Head Office: University of Padua

Department of Cardiac, Thoracic and Vascular Sciences and Public Health

Ph.D. COURSE: Translational Specialistic Medicine "G. B. Morgagni"

CURRICULUM: Cardiothoracic and Vascular Sciences

SERIES: XXXI

Genomics in Arrhythmogenic Cardiomyopathy: exploring the complexity

Coordinator: Prof. Annalisa Angelini

Supervisor: Prof. Kalliopi Pilichou

Ph.D. student: Rudy Celeghin

Index

Abstract

Background

Arrhythmogenic cardiomyopathy (AC) is a rare heart muscle disease characterized by fibrofatty myocardial replacement, prominent impairment of ventricular systolic function and arrhythmias. AC phenotypic spectrum was revealed wider than previously thought thanks to genotype–phenotype correlation. Combining multiple sources of clinical information, such as genetic, electrocardiographic, arrhythmic, morphofunctional, and histopathologic findings resulted the best approach to untangle the complexity of this disease.

Aim

The aim of our study was to assess AC genetic heterogeneity, applying appropriate genetic screening and performing a multiparametric genotype-phenotype correlation taking into account AC variants classification.

Materials and Methods

A total of 224 consecutive patients, with a clinical (n.192) or post mortem (n.32) diagnosis of AC, underwent genetic screening by using a 174 cardiac-related genes panel (Trusight Cardio, Illumina). The prevalence of Copy Number Variations (CNVs) and newly AC-associated genes such as *FLNC* and *CDH2*, were investigated. Detailed clinical data were obtained on 12-lead elettrocardiography, echocardiography, and cardiac magnetic resonance with the purpose of performing a multiparametric genotype-phenotype correlation. *TTN* variants were evaluated separately, due the magnitude of the gene, as well as rare variants in AC-unrelated genes. Finally, WES was carried out on 10 AC genotype negative patients in order to identify new candidate genes involved in the disease pathogenesis.

Results

We identified 95 different rare genetic variants in 97 (43%) of the 224 index cases. Of them, 79 variants were found in 5 major desmosomal genes (83%), whereas 16 in AC-related non desmosomal genes (17%). American College of Medical Genetics (ACMG)-based variant classification made evident that half of desmosomal variants (39/79, 49%) were classified as pathogenic/likely pathogenic and were predominantly radical (32/39, 78%). Comprehensive sequencing, including newly AC associated genes (*FLNC* and *CDH2*) and CNVs analysis, led to the identification of the genetic cause in 10 more patients

increasing the overall yield of genetic screening from 43% to 48%. No pathogenic variants were identified in 117 (52%) patients.

Overall we re-evaluated based on the current International Task Force Criteria (ITC) the clinical phenotype of 188 out of 224 AC (84%) index cases. Of these, 94 index cases received a definite AC diagnosis at the outpatient clinic, 16 were heart-transplanted (HTx) patients and 32 sudden death (SD) victims. The remaining 18 were borderline and 28 were possibly affected by AC. 78 of the 142 (55%) definite index cases carried at least a rare variant in AC related genes whereas, only 13 of the 46 (28%) borderline/possible index cases were genotype positive.

Genotype analysis focusing on ventricular involvement highlighted that patients with Left Dominant variant (LDAC) were significantly less positive for desmosomal variants (11/42, 26%) compared to the "classic" AC cases (75/182, 41%)(p-value 0.0065). More in deep, 19 of the 42 (45%) LDAC patients were SD victims, of whom only 4 cases (21%) were genotype positive for desmosomal rare variants.

Based on previous transcriptome studies from our laboratory we identified rare variants in *LGALS3*. Specifically, sequencing of 10 index cases through WES and 140 by direct sequencing, led to the identification of 5 *LGALS3* rare nucleotide variants in 7 probands (4%, 5 males, mean age 39±11 years). Of note, two missense variants occurred in the protein carbohydrate recognition domain (CRD) conferring the loss of its binding site.

Conclusions

Comprehensive genetic analysis revealed a genetic cause in nearly half (48%) of AC patients, of which only half could be classified as P/LP. A proper phenotypic characterization increased variant finding likelihood in definite AC patients (55%). Nevertheless, half of AC patients still missed a genetic cause. Specifically, genetic testing achieved to identify a causative variant in only ~25% of LDAC cases. Finally a new candidate gene was identified in 4% of AC cases, supporting the fact that other genetic factors might be involved in disease pathogenesis. Most of identified genetic variants were variants of unknown significance (VUS), highlighting that cascade genetic screening remains mandatory to understand their significance in disease pathogenesis.

1 Introduction

1.1 Background

Arrhythmogenic cardiomyopathy (OMIM #107970; ORPHA247) is a rare disease of the heart muscle characterized pathologically by fibrofatty myocardial replacement and clinically by prominent ventricular arrhythmias and impairment of ventricular systolic function [1-4]. The original description of this disorder, attributed to Marcus et al. [5] in 1982 although the clinical physiology of this condition was described few years earlier in French [6], characterized 24 individuals with extensive substitution of the right ventricle (RV) myocardium with fatty and fibrous tissue. Subsequently, the disease role in causing sudden death in athletes was described in a prospective clinical-pathologic study with detailed morphologic and histologic features as well as the prevalence of sudden cardiac death (SCD) in the young population of the Veneto region, North-eastern of Italy [1].

At first, myocyte loss with subsequent fibrofatty replacement was thought to be the result of a congenital defect of myocardial development contributing to the early designation of dysplasia. Thanks to the progress of the genetic and phenotypic characterization the term dysplasia was soon replaced by the designation of cardiomyopathy, which refers to a genetically determined heart muscle disorder, and this disease was named as arrhythmogenic right ventricular cardiomyopathy (ARVC) [1, 2].

Although the original disease phenotype hallmark was a predominant RV involvement, with minor and late left ventricle (LV) alteration, now we know that the disease spectrum is wider than previously thought. “Classic” ARVC is characterized by RV preponderance throughout the disease course; the “biventricular” pattern is defined by parallel involvement of both ventricles and the “left dominant” form LDAC is purported to mirror the “classic” pattern, with the LV consistently more severely affected than the RV [7]. These findings have led over the past few years to the use of the broader term “arrhythmogenic cardiomyopathy” (AC), which encompasses all the phenotypic expressions.

1.2 Epidemiology

The estimated prevalence of AC in the general population ranges from 1:2000 to 1:5000 [3, 4, 8]. In the past was considered an endemic disease in North East Italy ("Venetian disease"), where a systematic investigation of the causes of SCD in the young have identified affected AC individuals more commonly than in other countries, is now well recognized in human populations of different ethnicity. AC affects more frequently males than females (up to 3:1), and this has been ascribed to the direct influence of sex hormones on the phenotypic or to the sex-related differences in the amount and intensity of exercise [9-11]. It becomes clinically overt most often in the second-fourth decade of life [3, 4, 8]. More rarely, symptoms and signs can appear before puberty or in the elderly. However, occasionally the first clinical manifestations arise even in patients >70, but the diagnosis is often missed because clinicians do not take it into consideration this morbid entity in this older age-group [3].

1.3 Pathological findings

The hallmark lesion of AC is replacement of the ventricular myocardium by fibrofatty tissue [1, 2, 5]. This condition should not be confounded with Uhl disease, a congenital heart defect in which the RV myocardium fails to develop during embryonic life with the epicardium applied directly to endocardium in the absence of interposed fat [12]. In AC myocardial atrophy is a genetically determined process that occurs progressively with time, starts from the epicardium and extends toward the endocardium to become transmural, resulting into progressive wall thinning. As consequence, the pathognomonic gross features of AC consist of RV aneurysms, whether single or multiple, located in the so-called "triangle of dysplasia" (i.e. inflow, apex and outflow tract) [13] (Figure 1).

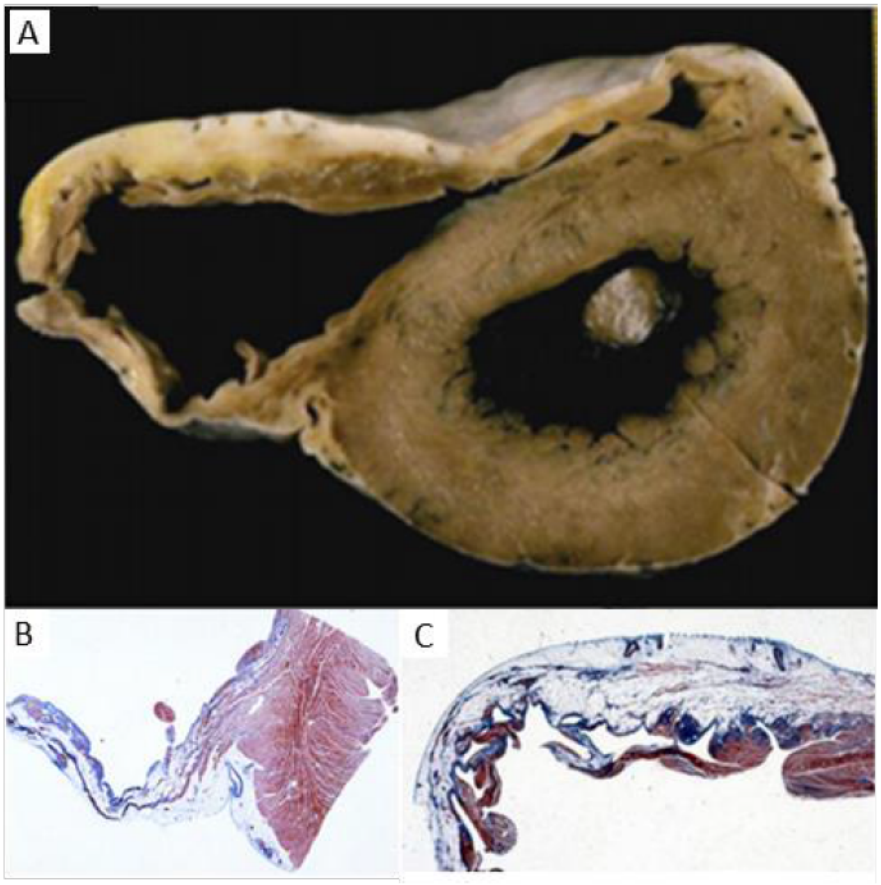


Figure 1: Pathologic features of arrhythmogenic cardiomyopathy. Classical right ventricular (RV) variant: A, Gross transverse section of the heart that shows anterior and posterior RV wall thinning because of myocardial atrophy and a subtricupid aneurysm. Fullthickness histology of the posterior (B) and anterior (C) RV free wall that shows fibrofatty tissue replacement. There is thinning and residual myocardium confined to the endocardial trabeculae (trichrome stain). (Modified from Corrado et al, 2017)

AC phenotypic spectrum has been revealed wider than previously thought thanks to genotype–phenotype correlation. Pathological features can vary from a grossly normal hearts at one end to hearts with massive biventricular disease involvement. LV involvement has been observed in up to 76% of the AC hearts studied at post-mortem, usually limited to the subepicardium or midmural layers of the free wall [14]. Hearts with end-stage disease leading to heart failure usually show huge biventricular chamber dilatation with multiple free-wall aneurysms [2, 14]. Moreover recently, an isolated, nonischemic LV fibrofatty scar, as seen either at postmortem examination or by post-contrast cardiac magnetic resonance (CMR) sequences, has been reported to be a not uncommon myocardial substrate of life-threatening ventricular arrhythmias and SCD in young people and athletes [15, 16].

Histological examination reveals islands of surviving myocytes, interspersed with fibrous and fatty tissue [1, 2]. Replacement-type fibrosis and myocyte degenerative changes should always be identified because fatty infiltration of the RV is not a sufficient morphologic hallmark of AC [17-19]. Of note intramyocardial fat is present fisiologically in the RV anterolateral and apical region and increases with age and body size. Myocyte degeneration and death are often associated with inflammatory infiltrates reported in up to 75% of hearts at autopsy. Rather than being a continuous, ongoing process, disease progression may occur through periodic acute bursts of an otherwise stable disease mimicking myocarditis or myocardial infarction with normal coronary arteries. The detection of viral genomes led to consideration of an infective viral cause, but it is most likely that either viruses are innocent bystanders or myocardial cell degeneration may serve as a milieu favouring viral attach.

1.4 Clinical findings

The phenotypic expression of AC varies considerably, ranging from the clinical profiles of asymptomatic family members with concealed structural abnormalities and no arrhythmias to symptomatic patients experiencing arrhythmic cardiac arrest or undergoing cardiac transplantation because of refractory heart failure [3, 14, 20-23]. The most common clinical presentation consists of ventricular arrhythmias and related symptoms/events, which include palpitations, syncopal episodes (mostly occurring during physical exercise), and cardiac arrest. Less-common presentations are RV or biventricular dilatation, with or without heart failure symptoms, mimicking dilated cardiomyopathy (DCM).

However, often non-specific clinical features comprise myocarditis or fake chest pain, dynamic ST-T wave changes on basal 12- lead elettrocardiogram (ECG) and myocardial enzymes release with normal coronary arteries.
Sudden cardiac death may occur unexpectedly as first symptom in previously asymptomatic individuals, mostly young people and competitive athletes, with a previously undiagnosed (and unsuspected) AC [1, 24-26].

The prognosis of AC is related either to ventricular electric instability, which may lead to arrhythmic SCD, or progression of ventricular muscle disease resulting in RV or biventricular systolic dysfunction.

The natural history of the disease can be divided into four phases, based on clinical and pathological observations [3, 27, 28]:

- A "concealed" phase typical of very early AC. Disease is frequently characterized by the absence of clinical symptoms, but subtle structural changes and minor ventricular arrhythmia may be recognized. Even if subjects are mainly asymptomatic, however they may be at risk of SCD, especially during physical exercise [24].
- In the "overt electrical disorder" phase, patients manifest symptomatic ventricular arrhythmias, palpitations, syncopes and pre-syncopes. This phase is characterized by the presence of functional and morphological abnormalities of the RV and/or Left ventricle (LV), usually detectable by cardiac imaging techniques.
- The "right ventricular dysfunction" phase is characterized by further extension of disease involving the RV myocardium, leadings to impaired contractility and isolated right heart failure.
- The most advanced phase is characterized by LV involvement leading to biventricular (BV) heart failure, which is difficult to distinguish from DCM. However, LV involvement may be detected also in earlier phases of the disease [7].

1.5 Diagnostic criteria

The best strategy to reach the clinical diagnosis consists in combining multiple sources of clinical information, such as genetic, electrocardiographic, arrhythmic, morphofunctional, and histopathologic findings. AC criteria were first proposed by an International Task Force (ITC) in 1994 [29] to facilitate disease diagnosis. The criteria were initially designed to provide adequate specificity for the right-dominant variant of AC among index cases with overt clinical manifestations. Based on their diagnostic sensitivity and/or specificity, criteria were classified as major and minor, and the diagnosis of AC was reached in the presence of 2 major criteria, 1 major plus 2 minor, or 4 minor criteria from different categories. Later, these criteria were modified in order to increase sensitivity in detecting milder forms of AC [30]. The modified criteria facilitated the recognition of patients' relatives affected based on different clinical features comprising structural, histological and arrhythmic abnormalities along with a detailed familial history of disease and SCD (Table 1) These diagnostic criteria in adults were demonstrated valid also in the pediatric age, exception made for the presence of inverted T wave on the right precordial leads in children < 12 years of age. To date, the AC diagnostic criteria include the presence of a pathogenetic variant in AC related genes as a major criterion to establish the diagnosis, different from all other forms of cardiomyopathy.

1.6 Limitations of current diagnostic criteria

Since both the 1994 and 2010 guidelines were developed to diagnose the original right-dominant disease phenotype they did not include specific criteria for diagnosing LV involvement and the more recently recognized left-sided phenotypic variants [7, 31]. Moreover, peculiarities of diagnosis in the paediatric population, which represents approximately one-sixth of the overall ARVC-population, were not addressed.

Table 1: Comparison of original and revised Task Force criteria for diagnosis of AC [29, 30]

Original task force criteria	Revised task force criteria
I. Global or regional dysfunction and structural alterations*	
Major	
	By 2D echo:
• Severe dilatation and reduction of RV ejection fraction with no (or only mild) LV impairment • Localized RV aneurysms (akinetic or dyskinetic areas with diastolic bulging) • Severe segmental dilatation of the RV	• 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) — *or* 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) — *or* RV ejection fraction ≤40%
	By RV angiography: • Regional RV akinesia, dyskinesia, or aneurysm
Minor	
	By 2D echo:
• Mild global RV dilatation and/or ejection fraction reduction with normal LV • Mild segmental dilatation of the RV • Regional RV hypokinesia	• 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/m^2) — PSAX RVOT ≥32 to <36 mm (corrected for body size [PSAX/BSA] ≥18 to <21 mm/m^2) — *or* 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) — *or* RV ejection fraction >40% to ≤45%
II. Tissue characterization of wall	
Major	
• Fibrofatty replacement of myocardium on endomyocardial biopsy	• 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. Repolarization abnormalities	
Major	• Inverted T waves in right precordial leads (V_1, V_2, and V_3) or beyond in individuals >14 years of age (in the absence of complete right bundle-branch block QRS ≥120 ms)
Minor	
• Inverted T waves in right precordial leads (V_2 and V_3) (people age >12 years, in absence of right bundle-branch block)	• Inverted T waves in leads V_1 and V_2 in individuals >14 years of age (in the absence of complete right bundle-branch block) or in V_4, V_5, or V_6 • Inverted T waves in leads V_1, V_2, V_3, and V_4 in individuals >14 years of age in the presence of complete right bundle-branch block

Continued

Table 1 *Continued*

Original task force criteria	Revised task force criteria
IV. Depolarization/conduction abnormalities	
Major	
• Epsilon waves or localized prolongation (>110 ms) of the QRS complex in right precordial leads (V_1 to V_3)	• Epsilon wave (reproducible low-amplitude signals between end of QRS complex to onset of the T wave) in the right precordial leads (V_1 to V_3)
Minor	
• Late potentials (SAECG)	• Late potentials by SAECG in ≥1 of 3 parameters in the absence of a QRS duration of ≥110 ms on the standard ECG • Filtered QRS duration (fQRS) ≥114 ms • Duration of terminal QRS <40 μV (low-amplitude signal duration) ≥38 ms • Root-mean-square voltage of terminal 40 ms ≤20 μV • Terminal activation duration of QRS ≥55 ms measured from the nadir of the S wave to the end of the QRS, including R′, in V_1, V_2, or V_3, in the absence of complete right bundle-branch block
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	
Minor	
• Left bundle-branch block-type ventricular tachycardia (sustained and nonsustained) (ECG, Holter, exercise) • Frequent ventricular extrasystoles (>1000 per 24 hours) (Holter)	• Nonsustained or sustained ventricular tachycardia of RV outflow configuration, left bundle-branch block 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	
• Familial disease confirmed at necropsy or surgery	• 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	
• Family history of premature sudden death (<35 years of age) due to suspected ARVC/D • Familial history (clinical diagnosis based on present criteria)	• 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

1.7 Clinical tests enabling AC diagnosis:

Twelve-lead ECG is a valuable diagnostic test in AC because it records repolarization and depolarization abnormalities in the majority of probands with a definitive diagnosis of AC. Negative T waves in the anterior precordial leads (V1 through V4) are the most common finding. Depolarization abnormalities include incomplete (rarely complete) right bundle branch block (RBBB), prolongation of right precordial QRS duration with a delayed S-wave upstroke (terminal activation delay >55 ms), QRS fragmentation, and post-excitation epsilon waves (ie, small amplitude potentials occurring at the end of QRS complex/beginning of the ST segment) [32, 33].

Signal-averaged electrocardiogram (SAECG) allows the registration of low amplitude potentials within the end of the QRS complex (late potential) that are not wide enough to be evident on the 12-lead ECG.

Holter ECG monitoring is used to register the electric activity of the cardiovascular system for a period of normally 24 h, with particular attention to the diurnal rhythm fluctuations.

Echocardiography (Echo) represents the first-line imaging approach for evaluating patients with suspected AC or for screening of family members, allowing serial examinations with the aim to assess the disease onset and progression.

CMR imaging is another non-invasive tissue characterization technique that allows the detection of morphological and structural abnormalities of the ventricular wall like micro aneurysms, calculation of RV and LV volumes and ejection fraction [34-36].

Endomyocardial biopsy (EMB) is used to detect myocytes in diverse stages of cell death and of fibrofatty replacement (major criterion). However, a negative biopsy is not enough to exclude AC because of the segmental nature of the disease, especially during early stages [37]. The sensitivity of EMB for AC is low if the myocardial samples are taken from the septum, which is a region uncommonly involved by the disease. EMB cannot be routinely recommended for diagnosis of AC and should be reserved for selected patients particularly probands with a sporadic form of AC, in whom the final diagnosis depends on histologic exclusion of phenocopies such as DCM, myocarditis, or sarcoidosis.

Three-dimensional electroanatomic voltage mapping by CARTO system (Biosense, Diamond Bar, CA) may be of significant added value for the diagnosis of AC because it has the potential to identify and quantify RV regions of scar with low-amplitude electric signals, which typically show fractionation, double potentials, or conduction delay [38, 39]. However, it is not recommended as a routine diagnostic tool because it is invasive, expensive, and highly operator dependent.

1.8 Left Dominant AC diagnosis

While the revised criteria easily catch the classical RV variant, there is lack of specific diagnostic guidelines for non-classical disease patterns, thus explaining the under-recognition of the LV one. ECG abnormalities such as lateral or inferolateral T-wave inversion (leads V5, V6, LI, and aVL), low voltage QRS complex on peripheral leads and RBBB/polymorphic ventricular arrhythmias suggest a left-sided involvement [7, 31, 40]. Gadolinium -enhanced CMR a far more-sensitive indicator of even early or minor left-sided disease, and is frequently detected in a wall segment without a concomitant morphofunctional abnormality, thus preceding the onset of LV dysfunction or dilatation [7, 41, 42]. Typically, LV late gadolinium enhancement involves the inferolateral and infero-septal regions, and affects the subepicardial or midwall layers.

1.9 Therapy

The most important objective of clinical treatment of AC patients is prevention of SCD. Current therapeutic options include lifestyle changes, β-blockers, antiarrhythmic drugs (AADs), catheter ablation, ICD, and heart transplantation (HTx). Recently, a task force of experts from both Europe and the United States produced a consensus document for treatment of AC [43]. Physical exercise is one of the most important risk factors, which promotes the phenotypic expression of the disease and triggers life-threatening ventricular arrhythmias in AC [11]. Accordingly, current guidelines recommended the exclusion of competitive or endurance sport activity of affected individuals but also desmosome gene variants carriers.

1.10 Molecular genetics of AC

In 1982, Marcus et al. advocated the inherited nature of AC, with the description of two affected adult cases in the same family. AC is currently considered an autosomal dominant disease with reduced penetrance and variable clinical expression [8] although, recurrent description of compound/ digenic heterozygotes and homozygotes patients have been associated with severe forms of the disease [9, 44] with or without cutaneous abnormalities [45-51].

The recognition of disease familial nature [52] led to the identification of the first disease-causing gene mutation in a recessive syndromic form of AC known as Naxos disease [53, 54]. Naxos disease was first described in 1986 as a familial, autosomal recessive disease characterized by hair and skin abnormalities (woolly hair and palmoplantar keratoderma) exhibiting an AC-like cardiomyopathy [55]. The typical hair phenotype is present at birth along with the erythema on the palms of the hands which gaffers within the first years of life, while the cardiac phenotype appears only during adolescence or early adulthood. In the original description, 9 affected subjects from 4 Greek families of the Naxos island were investigated by linkage analysis leading to the identification of a genetic locus on the long arm of chromosome 17 (17q21.2) [56]. Subsequent direct sequencing of the *Plakoglobin* (*JUP*) gene in all affected subjects identified a 2bp-deletion leading to a frame shift with consequent introduction of a premature stop codon after 11 amino acid [53].

Linkage analysis were also used to identify the first disease causing mutation in a dominant AC form [54]. Six affected subjects from an Italian family of the Veneto Region carried a heterozygous missense mutation c.897C>G this time in the *Desmoplakin* (*DSP*) gene, located on the short arm of chromosome 6 (6p24.3). Earlier, a homozygous *DSP* deletion was described for another syndromic form, known as Carvajal syndrome [57]. Carvajal syndrome was first described in 1998 as a familial, autosomal recessive disorder characterized by epidermolytic palmoplantar keratoderma, woolly hair, and heart anomalies. In the original description, 18 subjects from 3 Ecuador families, were investigated by linkage analysis and subsequent direct sequencing leading to the identification of a homozygous single-nucleotide deletion c.7901delG, which creates a premature stop codon depriving DSP of its C-domain [58, 59].

DSP and JUP are both components of the cardiac desmosome, therefore candidate gene approaches revealed the genetic heterogeneity of AC with the identification of 15 genetic loci (

Table 2).

Nowadays AC is defined as a disease of the desmosomes since causative variants are found in genes coding for desmosomal proteins: JUP, DSP, plakophilin-2 (PKP2), desmoglein-2 (DSG2), and democollin-2 (DSC2) [54, 60-64]. Isolated reports, accounting for less than 1-3% of cases, are caused by variants in nondesmosomal genes, such as transforming growth factor-β-3 (*TGFβ3*), cardiac ryanodine receptor (*RYR2*), transmembrane protein 43 (*TMEM43*), lamin A/C (*LMNA*), desmin (*DES*), catenin alpha 3 (*CTNNA3*), titin (*TTN*), phospholamban (*PLN*), filamin C (*FLNC*) and N-cadherin (*CDH2*) [65-74].

Table 2: AC genetic loci. Abbreviations AD: autosomal dominant; AR: autosomal recessive.

MIM entry	Locus	Gene ID	Gene name	Mode of transmission	Reference
#611528	17q21.2	JUP	Plakoglobin	AD	[60]
#601214				AR	[53]
#607450	6p24.3	DSP	Desmoplakin	AD	[54]
#605676				AR	[57]
#609040	12p11.21	PKP2	Plakophilin-2	AD/AR	[61]
#610193	18q12.11	DSG2	Desmoglein-2	AD/AR	[62]
#610476	18q12.1	DSC2	Desmocollin-2	AD/AR	[63]
Non-desmosomal genes					
#600996	1q43	RYR2	Cardiac Ryanodine Receptor 2	AD	[65]
#107970	14q24.3	TGF-β3	Transforming growth factor-beta 3	AD	[66]
#604400	3p25.1	TMEM43	Transmembrane Protein 43	AD	[67]
	2q35	DES	Desmin	AD	[68]
	6q22.31	PLN	Phospholamban	AD	[71]
	2q31.2	TTN	Titin	AD	[69]
	1q22	LMNA	Lamin A/C	AD	[70]
#615616	10q21.3	CTNNA3	alpha-1-catenin	AD	[72]
	7q32.1	FLNC	Filamin C	AD	[73]
	18q12.1	CDH2	Cadherin 2	AD	[74]

Independent studies identified large deletions in *PKP2* gene, suggesting a pathogenic role of copy number variants (CNVs) in the disease due to haploinsufficiency [75-78]. First, Cox et al.[75] described 3 large *PKP2* deletions in a 149 Dutch AC cases, reporting as prevalent as ≈2%. The authors highlighted that the CNVs analysis should be extended not only to *PKP2* but also in other desmosomal genes. Since then, single case carrying CNVs in *PKP2* have been reported [76-78] but only recently a systematic comprehensive CNVs screening of was performed by our group. Pilichou et al., [79] reported for the first time CNVs in desmosomal cadherin-related genes (*DSG2, DSC2*) and estimated the prevalence of *PKP2*-

related CNVs to 5.6% (9 of the 160) in genotype-negative proband. Although CNVs may confer haploinsufficiency, the reduced disease penetrance in family carriers (≈32%) was similar to the one observed in other point variants suggesting that other factors has been involved in the development and progression of the disease.

1.10.1 Desmosomes

Desmosome junctional complexes are particularly abundant in tissues subjected to mechanical stress like the epithelium and the myocardium, where these molecules mediate mechanical anchorage of cardiomyocytes by connecting the cytoskeleton to the cell membranes of adjacent cells (Figure 2). As well as may play a role in cell-cell communication, tissue differentiation, and apoptosis [80].

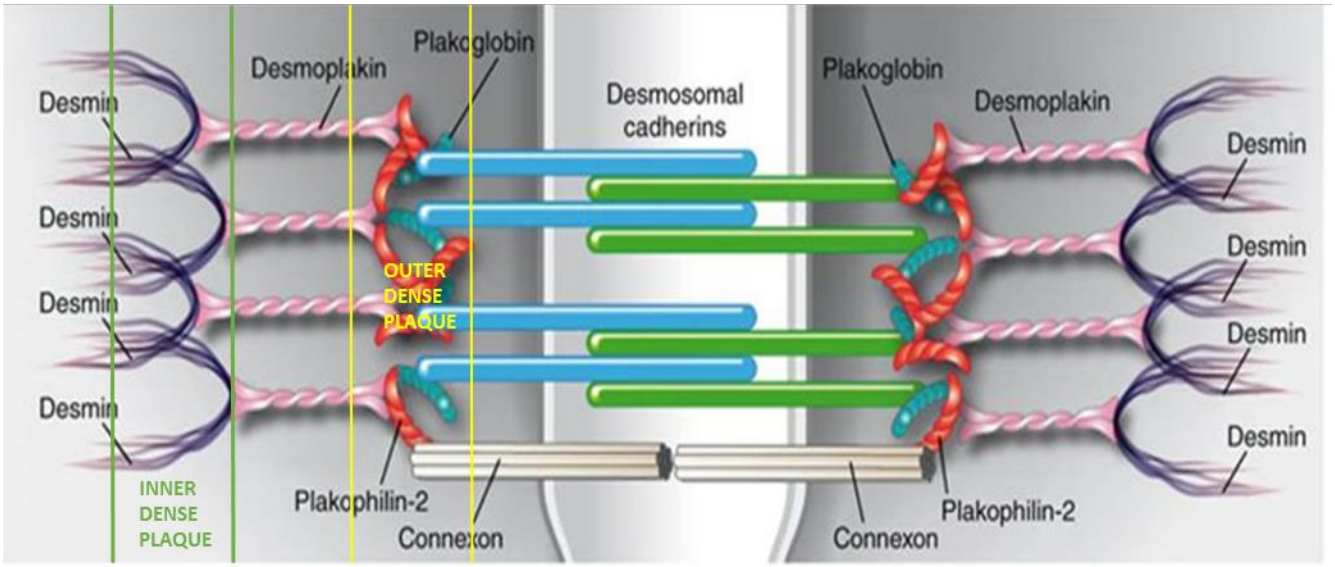


Figure 2: Schematic representation of desmosome modified from [79]

These electron-dense symmetrical structures that appear as dense membrane-associated plaques divided by a central mid-line of intercellular space are called desmoglea and intracellular plaque. The intracellular plaque is commonly described as composed of two areas: the outer dense plaque, and a dense inner plaque [81]. The desmosomal structure comprises transmembrane adhesive glycoproteins (components of the cadherin superfamily) and cytoplasmic proteins (components of the plakin and armadillo families). The outer dense plaque is where the cytoplasmic domains of the cadherins (DSG2, DSC2) attach to plakins (DSP) via armadillo proteins (PKP2 and JUP). The inner dense plaque is located where plakins attach to the intermediate filaments of the cell.

Desmoplakin_DSP (6p24.3) belongs to the plakin protein family which includes a large number of proteins such as plectin, envoplakin and periplakin, all mediating the link between different junctional complexes and the cytoskeleton. DSP is composed by an N-terminal domain required for both the localization to the desmosome and interaction with plakophilin and cadherin family proteins, a central coiled-coil rod domain involved in the protein dimerization and a C-terminal domain that interacts directly with the intermediate filaments. It is expressed in all tissues containing desmosomes [82] and an alternative splicing of the DSP precursor messenger RNA (mRNA) produces two isoforms, differing in the length of the central -helical domains: DSP I, predominantly expressed in heart, comprise 2871 amino acids whereas DSP II only 2271 amino acids.

The first pathogenic nucleotide variants described in *DSP* families with autosomal dominant striated palmoplantar keratoderma, were a heterozygous nonsense p.Gln331Ter and a splicing site c.939+1G>A variants [83] but cardiac alterations were not studied. Since then, more than 100 *DSP* pathogenic variants have been detected in 5 to 16% of AC cases [62, 75, 84].

Plakophilin-2_PKP2 (12p11.21) the predominant protein isotype expressed in heart, belongs to the armadillo family of proteins and interacts directly with both, desmosomal cadherins and DSP [85]. Two alternatively spliced mRNA transcripts give origin to two protein isoforms: transcript 2b (881 amino acid) and transcript 2a (837amino acid) [86]. PKP2 comprise an amino-terminal head domain and nine armadillo repeat motifs and it's essential for heart morphogenesis and proper localization of DSP in mice [87]. After the link to disease pathogenesis through a haploinsufficiency mechanism which bring to defect cell-cell contact [61], more than 120 *PKP2* pathogenic variants have been reported to AC accounting for approximately 10 to 45% of reported cases [61, 62, 75, 84]. Most variants show a dominant inheritance with reduced penetrance, but recessive and compound heterozygous variants have also been identified in several patients. Moreover, large deletions involving *PKP2* have also been described in subset of affected individuals [75-79].

Desmoglein-2_DSG2 (18q12.1) is a desmosomal cadherin belonging to the cadherin superfamily, mediating calcium dependent cell-cell adhesion. Desmosomes bears four isoforms of desmoglein (DSG 1 to 4), showing tissue specific expression patterns [88]. DSG2 is expressed in all tissues bearing desmosomes but seems to be the only isoform expressed in cardiac tissue [89, 90]. DSG2 has an intracellular anchoring domain interacting with DSP, a transmembrane domain, four extracellular cadherin domains each containing a calcium binding site that stabilizes the structure and function of cadherins, a small signal and a preprotein domain. Since 2006 when *DSG2* was linked for the first time to AC through the detection of 9 missense variants in 8 Italian families affected by AC affecting highly conserved amino acids, more than 50 *DSG2* variants have been found in 2 to 20% of AC patients [62, 75, 84]. Of note in 2006 the first compound heterozygote AC patients was reported.

Desmocollin-2_DSC2 (18q12.1), is another glycoprotein, member of the cadherin superfamily mediating calcium dependent cell-cell adhesion. Three desmocollin isoforms (DSC 1, 2 and 3) are currently known, of which DSC2 is the only isoform present in cardiac tissue even though ubiquitously expressed [88]. DSC2 is composed of a signal domain, a preprotein domain followed by four highly conserved extracellular subdomains and an extracellular anchor domain at the N-terminus. In 2006 Syrris and colleagues described the presence of heterozygous *DSC2* frameshift variants in 4 AC affected probands without variants in other desmosomal genes [63]. Since them, less than 50 *DSC2* nucleotide variants have been reported, accounting for 1 to 3% of AC cases [63, 75, 84, 91, 92].

Plakoglobin_JUP (17q21), is another armadillo protein of desmosomes. It is present both, in adherens junctions and desmosomes, where it binds to the cytoplasmic domain of cadherin acting as a linker molecule between the inner and outer parts of the desmosomal plaque. JUP comprise an N-terminal domain, a central domain containing highly conserved armadillo repeats and a C-terminal domain. JUP has only recently been linked to the dominant AC form, after the description of an in frame insertion of a serine residue p.Ser39_Lys40insSer in the N-terminus of the protein in a small German family [60]. To date, less than 20 *JUP* variants have been reported in approximately 1% of AC cases [60, 84] [75].

1.10.2 Non desmosomal genes

Isolated reports, accounts for less than 1-3%, associated variants in non-desmosomal genes with a clinical phenotype resembling AC

Transmembrane protein 43_TMEM43 (3p25.1) acts as a nuclear membrane organizer. *TMEM43* variants were first identified in Emery–Dreifuss muscular dystrophy patients. In contrast to classic AC, *TMEM43* variants carriers exhibit right ventricular aneurysms and ventricular arrhythmias before birth [67], the phenotypic penetrance of *TMEM43* variants is 100% and frequency is less than 1%. Recent studies reported the founder effect of *TMEM43* variants, probably caused by migration from continental Europe to Canada [93].

Desmin_DES (2q35) is a type of intermediate filament in myocytes, often related to myofibrillar myopathies. *DES* variants carriers often have both myopathy and cardiomyopathy [68]. The first *DES* variants p.Arg454Trp, affecting the localization of DSP and PKP2 at the intercalated disk, was identified in a family showing an early onset of conduction system disorder without variants in other desmosomal genes [94]. The frequency of *DES* is about 1–2%.

Titin_TTN (2q31.2) is a sarcomeric protein, the largest protein in mammals, which contributes to force transmission at the Z line and resting tension in the I band region. *TTN* variants were reported in other cardiomyopathies such as HypertrophicCardiomyopathy (HCM) and DCM. In AC, *TTN* variant carriers display various phenotypes, including biventricular dysfunction and conduction block. Eight unique missense *TTN* variants were identified in 7 families with AC-like phenotype and without desmosomal genes variants, including a prominent p. Thr2896Ile variant which showed complete cosegregation in one large family [69]. The role of *TTN* variants for AC is still a matter of study.

Lamin A/C_LMNA (1q22) is located in the inner nuclear membrane and is fundamental for cell stabilization. *LMNA* variants are reported in various systemic diseases, including Emery–Dreifuss muscular dystrophy, and in dilated cardiomyopathy patients with sinus bradycardia and conduction disturbance. In 2012, four *LMNA* variants were reported in AC-like patients with conduction disturbances [70] but variants frequency has not been yet determined as *LMNA* variants were often found in AC_DCM patients [95].

Phospholamban_PLN (6q22.31) is a protein necessary for calcium handling in cardiac contractions and PLN was known as a causative gene for DCM. The only reported *PLN* variant so far, p.Arg14del, was described in a Dutch patients with AC [71] which seems to be a founder one.

Alpha-T-catenin_CTNNA3 (10q21) is a cadherin which binds to plakophilins in cell adherens junctions between cardiomyocytes. Only two *CTNNA3* AC-related variants have been reported to date leading to a lack of interaction with beta-catenin [72].

Sodium voltage-gated channel alpha subunit 5_SCN5A (3p22.2) is a subunit of the cardiac sodium channel, and its mutations have been linked to various cardiac diseases, most 'sine materia', including long QT syndrome type 3, Brugada syndrome with non-ischaemic ST-segment elevation, progressive cardiac atrioventricular conduction disease, and DCM. In 2008, an *SCN5A* loss-of function variant was reported in a 58-year-old patient with AC and frequent non-sustained ventricular tachycardia in the absence of ST-segment elevation [96]. Recently, a large multicentre collaborative study reported that ~2% of AC probands have a pathogenic *SCN5A* mutations, expanding the spectrum of phenotypes associated with variants in this gene [97].

Filamin C_FLNC (7q32.1) is a muscle specific filamin which directly interacts with the dystrophin associated glycoproteins and the integrin complexes that link the subsarcolemmal actin cytoskeleton to the extracellular matrix. At intercalated discs, FLNC is located in the fascia adherens where myofibre ends reach the sarcolemma, adjacent to the position of desmosomal junctions. Its role in the attachment of the sarcomere's Z-disc to the sarcolemma and to the intercalated discs allows cell to-cell mechanical force transduction. Truncating *FLNC* variants in 28 families have been linked to particular overlapping phenotypes of dilated and left-dominant arrhythmogenic cardiomyopathies complicated by frequent premature sudden death, a dominant mode of transmission, and an unusual penetrance of higher than 97% in carriers older than 40 years of age [73].

Cadherin 2_CDH2 (18q12.1) also known as N-cadherin, is a protein that mediates calcium ion-dependent adhesion in the brain and skeletal and cardiac muscle. Recently, two different studies identified two variants in this gene linked to an AC phenotype [74, 98].

Variants in the regulatory region of ***transforming growth factor beta-3_TGFB3*** (14q24.3) gene have also been reported, but their pathogenicity is still a matter of debate [66].

Finally, the ***ryanodine receptor 2 _RYR2*** (1q43) gene encodes a protein of the sarcoplasmic reticulum, necessary for cardiac contraction by controlling the calcium ions. *RYR2* variants are nowadays associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) rather than with AC, as originally believed [65].

1.11 Pathogenesis

Several theories have been postulated to explain the pathogenesis of AC: dysontogenic, degenerative, inflammatory, transdifferentiational.

The dysontogenic theory [99] consider AC a milder form of Uhl's anomaly [12] which is a congenital heart defect characterized by hypoplasia of the RV myocardium at birth however, myocyte loss has been demonstrated to occur progressively starting from childhood [100]. The degenerative theory which was postulated in 1996, considered AC as a consequence of myocyte death, either by necrosis or apoptosis, due to inherited ultra-structural defects [101, 102]. Further, recent studies have demonstrated that the key initiating phenomenon in the cascade of events that lead to fibrofatty replacement of the normal myocardium is myocyte necrosis [103, 104].

In the inflammatory theory, the disease is considered the consequence of preceding myocarditis, since myocardial inflammation is a common feature in hearts with AC [105, 106] . The viral myocarditis may overlap an already affected heart accelerating the disease progression, rather than being a primary factor in the etiology of the disease [107].

The transdifferentiation theory suggests that cardiac myocytes undergo a metamorphosis leading to became adipocites [108]. This theory seems debatable due to the limited dedifferentiation capabilities of adult cardiomyocytes, indeed recent studies supported the idea that

adipocytes in AC derive from progenitor cells of the second heart field, which give rise to the bulbus cordis and the pulmonary infundibulum [109].

Transgenic animal models, that mimic the human AC phenotype (mice and zebrafish) and induced pluripotent stem cells (iPSCs) from affected patients, are useful tools to investigate mechanical and/or functional disruption of cell junctions by aberrant desmosomal proteins leading to cardiomyocyte death and subsequent repair with fibrous and fatty tissue

Reduced cell-cell adhesion has been demonstrated by using monolayers of neonatal rat ventricular myocytes in which *PKP2* was silenced [110]. However, when expressing mutant forms of either PKP2 or JUP, cells exhibited preserved intercellular adhesion and abnormal signalling in response to mechanical stress, advocating questions on a primary role of cell-cell adhesion in AC pathogenesis [111]. Parallel , Asimaki and colleagues demonstrated that a reduced junctional signal for JUP seems to be a hallmark of the disease in myocardial samples from AC patients, suggesting its possible role in intracellular signalling rather than adhesion [112], as propose by other groups [113, 114].

Marian group for the first time demonstrate in a Dsp-deficient mouse model that an abnormal distribution of intercalated disc protein leads to suppression of the canonical WNT/β-catenin/Tcf/Lef pathway a known regulator of adipogenesis, fibrogenesis and apoptosis [113]. Moreover, knocking down DSP in HL-1 cells causes the translocation of JUP into the nucleus, where it interferes with β-catenin/TCF transcriptional activity, leading to an adipogenic switch. Thereafter, the same group demonstrated that most of the adipocytes in AC originate from cardiac progenitors cells of the embryonic second heart field [109]. Furthermore, in mice overexpressing cardiac truncated JUP, suppression of the canonical WNT signaling led to adipogenesis in c-kit + cardiac progenitor cells [115].

A lower expression protein kinase C α, a signalling molecule which localises to the intercalated disc, has been reported analysing molecular remodelling of the intercalated disc [116] .As a result, neurofibromin-2 (or Merlin) becomes activated and in turn phosphorylates and inactivates the effector of the Hippo pathway, Yes -associated p rotein, YAP [117]. YAP is then free to bind to β-catenin and plakoglobin and therefore drives adipogenesis in myocardial tissue.The Hippo pathway, among other functions, regulates cell differentiation and homeostasis [118]. Interestingly, the activation of the Hippo pathway inhibits the canonical WNT pathway, reinforcing the hypothesis that the two pathways might be implicated in the pathogenesis of AC [119].

Further, among the others, Kim and colleagues successfully reprogrammed patient-derived dermal fibroblasts into iPSCs generating human cardiomyocytes for in vivo modelling. iPSCs-derived cardiomyocytes from AC patients with *PKP2* mutations, demonstrated that the abnormal JUP nuclear translocation and decreased β-catenin activity is insufficient to reproduce the pathologic phenotype [120].

Remarkably, transgenic experimental animal models and iPSC-derived cardiomyocytes demonstrated only abnormal "lipogenesis", but not adipocyte formation/transdifferentiation. Thus, cells other than cardiomyocytes must be involved in the abnormal adipogenesis and fibrosis which is an essential feature of AC phenotype [121]. A role of cardiac mesenchymal stromal cells as a source of adipocytes in AC has been recently suggested [122].

In most AC cases a reduction of connexin-43 expression at the intercellular junction has been highlighted, underlying that compromised mechanical coupling might also account for abnormal electrical coupling through gap-junction remodelling. Desmosomes, gap junctions and sodium channels are functionally coupled and modification of one constituent can affect the function and integrity of the others [4]. Moreover, cardiac sodium current was found to be reduced in experimental models of AC [104, 123-126]. These findings suggest that life-threatening ventricular arrhythmias precede structural abnormalities due to electrical uncoupling and reduced sodium current. However, this hypothesis remains to be proven in human AC patients.

Finally, high-throughput drug screening identified SB216763, a compound able to prevent heart failure and normalize survival in a transgenic zebrafish model of AC with cardiac specific expression of human *JUP* deletion [126]. Experiments in neonatal rat ventricular myocytes expressing the same *JUP* mutation and in cardiac myocytes derived from iPSCs of two *PKP2* mutation carriers further supported its efficacy in restoring the subcellular distribution of JUP, connexin-43 and SCNA5. Interestingly, SB216763 was already known as an activator of the canonical WNT signaling pathway and these data, together with those by Lombardi and Kim may led to the identification of a curative therapy in AC, by targeting a final common pathway of disease pathogenesis [109, 120].

1.12 Conventional Screening

Direct Sanger sequencing developed in the 70s [127] has been worldwide chosen for DNA sequencing until Next Generation Sequencing (NGS) era. Despite various modifications and improvements including its partial automation, it remains relatively time-consuming and expensive considering the growing need of sequencing larger DNA portions. In genetically heterogeneous diseases, the cost and effort of DNA sequencing is often considerable and numerous DNA pre-analytic techniques have been developed for the detection of point variants and small deletions. Pre-analytic techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), chemical cleavage method (CCM), denaturing high performance liquid chromatography (DHPLC), all followed by direct sequencing of aberrant conformers or elution profiles allowed to minimize sequencing to a subset of abnormal PCR products. DGGE is a technique developed by Fischer and Lerman [128] that can identify homoduplex molecules that differ by single bp substitutions using electrophoresed through a gradient of increasing concentration of a denaturing agent. SSCP analysis is based on the differential electrophoretic mobility of single-strand DNA molecules that differ by a single base [129]. Thermally denatured DNA is electrophoresed, and those single stranded DNA fragments that take up an altered conformation show up as aberrantly migrating bands on the electrophoretic gel. In CCM PCR heteroduplexes are incubated with two mismatch-specific reagents: Hydroxylamine modifies unpaired cytosine and potassium permanganate modifies unpaired thymine. Heteroduplexes are then cleaved at the site of the modified mismatched base and separated by electrophoresis. DHPLC technique is based on the detection of heteroduplexes containing a variants or polymorphism by reduced column retention of heteroduplexes compared to the respective homoduplexes under partially denaturing conditions [130] DHPLC, under optimized conditions, is costeffective, highly accurate, rapid, and efficient for variants detection. A disadvantage of DHPLC is the requirement and maintenance of a specialized and expensive equipment and optimization of each reaction required achieving the highest sensitivity for variant detection, moreover it can also be difficult to identify homozygotes unless the sample is spiked with a known control. The advent of NGS has revolutionized sequencing in term of cost, time and spectrum of genes analysed.

1.13 Next Generation Sequencing

Since the conclusion of the Human Genome Project in 2000, based on Sanger technology, which required about 13 years [131, 132] and 70 million dollars [133], deep sequencing technologies were developed to sequence in short range of time whole genomes and exomes.

NGS increases throughput and to decreases sequencing costs allowing to shift the interest from the research of variants in specific DNA regions to the identification of variants from a genome-wide sequencing data. This has led to advances in diagnostics and scientific research with the detection of variants linked to Mendelian and complex diseases [134-137]. NGS has been successfully applied for instance to the identification of genetic defects of a variety of disorders such as cancer [138, 139], neurological diseases [140, 141], intellectual disability [142, 143], mitochondrial dysfunctions [144, 145], cardiovascular diseases [146, 147]. Applications of NGS has the potential to focus on the analysis of entire genomes (Whole Genome Sequencing-WGS), on the sole coding part of the genome (Whole Exome Sequencing, WES) or on specific target genes (Targeted Resequencing, TR). WES is the selective sequencing of the coding sequence of the human genome and less expensive in comparison to WGS since the exome represents only about 1% of the genome. In the clinical setting, WES can offer a new approach to diagnose patients with an unclear clinical diagnosis and whom who are negative from classical screening of disease-related genes, enabling the research in genes and nucleotide variants not yet associated to the disease.

However, the introduction of WES for clinical laboratories remains challenging, especially because of the laborious and accurate process of bioinformatics analysis and data interpretation that are required to identify candidate genes and causal variants. For these reason in the clinical practice TR resulted the first screening strategy to reach higher coverage of exonic regions of interest while reducing the sequencing cost and time. These rapid, accurate, and relatively low cost method allows a high throughput, genotype-based approach to molecular diagnosis.TR may rapidly screen at once large panels of genes associated with a particular phenotype or may provide differential diagnosis in diseases that present with atypical manifestations, or for which not all genetic variants are known.

1.14 Next Generation Sequencing in Cardiomyopathies

The molecular analysis of genetically heterogeneous disorders, such as inherited cardiomyopathies, has received enormous benefits from the adoption of NGS screening. These diseases are linked to multiple causative genes, often showing rare and private variants, and old direct sequencing methods would be laborious, time consuming and would miss this complexity. In the last years linkage analysis performed in large families and genome-wide association studies performed in diverse populations have been successful in detecting causal loci, genes and variants of inherited cardiomyopathies. Although cardiomyopathies usually present different clinical features, they exhibit some level of genetic and, especially in their end-stage, phenotypic overlap. For example the detection of causative variants in desmosomal genes sometimes also occur in DCM patients in addition to AC patients [148-152], so that variants in the same gene or gene set correspond to a wider phenotypic spectrum. Thus, NGS may help differential diagnosis approach for correctly identifying patients with unclear clinical characteristics. With different NGS approaches genomic regions of interest can be targeted to enable clinical approach for the diagnostic purposes.

A diagnostic tool for cardiomyopathies in a clinical setup is represented by the sequencing of cardiomyopathy associated genes panel on NGS platforms [153]. For example, a study involving 223 unrelated probands with HCM, using targeted resequencing of 41 cardiovascular genes, reported 152 distinct candidate variants in sarcomeric or associated genes (89 novel) in 143 patients (64%), and an additional 94 candidate variants (73 novel) in desmosomal, and ion-channel genes in 96 patients (43%) [154]. Another study described the use of NGS in order to search for de novo genetic variants by WES in 2 unrelated genotype-negative infants presenting prolonged QTc interval. Genetic screening, focused on new candidate genes identified by WES, was then performed on an independent cohort of 82 subjects with long-QT (LQT) syndrome without mutations in LQT genes [155]. Further, Girolami et al., identified a novel disease-causing variant by targeted resequencing on 48 disease genes for HCM, which was shown to segregate with the cardiomyopathic trait in an Italian family with HCM [156], emphasizing the potential of NGS approach in diagnostic screening.

1.15 Genetic Testing in AC

The diagnosis of AC is based primarily on the structural, histopathologic, and arrhythmic manifestations of the disease in the proband within a family. Current guidelines recommend that the initial genetic testing within a family should only be applied to probands with an unequivocal phenotype. Like all forms of familial cardiomyopathy, AC is genetically heterogeneous. Yet, in contrast with other forms of cardiomyopathy, the presence of a gene mutation is a criterion to make the diagnosis.

The major advantage of a positive genetic founding consists in the possibility to identify early asymptomatic family member carriers by cascade genetic. Genetic testing increasingly identifies novel sequence variants with uncertain significance (VUS), nevertheless all first-degree relatives should undergo phenotypic screening in order to evaluate its role. Targeted testing for a VUS in other affected family members can exclude its primary role when a lack of cosegregation is recognized. Increasing levels of VUS cosegregation with the disease clinical phenotype in a family contribute to better clarify its possible pathogenicity.

Nowadays, the pathogenicity of missense and radical (nonsense, frameshifts, splice sites etc) mutations in cardiac diseases and specifically in AC is a matter of debate. Recent studies have shown that stop-coding in disease-causing genes are more pathogenic than missense variants, since the former are causing alteration of protein length and conformation, leading to haploinsufficiency due to protein instability [157-160]. Entire exons or even whole gene deletions of desmosomal genes have been recently described in AC families with a frequency of approximately 7 % and low penetrance (32%) within the family carriers [75, 76, 78, 79].These data are further stressing the question whether haploinsufficiency is enough to determine the disease phenotype.

Moreover the inheritance pattern of AC is more complex than previously appreciated, with frequent requirement for more than one 'hit' for fully penetrant disease [9, 44, 157, 161]. The low penetrance of AC may be explained by a "recessive-like" inheritance pattern, based on the fact that AC probands often carry homozygous or compound heterozygous variants in the same gene, or digenic/oligogenic variants in a cluster of desmosomal genes.

Genotyping success rate in AC varies according to cohort location and ethnicity, sequencing techniques, selection criteria and the stringency of the standards by which variants are considered causal. An allelic frequency lower than 0.02–0.05% is considered appropriate taking in account AC prevalence in the population.

1.16 Limitations of genetic testing

Despite the numerous advantages of the NGS technologies, there are still technical limitations. Reads produced by NGS do not completely cover the entire target region, either exome or a gene set: some sequences such as GC-rich sequences can be difficult to capture and some sequences cannot be targeted at all because of insufficient or inadequate probes. Moreover uneven capture efficiency across exons influences the reads depths of specific exons.

Another limitation of NGS is the difficult detection of structural variants, such as exon level copy-number variants, inversions and translocations. However, recent studies show the successful identification of CNV from NGS data [162-169]. Furthermore, one of the more evident challenges of NGS is the huge amount of data that require proper analysis and storing. Storage of computational data is becoming an expensive problem, hampered by increasing data volumes and frequent updates of analysis methods and tools.

With the routine use of NGS, the analysis of large panel of genes leads to the identification of a high number of VUS. Thus, genetic testing and its interpretation should be performed in dedicated AC cardio-genetic centers, with pre- and post-counseling facilities. Management, filtering and prioritization of variants and clinical interpretation of genetic findings require expertise, due to frequent genetic overlap between cardiomyopathies.

2 Aim of the study

The aim of our study was to assess AC genetic heterogeneity, applying appropriate genetic screening and variants classification, multiparametric genotype-phenotype correlation taking into account AC variants.

Firstly we performed a comprehensive genetic screening on 224 AC consecutive index cases, taking into account AC-related genes, CNVs and new recently reported genes. Then, we investigated with a stringent variants classification according to the ACMG criteria [170] in order to evaluated the yield of genetic testing after a comprehensive screening.

Secondly, we performed a multiparametric genotype-phenotype correlation comparing genetic screening results and clinical data obtained from CMR, ECG, Echo to investigated whether different genes produced a diverse phenotypic manifestations.

Finally, we investigated through WES 10 AC negative patients in order to identify new candidate genes involved in the disease pathogenesis.

3 Material and Methods

3.1 Cohort and Clinical Examination

The study involved a total of 224 consecutive patients with a clinical or post mortem diagnosis of AC referred to the Referential Clinical Genetic Centre of Arrhythmic Cardiomyopathies in Padua. Thirty-two probands were sudden death victims enrolled from the Registry of Cardio-Cerebro-Vascular Pathology, Veneto Region, Venice, Italy. For the rest 192 probands, of whom sixteen underwent cardiac transplantation AC clinical diagnosis was based on established major and minor criteria revised by the European Society of Cardiology and the Federation of Cardiology Task Force [30].

Clinical evaluation included physical examination, 12-lead ECG, signal-averaged ECG, cardiac magnetic resonance imaging, 24-hour ambulatory ECG monitoring. Exercise tests and electrophysiological studies were performed when considered necessary. AC diagnosis was considered definite when 2 major, or 1 major and 2 minor criteria, or 4 minor criteria from different categories, were fulfilled; borderline when 1 major and 1 minor or 3 minor criteria from different categories were fulfilled; possible: when 1 major or 2 minor criteria from different categories were fulfilled. First-degree and second-degree relatives from families were called for prospective evaluation and genetic screening if a disease-causing variant was detected. Written informed consent was obtained from all study participants before blood sampling and genetic screening.

All 224 patients underwent genetic screening through a 174 cardiac-related genes panel (Trusight Cardio, Illumina). The most recently reported genes *FLNC* and *CDH2* were not present in our NGS panel therefore the screening was performed by conventional direct sequencing

Moreover, variants on *TTN* were evaluated separately due the size of the gene and the large number of variants identified for each patients.

Whole exome sequencing was carried out on 10 AC genotype negative patients.

3.2 Nucleic acid and proteins extraction

Genomic DNA (gDNA) was isolated from whole blood and frozen myocardial tissue utilizing specific onboard protocols of MagNA Pure Compact System (Roche Applied Science, Mannheim, Germany), an automated benchtop device that uses the magnetic bead technology for nucleic acids isolation. The MagNA Pure Compact performs all nucleic acids isolation steps preventing cross-contamination between samples. It automatically pipets into the sample a pre-aliquoted volume of lysis buffer containing chaotropic salts and proteinase K, nucleic acids are bound to the surface of the magnetic glass beads and after several washing steps the purified DNA is eluted in DNase-free H_20.

3.3 DNA extraction from blood

gDNA extraction was carried out from 200 µl of whole blood using Nucleic acid isolation kit I (Roche Diagnostics GmbH, Mannheim, Germany) on the MagNA Pure Compact System by setting the "DNA_Blood_100_400" protocol on the machine software. The typical DNA yield from 200 µl of human whole blood using the "DNA_Blood_100_400" protocol and an elution volume of 100 µl is 6 µg.

3.4 DNA extraction from frozen tissue

gDNA extraction was performed from approximately 10 mg of snap frozen tissue by using Nucleic acid isolation kit I (Roche Applied Science, Mannheim, Germany) with the "DNA culture cells" protocol. An initial pre-treatment step was performed to enhance cell lysis. Briefly 200 µl of MagNA Pure Compact DNA Lysis buffer (Roche Applied Science, Mannheim, Germany) was added to the tissue sample and a 2' disruption on a Tissue Lyzer System (Qiagen, Hilden, Germany) was carried out at 25 Hz. The homogenized sample was then incubated with 20 µl of Proteinase K (20 mg/mL; Roche Diagnostics GmbH, Mannheim, Germany) for 10 min at 56 °C and transferred on the MagNA Pure Compact workstation. The typical DNA yield using the "DNA culture cells" protocol from 10 mg of snap frozen tissue and an elution volume of 50 µl is 14 µg.

3.5 DNA quantification

Nucleic acid isolation is followed by a quantity and quality (purity, integrity) check before down-stream preparation steps. DNA quantification allows standardization of the PCR input material and is crucial in NGS library preparation since it influence run quality and efficiency.

3.5.1 Spectrophotometric method

Nucleic acids quantification is performed on a spectrophotometer by measuring absorption of ultraviolet light at the wavelength of 260 nm and applying the Lambert-Beer law that correlates absorbance, molar extinction co-efficient and nucleic acids concentration. Absorbance measurements were carried out on a Nanovue spectrophotometer (GE Healthcare Life Sciences, UK) by directly pipetting onto the pedestals 2 µl of the DNA sample dissolved in DNase-free H_20, after an initial blank measurement. Nanovue automatically calculates the nucleic acids concentration by applying specific extinction coefficients (50 for dsDNA).

The ratios A260/280 and A260/230 are used as indicators of sample purity. The nucleic acid sample ratio A260/280 is generally used as indicator of protein contamination. Indeed the 280 nm is the absorbance wavelength of aromatic amino acid side chains and phenol groups. Pure DNA should present a 260/280 ratio between 1.8 and 2. The A260/230 ratio is generally used as indication of organic contaminants. 230 nm is the absorbance wavelength of many organic compounds (i.e. phenol, TRIzol, and chaotropic salts present in the most common lysis buffers). In pure DNA samples the ratio A260/230 should be between 2 and 2.2.

3.5.2 Qubit Fluorometer

The quantity and quality of DNA, was assessed by using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Walthman, MA, USA). According to the manufacturer's instructions analysis was conducted with the dsDNA HS Assay kit (Thermo Fisher Scientific, Walthman, MA, USA). Briefly, 1 µl of the sample is mixed with 199 µl of working solution containing specific fluorescent dyes, composed by 199 µl of dsDNA HS buffer and 1µl of Dye. After an incubation of 2 min the measurement is performed on the Qubit 2.0 by setting the DNA protocol. Moreover, as the DNA dye exclusively binds to double strand DNA (dsDNA), it further allows the selective quantification of dsDNA that will be exclusively used for downstream applications, minimizing the effect of contaminant RNA and ssDNA.

3.5.3 0.8% Agarose gel

To evaluated DNA integrity after extraction, standard 0.8% agarose gel electrophoresis was performed; 0.8 g of agarose (Thermo Fisher Scientific, Walthman, MA, USA) were added to 100 ml of 1X Tris-Acetate-EDTA (TAE) buffer. The solution was heated in a microwave to dissolve agarose, 5 µl of Nancy-520 DNA Gel Stain (Sigma-Aldrich, Saint Louis, MO, USA) was added to the gel, then it was cast in a sealed tray and a proper comb was inserted. Aliquots of 2 µl of DNA and 3 µl of bromophenol blue loading dye were mixed and loaded into each gel well. In addition, 2 µl of DNA Marker VIII (Roche Applied Science, Manheim, Germany) were loaded in order to determine the fragment sizes. Electrophoretic run was performed at 100 V in 1X TAE buffer. Visualization was achieved on a LAS mini 4000 (Fujifilm, Tokyo, Japan). After the run good quality DNA should appear as a compact band as shown in Figure 3.

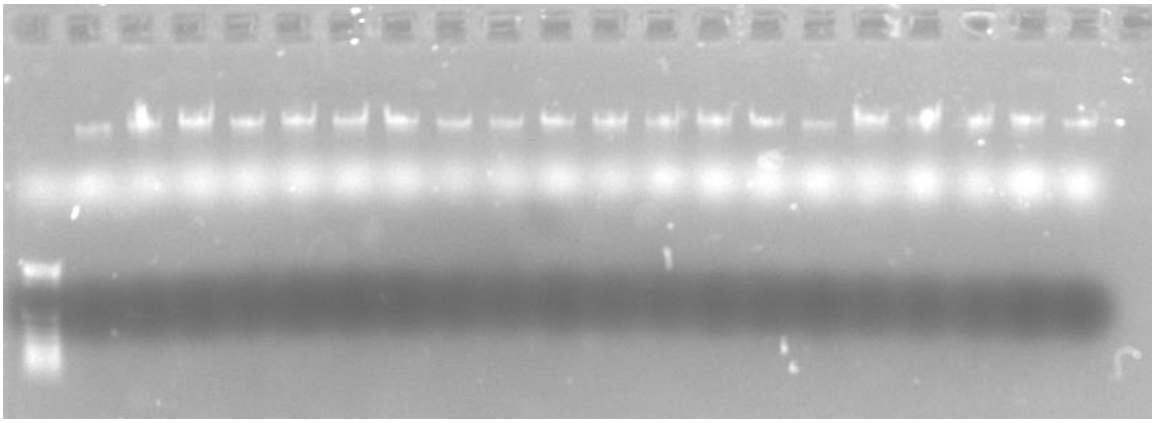


Figure 3: DNA quality analysis on agarose 0.8% gel

3.6 Polymerase chain reaction (PCR)

PCR, a method developed by Mullis in the 80s [171], allows the exponential amplification of specific targeted DNA regions. A PCR reaction requires template DNA, deoxynucleotide triphosphates (dNTPs), oligonucleotide primers flanking the target DNA sequence, DNA polymerase enzyme, reaction buffer and magnesium. PCR is composed of different consecutive reactions:

- Template denaturation by heating DNA at 95 °C;

- Annealing temperature (Ta) usually ranges from 55 to 65°C; oligonucleotide primers may align on the target region of the single strand DNA;

- Elongation at 72°C, the DNA polymerase synthesizes new strands of DNA starting from the 3' end of annealed primers. The newly synthesized DNA of the first cycle will be the template of the next and so on, reaching a million-fold increase of the DNA quantity at the end of the reaction.

3.6.1 Standard PCR

Amplification reactions were carried out in a final volume of 12.5 µl containing 50 ng of template (final concentration 4 ng/µl), 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM of dNTPs, 0.8 µM of forward and reverse primer and 0.32 U of Taq DNA polymerase (Thermo Fisher Scientific, Walthman, MA, USA). For some amplicons, amplification was enhanced by the use of 1 µl of Dimethyl sulfoxide (DMSO) in the reaction mix. DMSO destabilizes the double helix structure by hydrogen binding to the major and minor grooves of DNA and reduces secondary structure formation in the DNA template, thus facilitating primer annealing. Amplification reactions were performed on Mastercycler Pro (Eppendorf, Hamburg, Germany) at specific Ta for every PCR product, optimized based on the GC content of the sequence and melting temperatures (Tm) of primers. Briefly, each sample was denatured at 95°C for 10' to allow the activation of the hot start DNA polymerase, and exposed to 40 amplification cycles of denaturation for 30'' at 95°C, annealing for 30'' at a range of Ta comprised between 55°C and 65°C and extension at 72°C for 30''; followed by a final extension step of 7 min at 72°C to enhance the amplicon elongation.

The specificity (Sp) and the amplification yield of some PCR products was enhanced by the use of touch-down PCR protocol (TD-PCR). TD-PCR protocols set Ta above the anticipated melting temperature of a primer/template pair and then decrease the Ta of subsequent cycles stepwise. The initial Ta of the first

few cycles is higher than the expected Tm of primers, and then for the subsequent cycles the temperature progressively decreases to lower Ta allowing the correct hybridization of primer to the template. Thermal cycling conditions used for TD-PCR are as follows: an initial incubation at 95 °C for 10 min; followed by 10 cycles at 95 °C for 30'', Ta stepdown every cycle of 1 °C (from 70 °C to 60 °C); extension at 72 °C for 30''. The successive 30 cycles were performed at a Ta of 60 °C with denaturation and extension steps as above; followed by the final extension at 72 °C for 7'.

PCR products (ranging from 150-700 bp) were analysed on a 2% agarose gel.

3.6.2 GC rich PCR

GC rich amplicons were amplified by using AmpliTaq Gold 360 Master Mix kit (Thermo Fisher Scientific, Walthman, MA, USA) containing all premixed PCR components according to the manual. Amplification reactions were performed in a final volume of 12.5 µl by adding 7 µl of mix, 2.5 µl of GC solution provided, 2 µl of forward and reverse primer to 50 ng of template DNA.

3.6.3 PCR quality on 2% agarose gel

For standard 2% agarose gel electrophoresis, 2 g of agarose (Thermo Fisher Scientific, Walthman, MA, USA) were added to 100 ml of 1X Tris-Acetate-EDTA (TAE) buffer. The solution was heated in a microwave to dissolve agarose, gel was added with 5 µl of Nancy-520 DNA Gel Stain (Sigma-Aldrich, Saint Louis, MO, USA), cast in a sealed tray and a proper comb was inserted. Aliquots of 3 µl of PCR products and 3 µl of bromophenol blue loading dye were mixed and loaded into each gel well. Then the electrophoretic run was performed as for 0.8% agarose gel.

3.7 Purification of PCR products

Before sequencing, PCR products were purified by a treatment with Exonuclease 1 (EXO) and Shrimp Alkaline Phosphatase (SAP) enzymes, in order to eliminate primers and dNTPs residues from the previous PCR reaction, as they could interfere with the subsequent sequence reaction.

The purification reaction was carried out with the "Illustra ExoProStar 1-Step" kit (GE Healthcare Life Sciences, UK) by mixing 5 µl of PCR product and 2 µl of reaction mix, containing SAP enzyme that dephosphorylates dNTPs and EXO that hydrolyses residual primers and nonspecific single strand

oligonucleotides that could have been amplified during the PCR. The reaction conditions were: an incubation at 37 °C for 20' followed by 80°C for 15' to inactivate the enzymes.

3.8 Direct sequencing

The DNA sequencing method developed by Sanger in the 1970s [127] is based on the DNA chain-termination by chemically altered bases called di-deoxy nucleoside triphosphates (ddNTPs) carrying four different fluorophores. The method requires the presence of normal deoxy nucleoside triphosphates (dNTPs), and di-deoxy nucleotide triphosphates lacking a 3'-hydroxy group that, when incorporated into a newly synthesized DNA fragment, terminate the DNA strand elongation at specific bases (A, C, T, or G). This process produces DNA fragments with different sizes that can be separated by capillary electrophoresis and detected with laser-induced fluorescence.

Sequencing reactions were performed with the BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Walthman, MA, USA) following the manufacturer's instructions. 1 µl of each purified PCR products was mixed with 2 µl of sequencing buffer at a final concentration 1X, 1 µl of BigDye Terminators solution (containing dNTPs, fluorophore-conjugated ddNTPs, and polymerase), and 10 pmol of forward or reverse primer in a total volume of 10 µl. Sequencing reaction was performed as follows: 96°C for 1', 30 cycles of 96°C for 10'', 55 °C for 5'', 60°C for 2' 30''. Fluorescent-labeled sequences were then cleaned-up to eliminate unincorporated big dye terminators, dNTPs, and salts using ethanol/EDTA precipitation. Briefly, 25 µl of EtOH 96% , 1µl of EDTA 125 mM and 1µl of NaAc 3M pH5.2, were added to each sample and centrifuged at maximum speed for 30' at 20°C. Samples were washed with 35 µl of EtOH 96%, resuspended in 15 µl of formammide and then sequenced on an AB3500-Dx Genetic Analyzer (Thermo Fisher Scientific, Walthman, MA, USA).

3.9 Next Generation Sequencing

3.9.1 Library preparation

Libraries were constructed following the Illumina and Agilent recommendations present in Trusight Cardio kit manual and the SureSelect Clinical Research Exome V2 kit manual respectively. Although these two protocol are slightly different, there are based on the same principle and steps as briefly illustrated above. DNA was randomly fragmented by tagmentation and adapters sequences were added to the ends, allowing amplification by PCR in subsequent processes. Tagmented DNA was purified by using sample purification beads. Purified tagmented DNA was amplified through standard PCR reaction. Furthermore, indexes sequences needed for pooling libraries and sequencing were added, as well as common adapters required for cluster generation on the flow cell. PCR products were when purified in order to proceed with library pool that permits to combine multiple libraries with different indexes into a single well. DNA pooled library was mixed with capture probes to target regions of interest. Specifically, the Trusight Cardio kit contains RNA probes designed to capture 174 genes associated with inherited cardiomyopathies whereas the SureSelect Clinical Research Exome V2 kit was designed to capture the entire coding region of the human genome. After the hybridization library was quantified with the Qubit fluorimeter and the Tape Station platform (Agilent, Santa Clara, Carlsbad, USA). Afterwards the library was loaded on two Illumina platform: MiSeq platform was used for Targeted enrichment whereas Hiseq platform was used for WES.

3.9.2 Library Quality Check

The Agilent Tape Station 4200 (Agilent, Santa Clara, Carlsbad, USA) is a microfluidics-based platform that can perform quantification and quality control of nucleic acids by an electrophoretic separation of samples on micro channels containing fluorescent dyes. One µl of library is sufficient for analysis on Tape Station with the D5000 DNA kit (Agilent, Santa Clara, Carlsbad, USA). All the assays were performed according to the manufacturer's recommendations. Briefly, 1 µl of library was added to 9 µl of D5000 Sample Buffer and vortex. Then, D5000 ScreenTape and D5000 Ladder were loaded on the Tape Station as well as the sample. The D5000 DNA kit allow to estimate the average size of the fragment generated during the library preparation for NGS screening.

3.9.3 Sequencing by Synthesis

Library fragments are attached on the surface of a flow cell, that contained oligos that bound with the adaptors present on each fragment. DNA polymerase is used to produce clusters of approximately one million copies of the original fragment. Amplification of the clusters is called bridge amplification: During sequencing the four labeled nucleotides are simultaneously added to the flow cell channels with the DNA polymerase, to be incorporated into the cluster fragments. The four nucleotides are labeled with base-specific fluorescents dye containing -OH group to inhibits the addition of another nucleotides within each cycle. The polymerase ligates one fluorescent labeled nucleotides on the elongating complementary fragment and than fluorescence is detected. Afterwards the -OH group is detaches and the cycle can start again with the ligation of the following nucleotides.

3.9.4 Data Analysis

-Base calling

MiSeq Image Control: On board software that acquired and processed images. After intensity is extracted, it passes through intensity normalization and cross talk estimation. Bases are called and quality scores are determined: the results are stored in FASTQ files as the output of base calling. Files are demultiplexed (separated per sample).

Quality score is defined as a property logarithmically related to the base calling error probabilities ($Q=-10 \log_{10} P$). A quality score of 30 is typically accounted as good sequence quality: it represents a base calls accuracy of 99.9% ("Q30 quality"). The output FASTQ file contains exclusively bases scoring Q30 and above [172].

Burrows-Wheeler Aligner (BWA-MEM): Alignment algorithm for aligning sequence reads or long query sequences against a large reference genome such as human. It automatically chooses between local and end-to-end alignments, supports paired-end reads and performs chimeric alignment. The algorithm is robust to sequencing errors and applicable to a wide range of sequence lengths from 70bp to a few megabases. For mapping 100bp sequences, BWA-MEM shows better performance than several state-of-art read aligners to date [173]. BWA-MEM aligns FASTQ file to the reference genome generating the SAM/BAM files

Genome Analysis Toolkit (GATK): Algorithm that proceeds from BAM files and products the so call "variant calls". This involves identifying genomic variants in one or more individuals and applying filtering methods appropriate to the experimental design. The output is typically in VCF format. (https://software.broadinstitute.org/gatk/)

3.10 Variants Classification

The classification of variants is the last and most complex step in the diagnostic procedure. Clinical testing laboratories use guidelines to classify variants based upon the American College of Medical Genetics revised criteria, which include: pathogenic, likely pathogenic, VUS, likely benign, and benign [170].

To fulfil classification guidelines, individual whole-genome-sequencing databases across ancestries are crucial to determining pathogenicity to help delineate signal from noise, as common genetic variability is different among various populations [174, 175]. NGS high throughput allow the assembly of large reference cohorts that have greatly enhanced the ability to determine population-based variant frequencies. gnomAD (http://gnomad.broadinstitute.org) is the largest reference dataset, which nowadays contains 123 136 exome sequences and 15 496 whole-genome sequences from unrelated individuals. This database also provide further reliability to determine variant frequencies in specific racial or ethnicity groups.

Criteria to postulate causality are based on :

1. Minor allele frequency (MAF), extremely rare or unique alleles are less likely benign variants;
2. Type of variant identified (e.g. de novo, truncating, missense, nonsense, splice site, etc.), in most cases in coding sequence, or if at an intron/exon boundary or in intronic sequence, the ability to disrupt coding sequence;
3. Prior evidence of pathogenicity or lack thereof;
4. Evidence of segregation in a pedigree;
5. *In silico* tools of pathogenicity

3.11 Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a method based on the multiplex amplification of specific probes developed for the relative quantification of nucleic acid sequences (Schouten et al., 2002) that allows the detection of copy number variants of specific target regions.

To investigate for large deletions/duplications within desmosomal genes, MLPA was performed by using SALSA MLPA P168 ARVC-PKP2 probemix kit (MRC Holland, Amsterdam, The Netherland) instructions. The probemix contains oligonucleotide probes for every exon of *PKP2* (NM_004572.3) and its promoter, 6 probes for exons 1, 5, 7, 9, 21, 24 of *DSP* (NM_004415.2), 3 probes for exons 2, 9, 12 of *JUP* (NM_002230.2), 3 probes for exons 1, 7, 17 of *DSC2* (NM_004949.3), 3 probes for exons 1, 6, 15 of *DSG2* (.NM_001943.3). It also contains 3 probes for exons 1, 6, 7 of *TGFβ3* (NM_003239.2) and 2 probes for exons 3 and 97 of *RYR2* (NM_001035.2). It also contains nine control fragments and eight reference probes located on different chromosomes.

The technique is based on the simultaneous amplification of probes after hybridization on targets and can be divided in five distinct steps:

1) DNA denaturation
2) DNA hybridization with the oligonucleotide probes. MLPA probe pairs are composed by two oligonucleotides, that are contiguous to the region of interest and hybridize to immediately adjacent target sequences.
3) Ligation reaction. Only when both probes are hybridized can be ligated and trigger PCR reaction
4) PCR reaction. Probes contain at their 5' and 3'ends specific sequences complementary to universal PCR primers, so a universal pair of primers is used for the amplification step.
5) Analysis of PCR products by capillary electrophoresis.

One extremity of the 2 probes of every oligonucleotide pairs is conjugated with a fluorescent marker, and the other one contains a "stuffer" sequence different in length between the different probes, thus allowing the simultaneous analysis of several targets, as sequences differing for even one nucleotide can be recognized. The amount of ligation products is directly proportional to the input DNA due to the specific amplification of probes correctly hybridized and ligated The MLPA reaction products were run on ABI3500dx.

Data Analysis

Data were analyzed by using the Coffalyser Software (MRC Holland, Amsterdam, The Netherlands). This software first performs an evaluation of raw data by checking the DNA amount, denaturation, ligation efficiency and includes a baseline correction and peak identification. Subsequently it performs 2 normalization steps:

- Intra-sample normalization, where the probe peaks are compared to the ones of the reference probes, within each sample,

- Inter-sample normalization, where the probe peaks in the target sample are compared to the ones of the control samples.

This process allows the calculation of final probe ratios, called Dosage quotient (DQ). A DQ is close to 1 indicates a wild type sample, when it is close to 0.5 it indicates an heterozygous deletion (1 allele) and when it is close to 0 it indicates an homozygous deletion, as indicated by the MRC-Holland

Quantitative real-Time PCR (qPCR)

MLPA data validation was performed by relative quantitative PCR (qPCR) on a Light Cycler 480 II (Roche Applied Science, Mannheim, Germany) by SYBR Green-based quantification according to the manufacturer's protocol. Reactions were prepared in a final volume of 20 µl with 1x Master SYBR Green I (Roche Applied Science, Maanheim, Germany), 25 pmol of forward and reverse primers and 5 µl of DNA. The amplification was carried out under the following conditions: an initial preincubation of 95°C for 10' followed by 45 cycles of denaturation at 95°C for 30'', annealing at 60°C for 45'' and extension at 72°C for 10'', and a final extension at 72°C for 10'. qPCR experiments were carried out in triplicate and included a negative control. The housekeeping gene used as reference for human DNA samples was *GAPDH*. Primers were developed by the Primer-Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and designed to have a Tm around 60°C, to span inside exons and to produce amplicons approximately 150 bp long.

3.12 Data analysis tool

Software

Variant Studio (Illumina): Data analysis tools and access to curated variants with known biological associations design for large volumes of genomic data. Allows extensive filtering and efficient categorization of biologically relevant variants. (https://www.illumina.com/products/by-type/informatics-products/variantstudio.html)

Alamut Visual (Interactive Biosoftware, Rouen, France): an exploration software application for genomic variations that integrates genetic and genomic information from different sources into one consistent and convenient environment. It gathers in one place a wide set of external data and algorithms of recognized quality that are useful to the biologist for the clinical interpretation of genomic variants. (https://www.interactive-biosoftware.com/alamut-visual)

SeqMan (Lasergen): Provide quick and accurate sequence assembly and analysis of Sanger data. Discover variants, analyze coverage, annotate your consensus (https://www.dnastar.com/t-seqmanpro.aspx)

CHROMAS software (Technelysium): Sanger sequencing electropherograms visualization. (https://technelysium.com.au/wp/chromas/)

Polyphen-2: Tool that predicts the possible impact of an amino acid substitution on the structure and function of a protein [176]. Three outcomes are possible: probably damaging (the variant is expected with high confidence to affect protein structure), possibly damaging (the variant supposed to affect protein structure), benign (the variant is not expected to have any effect on protein structure). (http://genetics.bwh.harvard.edu/pph/)

Sorting Intolerant From Tolerant (SIFT): Tool that predicts the possible impact of an amino acid substitution on the protein function [177]. It classifies amino acids substitutions as tolerated or deleterious. (http://sift.jcvi.org/www/SIFT_enst_submit.html)

MutationTaster. The software performs a battery of *in-silico* tests to estimate the impact of the variant on the gene product/protein. Tests are made on both, protein and DNA level. Hence the MutationTaster is not limited to substitutions of single amino acids but can also handle synonymous alterations [98]. (http://www.mutationtaster.org/)

Combined Annotation Dependent Depletion (CADD): Tool for scoring the deleteriousness of single nucleotide variants as well as insertion/deletions variants in the human genome. Prioritize functional, deleterious, and disease causal variants across a wide range of functional categories, effect sizes and genetic architectures and can be used to prioritize causal variation in both research and clinical settings. (http://cadd.gs.washington.edu/)

Prism (GraphPad software): Statistical analysis software used to perform t tests, nonparametric comparisons, one-, two- and three-way ANOVA, analysis of contingency tables, and survival analysis. (https://www.graphpad.com/scientific-software/prism/)

Intronic variants located less than 50 bp from the exon boundaries were in-silico analysed by at least four different algorithms (**SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer**), in order to predict their possible effect on the splicing sites of the transcript.

Database

HGMD Professional (Qiagen): database that is implemented into Alamut Visual consisting of a complete archive of mutations validated and used by industry professionals for the clinical and molecular genetics, diagnostic tests and personalized genomic medicine. (http://www.hgmd.cf.ac.uk/ac/index.php)

dbSNP database: Central repository for both single base nucleotide substitutions and short deletion and insertion polymorphisms. (http://www.ncbi.nlm.nih.gov/SNP/)

Exome Aggregation Consortium (ExAC): Data set provided on this website spans 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies. (http://exac.broadinstitute.org/)

1000 Genomes Project : Created between 2008 and 2015, it's a public catalogue of human variation and genotype data. The final data set contains data for 2,504 individuals from 26 populations. (http://www.internationalgenome.org/)

Genome aggregation database (gnomAD): Resource developed by an international coalition of investigators, with the goal of aggregating and harmonizing both exome and genome sequencing data from a wide variety of large-scale sequencing projects, and making summary data available for the wider scientific community. The data set provided on this website spans 123,136 exome sequences and 15,496 whole-genome sequences from unrelated individuals sequenced as part of various disease-specific and population genetic studies. (http://gnomad.broadinstitute.org/) .

Statistical Analysis

Data are expressed as mean±SD and were analysed using Prism version 7 (GraphPad Software, La Jolla, USA). Comparisons were performed by univariate models using independent samples t tests for continuous variables and $\chi 2$ tests for categorical variables as appropriate, ANOVA and Kruskal Wallis for multiple comparison. A value of $P<0.05$ was considered significant.

4 Results

4.1 Cohort Analysis

Two-hundred twenty-four consecutive patients (169 males, 55 females; mean age 41y±15) were recruited at Referential Clinical Genetic Centre of Arrhythmic Cardiomyopathies and Cardiovascular Pathology unit (Registry of Cardio-Cerebro-Vascular Pathology, Veneto) of Padua. Among them, 32 (mean age 28y±9) were sudden death victims with a post mortem diagnosis of AC, all of them were males. The rest 192 probands (137 males, 55 females; mean age 39y±14) bared an AC clinical diagnosis based on major and minor criteria established by the European Society of Cardiology and the Federation of Cardiology Task Force [30]. Of note, 16 of the 192 (8%; 9 males, 7 females; mean age 45y±16) index cases underwent HTx.

Table 3: AC index cases classification based on Task Force Criteria

Number of patients	AC diagnosis
178	Definite
18	Borderline
28	Possible

As such, 178 index cases were classified with a definite AC diagnosis (139 males, 39 females, mean age 39±16), 18 borderline (12 males, 6 females; mean age 43±13) and 28 as possible affected (18 males, 10 females, mean age 43±13) (Table 3).

Forty-two of the 224 index cases (19%, 34 male, 8 females; mean age 37y±15) received an LDAC diagnosis, of whom 19 (45%) were sudden death victims.

4.2 Variants analysis

All 224 index cases underwent genetic screening through NGS. An average of 350 variants/patient were identified following sequence alignment and variant calling processes. These variants were subsequently filtered according to the ACMG criteria [170], which reduced their number to an average of 5 variants/patient.

Overall, we identified 95 different rare variants in 97 (43%) out of 224 index cases. Specifically, 67 patients carried a single rare variant on a desmosomal gene, 19 were multiple variants carriers (either digenic or compound) and 11 (5%) patients carried a single variant on AC-related non desmosomal genes (Figure 4).

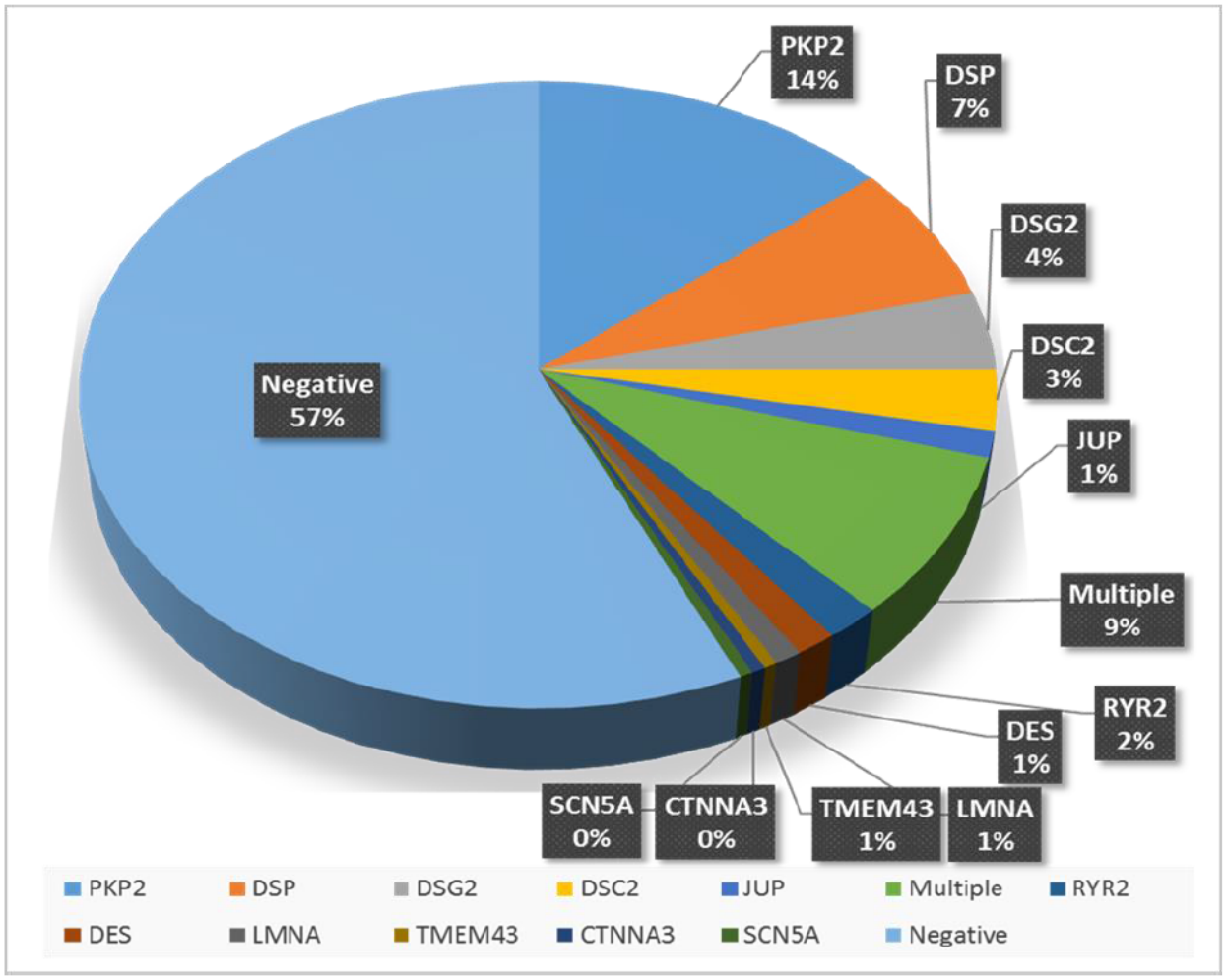


Figure 4: Yield of genetic screening and variants distribution

Among the 19 multiple variants carries, 17 patients (89%) were digenic heterozygotes and 2 (11%) were compound heterozygotes. Specifically, 13 out of 17 patients carried two variants in different desmosomal genes whereas four patients carried one variant in a desmosomal gene and an additional variant in a AC-related non desmosomal gene (Figure 5).

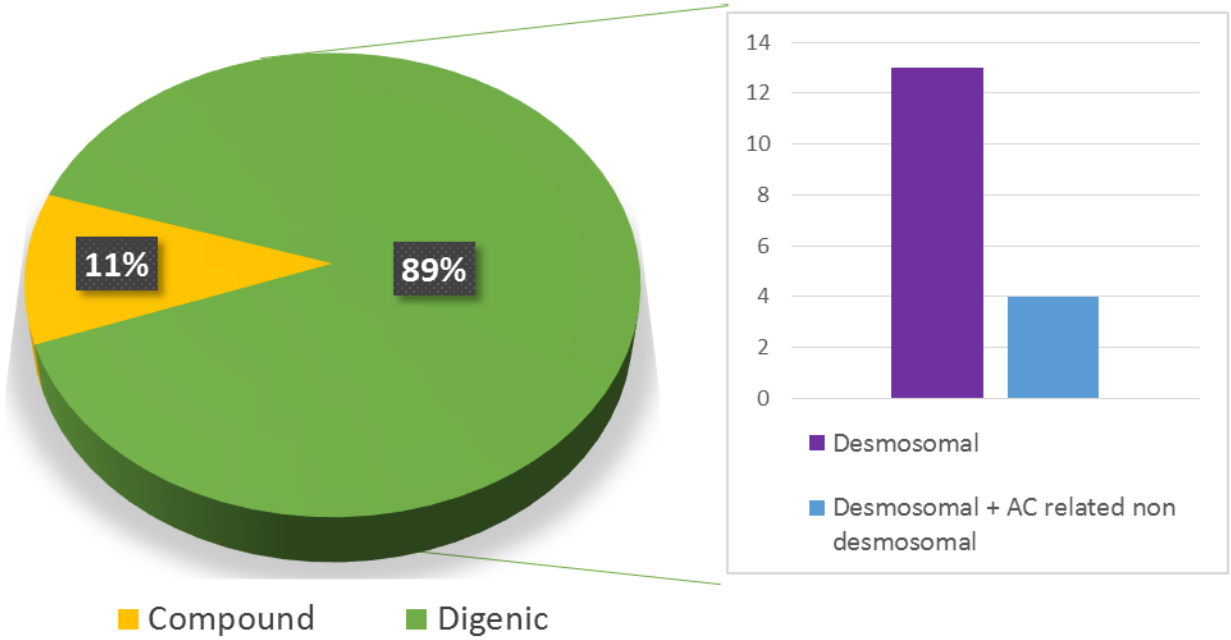


Figure 5: Multiple variant carriers analysis

DSP variants

DSP is a 24 exons gene encoding an anchoring protein. Sequencing led to the identification of 24 variants in 26 index cases, of whom 16 were single variant carriers . Three were splicing site variants, 9 were missense, 3 frameshift and 9 nonsense (Table 4). (see further for *in silico* evaluation)

Table 4: *DSP* variants. Pathogenic/Likely pathogenic variants are highlighted in bold (16/24).

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	**g.7580216G>T**	**c.3793G>T**	**p.Glu1265***	//	//	//	//	//
2	**g.7558412C>T**	**c.337C>T**	**p.Gln113***	//	//	//	//	//
3	**g.7579839dup**	**c.3416dupA**	**p.Tyr1139***	//	//	//	//	//
4	g.7579557G>A	c.3134G>A	p.Arg1045Gln	rs374263890	ALL:0.0040%	Deleterious (score: 0.04)	probably damaging (0.999)	Disease causing (p-value: 0.982)
5	**g.7580312C>T**	**c.3889C>T**	**p.Gln1297***	//	**ALL:0.00041 %**	//	//	//
6	**g.7565754G>A**	**c.939+1G>A**	//	**rs727504443**	**ALL:0.0032 %**	//	//	//
7	g.7566614G>C	c.944G>C	p.Arg315Pro	//	//	Tolerated (score: 0.07)	probably damaging (0.999)	Disease causing (p-value: 1)
8	**g.7579898G>T**	**c.3475G>T**	**p.Glu1159***	//	//	//	//	//
9	g.7567609C>T	c.1067C>T	p.Thr356Met	rs780626687	ALL:0.0012%	Deleterious (score: 0.02)	probably damaging (1)	Disease causing (p-value: 1)
10	**g.7584840_7584844del**	**c.7345_7349delAAGAA**	**p.Lys2449Glufs*15**	//	//	//	//	//
11	g.7585977G>A	c.8482G>A	p.Gly2828Ser	rs369682599	ALL:0.0048%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)
12	g.7585962C>G	c.8467C>G	p.Pro2823Ala	rs142717240	ALL:0.031%	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
13	g.7562934A>C	c.647A>C	p.Gln216Pro	//	//	Deleterious (score: 0.04)	possibly damaging (0.909)	Disease causing (p-value: 1)
14	**g.7559633G>A**	**c.597G>A**	**p.Arg199=**	//	//	//	//	//

15	g.7584196A>G	c.6701A>G	p.Asn2234Ser	//	//	Deleterious (score: 0.03)	Benign	Polymorphism (p-value: 1)
16	**g.7580197C>A**	**c.3774C>A**	**p.Asp1258Glu**	**rs748733750**	**ALL:0.0012 %**	**Tolerated (score: 0.93)**	**Benign**	**Polymorphism (p-value: 0.858)**
17	**g.7559484C>T**	**c.448C>T**	**p.Arg150***	//	//	//	//	//
18	**g.7579721_7579722insTGTT**	**c.3298_3299insTGTT**	**p.Cys1100Leufs*11**	//	//	//	//	//
19	**g.7583006dup**	**c.5511dup**	**p.Arg1838Serfs*19**	//	//	//	//	//
20	**g.7578090C>T**	**c.2956C>T**	**p.Gln986***	//	//	//	//	//
21	**g.7575029G>A**	**c.2436+1G>A**	//	//	//	//	//	//
22	**g.7584345C>T**	**c.6850C>T**	**p.Arg2284***	**rs794728130**	//	//	//	//
23	**g.7583295C>T**	**c.5800C>T**	**p.Arg1934***	**rs121912996**	//	//	//	//
24	g.7574391G>A	c.2203G>A	p.Gly735Ser	//	//	Tolerated (score: 0.93)	possibly damaging (0.909)	Disease causing (p-value: 1)

PKP2 variants

PKP2 is a 14 exons gene encoding for an armadillo protein. Sequencing led to the identification of 24 variants in 38 index cases, of whom 31 were single variant carriers. Two were splicing site variants, 12 missense, 6 frameshift and 4 nonsense (Table 5). (see further for *in silico* evaluation)

Table 5: *PKP2* variants. Pathogenic/Likely pathogenic variants are highlighted in bold (13/24)

NR	**g.DNA**	**c.DNA**	**Protein**	**dbSNPID**	**gnomAD**	**SIFT**	**Polyphen**	**Mutation taster**
1	**g.32975531A>G**	**c.1841T>C**	**L614P**	//	//	**Deleterious (score: 0)**	**possibly damaging (0.909)**	**Disease causing (p-value: 1)**
2	**g.33031183G>A**	**c.631C>T**	**p.Gln211***	//	//	//	//	//
3	**g.32974422del**	**c.2013delC**	**p.Lys672Argfs*12**	**rs764817683**	**ALL:0.00081 %**	//	//	//
4	**g.32994007del**	**c.1643delG**	**p.Gly548Valfs*15**	**rs794729137**	//	//	//	//
5	**g.33031955G>A**	**c.235C>T**	**p.Arg79***	**rs121434420**	**ALL:0.00041 %**	//	//	//

6	g.32949079C>A	c.2453G>T	p.Gly818Val		ALL:0.0012%	Deleterious (score: 0.02)	probably damaging (1)	Disease causing (p-value: 1)
7	**g.33031883G>T**	**c.307C>A**	**p.Pro103Thr**	**rs139215336**	**ALL:0.012%**	**Tolerated (score: 0.12)**	**possibly damaging (0.842)**	**Polymorphism (p-value: 1)**
8	g.33049482G>T	c.184C>A	p.Gln62Lys	rs199601548	ALL:0.017%	Tolerated (score: 0.21)	possibly damaging (0.842)	Disease causing (p-value: 0.89)
9	**g.33030849_33030850delinsG**	**c.964_965delinsC**	**p.Gly322Alafs*30**	//	//	//	//	//
10	**g.33049491G>A**	**c.175C>T**	**p.Gln59***	//	//	//	//	//
11	**g.32994096_32994097del**	**c.1553_1554delTC**	**p.Leu518Hisfs*22**	//	//	//	//	//
12	g.33031209A>G	c.605T>C	p.Val202Ala	rs751341106	ALL:0.0012%	Tolerated (score: 0.21)	Benign	Polymorphism (p-value: 1)
13	**g.33049515_33049518del**	**c.148_151delACAG**	**p.Thr50Serfs*61**	**rs397516997**	//	//	//	//
14	g.33031279G>T	c.535C>A	p.His179Asn	//	//	Tolerated (score: 0.42)	Benign	Polymorphism (p-value: 1)
15	g.33049557T>A	c.109A>T	p.Lys37*	//	//	//	//	//
16	g.32975552C>T	c.1820G>A	p.Cys607Tyr	//	//	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
17	g.32974324C>T	c.2111G>A	p.Gly704Glu	rs754656865	ALL:0.00041%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)
18	**g.33049582del**	**c.84del**	**p.Ser29Alafs*10**	//	//	//	//	//
19	g.32994074T>C	c.1576A>G	p.Thr526Ala	rs397516999	ALL:0.013%	Tolerated (score: 0.53)	Benign	Polymorphism (p-value: 1)
20	**g.32955336C>T**	**c.2299+1G>T**	//	**rs794729116**	//	//	//	//
21	g.32994067G>A	c.1583C>T	p.Thr528Met	rs774942476	ALL:0.0020%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)

22	g.32945603G>A	c.2552C>T	p.Thr851Met	rs146118033	ALL:0.0051%	Deleterious (score: 0.02)	probably damaging (0.999)	Polymorphism (p-value: 0.602)
23	g.33021938T>C	c.1093A>G	p.Met365Val	rs143900944	ALL:0.024%	Tolerated (score: 0.4)	Benign	Polymorphism (p-value: 1)
24	**g.32949042C>T**	**c.2489+1G>A**	**c.2489+1G>A**	**rs111517471**	**ALL:0.0022 %**	//	//	//

DSG2 variants

DSG2 is a 15 exons gene encoding for an ubiquitous desmosomal cadherin protein. Screening led to the identification of 18 variants in 18 index cases, of whom 9 were single variant carriers. Two were located on splicing sites, 12 were missense variants, 3 frameshift and only 1 nonsense (Table 6). (see further for *in silico* evaluation)

Table 6: *DSG2* variants. Pathogenic/Likely pathogenic variants are highlighted in bold (8/18)

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	**g.29104517A>G**	**c.797A>G**	**p.Asn266Ser**	**rs121913011**	**ALL:0.00041 %**	**Deleterious (score: 0)**	**probably damaging (0.997)**	**Disease causing (p-value: 1)**
2	g.29101074G>A	c.391G>A	p.Ala131Thr	rs373542380	ALL:0.0018%	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
3	g.29111007G>A	c.1072G>A	p.Ala358Thr	rs758537946	ALL:0.00081%	Deleterious (score: 0)		Disease causing (p-value: 1)
4	**g.29110973_29110975del**	**c.1038_1040delGAA**	**p.Lys346del**	**rs727502987**	**ALL:0.00081 %**			
5	**g.29122804del**	**c.2323delT**	**p.Tyr775Ilefs*33**		//			
6	g.29100818C>T	c.269C>T	p.Thr90Ile	rs772744115	ALL:0.010%	Tolerated (score: 0.1)	possibly damaging (0.909)	Disease causing (p-value: 0.886)
7	**g.29122517del**	**c.2032delG**	**p.Gly679Alafs*3**					
8	g.29104712G>A	c.875G>A	p.Arg292His	rs185821167	ALL:0.0083%	Deleterious (score: 0.04)	probably damaging (0.999)	Disease causing (p-value: 0.956)

9	g.29104504A>G	c.784A>G	p.Ile262Val			Tolerated (score: 0.36)	Benign	Disease causing (p-value: 0.62)
10	g.29121188G>A	c.1912G>A	p.Gly638Arg	rs201564919	ALL:0.011%	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
11	g.29104442C>T	c.722C>T	p.(Ala241Val)	rs753505150	ALL:0.00041%	Tolerated (score: 0.17)	prossibly damaging (0.909)	Polymorphism (p-value: 0.943
12	**g.29100929T>C**	**c.378+2T>G**	//	//	//	//	//	//
13	**g.29118734C>T**	**c.1672C>T**	**p.Gln558***					
14	g.29100794G>T	c.245G>T	p.Gly82Val	rs756081987	ALL:0.00041%	Deleterious (score: 0)	possibly damaging (0.909)	Polymorphism (p-value: 1)
15	**g.29118713G>A**	**c.1652-1G>T**			**ALL:0.0065%**			
16	g.29116383C>T	c.1642C>T	p.Arg548Cys	rs550400909	ALL:0.0022%	Tolerated (score: 0.15)	probably damaging (0.999)	Polymorphism (p-value: 1)
17	**g.29125783G>T**	**c.2434G>T**	**p.Gly812Cys**	**rs121913010**	**ALL:0.0012%**	**Deleterious (score: 0.01)**	**probably damaging (0.999)**	**Disease causing (p-value: 1)**
18	g.29125997C>T	c.2648C>T	p.Ser883Phe	rs371498622	ALL:0.0016%	Tolerated (score: 0.16)	Benign	Polymorphism (p-value: 0.702)

DSC2 variants

DSC2 is a 16 exons gene gene encoding for another desmosomal cadherin protein. Sequencing led to the identification of 9 variants in 14 index cases, of whom 8 were single variant carriers. All of them were missense variants (Table 7). (see further for *in silico* evaluation)

Table 7: *DSC2* variants. Pathogenic/Likely pathogenic variants are highlighted in bold (2/9)

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	**g.28669496T>C**	**c.536A>G**	**p.Asp179Gly**	**rs760185784**	**ALL:0.00041 %**	**Tolerated (score: 0.1)**	**probably damaging (1)**	**Disease causing (p-value: 0.999)**
2	g.28673591T>A	c.85A>T	p.Ser29Cys	rs764796476	ALL:0.00041%	Tolerated (score: 0.9)	Benign	Polymorphism (p-value: 0.877)
3	g.28666679T>C	c.802A>G	p.Thr268Ala	rs201015785	ALL:0.014%	Tolerated (score: 0.27	probably damaging (1)	Disease causing (p-value: 0.998)
4	g.28648893C>G	c.2475G>C	p.Glu825Asp	rs776401728	ALL:0.0014%	Tolerated (score: 0.48)	Benign	Disease causing (p-value: 0.799)
5	**g.28666646G>A**	**c.835C>T**	**p.Arg279Cys**	**rs193922708**	**ALL:0.0045%**	**Deleterious (score: 0.02)**	**probably damaging (1)**	**Polymorphism (p-value: 1)**
6	g.28662283A>G	c.1184T>C	p.Ile395Thr	rs768706276	ALL:0.00041%	Deleterious (score: 0.01)	probably damaging (0.911)	Disease causing (p-value: 1)
7	g.28648091C>G	c.2596G>C	p.Ala866Pro	//	//	Deleterious (score: 0.03)	probably damaging (0.911)	Disease causing (p-value: 1)
8	g.28648100C>T	c.2587G>A	p.Gly863Arg	rs147109895	ALL:0.026%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)
9	g.28648084G>A	c.2603C>T	p.Ser868Phe	rs141873745	ALL:0.0047%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)

JUP variants

JUP is a 14 exons gene encoding for an armadillo protein. Sequencing led to the identification of 4 variants in 4 index cases, of whom 3 were single variant carriers. All of them were missense variants (Table 8). (see further for *in silico* evaluation)

Table 8: *JUP* variants. Pathogenic/Likely pathogenic variants are highlighted in bold (0/4)

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	g.39912444T>C	c.2069A>G	p.Asn690Ser	rs147628503	ALL:0.0098	Tolerated (score: 0.55)	Benign	Polymorphism (p-value: 0.584)
2	g.39913746T>C	c.1967A>G	p.Lys656Arg	//	//	Tolerated (score: 0.5)	possibly damaging (0.909)	Disease causing (p-value: 0.996)
3	g.39919263T>C	c.1469A>G	p.Asn490Ser			Tolerated (score: 0.62)	Benign	Disease causing (p-value: 0.999)
4	g.39912110A>T	c.2124T>A	p.Asp708Glu			Tolerated (score: 1)	Benign	Polymorphism (p-value: 1)

AC-related non desmosomal variants

Genetic screening identified 16 different missense variants in AC-related non desmosomal genes in 16 index cases, of whom 11 were single variant carriers. Four variants were found in *SCN5A*, 4 in *RYR2*, 3 in *DES*, 2 in *TMEM43,* and 2 in *LMNA*, and 1 variant in *CTNNA3* gene (Table 9).

Table 9: AC-related non desmosomal variants

NR	Gene	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	SCN5A	g.38592078G>A	c.5785C>T	p.Arg1929Cys	rs267599786	ALL:0.0014%	Deleterious (score: 0.02)	possibly damaging (0.842)	Disease causing (p-value: 1)
2	SCN5A	g.38622661C>T	c.2989G>A	p.Ala997Thr	rs137854609	ALL:0.0082%	Tolerated (score: 0.9)	Benign	Disease causing (p-value: 0)
3	SCN5A	g.38592527G>A	c.5336C>T	p.Thr1779Met	rs199473634	ALL:0.0043%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)
4	SCN5A	g.38674719C>T	c.80G>A	p.Arg27His	rs199473045	ALL:0.024%	Deleterious (score: 0.01)	probably damaging (0.999)	Disease causing (p-value: 1)

5	RYR2	g.237804225A>G	c.7144A>G	p.Ile2382Val	//	//	Deleterious (score: 0)	Benign	Disease causing (p-value: 0.72)
6	RYR2	g.237886504C>G	c.10631C>G	p.Pro3544Arg	rs752032924	ALL:0.0018%	Deleterious (score: 0)	possibly damaging (0.842)	Disease causing (p-value: 1)
7	RYR2	g.237804198G>T	c.7117G>T	p.Asp2373Tyr	rs397516548	ALL:0.00082%	Deleterious (score: 0)	Benign	Disease causing (p-value: 1)
8	RYR2	g.237713944C>T	c.3167C>T	p.Thr1056Met	rs779146564	ALL:0.0021%	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
9	DES	g.220283530A>G	c.346A>G	p.Asn116Asp	//	//	deleterious (0)	probably damaging (1)	Disease causing (p-value: 1)
10	DES	g.220283506G>A	c.322G>A	p.Glu108Lys	rs62636490	//	deleterious (0)	probably damaging (1)	Disease causing (p-value: 1)
11	DES	g.220285661G>A	c.1009G>A	p.Ala337Thr	rs59962885	ALL:0.0069%	Deleterious (score: 0)	possibly damaging (0.909)	Disease causing (p-value: 1)
12	TMEM43	g.14180744G>C	c.947G>C	p.Trp316Ser	rs199526104	ALL:0.016%	Deleterious (score: 0)	probably damaging (0.999)	Disease causing (p-value: 1)
13	TMEM43	g.14172323G>A	c.164G>A	p.Gly55Asp	rs201453637	ALL:0.022%	Deleterious (score: 0)	probably damaging (1)	Disease causing (p-value: 1)
14	LMNA	g.156106165G>A	c.1318G>A	p.Val440Met	rs121912493	//	Deleterious (score: 0.04)	probably damaging (1)	Disease causing (p-value: 1)
15	LMNA	g.156104249G>A	c.569G>A	p.Arg190Gln	rs267607571	//	Deleterious (score: 0.03)	probably damaging (1)	Disease causing (p-value: 1)
16	CTNNA3	g.69366789A>G	c.118T>C	p.Cys40Arg	rs775169307	ALL:0.00082%	Tolerated (score: 0.08)	possibly damaging (0.842)	Disease causing (p-value: 1)

4.3 Variants distribution

79 of the 95 rare variants were found mostly in 5 major desmosomal genes (83%), whereas 16 of the 95 rare variants in AC-related non desmosomal genes (17%). The vast majority of variants were missense variants (62/95, 65%), but nonsense (14/95, 15%), frameshift (12/95, 13%) or splicing site variants were also found (7/95, 7%) (Figure 6).

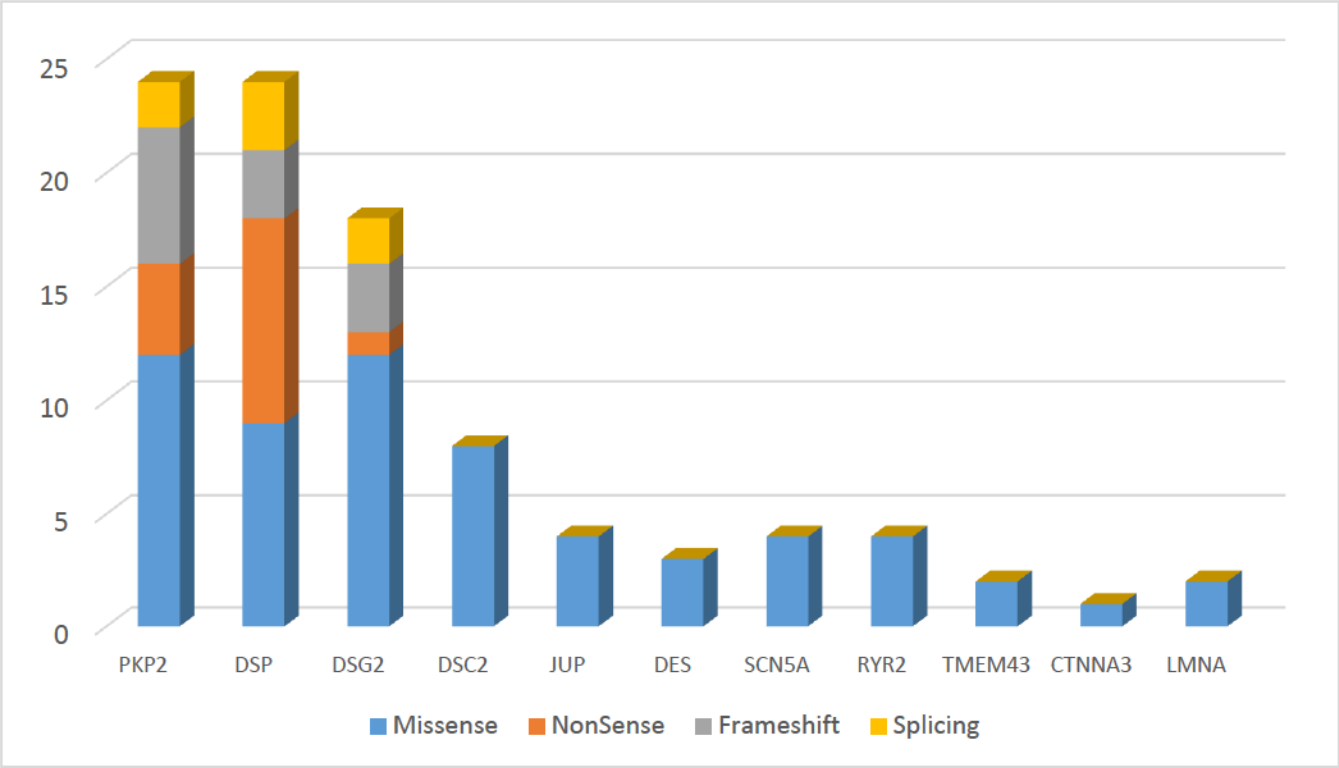


Figure 6. Variants distribution among genes

4.4 Variants classification

Thirty-nine of the 79 (49%) variants identified in desmosomal genes were classified as Pathogenic/Likely pathogenic, of which 32 (82%) were radical variants (nonsense, frameshift, canonical splicing sites) and 7 (18%) were missense variants.

The rest 40 of the 79 (51%) desmosomal variants were classified as VUS, of which 1 (3%) was a nonsense variant and 39 (98%) were missense variants.

All the 16 variants identified in AC-related non desmosomal genes were classified as VUS and were all missense variants (Figure 7).

The majority of pathogenic/likely pathogenic variants were radical variants, whereas VUS were almost all missense variants (p-value <0.0001).

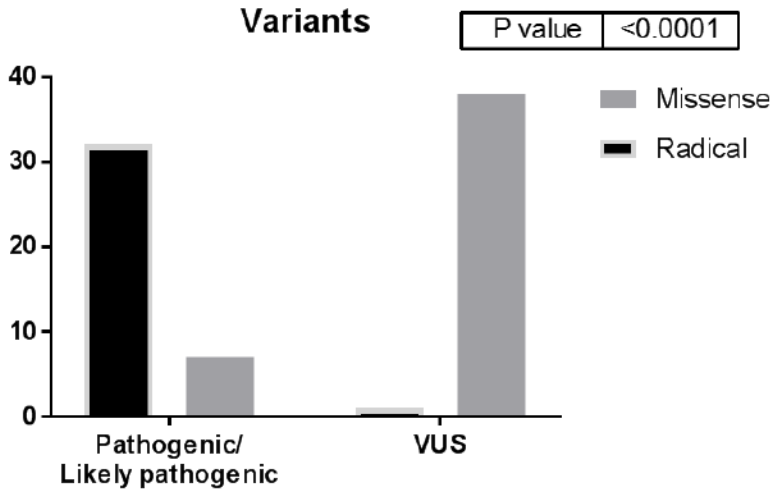


Figure 7: Variants classification according to ACMG criteria (Richards et al. 2015)

4.5 Recurrent variants

Conventionally genetics of AC is characterised by rare and private variants, nevertheless within our cohort 4 variants revealed a higher than expected incidence among index cases.

***PKP2*: c.148_151delACAG p.Thr50Serfs*61**

A four-base pair deletion in exon 1 (c.148_151delACAG) of *PKP2* is predicted to shift the reading frame of the protein (p.Thr50Serfs*61) keeping intact the first 50 amino acids and introducing a premature stop codon after 61 amino acids. The predicted truncated protein should maintain the N-terminal domain, but it would lack the armadillo type domanin.

It has already been reported 7 times in the literature [44, 61, 178-182] but its frequency is not available. In our cohort we found 5 unrelated heterozygotes index cases (5%) carrying this variant.

***PKP2* c.1643delG p.Gly548Valfs*15**

A guanidine deletion in exon 7 (c.1643delG) of *PKP2* is predicted to shift the reading frame of the protein (p.Gly548Valfs*15) keeping intact 548 of the 881 amino acids and introducing a premature stop codon after 15 amino acids. The predicted truncated protein should maintain the N-terminal domain, but is predicted to loose half of the armadillo domain.

It has already been reported 3 times in the literature [61, 178, 179] but its frequency is not available. In our cohort we found 4 unrelated heterozygotes index cases (4%) carrying this variant.

***PKP2*: c.2013delC p.Lys672Argfs*12**

A cystein deletion in exon 10 (c.2013delC) of *PKP2* is predicted to shift the reading frame of the protein (p.Lys672Argfs*12) keeping intact 672 of the 881 amino acids and introducing a premature stop codon after 12 amino acids. The predicted truncated protein should maintain the N-terminal domain, but it would lack more than 60% of the armadillo domain.

It has already been reported 5 times in the literature [9, 120, 178, 181, 183] and it displays a MAF of 0.008% in gnomAD database. In our cohort we found 6 unrelated heterozygotes index cases (6%) carrying this variant, representing the most recurrent variant.

DSC2 **c.536A>G p.Asp179Gly**

A missense variant c.536A>G caused by the substitution of adenosine to guanidine, is located in exon 5 of *DSC2* resulting in the 179th amino acid change of Aspartic acid to Glycine. It has already been reported as a founder mutation [50] and it displays a MAF of 0.00041% in gnomAD database. In our cohort we found 4 unrelated homozygotes index cases (4%) carrying this variant. Interestingly, all patients carrying this mutation originated form a small town of the North East of Italy named Chioggia.

4.6 Copy number variants

The entire cohort of 224 AC patients who underwent screening in research of medium intragenic rearrangements of desmosomal genes, displayed 8 (4%) aberrant MLPA profiles, indicating a deletion or duplication of one or more exons, or eventually the entire gene. These 8 CNVs carriers were considered as positively genotyped AC cases (Table 10).

Table 10: CNVs identified by MLPA

Nr.	Copy number variants (CNVs)
2	DEL PKP2 ex4 (chr 12, ~200bp)
4	DEL PKP2 (chr 12, ~120 kb)
1	DEL PKP2 ex6-14 (chr12, ~60kb)
1	DEL DSG2 and DSC2 (chr 18, ~482 kb)

Specifically, a single-exon heterozygous deletion was detected in 2 patients. The exon 4 *PKP2* gene deletion (c.1035_1170del) is predicted to shift the reading frame of the protein (p.Asn346Leufs*14) keeping intact the first 346 amino acids and introducing a premature stop codon after 14 amino acids. A 360 amino acid peptide should be generated instead of a 881 amino acid protein.

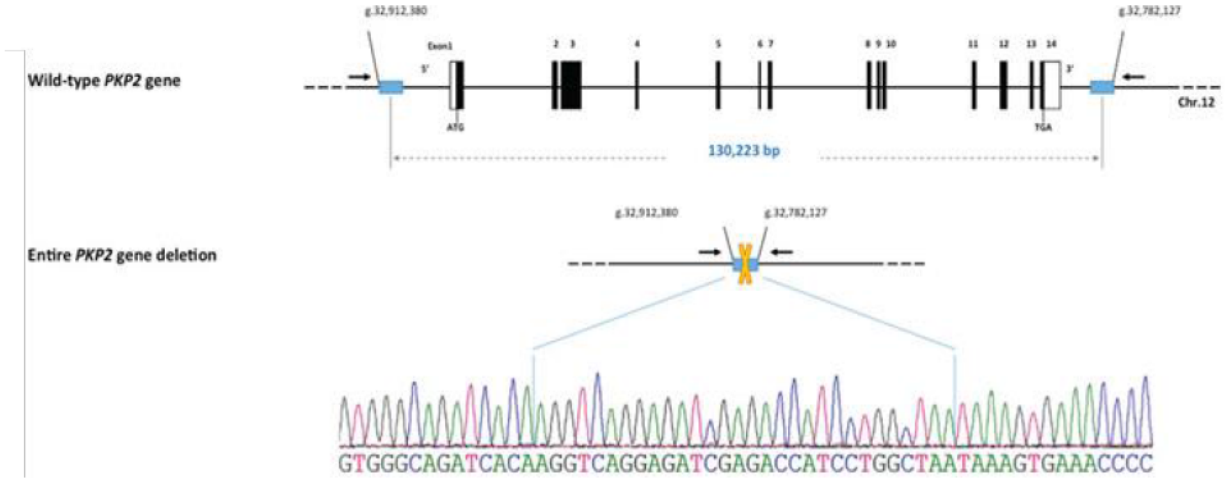


Figure 8: Schematic representation of entire *PKP2* deletion

Further, fine mapping by high-density single-nucleotide polymorphism arrays on chr12 identified the breakpoints of a 120kb deletion which contains the entire *PKP2* gene detected in 4 patients. Bioinformatics tools estimated the effect of this deletion in a gene dosage reduction ranging from 0.3 to 0.6 (Figure 8).

A 60kb-*PKP2* heterozygous deletion on chr12 was found in one patient, leading to the deletion of all exons from 6 to 14 of the protein. This 9-exon deletion is predicted to generate a truncated peptide composed by 460 of 881 amino acids.

Finally, a large heterozygous deletion on chr18 (chr18:30,945,216-31,669,766) comprising at least 482kb was found in one patient. This multiple-gene deletion comprise most members of the DSGs and DSCs subfamilies (*DSG1, DSG2, DSG3, DSG4 and DSC1, DSC2*) including the entire *DSG2* and *DSC2* genes linked to disease pathogenesis. Bioinformatics evaluation estimated the gene dosage reduction ratios of 0.35 and 0.51 respectively. Of note, the same patient carried also a heterozygous variant in *DSC2* c.536A>G (p.Asp179Gly) in the other allele, reported previously as a Italian founder mutation.

4.7 CDH2 screening

CDH2 gene was most recently associated with AC [74, 98]; it is encoding for cadherin 2 protein, or better known also as N-cadherin, which is a cell surface transmembrane protein with a vital structural and functional role in the intercalated discs and major contributor in cell-to-cell adhesion. All 224 AC patients underwent direct Sanger sequencing in order to estimate the prevalence of *CDH2* variants in our cohort.

We identified 3 different missense variants in *CDH2*, none of them classified as pathogenic/likely pathogenic (Table 11).

Table 11: *CDH2* variants

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	g.25756958G>T	c.29C>A	p.(Thr10Asn)	rs773400550	ALL:0.0011%	Tolerated (score: 0.23	benign	//
2	g.25756947G>C	c.40C>G	p.(Leu14Val)	//	//	Deleterious (score: 0)	possibly damaging (0.4589)	
3	g.25593772T>C	c.274A>G	p.(Ser92Gly)	rs150017015	ALL:0.040%	Tolerated (score: 0.35)	benign	

Variant filtering excluded c.29C>A variant located in exon 1 due to its weak amino acid conservation across species and a low score displayed in all *in silico* pathogenicity algorithms. Further, c.274A>G variant was excluded due to its high frequency in the general population and its weak *in silico* association.

Based on the ACMG -2015 criteria only c.40C>G variant located on exon 1 could be considered a VUS. Of note, the same proband carried also another VUS c.3134G>A in *DSP* (p.Arg1045Gln). Cascade genetic screening in the family was not available (Figure 9).

c.40C>G missense variant is located on a highly conserved amino acid across species and affected the N terminal signaling domain of CDH2.

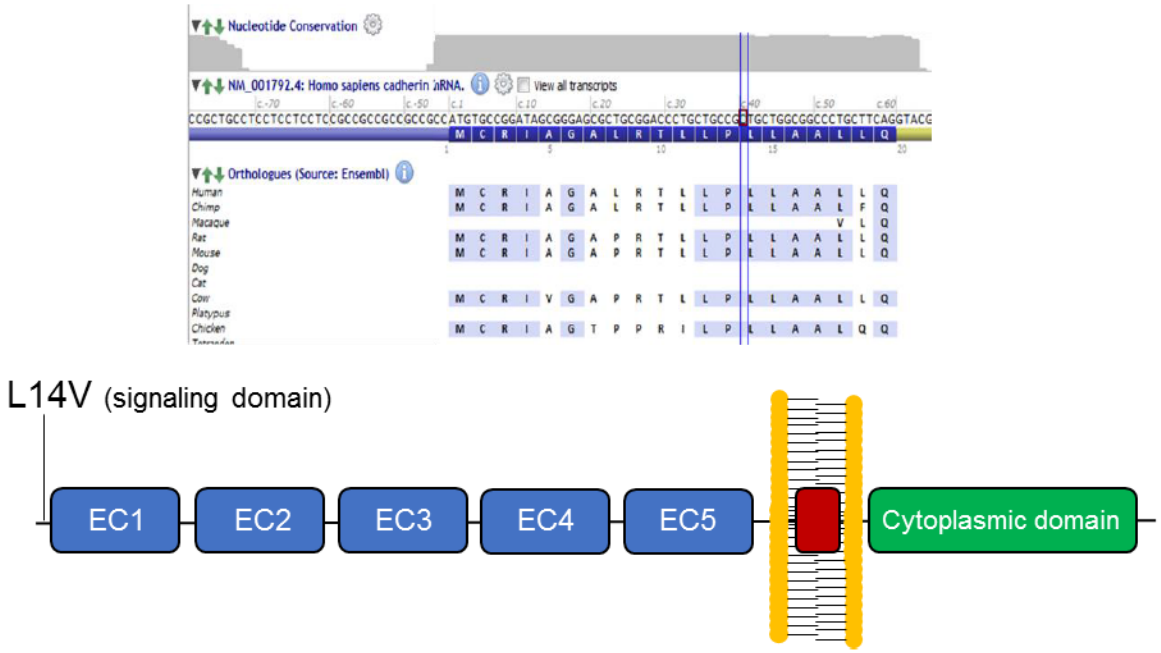


Figure 9: *CDH2* amino acid conservation and variant localization

4.8 *FLNC* screening

Filamin C protein directly interacts with the dystrophin associated glycoprotein and the integrin complexes, this last link the subsarcolemmal actin cytoskeleton to the extracellular matrix. Truncating *FLNC* mutations were found in 28 families exhibiting both LDAC and an overlapping phenotype known as dilated-AC; this last, shares with the 'classic' AC form a dominant trait of transmission and frequent premature sudden death but is complicated by an unusual high penetrance (97%) in carriers older than 40 years [73]. Up to date, 100 out of 224 AC index cases have been analysed by direct Sanger sequencing, identifying 3 different variants in *FLNC* (Table 12).

Table 12: *FLNC* variants

NR	g.DNA	c.DNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation taster
1	g.128478819C>G	c.1373C>G	p.Pro458Arg	rs773400550	//	Tolerated (score: 0.08)	Benign	Disease causing
2	g.128480675_128480676insT	c.1623_1624insT	p.Pro542Serfs*21	//	//	//	//	//
3	g.128492728C>T	c.5926C>T	p.Gln1976*	//	//	//	//	//

A missense c.1373C>G variant in exon 8 is causing the amino acid substitution of Proline to Arginine in position 458 of the protein. This variant is not present in gnomAD database and it is predicted as pathogenic only from one *in silico* algorithm. The proband carrying this variant was negative for other genetic variants both in desmosomal or AC-related non desmosomal genes.

A single-base pair insertion in exon 10 (c.1623_1624insT) is predicted to shift the reading frame of the protein (p.Pro542Serfs*21) keeping intact the first 542 of the 2725 amino acids and introducing a premature stop codon after 21 amino acids. The predicted truncated protein should maintain the calpoin homology domain, but it would lack the filamin/ABP280 repeated like domain. This variant was absent in current databases and was found in a proband negative for other genetic variants in AC-related genes.

A nonsense c.5926C>T variant in exon 36 is causing the Glutamine substitution by a premature stop codon in the protein position 1976 of the 2725 aminoacids(p.Gln1976*). This variant, absent in current databases, was found in a proband without putative pathogenic variants in AC-related genes.

4.9 Comprehensive yield of genetic screening

Deep sequencing, including new recently AC associated genes and CNVs analysis, led to the identification of the genetic cause in 10 more patients previously considered negative to genetic screening. In this way, the overall yield of genetic screening increased from 43% to 48%, 107 patients out of 224. No pathogenic variants were identified in 117 (52%) patients (Figure 10). Of note *TTN* variants were not included in this computation.

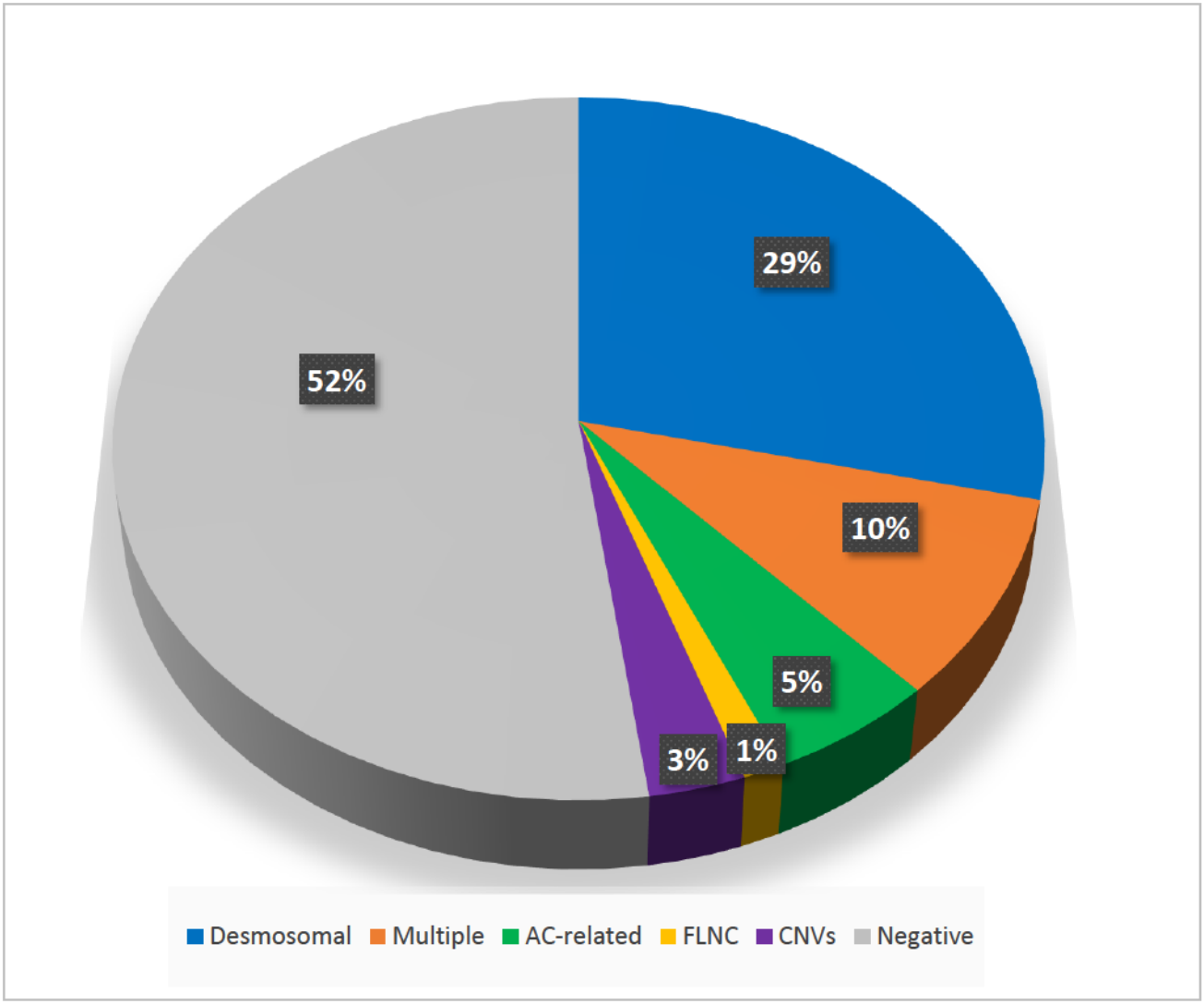


Figure 10: Comprehensive yield of genetic screening

4.10 Genotype-Phenotype correlation analysis

Overall we re-evaluated the clinical phenotype of 188 out of 224 AC (84%) index cases.

Detailed instrumental data from CMR, Echo, and twelve-lead ECG were collected for 156 of 188 AC index cases, of whom 16 underwent HTx. The rest 32 index cases received a post mortem diagnosis of AC.

Of these, based on the current ITC criteria, 94 index cases received a definite AC diagnosis at the outpatient clinic, 16 were HTx patients and 32 SD victims. The rest 18 were borderline and 28 were possibly affected by AC. Total number of index cases with definite AC diagnosis: 142.

Genotype/Phenotype correlation has been carried out on 3 different levels (Figure 11):

I. **Clinical diagnosis/Genotype**
II. **Desmosomal variant carriers/ AC-related non desmosomal variant carriers**
III. **Adverse Outcome/Genotype**

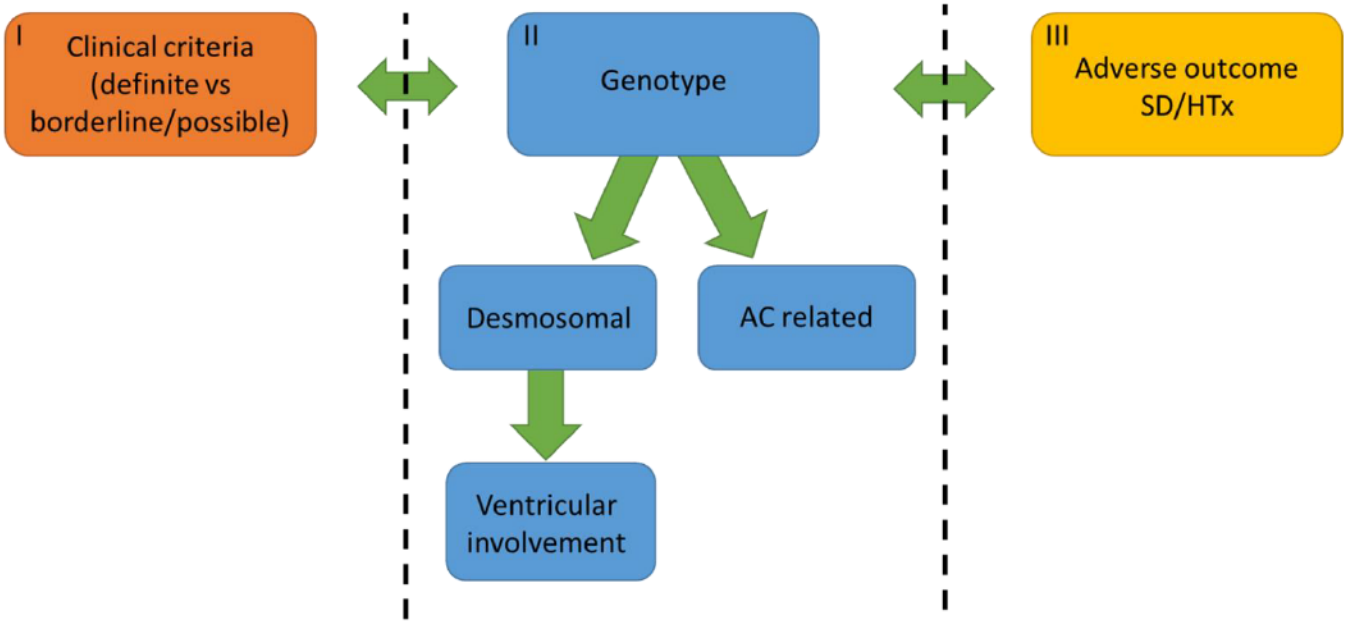


Figure 11: Genotype-Phenotype correlation workflow

I. Clinical diagnosis/Genotype

ITC criteria and patients genotype correlation highlighted that 78 of the 142 (55%) definite index cases carried at least a rare variant in AC-related genes whereas, 13 of the 46 (28%) borderline/possible index cases were genotype positive (Figure 13).

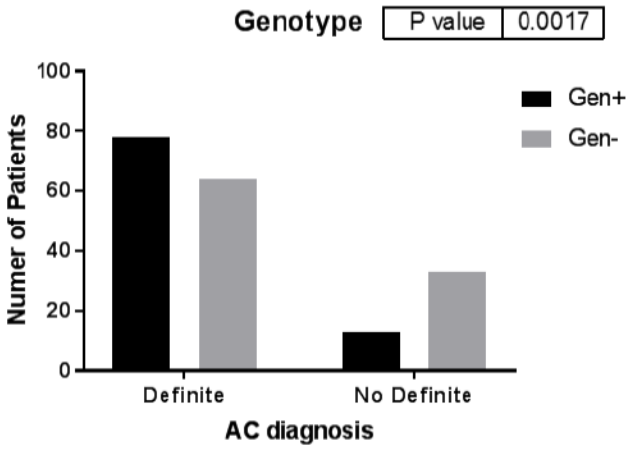


Figure 13: Comparison between definite AC patients genotype and non definite ones. (Gen+: genotype positive, Gen-: genotye negative)

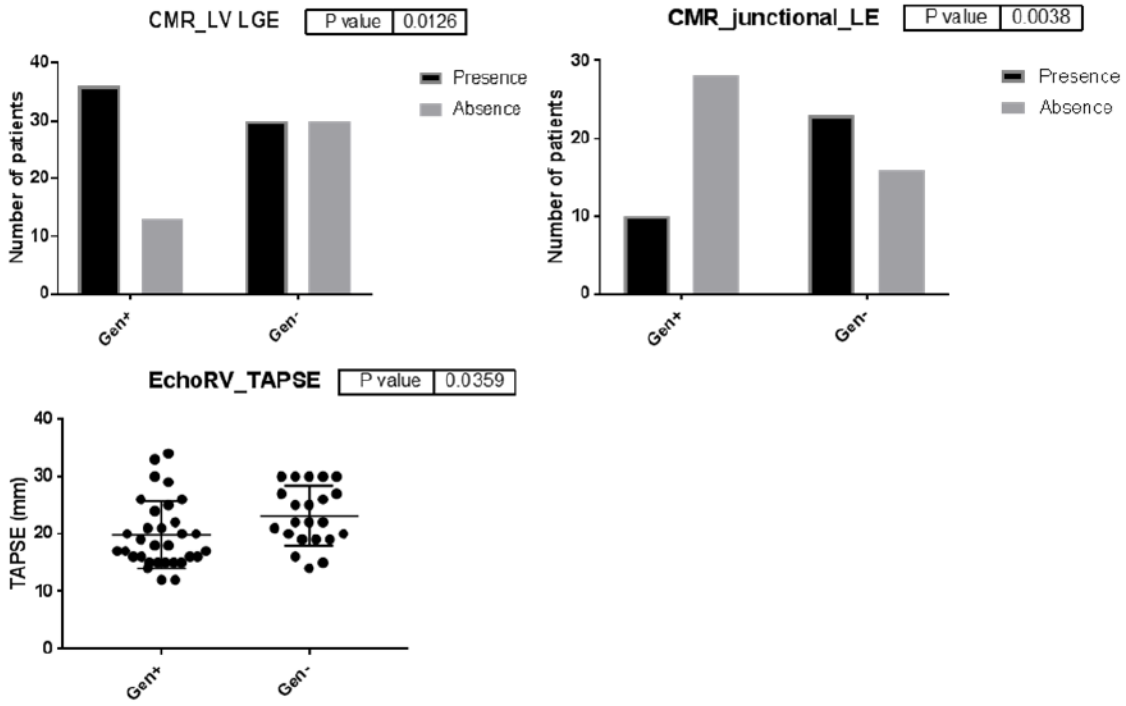


Figure 12: Comparison between Genotype positive (Gen+) and Genotype negative (Gen-) AC patients. (CMR_LV_LGE: Cardiac magnetic resonance late gadolinium enhancement of the left ventricle, CMR_juncitonal_LE: Cardiac magnetic resonance junctional late gadolinium enh

Interestingly, among clinical parameters assessed, late gadolinium enhancement on the LV was found more often in genotype positive index cases (36/49, 74%) compared to genotype negative cases (30/30, 50%) (p-value 0.0126). Junctional gadolinium enhancement was less frequent in genotype positive cases (10/38, 26%) than in genotype negative cases (23/39, 59%) (p-value 0.0038). Further, genotype positive cases (19mm ±1)(p-value 0.0359) exhibited a lower tricuspid annular plane systolic excursion compared to genotype (Figure 12).

Further, 70 of the 78 (89%) genotype positive definite cases were desmosomal rare variant carriers. Specifically 32 carried a single radical variant, 22 a missense variant, 3 a CNVs and 13 were multiple desmosomal variant carrier.

Eight of the 78 (11%) genotype positive definite cases were AC-related non desmosomal variant carriers. Specifically, 7 carried a missense variants and 1 a radical variant (Figure 14).

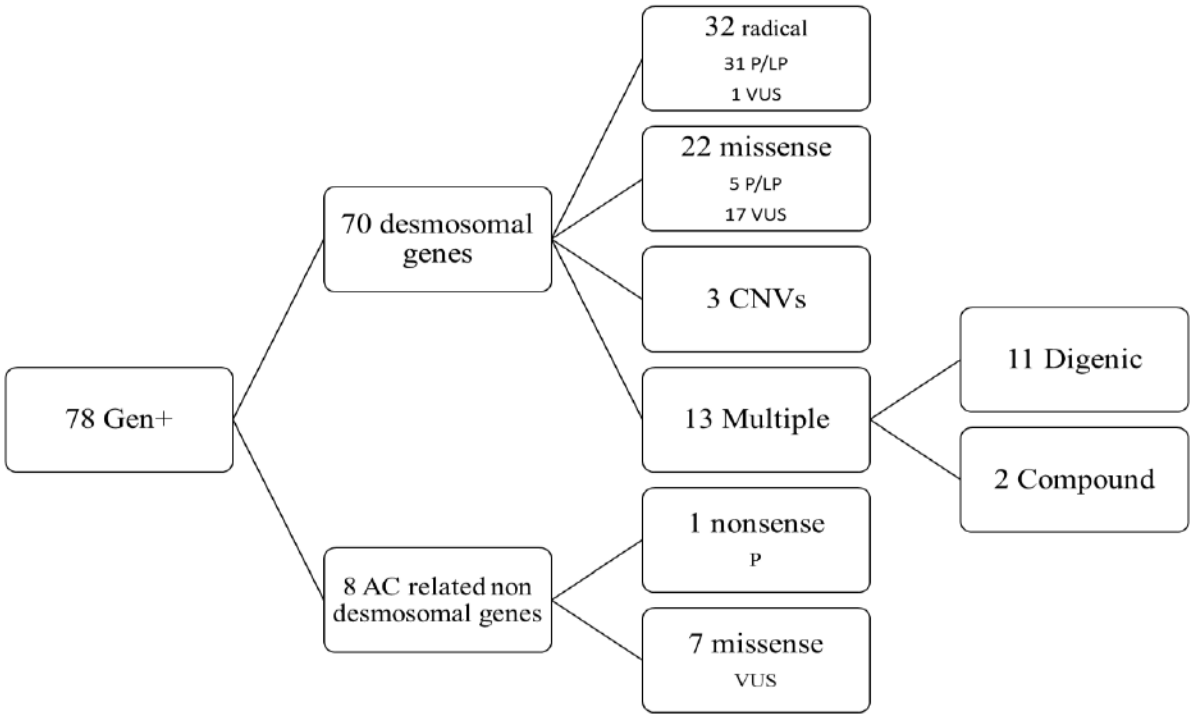


Figure 14: Genotype positive definite AC cases variants distribution. (P/LP: Pathogenic/Likely pathogenic, VUS: Variant of uncertain significance

Ten of the 13 (86%) genotype positive borderline/possible cases were desmosomal variants carriers. Specifically, 4 carried a radical variant and 6 a rare missense variant. Three of the 13 (14%) genotype positive borderline/possible cases carried 3 different missense variants in AC-related non desmosomal genes (Figure 15).

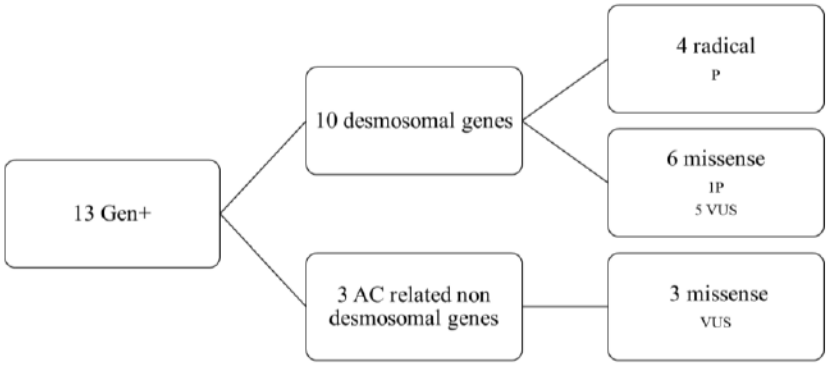


Figure 15: Genotype positive bordeline/possible AC cases variants distribution. (P/LP: Pathogenic/Likely pathogenic, VUS: Variant of uncertain significance

II. Desmosomal variant carriers/ AC-related non desmosomal variant carriers

Clinical parameters analysis, on genotype positive patients, revealed that desmosomal rare variant carriers displayed a similar LV ejection fraction (57.28% ± 1.27) compared to AC-related non desmosomal carriers (51.63% ± 3.32) (p-value 0.090). Interestingly, desmosomal rare variant carriers exhibit less often complete right bundle branch block (4/46, 9%) compared to AC-related non desmosomal variant carriers (3/5, 60%) (p-value 0.0174) (Figure 16).

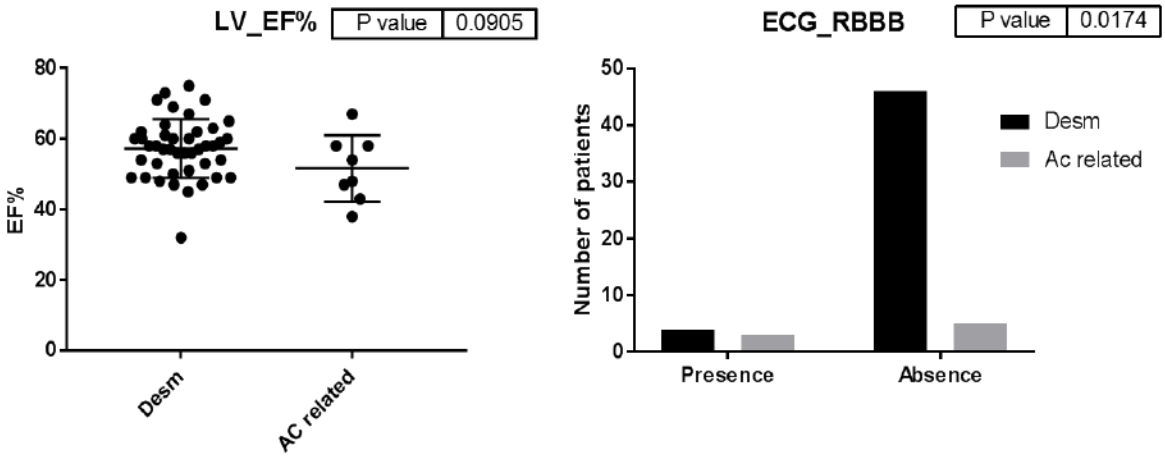


Figure 16: Comparison between AC desmosomal and non desmosomal phenotypes. (LV_EF%: Left ventricle ejection fraction percentage, ECG_RBBB: Elettrocardiographic complete Right Bundle Branch Block)

Of note, clinical parameters are unable to distinguish desmosomal gene-specific phenotype suggesting a clinical homogeneity among desmosomal variant carriers.

Genotype analysis focused on ventricular involvement highlighted that LDAC patients were significantly less positive for desmosomal variants (11/42, 26%) compared to the "classic" AC cases (75/182, 41%)(p-value 0.0065) (Figure 17).

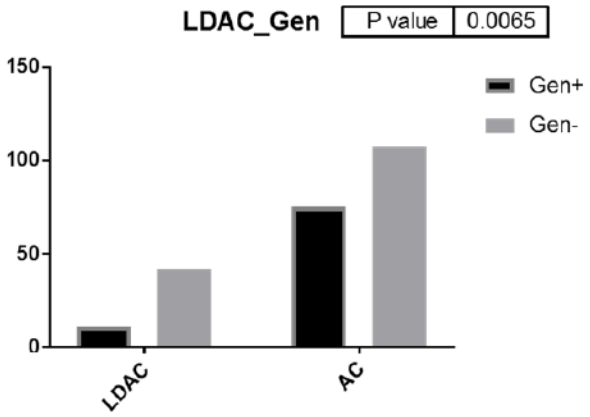


Figure 17: Sudden death victims (SD) genotype-phenotype correlation. (SD_LDAC_Gen: Left Dominan Arrhythmogenic Cardiomyopathy sudden death victims genotype, Gen+: genotype positive, Gen-: genotype negative)

In more detail, 19 of the 42 (45%) LDAC patients were SD victims, of whom only 4 cases (21%) were genotype positive for desmosomal rare variants (Figure 18). Of note, 4 of 11 (36%) desmosomal variants were classified as Pathogenic/Likely pathogenic

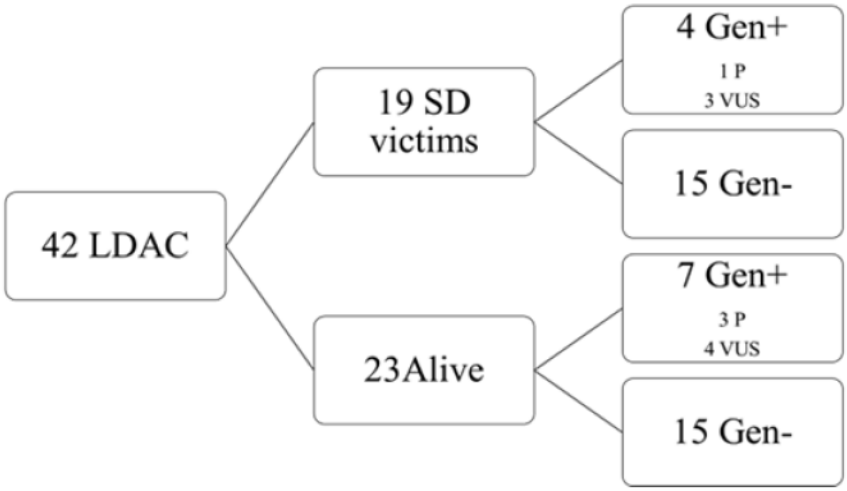


Figure 18: GenotypeVentricular involvement analysis. (LDAC_Gen: Left Dominant Arrhythmogenic Cardiomyopathy patients genotype, SD: sudden death, Gen+: genotype positive, Gen-: genotype negative, P/LP: Pathogenic/Likely pathogenic, VUS: Variant of uncertain significance.

III. Adverse Outcome/Genotype

Analyses focused on disease adverse outcomes highlighted that 32 SD victims exhibiting a lower mean age 29y ± 2 compared to 16 HTx patients (44y ± 4) (p-value 0.0053) and SD victims were all males (32/32, p-value <0.0001). Further, focusing on the genotype 10/32 (28%) SD victims carried rare variants in desmosomal genes compared to HTx patients 10/16 (63%) (p-value 0.0217) (Figure 19).

Specifically, 5 SD victims carried radical variants, 3 missense variants, 1 CNVs and 1 SD victim carried one desmosomal variant in two different genes (digenic carrier). Four HTx cases carried radical variants, 3 missense variants, 1 CNVs and 2 HTx cases carried multiple rare variants.

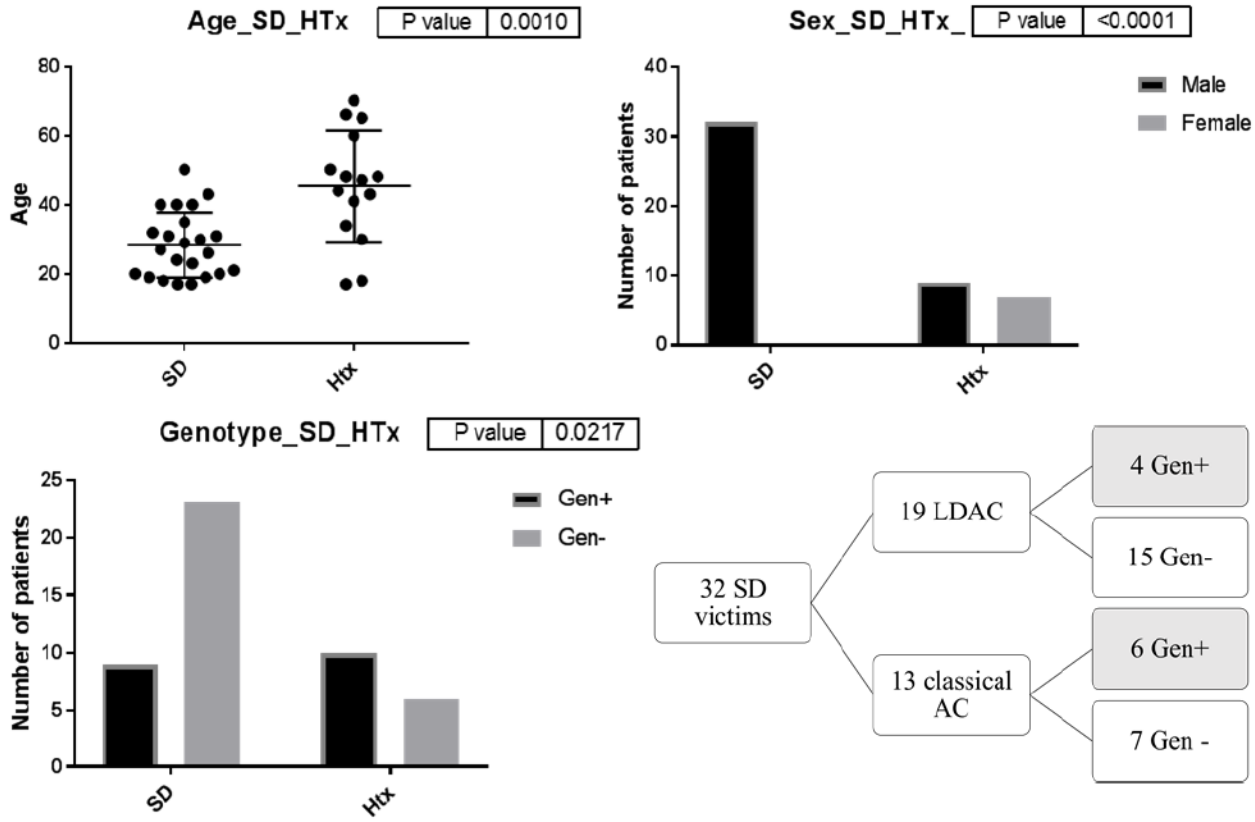


Figure 19: Comparison between adverse outcome. (SD: Sudden death, HTx heart transplantation, Gen+: genotype positive, Gen-: genotye negative)

4.11 *TTN* variants

Titin is the largest protein in mammals and is expressed in both cardiac and skeletal muscle bridging the sarcomere along its longitudinal axis and forming a contiguous filament along the myofibril. Several isoforms ranging in size from 2970 to 3900 kDa are produced from the single *TTN* gene, which is located on chromosome region 2q31 and is comprised of 363 exons. [184, 185] Eight unique missense *TTN* variants were identified in 7 families with AC-like phenotype and lacking other desmosomal genes mutations [69]. However *TTN* missense variants nowadays are considered mostly benign, with very few exceptions. [186]. Up to date, we investigated 151 of 224 AC index cases in our cohort, identifying 50 unique *TTN* rare variants in 40 patients (26%), of whom, nearly half (18/40, 45%) already carrying a rare desmosomal variant (Figure 20). Single or multiple rare variants were located on highly conserved amino acids and predicted to be pathogenic by at least 2 *in silico* algorithms. (Appendix A)

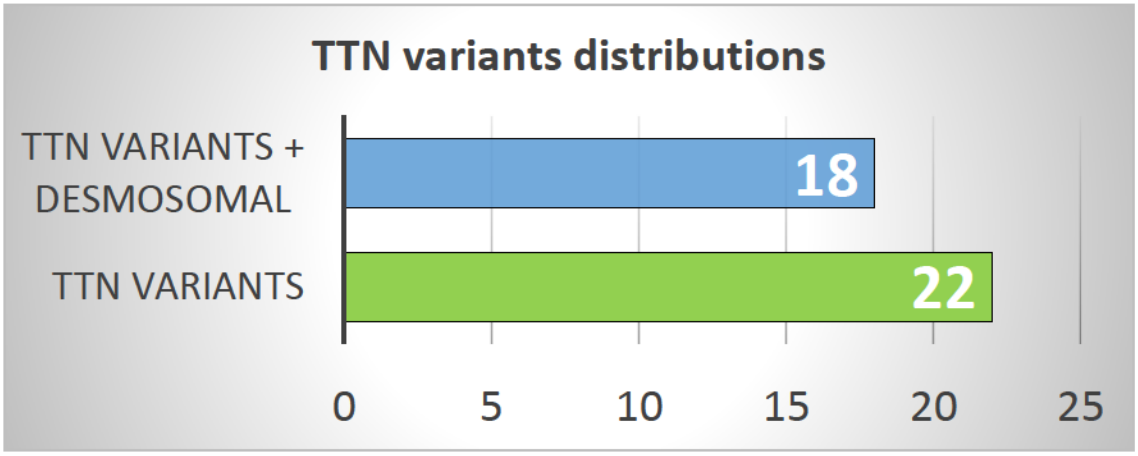


Figure 20: *TTN* variants distribution

Most *TTN* variants identified were missense 48/50 (96%) and 2 variants were located on critical splicing sites. Regarding these last,

- c.32471-1G>A variant affects the acceptor slice site of intron 130 and is predicted to alter mRNA maturation as previously reported in the literature associated with titinopaties. [187].

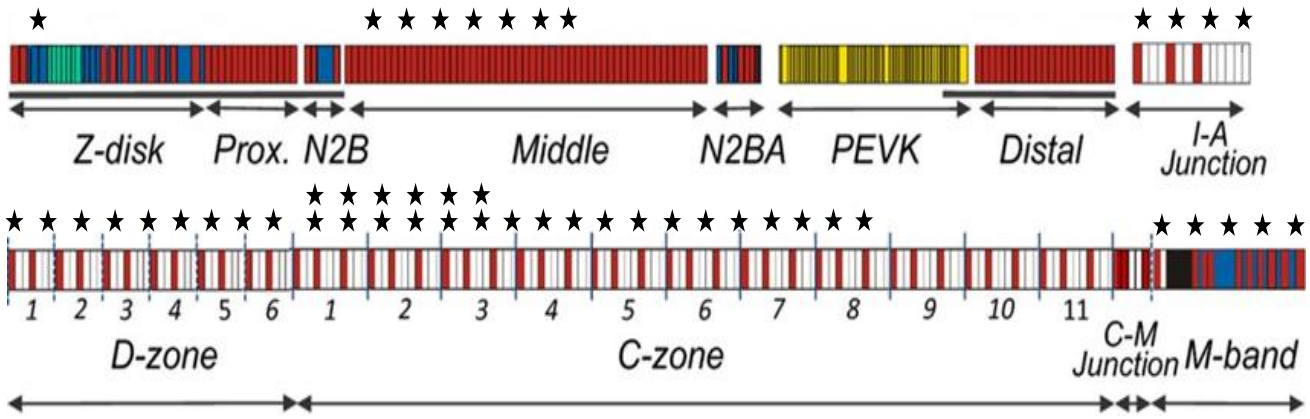
  Interestingly the proband carried three additional rare missense variants on *TTN* therefore co-segregation studies are crucial to determine their role in the disease.

- c.39817G>C variant is changing the last nucleotide of exon 211 affecting the donor site of the protein. It is not reported in the literature but is predicted to alter the splicing process by *in silico*

algorithm. The patient carrying this variant did not carry other AC-related variants. Cascade genetic screening is mandatory to determine its pathogenic role.

TTN variants localization

TTN amino acid substitutions were localized predominantly on the A-Band (33/50, 66%) which contains D-zone, C-zone and C-M junction; specifically 9 of the 50 substitutions (18%) were localized in the D-zone repeated domain and 24 of the 50 substitutions (48%) in the C-zone repeated domain. (Figure 21)



Domain	Exons	AA	Observed SNPs
Zline	1_28	2169	1
I band	29_250	11755	7
I/A junction	251_270	1387	4
D zone	271_306	4217	9
C zone	307_355	11927	24
C/M junction	356_358	492	0
Mline	359	2403	5
		34350	50

Figure 21: Schematic TTN structure and variants localisation

Rare amino acid substitutions in the C-zone repeats resulted overrepresented (p-value 0.0213) based on Pearson's $\chi 2$ goodness-of-fit tests which was used to test if the distribution was random considering the size in amino acids of each protein region tested.

4.12 Rare variants in disease_unrelated genes

Seven genotype negative but also 6 genotype positive patients carried additional rare variants in genes encoding sarcomeric proteins (MYBPC3, MYH7) and ion channel subunits (CACNA1B, SCN10A), usually associated with other forms of inherited cardiomyopathies (i.e HCM, DCM, Brugada Syndrome, QT syndromes). Their role as possible modifiers is still under investigation (Figure 22).

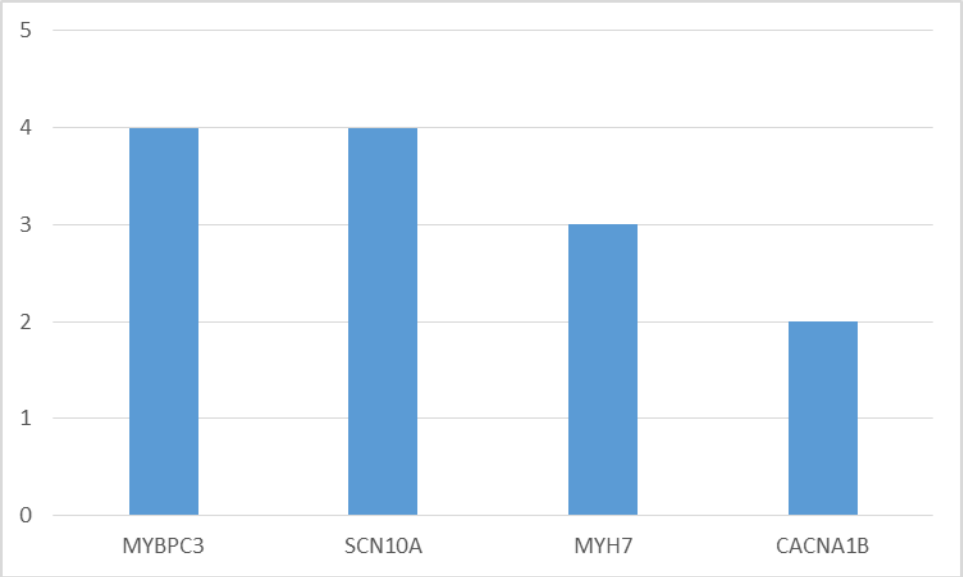


Figure 22: Additional rare variants identified in our cohort

4.13 WES screening

Whole exome sequencing carried on 10 genotype-negative AC probands identified ~80.000 variants/patient. Gene and variants prioritization led to reduce their number to an average of 200 rare variants/patient.

Recent transcriptome data from another study carried in our laboratory revealed the under expression of *galectin 3* (*LGALS3)*, both in a transgenic mouse model and AC patients, prior structural abnormalities pathognomonic features of the disease *(unpublished)*. Fascinatingly WES highlighted the presence of rare genomic variants in this gene.

4.13.1 Galectin 3 (LGALS3)

Lectin, galactoside-binding, soluble 3 (*LGALS3*) is situated on chromosome 14, locus q21-22, and is composed of six exons and five introns spanning about 17 kilobases. *LGALS3* encodes for Galectin-3 protein which belongs to the family of animal-lectins proteins, a group of water soluble, non-glycosylated globular proteins that can interact with carbohydrates in a divalent cation-independent manner.

Galectin-3 protein consists of 250 amino acids, separated into three distinct domains (Figure 24). The carbohydrate recognition domain (CRD), accounting for approximately 130 amino acids, contains Slectin motifs that provide the ability to bind β-galactosides, a property shared by all proteins in the Galectin family [188, 189]. In addition to its CRD, galectin-3 has a central region spanning approximately 120 amino acids which contains a highly conserved tandem repeat domain rich in Proline, Glycine and Tyrosine [190, 191]. The N-terminal domain contains a 12 amino acid leader sequence that is required for galectin-3 secretion [191]. Within this leader sequence, Serine can be phosphorylated reducing significantly Galactin-3 binding to two of its ligands, laminin and mucin; this process may act as an on/off switch for Galactin-3 ability to bind to sugars [192]. The N-terminal domain also enables the formation of oligomers and is required for full biological function of the protein, including its role in modulating cell adhesion and inducing intracellular signaling [190, 193]. Galectin-3 has multiple functions within cells, localized in the nucleus and cytoplasm, as well as outside of the cell [191, 194].

DNA sequencing in 150 AC index cases, 10 through WES and 140 through direct sequencing, led to the identification of 5 *LGALS3* rare nucleotide variants in 7 probands (4%, 5 males, mean age 39±11 years). Of note, 3 of the 7 *LGALS3* carried also pathogenic/likely pathogenic desmosomal genetic variants(Table 13):

Table 13: *LGALS3* rare variants

N°	cDNA	Protein	dbSNPID	gnomAD	SIFT	Polyphen	Mutation Taster	Other variants
1	c.29 C>T	p.Ala10Val	rs202159462	0. 025%	Tolerated (score: 0.06)	Probably damaging	Disease causing (p-value: 0.97)	DSG2
2	c.137C>G	p.Pro46Arg	rs200440596	0. 02%	Deleterious (score: 0)	Probably damaging	Disease causing (p-value: 1)	PKP2; DSG2
3	c.137C>G	p.Pro46Arg	rs200440596	0. 02%	Deleterious (score: 0)	Probably damaging	Disease causing (p-value: 1)	DSP; DSC2
4	c.137C>G	p.Pro46Arg	rs200440596	0. 02%	Deleterious (score: 0)	Probably damaging	Disease causing (p-value: 1)	NEG
5	c.485G>A	p.Arg162His	rs201865041	0. 002%	Deleterious (score: 0)	Probably damaging	Disease causing (p-value: 1)	NEG
6	c.520A>G	p.Asn174Asp	//	//	Deleterious (score: 0)	Probably damaging	Disease causing (p-value: 1)	NEG
7	c.18+1_18+4delGTAA	//	rs745877914	0.0083%	//	//	//	NEG

A four-base deletion (c.18+1_18+4delGTAA) affecting the donor site of exon 2 is presumed to alter the mRNA maturation by different *in silico* algorithms. This 4-base pair deletion displayed a MAF of 0.0083% in gnomAD database and was located on the N terminal domain of the protein.

A missense variant (c.29 C>T) located in exon 2 causing the substitution of cytosine to thymidine results in the 10th amino acid change from Alanine to Valine. This single nucleotide rare variant displayed a MAF of 0.025% in gnomAD database and was located on the N terminal domain of the protein. This *LGALS3* rare variant carrier harboured also a pathogenic variant in *DSG2*.

A missense variant (c.137C>G) located on exon 2 causing the substitution of cytosine to guanidine results in the 46th amino acid change from Proline to Arginine. This single nucleotide rare variant displayed a MAF of 0.020% in gnomAD database and was located on the highly conserved tandem repeated domain of the protein. This rare variant was shared among 3 AC index cases, two of which had primary pathogenic variants in desmosomal encoding genes.

A missense variant (c.485G>A) located on exon 4 causing the substitution of guanidine to adenine results in the 162th amino acid change from Arginine to Histidine. This single nucleotide rare variant displayed a MAF of 0.002% in gnomAD database and was located on the CRD of the protein.

A missense variants (c.520G>A) located on exon 4 causing the substitution of guanidine to adenine results in the 174th amino acid change from Asparagine to Aspartatic acid. This single nucleotide rare variant was absent from gnomAD database and was located on the CRD of the protein.

Remarkably, both c.485G>A and c.520A>G *LGALS3* missense variants disrupt the CRD binding site of galectin-3 protein. (Figure 23)

LGALS3 variants localization

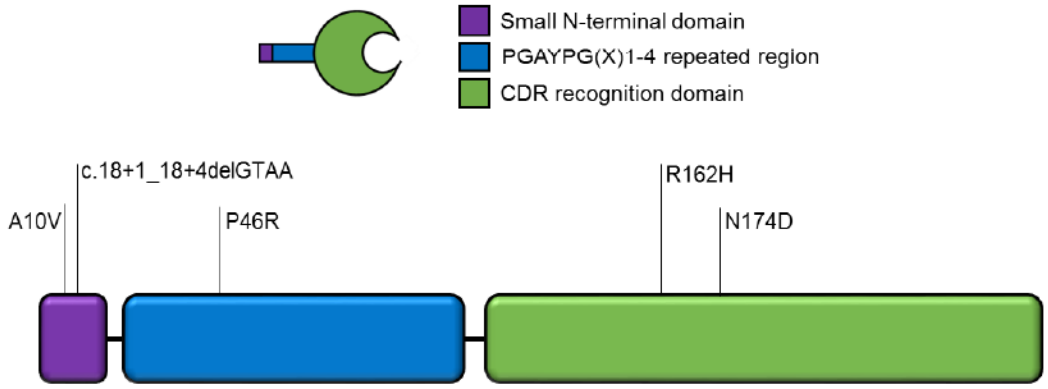


Figure 24: *LGALS3* structure and variants localisation

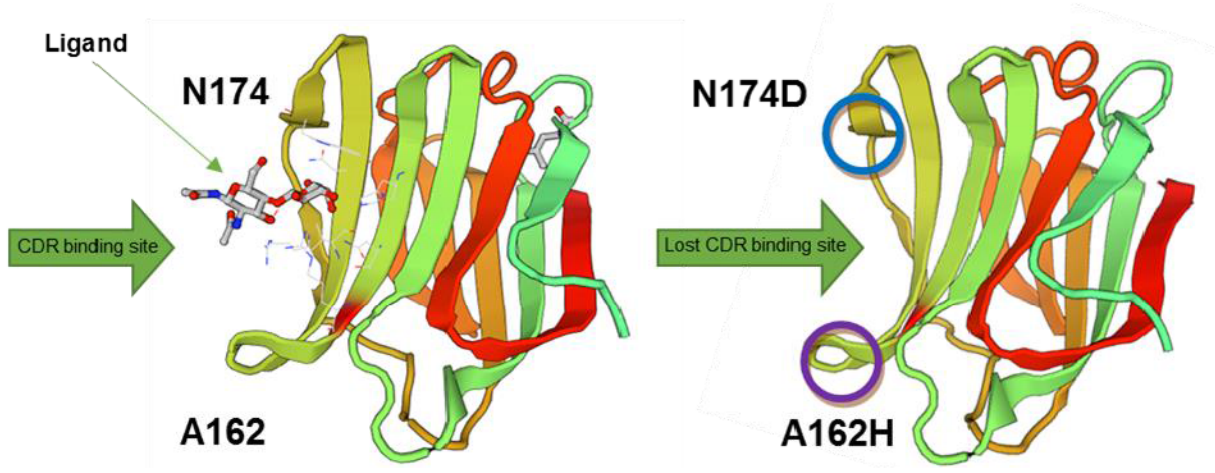


Figure 23: CRD binding site disruption

5 Discussion

Arrhythmogenic cardiomyopathy cardinal pathognomonic features are fibrofatty myocardial replacement, impairment of ventricular systolic function and prominent life-threatening ventricular arrhythmias. AC phenotypic spectrum was broader than previously thought and diagnosis may be achieved by combining multiple sources of clinical information, such as genetic data, arrhythmic, morphofunctional, and histopathologic findings.

To address the issue of investigate genetic heterogeneity in AC, we focused on appropriate genetic screening, stringent variants classification and multiparametric genotype-phenotype correlation followed by the search of new disease causative genes.

Variants distribution and classification

NGS screening led to identification of 95 different rare variants in 97 index cases (43%). Specifically 78 patients carried a single rare variant, whereas 19 were multiple variants carriers (either digenic or compound). Seventy-nine of the 95 rare variants were found in 5 major desmosomal genes (83%), and 16 in AC-related non desmosomal genes (17%) suggesting a significant contribution of non desmosomal genes in disease pathogenesis. Variants classification according to the ACMG criteria highlighted that half of desmosomal variants (39/79, 50%) were classified as Pathogenic/Likely pathogenic and were mostly radical variants (32/39, 78%). VUS were missense variants either desmosomal or non desmosomal making evident the need of cascade genetic screening to better understand their role in disease pathogenesis.

Four rare variants in desmosomal genes, displayed a higher prevalence among genotype positive patients in our cohort (up to 6%) supporting the theory of a possible founder effect in the Veneto population although further haplotype studies are needed to investigate their segregation.

Searching for genetic rearrangements in desmosomal genes identified 4 different heterozygous deletions in 7 genotype negative patients, highlighting the potential of CNVs analysis to increase the diagnostic yield of genetic testing. Even though genotype-phenotype correlation demonstrated a low disease penetrance in family members carrying a CNV [79], emphasizing that haploinsufficiency alone may not lead to AC phenotype.

The prevalence of additional AC associated genes was also investigated. Specifically, 3 different Pathogenic/Likely pathogenic *FLNC* variants were identified in 3 of 100 AC index patients evaluated thus far. Only 1 *CDH2* VUS was found in a desmosomal variant carrier . *FLNC* prevalence, still under evaluation (100/224 index cases analyzed) but it seems to be as high as *DSC2* (3%). The prevalence of *CDH2* in our cohort is under <0.5% (1 out of 224 index cases) suggesting a limited disease association of this gene. To this regard, cascade genetic screening on large families is required to evaluate the role of these variants on disease pathogenesis.

Deep sequencing and CNVs analysis allowed to positively genotype 10 more AC patients, previously considered negative to genetic testing. In this way the overall yield of genetic screening increased from 43% to 48%, (107 patients out of 224 AC index cases). No pathogenic variants were identified in 52% (117/224) of AC index patients showing that other factors, genetic or not, might be involved in the development and progression of the disease.

Noteworthy, our analysis made evident the presence of additional rare variants encoding sarcomeric proteins (MYBPC3, MYH7), ion channel subunits (CACNA1B, SCN10A) as well as cytoskeletal (TTN) proteins, in both genotype negative and positive patients. Specifically we investigated the prevalence of *TTN* rare variants in AC finding 50 different VUS in 40 patients, of whom, nearly half (18/40, 45%) already carried a rare desmosomal variant. The role of these variants in disease pathogenesis is still under investigation but the presence of VUS in genes related with other forms of inherited cardiomyopathies underlined the genetic overlap between cardiomyopathies. These clinical entities previously regarded as monogenic traits should be probably envisioned as a group of conditions with variable inheritance patterns, molecular aetiologies and clinical presentations. In fact, homozygous, compound heterozygous, digenic/oligogenic variants, or a combination of genetic and exogenetic factors in desmosomal genes are frequently described in patients with AC [9, 44, 161]. Gene dosage effects that may occur due to loss-of-function mutations have been supported also by experimental models in AC, and may underlie oligo- and multigenic inheritance [103, 195]. Stoichiometric alterations of macromolecular complexes (and cellular networks) or a combination of innocent single variants at 2 loci encoding interacting proteins may lead to a fitness defect, increasing the risk of having an overt AC phenotype [9, 44, 161]. Thus, special attention should be paid to variants appearing in genes encoding subunits of the same macromolecular complexes or involved in the same biological pathways because they might contribute to the phenotypic variability.

This phenomenon has been also described in other cardiomyopathies as to demonstrate that these rare cardiac disorders with low/absent disease penetrance, previously considered monogenic, may be the result of the cumulative effect of more loci (risk alleles) suggesting a more complex inheritance model.

Genotype-phenotype correlation.

Detailed clinical data on twelve-lead ECG, echocardiography and CMR were collected for 156 of the 188 AC index cases, 16 of whom underwent HTx, whereas 32 of the 188 index cases received a post mortem diagnosis of AC. Overall, based on the ITC criteria 142 patient received a definite AC diagnosis, 18 were borderline and 28 were possibly affected. Of note, all HTx patients as well as SD victims received a definite diagnosis. Genetic screening highlighted that AC patients with a definite diagnosis were more frequently (55%) Pathogenic/Likely pathogenic variant carriers compared to borderline/possible affect ones (27%). AC definite patients carried desmosomal variants (89%) and more of a half of these desmosomal variants were Pathogenic/Likely pathogenic. On the other hand, variants in AC-related non desmosomal genes were exclusively VUS, exception made for a nonsense *FLNC* variant carried by a SD victim with a post mortem definite diagnosis of AC. Our data underlined that a proper clinical phenotype characterization is essential to increase the likelihood of identifying a genetic cause in AC patients. Positive genetic testing in ~ 30% of borderline/possible AC index patients confirmed the clinical suspicion. Still, half of AC index patients lacked genetic causes suggesting that probably other factors, genetic or epigenetic, may contribute to disease development.

Further, comparison of genotype positive and genotype negative index cases revealed an higher rate of LV gadolium enhancement in genotype positive patients (74%) compared to negative ones (50%) reflecting a LV involvement due to the disease progression from the RV to biventricular. On the other hand, genotype negative patients displayed frequently junctional late enhancement (59%) as demonstrated to be an aspecific finding common also in athletes. Of note, genotype negative cases presented a higher tricuspid annular plane systolic excursion (23mm ±19) compared to genotype positive cases (19mm ±1).

Further analyses considering desmosomal variant carriers as a homogeneous subgroup of the global genotype positive classification, confirmed literature data that “classic”desmosomal AC patients rarely (9%) present complete RBBB [32, 33]. Up to date none of the clinical parameters can clearly distinguish a desmosomal gene-specific related phenotype underling a similar clinical manifestations.

Based on assessing the ventricular involvement of ITC index cases we found desmosomal variants in 25% of LDAC patients; no gene-specific prevalence was detected in contrast to current literature reports [196-198]. This finding was confirmed on 19 LDAC SD victims, of whom only 4 (21%) a carried rare variant in desmosomal genes. Thus, other genes/factors may be involved in the LDAC pathogenesis as to expand the genetic spectrum of AC.

Focusing on the adverse outcome in AC patients (SD or HTx), we found that SD victims were all males and younger in age than the HTx counterpart. From the genetic point of view, only 28% of the SD victims carried a rare desmosomal variant compared to 63% in HTx patients. These data stress further the fact that HTx patients reaching the overt phase of the disease, characterized by heart failure, and biventricular involvement, exhibited the classic desmosomal AC genotype, whereas SD victims, most often LDAC patients, exhibited a lower prevalence of desmosomal variants as to suggest other genetic factors related to this AC subtype.

Identification of a new candidate gene

LGALS3 was prioritized among ~300 genes emerged from whole exome sequencing in 10 biventricular genotype negative AC index cases, due to earlier findings from transcritpional studies. Together with subsequent direct sequencing of *LGALS3* in a larger cohort of 140 more AC index patients, 5 rare missense variants in 7 probands (~5%, 5 males, mean age 39±11 years) were identified.

LGALS3 gene, which encodes for a member of the adhesion/growth-regulatory galectin proteins family GAL3, is involved in a wide array of cellular activities among which regulation of cellular trafficking of glycoproteins, signaling, and cell adhesion. *LGALS3* is also an upstream regulator gene of GSK3β. Previous studies demonstrated that WNT/β-catenin signaling pathway inhibition is a consequence of genetically abnormal desmosomes leading to the release of JUP protein from intercalated disks with subsequent transfer to the nuclear pool [113]. Suppression of WNT/β-catenin signaling was also confirmed by the subsequent identification of a WNT signaling inhibitor molecule of glycogen synthase kinase 3 β (GSK3β) in a AC zebrafish model and the aberrant activation of the Hippo/YAP signaling pathway [199, 200]. Recent studies demonstrated that lower expression of GAL3 leads to reduced levels of phospho-GSK3β at serine 9, thus increasing GSK3β activity; phospho-GSK-3β leads to phosphorylation of β -catenin and its degradation, suppressing WNT signaling [201].

Moreover, GAL3 has the potential to enhance intercellular adhesion through CRD direct binding to the extracellular domain of cadherins and specifically to DSG2 (N-linked β-galactosides ectodomain), as previously demonstrated by *in vitro* experiments in intestinal, corneal and conjunctival epithelia [202-

204]. Further, GAL3-reactive sites have been also found to co-localize with the desmosomal proteins DSP and DSG in primary squamous carcinomas, pointing to the potential role of GAL3 in mediating intercellular contacts [205]. Indeed, transcriptome data from our group demonstrated the under expression of *LGALS3*, both in a transgenic mouse model and AC patients, before the onset of the disease. [*unpublished*]. Our data supported that dysregulation of *LGALS3* may be another key factor in the cascade reaction toward WNT signaling inhibition, as well as lack of desmosomal binding adhesion and stabilization. All these data support the involvement of this molecule in AC pathogenesis, although further studies are needed to better elucidate its role and function.

6 Bibliography

[1] Thiene, G., A. Nava, D. Corrado, L. Rossi, N. Pennelli, Right ventricular cardiomyopathy and sudden death in young people, N Engl J Med 318 (1988) 129-33.

[2] Basso, C., G. Thiene, D. Corrado, A. Angelini, A. Nava, M. Valente, Arrhythmogenic right ventricular cardiomyopathy. Dysplasia, dystrophy, or myocarditis?, Circulation 94 (1996) 983-91.

[3] Basso, C., D. Corrado, F.I. Marcus, A. Nava, G. Thiene, Arrhythmogenic right ventricular cardiomyopathy, Lancet 373 (2009) 1289-300.

[4] Basso, C., B. Bauce, D. Corrado, G. Thiene, Pathophysiology of arrhythmogenic cardiomyopathy, Nat Rev Cardiol 9 (2011) 223-33.

[5] Marcus, F.I., G.H. Fontaine, G. Guiraudon, R. Frank, J.L. Laurenceau, C. Malergue, Y. Grosgogeat, Right ventricular dysplasia: a report of 24 adult cases, Circulation 65 (1982) 384-98.

[6] Frank, R., G. Fontaine, J. Vedel, G. Mialet, C. Sol, G. Guiraudon, Y. Grosgogeat, [Electrocardiology of 4 cases of right ventricular dysplasia inducing arrhythmia], Arch Mal Coeur Vaiss 71 (1978) 963-72.

[7] Sen-Chowdhry, S., P. Syrris, S.K. Prasad, S.E. Hughes, R. Merrifield, D. Ward, D.J. Pennell, W.J. McKenna, Left-dominant arrhythmogenic cardiomyopathy: an under-recognized clinical entity, J Am Coll Cardiol 52 (2008) 2175-87.

[8] Nava, A., B. Bauce, C. Basso, M. Muriago, A. Rampazzo, C. Villanova, L. Daliento, G. Buja, D. Corrado, G.A. Danieli, G. Thiene, Clinical profile and long-term follow-up of 37 families with arrhythmogenic right ventricular cardiomyopathy, J Am Coll Cardiol 36 (2000) 2226-33.

[9] Rigato, I., B. Bauce, A. Rampazzo, A. Zorzi, K. Pilichou, E. Mazzotti, F. Migliore, M.P. Marra, A. Lorenzon, M. De Bortoli, M. Calore, A. Nava, L. Daliento, D. Gregori, S. Iliceto, G. Thiene, C. Basso, D. Corrado, Compound and digenic heterozygosity predicts lifetime arrhythmic outcome and sudden cardiac death in desmosomal gene-related arrhythmogenic right ventricular cardiomyopathy, Circ Cardiovasc Genet 6 (2013) 533-42.

[10] Choudhary, N., C. Tompkins, B. Polonsky, S. McNitt, H. Calkins, N.A. Mark Estes, 3rd, A.D. Krahn, M.S. Link, F.I. Marcus, J.A. Towbin, W. Zareba, Clinical Presentation and Outcomes by Sex in Arrhythmogenic Right Ventricular Cardiomyopathy: Findings from the North American ARVC Registry, J Cardiovasc Electrophysiol 27 (2016) 555-62.

[11] James, C.A., A. Bhonsale, C. Tichnell, B. Murray, S.D. Russell, H. Tandri, R.J. Tedford, D.P. Judge, H. Calkins, Exercise increases age-related penetrance and arrhythmic risk in arrhythmogenic right ventricular dysplasia/cardiomyopathy-associated desmosomal mutation carriers, J Am Coll Cardiol 62 (2013) 1290-7.

[12] Uhl, H.S., A previously undescribed congenital malformation of the heart: almost total absence of the myocardium of the right ventricle, Bull Johns Hopkins Hosp 91 (1952) 197-209.

[13] Fontaine, G., R. Frank, G. Guiraudon, A. Pavie, Y. Tereau, G. Chomette, Y. Grosgogeat, [Significance of intraventricular conduction disorders observed in arrhythmogenic right ventricular dysplasia], Arch Mal Coeur Vaiss 77 (1984) 872-9.

[14] Corrado, D., C. Basso, G. Thiene, W.J. McKenna, M.J. Davies, F. Fontaliran, A. Nava, F. Silvestri, C. Blomstrom-Lundqvist, E.K. Wlodarska, G. Fontaine, F. Camerini, Spectrum of clinicopathologic

manifestations of arrhythmogenic right ventricular cardiomyopathy/dysplasia: a multicenter study, J Am Coll Cardiol 30 (1997) 1512-20.

[15] Zorzi, A., M. Perazzolo Marra, I. Rigato, M. De Lazzari, A. Susana, A. Niero, K. Pilichou, F. Migliore, S. Rizzo, B. Giorgi, G. De Conti, P. Sarto, L. Serratosa, G. Patrizi, E. De Maria, A. Pelliccia, C. Basso, M. Schiavon, B. Bauce, S. Iliceto, G. Thiene, D. Corrado, Nonischemic Left Ventricular Scar as a Substrate of Life-Threatening Ventricular Arrhythmias and Sudden Cardiac Death in Competitive Athletes, Circ Arrhythm Electrophysiol 9 (2016).

[16] di Gioia, C.R., C. Giordano, B. Cerbelli, A. Pisano, E. Perli, E. De Dominicis, B. Poscolieri, V. Palmieri, C. Ciallella, P. Zeppilli, G. d'Amati, Nonischemic left ventricular scar and cardiac sudden death in the young, Hum Pathol 58 (2016) 78-89.

[17] Basso, C., G. Thiene, Adipositas cordis, fatty infiltration of the right ventricle, and arrhythmogenic right ventricular cardiomyopathy. Just a matter of fat?, Cardiovasc Pathol 14 (2005) 37-41.

[18] Basso, C., M. Burke, P. Fornes, P.J. Gallagher, R.H. de Gouveia, M. Sheppard, G. Thiene, A. van der Wal, P. Association for European Cardiovascular, Guidelines for autopsy investigation of sudden cardiac death, Virchows Arch 452 (2008) 11-8.

[19] Marcus, F., C. Basso, K. Gear, V.L. Sorrell, Pitfalls in the diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia, Am J Cardiol 105 (2010) 1036-9.

[20] Marcus, F.I., W. Zareba, H. Calkins, J.A. Towbin, C. Basso, D.A. Bluemke, N.A. Estes, 3rd, M.H. Picard, D. Sanborn, G. Thiene, T. Wichter, D. Cannom, D.J. Wilber, M. Scheinman, H. Duff, J. Daubert, M. Talajic, A. Krahn, M. Sweeney, H. Garan, S. Sakaguchi, B.B. Lerman, C. Kerr, J. Kron, J.S. Steinberg, D. Sherrill, K. Gear, M. Brown, P. Severski, S. Polonsky, S. McNitt, Arrhythmogenic right ventricular cardiomyopathy/dysplasia clinical presentation and diagnostic evaluation: results from the North American Multidisciplinary Study, Heart Rhythm 6 (2009) 984-92.

[21] Zorzi, A., I. Rigato, K. Pilichou, M. Perazzolo Marra, F. Migliore, E. Mazzotti, D. Gregori, G. Thiene, L. Daliento, S. Iliceto, A. Rampazzo, C. Basso, B. Bauce, D. Corrado, Phenotypic expression is a prerequisite for malignant arrhythmic events and sudden cardiac death in arrhythmogenic right ventricular cardiomyopathy, Europace 18 (2016) 1086-94.

[22] Protonotarios, A., A. Anastasakis, D.B. Panagiotakos, L. Antoniades, P. Syrris, A. Vouliotis, C. Stefanadis, A. Tsatsopoulou, W.J. McKenna, N. Protonotarios, Arrhythmic risk assessment in genotyped families with arrhythmogenic right ventricular cardiomyopathy, Europace 18 (2016) 610-6.

[23] Groeneweg, J.A., A. Bhonsale, C.A. James, A.S. te Riele, D. Dooijes, C. Tichnell, B. Murray, A.C. Wiesfeld, A.C. Sawant, B. Kassamali, D.E. Atsma, P.G. Volders, N.M. de Groot, K. de Boer, S.L. Zimmerman, I.R. Kamel, J.F. van der Heijden, S.D. Russell, M. Jan Cramer, R.J. Tedford, P.A. Doevendans, T.A. van Veen, H. Tandri, A.A. Wilde, D.P. Judge, J.P. van Tintelen, R.N. Hauer, H. Calkins, Clinical Presentation, Long-Term Follow-Up, and Outcomes of 1001 Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Patients and Family Members, Circ Cardiovasc Genet 8 (2015) 437-46.

[24] Corrado, D., G. Thiene, A. Nava, L. Rossi, N. Pennelli, Sudden death in young competitive athletes: clinicopathologic correlations in 22 cases, Am J Med 89 (1990) 588-96.

[25] Corrado, D., C. Basso, G. Rizzoli, M. Schiavon, G. Thiene, Does sports activity enhance the risk of sudden death in adolescents and young adults?, J Am Coll Cardiol 42 (2003) 1959-63.

[26] Corrado, D., C. Basso, A. Pavei, P. Michieli, M. Schiavon, G. Thiene, Trends in sudden cardiovascular death in young competitive athletes after implementation of a preparticipation screening program, JAMA 296 (2006) 1593-601.

[27] Corrado, D., G. Fontaine, F.I. Marcus, W.J. McKenna, A. Nava, G. Thiene, T. Wichter, Arrhythmogenic right ventricular dysplasia/cardiomyopathy: need for an international registry. European Society of Cardiology and the Scientific Council on Cardiomyopathies of the World Heart Federation, J Cardiovasc Electrophysiol 11 (2000) 827-32.

[28] Thiene, G., D. Corrado, C. Basso, Arrhythmogenic right ventricular cardiomyopathy/dysplasia, Orphanet J Rare Dis 2 (2007) 45.

[29] McKenna, W.J., G. Thiene, A. Nava, F. Fontaliran, C. Blomstrom-Lundqvist, G. Fontaine, F. Camerini, Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology, Br Heart J 71 (1994) 215-8.

[30] Marcus, F.I., W.J. McKenna, D. Sherrill, C. Basso, B. Bauce, D.A. Bluemke, H. Calkins, D. Corrado, M.G. Cox, J.P. Daubert, G. Fontaine, K. Gear, R. Hauer, A. Nava, M.H. Picard, N. Protonotarios, J.E. Saffitz, D.M. Sanborn, J.S. Steinberg, H. Tandri, G. Thiene, J.A. Towbin, A. Tsatsopoulou, T. Wichter, W. Zareba, Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the Task Force Criteria, Eur Heart J 31 (2010) 806-14.

[31] Sen-Chowdhry, S., P. Syrris, D. Ward, A. Asimaki, E. Sevdalis, W.J. McKenna, Clinical and genetic characterization of families with arrhythmogenic right ventricular dysplasia/cardiomyopathy provides novel insights into patterns of disease expression, Circulation 115 (2007) 1710-20.

[32] Cox, M.G., M.R. Nelen, A.A. Wilde, A.C. Wiesfeld, J.J. van der Smagt, P. Loh, M.J. Cramer, P.A. Doevendans, J.P. van Tintelen, J.M. de Bakker, R.N. Hauer, Activation delay and VT parameters in arrhythmogenic right ventricular dysplasia/cardiomyopathy: toward improvement of diagnostic ECG criteria, J Cardiovasc Electrophysiol 19 (2008) 775-81.

[33] Platonov, P.G., H. Calkins, R.N. Hauer, D. Corrado, J.H. Svendsen, T. Wichter, E.K. Biernacka, A.M. Saguner, A.S. Te Riele, W. Zareba, High interobserver variability in the assessment of epsilon waves: Implications for diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia, Heart Rhythm 13 (2016) 208-16.

[34] Tandri, H., M. Saranathan, E.R. Rodriguez, C. Martinez, C. Bomma, K. Nasir, B. Rosen, J.A. Lima, H. Calkins, D.A. Bluemke, Noninvasive detection of myocardial fibrosis in arrhythmogenic right ventricular cardiomyopathy using delayed-enhancement magnetic resonance imaging, J Am Coll Cardiol 45 (2005) 98-103.

[35] Sen-Chowdhry, S., S.K. Prasad, P. Syrris, R. Wage, D. Ward, R. Merrifield, G.C. Smith, D.N. Firmin, D.J. Pennell, W.J. McKenna, Cardiovascular magnetic resonance in arrhythmogenic right ventricular cardiomyopathy revisited: comparison with task force criteria and genotype, J Am Coll Cardiol 48 (2006) 2132-40.

[36] Perazzolo Marra, M., G. Thiene, S. Rizzo, M. De Lazzari, E. Carturan, F. Tona, A.L. Caforio, L. Cacciavillani, R. Marcolongo, G. Tarantini, F. Corbetti, S. Iliceto, C. Basso, Cardiac magnetic resonance features of biopsy-proven endomyocardial diseases, JACC Cardiovasc Imaging 7 (2014) 309-12.

[37] Basso, C., F. Ronco, F. Marcus, A. Abudureheman, S. Rizzo, A.C. Frigo, B. Bauce, F. Maddalena, A. Nava, D. Corrado, F. Grigoletto, G. Thiene, Quantitative assessment of endomyocardial biopsy in arrhythmogenic right ventricular cardiomyopathy/dysplasia: an in vitro validation of diagnostic criteria, Eur Heart J 29 (2008) 2760-71.

[38] Migliore, F., A. Zorzi, M. Silvano, M. Bevilacqua, L. Leoni, M.P. Marra, M. Elmaghawry, L. Brugnaro, C. Dal Lin, B. Bauce, I. Rigato, G. Tarantini, C. Basso, G. Buja, G. Thiene, S. Iliceto, D. Corrado, Prognostic value of endocardial voltage mapping in patients with arrhythmogenic right ventricular cardiomyopathy/dysplasia, Circ Arrhythm Electrophysiol 6 (2013) 167-76.

[39] Denis, A., F. Sacher, N. Derval, H.S. Lim, H. Cochet, A.J. Shah, M. Daly, X. Pillois, K. Ramoul, Y. Komatsu, A. Zemmoura, S. Amraoui, P. Ritter, S. Ploux, P. Bordachar, M. Hocini, P. Jais, M. Haissaguerre, Diagnostic value of isoproterenol testing in arrhythmogenic right ventricular cardiomyopathy, Circ Arrhythm Electrophysiol 7 (2014) 590-7.

[40] Bauce, B., C. Basso, A. Rampazzo, G. Beffagna, L. Daliento, G. Frigo, S. Malacrida, L. Settimo, G. Danieli, G. Thiene, A. Nava, Clinical profile of four families with arrhythmogenic right ventricular cardiomyopathy caused by dominant desmoplakin mutations, Eur Heart J 26 (2005) 1666-75.

[41] Marra, M.P., L. Leoni, B. Bauce, F. Corbetti, A. Zorzi, F. Migliore, M. Silvano, I. Rigato, F. Tona, G. Tarantini, L. Cacciavillani, C. Basso, G. Buja, G. Thiene, S. Iliceto, D. Corrado, Imaging study of ventricular scar in arrhythmogenic right ventricular cardiomyopathy: comparison of 3D standard electroanatomical voltage mapping and contrast-enhanced cardiac magnetic resonance, Circ Arrhythm Electrophysiol 5 (2012) 91-100.

[42] Perazzolo Marra, M., S. Rizzo, B. Bauce, M. De Lazzari, K. Pilichou, D. Corrado, G. Thiene, S. Iliceto, C. Basso, Arrhythmogenic right ventricular cardiomyopathy. Contribution of cardiac magnetic resonance imaging to the diagnosis, Herz 40 (2015) 600-6.

[43] Corrado, D., T. Wichter, M.S. Link, R.N. Hauer, F.E. Marchlinski, A. Anastasakis, B. Bauce, C. Basso, C. Brunckhorst, A. Tsatsopoulou, H. Tandri, M. Paul, C. Schmied, A. Pelliccia, F. Duru, N. Protonotarios, N.M. Estes, 3rd, W.J. McKenna, G. Thiene, F.I. Marcus, H. Calkins, Treatment of Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia: An International Task Force Consensus Statement, Circulation 132 (2015) 441-53.

[44] Bauce, B., A. Nava, G. Beffagna, C. Basso, A. Lorenzon, G. Smaniotto, M. De Bortoli, I. Rigato, E. Mazzotti, A. Steriotis, M.P. Marra, J.A. Towbin, G. Thiene, G.A. Danieli, A. Rampazzo, Multiple mutations in desmosomal proteins encoding genes in arrhythmogenic right ventricular cardiomyopathy/dysplasia, Heart Rhythm 7 (2010) 22-9.

[45] Alcalai, R., S. Metzger, S. Rosenheck, V. Meiner, T. Chajek-Shaul, A recessive mutation in desmoplakin causes arrhythmogenic right ventricular dysplasia, skin disorder, and woolly hair, J Am Coll Cardiol 42 (2003) 319-27.

[46] Simpson, M.A., S. Mansour, D. Ahnood, K. Kalidas, M.A. Patton, W.J. McKenna, E.R. Behr, A.H. Crosby, Homozygous mutation of desmocollin-2 in arrhythmogenic right ventricular cardiomyopathy with mild palmoplantar keratoderma and woolly hair, Cardiology 113 (2009) 28-34.

[47] Cabral, R.M., L. Liu, C. Hogan, P.J. Dopping-Hepenstal, B.C. Winik, R.A. Asial, R. Dobson, C.A. Mein, P.A. Baselaga, J.E. Mellerio, A. Nanda, C. Boente Mdel, D.P. Kelsell, J.A. McGrath, A.P. South, Homozygous mutations in the 5' region of the JUP gene result in cutaneous disease but normal heart development in children, J Invest Dermatol 130 (2010) 1543-50.

[48] Gerull, B., F. Kirchner, J.X. Chong, J. Tagoe, K. Chandrasekharan, O. Strohm, D. Waggoner, C. Ober, H.J. Duff, Homozygous founder mutation in desmocollin-2 (DSC2) causes arrhythmogenic cardiomyopathy in the Hutterite population, Circ Cardiovasc Genet 6 (2013) 327-36.

[49] Al-Sabeq, B., A.D. Krahn, S. Conacher, G.J. Klein, Z. Laksman, Arrhythmogenic right ventricular cardiomyopathy with recessive inheritance related to a new homozygous desmocollin-2 mutation, Can J Cardiol 30 (2014) 696 e1-3.

[50] Lorenzon, A., K. Pilichou, I. Rigato, G. Vazza, M. De Bortoli, M. Calore, G. Occhi, E. Carturan, E. Lazzarini, M. Cason, E. Mazzotti, G. Poloni, M.L. Mostacciuolo, L. Daliento, G. Thiene, D. Corrado, C. Basso, B. Bauce, A. Rampazzo, Homozygous Desmocollin-2 Mutations and Arrhythmogenic Cardiomyopathy, Am J Cardiol 116 (2015) 1245-51.

[51] Qadri, S., O. Anttonen, J. Viikila, E.H. Seppala, S. Myllykangas, T.P. Alastalo, M. Holmstrom, T. Helio, J.W. Koskenvuo, Case reports of two pedigrees with recessive arrhythmogenic right ventricular cardiomyopathy associated with homozygous Thr335Ala variant in DSG2, BMC Med Genet 18 (2017) 86.

[52] Nava, A., G. Thiene, B. Canciani, R. Scognamiglio, L. Daliento, G. Buja, B. Martini, P. Stritoni, G. Fasoli, Familial occurrence of right ventricular dysplasia: a study involving nine families, J Am Coll Cardiol 12 (1988) 1222-8.

[53] McKoy, G., N. Protonotarios, A. Crosby, A. Tsatsopoulou, A. Anastasakis, A. Coonar, M. Norman, C. Baboonian, S. Jeffery, W.J. McKenna, Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease), Lancet 355 (2000) 2119-24.

[54] Rampazzo, A., A. Nava, S. Malacrida, G. Beffagna, B. Bauce, V. Rossi, R. Zimbello, B. Simionati, C. Basso, G. Thiene, J.A. Towbin, G.A. Danieli, Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy, Am J Hum Genet 71 (2002) 1200-6.

[55] Protonotarios, N., A. Tsatsopoulou, P. Patsourakos, D. Alexopoulos, P. Gezerlis, S. Simitsis, G. Scampardonis, Cardiac abnormalities in familial palmoplantar keratosis, Br Heart J 56 (1986) 321-6.

[56] Coonar, A.S., N. Protonotarios, A. Tsatsopoulou, E.W. Needham, R.S. Houlston, S. Cliff, M.I. Otter, V.A. Murday, R.K. Mattu, W.J. McKenna, Gene for arrhythmogenic right ventricular cardiomyopathy with diffuse nonepidermolytic palmoplantar keratoderma and woolly hair (Naxos disease) maps to 17q21, Circulation 97 (1998) 2049-58.

[57] Carvajal-Huerta, L., Epidermolytic palmoplantar keratoderma with woolly hair and dilated cardiomyopathy, J Am Acad Dermatol 39 (1998) 418-21.

[58] Norgett, E.E., S.J. Hatsell, L. Carvajal-Huerta, J.C. Cabezas, J. Common, P.E. Purkis, N. Whittock, I.M. Leigh, H.P. Stevens, D.P. Kelsell, Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma, Hum Mol Genet 9 (2000) 2761-6.

[59] Kaplan, S.R., J.J. Gard, L. Carvajal-Huerta, J.C. Ruiz-Cabezas, G. Thiene, J.E. Saffitz, Structural and molecular pathology of the heart in Carvajal syndrome, Cardiovasc Pathol 13 (2004) 26-32.

[60] Asimaki, A., P. Syrris, T. Wichter, P. Matthias, J.E. Saffitz, W.J. McKenna, A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy, Am J Hum Genet 81 (2007) 964-73.

[61] Gerull, B., A. Heuser, T. Wichter, M. Paul, C.T. Basson, D.A. McDermott, B.B. Lerman, S.M. Markowitz, P.T. Ellinor, C.A. MacRae, S. Peters, K.S. Grossmann, J. Drenckhahn, B. Michely, S. Sasse-Klaassen, W. Birchmeier, R. Dietz, G. Breithardt, E. Schulze-Bahr, L. Thierfelder, Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy, Nat Genet 36 (2004) 1162-4.

[62] Pilichou, K., A. Nava, C. Basso, G. Beffagna, B. Bauce, A. Lorenzon, G. Frigo, A. Vettori, M. Valente, J. Towbin, G. Thiene, G.A. Danieli, A. Rampazzo, Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy, Circulation 113 (2006) 1171-9.

[63] Syrris, P., D. Ward, A. Evans, A. Asimaki, E. Gandjbakhch, S. Sen-Chowdhry, W.J. McKenna, Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2, Am J Hum Genet 79 (2006) 978-84.

[64] Thiene, G., The research venture in arrhythmogenic right ventricular cardiomyopathy: a paradigm of translational medicine, Eur Heart J 36 (2015) 837-46.

[65] Tiso, N., D.A. Stephan, A. Nava, A. Bagattin, J.M. Devaney, F. Stanchi, G. Larderet, B. Brahmbhatt, K. Brown, B. Bauce, M. Muriago, C. Basso, G. Thiene, G.A. Danieli, A. Rampazzo, Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2), Hum Mol Genet 10 (2001) 189-94.

[66] Beffagna, G., G. Occhi, A. Nava, L. Vitiello, A. Ditadi, C. Basso, B. Bauce, G. Carraro, G. Thiene, J.A. Towbin, G.A. Danieli, A. Rampazzo, Regulatory mutations in transforming growth factor-beta3 gene cause arrhythmogenic right ventricular cardiomyopathy type 1, Cardiovasc Res 65 (2005) 366-73.

[67] Merner, N.D., K.A. Hodgkinson, A.F. Haywood, S. Connors, V.M. French, J.D. Drenckhahn, C. Kupprion, K. Ramadanova, L. Thierfelder, W. McKenna, B. Gallagher, L. Morris-Larkin, A.S. Bassett, P.S. Parfrey, T.L. Young, Arrhythmogenic right ventricular cardiomyopathy type 5 is a fully penetrant, lethal arrhythmic disorder caused by a missense mutation in the TMEM43 gene, Am J Hum Genet 82 (2008) 809-21.

[68] van Tintelen, J.P., I.C. Van Gelder, A. Asimaki, A.J. Suurmeijer, A.C. Wiesfeld, J.D. Jongbloed, A. van den Wijngaard, J.B. Kuks, K.Y. van Spaendonck-Zwarts, N. Notermans, L. Boven, F. van den Heuvel, H.E. Veenstra-Knol, J.E. Saffitz, R.M. Hofstra, M.P. van den Berg, Severe cardiac phenotype with right ventricular predominance in a large cohort of patients with a single missense mutation in the DES gene, Heart Rhythm 6 (2009) 1574-83.

[69] Taylor, M., S. Graw, G. Sinagra, C. Barnes, D. Slavov, F. Brun, B. Pinamonti, E.E. Salcedo, W. Sauer, S. Pyxaras, B. Anderson, B. Simon, J. Bogomolovas, S. Labeit, H. Granzier, L. Mestroni, Genetic variation in titin in arrhythmogenic right ventricular cardiomyopathy-overlap syndromes, Circulation 124 (2011) 876-85.

[70] Quarta, G., P. Syrris, M. Ashworth, S. Jenkins, K. Zuborne Alapi, J. Morgan, A. Muir, A. Pantazis, W.J. McKenna, P.M. Elliott, Mutations in the Lamin A/C gene mimic arrhythmogenic right ventricular cardiomyopathy, Eur Heart J 33 (2012) 1128-36.

[71] van der Zwaag, P.A., I.A. van Rijsingen, A. Asimaki, J.D. Jongbloed, D.J. van Veldhuisen, A.C. Wiesfeld, M.G. Cox, L.T. van Lochem, R.A. de Boer, R.M. Hofstra, I. Christiaans, K.Y. van

Spaendonck-Zwarts, R.H. Lekanne dit Deprez, D.P. Judge, H. Calkins, A.J. Suurmeijer, R.N. Hauer, J.E. Saffitz, A.A. Wilde, M.P. van den Berg, J.P. van Tintelen, Phospholamban R14del mutation in patients diagnosed with dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy: evidence supporting the concept of arrhythmogenic cardiomyopathy, Eur J Heart Fail 14 (2012) 1199-207.

[72] van Hengel, J., M. Calore, B. Bauce, E. Dazzo, E. Mazzotti, M. De Bortoli, A. Lorenzon, I.E. Li Mura, G. Beffagna, I. Rigato, M. Vleeschouwers, K. Tyberghein, P. Hulpiau, E. van Hamme, T. Zaglia, D. Corrado, C. Basso, G. Thiene, L. Daliento, A. Nava, F. van Roy, A. Rampazzo, Mutations in the area composita protein alphaT-catenin are associated with arrhythmogenic right ventricular cardiomyopathy, Eur Heart J 34 (2013) 201-10.

[73] Ortiz-Genga, M.F., S. Cuenca, M. Dal Ferro, E. Zorio, R. Salgado-Aranda, V. Climent, L. Padron-Barthe, I. Duro-Aguado, J. Jimenez-Jaimez, V.M. Hidalgo-Olivares, E. Garcia-Campo, C. Lanzillo, M.P. Suarez-Mier, H. Yonath, S. Marcos-Alonso, J.P. Ochoa, J.L. Santome, D. Garcia-Giustiniani, J.L. Rodriguez-Garrido, F. Dominguez, M. Merlo, J. Palomino, M.L. Pena, J.P. Trujillo, A. Martin-Vila, D. Stolfo, P. Molina, E. Lara-Pezzi, F.E. Calvo-Iglesias, E. Nof, L. Calo, R. Barriales-Villa, J.R. Gimeno-Blanes, M. Arad, P. Garcia-Pavia, L. Monserrat, Truncating FLNC Mutations Are Associated With High-Risk Dilated and Arrhythmogenic Cardiomyopathies, J Am Coll Cardiol 68 (2016) 2440-51.

[74] Mayosi, B.M., M. Fish, G. Shaboodien, E. Mastantuono, S. Kraus, T. Wieland, M.C. Kotta, A. Chin, N. Laing, N.B. Ntusi, M. Chong, C. Horsfall, S.N. Pimstone, D. Gentilini, G. Parati, T.M. Strom, T. Meitinger, G. Pare, P.J. Schwartz, L. Crotti, Identification of Cadherin 2 (CDH2) Mutations in Arrhythmogenic Right Ventricular Cardiomyopathy, Circ Cardiovasc Genet 10 (2017).

[75] Cox, M.G., P.A. van der Zwaag, C. van der Werf, J.J. van der Smagt, M. Noorman, Z.A. Bhuiyan, A.C. Wiesfeld, P.G. Volders, I.M. van Langen, D.E. Atsma, D. Dooijes, A. van den Wijngaard, A.C. Houweling, J.D. Jongbloed, L. Jordaens, M.J. Cramer, P.A. Doevendans, J.M. de Bakker, A.A. Wilde, J.P. van Tintelen, R.N. Hauer, Arrhythmogenic right ventricular dysplasia/cardiomyopathy: pathogenic desmosome mutations in index-patients predict outcome of family screening: Dutch arrhythmogenic right ventricular dysplasia/cardiomyopathy genotype-phenotype follow-up study, Circulation 123 (2011) 2690-700.

[76] Li Mura, I.E., B. Bauce, A. Nava, M. Fanciulli, G. Vazza, E. Mazzotti, I. Rigato, M. De Bortoli, G. Beffagna, A. Lorenzon, M. Calore, E. Dazzo, C. Nobile, M.L. Mostacciuolo, D. Corrado, C. Basso, L. Daliento, G. Thiene, A. Rampazzo, Identification of a PKP2 gene deletion in a family with arrhythmogenic right ventricular cardiomyopathy, Eur J Hum Genet 21 (2013) 1226-31.

[77] Sonoda, K., S. Ohno, S. Otuki, K. Kato, N. Yagihara, H. Watanabe, T. Makiyama, T. Minamino, M. Horie, Quantitative analysis of PKP2 and neighbouring genes in a patient with arrhythmogenic right ventricular cardiomyopathy caused by heterozygous PKP2 deletion, Europace 19 (2017) 644-50.

[78] Roberts, J.D., J.C. Herkert, J. Rutberg, S.M. Nikkel, A.C. Wiesfeld, D. Dooijes, R.M. Gow, J.P. van Tintelen, M.H. Gollob, Detection of genomic deletions of PKP2 in arrhythmogenic right ventricular cardiomyopathy, Clin Genet 83 (2013) 452-6.

[79] Pilichou, K., E. Lazzarini, I. Rigato, R. Celeghin, M. De Bortoli, M. Perazzolo Marra, M. Cason, J. Jongbloed, M. Calore, S. Rizzo, D. Regazzo, G. Poloni, S. Iliceto, L. Daliento, P. Delise, D. Corrado, J.P. Van Tintelen, G. Thiene, A. Rampazzo, C. Basso, B. Bauce, A. Lorenzon, G. Occhi, Large Genomic Rearrangements of Desmosomal Genes in Italian Arrhythmogenic Cardiomyopathy Patients, Circ Arrhythm Electrophysiol 10 (2017).

[80] Merritt, A.J., M.Y. Berika, W. Zhai, S.E. Kirk, B. Ji, M.J. Hardman, D.R. Garrod, Suprabasal desmoglein 3 expression in the epidermis of transgenic mice results in hyperproliferation and abnormal differentiation, Mol Cell Biol 22 (2002) 5846-58.

[81] North, A.J., W.G. Bardsley, J. Hyam, E.A. Bornslaeger, H.C. Cordingley, B. Trinnaman, M. Hatzfeld, K.J. Green, A.I. Magee, D.R. Garrod, Molecular map of the desmosomal plaque, J Cell Sci 112 (Pt 23) (1999) 4325-36.

[82] Leung, C.L., K.J. Green, R.K. Liem, Plakins: a family of versatile cytolinker proteins, Trends Cell Biol 12 (2002) 37-45.

[83] Armstrong, D.K., K.E. McKenna, P.E. Purkis, K.J. Green, R.A. Eady, I.M. Leigh, A.E. Hughes, Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma, Hum Mol Genet 8 (1999) 143-8.

[84] Fressart, V., G. Duthoit, E. Donal, V. Probst, J.C. Deharo, P. Chevalier, D. Klug, O. Dubourg, E. Delacretaz, P. Cosnay, P. Scanu, F. Extramiana, D. Keller, F. Hidden-Lucet, F. Simon, V. Bessirard, N. Roux-Buisson, J.L. Hebert, A. Azarine, D. Casset-Senon, F. Rouzet, Y. Lecarpentier, G. Fontaine, C. Coirault, R. Frank, B. Hainque, P. Charron, Desmosomal gene analysis in arrhythmogenic right ventricular dysplasia/cardiomyopathy: spectrum of mutations and clinical impact in practice, Europace 12 (2010) 861-8.

[85] Chen, X., S. Bonne, M. Hatzfeld, F. van Roy, K.J. Green, Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and beta -catenin signaling, J Biol Chem 277 (2002) 10512-22.

[86] Gandjbakhch, E., P. Charron, V. Fressart, G. Lorin de la Grandmaison, F. Simon, F. Gary, A. Vite, B. Hainque, F. Hidden-Lucet, M. Komajda, E. Villard, Plakophilin 2A is the dominant isoform in human heart tissue: consequences for the genetic screening of arrhythmogenic right ventricular cardiomyopathy, Heart 97 (2011) 844-9.

[87] Grossmann, K.S., C. Grund, J. Huelsken, M. Behrend, B. Erdmann, W.W. Franke, W. Birchmeier, Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation, J Cell Biol 167 (2004) 149-60.

[88] Green, K.J., C.L. Simpson, Desmosomes: new perspectives on a classic, J Invest Dermatol 127 (2007) 2499-515.

[89] Schafer, S., P.J. Koch, W.W. Franke, Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins, Exp Cell Res 211 (1994) 391-9.

[90] Nuber, U.A., S. Schafer, A. Schmidt, P.J. Koch, W.W. Franke, The widespread human desmocollin Dsc2 and tissue-specific patterns of synthesis of various desmocollin subtypes, Eur J Cell Biol 66 (1995) 69-74.

[91] Heuser, A., E.R. Plovie, P.T. Ellinor, K.S. Grossmann, J.T. Shin, T. Wichter, C.T. Basson, B.B. Lerman, S. Sasse-Klaassen, L. Thierfelder, C.A. MacRae, B. Gerull, Mutant desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy, Am J Hum Genet 79 (2006) 1081-8.

[92] Beffagna, G., M. De Bortoli, A. Nava, M. Salamon, A. Lorenzon, M. Zaccolo, L. Mancuso, L. Sigalotti, B. Bauce, G. Occhi, C. Basso, G. Lanfranchi, J.A. Towbin, G. Thiene, G.A. Danieli, A. Rampazzo, Missense mutations in desmocollin-2 N-terminus, associated with arrhythmogenic right

ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro, BMC Med Genet 8 (2007) 65.

[93] Haywood, A.F., N.D. Merner, K.A. Hodgkinson, J. Houston, P. Syrris, V. Booth, S. Connors, A. Pantazis, G. Quarta, P. Elliott, W. McKenna, T.L. Young, Recurrent missense mutations in TMEM43 (ARVD5) due to founder effects cause arrhythmogenic cardiomyopathies in the UK and Canada, Eur Heart J 34 (2013) 1002-11.

[94] Otten, E., A. Asimaki, A. Maass, I.M. van Langen, A. van der Wal, N. de Jonge, M.P. van den Berg, J.E. Saffitz, A.A. Wilde, J.D. Jongbloed, J.P. van Tintelen, Desmin mutations as a cause of right ventricular heart failure affect the intercalated disks, Heart Rhythm 7 (2010) 1058-64.

[95] Valtuille, L., I. Paterson, D.H. Kim, J. Mullen, C. Sergi, G.Y. Oudit, A case of lamin A/C mutation cardiomyopathy with overlap features of ARVC: a critical role of genetic testing, Int J Cardiol 168 (2013) 4325-7.

[96] Erkapic, D., T. Neumann, J. Schmitt, J. Sperzel, A. Berkowitsch, M. Kuniss, C.W. Hamm, H.F. Pitschner, Electrical storm in a patient with arrhythmogenic right ventricular cardiomyopathy and SCN5A mutation, Europace 10 (2008) 884-7.

[97] Te Riele, A.S., E. Agullo-Pascual, C.A. James, A. Leo-Macias, M. Cerrone, M. Zhang, X. Lin, B. Lin, N.L. Sobreira, N. Amat-Alarcon, R.F. Marsman, B. Murray, C. Tichnell, J.F. van der Heijden, D. Dooijes, T.A. van Veen, H. Tandri, S.J. Fowler, R.N. Hauer, G. Tomaselli, M.P. van den Berg, M.R. Taylor, F. Brun, G. Sinagra, A.A. Wilde, L. Mestroni, C.R. Bezzina, H. Calkins, J. Peter van Tintelen, L. Bu, M. Delmar, D.P. Judge, Multilevel analyses of SCN5A mutations in arrhythmogenic right ventricular dysplasia/cardiomyopathy suggest non-canonical mechanisms for disease pathogenesis, Cardiovasc Res 113 (2017) 102-11.

[98] Turkowski, K.L., D.J. Tester, J.M. Bos, K.H. Haugaa, M.J. Ackerman, Whole exome sequencing with genomic triangulation implicates CDH2-encoded N-cadherin as a novel pathogenic substrate for arrhythmogenic cardiomyopathy, Congenit Heart Dis 12 (2017) 226-35.

[99] Dokuparti, M.V., P.R. Pamuru, B. Thakkar, R.R. Tanjore, P. Nallari, Etiopathogenesis of arrhythmogenic right ventricular cardiomyopathy, J Hum Genet 50 (2005) 375-81.

[100] Daliento, L., P. Turrini, A. Nava, G. Rizzoli, A. Angelini, G. Buja, R. Scognamiglio, G. Thiene, Arrhythmogenic right ventricular cardiomyopathy in young versus adult patients: similarities and differences, J Am Coll Cardiol 25 (1995) 655-64.

[101] Mallat, Z., A. Tedgui, F. Fontaliran, R. Frank, M. Durigon, G. Fontaine, Evidence of apoptosis in arrhythmogenic right ventricular dysplasia, N Engl J Med 335 (1996) 1190-6.

[102] Valente, M., F. Calabrese, G. Thiene, A. Angelini, C. Basso, A. Nava, L. Rossi, In vivo evidence of apoptosis in arrhythmogenic right ventricular cardiomyopathy, Am J Pathol 152 (1998) 479-84.

[103] Pilichou, K., C.A. Remme, C. Basso, M.E. Campian, S. Rizzo, P. Barnett, B.P. Scicluna, B. Bauce, M.J. van den Hoff, J.M. de Bakker, H.L. Tan, M. Valente, A. Nava, A.A. Wilde, A.F. Moorman, G. Thiene, C.R. Bezzina, Myocyte necrosis underlies progressive myocardial dystrophy in mouse dsg2-related arrhythmogenic right ventricular cardiomyopathy, J Exp Med 206 (2009) 1787-802.

[104] Rizzo, S., E.M. Lodder, A.O. Verkerk, R. Wolswinkel, L. Beekman, K. Pilichou, C. Basso, C.A. Remme, G. Thiene, C.R. Bezzina, Intercalated disc abnormalities, reduced Na(+) current density, and

conduction slowing in desmoglein-2 mutant mice prior to cardiomyopathic changes, Cardiovasc Res 95 (2012) 409-18.

[105] Bowles, N.E., J. Ni, F. Marcus, J.A. Towbin, The detection of cardiotropic viruses in the myocardium of patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy, J Am Coll Cardiol 39 (2002) 892-5.

[106] Thiene, G., D. Corrado, A. Nava, L. Rossi, A. Poletti, G.M. Boffa, L. Daliento, N. Pennelli, Right ventricular cardiomyopathy: is there evidence of an inflammatory aetiology?, Eur Heart J 12 Suppl D (1991) 22-5.

[107] Calabrese, F., C. Basso, E. Carturan, M. Valente, G. Thiene, Arrhythmogenic right ventricular cardiomyopathy/dysplasia: is there a role for viruses?, Cardiovasc Pathol 15 (2006) 11-7.

[108] d'Amati, G., C.R. di Gioia, C. Giordano, P. Gallo, Myocyte transdifferentiation: a possible pathogenetic mechanism for arrhythmogenic right ventricular cardiomyopathy, Arch Pathol Lab Med 124 (2000) 287-90.

[109] Lombardi, R., J. Dong, G. Rodriguez, A. Bell, T.K. Leung, R.J. Schwartz, J.T. Willerson, R. Brugada, A.J. Marian, Genetic fate mapping identifies second heart field progenitor cells as a source of adipocytes in arrhythmogenic right ventricular cardiomyopathy, Circ Res 104 (2009) 1076-84.

[110] Sato, P.Y., W. Coombs, X. Lin, O. Nekrasova, K.J. Green, L.L. Isom, S.M. Taffet, M. Delmar, Interactions between ankyrin-G, Plakophilin-2, and Connexin43 at the cardiac intercalated disc, Circ Res 109 (2011) 193-201.

[111] Hariharan, V., A. Asimaki, J.E. Michaelson, E. Plovie, C.A. MacRae, J.E. Saffitz, H. Huang, Arrhythmogenic right ventricular cardiomyopathy mutations alter shear response without changes in cell-cell adhesion, Cardiovasc Res 104 (2014) 280-9.

[112] Asimaki, A., H. Tandri, H. Huang, M.K. Halushka, S. Gautam, C. Basso, G. Thiene, A. Tsatsopoulou, N. Protonotarios, W.J. McKenna, H. Calkins, J.E. Saffitz, A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy, N Engl J Med 360 (2009) 1075-84.

[113] Garcia-Gras, E., R. Lombardi, M.J. Giocondo, J.T. Willerson, M.D. Schneider, D.S. Khoury, A.J. Marian, Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy, J Clin Invest 116 (2006) 2012-21.

[114] Swope, D., J. Li, E.J. Muller, G.L. Radice, Analysis of a Jup hypomorphic allele reveals a critical threshold for postnatal viability, Genesis 50 (2012) 717-27.

[115] Lombardi, R., M. da Graca Cabreira-Hansen, A. Bell, R.R. Fromm, J.T. Willerson, A.J. Marian, Nuclear plakoglobin is essential for differentiation of cardiac progenitor cells to adipocytes in arrhythmogenic right ventricular cardiomyopathy, Circ Res 109 (2011) 1342-53.

[116] Chopra, A., E. Tabdanov, H. Patel, P.A. Janmey, J.Y. Kresh, Cardiac myocyte remodeling mediated by N-cadherin-dependent mechanosensing, Am J Physiol Heart Circ Physiol 300 (2011) H1252-66.

[117] Zhou, L., C.O. Hanemann, Merlin, a multi-suppressor from cell membrane to the nucleus, FEBS Lett 586 (2012) 1403-8.

[118] Yu, F.X., K.L. Guan, The Hippo pathway: regulators and regulations, Genes Dev 27 (2013) 355-71.

[119] Chen, S.N., P. Gurha, R. Lombardi, A. Ruggiero, J.T. Willerson, A.J. Marian, The hippo pathway is activated and is a causal mechanism for adipogenesis in arrhythmogenic cardiomyopathy, Circ Res 114 (2014) 454-68.

[120] Kim, C., J. Wong, J. Wen, S. Wang, C. Wang, S. Spiering, N.G. Kan, S. Forcales, P.L. Puri, T.C. Leone, J.E. Marine, H. Calkins, D.P. Kelly, D.P. Judge, H.S. Chen, Studying arrhythmogenic right ventricular dysplasia with patient-specific iPSCs, Nature 494 (2013) 105-10.

[121] Wen, J.Y., C.Y. Wei, K. Shah, J. Wong, C. Wang, H.S. Chen, Maturation-Based Model of Arrhythmogenic Right Ventricular Dysplasia Using Patient-Specific Induced Pluripotent Stem Cells, Circ J 79 (2015) 1402-8.

[122] Sommariva, E., S. Brambilla, C. Carbucicchio, E. Gambini, V. Meraviglia, A. Dello Russo, F.M. Farina, M. Casella, V. Catto, G. Pontone, M. Chiesa, I. Stadiotti, E. Cogliati, A. Paolin, N. Ouali Alami, C. Preziuso, G. d'Amati, G.I. Colombo, A. Rossini, M.C. Capogrossi, C. Tondo, G. Pompilio, Cardiac mesenchymal stromal cells are a source of adipocytes in arrhythmogenic cardiomyopathy, Eur Heart J 37 (2016) 1835-46.

[123] Cerrone, M., M. Noorman, X. Lin, H. Chkourko, F.X. Liang, R. van der Nagel, T. Hund, W. Birchmeier, P. Mohler, T.A. van Veen, H.V. van Rijen, M. Delmar, Sodium current deficit and arrhythmogenesis in a murine model of plakophilin-2 haploinsufficiency, Cardiovasc Res 95 (2012) 460-8.

[124] Zhang, Q., C. Deng, F. Rao, R.M. Modi, J. Zhu, X. Liu, L. Mai, H. Tan, X. Yu, Q. Lin, D. Xiao, S. Kuang, S. Wu, Silencing of desmoplakin decreases connexin43/Nav1.5 expression and sodium current in HL1 cardiomyocytes, Mol Med Rep 8 (2013) 780-6.

[125] Noorman, M., S. Hakim, A. Asimaki, A. Vreeker, H.V. van Rijen, M.A. van der Heyden, N. de Jonge, R.A. de Weger, R.N. Hauer, J.E. Saffitz, T.A. van Veen, Reduced plakoglobin immunoreactivity in arrhythmogenic cardiomyopathy: methodological considerations, Cardiovasc Pathol 22 (2013) 314-8.

[126] Asimaki, A., S. Kapoor, E. Plovie, A. Karin Arndt, E. Adams, Z. Liu, C.A. James, D.P. Judge, H. Calkins, J. Churko, J.C. Wu, C.A. MacRae, A.G. Kleber, J.E. Saffitz, Identification of a new modulator of the intercalated disc in a zebrafish model of arrhythmogenic cardiomyopathy, Sci Transl Med 6 (2014) 240ra74.

[127] Sanger, F., S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc Natl Acad Sci U S A 74 (1977) 5463-7.

[128] Fischer, S.G., L.S. Lerman, DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory, Proc Natl Acad Sci U S A 80 (1983) 1579-83.

[129] Sidransky, D., Nucleic acid-based methods for the detection of cancer, Science 278 (1997) 1054-9.

[130] Oefner, P.J., P.A. Underhill, Comparative DNA-Sequencing by Denaturing High-Performance Liquid-Chromatography (Dhplc), American Journal of Human Genetics 57 (1995) 1547-.

[131] Lander, E.S., L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, Y. Stange-Thomann, N. Stojanovic, A.

Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A. Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, R.A. Gibbs, D.M. Muzny, S.E. Scherer, J.B. Bouck, E.J. Sodergren, K.C. Worley, C.M. Rives, J.H. Gorrell, M.L. Metzker, S.L. Naylor, R.S. Kucherlapati, D.L. Nelson, G.M. Weinstock, Y. Sakaki, A. Fujiyama, M. Hattori, T. Yada, A. Toyoda, T. Itoh, C. Kawagoe, H. Watanabe, Y. Totoki, T. Taylor, J. Weissenbach, R. Heilig, W. Saurin, F. Artiguenave, P. Brottier, T. Bruls, E. Pelletier, C. Robert, P. Wincker, D.R. Smith, L. Doucette-Stamm, M. Rubenfield, K. Weinstock, H.M. Lee, J. Dubois, A. Rosenthal, M. Platzer, G. Nyakatura, S. Taudien, A. Rump, H. Yang, J. Yu, J. Wang, G. Huang, J. Gu, L. Hood, L. Rowen, A. Madan, S. Qin, R.W. Davis, N.A. Federspiel, A.P. Abola, M.J. Proctor, R.M. Myers, J. Schmutz, M. Dickson, J. Grimwood, D.R. Cox, M.V. Olson, R. Kaul, C. Raymond, N. Shimizu, K. Kawasaki, S. Minoshima, G.A. Evans, M. Athanasiou, R. Schultz, B.A. Roe, F. Chen, H. Pan, J. Ramser, H. Lehrach, R. Reinhardt, W.R. McCombie, M. de la Bastide, N. Dedhia, H. Blocker, K. Hornischer, G. Nordsiek, R. Agarwala, L. Aravind, J.A. Bailey, A. Bateman, S. Batzoglou, E. Birney, P. Bork, D.G. Brown, C.B. Burge, L. Cerutti, H.C. Chen, D. Church, M. Clamp, R.R. Copley, T. Doerks, S.R. Eddy, E.E. Eichler, T.S. Furey, J. Galagan, J.G. Gilbert, C. Harmon, Y. Hayashizaki, D. Haussler, H. Hermjakob, K. Hokamp, W. Jang, L.S. Johnson, T.A. Jones, S. Kasif, A. Kaspryzk, S. Kennedy, W.J. Kent, P. Kitts, E.V. Koonin, I. Korf, D. Kulp, D. Lancet, T.M. Lowe, A. McLysaght, T. Mikkelsen, J.V. Moran, N. Mulder, V.J. Pollara, C.P. Ponting, G. Schuler, J. Schultz, G. Slater, A.F. Smit, E. Stupka, J. Szustakowki, D. Thierry-Mieg, J. Thierry-Mieg, L. Wagner, J. Wallis, R. Wheeler, A. Williams, Y.I. Wolf, K.H. Wolfe, S.P. Yang, R.F. Yeh, F. Collins, M.S. Guyer, J. Peterson, A. Felsenfeld, K.A. Wetterstrand, A. Patrinos, M.J. Morgan, P. de Jong, J.J. Catanese, K. Osoegawa, H. Shizuya, S. Choi, Y.J. Chen, J. Szustakowki, C. International Human Genome Sequencing, Initial sequencing and analysis of the human genome, Nature 409 (2001) 860-921.

[132] Venter, J.C., M.D. Adams, E.W. Myers, P.W. Li, R.J. Mural, G.G. Sutton, H.O. Smith, M. Yandell, C.A. Evans, R.A. Holt, J.D. Gocayne, P. Amanatides, R.M. Ballew, D.H. Huson, J.R. Wortman, Q. Zhang, C.D. Kodira, X.H. Zheng, L. Chen, M. Skupski, G. Subramanian, P.D. Thomas, J. Zhang, G.L. Gabor Miklos, C. Nelson, S. Broder, A.G. Clark, J. Nadeau, V.A. McKusick, N. Zinder, A.J. Levine, R.J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A.E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R.R. Ji, Z. Ke, K.A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G.V. Merkulov, N. Milshina, H.M. Moore, A.K. Naik, V.A. Narayan, B. Neelam, D. Nusskern, D.B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M.L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J.

Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y.H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N.N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J.F. Abril, R. Guigo, M.J. Campbell, K.V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y.H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh, X. Zhu, The sequence of the human genome, Science 291 (2001) 1304-51.

[133] Metzker, M.L., Sequencing technologies - the next generation, Nat Rev Genet 11 (2010) 31-46.

[134] Sobreira, N.L., E.T. Cirulli, D. Avramopoulos, E. Wohler, G.L. Oswald, E.L. Stevens, D. Ge, K.V. Shianna, J.P. Smith, J.M. Maia, C.E. Gumbs, J. Pevsner, G. Thomas, D. Valle, J.E. Hoover-Fong, D.B. Goldstein, Whole-genome sequencing of a single proband together with linkage analysis identifies a Mendelian disease gene, PLoS Genet 6 (2010) e1000991.

[135] Yang, Y., D.M. Muzny, J.G. Reid, M.N. Bainbridge, A. Willis, P.A. Ward, A. Braxton, J. Beuten, F. Xia, Z. Niu, M. Hardison, R. Person, M.R. Bekheirnia, M.S. Leduc, A. Kirby, P. Pham, J. Scull, M. Wang, Y. Ding, S.E. Plon, J.R. Lupski, A.L. Beaudet, R.A. Gibbs, C.M. Eng, Clinical whole-exome sequencing for the diagnosis of mendelian disorders, N Engl J Med 369 (2013) 1502-11.

[136] Krawitz, P.M., D. Schiska, U. Kruger, S. Appelt, V. Heinrich, D. Parkhomchuk, B. Timmermann, J.M. Millan, P.N. Robinson, S. Mundlos, J. Hecht, M. Gross, Screening for single nucleotide variants, small indels and exon deletions with a next-generation sequencing based gene panel approach for Usher syndrome, Mol Genet Genomic Med 2 (2014) 393-401.

[137] Nijman, I.J., J.M. van Montfrans, M. Hoogstraat, M.L. Boes, L. van de Corput, E.D. Renner, P. van Zon, S. van Lieshout, M.G. Elferink, M. van der Burg, C.L. Vermont, B. van der Zwaag, E. Janson, E. Cuppen, J.K. Ploos van Amstel, M.E. van Gijn, Targeted next-generation sequencing: a novel diagnostic tool for primary immunodeficiencies, J Allergy Clin Immunol 133 (2014) 529-34.

[138] Mwenifumbo, J.C., M.A. Marra, Cancer genome-sequencing study design, Nat Rev Genet 14 (2013) 321-32.

[139] Kougioumtzi, A., P. Tsaparas, A. Magklara, Deep sequencing reveals new aspects of progesterone receptor signaling in breast cancer cells, PLoS One 9 (2014) e98404.

[140] Della Mina, E., R. Ciccone, F. Brustia, B. Bayindir, I. Limongelli, A. Vetro, M. Iascone, L. Pezzoli, R. Bellazzi, G. Perotti, V. De Giorgis, S. Lunghi, G. Coppola, S. Orcesi, P. Merli, S. Savasta, P. Veggiotti, O. Zuffardi, Improving molecular diagnosis in epilepsy by a dedicated high-throughput sequencing platform, Eur J Hum Genet 23 (2015) 354-62.

[141] Zhao, W., Q.Y. Zhu, J.T. Zhang, H. Liu, L.J. Wang, Z.Q. Chen, L.P. Guan, X.S. Huang, L. Yang, S.Y. Yu, Exome sequencing identifies novel compound heterozygous mutations in SPG11 that cause autosomal recessive hereditary spastic paraplegia, J Neurol Sci 335 (2013) 112-7.

[142] Iqbal, Z., K. Neveling, A. Razzaq, M. Shahzad, M.Y. Zahoor, M. Qasim, C. Gilissen, N. Wieskamp, M.P. Kwint, S. Gijsen, A.P. de Brouwer, J.A. Veltman, S. Riazuddin, H. van Bokhoven, Targeted next generation sequencing reveals a novel intragenic deletion of the TPO gene in a family with intellectual disability, Arch Med Res 43 (2012) 312-6.

[143] Tsurusaki, Y., H. Osaka, H. Hamanoue, H. Shimbo, M. Tsuji, H. Doi, H. Saitsu, N. Matsumoto, N. Miyake, Rapid detection of a mutation causing X-linked leucoencephalopathy by exome sequencing, J Med Genet 48 (2011) 606-9.

[144] Dames, S., L.S. Chou, Y. Xiao, T. Wayman, J. Stocks, M. Singleton, K. Eilbeck, R. Mao, The development of next-generation sequencing assays for the mitochondrial genome and 108 nuclear genes associated with mitochondrial disorders, J Mol Diagn 15 (2013) 526-34.

[145] Tang, S., J. Wang, V.W. Zhang, F.Y. Li, M. Landsverk, H. Cui, C.K. Truong, G. Wang, L.C. Chen, B. Graham, F. Scaglia, E.S. Schmitt, W.J. Craigen, L.J. Wong, Transition to next generation analysis of the whole mitochondrial genome: a summary of molecular defects, Hum Mutat 34 (2013) 882-93.

[146] Mook, O.R., M.A. Haagmans, J.F. Soucy, J.B. van de Meerakker, F. Baas, M.E. Jakobs, N. Hofman, I. Christiaans, R.H. Lekanne Deprez, M.M. Mannens, Targeted sequence capture and GS-FLX Titanium sequencing of 23 hypertrophic and dilated cardiomyopathy genes: implementation into diagnostics, J Med Genet 50 (2013) 614-26.

[147] Dorn, C., M. Grunert, S.R. Sperling, Application of high-throughput sequencing for studying genomic variations in congenital heart disease, Brief Funct Genomics 13 (2014) 51-65.

[148] Posch, M.G., M.J. Posch, C. Geier, B. Erdmann, W. Mueller, A. Richter, V. Ruppert, S. Pankuweit, B. Maisch, A. Perrot, J. Buttgereit, R. Dietz, W. Haverkamp, C. Ozcelik, A missense variant in desmoglein-2 predisposes to dilated cardiomyopathy, Mol Genet Metab 95 (2008) 74-80.

[149] Elliott, P., C. O'Mahony, P. Syrris, A. Evans, C. Rivera Sorensen, M.N. Sheppard, G. Carr-White, A. Pantazis, W.J. McKenna, Prevalence of desmosomal protein gene mutations in patients with dilated cardiomyopathy, Circ Cardiovasc Genet 3 (2010) 314-22.

[150] Zhang, M., F. Tavora, A. Burke, Desmosomal protein gene mutations in patients with idiopathic DCM, Heart 97 (2011) 2090.

[151] Garcia-Pavia, P., P. Syrris, C. Salas, A. Evans, J.G. Mirelis, M. Cobo-Marcos, C. Vilches, B. Bornstein, J. Segovia, L. Alonso-Pulpon, P.M. Elliott, Desmosomal protein gene mutations in patients with idiopathic dilated cardiomyopathy undergoing cardiac transplantation: a clinicopathological study, Heart 97 (2011) 1744-52.

[152] Garcia-Pavia, P., M. Cobo-Marcos, G. Guzzo-Merello, M. Gomez-Bueno, B. Bornstein, E. Lara-Pezzi, J. Segovia, L. Alonso-Pulpon, Genetics in dilated cardiomyopathy, Biomark Med 7 (2013) 517-33.

[153] Biswas, A., V.R. Rao, S. Seth, S.K. Maulik, Next generation sequencing in cardiomyopathy: towards personalized genomics and medicine, Mol Biol Rep 41 (2014) 4881-8.

[154] Lopes, L.R., A. Zekavati, P. Syrris, M. Hubank, C. Giambartolomei, C. Dalageorgou, S. Jenkins, W. McKenna, C. Uk10k, V. Plagnol, P.M. Elliott, Genetic complexity in hypertrophic cardiomyopathy revealed by high-throughput sequencing, J Med Genet 50 (2013) 228-39.

[155] Crotti, L., C.N. Johnson, E. Graf, G.M. De Ferrari, B.F. Cuneo, M. Ovadia, J. Papagiannis, M.D. Feldkamp, S.G. Rathi, J.D. Kunic, M. Pedrazzini, T. Wieland, P. Lichtner, B.M. Beckmann, T. Clark, C. Shaffer, D.W. Benson, S. Kaab, T. Meitinger, T.M. Strom, W.J. Chazin, P.J. Schwartz, A.L. George, Jr., Calmodulin mutations associated with recurrent cardiac arrest in infants, Circulation 127 (2013) 1009-17.

[156] Girolami, F., M. Iascone, B. Tomberli, S. Bardi, M. Benelli, G. Marseglia, C. Pescucci, L. Pezzoli, M.E. Sana, C. Basso, N. Marziliano, P.A. Merlini, A. Fornaro, F. Cecchi, F. Torricelli, I. Olivotto, Novel alpha-actinin 2 variant associated with familial hypertrophic cardiomyopathy and juvenile atrial arrhythmias: a massively parallel sequencing study, Circ Cardiovasc Genet 7 (2014) 741-50.

[157] Bao, J., J. Wang, Y. Yao, Y. Wang, X. Fan, K. Sun, D.S. He, F.I. Marcus, S. Zhang, R. Hui, L. Song, Correlation of ventricular arrhythmias with genotype in arrhythmogenic right ventricular cardiomyopathy, Circ Cardiovasc Genet 6 (2013) 552-6.

[158] Kapplinger, J.D., A.P. Landstrom, B.A. Salisbury, T.E. Callis, G.D. Pollevick, D.J. Tester, M.G. Cox, Z. Bhuiyan, H. Bikker, A.C. Wiesfeld, R.N. Hauer, J.P. van Tintelen, J.D. Jongbloed, H. Calkins, D.P. Judge, A.A. Wilde, M.J. Ackerman, Distinguishing arrhythmogenic right ventricular cardiomyopathy/dysplasia-associated mutations from background genetic noise, J Am Coll Cardiol 57 (2011) 2317-27.

[159] Andreasen, C., J.B. Nielsen, L. Refsgaard, A.G. Holst, A.H. Christensen, L. Andreasen, A. Sajadieh, S. Haunso, J.H. Svendsen, M.S. Olesen, New population-based exome data are questioning the pathogenicity of previously cardiomyopathy-associated genetic variants, Eur J Hum Genet 21 (2013) 918-28.

[160] Rasmussen, T.B., P.H. Nissen, J. Palmfeldt, K. Gehmlich, S. Dalager, U.B. Jensen, W.Y. Kim, L. Heickendorff, H. Molgaard, H.K. Jensen, U.T. Baandrup, P. Bross, J. Mogensen, Truncating plakophilin-2 mutations in arrhythmogenic cardiomyopathy are associated with protein haploinsufficiency in both myocardium and epidermis, Circ Cardiovasc Genet 7 (2014) 230-40.

[161] Xu, T., Z. Yang, M. Vatta, A. Rampazzo, G. Beffagna, K. Pilichou, S.E. Scherer, J. Saffitz, J. Kravitz, W. Zareba, G.A. Danieli, A. Lorenzon, A. Nava, B. Bauce, G. Thiene, C. Basso, H. Calkins, K. Gear, F. Marcus, J.A. Towbin, I. Multidisciplinary Study of Right Ventricular Dysplasia, Compound and digenic heterozygosity contributes to arrhythmogenic right ventricular cardiomyopathy, J Am Coll Cardiol 55 (2010) 587-97.

[162] Zhao, M., Q. Wang, Q. Wang, P. Jia, Z. Zhao, Computational tools for copy number variation (CNV) detection using next-generation sequencing data: features and perspectives, BMC Bioinformatics 14 Suppl 11 (2013) S1.

[163] Wang, H., D. Nettleton, K. Ying, Copy number variation detection using next generation sequencing read counts, BMC Bioinformatics 15 (2014) 109.

[164] Duan, J., H.W. Deng, Y.P. Wang, Common copy number variation detection from multiple sequenced samples, IEEE Trans Biomed Eng 61 (2014) 928-37.

[165] Horpaopan, S., I. Spier, A.M. Zink, J. Altmuller, S. Holzapfel, A. Laner, S. Vogt, S. Uhlhaas, S. Heilmann, D. Stienen, S.M. Pasternack, K. Keppler, R. Adam, K. Kayser, S. Moebus, M. Draaken, F.

Degenhardt, H. Engels, A. Hofmann, M.M. Nothen, V. Steinke, A. Perez-Bouza, S. Herms, E. Holinski-Feder, H. Frohlich, H. Thiele, P. Hoffmann, S. Aretz, Genome-wide CNV analysis in 221 unrelated patients and targeted high-throughput sequencing reveal novel causative candidate genes for colorectal adenomatous polyposis, Int J Cancer 136 (2015) E578-89.

[166] Lopes, L.R., C. Murphy, P. Syrris, C. Dalageorgou, W.J. McKenna, P.M. Elliott, V. Plagnol, Use of high-throughput targeted exome-sequencing to screen for copy number variation in hypertrophic cardiomyopathy, Eur J Med Genet 58 (2015) 611-6.

[167] Ceyhan-Birsoy, O., T.J. Pugh, M.J. Bowser, E. Hynes, A.L. Frisella, L.M. Mahanta, M.S. Lebo, S.S. Amr, B.H. Funke, Next generation sequencing-based copy number analysis reveals low prevalence of deletions and duplications in 46 genes associated with genetic cardiomyopathies, Mol Genet Genomic Med 4 (2016) 143-51.

[168] Mademont-Soler, I., J. Mates, R. Yotti, M.A. Espinosa, A. Perez-Serra, A.I. Fernandez-Avila, M. Coll, I. Mendez, A. Iglesias, B. Del Olmo, H. Riuro, S. Cuenca, C. Allegue, O. Campuzano, F. Pico, C. Ferrer-Costa, P. Alvarez, S. Castillo, P. Garcia-Pavia, E. Gonzalez-Lopez, L. Padron-Barthe, A. Diaz de Bustamante, M.T. Darnaude, J.I. Gonzalez-Hevia, J. Brugada, F. Fernandez-Aviles, R. Brugada, Additional value of screening for minor genes and copy number variants in hypertrophic cardiomyopathy, PLoS One 12 (2017) e0181465.

[169] Kerkhof, J., L.C. Schenkel, J. Reilly, S. McRobbie, E. Aref-Eshghi, A. Stuart, C.A. Rupar, P. Adams, R.A. Hegele, H. Lin, D. Rodenhiser, J. Knoll, P.J. Ainsworth, B. Sadikovic, Clinical Validation of Copy Number Variant Detection from Targeted Next-Generation Sequencing Panels, J Mol Diagn 19 (2017) 905-20.

[170] Richards, S., N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, W.W. Grody, M. Hegde, E. Lyon, E. Spector, K. Voelkerding, H.L. Rehm, A.L.Q.A. Committee, Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology, Genet Med 17 (2015) 405-24.

[171] Mullis, K.B., The unusual origin of the polymerase chain reaction, Sci Am 262 (1990) 56-61, 4-5.

[172] Ewing, B., P. Green, Base-calling of automated sequencer traces using phred. II. Error probabilities, Genome Res 8 (1998) 186-94.

[173] Li, H., R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform, Bioinformatics 25 (2009) 1754-60.

[174] Genomes Project, C., G.R. Abecasis, D. Altshuler, A. Auton, L.D. Brooks, R.M. Durbin, R.A. Gibbs, M.E. Hurles, G.A. McVean, A map of human genome variation from population-scale sequencing, Nature 467 (2010) 1061-73.

[175] Tennessen, J.A., A.W. Bigham, T.D. O'Connor, W. Fu, E.E. Kenny, S. Gravel, S. McGee, R. Do, X. Liu, G. Jun, H.M. Kang, D. Jordan, S.M. Leal, S. Gabriel, M.J. Rieder, G. Abecasis, D. Altshuler, D.A. Nickerson, E. Boerwinkle, S. Sunyaev, C.D. Bustamante, M.J. Bamshad, J.M. Akey, G.O. Broad, G.O. Seattle, N.E.S. Project, Evolution and functional impact of rare coding variation from deep sequencing of human exomes, Science 337 (2012) 64-9.

[176] Adzhubei, I., D.M. Jordan, S.R. Sunyaev, Predicting functional effect of human missense mutations using PolyPhen-2, Curr Protoc Hum Genet Chapter 7 (2013) Unit7 20.

[177] Kumar, P., S. Henikoff, P.C. Ng, Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm, Nat Protoc 4 (2009) 1073-81.

[178] Dalal, D., L.H. Molin, J. Piccini, C. Tichnell, C. James, C. Bomma, K. Prakasa, J.A. Towbin, F.I. Marcus, P.J. Spevak, D.A. Bluemke, T. Abraham, S.D. Russell, H. Calkins, D.P. Judge, Clinical features of arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in plakophilin-2, Circulation 113 (2006) 1641-9.

[179] Perrin, M.J., P. Angaran, Z. Laksman, H. Zhang, L.F. Porepa, J. Rutberg, C. James, A.D. Krahn, D.P. Judge, H. Calkins, M.H. Gollob, Exercise testing in asymptomatic gene carriers exposes a latent electrical substrate of arrhythmogenic right ventricular cardiomyopathy, J Am Coll Cardiol 62 (2013) 1772-9.

[180] Tisma-Dupanovic, S., J.B. Wagner, S. Shah, D.T. Huang, A.J. Moss, An adolescent with possible arrhythmogenic right ventricular dysplasia and long QT syndrome: evaluation and management, Ann Noninvasive Electrocardiol 18 (2013) 75-8.

[181] Philips, B., S. Madhavan, C.A. James, A.S. te Riele, B. Murray, C. Tichnell, A. Bhonsale, S. Nazarian, D.P. Judge, H. Calkins, H. Tandri, A. Cheng, Arrhythmogenic right ventricular dysplasia/cardiomyopathy and cardiac sarcoidosis: distinguishing features when the diagnosis is unclear, Circ Arrhythm Electrophysiol 7 (2014) 230-6.

[182] Walsh, R., K.L. Thomson, J.S. Ware, B.H. Funke, J. Woodley, K.J. McGuire, F. Mazzarotto, E. Blair, A. Seller, J.C. Taylor, E.V. Minikel, C. Exome Aggregation, D.G. MacArthur, M. Farrall, S.A. Cook, H. Watkins, Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples, Genet Med 19 (2017) 192-203.

[183] Konig, E., C.B. Volpato, B.M. Motta, H. Blankenburg, A. Picard, P. Pramstaller, M. Casella, W. Rauhe, G. Pompilio, V. Meraviglia, F.S. Domingues, E. Sommariva, A. Rossini, Exploring digenic inheritance in arrhythmogenic cardiomyopathy, BMC Med Genet 18 (2017) 145.

[184] Bang, M.L., T. Centner, F. Fornoff, A.J. Geach, M. Gotthardt, M. McNabb, C.C. Witt, D. Labeit, C.C. Gregorio, H. Granzier, S. Labeit, The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system, Circ Res 89 (2001) 1065-72.

[185] LeWinter, M.M., Y. Wu, S. Labeit, H. Granzier, Cardiac titin: structure, functions and role in disease, Clin Chim Acta 375 (2007) 1-9.

[186] Paldino, A., G. De Angelis, M. Merlo, M. Gigli, M. Dal Ferro, G.M. Severini, L. Mestroni, G. Sinagra, Genetics of Dilated Cardiomyopathy: Clinical Implications, Curr Cardiol Rep 20 (2018) 83.

[187] Punetha, J., A. Kesari, P. Uapinyoying, M. Giri, N.F. Clarke, L.B. Waddell, K.N. North, R. Ghaoui, G.L. O'Grady, E.C. Oates, S.A. Sandaradura, C.G. Bonnemann, S. Donkervoort, P.H. Plotz, E.C. Smith, C. Tesi-Rocha, T.E. Bertorini, M.A. Tarnopolsky, B. Reitter, I. Hausmanowa-Petrusewicz, E.P. Hoffman, Targeted Re-Sequencing Emulsion PCR Panel for Myopathies: Results in 94 Cases, J Neuromuscul Dis 3 (2016) 209-25.

[188] Cherayil, B.J., S. Chaitovitz, C. Wong, S. Pillai, Molecular cloning of a human macrophage lectin specific for galactose, Proc Natl Acad Sci U S A 87 (1990) 7324-8.

[189] Barondes, S.H., D.N. Cooper, M.A. Gitt, H. Leffler, Galectins. Structure and function of a large family of animal lectins, J Biol Chem 269 (1994) 20807-10.

[190] Seetharaman, J., A. Kanigsberg, R. Slaaby, H. Leffler, S.H. Barondes, J.M. Rini, X-ray crystal structure of the human galectin-3 carbohydrate recognition domain at 2.1-A resolution, J Biol Chem 273 (1998) 13047-52.

[191] Dumic, J., S. Dabelic, M. Flogel, Galectin-3: an open-ended story, Biochim Biophys Acta 1760 (2006) 616-35.

[192] Mazurek, N., J. Conklin, J.C. Byrd, A. Raz, R.S. Bresalier, Phosphorylation of the beta-galactoside-binding protein galectin-3 modulates binding to its ligands, J Biol Chem 275 (2000) 36311-5.

[193] Ahmad, N., H.J. Gabius, S. Andre, H. Kaltner, S. Sabesan, R. Roy, B. Liu, F. Macaluso, C.F. Brewer, Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes, J Biol Chem 279 (2004) 10841-7.

[194] Frigeri, L.G., F.T. Liu, Surface expression of functional IgE binding protein, an endogenous lectin, on mast cells and macrophages, J Immunol 148 (1992) 861-7.

[195] Zhang, Z., M.J. Stroud, J. Zhang, X. Fang, K. Ouyang, K. Kimura, Y. Mu, N.D. Dalton, Y. Gu, W.H. Bradford, K.L. Peterson, H. Cheng, X. Zhou, J. Chen, Normalization of Naxos plakoglobin levels restores cardiac function in mice, J Clin Invest 125 (2015) 1708-12.

[196] Norman, M., M. Simpson, J. Mogensen, A. Shaw, S. Hughes, P. Syrris, S. Sen-Chowdhry, E. Rowland, A. Crosby, W.J. McKenna, Novel mutation in desmoplakin causes arrhythmogenic left ventricular cardiomyopathy, Circulation 112 (2005) 636-42.

[197] Lopez-Ayala, J.M., I. Gomez-Milanes, J.J. Sanchez Munoz, F. Ruiz-Espejo, M. Ortiz, J. Gonzalez-Carrillo, D. Lopez-Cuenca, M.J. Oliva-Sandoval, L. Monserrat, M. Valdes, J.R. Gimeno, Desmoplakin truncations and arrhythmogenic left ventricular cardiomyopathy: characterizing a phenotype, Europace 16 (2014) 1838-46.

[198] Bhonsale, A., J.A. Groeneweg, C.A. James, D. Dooijes, C. Tichnell, J.D. Jongbloed, B. Murray, A.S. te Riele, M.P. van den Berg, H. Bikker, D.E. Atsma, N.M. de Groot, A.C. Houweling, J.F. van der Heijden, S.D. Russell, P.A. Doevendans, T.A. van Veen, H. Tandri, A.A. Wilde, D.P. Judge, J.P. van Tintelen, H. Calkins, R.N. Hauer, Impact of genotype on clinical course in arrhythmogenic right ventricular dysplasia/cardiomyopathy-associated mutation carriers, Eur Heart J 36 (2015) 847-55.

[199] Chelko, S.P., A. Asimaki, P. Andersen, D. Bedja, N. Amat-Alarcon, D. DeMazumder, R. Jasti, C.A. MacRae, R. Leber, A.G. Kleber, J.E. Saffitz, D.P. Judge, Central role for GSK3beta in the pathogenesis of arrhythmogenic cardiomyopathy, JCI Insight 1 (2016).

[200] Giuliodori, A., G. Beffagna, G. Marchetto, C. Fornetto, F. Vanzi, S. Toppo, N. Facchinello, M. Santimaria, A. Vettori, S. Rizzo, M. Della Barbera, K. Pilichou, F. Argenton, G. Thiene, N. Tiso, C. Basso, Loss of cardiac Wnt/beta-catenin signalling in desmoplakin-deficient AC8 zebrafish models is rescuable by genetic and pharmacological intervention, Cardiovasc Res 114 (2018) 1082-97.

[201] Song, S., N. Mazurek, C. Liu, Y. Sun, Q.Q. Ding, K. Liu, M.C. Hung, R.S. Bresalier, Galectin-3 mediates nuclear beta-catenin accumulation and Wnt signaling in human colon cancer cells by regulation of glycogen synthase kinase-3beta activity, Cancer Res 69 (2009) 1343-9.

[202] Boscher, C., Y.Z. Zheng, R. Lakshminarayan, L. Johannes, J.W. Dennis, L.J. Foster, I.R. Nabi, Galectin-3 protein regulates mobility of N-cadherin and GM1 ganglioside at cell-cell junctions of mammary carcinoma cells, J Biol Chem 287 (2012) 32940-52.

[203] Jiang, K., C.R. Rankin, P. Nava, R. Sumagin, R. Kamekura, S.R. Stowell, M. Feng, C.A. Parkos, A. Nusrat, Galectin-3 regulates desmoglein-2 and intestinal epithelial intercellular adhesion, J Biol Chem 289 (2014) 10510-7.

[204] Argueso, P., J. Mauris, Y. Uchino, Galectin-3 as a regulator of the epithelial junction: Implications to wound repair and cancer, Tissue Barriers 3 (2015) e1026505.

[205] Plzak, J., K. Smetana, Jr., E. Hrdlickova, R. Kodet, Z. Holikova, F.T. Liu, B. Dvorankova, H. Kaltner, J. Betka, H.J. Gabius, Expression of galectin-3-reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation, Int J Oncol 19 (2001) 59-64.

7 Appendix A

NR	g.DNA	c.DNA	Protein	dbSNP ID	gnomAD	SIFT	Polyphen	Mutation Taster
1	179466859	c.55139T>C	p.Ile18380Thr	rs72646819	ALL:0.0070%	Deleterious (score: 0, median: 4.00)	possibly damaging (0.909)	
2	179427578	c.83281G>C	p.Val27761Leu	rs371788070	ALL:0.0077%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 1)
3	179454354	c.62098A>G	p.Asn20700Asp	rs151193056	ALL:0.0083%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	
4	179396778	c.104564C>A	p.Ser34855Tyr	//	//	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	
5	179409150	c.95806G>A	p.Asp31936Asn	rs267599025	ALL:0.0025%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	
6	179429821	c.81038G>A	p.Arg27013Gln	rs376374751	ALL:0.0058%	Deleterious (score: 0, median: 4.00)	Benign	
7	179403936	c.98726T>C	p.Val32909Ala	rs368877793	ALL:0.014%	Deleterious (score: 0.01, median: 4.00)	possibly damaging (0.909)	disease causing (p-value: 1)
8	179463292	c.57052T>G	p.Ser19018Ala	rs367822879	ALL:0.0013%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 0.965)
9	179468673	c.54741G>A	p.Met18247Ile	rs368858337	ALL:0.0016%	Deleterious (score: 0.03, median: 4.00)	Benign	disease causing (p-value: 1)
10	179576880	c.27677G>A	p.Cys9226Tyr	rs369108107	ALL:0.0012%	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
11	179549717	c.324711G>A		rs371725574		splice		

12	179436313	c.74546G>A	p.Arg24849Gln	rs745488276	ALL:0.0029%	Deleterious (score: 0, median: 4.00)	possibly damaging (0.909)	
13	179403702	c.98960C>T	p.Ser32987Phe	rs746380940	ALL:0.0087%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 1)
14	179403703	c.98959T>C	p.Ser32987Pro	rs758494581	ALL:0.0087%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 1)
15	179483575	c.46702C>T	p.Pro15568Ser	rs561728671	ALL:0.0033%	Deleterious (score: 0, median: 4.01)	probably damaging (0.999)	disease causing (p-value: 1)
16	179418241	c.89491A>G	p.Lys29831Glu	rs774632104	ALL:0.0033%	Deleterious (score: 0.01, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 1)
17	179435027	c.75832C>T	p.Arg25278Cys	rs777445126	ALL:0.0041%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 0.989)
18	179395699	c.105643A>C	p.Thr35215Pro	rs769195414	ALL:0.0016%	Deleterious (score: 0.01, median: 4.01)	probably damaging (0.999)	polymorphism (p-value: 1)
19	179454489	c.61963G>A	p.Glu20655Lys	rs776314797	ALL:0.0020%	Deleterious (score: 0, median: 4.00)	probably damaging (0.999)	disease causing (p-value: 0.999)
20	179582475	c.25126C>T	p.Pro8376Ser	rs375209098	ALL:0.011%	Deleterious (score: 0, median: 4.32)	possibly damaging (0.909)	
21	179435332	c.75527G>A	p.Arg25176His	rs375693396	ALL:0.010%	Deleterious (score: 0.02, median: 4.01)	probably damaging (0.999)	
22	179514891	c.39817G>C	p.Val13273Leu		//	splice		
23	179469578	c.54238G>A	p.Glu18080Lys	rs756321267	T=0.000008/1 (ExAC)	Deleterious (score: 0.01, median: 4.01)	Benign	
24	179431879	c.78980G>A	p.Arg26327Gln	rs370367786	T=0.00005/6 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)

25	179452473	c.63563C>T	p.Ala21188Val	rs746456662	C=0.000008/1 (ExAC)	Deleterious (score: 0, median: 4.32)	Benign	disease causing (p-value: 1)
26	179571284	c.29317G>A	p.Ala9773Thr	rs371163094	T=0.00006/7 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
27	179443367	c.68300C>T	p.Pro22767Leu	rs775953148	A=0.00002/2 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
28	179398728	c.102614G>A	p.Gly34205Asp	rs367926247	T=0.00007/8 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
29	179435113	c.75746G>A	p.Arg25249His	rs773286477	T=0.00004/5 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
30	179429826	c.81033G>T	p.Glu27011Asp	rs765653425	A=0.000008/1 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 0.999)
31	179436796	c.74063G>A	p.Arg24688His	rs376988498	T=0.00004/5 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
32	179398501	c.102841A>C	p.Ile34281Leu	rs397517786	G=0.00007/9 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
33	179395926	c.105416C>T	p.Thr35139Ile	rs200782068	A=0.00005/6 (ExAC)	Deleterious (score: 0, median: 4.32	probably damaging (0.999)	disease causing (p-value: 1)
34	179428726	c.82133A>C	p.Lys27378Thr	//	//	Deleterious (score: 0, median: 4.32)	possibly damaging (0.909)	disease causing (p-value: 0.998)
35	179428451	c.82408C>A	p.Pro27470Thr	//	//	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
36	179478508	c.49502A>G	p.Asn16501Ser	//	//	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
37	179474876	c.51377A>G	p.Lys17126Arg	rs757728544	C=0.00002/2 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)

38	179441340	c.69631G>A	p.Glu23211Lys	//	//	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
39	179457733	c.59113C>T	p.Arg19705Cys	rs72646839	A=0.0002/25 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
40	179464037	c.56483A>C	p.Asn18828Thr	rs71423569	//	Deleterious (score: 0, median: 4.32)	possibly damaging (0.909)	disease causing (p-value: 1)
41	179667003	c.157G>A	p.Gly53Ser	rs150902810	T=0.00005/6 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
42	179638729	c.7166T>A	p.Met2389Lys	rs748735241	T=0.000008/1 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 0.993)
43	179417266	c.90361A>G	p.Thr30121Ala	//	//	Tolerated (score: 0.07, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 0.944)
44	179479231	c.49010G>A	p.Cys16337Tyr		//	Deleterious (score: 0, median: 4.32)	possibly damaging (0.909)	disease causing (p-value: 0.626)
45	179486630	c.45019G>C	p.Asp15007His	//	//	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
46	179417680	c.89947G>A	p.Val29983Met	rs397517746	T=0.00010/12 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
47	179417193	c.90434T>C	p.Ile30145Thr	rs751359750	G=0.000008/1 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
48	179479583	c.48751G>A	p.Asp16251Asn	rs199954570	T=0.00005/2 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
49	179443674	c.68083G>A	p.Ala22695Thr	rs767279296	T=0.00002/2 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 1)
50	179451282	c.64346T>C	p.Val21449Ala	rs748888420	G=0.000008/1 (ExAC)	Deleterious (score: 0, median: 4.32)	probably damaging (0.999)	disease causing (p-value: 0.861)