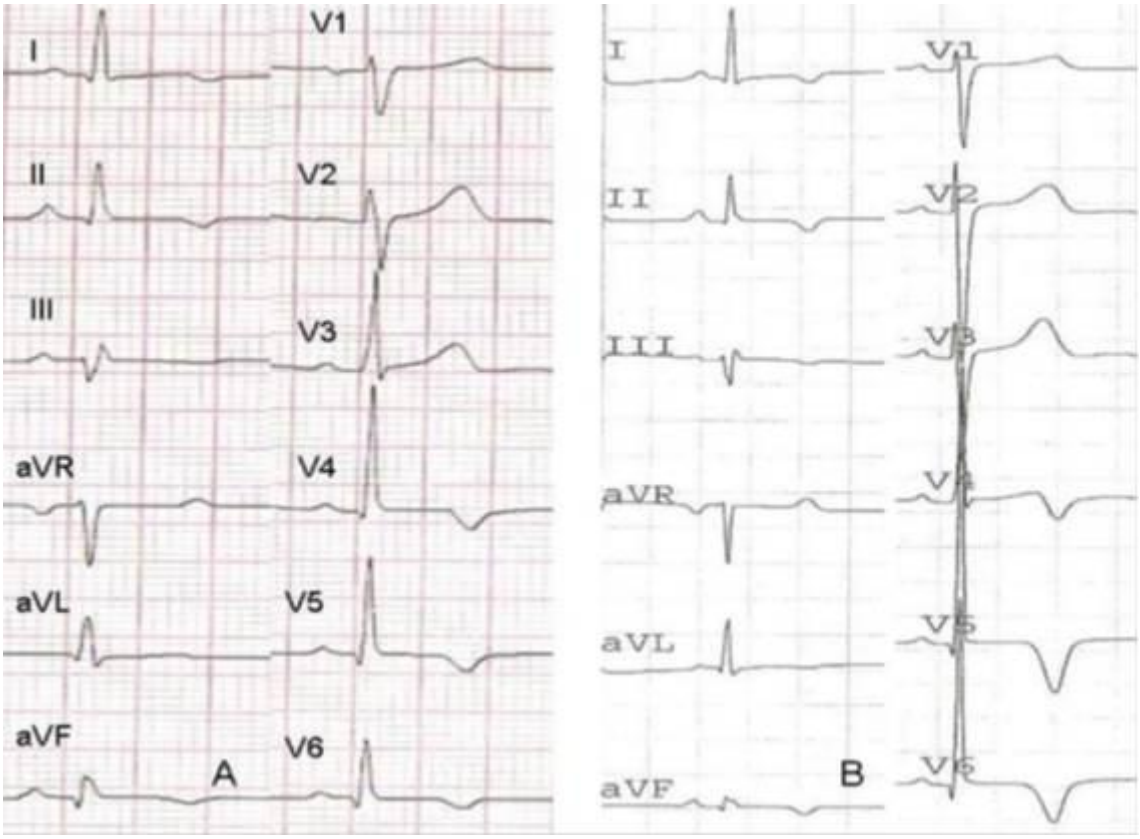

Figure 1: ECG from patient 2. Before (A), six months later (B) and one year after the first acute episode (C). Note the dynamic repolarisation abnormalities (III, aVF and V2) as well as dynamic QRS prolongation.

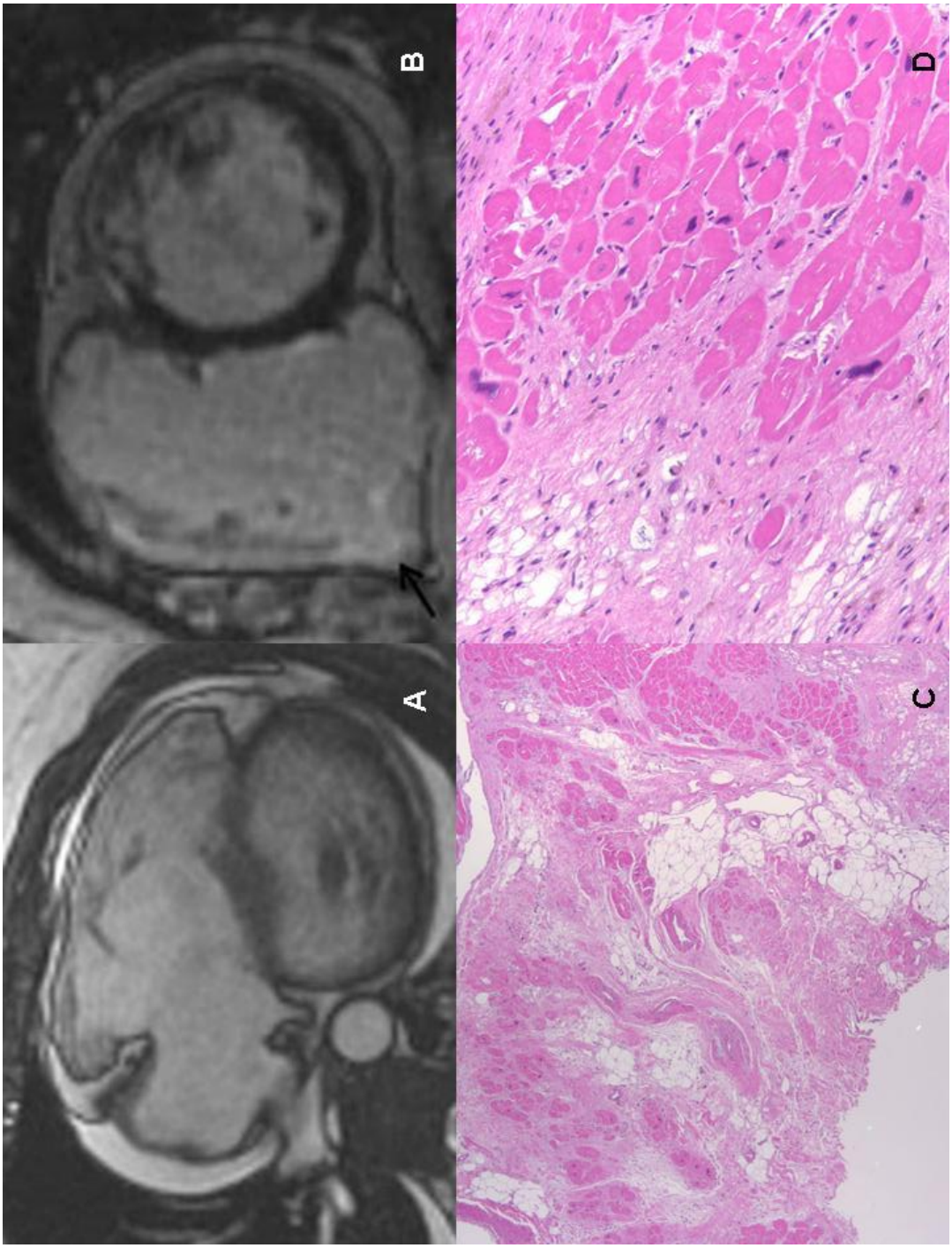

Figure 2: CMR and histology findings of patient 6. A and B: Severe RV enlargement and severe biventricular impairment was observed. Arrows point to dissynchrony areas. C: Histology of a RV trabeculation (left) arising from the free wall (right): Hematoxilin-eosin staining (H-E), 4X. There was extensive fibrofatty replacement. D: Mid apical septum (right septum is on the left) (H-E, 20X). There is extensive fibrosis and myocitolisis.

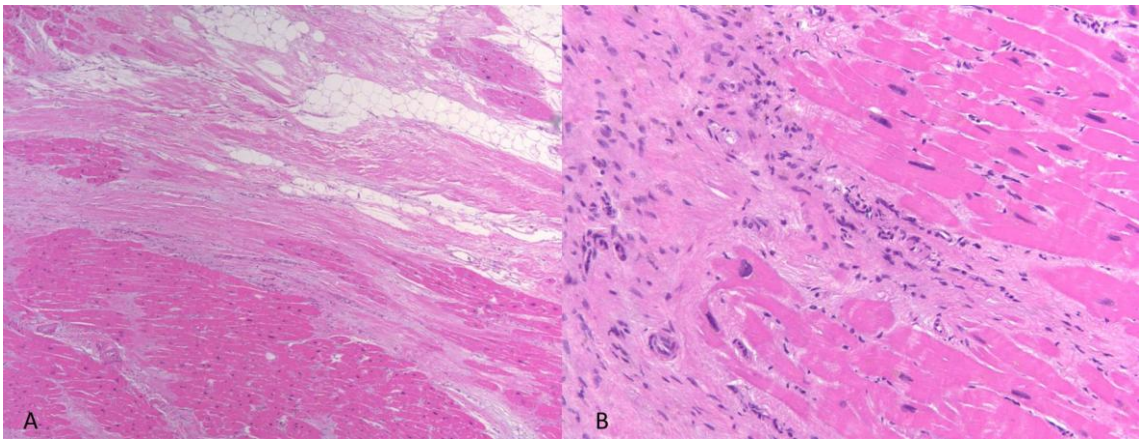

Figure 3: A. Inferolateral wall of LV (4X). H-E, 4X. There is fibrofatty replacement from epicardium (top) to endocardium (bottom). There are areas of hyaline fibrosis. B: At higher magnification (20X), clusters of lymphocytes inside fibrosis are observed, which suggests previous recurrent myocarditis.

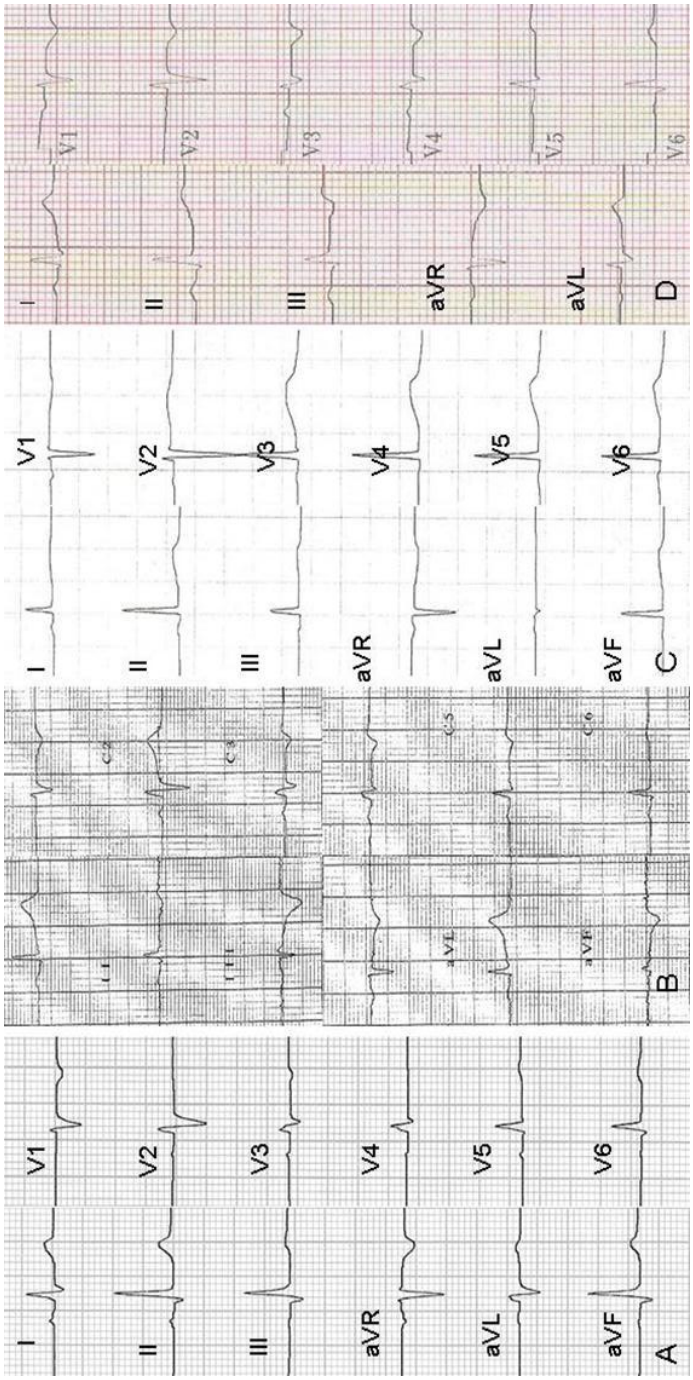

Figure 4: ECG from Patient 6. Before myocarditis (A), during myocarditis (B), six months later (T wave pseudonormalization) (C) and one year after (deeper T wave inversion and lower QRS voltage (D).

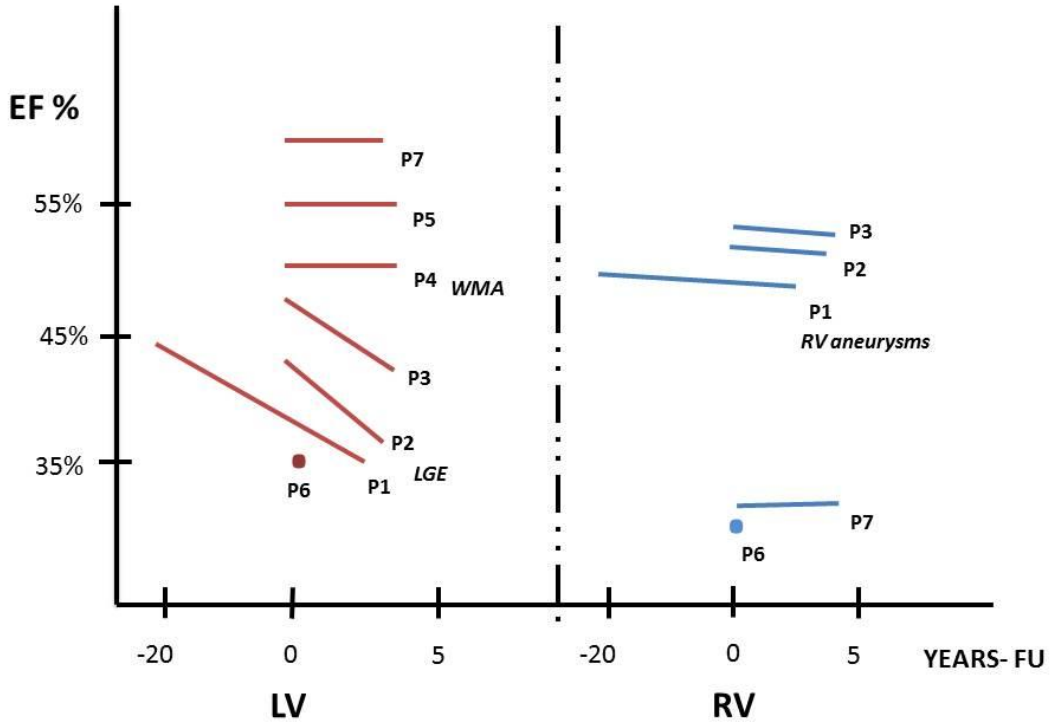

Figure 5: Evolution of the LV and RV ejection fraction pre and post- acute myocarditis. Only RV data are presented if CMR data was available. WMA: wall motion abnormalities.

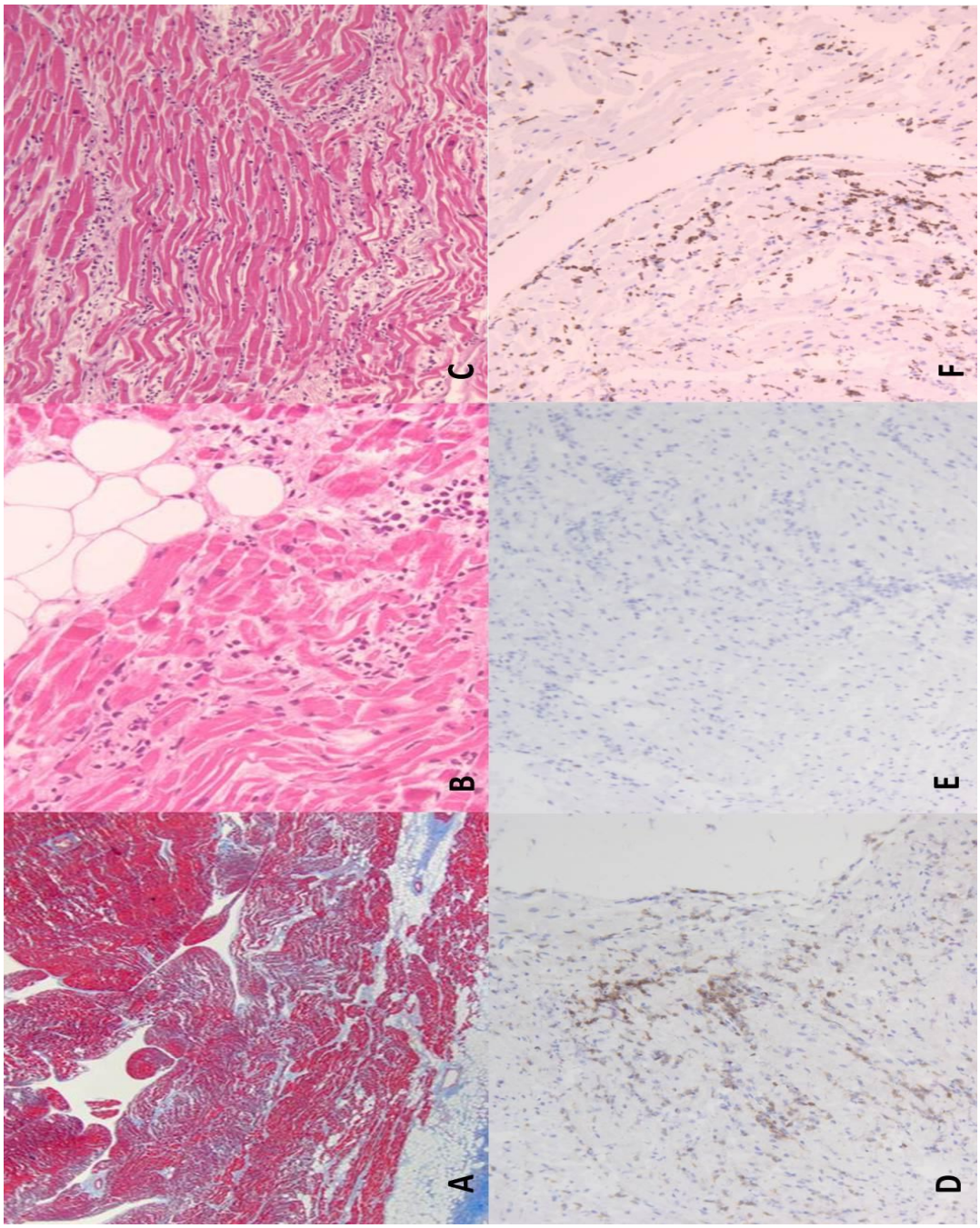

Figure 6: Postmortem of a 40 year old woman with an unremarkable past medical history, who died suddenly at home. Macroscopic study showed mild ventricular hypertrophy (heart weight 350 g) as well as mild biventricular dilatation. **A**: Masson trichromic of the lateral wall of the RV (4X) shows mild fibrofatty replacement of lateral wall of the right ventricle, from epicardium (bottom) to endocardium (top). **B**: H-E at higher magnification (20X) of the mid wall shows lymphocytic clusters, fibrosis and isolated adipocytes. **C**: Extensive areas of lymphocytic myocarditis localized all

over the RV free wall. Cardiotrophic viruses (Parvovirus B19, Adenovirus, Enterovirus, Citomegalovirus, Epstein Barr, Herpesvirus-6) were excluded by myocardial PCR.

D-F depicts the immunohistochemistry findings of the RV: lymphocytes T CD4+ (D) and macrophages CD68+ were present. On the contrary, no lymphocytes TCD8+ were observed (E). This inflammatory pattern found in an early stage of the disease was reminiscent of that found in type IV hypersensitivity reactions.

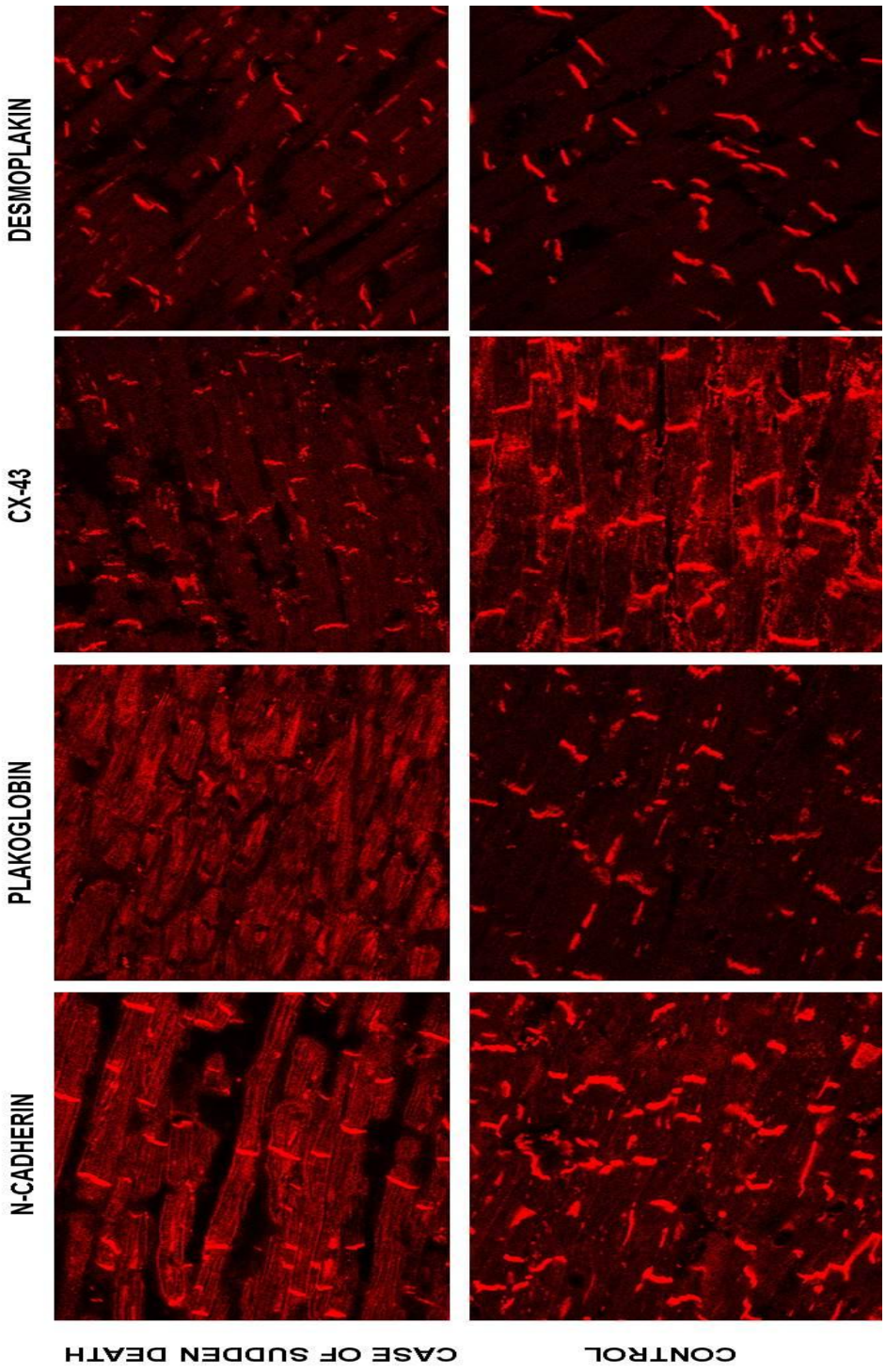

Figure 7: Immunohistochemistry of N-cadherin, plakoglobin, desmoplakin and connexin-43. Plakoglobin is not present in the intercellular junctions in the ARVD case.

TABLE

Patient	Sex	Age	First symptom	TnI rise	CK-MB rise	Recurrent myocarditis	Phenotype	Gene	Mutation	Outcomes after myocarditis
1	M	20	Chest pain	Yes	Yes	Yes	ALVC	*DSP*	*c.531SdelT*	Worsening in LV systolic function.
2	M	14	Chest pain	Yes	No	Yes	ALVC	*DSP*	*c.1339C>T*	Worsening in LV systolic function. Changes in repolarization
3	M	19	Chest pain	Yes	No	Yes	ALVC	*DSP*	*c.1339C>T*	Worsening in LV systolic function
4	F	37	Chest pain and syncope	Yes	No	Yes	ALVC	*DSP*	*c.1339C>T*	Ventricular tachycardia
5	F	38	Chest pain	Yes	Yes	No	Normal	*DSP*	*c.1339C>T*	No change
6	F	40	Palpitations	Yes	Yes	No	ARVC	*LDB3*	*c.1051A>G*	Changes in repolarization
7	F	45	Chest pain and palpitations	Yes	Yes	No	ARVC-Biv	*LDB3*	*c.1051A>G*	Syncope. Ventricular tachycardia

Table 1: Summary of the main clinical features of the patients suffering myocarditis-like episodes

SUPPLEMENTARY MATERIAL

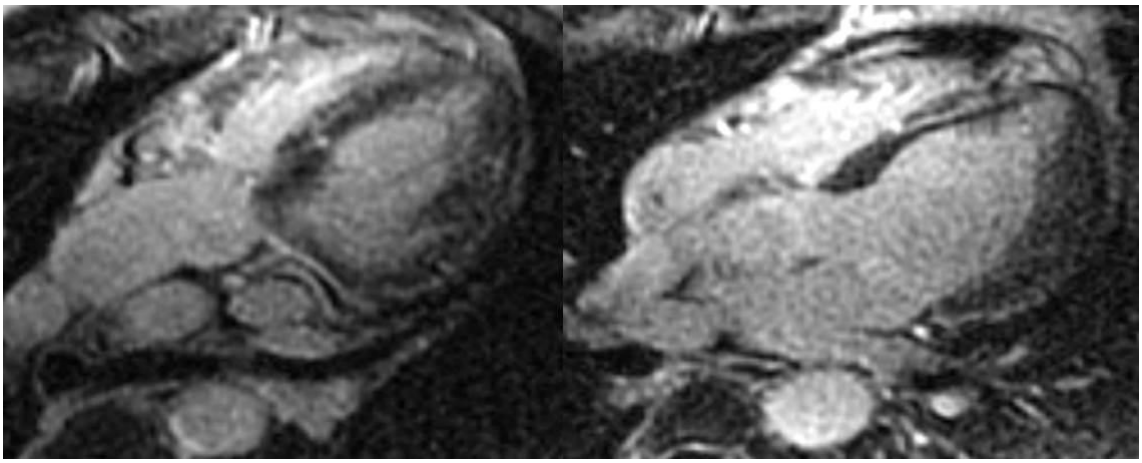

Figure 1: CMR findings of Patient 1. It demonstrated LVNC, severe systolic dysfunction and widespread LGE.

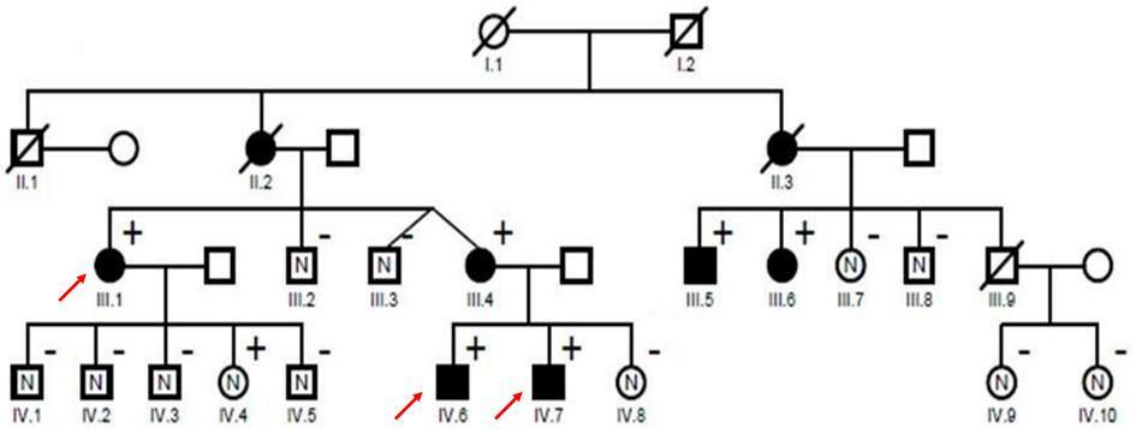

Figure 2: Family tree of Patients 2 and 3.

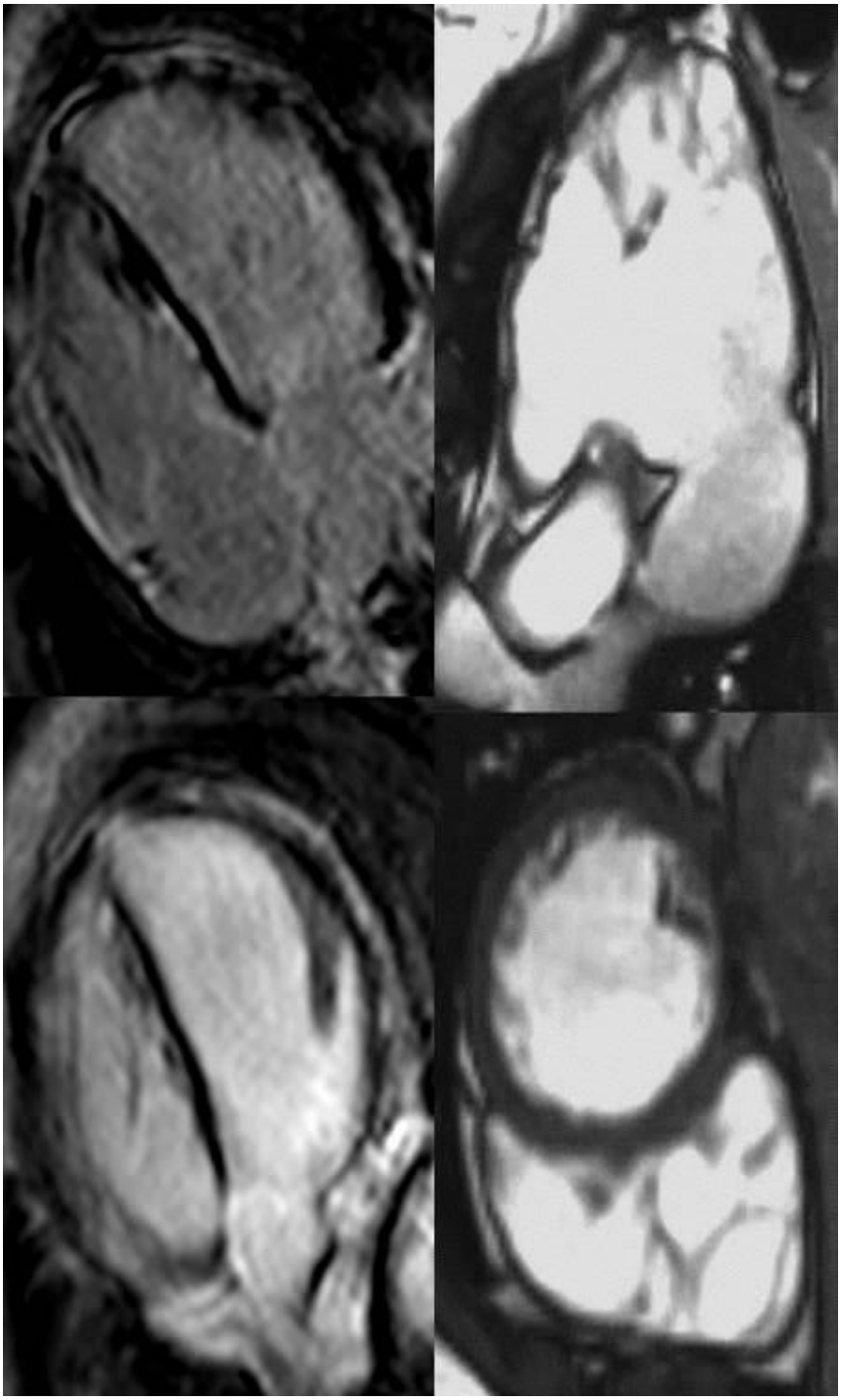

Figure 3: CMR of Patient 2 (top) and 7 (bottom).

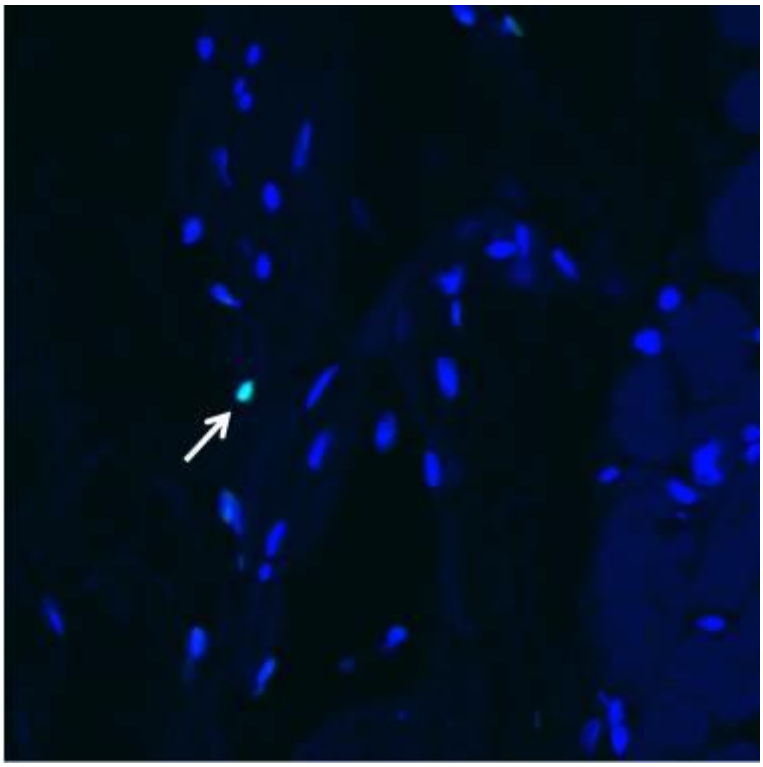

Figure 4: TUNEL analysis for apoptosis (arrow) in Patient 6.

TABLES

Cromosome	Gene	Cromosome	Gene	Cromosome	Gene	Cromosome	Gene
chr12	ABCC9	chr6	EYA4	chr11	KCNQ1	chr3	RAF1
chr10	ACTA2	chr15	FBN1	chr20	KCNQ2	chr17	RANGRF
chr15	ACTC1	chr5	FBN2	chr12	KRAS	chr10	RBM20
chr1	ACTN2	chrX	FHL1	chr6	LAMA4	chr1	RYR2
chr10	ADRB1	chr2	FHL2	chrX	LAMP2	chr19	SCN1B
chr5	ADRB2	chr9	FKTN	chr10	LDB3	chr11	SCN2B
chr8	ADRB3	chr7	FLNC	chr19	LDLR	chr11	SCN3B
chr1	AGL	chr9	FXN	chr1	LMNA	chr11	SCN4B
chr7	AKAP9	chr17	GAA	chr12	LRP6	chr3	SCN5A
chr4	ANK2	chr8	GATA4	chr15	MAP2K1	chr16	SCNN1B
chr10	ANKRD1	chr6	GJA1	chr19	MAP2K2	chr16	SCNN1G
chr2	APOB	chr1	GJA5	chr11	MYBPC3	chr5	SGCD
chr10	BAG3	chrX	GLA	chr14	MYH6	chr10	SHOC2
chr2	BMPR2	chr3	GPD1L	chr14	MYH7	chr4	SLC25A4
chr7	BRAF	chr5	HCN1	chr12	MYL2	chr20	SNTA1
chr9	CACNA1B	chr15	HCN4	chr3	MYL3	chr2	SOS1
chr12	CACNA1C	chr11	HRAS	chr20	MYLK2	chrX	TAZ
chr3	CACNA1D	chr20	JAG1	chr5	MYOT	chr7	TBX20
chr7	CACNA2D1	chr20	JPH2	chr4	MYOZ2	chr17	TCAP
chr10	CACNB2	chr17	JUP	chr10	MYPN	chr14	TGFB3
chr19	CALR3	chr12	KCNA5	chr1	NEXN	chr9	TGFBR1
chr1	CASQ2	chr1	KCND3	chr5	NKX2-5	chr3	TGFBR2
chr3	CAV3	chr21	KCNE1	chr1	NPPA	chr1	TGFBR3
chr11	CRYAB	chrX	KCNE1L	chr1	NRAS	chr3	TMEM43
chr11	CSRP3	chr21	KCNE2	chr4	PDLIM3	chr12	TMPO
chr16	CTF1	chr11	KCNE3	chr12	PKP2	chr3	TNNC1
chr2	DES	chr2	KCNE4	chr2	PKP4	chr19	TNNI3
chrX	DMD	chr7	KCNH2	chr8	PLEC	chr1	TNNT2
chr18	DSC2	chr11	KCNJ11	chr6	PLN	chr15	TPM1
chr18	DSG2	chr17	KCNJ12	chr14	PNN	chr2	TTN
chr6	DSP	chr17	KCNJ2	chr7	PRKAG2	chr18	TTR
chr18	DTNA	chr2	KCNJ3	chr14	PSEN1	chr10	VCL
chr7	ELN	chr11	KCNJ5	chr1	PSEN2		
chrX	EMD	chr12	KCNJ8	chr12	PTPN11		

Table 1, supplementary material: List of genes analysed by means of NGS. This test includes all genes previously associated with the development of cardiomyopathies, channelopathies, familial aortic syndromes, familial pulmonary hypertension and familial hypercholesterolemia. The associations of most of those genes were demonstrated in clinical cases; nevertheless, some of them are considered "candidate genes" according to the function of the codified protein.

CLINICAL PERSPECTIVES

This manuscript establishes for the first time a link between ARVD, genetics and myocarditis. In this short series, this research demonstrates that:

1. Desmoplakin mutations look related to unique as well as recurrent episodes of superimposed acute episodes of myocarditis.
2. A new mutation in the LIM domain seems also susceptible to possible viral susceptibility. The extension of this concept to other ARVD mutations and other cardiomyopathies remains to be demonstrated.
3. Chest pain and troponin release independent of coronary disease should draw attention of a possible manifestation of a concealed form of ARVD.
4. Lymphocytic myocarditis may be present at an early stage in ARVD, even before the fibrofatty replacement is evident which should raise the suspicion when assessing sudden death cases attributed to right ventricular myocarditis.

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SUMMARY

The goal of this thesis and all the scientific production derived from this research is to characterize the whole spectrum of arrhythmogenic cardiomyopathy (genetic background, immunohistochemistry of the main proteins of the intercalated disks, epidemiology and arrhythmic outcomes). This multidisciplinary approach is essential to improve the diagnosis, risk stratification and clinical management of this disease.

Patients diagnosed with ACM were consecutively enrolled in the Inherited Cardiac Disease Unit of the Virgen de la Arrixaca University Hospital between the years 2003-2014. The investigations carried out included 12 leads ECG, signal averaged-ECG, exercise test, 24-hour Holter monitor, 2D-echocardiogram, cardiac magnetic resonance, electrophysiological study and endomyocardial biopsy (the last two tests were performed in a minority of cases). Immunohistochemistry of the intercellular junctions were carried out in the myocardial samples available in addition to the conventional histology techniques. Probands underwent genetic testing (Sanger sequencing before 2012 and Next Generation Sequencing from 2012 onwards).

The proposed aims for this research have been accomplished as it is explained throughout this thesis.

- The first chapter **(Aim 1)** demonstrates the importance of the family screening for the correct diagnosis of arrhythmogenic cardiomyopathy not only amongst the relatives but also in probands, in whom the definitive diagnosis is usually not made in the initial evaluation.

  In the absence of a gold standard test for the diagnosis of this disease, only a detailed clinical evaluation comprising electrocardiographic, arrhythmic and morphological abnormalities, genetics and a detailed family history will allow to reach the final diagnosis.
- The second chapter **(Aims 2-4)** is focused on the differences between right and left dominant phenotypes. Since biventricular involvement is often found in both groups (either in imaging or pathologic studies) the terms right dominant

(RDACM) and left dominant arrhythmogenic cardiomyopathy (LDACM) were adopted to illustrate the spectrum of the disease.

Similarities and differences in the clinical profile, arrhythmic events, genetic background and pathological findings between phenotypes are analyzed in detail:

- **Aim 2**:

  -The survival free from sudden death in arrhythmogenic cardiomyopathy patients is compared with that found in the most prevalent cardiomyopathies (hypertrophic cardiomyopathy and dilated cardiomyopathy).

  This analysis shows a remarkably higher incidence of sudden death in the arrhythmogenic cardiomyopathy group, particularly after the fourth decade of life.

  -There is a high prevalence of females amongst LDACM patients (contrary to the higher prevalence of males in RDACM). Females are usually diagnosed later in life.

  -Females usually suffer malignant arrhythmias later in life than their male counterparts.

  -The association of malignant arrhythmias and sudden death with exercise was low. In only one third of the cases that suffered the composite sudden death/ventricular fibrillation/sustained ventricular tachycardia was the event exercise related.

  - A RV/LV volume ratio <1 in cardiac magnetic resonance, a mildly dilated LV and a high prevalence of LGE and LVNC were distinctive features in LDACM compared to RDACM.

  -Superimposed LVNC was present in up to 30% of the LDACM patients. Neither LGE nor LVNC were associated with a higher risk of sudden death, sustained ventricular tachycardia or syncope. However, the presence of LVNC was associated with a more severe systolic disfunction.

  -A LVEF< 45% was found to be a sensitive cut-off value for the identification of subjects at risk of ventricular arrhythmias in LDACM.

  -Some families presented a high prevalence of cancer, which initially led to the diagnosis of anthracycline cardiomyopathy. The question whether

anthracycline exposure may accelerate the development of the cardiomyopathy or modulate its expression resulting in a more severe phenotype will require further research.

-The genetic testing demonstrated differences in the genetics between phenotypes: Desmoplakin truncating mutations and the European founder mutation *PLN p.R14del* were frequently identified in LDACM patients. These mutations segregated with a high prevalence in these families.

On the other hand, a wider range of mutations were found in RDACM, many of them, bearing missense mutations and variants on unknown significance.

-The difference in the molecular basis of the disease is not only present in their genetic basis but also did they exist at a tissue level. These finding was observed in the immunohistochemical analysis of some important proteins present in the intercalated disks:

Normal Cx43 (one of the component of the gap junctions and a key responsible for the intercellular coupling) and plakoglobin patterns (a component of the desmosomes, responsible for the mechanical intercellular coupling and regulator of important intracellular signalling pathways) was consistently normal in LDACM, contrary to the abnormal expression found in RDACM samples. Therefore, the reduced junctional signal for plakoglobin and Cx43 appears to track with the predominant phenotype of the disease.

-The redistribution of the plakoglobing (also known as ϒ-catenin) to the nucleous may interfere with the transcriptional activity regulated by the β-catenin/T-cell factor/lymphoid enhancer factor complex. However, the precise role of the interaction β-catenin/ ϒ-catenin in the pathogenesis of arrhythmogenic cardiomyopathy remains to be clarified.

The electrical intercellular coupling between neighbour cells depends on the integrity of the gap junctions. Aside from a reduced Cx43 and Nav1.5 (the major protein of the Na channel), experimental models show abnormal ion currents (reduced I_{Na} and I_{K1}) which could affect the electrical propagation of the electrical impulse. The role of these abnormalities in the arrhythmogenesis remains to be clarified.

- **Aim 3**:

  -LV involvement in arrhythmogenic cardiomyopathy has been traditionally considered a sign of advanced disease and bad prognosis leading to heart failure and ventricular arrhythmias. However, the incidence of ventricular arrhythmias was similar in both phenotypes.

  -Special attention was paid to the carriers of an ICD in whom the annual rate of appropriate therapies was similar. Only patients fitted with an ICD for secondary prevention, hemodinamically stable sustained ventricular tachycardia or syncope suffered appropriate therapies during the follow-up.

  -The recognition of this fact in important in order to optimize the indications for an ICD, since this group of patient present a high rate of complications (almost 1 in 5 patients), either derived from the implantation or during the follow-up.

  -It is important to highlight the strong association between reduced tricuspid DTI values (<10 cm/s) in carriers of an ICD and sustained ventricular arrhythmias during the follow-up in both RDACM and LDACM patients.

  -This association may be explained by the fact that that the most severe phenotypes (severe ventricular dilatation, severe systolic dysfunction and extensive biventricular involvement) shows reduced DTI values.

  -The presence of non sustained ventricular tachycardia in the ICD electrograms was more frequently found in patients with the most severe phenotypes. However, they did not predict appropriate therapies during the follow-up.

- **Aim 4:**

  -The identification of early signs of disease is healthy relatives of patients affected with arrhythmogenic cardiomyopathy is paramount to optimize the cardiac evaluation and the identification of individuals potentially at risk.

  -A novel informatic software was designed and implemented in order to analyze early electrocardiographic abnormalities in genotype positive-phenotype negative individuals. QRS, ST and T wave morphologies were analysed in detail.

  -This analysis allowed the identification of final inferolateral QRS fragmentation associated with a flat or negative ST slope as a frequent electrocardiographic

finding found in healthy carriers. On the contrary, the ST slope presented a positive slope in healthy wild-type individuals. This is a useful tool in the family screening since it may indentify genetic carriers.

-The question whether this finding will progress towards a future T wave inversion should be evaluated in future studies.

- The third chapter focuses on the genetic background of the arrhythmogenic cardiomyopathy patients and its phenotypic correlation. It is focused on three issues: the genotype-phenotype correlation in desmoplakin truncating mutations, the identification of new genes involved in arrhythmogenic cardiomyopathy and the genotype-phenotype correlation of the European founder mutation *PLN p.R14del.*

- **Aim 5:**

  -The first part describes the clinical characterization of LDACM caused by the novel *DSP p.Q447** mutation and all the previously reported desmoplakin truncating mutations.

  -Desmoplakin comprises of three major functional domains: head, body and tail. The carboxy-terminal domain is responsible for binding to desmin intermediate filaments whilst the N-terminal domain links desmoplakin to plakoglobin and plakophilin-2. The body is responsible for the formation of protein dimmers.

  -The mutation was a C to T transition at exon 11 in the *DSP* gene leading to a premature stop codon. This change occurred at the N-terminal region, and it is thought to generate a truncated peptide (85% in length). The affected amino-acid at position *p.Q447** is located in one of the globular head domains of desmoplakin.

  -As previously stated **(Aim 2)**, myocardial samples from endomyocardial biopsy failed to demonstrate plakoglobin, desmoplakin, Cx43 or desmin remodelling. The presence of a normal desmoplakin pattern in the intercalated disk suggests a negative-dominant effect.

-Truncating mutations in desmoplakin, regardless of their position on the protein, behave aggressively and are characterized by early clinical presentation, a high burden of arrhythmias and sudden death.

-LDACM caused by *DSP* truncating mutations should be suspected in the presence of dilated cardiomyopathy and/or left ventricular non compaction associated with ventricular arrhythmias or family background of ventricular arrhythmias or sudden death. This phenotype frequently associates inferolateral T wave inversion and late gadolinium enhancement in cardiac magnetic resonance.

-In view of the highly arrhythmic burden present amongst patients harbouring *DSP p.Q447** or other desmoplakin truncating mutations, the indication of an ICD should be considered before reaching a severe systolic dysfunction.

- **Aim 6:**

-The implementation of Next Generation Sequencing allows the identification of new genes involved in cardiomyopathies. For the first time, we described a mutation in the Z-line protein (*LDB3 p.T351A)* in a family affected with classic RDACM. This missense mutation is localized in exon 7 has been previously reported to cause dilated cardiomyopathy and left ventricular non compaction.

-Cypher/ZASP is a cytoskeletal protein which binds to α-actinin in the Z-line of skeletal and cardiac myocites. Aside from cardiomyopathies, mutations in this protein have been linked to neuromuscular diseases. This protein plays a key role in maintaining the cardiac structure through the interaction with other Z-line proteins.

-The molecular mechanism that explains the link between *LDB3* and arrhythmogenic cardiomyopathy will require further research. The present finding highlight the complexity of the genetic basis of the disease since a mutation in another non-desmosomal gene emerges as a cause of the disease.

-*LDB3* should be included in the screening panel for genetic testing alongside other non-desmosomal genes.

- **Aim 7:**

  -A family affected with arrhythmogenic cardiomyopathy caused by the European founder mutation *PLN p.R14del* is studied.

  -Phospholamban is a 52 amino acid protein that regulates the sarcoplasmic reticulum Ca^{2+} (SERCA2a) pump in cardiac muscle. This protein is an inhibitor of SERCA2a in its unphosphorylated state. The inhibition is relieved upon phosphorylation. Phospholamban is essential for maintaining Ca^{2+} homeostasis and the cardiac output in response in response to β-adrenergic stimuli.

  -*PLN p.R14del* causes the deletion of three nucleotides that does not affect the reading frame. It is a European founder mutation that causes RDACM and dilated cardiomyopathy usually with biventricular involvement.

  -The microsatellite analysis suggests the existence of a common European ancestor.

  -This family is a clear example of the variability of the clinical phenotype of the disease in relatives. This fact highlights the concept of arrhythmogenic cardiomyopathy as a broad spectrum of disease.

  -The presence of Low QRS voltages in patients affected with arrhythmogenic cardiomyopathy is a red flag that may suggest an underlying mutation in *PLN.*

- The fourth chapter **(Aim 8)** is focused on the genetic basis of inflammation and the clinical implications of myocarditis in arrhythmogenic cardiomyopathy. From a clinical perspective it is important to recognize the following facts:

  -Myocarditis may be the first clinical presentation of arrhythmogenic cardiomyopathy. This fact should be draw attention to the possible diagnosis of arrhythmogenic cardiomyopathy in relatives.

  -These myocarditis-like episodes reflect a progression of the disease and it may associate impairment of the systolic function, gadolinium pattern on cardiac magnetic resonance or ventricular arrhythmias.

  -The myocarditis cluster found in relatives harbouring the same mutations suggests a genetically determined vulnerability to these episodes. The aetiology

of these episodes (viral versus primary myocardial degeneration remains to be elucidated).

-We failed to demonstrate the presence of viral DNA in myocardial samples.

- Immunohistochemistry demonstrated the presence of lymphocytes CD8+ and macrophages CD68+, a pattern similar to that found in type 4-hypersensitivity response.

- The recognition of the fact that myocarditis may be the first clinical manifestation of arrhythmogenic cardiomyopathy is not only important in clinical practise, but also in the pathological arena:
    - Lymphocytic myocarditis circumscribed to the RV should rise the suspicion of a very early stage RDACM. In this scenario, the remodelling of plakoglobin and Cx43 from the intercellular junctions suggests underlying RDACM.
    - Plakoglobin remodelling has been reported in highly arrhythmic granulomatous myocarditis such as giant cells myocarditis or cardiac sarcoidosis. However, this abnormality was not observed in cases of lymphocytic myocarditis.

RESUMEN

El objetivo de esta tesis doctoral y de la producción científica derivada de la misma es la caracterización clínica del espectro de la miocardiopatía arritmogénica (bases genéticas, hallazgos inmunohistoquímicos de la unión intercelular, epidemiología y eventos arrítmicos). Esta aproximación es esencial para la mejora en el diagnóstico, la estratificación del riesgo y el manejo clínico de la enfermedad.

Los pacientes con el diagnóstico de miocardiopatía arritmogénica fueron reclutados y seguidos clínicamente en la Unidad de Cardiopatías Hereditarias del Hospital Universitario Virgen de la Arrixaca entre los años 2003-2014. Las pruebas complementarias realizadas incluyeron ECG de 12 derivaciones, ECG de señal promediada, prueba de esfuerzo, Holter de 24 horas, ecocardiograma, resonancia magnética cardiaca, estudio electrofisiológico y biopsia endomiocárdica (estas dos últimas pruebas fueron únicamente realizada en una minoría de pacientes). El estudio inmunohistoquímico de las proteínas de la unión intercelular se realizó en las muestra de tejido miocárdico disponibles. A los probandos se les realizó estudio genético mediante secuenciación Sanger (hasta el año 2012) y Next Generation Sequencing a partir de dicha fecha.

Los objetivos de esta tesis doctoral se han conseguido tal y como se ha expuesto a lo largo de los diferentes capítulos.

- El primer capítulo **(Objetivo 1)** demuestra la importancia del screening familiar para alcanzar el diagnóstico correcto de miocardiopatía arritmogénica no solo entre los familiares sino también en el probando dado que en muchas ocasiones, no es posible realizar el diagnóstico clínico en una primera valoración. Dada la ausencia de una prueba definitiva para el diagnóstico de la miocardiopatía arritmogénica, solo una evaluación clínica minuciosa que englobe los hallazgos arrítmicos y electrocardiográficos, las alteraciones morfológicas, la genética y la historia familiar permitirá alcanzar un diagnóstico de certeza.

- El segundo capítulo estudia las diferencias entre la miocardiopatía arritmogénica predominantemente derecha y la predominantemente izquierda. Dado que estos pacientes presentan a menudo afectación biventricular, esta terminología ilustra de forma adecuada el amplio espectro de enfermedad. Las similitudes y diferencias en el perfil clínico, los eventos arrítmicos, bases genéticas y hallazgos patológicos entre ambos fenotipos se analizan en detalle:

- **Objetivo 2:**

- La supervivencia libre de muerte súbita en miocardiopatía arritmogénica es comparada con la de las miocardiopatías más prevalentes (miocardiopatía hipertrófica y miocardiopatía dilatada).
- Este análisis muestra una incidencia de muerte súbita mucho más elevada en patientes con miocardiopatía arritmogénica, particularmente a partir de la cuarta década de la vida.
- Se observa una alta prevalencia de mujeres entre los pacientes afectados con miocardiopatía predominantemente izquierda (al contrario de la mayoría de varones que se observa entre los pacientes afectados predominantemente con miocardiopatía arritmogénica predominantemente derecha).
- Las mujeres presentan arritmias ventriculares a una mayor edad que los hombres.
- Solamente un tercio de las arritmias ventriculares sostenidas y casos de muerte súbita se relacionaron con el ejercicio.
- Un ratio volumen de ventrículo derecho/volumen de ventrículo izquierdo <1, dilación leve de ventrículo izquierdo, la presencia de realce tardío en la resonancia magnética y la no compactación de ventrículo izquierdo son características típicas de la miocardiopatía arritmogénica predominantemente izquierda.
- La no compactación de ventrículo izquierdo se detecta hasta en el 30% de los pacientes afectados con miocardiopatía arritmogénica predominantemente izquierda.

- Ni la presencia de no compactación ni la presencia de realce tardío se asoció con un mayor riesgo de muerte súbita, taquicardia ventricular sostenida o síncope. Sin embargo la prevalencia de no compactación fue mayor en los casos con disfunción ventricular más severa.
- -Una fracción de eyección inferior al 45% en miocardiopatía predominantemente izquierda identifica a pacientes con un mayor riesgo de sufrir arritmias ventriculares.
- Algunas de las familias estudiadas presentan una elevada prevalencia de cáncer. Esta combinación llevó al diagnóstico inicial erróneo de miocardiopatía dilatada secundaria a antraciclinas. La hipótesis de la exposición a antraciclinas como modulador de este fenotipo requiere ser investigada.
- El estudio genético demostró importantes diferencias entre ambos fenotipos: Mutationes que causan el truncamiento en desmoplakina y la mutación fundadora Europea *PLN p.R14del* fueron frecuentemente identificadas entre los pacientes afectados con miocardiopatía predominantemente izquierda. Estas mutaciones muestran una elevada penetrancia. Por otro lado, los pacientes afectados con miocardiopatía arritmogénica predominantemente derecha presentaron una mayor variedad mutacional. Muchos de ellos eran portadores de mutationes missense o variantes de significado incierto.
- La diferencia en las bases moleculares entre los dos fenotipos se aprecian también a nivel tisular. Estos hallazgos se descubrieron en el estudio inmunohistoquímico de algunos importantes componentes de la unión intercelular.
- Un patrón inmunohistoquímico normal de Cx43 (uno de los componentes de las gap junctions y uno de los elementos clave para el intercelular coupling) y de en plakoglobina (una proteína desmosómica, responsable del anclaje mecánico y regulados de importantes vías de señalización) se observó en las muestras de miocardiopatía predominantemente izquierda. Este hallazgo contrasta con el remodelado frecuentemente observado en muestras provenientes de miocardiopatía predominantemente derecha.

-La reducción en la expresión de plakoglobina y Cx43 en la unión intercelular depende del fenotipo predominante de enfermedad.

-La redistribución de la plakoglobina (también conocida como ϒ-catenina) hacia el núcleo celular puede interferir con la actividad transcripcional regulada por el complejo β-catenina/T-cell factor/lymphoid enhancer factor. Sin embargo, el papel concreto de la interación β-catenina/ ϒ-catenina en la fisiopatología de la miocardiopatía arritmogénica debe ser aclarado.

-El acoplamiento eléctrico entre células vecinas depende de la integridad de las gap junctions. Además de la reducción en la expresión de la Cx43 y de Nav1.5 (principal componente del canal de sodio), los modelos de miocardiopatía arritmogénica muestran una reducción de las corrientes iónicas I_{Na} y I_{K1}, lo que puede afectar a la propagación del impulso eléctrico. La significación de estas alteraciones en la arritmogénisis de la enferemdad deberá ser investigada.

- **Objetivo 3**

-La afectación de ventrículo izquierdo en miocardiopatía arritmogénica ha sido considerado tradicionalmente un signo de enfermedad avanzada y signo de mal prognóstico, debido a la progresión a insuficiencia y arritmias ventriculares. Este estudio demuestra una incidencia de muerte súbita y arritmias ventriculares similar entre ambos fenotipos.

- Especial interés se prestó a aquellos pacientes portadores de DAI. En este grupo, la incidencia anual de terapias apropiadas fue similar. Solamente aquellos pacientes portadores de DAI en prevención secundaria o en aquellos en los que la indicación fue una arritmias ventricular sostenida bien tolerada o síncope sufrieron terapias durante el seguimiento.

-Este hallazgo es importante para optimizar la indicación de implante de DAI dada la elevada prevalencia de complicaciones (1 de cada 5 pacientes) relacionadas con el implante o detectadas durante el seguimiento.

-Es importante subrayar la asociación entre valores reducidos de DTI tricuspídeo (<10 cm/s) y la presencia de arritmias ventriculares durante el

seguimiento en pacientes portadores de DAI (tanto en casos de miocardiopatía predominantemente derecha como predominantemente izquierda). Esta asociación puede ser explicada por el hecho de que los fenotipos más severos (aquellos casos con mayor dilatación ventricular, mayor disfunción sistólica y afectación biventricular importante típicamente presentan DTI tricuspídeo reducido.

-Del mismo modo, los casos más severos presentaron más taquicardias ventriculares no sostenidas en los electrogramas. Sin embargo, este hallazgo no se asoció con una mayor incidencia de terapias apropiadas durante el seguimiento.

- **Objetivo 4:**

  -La identificación de signos precoces de enfermedad en familiares sanos es muy importante para la planificación del seguimiento cardiolólógico y la identificación de individuos potencialmente en riesgo.

  - Se diseñó un nuevo software para el análisis del ECG digital en estos pacientes y en sus familiares. El objetivo fue identificar signos de enfermedad precoz mediante el análisis detallado de la morfología del QRS, ST y onda T.

  -Se identificó la fragmentación del final del QRS asociada a una morfología plana o descendente del segmento ST en derivaciones inferolaterales como un hallazgo frecuente en portadores genéticos sanos. Por el contrario, la pendiente del segmento ST presenta una pendiente ascendente en el grupo control.

  -Esta sencilla herramienta diagnóstica permite optimizar el estudio familiar en miocardiopatía arritmogénica, señalando posibles potadores genéticos.

  - La hipótesis de la fragmentación final del QRS asociada a una morfología plana o ligeramente descendente del segmento ST como un signo que precede la inversión de la onda T deberá estudiarse en futuros estudios.

- El tercer capítulo estudia las bases genéticas de la miocardiopatía arritmogénica y su correlación fenotípica. La investigación se centra en tres objetivos: el estudio genotipo-fenotipo en portadores de mutaciones que originan truncamientos en desmoplakina, la identificación de nuevos genes involucrados en miocardiopatía arritmogénica y la correlación genotipo-fenotipo de la mutación fundadora *PLN p.R14del.*
- Objetivo 5:

  -Se centra en la caracterización clínica de la miocardiopatía predominantemente izquierda causada por la una mutación no descrita previamente en *DSP (p.Q447*)* y en todas la mutaciones de truncamiento en desmoplakina descritas hasta la fecha.

- La desmoplakina se encuentra formada por tres dominios funcionales: cabeza, cuerpo y cola. El dominio carboxi-terminal (cola) es reponsable de la unión a los filamentos intermedios de desmina, mientras que el amino-terminal (cabeza) es responsable de la unión a la plakoglobina y plakofilina-2. El cuerpo es responsable de la formación de los dímeros de desmoplakina.

  -Esta mutación origina una sustitución C por T en el exón 11 del gen *DSP* lo que origina un codón de stop. Esta alteración se produce en el extremo amino-terminal, produciendo un truncamiento del 85% de la longitud de la proteína. El aminoácido afectado se encuentra en la posición 447, en la cabeza de la proteína. Como se ha señalado anteriormente, el análisis inmunohistoquímico realizado en biopsia endomiocárdica demostró un patrón normal de plakoglobina, desmoplakina, Cx43 y desmina. La presencia de patrón normal de desmoplakin sugiere un efecto dominante-negativo de esta mutación.

  -Los truncamientos de desmoplakina, independientemente de su posición en la proteína se comportan agresivamente causando una miocardiopatía generalmente precoz, que asocia elevada prevalencia de arritmias ventriculares y muerte súbita.

-El diagnóstico de miocardiopatía predominantemente izquierda causada por un truncamiento en desmoplakina debería ser sospechada en presencia de miocardiopatía dilatada y/o miocardiopatía no compactada asociada a arritmias ventriculares e historia familiar de arritmias ventriculares o muerte súbita. Esta miocardiopatía presenta frecuentemente inversión de la onda T en derivaciones inferolaterales y realce tardío en la resonancia magnética cardiaca.

-El reconocimiento de miocardiopatía arritmogénica predominantemente izquierda causada por *DSP p.447** u otras mutaciones de truncamiento en desmoplakina es importante debido a la elevada incidencia de arritmias ventriculares que presentan estos pacientes. Por este motivo, el implante de un DAI en prevención primaria debe ser considerado antes de alcanzar la disfunción sistólica severa.

- **Objetivo 6**

- La utilización de Next Generation Sequencing permite la identificación de nuevos genes involucrados en el desarrollo de miocardiopatías. Por vez primera se describe una mutación (*LDB3 p.T351A)* in Cypher/ZASP, una proteína sarcomérica de la línea Z, en una familia afectada por miocardiopatía arritmogénica predominantemente derecha.

  -Esta mutación de tipo missense se localiza en el exon 7 y ha sido previamente asociada al desarrollo de miocardiopatía dilatada y miocardiopatía no compactada.

  -Cypher/ZASP se une a α-actinina en la línea Z del músculo esquelético y cardiaco y desempeña un importante papel en el mantenimiento de la estructura celular. Además de ser causa de miocardiopatía, mutaciones en este gen pueden producir afectación neuromuscular.

  -El mecanismo patogénico por el que esta mutación en *LDB3* causa miocardiopatía arritmogénica requiere ser investigado. Este hallazgo subraya la complejidad de la base genética de la miocardiopatía arritmogénica al ser este un nuevo gen no desmosómico involucrado en esta enfermedad.

-*LDB3* debería ser incluido en el panel de genes a estudio en casos de miocardiopatía arritmogénica.

- **Objetivo 7**

  - Se identifica por primera vez una familia española afectada por miocardiopatía arritmogénica causada por la mutación Europea fundadora *PLN p.R14del*.

  -Fosfolamban es una proteina formada por 52 aminoácidos que regula la función de la bomba transportadora de calcio (SERCA2a) en el retículo sarcoplásmico del musculo cardiaco. Esta proteína inhibe SERCA2a en su forma no fosforilada. Esta inhibición cesa con su fosforilación. Fosfolamban es esencial en la regulación de la homeostasis del calcio y en la regulación del gasto cardiaco ante la respuesta del estímulo β-adrenergico.

  - *PLN p.R14del* causa una delección que no afecta al marco de lectura. Esta mutación causa miocardiopatía arritmogénica, miocardiopatía dilatada con frecuente afectación biventricular.

  -El estudio de microsatélites sugiere la existencia de un ancestro común Europeo. Esta familia es un ejemplo claro de la variabilidad fenotípica observada entre familiares y enfatiza el concepto de miocardiopatía arritmogénica como un amplio espectro de enfermedad.

  -La presencia de QRS de bajo voltaje en pacientes afectados con miocardiopatía arritmogénica es un “signo de alerta” que sugiere una posible mutación en *PLN*.

- El cuarto capítulo **(Objetivo 8)** describe las bases genéticas de la inflamación en la miocardiopatía arritmogénica, así como sus implicaciones clínicas. Desde un punto de vista clínico, es importante reconocer los siguientes puntos de interés:

  -La miocarditis puede ser la primera manifestación clínica de la miocardiopatía arritmogénica. Este hecho debe ser tenido en cuenta especialmente cuando se la miocarditis se produce en familiares afectados por miocardiopatía arritmogénica.

- Estos miocarditis-like episodios manifiestan una progresión de la enfermedad y pueden acompañarse de empeoramiento de la función sistólica, aumento del patrón de realce tardío en la resonancia magnética o arritmias ventriculares.

-La concentración de episodios de miocarditis entre familiares portadores de la misma mutación sugiere una vulnerabilidad miocárdica determinada genéticamente. La etiología de estos episodios (origen vírico versus degeneración miocárdica primaria deberá ser investigada).

- El estudio microbiológico de tejido miocárdico no demostró la presencia de ADN viral.

-El estudio inmunohistoquímico demostró la presencia de linfocitos CD4+ y macrófagos CD68+ en el infiltrado inflamatorio. No se identificaron linfocitos CD8+. Este patrón inflamatorio es similar al encontrado en las reacciones de hipersensibilidad tipo IV.

-El reconocimiento de que la miocarditis puede ser la manifestación inicial de una miocardiopatía arritmogénica es importante no sólo desde el punto de vista clínico, sino también desde el punto de vista anatomopatológico:

- La presencia de una miocarditis linfocítica de afectación predominantemente derecha debería hacer sospechar el diagnóstico de miocardiopatía arritmogénica en un estadío precoz, antes que se produzca la sustitución fibroadiposa. En este contexto, el remodelado de plakoglobina y Cx43 de las uniones intercelulares sugiere el diagnóstico de miocardiopatía arritmogénica derecha.
- El remodelado de plakoglobina ha sido descrito en casos de miocarditis altamente arritmogénica como la miocarditis de células gigantes y la sarcoidosis cardiaca. Sin embargo, esta alteración no ha sido descrita en miocarditis linfocítica.

CONCLUSIONS

1. The survival free from sudden death in arrhythmogenic cardiomyopathy patients is significantly lower compared with that found in the most prevalent cardiomyopathies (hypertrophic cardiomyopathy and dilated cardiomyopathy).

2. An right ventricle/left ventricle end-diastolic volume ratio <1 in cardiac magnetic resonance, a mildly dilated left ventricle, a high prevalence of late gadolinium enhancement and left ventricular non compaction were distinctive features in left dominant arrhythmogenic cardiomyopathy.

3. The reduced signal for plakoglobin and connexin-43 appears to track with the predominant phenotype of the disease.

4. The incidence of sudden death and sustained ventricular arrhythmias is similar in both phenotypes of arrhythmogenic cardiomyopathy.

5. In view of the high prevalence of sustained ventricular arrhythmias in patients harbouring *DSP p.Q447** and other desmoplakin truncating mutations, the indication of an ICD should be considered before reaching a severe systolic dysfunction.

6. LDB3 is a new non desmosomal gene involved in arrhythmogenic cardiomyopathy

7. The European founder mutation *PLN p.R14del* has been identified in a family in Murcia. The presence of low QRS voltages in families affected with arrhythmogenic cardiomyopathy is a red flag of this mutation.

8. Myocarditis may be the first clinical presentation of arrhythmogenic cardiomyopathy. It reflects an active phase of the disease and it is usually associated with ventricular arrhythmias and worsening of the systolic function.

9. Immunohistochemistry of the intercellular junctions indentifies cases of arrhythmogenic cardiomyopathy amongst cases of lymphocytic myocarditis circumscribed to the right ventricle.

CONCLUSIONES

1. La supervivencia libre de muerte súbita en pacientes afectados con miocardiopatía arritmogénica es significativamente inferior que en la encontrada en pacientes diagnosticados con otras miocardiopatía más comunes (miocardiopatía hipertrófica y miocardiopatía dilatada)

2. Una relación entre el volumen telediastólico ventrículo derecho y de ventrículo izquierdo <1 en la resonancia magnética cardiaca, una dilatación leve de ventrículo izquierdo, una elevada prevalencia de realce tardío y la no compactación del ventrículo izquierdo fueron hallazgos típicos de la miocardiopatía arritmogénica predominantemente izquierda.

3. La alteración de la expresión de la plakoglobina y de la conexina-43 depende del fenotipo de miocardiopatía arritmogénica, siendo un hallazgo habitual en las formas predominantemente derechas.

4. La incidencia de muerte súbita y de arritmias ventriculares sostenidas es similar en ambas formas de miocardiopatía arritmogénica.

5. Dada la elevada la elevada incidencia de arritmias ventriculares y muerte súbita en pacientes portadores de *DSP p.Q447** y otras mutaciones de truncamiento en desmoplakina, la indicación de desfibrilador debería considerarse antes de alcanzar una disfunción ventricular severa.

6. *LDB3* es un nuevo gen no desmosómico involucrado en el desarrollo de miocardiopatía arritmogénica.

7. La mutación fundadora Europea *PLN p.R14del* ha sido identificada en Murcia. La presencia de bajos voltajes en el ECG es una característica distintiva de esta mutación.

8. La miocarditis puede ser la primera manifestación de una miocardiopatía arritmogénica. Es signo de actividad de la enfermedad y en ocasiones se asocia a arritmias ventriculares o deterioro de la función sistólica.

9. El estudio inmunohistoquímico de las uniones intercelulares permite identificar casos de miocardiopatía arritmogénica entre casos de miocarditis linfocítica del ventrículo derecho.

PUBLICACIONES

Arrhythmogenic right ventricular cardiomyopathy

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See Online for appendix

A 21-year-old white semiprofessional footballer presented in April, 2012, with recurrent palpitations and chest pain on exertion. Medical and family history were unremarkable. ECG showed ventricular trigemini with left bundle branch block morphology, inferior axis, and late transition suggestive of right ventricular outflow tract origin, with normal repolarisation (figure). 5 days later, repeat ECG showed transient flat and notched T waves in left precordial leads (appendix). Blood tests including troponin, echocardiogram, and cardiac magnetic resonance (CMR) were normal. Signal averaged-ECG showed late potentials. The ventricular ectopics disappeared during an exercise test. Days later the patient had a non-sustained ventricular tachycardia associated with chest pain and light-headedness.

Idiopathic right outflow ventricular tachycardia was the most likely diagnosis but we had to rule out myocarditis[1] and arrhythmogenic right ventricular cardiomyopathy, a cause of malignant ventricular arrhythmias and sudden death.[2] Diagnosis of arrhythmogenic right ventricular cardiomyopathy is based on imaging, morphology of the ventricular tachycardia on ECG, number of ventricular ectopics on 24 h Holter monitoring, histology, genetics, and family history.[3] The next investigations are endomyocardial biopsy, an invasive procedure with a modest diagnostic yield, and genetic tests (positive in 50% of cases).[4]

On family screening the ECG of his asymptomatic 50-year-old father was highly suspicious of arrhythmogenic right ventricular cardiomyopathy (appendix). His echocardiogram (appendix) and CMR showed a very dilated right ventricular outflow tract and impaired right ventricle (ejection fraction 30%, fractional area change [FAC] 32%). He was diagnosed with arrhythmogenic right ventricular-cardiomyopathy. We started our patient on sotalol, and fitted an implantable defibrillator (ICD) because of newly documented sustained ventricular tachycardia. He gave up football and was discouraged from strenuous exercise. His father was started on β blockers. In May, 2013, our patient was found to have inverted T waves in leads V1–V3 (figure) and a non-dilated but impaired right ventricle (FAC 35%). Genetic testing did not show a causative mutation.

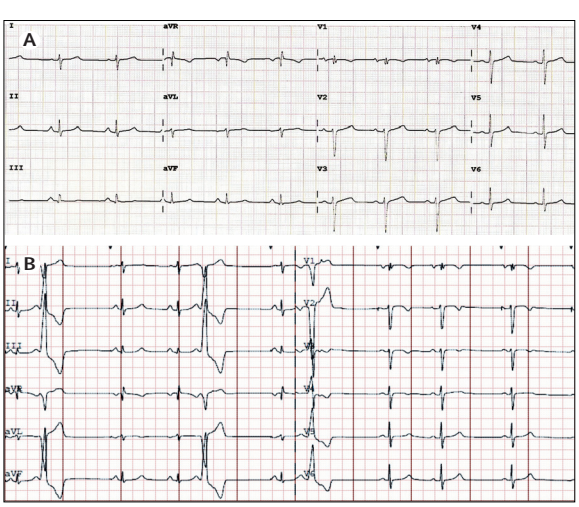

Figure: **Arrhythmogenic right ventricular cardiomyopathy**
(A) Admission ECG showing low voltage in the limb leads with normal repolarisation. (B) New T wave inversion in leads V1–V4, 1 year later.

Our patient met two major criteria for diagnosis of arrhythmogenic right ventricular cardiomyopathy (ECG, arrhythmogenic right ventricular cardiomyopathy in a first degree relative) and three minor criteria (>500 ventricular ectopics in 24 h, late potentials, and NS-VT of inferior axis). During a 2-year follow-up both patients have been stable. The proband remains on sotalol and his ICD has not had to treat any malignant ventricular arrhythmias. His father remains asymptomatic on β blockers.

Arrhythmogenic right ventricular cardiomyopathy ranges from overt to mild phenotypes (concealed stage) and sudden death can occur at any stage, sometimes in the more subtle forms of arrhythmogenic right ventricular cardiomyopathy. A very dilated right ventricle is not a strong predictor of ventricular arrhythmias.[5] Family screening is essential for the diagnosis of relatives. A positive family history increases the probability of the diagnosis from 1:5000 to 1:2[3] and allows diagnosis in mild phenotypes. Diagnosis of arrhythmogenic right ventricular cardiomyopathy can be challenging in athletes, in whom right ventricular dilatation and T-wave inversion in the right praecordial leads might be a physiological adaptation to exercise, particularly in black Caribbean athletes. Because sudden death is often the first presentation, early diagnosis in asymptomatic patients is essential. ECG is widely available and is therefore a valuable means of diagnosis. Special attention should be paid to T-wave inversion in leads V1–V3 or V5–V6, which should raise the suspicion of arrhythmogenic right ventricular cardiomyopathy.

Contributors
All authors contributed to management of the patient and writing the report. Consent to publication was obtained.

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Desmoplakin truncations and arrhythmogenic left ventricular cardiomyopathy: characterizing a phenotype

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Aims Risk stratification for sudden death in arrhythmogenic right ventricular cardiomyopathy (ARVC) is challenging in clinical practice. We lack recommendations for the risk stratification of exclusive left-sided phenotypes. The aim of this study was to investigate genotype–phenotype correlations in patients carrying a novel *DSP* c.1339C>T, and to review the literature on the clinical expression and the outcomes in patients with *DSP* truncating mutations.

Methods and results Genetic screening of the *DSP* gene was performed in 47 consecutive patients with a phenotype of either an ARVC ($n = 24$) or an idiopathic dilated cardiomyopathy (DCM), who presented with ventricular arrhythmias or a family history of sudden death ($n = 23$) (aged 40 ± 19 years, 62% males). Three unrelated probands with DCM were found to be carriers of a novel mutation (c.1339C>T). Cascade family screening led to the identification of 15 relatives who are carriers. Penetrance in c.1339C>T carriers was 83%. Sustained ventricular tachycardia was the first clinical manifestation in six patients and nine patients were diagnosed with left ventricular impairment (two had overt severe disease and seven had a mild dysfunction). Cardiac magnetic resonance revealed left ventricular involvement in nine cases and biventricular disease in three patients. Extensive fibrotic patterns in six and non-compaction phenotype in five patients were the hallmark in imaging.

Conclusion *DSP* c.1339C>T is associated with an aggressive clinical phenotype of left-dominant arrhythmogenic cardiomyopathy and left ventricular non-compaction. Truncating mutations in desmoplakin are consistently associated with aggressive phenotypes and must be considered as a risk factor of sudden death. Since ventricular tachycardia occurs even in the absence of severe systolic dysfunction, an implantable cardioverter-defibrillator should be indicated promptly.

Keywords Arrhythmogenic cardiomyopathy • Dilated cardiomyopathy • Sudden death • Implantable cardioverter-defibrillator • Desmoplakin

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary myocardial disorder clinically characterized by ventricular arrhythmias, sudden cardiac death (SCD), and end-stage heart failure. It is a common cause of SCD among young people and athletes,[1] and fatal arrhythmia may even occur in the absence of significant ventricular remodelling.

Arrhythmogenic right ventricular cardiomyopathy was thought to primarily affect the right ventricle (RV) although in a proportion of cases, the disease has been shown to affect the left ventricle (LV) not only at the end stage but also as a primary target. In this scenario, isolated arrhythmogenic left ventricular cardiomyopathy (ALVC) is frequently misdiagnosed as dilated cardiomyopathy (DCM) or myocarditis.[2,3]

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What's new?

- We identified a new desmoplakin mutation (*DSP* c.1339C>T) associated with a severe phenotype of arrhythmogenic cardiomyopathy and a high burden of ventricular arrhythmia.
- Left ventricular non-compaction with high personal and familiar arrhythmic burden should arouse suspicion towards desmosomal disease.
- Vast majority of desmoplakin truncated mutations reported in the literature are associated with severe phenotypes.

Mutations in genes encoding desmosomal proteins (desmoplakin, plakophilin-2, plakoglobin, desmocollin-2, and desmoglein-2)[4] were first identified in classical ARVC and were later found in left dominant forms.[5,6] Other non-desmosomal genes have recently been associated with arrhythmogenic cardiomyopathy (ACM).[7]

The aim of this study was to investigate genotype–phenotype correlations in patients carrying a novel *DSP* c.1339C>T, and to review the literature on the clinical expression and the outcomes in patients with *DSP* truncating mutations.

Methods

The study was approved by the Ethics Committee of our hospital (Virgen de la Arrixaca University Hospital, Murcia) and it complied with the ethical principles of the Declaration of Helsinki. All participants gave their written consent. A genealogical pedigree of each family was obtained. Clinical assessment consisted of a 12-lead digital electrocardiogram (ECG), a signal-averaged ECG (SA-ECG), a 2D-Doppler echocardiogram, cardiac magnetic resonance (CMR), an exercise test, and 24 h Holter monitoring. In addition, an electrophysiological study (EPS) was performed in three patients. Patients were followed up annually [median 26 (13–87) months].

Study population

The population studied included 47 unrelated patients [29 (62%) males, aged 40 ± 19 years]. Twenty three (49%) met the criteria for ARVC and 24 for DCM, showing either sustained ventricular tachycardia (S-VT) or a history of SCD in a first- or second-degree relative under 35 years of age. We chose a DCM population with such a particular aggressive arrhythmic burden that arouse the suspicion of arrhythmogenic left ventricular cardiomyopathy as the first diagnosis to be considered.

A genetic study of the five desmosomal genes (*DSP, DSG2, DSC2, PKP2, PKG), PLN*, and *LDB3* was performed in the 47 patients. A novel *DSP* c.1339C>T mutation was identified in three unrelated probands. First-degree relatives from these three families were invited to participate in a clinical and genetic investigation. Fifteen relatives were found to carry *DSP* c.1339C>T. Two additional patients previously diagnosed with DCM, mild systolic dysfunction, and ventricular arrhythmia died before the study started and in light of their family history, they were considered to be obligatory carriers.

A LV end-diastolic diameter (LVEDd) of >117% from normal and/or LV systolic impairment (<45%) was used for the diagnosis of DCM in probands. Familial criteria were applied in the remaining carriers.[8] Arrhythmogenic right ventricular cardiomyopathy diagnosis was made according to the revised Task Force Criteria (TFC).[9] Jenni *et al.*[10] criteria were employed for the definition of left ventricular non-compaction (LVNC).

Genetic analysis

Deoxyribonucleic acid was extracted from peripheral blood samples using the Maxwell 16 Blood Purification kit. Previously published *DSP, DSC2, DSG2, PKP2, PKG, PLN* primer sequences were used for amplification. Results were compared with the sequences available in Gen Bank (RefSeq: NC_000006.11) and analysed by SeqScape software (Applied-Biosystem).[11] MYBPC3 and MYH7 were studied in two selected individuals from families with *DSP* c.1339C>T with LVNC phenotype. *DSP* c.1339C>T variant was absent in 500 Spanish control samples and was not present in the 5000 exome project [Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP) (URL: http://evs.gs.washington.edu/EVS/) [1/12/2013].] Four published markers that flank the *DSP* gene were used to investigate the presence of a potential founder effect in carriers of *DSP* c.1339C>T. All of them are located within the DSP gene. Microsatellite 1 (Des.mic.1) is located in intron 1 and Microsatellite 2 (Des.mic.23) is located in intron 23 of the *DSP* gene. Both of them were indentified in a sequence of a BAC clone mapping to 6p23-p24 (GeneBank accession no. AL031058). The primer sequences for Des.mic 1 and 3 are as follows:

Des.mic.1 forward, 5′-CCCATCTATGCATAATGCAACC-3′; reverse, 5′-GTCCTCACGGATGTGCTACAAG-3′

Des.mic.23 forward, 5′-CGCTTTTGATCATGGCCCTAGTG-3′; reverse, 5′-CTCACCTGTTACAGCTAGATG-3′′′

Immunohistochemistry

We analysed endomyocardial biopsy (EMB) samples from right ventricular (RV) septum in one case (C.III.12). They were formalin fixed, paraffin embedded, cut at a thickness of 4 mm, and mounted on slides. We incubated the slides with a primary antibody [monoclonal mouse anti-N-cadherin (1 : 400) (Sigma); monoclonal mouse anti-plakoglobin (1 : 1000) (Sigma); monoclonal rabbit anti-connexin 43 (1 : 400) (Sigma) and monoclonal rabbit anti-desmin (1 : 200)(Abcam)]. The slides were incubated with secondary goat anti-mouse or goat anti-rabbit (1 : 400) (Jackson ImmunoResearch). Stained slides were checked using a confocal microscope (LSM510, Meta Zeiss). Findings were compared with a control sample from a patient deceased for no cardiovascular reason in which necropsy did not show any evidence of cardiac disease. Case and control samples were fixed and embedded according to the same protocol and stained under similar conditions.

Electrocardiogram monitoring tests

One or more abnormal parameters in SA-ECG were considered as pathological (MAC 5500 ECG Diagnosis System—GE Healthcare). Patients with right bundle branch block (RBBB) or left bundle branch block (LBBB) were excluded from the SA-ECG analysis. Tracings from ambulatory 24 h Holter monitoring (Marquette Electronics and Synetec, ELA Medical) and treadmill exercise tests (Marquette Electronics, Inc. and General Electric T-2100) were reviewed. Non-sustained ventricular tachycardia (NS-VT) was defined as the presence of three or more ventricular beats at a rate of >100 b.p.m. Sustained ventricular tachycardia was defined as VT lasting >30 s or lasting less if it was poorly tolerated.

Echocardiogram

All participants underwent 2D-Doppler echocardiogram (Hewlett-Packard Sonos 7500, Hewlett-Packard). The standard analysis of the LV included the measurement of end systolic and end diastolic diameters and volumes, ejection fraction, wall motion abnormalities, and

pathological valvular flows. Right ventricle analysis consisted of outflow tract diameter calculations (measured on both the long and short axis) and in order to assess RV performance, TAPSE, Doppler tissue imaging (DTI) and the fractional shortening ratios were both measured.

Cardiac magnetic resonance

Cardiac magnetic resonance was performed on 10 *DSP* c.1339C>T carriers using a 1.5T magnet (Achieva CV, Philips Medical Systems). SSFP end-expiratory breath-hold cine imaging was acquired. After a bolus injection of Gadobutrol (0.1 mmol/kg, Gadovist; Bayer Shering Pharma, Berlin, Germany) the T1 measurement was calculated using standard late gadolinium enhancement (LGE) sequences. The analysis was performed using a personal computer and semi-automated software (Philips, Software work space 2.6.3.2). Five patients were excluded from this analysis [three already carried an implantable cardioverter-defibrilator (ICD) and two rejected the test].

Results

A novel heterozygous *DSP* variant (c.1339C>T) inherited in an autosomal dominant manner with high penetrance (83%) was identified in 3 patients. The mutation was a C to T transition at exon 11 in the *DSP* gene leading to a premature stop codon. This change occurred at the N-terminal region, and it is thought to generate a truncated peptide (85% in length). The affected amino acid at position c.1339C>T is located in one of the globular head domains of desmoplakin. This domain is shown to mediate the interaction of desmoplakin with two catenin proteins: plakoglobin and plakophilin-2[12] (*Figure 1*). The microsatellite study suggested a founder effect in the three families and the presence of a common ancestor.

Family A

The 37-year-old proband (A.III.5) (*Figure 2*) was diagnosed after an episode of S-VT at rest. An echocardiogram demonstrated severe LV dilatation and systolic impairment. The patient had a history of longstanding alcohol drinking, smoking, and occasional cocaine use. The angiogram excluded coronary lesions and an ICD was implanted.

His mother (A.II.3) had died suddenly at 67 years of age. She had been diagnosed with DCM after ruling out coronary disease. The maternal grandfather suffered from heart failure and died in his sleep at the age of 37 years (A.I.1). A maternal aunt had previously died of heart failure (A.II.2).

Prior to the proband's admission, a cousin aged 55 years (A.III.1) who presented with recurrent S-VT and a structurally normal heart underwent ICD implantation. Familial cascade screening led to a new diagnosis of four asymptomatic, though affected, individuals (A.III.4, A.III.6, A.IV.6, and A.IV.7). It should be noted that the diagnoses of A.III.4 and A.III.6 were LVNC. A severely increased LGE pattern was observed in A.IV.6 and A.IV.7 alongside mild systolic dysfunction. During the follow-up period, both siblings have suffered multiple episodes of chest pains with troponin rise. Coronary artery disease was ruled out in both cases. They had an ICD implanted for primary prevention in the view of progressive systolic dysfunction.

Family B

The proband (B.II.1), a 42-year-old woman, was admitted after a presyncopal S-VT episode. Echocardiography revealed global left ventricular hypokinesia and a moderately impaired systolic function.

The mother (B.I.1), aged 72 years, had a history of presyncopal episodes associated with S-VT. An echocardiogram showed severe systolic impairment and angiography ruled out coronary disease. She underwent an EPS where poorly tolerated S-VT of a different morphology was induced. Both patients received an ICD.

A maternal aunt had previously died suddenly at the age of 40 years. Family genetic screening led to diagnosis of an asymptomatic non-affected carrier: a sister (B.II.2) aged 50 years. The echocardiogram was normal and the patient rejected CMR due to claustrophobia. Four maternal aunts, one maternal uncle, and a nephew refused a cardiac and genetic study.

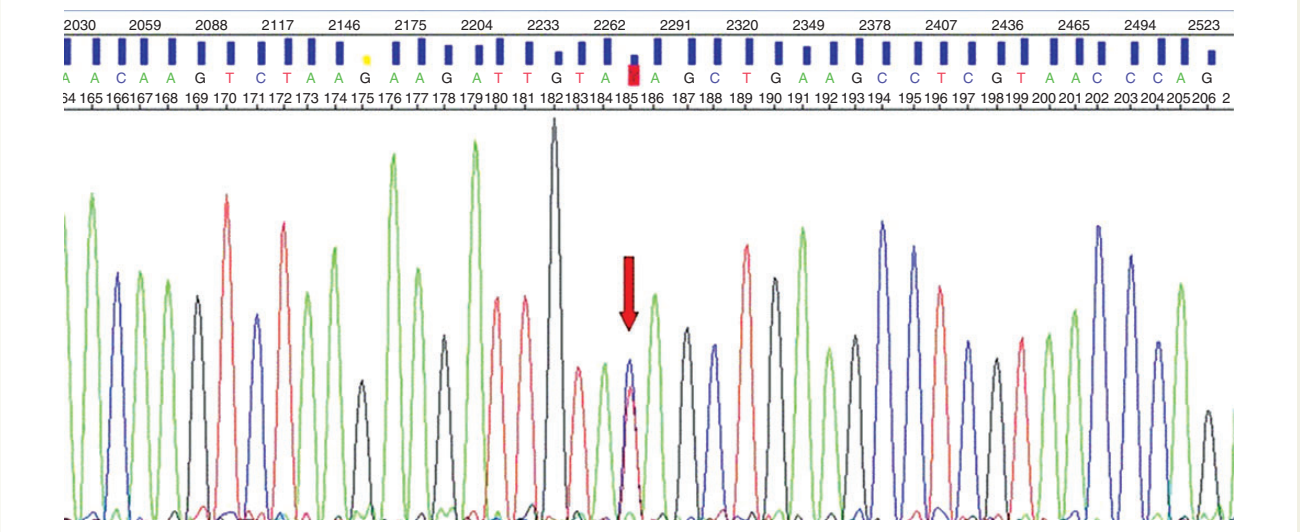

Figure 1 Electropherogram. The mutation was a C -> T transition at exon 11 in the *DSP* gene leading to a premature stop codon.

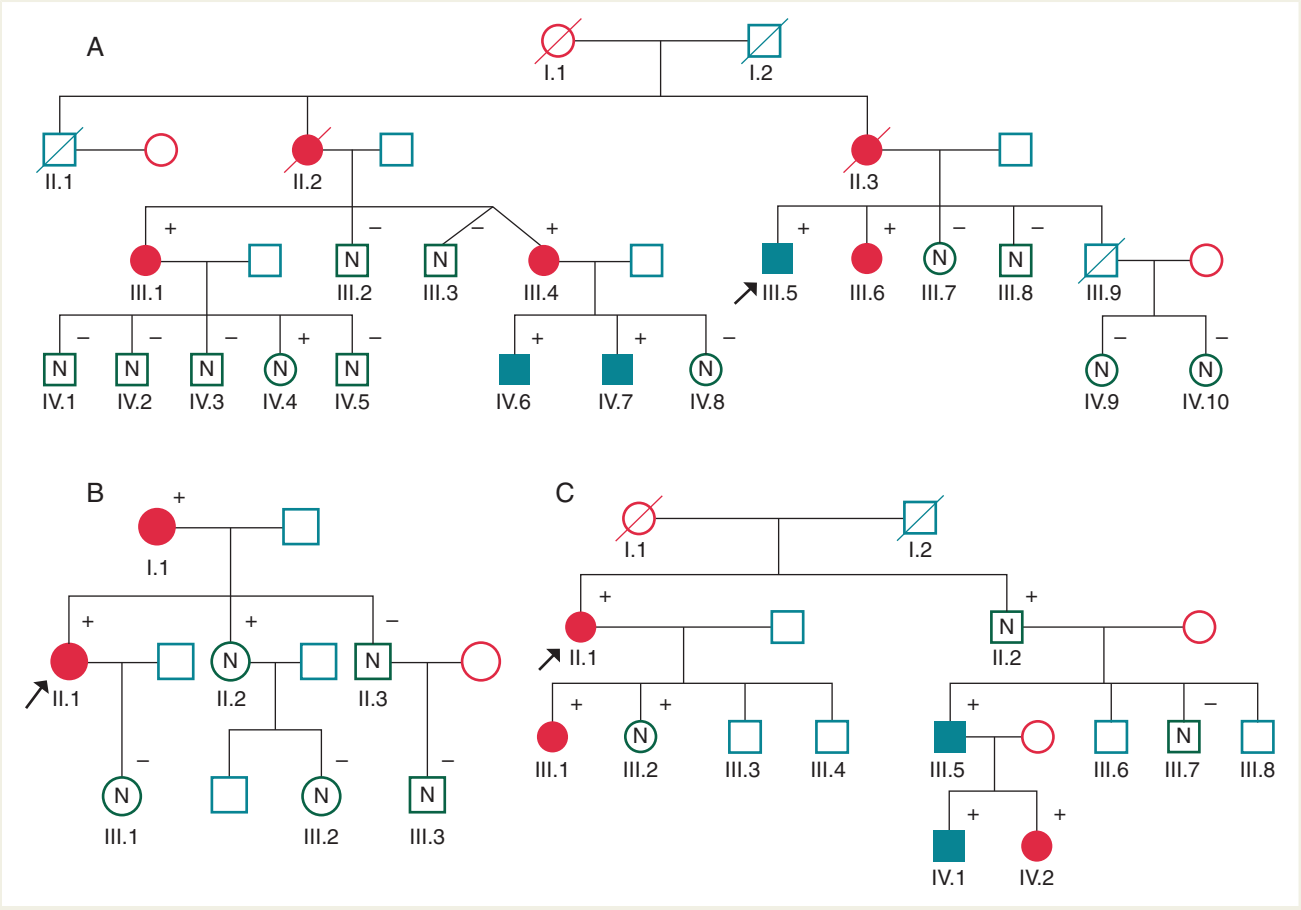

Figure 2 Family tree of families A, B, and C. Arrow points to probands. Black filled symbols stand for affected carriers.

Family C

The 62-year-old female proband was admitted to hospital after a first episode of acute heart failure (C.II.1). Echocardiography demonstrated moderately impaired systolic function and MRI results led to the diagnosis of LVNC.

His daughter (C.III.1) had suffered an episode of acute myocarditis at the age of 32 years.

Six years after the diagnosis of the proband, a 45-year-old nephew (C.III.5) presented with cardiac arrest while walking. He was diagnosed with LVNC and moderate systolic dysfunction. One month after having an ICD implanted, he received four shocks due to S-VT during mild exertion (*Figure* 5). Both his 13-year old son (C.IV.1) and his 17-year-old daughter (C.IV.2) were asymptomatic and they were diagnosed with LVNC in familial screening. The latter was found to have frequent ventricular ectopics and couplets on holter monitoring.

Clinical presentation and therapy

The first clinical presentation was presyncopal S-VT in five patients, resuscitated SCD in one, and heart failure in two patients. Cardiac and genetic work-up led to the identification of seven asymptomatic patients, as well as three non-affected asymptomatic carriers. Eight carriers met the criteria for DCM, four for LVNC and seven carriers also fulfilled the diagnostic criteria for the definitive diagnosis of ARVC (*Table* 1).

Beta-blockers were started in all patients. Nine patients with systolic impairment were on angiotensin-converting enzyme inhibitors or ARB-II blockers. Spironolactone was started in four patients. Five patients received an ICD for secondary prevention of sudden death (A.III.1; A.III.5; B I.1; B II.1; and C.III.5) and four for its primary prevention (A III.4; AIII.6; A.IV.6, and A.IV.7). Primary prevention was considered in the presence of any of the following events: moderate systolic impairment; frequent ventricular ectopic beats; and/or couplets on Holter monitoring despite beta-blocker administration. Two siblings (A.IV.6 and A.IV.7) had the ICD implanted for recurrent chest pains and troponin rise anlongside with worsening of the ejection fraction. During follow-up, two patients, B III.1 and C.III.5, had an appropriate ICD shock due to S-VT.

Twelve-lead electrocardiogram and signal-averaged electrocardiogram

Electrocardiograms from 16 mutation carriers were available for analysis. Nine (56%) showed T wave inversion in the precordial and/or frontal leads. Intraventricular conduction abnormalities were present in 4 patients (LBBB in two patients, bi-fascicular block in one, and non-specific conduction abnormalities in another case). A SA-ECG was performed in 14 patients (two had died before the study and two were excluded due to the existence of baseline wide QRS). Six showed late potentials (43%) (*Figure* 3).

Table 1 Clinical characterization of DSP c.1339 C>T carriers

Case	Family	Pedigree	Age/ Sex	Syncope	Sustained VT	Morphology VT	CL (ms)	Non sustained VT	NYHA	ARVC diagnosis	LVEF	LGE distribution	Hypertrabeculation (CMR)
1	A	II.2	59F	Yes	Yes	RBBB	330	Yes	I	Borderline	40	NA	NA
2	A	II.3	65F	No	No	NA	No	No	II	Possible	40	NA	NA
3	A	III.1	55F	No	Yes	NA	NA	No	I	Definitive	64	NA	NA
4	A	III.6	45F	No	No	No	No	No	I	Definitive	40	Inferoposterior and lateral	Yes
5	A	III.4	44F	No	No	NA	NA	Yes	I	Borderline	50	Global	Yes
6	**A**	**III.5**	**36M**	**No**	**Yes**	**NA**	**NA**	**Yes**	**I**	**Definitive**	**30**	**NA**	**NA**
7	A	IV.4	38F	No	No	No	No	No	I	Possible	60	No	NA
8	A	IV.6	14M	No	No	No	No	No	I	Possible	45	Lateral	No
9	A	IV.7	19M	No	No	No	No	No	I	Possible	55	Lateral	No
10	B	I.1	72F	No	Yes	LBBB. Axis −30°	320	Yes	II	Definitive	30	NA	NA
11	B	II.2	49F	No	No	No	No	No	I	Possible	58	NA	NA
12	**B**	**II.1**	**42F**	**No**	**Yes**	**NA**	**NA**	**No**	**I**	**Definitive**	**40**	**NA**	**NA**
13	**C**	**II.1**	**62F**	**No**	**Yes**	**NA**	**NA**	**Yes**	**II**	**Definitive**	**43**	**Inferoposterior and lateral**	**No**
14	C	III.5	45M	No	Yes	NA	220	No	I	Definitive	42	Global	Yes
15	C	II.3	73M	No	No	No	No	No	I	Borderline	60	No	No
16	C	III.1	38F	No	No	No	No	No	I	Borderline	60	No	No
17	C	IV.1	13M	No	No	No	No	No	I	Borderline	66	No	Yes
18	C	IV.2	17F	No	No	No	No	No	I	Borderline	60	No	Yes

F, female; M, male; CL, cycle length; NA, not available. Probands are in bold.

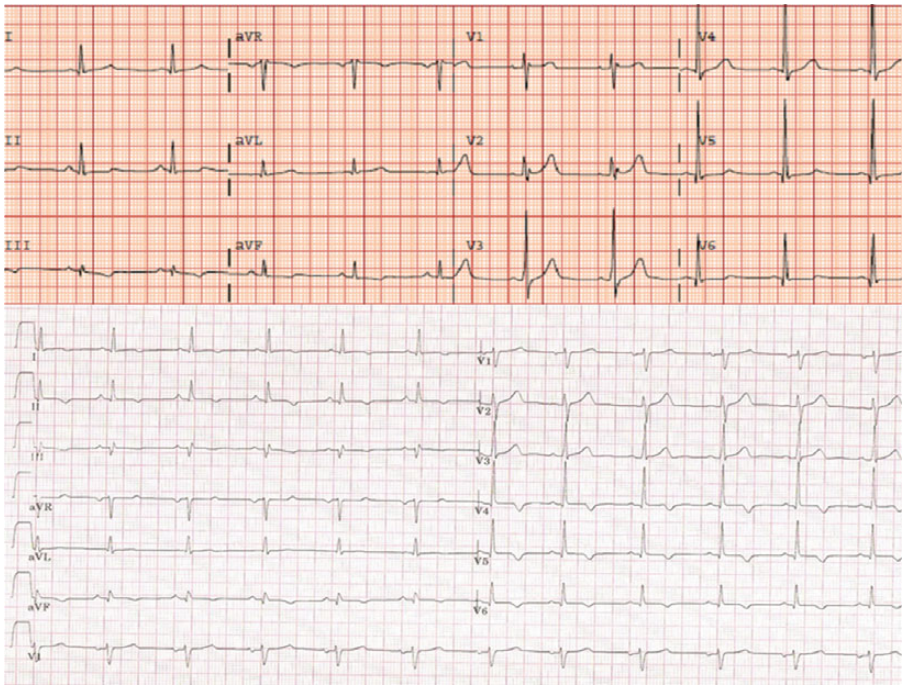

Figure 3 ECG from a LVNC patient (top) (C.III.5) and a DCM patient (bottom) (A.IV.7) T wave inversion in inferolateral leads was the most common abnormality in ECG.

Twenty-four hour electrocardiogram-Holter monitoring, an exercise test and ICD recordings

NS-VT was evidenced in four patients using 24 h Holter monitoring. Two additional patients had a NS-VT episode on ICD recordings, one of which had an episode of NS-VT while undergoing an exercise treadmill test. One patient suffered SVT one month after ICD implantation requiring four shocks to restore sinus rhythm. Frequent ventricular ectopics occurred during the treadmill test in two cases. Another young carrier without ECG or MRI abnormalities presented frequent ventricular ectopics and couplets.

Electrophysiological study

An EPS was performed on 3 patients, 2 of which (B.I.1 and B.II.1) were prior to ICD implantation. During the procedure the proband (B.I.1) developed S-VT different from the one previously recorded during hospitalization. Her daughter (B.II.1) developed S-VT during the EPS similar to the one originally recorded. Successful ablation was achieved. However, she did suffer a relapse 4 years later and further VT ablation was required. Endocardial voltage mapping using the CARTO navigator system in this later case showed no low voltage areas.

Echocardiogram

An echocardiographic study showed global or inferoposterior hypokinesia in 11 of 18 (61%) carriers. Two patients had severe systolic dysfunction and five had moderate systolic impairment. In three cases, LV apical trabeculae fulfilling the diagnostic criteria for LVNC were present. Neither RV enlargement nor dysfunction was detected in any case.

Cardiac magnetic resonance

Cardiac magnetic resonance was performed in 10 patients. Pathological findings were found in eight of them. Biventricular disease was observed in three patients and isolated LV involvement was present in six. Sub-epicardial myocardial fibrosis at the LV was the hallmark and it was detected in six patients. Fibrosis was distributed in the lateral wall in three cases, the inferoposterior wall in two, and it was globally distributed two more patients. Five patients were diagnosed with LVNC (*Figure 4*).

Immunohistochemistry

An EMB was analysed from C.III.5 during ICD implantation after cardiac arrest. Histology showed normal cardiomyocytes and no trace of either fibrofatty replacement, disarray, granuloma, inflammatory infiltrates or apoptosis (*Figure 5*). Staining for N-cadherin, desmoplakin, plakoglobin, connexin 43, and desmin was normal and no differences when compared the case with a control were observed (*Figure 6*).

Discussion

To date, 42 *DSP* mutations leading to premature termination of translation have been reported. There is clinical information available on 120 carriers from 44 families. Of these, 86 (72%) are affected, with

18 (15%) SCD cases reported in carriers of one of these mutations. In the 14 families where clinical cascade screening was possible, disease penetrance was >50% in ten families (71%) and >90% in seven (50%) (*Figure 7*).

Herein, we present the largest series of carriers of the same *DSP* truncating mutation reported to date. The microsatellite study suggests *DSP* c.1339C>T to have a founder effect. Several ARVC and/or ALVC families bearing truncating *DSP* mutations have been published, some of them, suffering SCD as a first clinical manifestation.[13–15] These studies have reported highly penetrant disease phenotypes, with predominant LV involvement, a high incidence of malignant arrhythmias, and a poor prognosis. In keeping with these studies, the novel mutation herein presented is also associated with high disease penetrance (83%), LV dysfunction, extensive fibrosis, mild/no LV enlargement, left/anterior precordial lead repolarization abnormalities, and high prevalence of ventricular arrhythmias. It is notable that myocardial fibrosis was present even in young carriers (14 and 19 year olds). Such extensive fibrosis at an early age is an uncommon finding in cardiomyopathies and particularly rare in heterozygous carriers of autosomal dominant mutations underlying ACM.

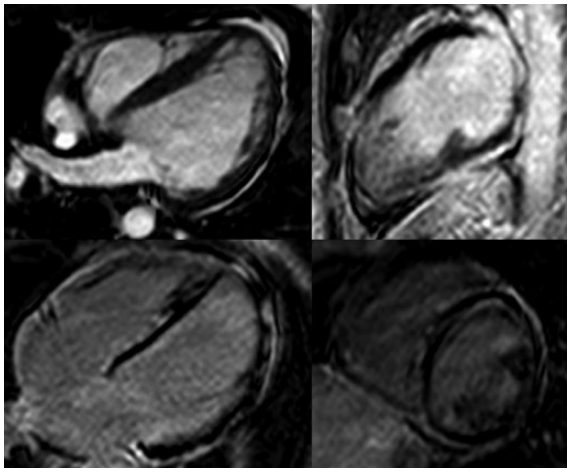

Figure 4 Magnetic resonance of a LVNC (C.III.5) (top) and DCM patient (A.IV.7)(bottom).

Characterizing a phenotype using an electrocardiogram

Six percent of the patients showed ECG abnormalities, even in the absence of overt structural disease. New diagnostic criteria for the diagnosis of ARVC include T wave inversion in V4–V6 as a marker of LV involvement. In the light of our results and those previously reported in other series[13] we would like to point out that T-wave inversion in inferior leads may be sensitive markers of ALVC even in the absence of ventricular remodelling. Signal-averaged ECG findings failed to correlate with ECG or imaging abnormalities.

Characterizing a phenotype with cardiac magnetic resonance

Heterogeneous myocardial fibrosis pattern evidenced by means of gadolinium on CMR has been associated with progression to heart failure in patients with hypertrophic cardiomyopathy, and, additionally, ventricular arrhythmias have been reported to be more frequent in patients with LGE in ischemic and idiopathic DCM.[16]

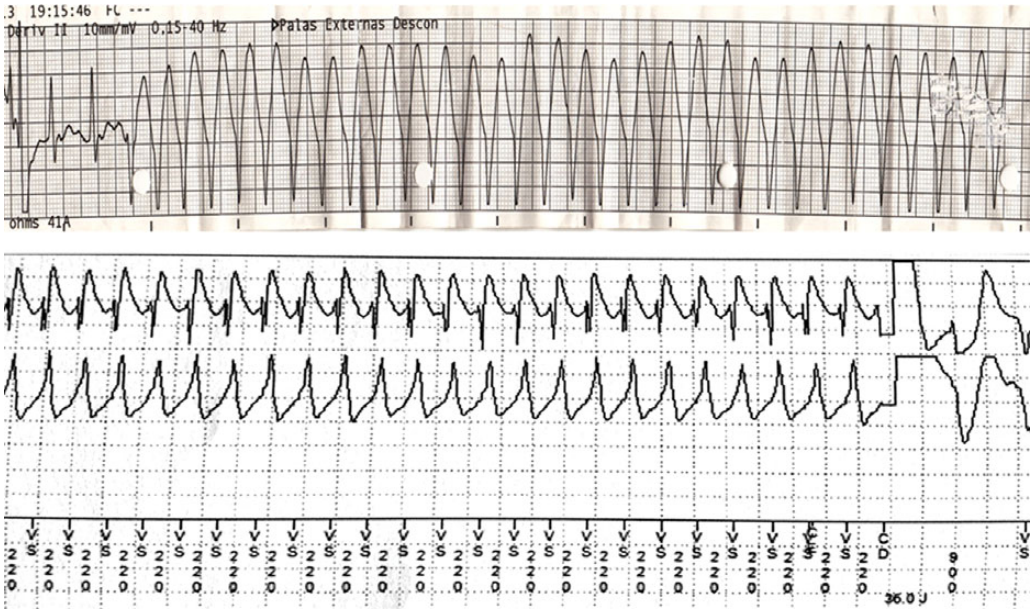

Figure 5 Sustained ventricular tachycardia (S-VT) recorded at the emergency department during resuscitation manoeuvres (top). Implantable cardioverter-defibrilator recordings showing S-VT and the beginning of another S-VT of a different morphology after shock delivery (bottom). Four shocks were necessary to restore sinus rhythm (C.III.5).

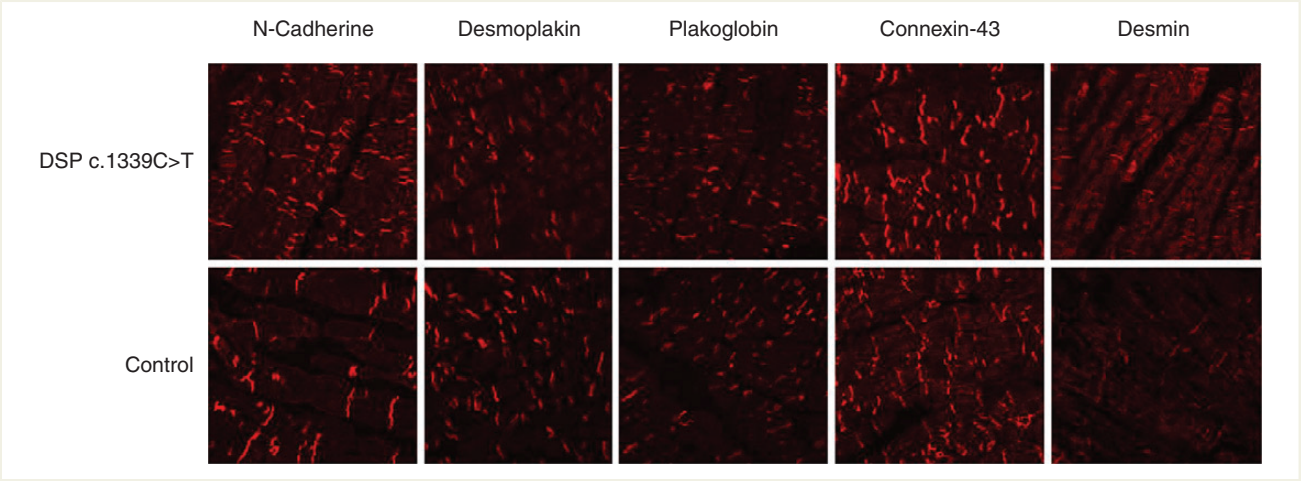

Figure 6 Immunohistochemistry from an EMB. No difference in the distribution of desmoplakin present in DSP c.1339C>T compared with the control. Plakoglobin, connexin-43 and desmin also show an identical distribution.

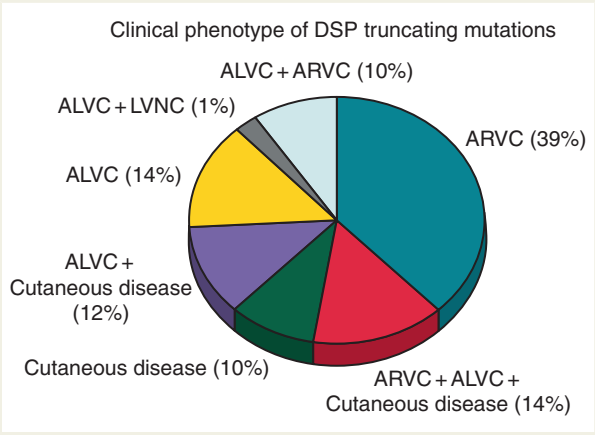

Figure 7 Clinical phenotypes of the 42 *DSP* truncating mutations reported to date. Plus indicates overlap syndrome.

The vast majority of the *DSP* c.1339C>T carriers showed extensive fibrosis in the LV, particularly at the sub-epicardium, similar to that seen in localized myocarditis. During a 'hot phase', ACM may mimic myocarditis in its clinical presentation or CMR appearance.[2,17] In our series, CMR was performed on clinically stable patients. Despite morphological findings being compatible with the diagnosis of LVNC in 5 patients, the presence of a truncating mutation in *DSP*, arrhythmic burden being out of proportion with the degree of systolic dysfunction and the family history led us to the diagnosis of ACM.

Implications for prevention of malignant arrhythmias and sudden death

Identifying individuals at high risk of SCD is of undisputed importance. Whether ALVC is associated with a poorer prognosis than ARVC or DCM remains a matter of debate. In terms of risk stratification in ARVC, several factors have been proposed over the past few years, the presence of which would prompt ICD implantation. These include resuscitated cardiac arrest, syncope, recurrent or syncopal S-VT, and severe RV or LV involvement.[18,19] Other markers such as family history of sudden death, induction of VT upon EPS, extensive bipolar low voltage area in RV,[20] fragmented potentials within the scar,[21] T wave inversion in three or more leads[22] and dispersion of depolarization and repolarization have been also reported.[23,24] On the other hand, primary ICD implantation in DCM is based almost exclusively on the severity of impairment of LV ejection fraction and functional status.[25] The decision on ICD implantation in ALVC poses a real challenge. To date, there is no general consensus in support of the use of genetic studies for ICD implantation. There is, however, no doubt that some genetic conditions such as laminopathies are associated with a high incidence of arrhythmic events and SCD, which may precede severe myocardial remodelling and dysfunction.

In recent years, the classic concept or ARVC has been broadened to the general concept of ACM. We have found a high prevalence of a LVNC-like phenotype in our families, leading us to recommend screening for *DSP* mutations in LVNC patients affected by a high burden of personal and familial arrhythmia. In this particular scenario, LVNC may be another phenotype to be considered in the broad spectrum of ACM.

Desmoplakin c.1339C>T: an unexpected immunohistochemistry pattern

Through its N-terminus, desmoplakin associates with armadillo proteins at the cell membrane. Through its C-terminus, it anchors intermediate filaments tethering them to junctional sites. Herein, we describe a novel truncating mutation in the N-terminal domain, anticipated to disrupt the synthesis of desmoplakin.

Confocal microscopy revealed no difference in the expression of desmoplakin, plakoglobin, connexine-43 and desmin compared with the control (*Figure 6*). It is notable that the hallmark in ARVC

immunohistochemistry; plakoglobin shifting from junctions[26] was not present. This finding is in keeping with a series of phospholamban mutation carriers where the plakoglobin distribution significantly differed in the DCM phenotype compared with the ARVC phenotype.[7]

This difference in plakoglobin remodelling leads us to rise the question whether the signalling pathways differs in ALVC and ARVC. Further studies will be required to shed light on the pathophysiology of this particular phenotype.

Conclusion

We have reported on a novel mutation in desmoplakin (c.1339C>T) associated with a phenotype of ALVC and LVNC. Truncating mutations in desmoplakin seem to consistently cause extensive LV fibrosis with near normal RV performance. Ventricular arrhythmias and SCD occur in the absence of overt LV dysfunction or dilatation, and as a consequence, ICD implantation must be considered promptly. Genetic information seems to be of paramount prognostic value in this setting.

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Conflict of interest: Dr Lorenzo Monserrat is shareholder of Health in Code S.L.

None of the authors have any conflict of interest to declare.

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Short Report

A mutation in the Z-line Cypher/ZASP protein is associated with arrhythmogenic right ventricular cardiomyopathy

Lopez-Ayala J.M., Genga M.O., Gomez-Milanes I., Lopez-Cuenca D., Ruiz-Espejo F., Sanchez-Munoz J.J., Oliva-Sandoval M.J., Monserrat L., Gimeno J.R. A mutation in the Z-line Cypher/ZASP protein is associated with arrhythmogenic right ventricular cardiomyopathy.
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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an important cause of malignant arrhythmia and sudden death particularly in young people. Although it is considered a desmosomal disease, mutations in non-desmosomal genes have also been identified. We report on a family where a mutation in LDB3 is associated with this condition. The index case and first and second degree relatives underwent a complete clinical evaluation: physical examination, electrocardiography (ECG), signal-averaged ECG, 2D echocardiogram, cardiac magnetic resonance and 24-h monitoring. After ruling out mutations in the five desmosomal genes, genetic testing by means of Next Generation Sequencing was carried out on the proband. A heterozygous missense mutation in LDB3 c.1051A>G was identified. This result was confirmed by subsequent Sanger DNA sequencing. Another six carriers were identified amongst her relatives. Three subjects fulfilled the criteria for a definitive diagnosis of ARVC and one reached a borderline diagnosis. In conclusion, this is the first family with ARVC where a mutation in LDB3 is associated with ARVC. Next generation sequencing arises as a particular useful tool to point to new causative genes in ARVC.

Conflict of interest

Dr Lorenzo Monserrat is shareholder of Health in Code S.L. The others authors have no conflicts of interest to declare.

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Key words: arrhythmogenic right ventricular cardiomyopathy – Cypher/ZASP – LDB3 – next generation sequencing

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a familiar cardiomyopathy inherited in a dominant trait which is characterized by a high incidence of ventricular arrhythmia and sudden death, particularly amongst young patients and athletes (1). In some cases, sudden death is the first clinical presentation and it is usually diagnosed in the post-mortem study. ARVC not only affects the right ventricle (RV) but also the left ventricle (LV) even as a primary target. Despite being initially considered as a merely desmosomal disease caused by mutations in any of the five desmosomal genes, recent investigations have reported that other non-desmosomal genes (such as TMEM43 (2), PLN (3), desmin (4), lamin A/C (5), titin (6) and CTNNA3 (7), TGF-β3 (8) and RyR2 (9)) are also involved in the development of ARVC. Identification of a causative heterozygous mutation is only reached in the order of 50% of the cases (10, 11).

Herein we report on a family where a mutation in *LDB3* (Lim-domain binding protein 3) is linked to the condition.

Material and methods

The proband and the first and second degree relatives keen to be screened underwent a complete clinical

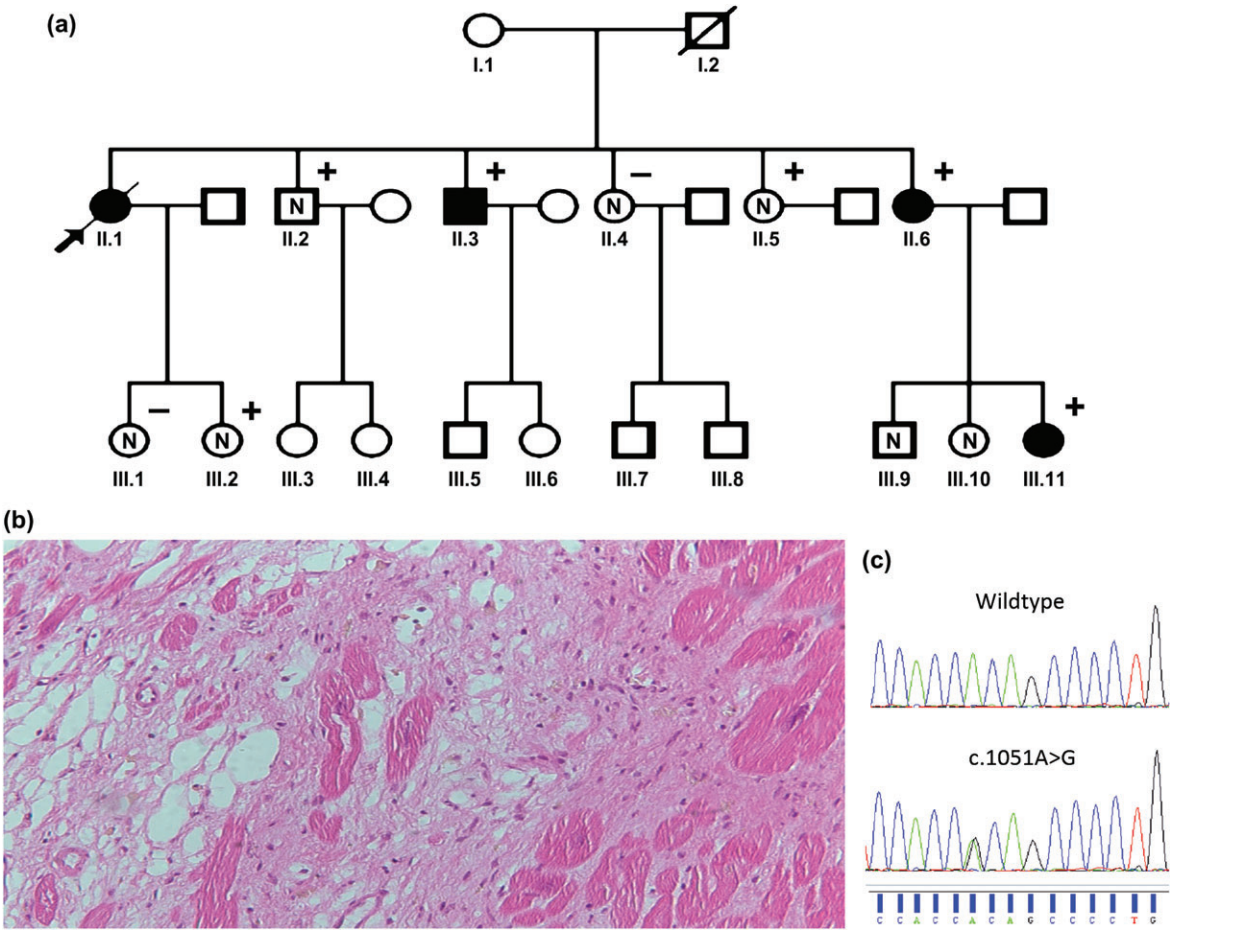

Fig. 1. (a) The black arrow points to the proband. Black filled signals stand for affected carriers; + stand for carriers. (b) Histology from the free lateral wall of the right ventricle (RV). There is important myocardial degeneration, fibro-fatty replacement and lymphocytic inflammatory patches. (c) The electropherogram shows the partial sequence of exon 7 of the LBD3 gene.

evaluation which consisted of a 12-lead digital ECG, a signal averaged ECG (SA-ECG), a 2D-doppler echocardiogram, cardiac magnetic resonance (CMR), an exercise test and 24-h Holter. Genetic testing was approved by the Ethics Committee of our hospital. All participants gave their written consent. DNA was extracted from peripheral blood samples using the Maxwell 16 Blood Purification Kit (Promega, Madison, WI). Sanger DNA sequencing failed to find a disease-causing mutation in any of the five desmosomal genes in the proband (DSP, JUP, DSC-2, DSG-2, PKP-2). We performed Next Generation Sequencing (NGS) with a selection of 134 related and non-related genes in order to discover another hypothetical disease-causing gene (Table S1, Supporting Information). These genes are included in a commercial Inherited Cardiac Disease library. Pathogenicity of the identified variants was assessed by means of three softwares (Polymorphism Phenotyping-2, P-Mut and Sorting Intolerant from Tolerant) and genotype–phenotype correlation.

Results

A missense heterozygous mutation (*c.1051A > G*) was identified in LDB3. Sanger DNA sequencing was used to confirm the presence of this variant in the proband. Bidirectional sequencing was performed using BigDye v1.1 chemistry in an ABI3130 analyser (Applied Biosystems, Foster City, CA). The sequence of the patient was compared with the *LDB3* sequence in the National Center for Biotechnology Information (NCBI) sequence database (NM_001080114.1). We aimed to assess its segregation within the family as well as its genotype–phenotype correlation.

The proband was a caucasian 45-year-old female with a long-standing medical history of presyncope. She required medical attention after suffering a syncopal sustained ventricular tachycardia that required electrical cardioversion to restore sinus rhythm. Her electrocardiography (ECG) showed complete right bundle branch block (RBBB) and inverted and flat T waves in precordial leads. An echocardiogram and CMR showed biventricular dilatation as well as severe biventricular systolic dysfunction. Midwall late gadolinium enhancement (LGE) affecting the LV was also identified (Fig. 2). In the view of these findings, a definitive diagnosis of ARVC was made. An angiogram ruled out coronary artery disease and an implantable cardioverter defibrillator (ICD) was recommended. Tragically, she

Table 1. Clinical features of *LDB3 c.1051A > G* carriers

Number	Pedigree ID	Sex	Age at diagnosis	ARVC criteria (Task Force Criteria 2010)	Symptoms	ECG	SA-ECG	CMR	VT	Ventricular ecopics
1	II.1	Female	45	Definitive	Syncope	RBBB + ITW	NA	Biventricular dilatation and failure	Yes	>500
2	II.6	Female	43	Definitive	Palpitations	ITW inferior and RPL	3/3	RV dilatation and failure	No	<200
3	II.3	Male	57	Definitive	No	Incomplete RBBB	3/3	RV dilatation and failure	No	<200
4	III.11	Female	17	Definitive	No	IWT in RPL	0/3	Normal	No	<200
5	II.2	Male	54	Possible	No	Normal	0/3	Normal	No	<200
6	II.5	Female	59	Borderline	No	Normal	2/3	Normal	No	<200
7	III.2	Female	16	Possible	No	Normal	0/3	Normal	No	>200

ARVC, arrhythmogenic right ventricular cardiomyopathy; CMR, cardiac magnetic resonance; ITW, inverted T waves; NA, not available; RBBB, right bundle branch block; RV, right ventricle; SA-ECG, signal averaged electrocardiography.

died in the operating theater during the surgical intervention as a result of an anaesthetic complication. The post-mortem examination revealed extensive fibro-fatty replacement in the RV, extensive fibrosis in the LV and limited inflammatory patches. There were neither signs of superimposed acute myocarditis in keeping with a 'hot phase' (see Fig. 1B).

Cascade screening identified another six carriers (four women; median age 47 years) of *LDB3 c.1051A>G* (Fig. 1A). Having not considered the carrier status as a major criterion for the diagnosis of ARVC, the clinical work-up led to a definitive diagnosis in two carriers (a brother and a sister) and a borderline diagnosis in one niece who also carried the mutation (Table 1).

A 43-year old sister (II.6) complained of frequent palpitations. Her ECG showed inverted T waves in right precordial and inferior leads and the SA-ECG showed late potentials. Several 24-h holter monitoring showed a run of idioventricular rhythm and 100 ventricular ectopic beats per day. The CMR revealed a dilated RV with severe systolic dysfunction and normal LV. No LGE was observed (Fig. 2). She was started on betablockers.

The 57-year-old brother (II.3) was asymptomatic. His ECG did not show repolarization abnormalities and his SA-ECG showed late potentials. The CMR revealed a hypokinetic RV free-wall and moderate systolic dysfunction. The LV was normal and there was no LGE (Fig. 2).

A 17-year-old niece (III.11) was also found to be a carrier. Although in the first evaluation she was asymptomatic and her ECG, SA-ECG, echocardiogram, CMR and holter failed to show any sign of the condition, she developed inverted T waves in precordial leads 2 years later. Therefore a borderline diagnosis of ARVC was accomplished.

The 59 years-old sister (II.5) was asymptomatic. Cardiac evaluation was normal although she developed late potentials in the SA-ECG during the follow up.

The 16-year old proband's daughter (III.2) and the 54 year-old brother (II.2) were asymptomatic and all tests were normal. None of the wild-type relatives presented signs in suggestive of ARVC.

Discussion

Despite the fact that ARVC is a disease caused by disruptions to the desmosomes and that mutations in desmosomal genes are identified in 50% of patients, the physiopathology of this condition remains unclear.

Recent studies support a more holistic approach to the condition in which not only desmosomes but GAP junctions, ion channels, ion currents and area composita proteins have been found to be involved in the development of ARVC and arrhythmogenesis (7, 12).

Although a particular type of ARVC associated with myofibrillar myopathy and conduction disturbances is linked to the chromosome 10q (the locus where *LDB3* is located), no mutation in any of the candidate genes proposed was identified (13).

For the first time we identified a mutation in *LDB3* in a family suffering ARVC.

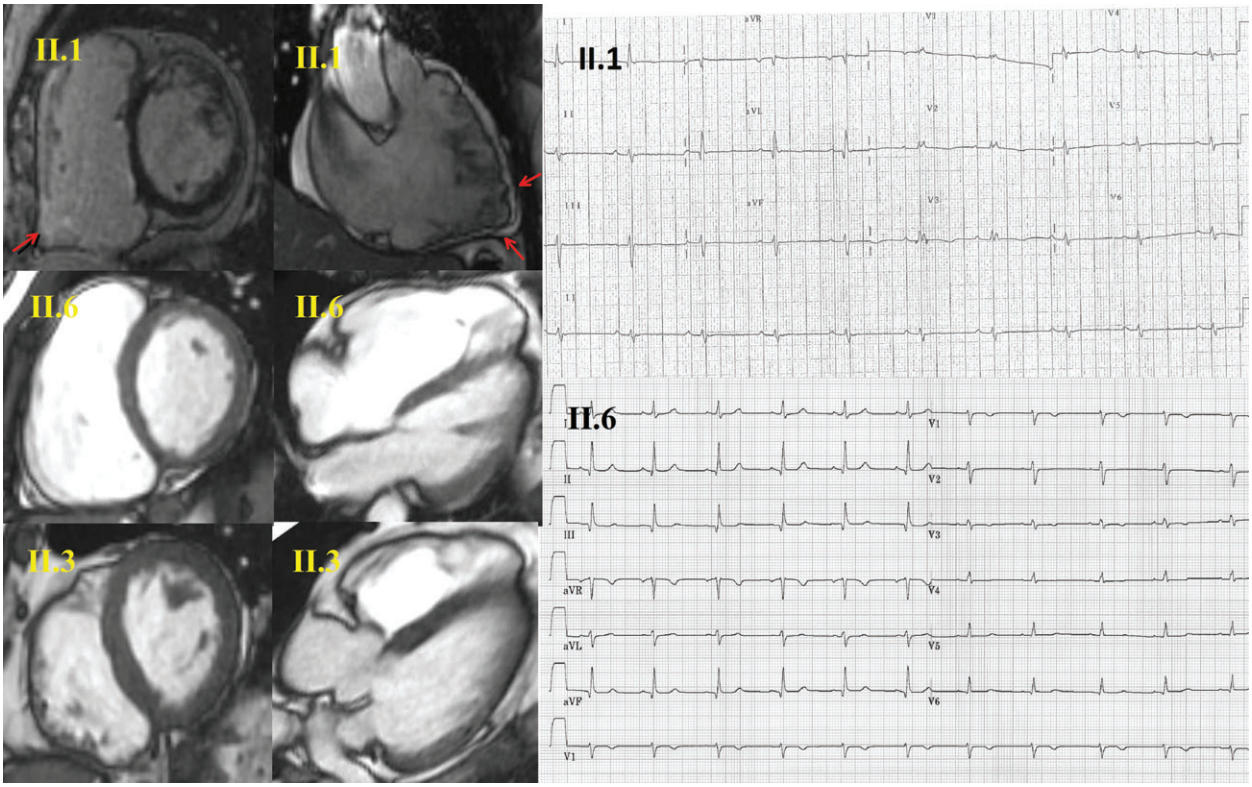

Fig. 2. On the left, cardiac magnetic resonance (CMR) from the proband (II.1), the sister (II.6) and the brother (II.3). Arrows point to dyscinetic areas ('accordion sign'). At the top right, the proband's electrocardiography (ECG) depicts right bundle branch block (RBBB) and inverted and flat T waves in precordial leads. At the bottom, the sister's ECG (II.6) also shows inverted and flat T waves in precordial leads.

LDB3 encodes the ZASP protein in humans (homologous of the Cypher protein in mice). It is a cardiac and skeletal protein expressed in the cytoplasm and it plays an essential role stabilizing the Z-line. Alternative splicing of the protein leads to multiple isoforms, with the PDZ domain being common to all of them (it interacts with the last 150 amino acids of α-actinin), whereas the ZM domain and the C-terminal LIM are isoform-specific (14–16). Previous studies have shown that the suppression of Cypher in mice is associated with DCM alongside with death because of heart failure and sudden arrhythmic death. Notably, in this knockout model, there was an overt cardiac dilatation of the RV (17). Sudden death may occur in the setting of missense mutation in *LDB3* even in the absence of apparent cardiomyopathy (18). Other study recapitulated a DCM phenotype in a transgenic mouse model (S196L). Not only ECG abnormalities may be present at an early stage but disturbances in calcium and sodium currents and electrical abnormalities are also present (14). Although the evidence supported by genotype–phenotype studies is very limited, there is some data on mutations in exon 4 causing left ventricular non-compaction (LVNC) and mutations in exon 6 causing mixed phenotypes (LVNC and DCM) (18). Other mutations in LDB3 have been also associated with autosomal dominant dystrophy (19) and hypertrophic cardiomyopathy (20).

LDB3 c.1051A > G is localized in the exon 7. The frequency of this genetic variant could be very low in general population (less than 0.5%) according to the Exome Variant Server, were T351A was identified to date in 5 of 8595 alleles from European–American individuals and in none of 4406 alleles from Afro-American subjects (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/).

LDB3 c.1051A > G affects a moderately conserved residue and the change of a polar amino acid (T) for a polar amino acid (A) (Figure 1C) is predicted to modify the polarity, mass and hydrophobicity of the protein, although Polyphen-2, PMut and SIFT predicts a tolerate protein function.

This mutation has been previously identified in patients with DCM and LVNC which led to its inclusion in a DCM genetic testing panel (21, 22).

Amongst the carriers reported in the family herein described, three fulfilled criteria for a definitive diagnosis of ARVC and one reached a borderline diagnosis. The penetrance of the condition was 4/7 carriers. The phenotype ranged from severe biventricular systolic dysfunction with RV aneurysms in the proband to repolarization abnormalities with normal CMR in the youngest case. There was no evidence of skeletal muscular disease in any carrier and there was no identifiable 'red flags' in any patient as previously reported in ARVC associated to other non-desmosomal genes (e.g.: atrial arrhythmias and conduction disturbances in lamin A/C mutation carriers (5). CK levels were normal in all cases. Serial ECG

and holter monitoring did not now show AV block or pathological pauses in any carrier (13).

This findings highlight the complexity of the genetic basis of the disease because a mutation in a new non-desmosomal gene emerges as a cause of ARVC. Further studies will be required to clarify the causative relationship between *LDB3* mutations and ARVC.

In view of the discrete yield of genetic testing in ARVC, the implementation of NGS not only in research but in clinical practice has arisen as a new tool for the identification of candidate genes associated with the disease. Having said that, and in order to avoid any confusion due to 'genetic noise', unnecessary medical tests, and emotional stress in patients and relatives, these results should ideally be validated by functional studies, animal models and a detailed family cascade screening to assess its co-segregation.

In conclusion, for the first time we have reported on a mutation in *LDB3* which causes ARVC. This finding highlights the blurry boundary that exists not only in the phenotype but also in the genetic basis of different cardiomyopathies.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Acknowledgements

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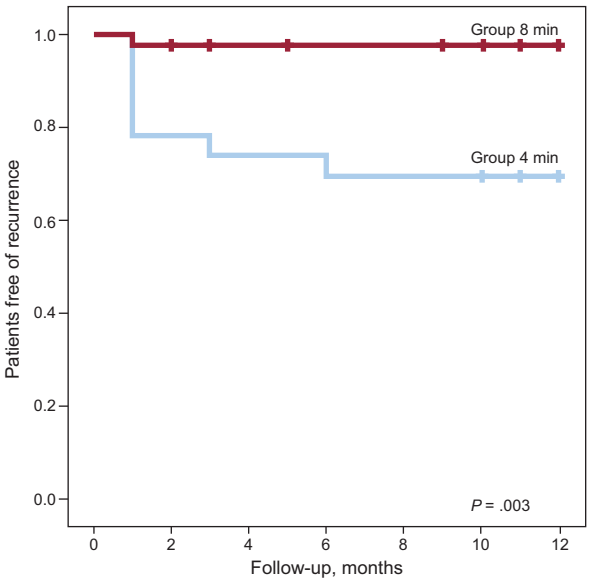

Figure. Outcomes presented as a Kaplan-Meier plot of recurrence after cryoablation. Red line: 8-minute application group; blue line: 4-minute application group.

21 patients with complete abolition of conduction through the slow pathway (recurrence rate of 9.5%) were compared with the 41 patients with residual conduction through the slow pathway with a single echo beat (recurrence rate of 14.6%, $P = .4$). The other variables analyzed were not associated with a higher recurrence rate.

The main findings in this series of patients with nodal reentrant tachycardia treated with cryoablation with an 8-mm catheter are as follows: *a)* we confirmed that the initial efficacy in an adult population is very high, with similar success rates to those obtained with radiofrequency ablation; *b)* the safety profile was excellent, even with 8-mm catheters, which do not allow cryomapping; *c)* our results suggest that the recurrence rate during the first year can be reduced by extending the duration of initially successful application to 8 minutes; *d)* modification of the slow pathway was not found to be important, as failure to detect anterograde conduction was not associated with better clinical outcome than residual conduction inducing a single echo beat.

The most controversial aspect of cryoablation is the higher recurrence rate associated with this technique compared with radiofrequency (10% vs 4%, respectively).[1] There is a lack of comparative studies, but the recurrence rates in the published series with 8-mm catheters of around 5% are lower than those reported with cryoablation with 4- and 6-mm catheters and are similar to those reported with radiofrequency ablation. These results may have been influenced by the conditions in which cryoablation was applied. For example, Chan et al,[3] who reported a relapse rate of 5.6%, performed a security freeze in the same area as the successful application, and Peyrol et al,[4] who reported a recurrence rate of 4.9%, also mention that they performed an additional application of 4 minutes at the site of successful application. Bearing in mind that tissue adherence is lost during periods of heating and thus precision may be lost, we decided to simply extend the duration of cryoapplications from 4 minutes to 8 minutes in our patients.

If low recurrence rates were to be confirmed in larger prospective series when applications are extended to 8 minutes, this catheter and application method could improve the outcomes of treatment of nodal reentrant tachycardia with cryoablation.

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Phospholamban p.arg14del Mutation in a Spanish Family With Arrhythmogenic Cardiomyopathy: Evidence for a European Founder Mutation

Mutación p.Arg14del en fosfolambán en una familia española con miocardiopatía arritmogénica: evidencia de una mutación europea fundadora

To the Editor,

Phospholamban is an inhibitor of the sarcoplasmic calcium pump, which regulates contractility and relaxation. Mutations in its gene, *PLN*, have been associated with aggressive phenotypes of both dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy.[1,2]

Herein we report a family diagnosed with arrhythmogenic cardiomyopathy with some peculiar features, carrying a Dutch founder mutation in phospholamban (*PLN* c.40_42delAGA; p.Arg14del).[3] The genotype-phenotype correlation allowed the identification of some red flags, which should lead to suspicion of this mutation in the clinical work-up.

The proband (III.2, Figure 1) was a 28-year-old woman with a past medical history of presyncopes. The electrocardiogram (ECG) showed QS inferiorly and striking low voltages throughout all leads (Figure 2).

Echocardiography showed a nondilated left ventricle with global hypokinesia and a left ventricular ejection fraction of 40%.

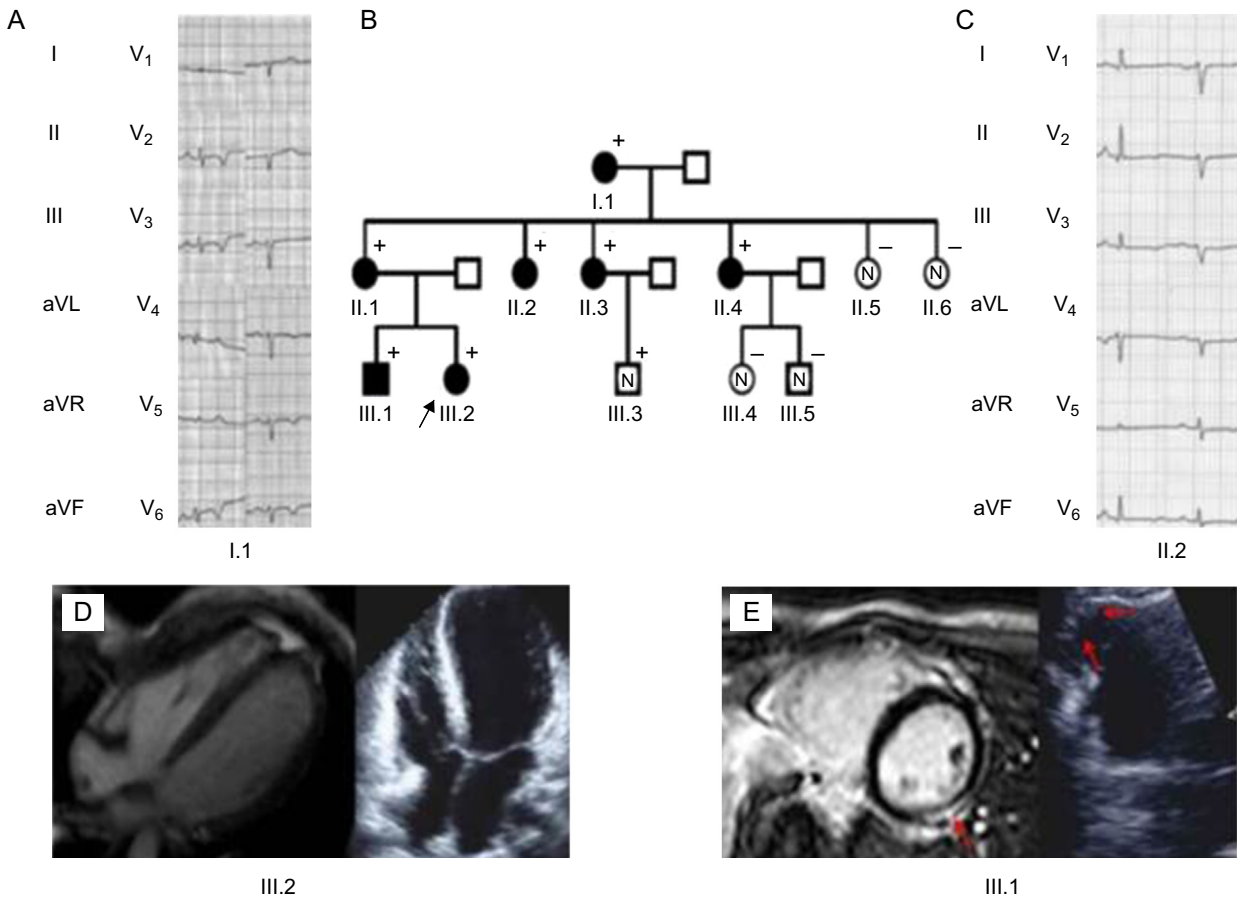

Figure 1. A and C: Electrocardiograph of the carriers: Low voltages and poor R wave progression are the hallmark. Notice the inverted T waves in let precordial leads. B: Family tree. The black arrow points to the proband (III.2). + represent carriers. Filled black figures represent affected carriers. D: The proband's echocardiogram and cardiac magnetic resonance revealed normal biventricular volumes, severe biventricular dysfunction, and absence of late gadolinium enhancement. E: Cardiac imaging and echocardiographic findings of III.1. Red arrows indicate left ventricle lateral late gadolinium enhancement patch. On the left, a right ventricle aneurysm of the free wall is observed.

Cardiac magnetic resonance with gadolinium did not show late enhancement. The right ventricle was not dilated and had global normal systolic (right ventricular ejection fraction of 51%) function, although the apex was remarkably hypokinetic.

Blood tests were unremarkable. A coronary computed tomography scan showed normal coronaries. The results of 24-hour Holter monitoring were normal. The patient was discharged on beta-blockers, an angiotensin-converting enzyme inhibitor, and spironolactone.

She was readmitted for a new collapse 3 months later. During that admission, sustained ventricular tachycardia (Figure 2) was documented, prompting implantation of an implantable cardioverter-defibrillator. The patient remained in New York Heart Association (NYHA) functional class I. Genetic analysis ruled out pathogenic mutations in the 5 desmosomal genes, *LMNA* and *MYBPC3*. However, a pathogenic mutation in *PLN*, p.Arg14del, was identified. There was no other relevant family history of cardiomyopathy or sudden death.

Cascade family screening identified 7 additional carriers of the *PLN* p.Arg14del mutation:

1. An asymptomatic 25-year-old brother (III.1, Figure 1) had frequent (>1000/24 h) ventricular ectopics. His ECG showed late R wave transition in precordial leads. Although the echocardiogram was normal, cardiac magnetic resonance revealed a hypokinetic right ventricle apex and a subepicardic late gadolinium enhancement patch in the lateral wall of the left ventricle. Biventricular systolic function was normal. Recurrent episodes of symptomatic nonsustained ventricular tachycardia (15-20 beats) were documented. Beta-blockers were started and an implantable cardioverter-defibrillator was implanted.
2. The proband's 52-year-old mother (II.1) was asymptomatic. Her ECG showed late R transition in the precordial leads. The results of echocardiogram and cardiac magnetic resonance were normal.
3. The maternal grandmother (I.1) was incidentally diagnosed at the age of 74 years in a preoperative cardiac evaluation. Her ECG demonstrated negative T waves in inferior and lateral leads. The echocardiogram showed a left ventricular ejection fraction of 45% with normal-sized left ventricular diameters. An exercise echocardiogram was negative for ischemia, and Holter monitoring failed to demonstrate any arrhythmia. She had a good response to medical treatment with left ventricular ejection fraction normalization.
4. Three asymptomatic maternal aunts (II.2, II.3, and II.4) showed poor R wave progression and flat T waves throughout the ECG. The results of echocardiograms and cardiac magnetic resonance were normal
5. A 16-year-old cousin (III.3) was asymptomatic and the clinical work-up was normal.

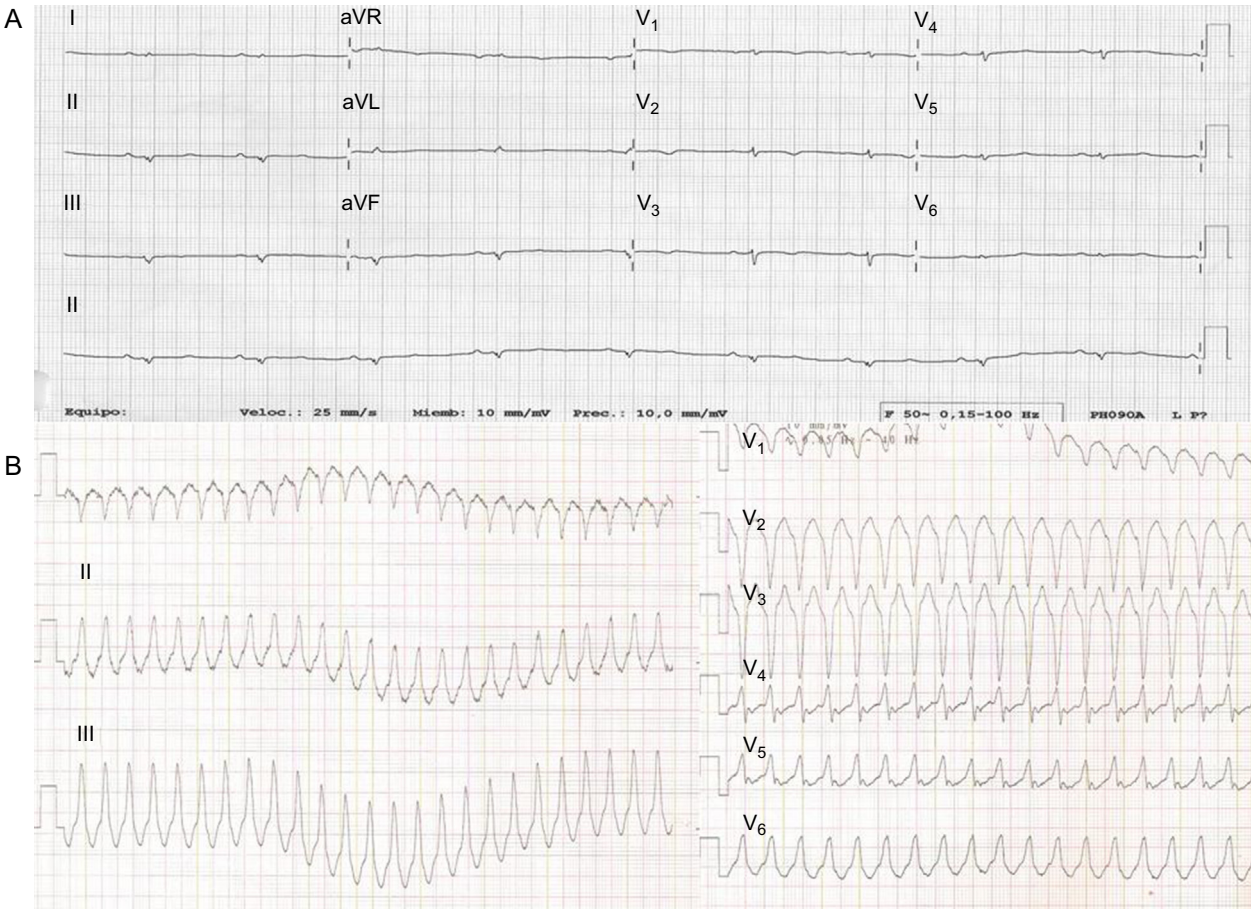

Figure 2. A: The proband's electrocardiogram. Notice the strikingly low voltages throughout and inverted T waves in right precordial leads. B: Sustained ventricular tachycardia suggestive of a right ventricle outflow tract origin.

Due to the morphology of ventricular tachycardia and ECG abnormalities and their status as carriers, 2 patients met the criteria for definitive arrhythmogenic right ventricular cardiomyopathy whilst 3 had a borderline diagnosis.

Haplotype analyses of markers around *PLN*, in 2 affected Spanish *PLN* mutation carriers, were compared with the Dutch series. Interestingly, the Spanish patients shared 4 out of 5 markers from the shared Dutch haplotype, suggesting a common founder ancestor.

In the family presented herein, the penetrance of the disease is 6/8 (75%). Because *PLN* p.Arg14del is a pathogenic mutation, this family is a clear example of the variability of the clinical phenotype in relatives with arrhythmogenic right ventricular cardiomyopathy.[4] Of note, recent evidence shows a trend for female carriers to show milder phenotypes than males yet they nevertheless show malignant ventricular arrhythmias.[5] A notable finding was the wide variety of ECG abnormalities found in the family: widespread low QRS voltage (even reminiscent of restrictive cardiomyopathy) in the proband, inverted T waves in inferolateral leads (typical of arrhythmogenic left ventricular cardiomyopathy), and poor R wave progression in all but 1 carrier.

Low voltage and poor R wave progression in ECG is a red flag in arrhythmogenic right ventricular cardiomyopathy or dilated cardiomyopathy patients and should lead to suspicion of a *PLN* mutation as a cause of the disease. This is paramount, particularly when assessing the timing of implantable cardioverter-defibrillator implantation, which, in view of the current data, should be indicated before the patient has severely depressed systolic dysfunction.[5]

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Radial Artery Pseudoaneurysm Following Cardiac Catheterization: Clinical Features and Nonsurgical Treatment Results

Seudoaneurisma de la arteria radial tras cateterización cardiaca: características clínicas y resultados del tratamiento no quirúrgico

To the Editor,

The use of radial access for catheterization and cardiac intervention is becoming increasingly popular, mainly because of its lack of complications.[1] Radial artery pseudoaneurysm (RAp)[2] is an extremely rare complication, so many of its clinical features are unknown and treatment is not systematic. In the few reported cases of RAp, surgical repair was the most commonly used treatment,[3] although recently there have been reports of successful nonsurgical treatment in single patients.[4,5] During the period 2004-2013, we prospectively collected all cases of RAp occurring in our center. In this article, we describe their clinical characteristics and outcomes following initial nonsurgical treatment.

During this period, 16 808 catheterizations were performed (96.5% transradial), and 5 radial artery RAp were detected (incidence, 3 of 10 000 catheterizations). The Table shows the characteristics of the RAp and the treatment applied. All cases presented as a pulsatile erythematous mass at the puncture site (Figure A). One patient (case 5) presented with pulsatile bleeding through an ulceration/erosion of the RAp. In another patient (case 4) we observed crusted erosion of the RAp without spontaneous bleeding (Figure A). All RAp were confirmed by vascular ultrasound (Figure B). The most common factors associated with the occurrence RAp were the use of coumarin anticoagulation during the procedure (4 patients) and the occurrence of hematoma in the forearm during/after compression (4 patients). Nonsurgical treatment was effective in all patients (mechanical compression was successful in 3 and failed in 2; in these patients, thrombin injection was performed, occluding the RAp in both). Direct mechanical compression of the RAp resulted in iatrogenic rupture of the outer wall (Figure C) in 2 patients (cases 2 and 5). In one patient (case 2), thrombin injection produced acute occlusion of the radial artery, which was asymptomatic.

With this series of radial artery RAp, the most extensive published to date, we highlight 3 as yet undescribed features that we consider important for preventing and treating future cases:

1. The presence of a hematoma in the forearm during/after compression (probably due to inadequate compression), along with the presence of predisposing factors (anticoagulation), is a

Table
Clinical Characteristics of the Series

	Case 1	Case 2	Case 3	Case 4	Case 5
Age, y	55	76	79	88	81
Sex	Male	Male	Male	Male	Female
Body surface, m^2	1.9	1.8	1.9	1.8	1.7
Anticoagulation with coumarin	Yes	Yes	Yes	No	Yes
Anti-GPIIb/IIIa	No	No	No	No	No
Sheath size, Fr	6	6	5	6	6
Diagnosis time, d	15	5	4	17	10
Clinical features apart from pulsatile swelling	No	No	No	Encrusted lesion at the apex	Ulceration with spontaneous bleeding
Interruption of OAC during procedure	No	No	No	Not applicable	No
Prolonged direct mechanical compression (> 12 h)	With pneumatic device	With pneumatic device	With pneumatic device but only 5 h	With pneumatic device	With direction compression with the echo probe
Initial success	No	No	Yes	No	No
Complications	No	Rupture of RAp	No	No	Rupture of RAp
Final treatment	Compression with elastic bandages 48 h	Thrombin injection	Not recorded	Thrombin injection	Compression proximal to the RAp
Interruption of OAC during follow up	Yes	No	Not applicable	Not applicable	Yes
Ultimately successful	Yes	Yes	Yes	Yes	Yes
Complications	None	Asymptomatic radial occlusion	None	None	None

OAC, oral anticoagulants; Anti-GPIIb/IIIa, glucoprotein IIb/IIIa inhibitors; RAp, radial artery pseudoaneurysm.

Genetics of myocarditis in arrhythmogenic right ventricular dysplasia

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BACKGROUND Myocarditis occasionally is related to arrhythmogenic right ventricular dysplasia (ARVD) and sometimes overlaps during the early stages, which may lead to misdiagnosis. Acute myocarditis may reflect an active phase of ARVD.

OBJECTIVE The purpose of this study was to evaluate the genetic basis of myocarditis in ARVD and to investigate the association with a poorer prognosis and a higher risk of ventricular arrhythmias.

METHODS Two groups were analyzed: group A, which consisted of 131 affected patients—84 with ARVD (62% male, age 45 years [range 33–55 years]) and 47 with left-sided forms (arrhythmogenic left ventricular dysplasia [ALVD]) (47% male, age 45 years [range 25–61 years]); and group B, which consisted of 64 nonaffected mutation-carrying relatives (36% male, age 42 years [range 22–56 years]; 23 from classic ARVD families and 41 from ALVD families).

RESULTS Seven patients (3.5%) presented with a clinical diagnosis of acute myocarditis over median follow-up of 34 months. Myocarditis was the first clinical presentation in 6 of 7 cases. In 2 patients, acute myocarditis preceded worsening of left ventricular systolic function. In 1 case, myocarditis was associated with an increased gadolinium pattern in cardiac magnetic resonance. Two patients presented with ECG changes weeks after myocarditis resolution. Myocarditis preceded the development of ventricular tachycardia in 2 other patients. Myocarditis clustered in families bearing *DSP Q447** and *LDB3 c.1051A>G*.

CONCLUSION Acute myocarditis reflects an active phase of ARVD that leads to changes in phenotype and abrupt progression of the disease. An active phase should be suspected in a patient with myocarditis associated with a family history of ARVD. Certain mutations may increase the susceptibility to superimposed myocarditis in ARVD.

KEYWORDS Arrhythmogenic right ventricular dysplasia; Myocarditis; Genetics; Desmoplakin; LDB3

ABBREVIATIONS **ALVD** = arrhythmogenic left ventricular dysplasia; **ARVD** = arrhythmogenic right ventricular dysplasia; **CMR** = cardiac magnetic resonance; **DCM** = dilated cardiomyopathy; **LGE** = late gadolinium enhancement; **LV** = left ventricle; **RV** = right ventricle; **SVT** = supraventricular ventricular tachycardia; **TrI** = troponin I

(Heart Rhythm 2015;12:766–773)

Introduction

Arrhythmogenic right ventricular dysplasia (ARVD) is a frequently inherited myocardial disease. The clinical spectrum includes palpitations, syncope, ventricular arrhythmias, heart failure, and sudden death.[1] The initial hypothesis of a "trouble of development" recently was confirmed *in vitro* as the underlying mechanism of the disease.[2] The estimated prevalence of ARVD in the general population ranges from 1:1000 to 1:5000.[3] The condition is an important cause of sudden death, particularly in young people and athletes.[4] ARVD may also extend to the left ventricle (LV) during the late stages[5,6] or affect the LV as an initial target (arrhythmogenic left ventricular dysplasia [ALVD]).[7]

Since the initial description of a recessive mutation in plakoglobin as a cause of ARVD,[8] widespread implementation of genetic testing has led to the identification of desmosomal gene mutations in >50% of ARVD patients.[9] Moreover, mutations in other nondesmosomal genes have been associated with ARVD and ARVD-like phenotypes (*PLN*,[10] *TMEM43*,[11] *LMNA A/C*,[12] *DES*,[13] *CTNNA*,[14] *TTN*[15]).

Other factors such as viral infection have been reported as cofactors of morbidity.[5,16] However, not only is myocarditis known to be related to ARVD, but possible myocarditis (chest pain and troponin level increases) also seems to be part of the clinical presentation as a superimposed phenomenon during the natural history of the disease.[17]

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The aim of this study was to evaluate the clinical features of these so-called active phases and to investigate whether they are associated with poor prognosis and higher risk for ventricular arrhythmias. The study consisted of 2 groups of patients: (1) ARVD/ALVD patients and (2) the gene-positive but phenotype-negative relatives of the ARVD patients. The rationale for including the second group was to assess whether carrier status (in otherwise asymptomatic relatives with a vulnerable myocardium) is associated with a higher prevalence of myocarditis.

Methods

We evaluated the medical histories of a cohort of ARVD patients and their relatives. All patients provided written consent. The study was approved by the ethical committee of our hospital.

Patients were classified into 2 groups. Group A consisted of patients with a definitive diagnosis of ARVD (Task Force Criteria 2010[18]) or ALVD, defined as ARVD mutation carriers with a phenotype of dilated cardiomyopathy (DCM), systolic dysfunction, and a past medical history of ventricular arrhythmias and/or a family history of sudden death. Group B consisted of nonaffected mutation carriers, all relatives of patients diagnosed with ARVD or ALVD. Patients with ischemic heart disease were excluded from the final analysis.

Clinical evaluation

Clinical evaluation consisted of 12-lead digital ECG, signal-averaged ECG, 2-dimensional Doppler echocardiography, exercise test, and 24-hour Holter monitoring. Cardiac magnetic resonance (CMR) with late gadolinium enhancement (LGE) was performed in 5 of 7 patients with probable myocarditis.

Genetic analysis

Mutation analysis was performed in all patients by di-deoxy Sanger sequencing of all exons and flanking intron–exon boundaries in *DSP*, *DSC2, DSG2, PKP2, JUP, TMEM43, LMNA A/C,* and *PLN*. For patients in whom no conclusive mutations were found, the study was expanded with inclusion of a panel of 124 inherited cardiac genes that were analyzed by next-generation sequencing (see Online Supplementary Table 1).

Inflammatory and myocardial damage biomarkers

Blood tests analyzed blood count and inflammation markers, including C-reactive protein, creatine kinase (CK), CK-MB, and troponin I (TrI).

Histology and immunohistochemistry

Myocardial samples from the right ventricular (RV) free wall, septum, and LV (2 samples from each location) were analyzed. The samples were fixed in formalin, embedded in paraffin, cut into 5-μm slices, and mounted on slides. Sections were stained with hematoxylin–eosin and Masson trichrome. The lesions were studied for fibrofatty evidence, inflammation, necrosis, and apoptosis. Lymphocytic population was characterized by immunohistochemistry.

In order to make a definite diagnosis of ARVD in the patient with massive lymphocytic myocarditis and subtle fibrofatty evidence, patterns of plakoglobin, connexin-43, and desmoplakin were analyzed. Slides were incubated with a primary antibody [monoclonal mouse anti–N-cadherin (1:400; Sigma, UK); monoclonal mouse anti-plakoglobin (1:1000; Sigma); monoclonal rabbit anti-connexin43 (1:400; Sigma), and monoclonal mouse anti-desmoplakin (1:10; Fitzgerald, UK)]. The slides were incubated with goat anti–mouse or goat anti–rabbit (1:400; Jackson ImmunoResearch, USA) secondary antibodies. Stained slides were checked using a confocal microscope (LSM510, Meta Zeiss, Germany). The results were compared to a control sample from a patient who died of no cardiovascular reason, and both the macroscopic and microscopic examinations of the heart were normal. Apoptosis was analyzed by TUNEL assay and caspase-3 activity.

Results

Group A consisted of 131 patients affected with ARVD or ALVD: 84 classic ARVD (62% male, age 45 years [range 33–55 years]) and 47 ALVD (47% male, age 45 years [range 25–61 years]). Group B consisted of 64 nonaffected mutation-carrying relatives (36% male, age 42 years [range 22–56 years]): 23 from classic ARVD families and 41 from predominant ALVD families. Baseline clinical characteristics are summarized in Online Supplementary Table 2.

Seven patients (3.5%; 2 ARVD, 4 predominant, 1 gene-positive and phenotype-negative) presented with a clinical diagnosis of acute myocarditis over median follow-up of 34 months. Six cases (4.5%) occurred in the group of affected ARVD/ALVD patients and 1 (1.5%) in the group of nonaffected mutation carriers.

Patient 1 *(DSP c.5318delT)*

A 20-year-old male patient was diagnosed with probable acute myocarditis after an episode of chest pain and increased levels of TrI, CK, and MB-CK. ECG at the time did not show repolarization abnormalities. Echocardiography revealed mild LV dilation and dysfunction (LV ejection fraction 45%). Angiography ruled out coronary artery disease. Since this initial episode, he presented with recurrent episodes of chest pain, elevated troponin level, and progressive biventricular dysfunction (see Online Supplementary Figure 1). Genetic testing identified a nonsense mutation in desmoplakin *(DSP c.5318delT),* which is predicted to truncate desmoplakin in the middle of the C-terminal domain of the protein.

Patients 2 and 3 *(DSP c.1339C>T)*

Two siblings aged 14 and 19 years (see Online Supplementary Figure 2) were diagnosed during family screening with ALVD. *DSP c.1339C>T*, a dominant

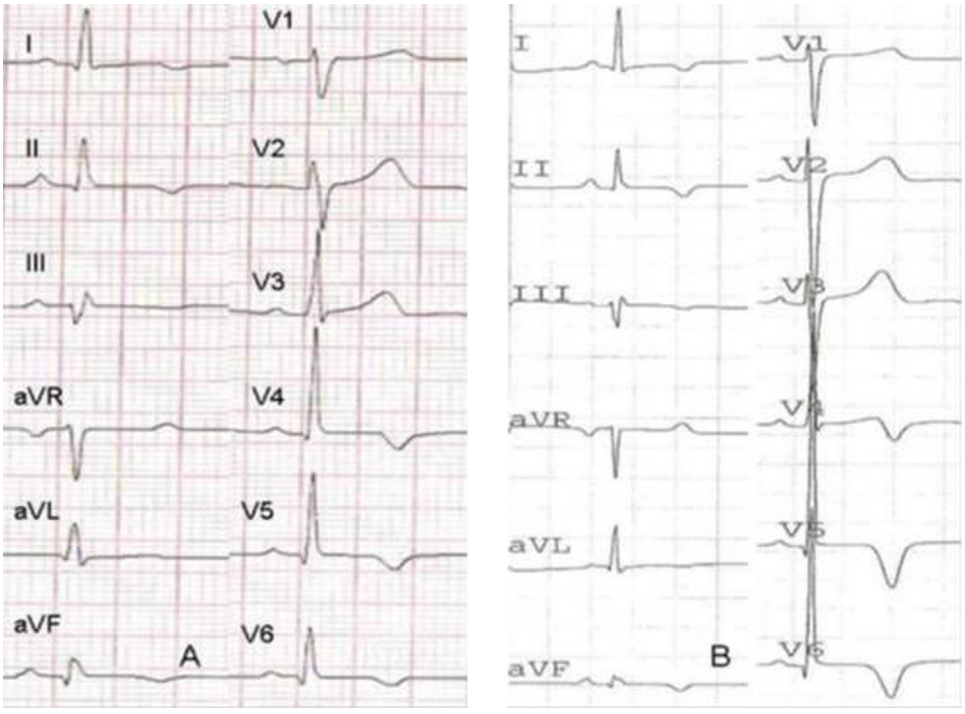

Figure 1 ECG from patient 2. Before **(A),** and 6 months after the acute episode. **(B)**. Note: repolarization changes.

mutation leading to a stop codon, was identified in both siblings. ECG showed dynamic T-wave inversion in the inferolateral leads in the younger sibling (Figure 1) and flat T waves in the older sibling.

Echocardiography revealed mild systolic dysfunction in the younger sibling and normal function in the older sibling. In both cases, CMR depicted similar findings consisting of a widespread LGE pattern (see Online Supplementary Figure 3). Both siblings were admitted because of episodes of chest pain and elevated TrI levels after the initial diagnosis. They suffered progressive LV impairment that was documented during follow-up.

Patient 4 (*DSP c.1339C > T*)

A maternal aunt of patients 2 and 3 had been diagnosed with myocardial infarction with normal coronary arteries at the age of 48 years. ECG had shown inverted T waves in the inferolateral leads. The inferior wall was hypokinetic, but the left ventricular volumen (end diastolic 170 ml) and LV ejection fraction (LVEF 55%) were normal. Six years later, she was admitted with chest pain and palpitations. Supraventricular ventricular tachycardia (SVT) with left bundle branch block and axis +30º was recorded, and electrical cardioversion successfully restored sinus rhythm. The angiogram was normal. Electrophysiologic study failed to induce ventricular tachycardia. Voltage mapping did not reveal low-voltage areas. She was discharged on sotalol. One year later, she presented with a new episode of chest pain on exertion and hemodynamically unstable SVT, which prompted placement of an implantable cardioverter-defibrillator for secondary prevention. No CMR was performed at that time. Years later she was found to be a carrier of *DSP c.1339C > T*. She subsequently developed new motion abnormalities that affected the interventricular septum.

Patient 5 (*DSP c.1339C > T*)

A 38-year-old woman was admitted for acute chest pain and elevated CK-MB and TrI levels 2 weeks after she gave birth. Her medical history was unremarkable, but interestingly her mother had previously been diagnosed with ALVD. Both women were found to carry *DSP c.1339C > T,* although they were not related to patients 2, 3, and 4. ECG, echocardiography, and CMR were normal.

Patients 6 and 7 (*LIM-domain Binding Protein-3, LDB3c.1051A > G*)

A 45-year-old woman was admitted for recurrent chest pain and presyncope. Blood tests showed increased CK-MB and TrI levels. While being monitored, she suffered a poorly tolerated SVT that was successfully cardioverted. The angiogram was normal. Echocardiography and CMR showed severe biventricular dilation and dysfunction with multiple dyskinetic areas in the RV and extensive LGE. An implantable cardioverter-defibrillator was indicated, but the patient died in the operating room of a neurologic complication related to the anesthetic procedure. Postmortem examination revealed severe RV fibrofatty evidence, myocytolysis, and scattered lymphocytic patches (Figures 2 and 3).

The patient's 40-year-old sister reported a longstanding history of palpitations. Her ECG showed a narrow QRS and inverted T waves in the right precordial and inferior leads as well as Q waves in the inferior leads (Figure 4). CMR

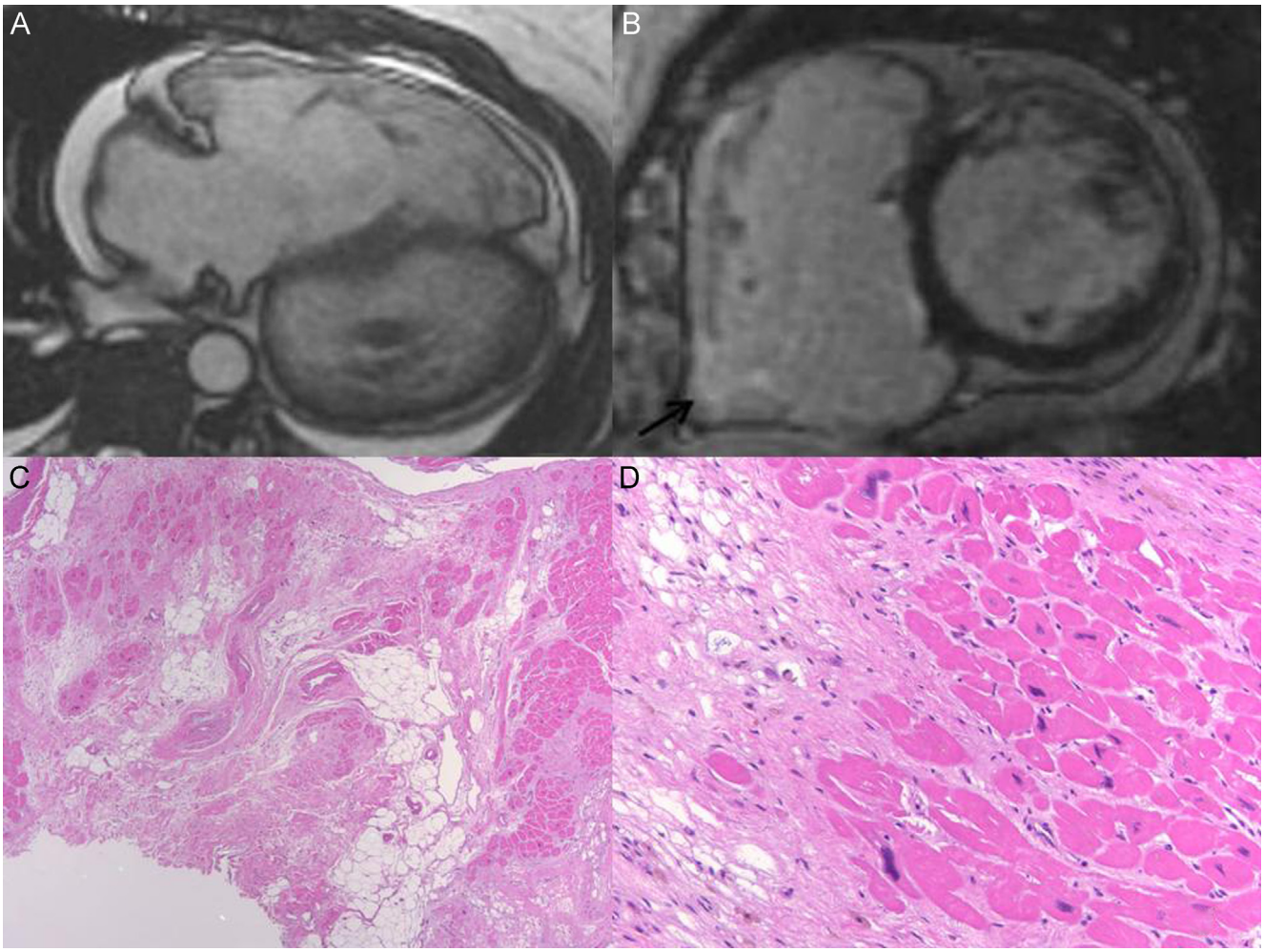

Figure 2 Cardiac magnetic resonance and histology findings of patient 6. **A, B:** Severe right ventricular (RV) enlargement and severe biventricular impairment were observed. *Arrow* points to dyssynchronous areas. **C:** Histology of an RV trabeculation *(left)* arising from the free wall *(right)* (hematoxylin–eosin ×4). Extensive fibrofatty replacement is present. **D:** Mid-apical septum (right septum on the *left*) (hematoxylin–eosin ×20). Extensive fibrosis and myocytolysis are present.

showed a dilated and impaired RV with multiple hypokinetic areas. Three years after the initial diagnosis, she presented with an episode of chest pain and ST elevation in the lateral leads as well as a rise in TrI level. Angiography ruled out coronary lesions, and a diagnosis of acute myopericarditis was made. Another CMR performed 4 weeks after the acute

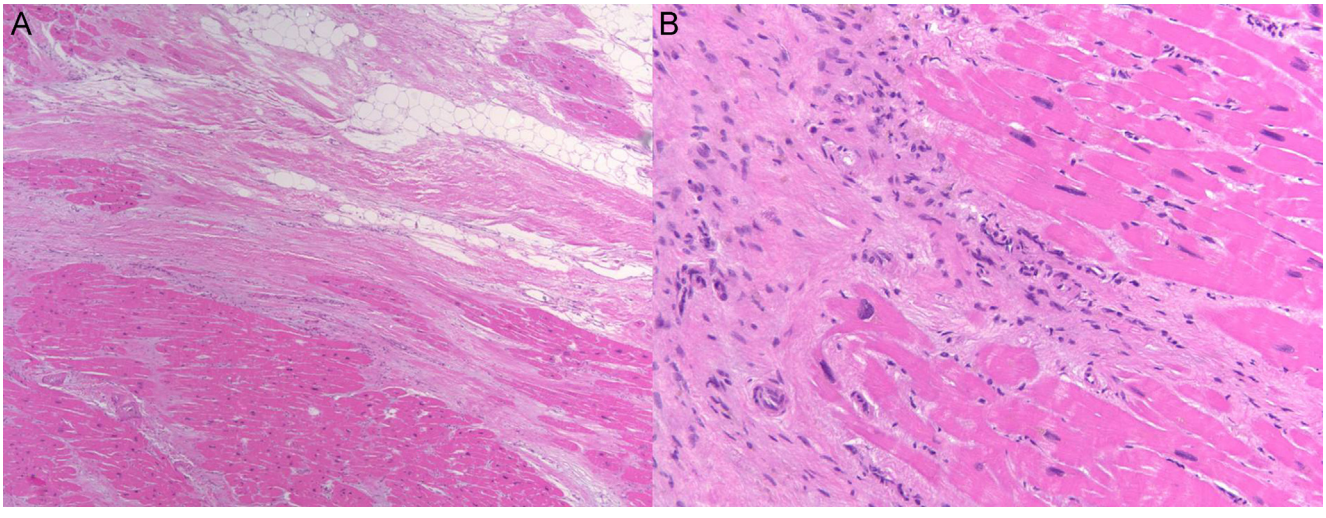

Figure 3 **A:** Inferolateral wall of LV (hematoxylin–eosin ×4). Fibrofatty tissue is present from epicardium *(top)* to endocardium *(bottom).* There are areas of hyaline fibrosis. **B:** Clusters of lymphocytes inside the fibrosis suggest previous recurrent myocarditis (×20).

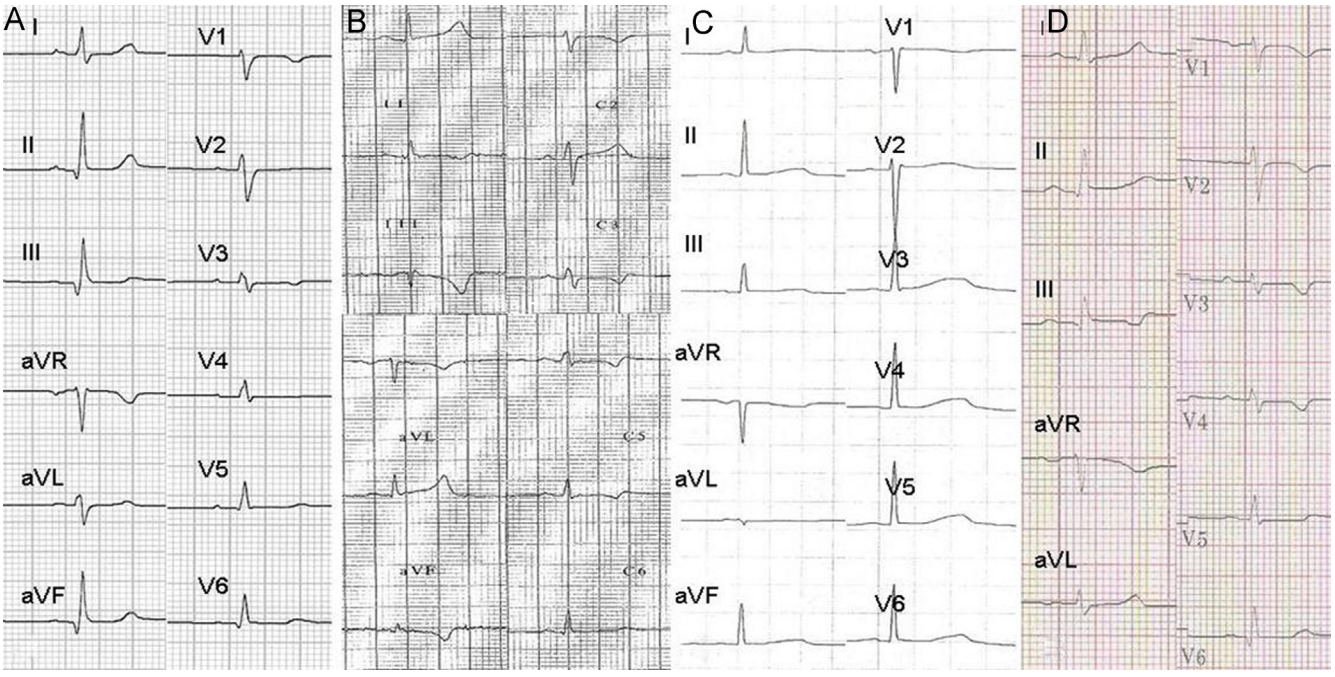

Figure 4 ECG from patient 6, before myocarditis **(A),** during myocarditis **(B),** 6 months later (T-wave pseudonormalization) **(C),** and 1 year later (deeper T-wave inversion and lower QRS voltage) **(D).**

episode showed no change in RV volume or function. Neither edema nor LGE was observed (see Online Supplementary Figure 3).

After this episode, ECG showed pseudonormalization of T waves in the right precordial leads and Q-wave disappearance that remained for the next 6 months. A new T-wave inversion occurred thereafter (Figure 4).

Next-generation sequencing identified a missense mutation in *LDB3 (LIM-domain Binding Protein-3)* (*c.1051A>G*), which was found to segregate in this family.

In no case was there a history of fever or infection in the weeks before acute myocarditis. Notably, the results of blood tests performed at the time of the episodes did not show elevations in inflammatory markers such as C-reactive protein or leukocytosis. On CMR, none of the patients demonstrated pericardial effusion or myocardial edema.

Discussion

The link between ARVD and inflammation is well known. Inflammatory infiltrates are present in 60% to 88% of ARVD samples, and there is a higher prevalence of inflammation on postmortem examination of sudden death cases than in patients who died because of heart failure and among patients with LV involvement.[5,6] These findings led to the proposal that viral infections (a cofactor of morbidity and mortality in ARVD) have a specific genetic background.[5]

We identified 7 patients who presented with acute myocarditis (4/47 with ALVD, 2/84 with classic ARVD, and 1/23 nonaffected ALVD mutation carrier [from among 23 genotype-positive, phenotype-negative carriers]). These episodes of acute myocarditis were recurrent in 4 cases (Table 1). Five patients carried *DSP c.1339C>T* and *c.5318delT,* and 2 patients carried *LDB3 c.1051A>G*. All of these patients were relatives or stemmed from a common ancestor (assessed by microsatellite analysis), which points to a familial distribution of myocarditis.

Chest pain was the first clinical presentation in 5 cases. Two patients who were diagnosed had acute chest pain but normal angiogram, and only detailed clinical workup along with the family history led to a correct diagnosis.

In 3 patients (no. 1, 2, and 3), probable myocarditis preceded worsening in LV systolic function. In 1 case (no. 3), it was associated with an increase in LGE (Figure 5). Of note in this patient is the absence of signs, which is in keeping with acute myocarditis, on CMR, like myocardial edema. In 2 patients (no. 4 and 6), chest pain preceded the development of SVT.

Because myocarditis frequently was observed as the first clinical manifestation in ARVD/ALVD patients in this study, we also aimed to evaluate the prevalence of myocarditis in a subset of nonaffected gene-positive carriers to determine whether they would reveal an initial ARVD/ALVD phenotype. Only 1 patient in this group (no. 5) suffered an episode of chest pain and elevated TrI that was not associated with a later change in ECG, Holter monitoring, or imaging. The small sample comprising the genotype-positive, phenotype-negative relatives prevented us from drawing conclusions on the susceptibility to myocarditis of healthy carriers. Further investigations are need to shed light on this point.

Dynamic changes in repolarization previously have been described as a sign of early disease in ARVD,[19] with these changes postulated as a sign of gap junction remodeling. In this series, 2 ARVD patients with severe RV dysfunction and 1 with ALVD (no. 2, 3, and 7) presented with dynamic

Table 1 Main clinical characteristics of the 7 patients included in the study

Patient no.	Sex	Age (years)	First symptom	TrI rise	CK-MB rise	Recurrent myocarditis	Phenotype	Gene	Mutation	Outcome after myocarditis
1	M	20	Chest pain	Yes	Yes	Yes	ALVD	*DSP*	*c.5318delT*	Worsening LV systolic function
2	M	14	Chest pain	Yes	No	Yes	ALVD	*DSP*	*c.1339C>T*	Worsening LV systolic function, changes in repolarization
3	M	19	Chest pain	Yes	No	Yes	ALVD	*DSP*	*c.1339C>T*	Worsening LV systolic function
4	F	37	Chest pain and syncope	Yes	No	Yes	ALVD	*DSP*	*c.1339C>T*	Ventricular tachycardia
5	F	38	Chest pain	Yes	Yes	No	Normal	*DSP*	*c.1339C>T*	No change
6	F	40	Palpitations	Yes	Yes	No	ARVD	*LDB3*	*c.1051A>G*	Changes in repolarization
7	F	45	Chest pain and palpitations	Yes	Yes	No	ARVD-biventricular	*LDB3*	*c,1051A>G*	Syncope, ventricular tachycardia

ALVD = arrhythmogenic left ventricular dysplasia; ARVD = arrhythmogenic right ventricular dysplasia; CK = creatine kinase; TrI = troponin I.

changes (including T-wave pseudonormalization in 1 case) a few weeks after acute myocarditis.

Although useful for making an accurate diagnosis, there was no consensus on performing endomyocardial biopsy in all patients because they all had responded to medical treatment and were clinically stable. However, a myocardial sample from postmortem examination was available for 1 patient (no. 7). Hematoxylin–eosin staining showed fibrofatty evidence, myocyte necrosis, and scattered apoptotic myocytes (see Online Supplementary Figure 4). The extensive hyaline fibrosis found in this case along with lymphocytic clusters inside the fibrotic areas suggests recurrent superimposed myocarditis throughout the natural history of the disease (Figure 3).

Despite chronic myocarditis being considered the cause of some cases of DCM, such a dilemma remains to be clarified for ARVD. Despite the variable prevalence of a viral genome in ARVD patients,[20,21] the presence of lymphocytic infiltrates in both ventricles is a common postmortem finding in ARVD.[5,6]

Plakoglobin remodeling at cardiac myocyte junctions occurs in ARVD as well as in granulomatous myocarditis (cardiac sarcoidosis and giant cell myocarditis). On the contrary, plakoglobin signal is not depressed in viral lymphocytic myocarditis.[22] Interestingly, we documented that lymphocytic myocarditis may be the predominant histologic pattern during the initial stages of disease, where fibrofatty tissue is scarce or even absent, and complete plakoglobin shifting has already occurred at the intercellular junctions (Figures 6 and 7).

In our series, 5 of 7 patients, including several members from a single family, presented with signs of acute myocarditis at different times. From a clinical perspective, this superimposed phenomenon may occur during the natural history of the disease, which reinforces the concept of a superimposed phenomenon in a genetically demonstrated ARVD pattern. These episodes may mirror a histologic active phase and progression of myocardial disease.

Although the evidence is based on a small series of patients, there seems to be a higher incidence of myocarditis in *DSP* mutation carriers affected by ALVD,[17] which should prompt clinicians to look for mutations in *DSP* in this clinical scenario.

This study raises awareness of an underrecognized clinical presentation of ARVD that consists of chest pain and a rise in myocardial biomarkers either as the first sign of the disease or as a marker of progression.

Although rare in the entire ARVD population, signs of acute myocarditis should raise the possibility of ARVD, especially when it occurs in the patient's relatives. This clinical presentation is particularly suggestive when it occurs in the absence of specific causes, infection, fever, pericardial effusion, or increased levels of blood inflammation biomarkers.

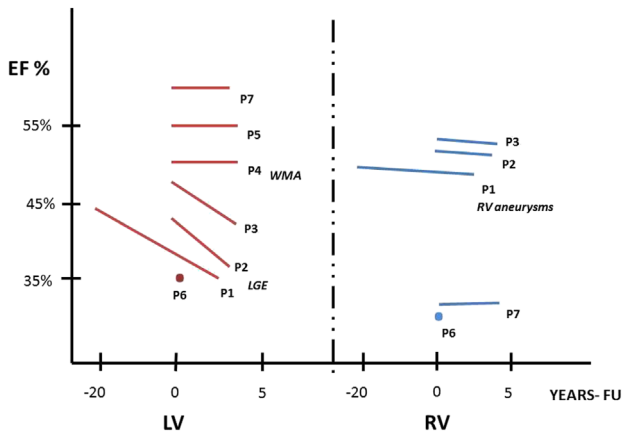

Figure 5 volution of left ventricular (LV) and right ventricular (RV) ejection fraction (EF) before and after acute myocarditis. Only RV data are presented if cardiac magnetic resonance data were available. FU = follow-up; LGE = late gadolinium enhancement; WMA = wall-motion abnormality.

Study limitations

This is a retrospective study based on a small series of cases. Because of the small sample of healthy relative carriers, results from this subgroup should be interpreted with caution. Extension of this concept to other ARVD mutations or cardiomyopathies remains to be demonstrated. Another limitation is the absence of virus in myocardial samples (a technique that is difficult to perform), which may explain the failure of viral identification reported by some groups.[21]

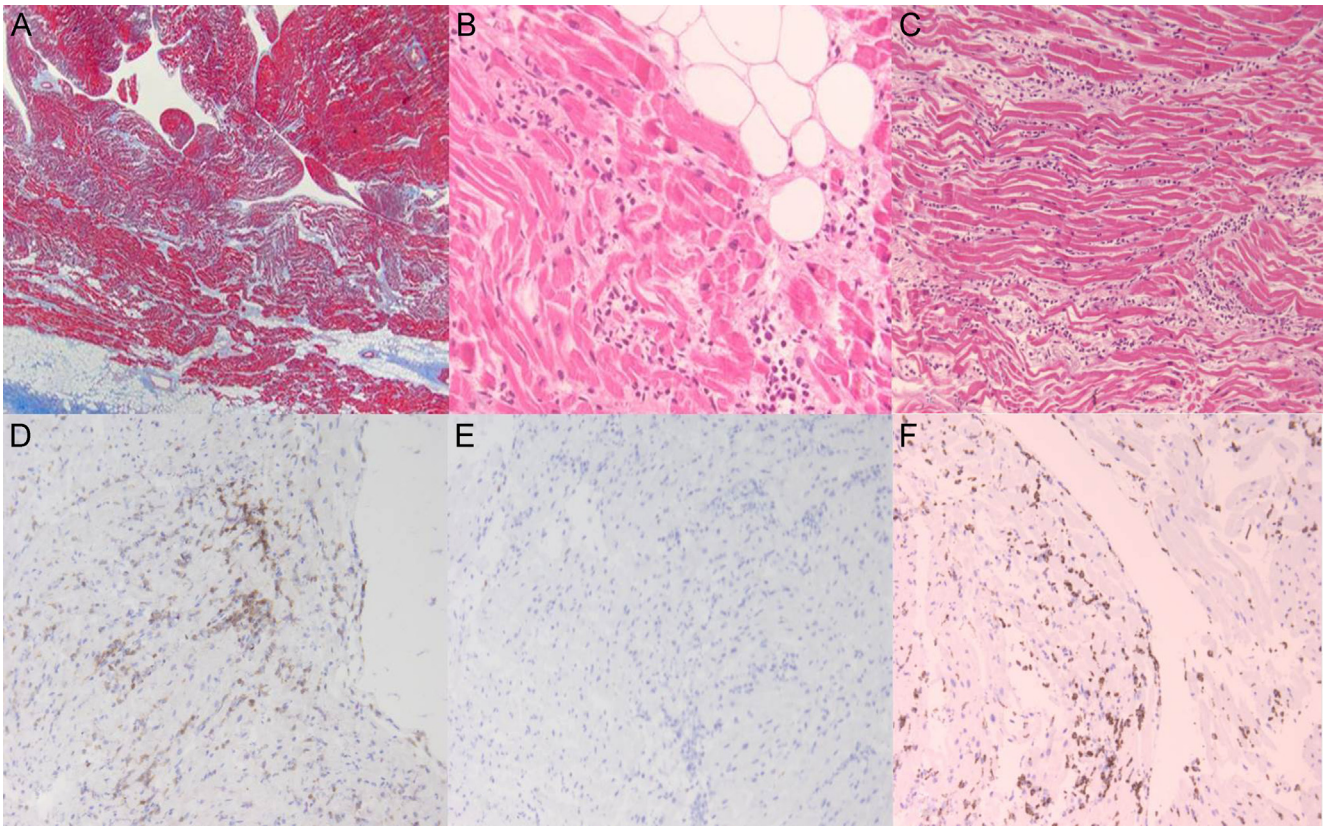

Figure 6 Postmortem sample from a 40-year-old woman with an unremarkable medical history who died suddenly at home. Macroscopic study showed mild ventricular hypertrophy (heart weight 350 g) as well as mild biventricular dilation. **A:** Lateral wall of the right ventricle (RV) shows mild fibrofatty replacement from epicardium *(bottom)* to endocardium *(top)* (Masson trichrome ×4). **B:** Mid-wall shows lymphocytic clusters, fibrosis, and isolated adipocytes (hematoxylin–eosin ×20). **C:** Extensive areas of lymphocytic myocarditis localized all over the RV free wall. Cardiotrophic viruses (parvovirus B19, adenovirus, enterovirus, cytomegalovirus, Epstein–Barr, herpesvirus-6) were excluded by myocardial polymerase chain reaction. **D–F:** Immunohistochemistry findings of the RV. Lymphocytes T CD4+ **(D)** and macrophages CD68+ **(F)** were present. In contrast, no lymphocytes TCD8+ were observed **(E).** This inflammatory pattern, found in an early stage of the disease, is reminiscent of that found in type IV hypersensitivity reactions.

Conclusion

This is the first work to establish a direct link between genetics, myocarditis, and ARVD. Relatives bearing the same disease-causing genes show a particular susceptibility to myocarditis, a concept that is well known in DCM. Moreover, this concept may be further extended to other cardiomyopathies.

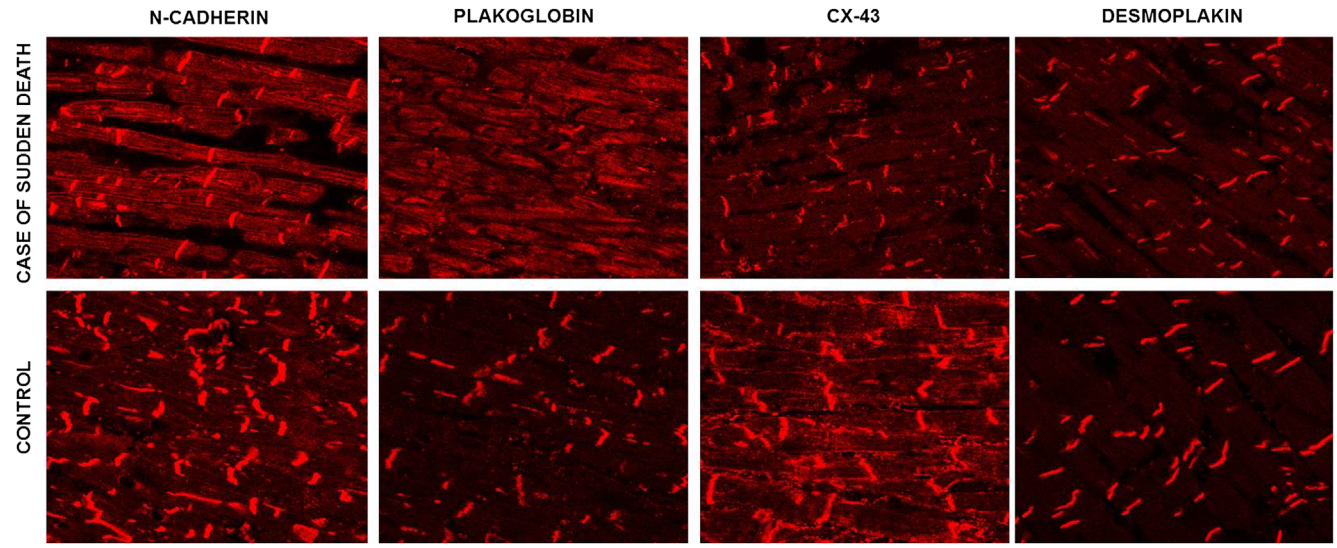

Figure 7 Immunohistochemistry of N-cadherin, plakoglobin, connexin-43 (Cx43), and desmoplakin. Plakoglobin is not present in the intercellular junctions in the arrhythmogenic right ventricular dysplasia case.

Myocarditis reflects an active phase of ARVD and may be the first clinical presentation. Changes in the phenotype could occur during these phases and range from isolated ECG changes to worsening of systolic function or ventricular arrhythmia. An active phase should be suspected in a patient with myocarditis (especially if recurrent) associated with a family history of ARVD.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.hrthm.2015.01.001

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CLINICAL PERSPECTIVES

This study establishes for the first time a link between ARVD, genetics, and myocarditis. The research performed in this short series demonstrates the following. (1) The genetic background is important for the development of superimposed myocarditis in ARVD. Desmoplakin mutations seem to be related to unique as well as recurrent episodes of myocarditis. Moreover, a new mutation in the LIM domain also seems to be prone to possible viral susceptibility. However, the extension of this concept to other ARVD mutations and cardiomyopathies remains to be demonstrated. (2) Chest pain and troponin release independent of coronary disease should draw attention to a possible manifestation of a concealed form of ARVD. This early recognition is of paramount importance because it may be a sign of active disease, which sometimes is associated with ventricular arrhythmia as well as ECG and imaging changes. (3) Lymphocytic myocarditis may be present at an early stage of ARVD, even before fibrofatty tissue is evident. This finding should raise the suspicion of ARVD when assessing sudden death cases attributed to RV myocarditis. (4) The results of this study provide practical guidance in the diagnostic workup necessary to deal with these complex patients. It is of paramount importance for both clinicians and pathologists to obtain a detailed family history focused on ARVD, ALVD, and myocarditis. A positive family history and familial clustering may raise the necessary suspicion to make the final diagnosis of this disease.