



# Genetics of arrhythmogenic right ventricular cardiomyopathy

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## ABSTRACT

Arrhythmogenic right ventricular cardiomyopathy is a rare clinical entity characterised by fibro-fatty replacement of myocardium, mainly involving right ventricular free wall, leading to malignant electrical instability and sudden cardiac death. The disease is inherited in up to 50% of cases, with incomplete penetrance and variable phenotypic expression. To date, more than 300 pathogenic mutations have been identified in 12 genes, mainly with autosomal dominant inheritance. Here, we focus on recent advances in the genetics of arrhythmogenic right ventricular cardiomyopathy. Despite continuous improvements, current genotype–phenotype studies have not contributed yet to establish a genetic risk stratification of the disease.

## INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC, Online Mendelian Inheritance in Man (OMIM) 107970) is an inheritable structural heart disease first described by Frank *et al.*<sup>1</sup> The first comprehensive clinical description of a cohort of ARVC patients was reported in 1982 by Marcus *et al.*<sup>2</sup> among adults with ventricular tachyarrhythmias of left bundle branch block morphology. ARVC is characterised by progressive replacement of cardiac myocytes by fibro-fatty tissue, predominantly of the right ventricular (RV),<sup>3</sup> but in up to 50% of cases, the left ventricular (LV) may be also involved.<sup>4–6</sup> Structural alterations are responsible for electrical abnormalities, with or without impaired mechanical function, subsequent ventricular arrhythmias, syncope and sudden cardiac death (SCD).<sup>7–8</sup>

Current diagnosis is based on the presence of a series of diagnostic criteria (minor and major). Therefore, the RV outflow tract, apex and inflow tract (subtricuspid area)—the ‘triangle of dysplasia’—show localised/diffuse atrophy with progressive fibro-fatty infiltration. The interventricular septum and also frequently the LV myocardium are often involved on a histopathological level. These structural alterations are evaluated by bidimensional echocardiography, cardiac resonance or RV angiography and biopsy characterisation of the myocardial wall. As a result of these morphological alterations, depolarisation and repolarisation abnormalities, ventricular arrhythmias and family history of ARVC and SCD in young individuals (less than 35-years-old) are clinical findings and manifestations of the pathology. Thus, the diagnostic criteria were suggested by a Task Force

(TFC) on ARVC in 1994,<sup>9</sup> and revised in 2010.<sup>10</sup> The prevalence range is estimated around 1 : 2500/1 : 5000 in the general population, but in Northern Italy and Germany the range increases to 20/5000.<sup>2–11</sup> ARVC is recognised as a main cause of SCD in young population under 35 years of age (15%–25%),<sup>12–13</sup> especially among athletes, as exercise is a well-known trigger of ventricular arrhythmias and accelerates the development of structural abnormalities in ARVC.<sup>14</sup> The disease is more penetrant in men (1.6 : 1), although gender appears a priori not to harbour adverse effects on long-term survival.<sup>15</sup> Syncope can be the first manifestation of the disease.<sup>16–17</sup> If the disease is recognised at an early stage and if appropriate treatment is instituted, SCD can be prevented. Recent studies have demonstrated the efficacy of the implantable cardioverter-defibrillator therapy in the prevention of SCD in patients affected by ARVC and history of syncope, haemodynamically unstable sustained ventricular tachycardia (VT) and heart failure<sup>7–18</sup> although little data are available on the prophylactic implantation of an implantable cardioverter-defibrillator in patients considered to be at intermediate arrhythmic risk (haemodynamically stable sustained VT, non-sustained VT, severe dilatation and/or dysfunction of the RV, LV or both, and early onset of severe structural disease).<sup>19–21</sup>

In recent years, while there has been continuous progress in genetics of ARVC, little is known about the mechanistic process responsible for the disease. Despite several hypotheses proposed in recent years, the main hypothesis currently accepted focuses on irregular cell–cell adhesion that promotes myocyte death and subsequent replacement by fibro-fatty tissue. Concretely, this degenerative theory focuses on desmosomal mutations that inhibit Wnt signalling and, thereby, modulate pathological gene expression, promote cardiac myocytes apoptosis and mediate expression of a fibro-adipogenic phenotype.<sup>22</sup> Desmosomal proteins (particularly plakoglobin (PG)) play a key role both as structural proteins and as nuclear signalling molecules,<sup>23</sup> PG being a major mediator of adipogenesis. Thus, the pathogenesis of ARVC involves altered cell biomechanical behaviour and altered signalling that lead to cardiac myocytes injury and death.<sup>24</sup>

ARVC is frequently a familial disease,<sup>12</sup> and recent studies conclude a familial affection in up to 50% of cases, usually with an autosomal dominant pattern of inheritance, incomplete and age-related

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penetrance<sup>25</sup> and polymorphic phenotypic expression.<sup>26–27</sup> Autosomal recessive forms of ARVC have been also reported although they are less frequent and linked to mutations in different genes.<sup>28–30</sup> As the overall rate of mutation discovery in ARVC is approximately 60%–65%, genetic testing has been incorporated in the revised TFC, serving as a critical tool to aid in diagnostic confirmation. Genetic testing allows cascade screening of relatives of a positive index case, identifying other genetic carriers who may be at risk of developing the disease and suffer SCD.<sup>25–26–31</sup> Familial genetic testing of sudden unexpected death victims with negative autopsy (SUDNA) identified ARVC-linked pathogenic mutations in 5%–10% of cases,<sup>32</sup> and recent studies in SUDNA samples showed that approximately 25% of patients carried a mutation in the *PKP2* gene.<sup>33</sup> However, it still remains to be clarified whether definitive risk stratification can be based on mutation–phenotype relationship in the ARVC population. Even if genetic variation is identified in desmosomal genes, it does not indicate that the patient is going to be affected because of the variable penetrance.<sup>34</sup>

### DISEASE-RELATED GENES

Three different structures are involved in cell–cell adhesion in the myocardium: desmosomes, adherens junctions (zonula adherens/fasciae adherens) and gap junctions. Although genetic modifications of any of these three structures could theoretically induce the disease, only mutations in genes encoding desmosomal proteins have been associated with ARVC. So far, more than 800 genetic variants have been identified in 12 genes, but only around 300 have been classified as damaging,<sup>35</sup> responsible for around 60%–65% of all ARVC cases. The 12 ARVC-related genes encode five desmosomal proteins (desmoplakin –*DSP*-, plakophilin-2 –*PKP2*-, desmoglein-2 –*DSG2*-, desmocollin-2 –*DSC2*- and PG –*JUP*-), and seven non-desmosomal proteins (desmin –*DES*-, transmembrane protein 43 –*TMEM43*-, transforming growth factor  $\beta$ -3 –*TGFB3*-, lamin A/C –*LMNA*-, titin –*TTN*-, phospholamban –*PLN*- and  $\alpha$ T-catenin –*CTNNA3*-) (table 1). In 2001, a mutation in the cardiac ryanodine receptor, encoded by the ryanodine gene (*RyR2*) was identified in a patient affected by ARVC;<sup>36</sup> however, this case has been recently classified as an ARVC phenocopy.<sup>37</sup> To date, the majority of pathogenic mutations have been identified in genes coding for desmosomal proteins, with only the *PKP2* gene being responsible for approximately 35%–40% of cases. Mutations in

the genes *DSP*, *DSG2* and *DSC2* are responsible for nearly 15%–20% of ARVC cases.<sup>38–40</sup>

### Desmosomal genes

Desmosomes are complex multiprotein structures of the cell membrane that provide structural and functional integrity to adjacent cells in different tissues, such as myocardium. Desmosome configuration includes adhesion molecules of the cadherin family (*DSG* and *DSC*) and proteins of the plakin and catenin families (desmoplakin, plakophilin and PG) which link intermediate filaments of the cytoskeleton to the desmosomal cadherins (figure 1).

### Plakophilin-2

The most prevalent form of ARVC (type 9) is caused by mutations in the *PKP2* gene (ENSG00000057294), which encode plakophilin-2 protein (*PKP2*, ENSP00000070846), an essential armadillo repeat protein located in the outer dense plaque of cardiac desmosomes that interacts with multiple other cell adhesion proteins<sup>41</sup> (figure 2). Gerull *et al*<sup>42</sup> suggested that absence and/or alteration of *PKP2* structure in the cardiac desmosomes impair myocytes interactions among myocytes, inducing myocardium disruption, particularly in response to mechanical stress. Therefore, this may be a plausible explanation as to why exercise is a main inductor of ventricular tachyarrhythmias and SCD.

To date, more than 150 pathogenic mutations in *PKP2* have been identified making it the main gene responsible for the disease (it represents around 35%–40% of total ARVC cases). The most prevalent variations in the *PKP2* gene correspond to small deletion/insertion with frameshift pattern (40%),<sup>35</sup> followed by nonsense mutation in 25% of cases, missense variations in 20% and pathogenic splice site variations in 15%. Most of the ARVC-linked pathogenic mutations identified in the *PKP2* gene have an autosomal dominant pattern of inheritance (haploinsufficiency mechanism) although a recessive form was also described in 2006.<sup>30</sup> In this last study, genetic screening identified a novel homozygous 7-base-pair deletion in the *PKP2* gene, which was predicted to be translationally silent.

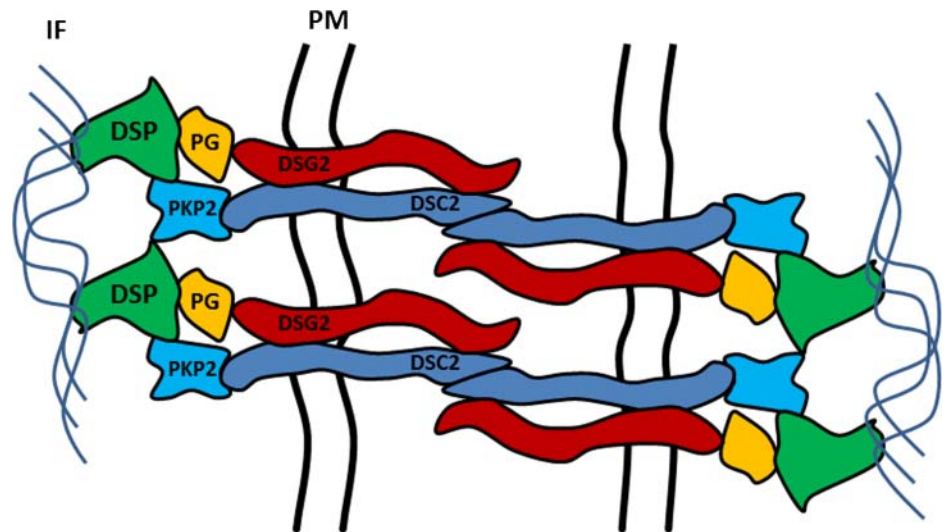
As mentioned above, genetic analysis of all ARVC-known genes by conventional Sanger resequencing identifies one genetic mutation in around 60%–65% of ARVC cases. Although the remaining cases may be secondary to pathogenic mutations within undiscovered genes, an alternative possibility is the presence of copy number variants (CNV): large deletions/insertions in known genes

**Table 1** Genes associated with ARVC

Gene	Locus	MIM	Gene ID	Protein	Inheritance
<i>CTNNA3</i>	10q21.3	607667	29119	$\alpha$ T-catenin	AD
<i>DES</i>	2q35	125660	1674	Desmin	AD
<i>DSC2</i>	18q21	125645	1824	Desmocollin-2	AD
<i>DSG2</i>	18q12.1	125671	1829	Desmoglein-2	AD/AR
<i>DSP</i>	6p24	125647	1832	Desmoplakin	AD/AR
<i>JUP</i>	17q21	173325	3728	Junction plakoglobin	AD/AR
<i>LMNA</i>	1q22	150330	4000	Lamin A/C	AD
<i>PKP2</i>	12p11	609040	5318	Plakophilin-2	AD/AR
<i>PLN</i>	6q22.1	172405	5350	Phospholamban	AD
<i>TGFB3</i>	14q24.3	190230	7043	Transforming growth factor $\beta$ 3	AD
<i>TMEM43</i>	3p25.1	612048	79188	Transmembrane protein 43	AD
<i>TTN</i>	2q31.2	188840	7273	Titin	AD

AD, autosomal dominant; AR, autosomal recessive; ARVC, arrhythmogenic right ventricular cardiomyopathy; MIM, Mendelian Inheritance in Man.

**Figure 1** Diagram of desmosomal proteins related to arrhythmogenic right ventricular cardiomyopathy. DSC2, desmocollin-2; DSG2, desmoglein-2; DSP, desmoplakin; IF, intermediate filaments; PG, plakoglobin; PKP2, plakophilin-2; PM, plasmatic membrane.



that are not detected by conventional genetic testing methods (Sanger sequencing). In 2010, Christensen reported a Danish cohort in which *PKP2* large genomic insertions and deletions were excluded in all patients after multiplex ligation-dependent probe amplification.<sup>40</sup> Recently, Roberts *et al*<sup>43</sup> published two cases that developed ARVC due to large genomic *PKP2* deletions, to date, the only CNVs study in ARVC cases. Thus, we strongly support that clinical genetic testing in ARVC cases should include screening for CNV when conventional sequencing analysis does not reveal pathogenic mutations after conventional Sanger resequencing of all ARVC-related genes.

#### Desmocollin-2

The desmosomal cadherins proteins, such as DSC and DSG, are the major constituents of the desmosomal plaque. Both proteins are type I integral membrane glycoproteins with four conserved extracellular subdomains, variable extracellular anchor domain, a single transmembrane domain, an intracellular anchor domain, and additional cytoplasmic subdomains (figure 2). Both proteins participate in calcium-dependent cell adhesion, regulation of tissue morphogenesis and intracellular signalling processes.<sup>44</sup> The *DSC* gene contains 17 exons ranging in size from 46 to 258 bp and spans more than 32 kb of DNA. Exon 16 is alternatively spliced, giving rise to the  $\alpha$  and  $\beta$  forms of the protein. So, it encodes two products derived from alternatively spliced

transcripts from single genes (*DSC1* and *DSC2*). The human *DSC2* gene (ENSG00000134755) encodes the most widely distributed form of DSC proteins, DSC2 (ENSP00000280904).<sup>45</sup>

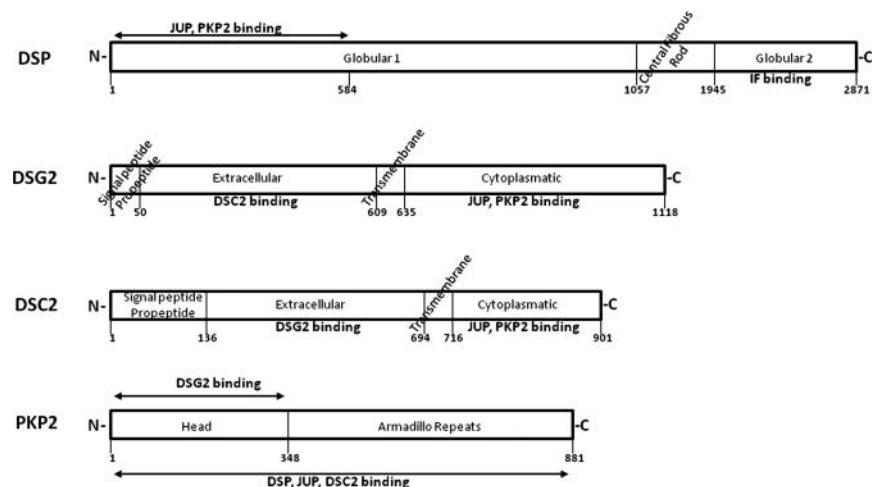
In 2006, Heuser *et al* published the first *DSC2* gene mutations in humans affected by ARVC (type 11). They also established that physiological levels of the DSC2 protein are essential for cardiac desmosome formation, early cardiac morphogenesis and cardiac function.<sup>46</sup> To date, less than 50 pathogenic mutations on *DSC2* gene have been linked to ARVC, and 50% of them are missense mutations, while the rest correspond to non-sense mutations and small deletions/insertions that induce frameshift variations. In addition, four splice site pathogenic variations have been identified in the *DSC2* gene in ARVC cases that fulfil TFC diagnostic criteria.<sup>35</sup>

#### Desmoglein-2

DSGs are desmosomal cadherins and, together with DSCs, form the two essential transmembrane components of the desmosome (figure 2). Each member of the DSG protein family (DSG type 1–4) has four extracellular cadherin domains and a transmembrane domain.<sup>44</sup> DSG2 (ENSP00000261590) is the only isoform expressed in cardiac myocytes.

In 2006, two reports linked pathogenic mutations in the *DSG2* gene (ENSG00000046604) to ARVC.<sup>47–48</sup> Since then, more than 50 pathogenic variants have been associated with

**Figure 2** Structural characteristics of main desmosomal proteins responsible for arrhythmogenic right ventricular cardiomyopathy. C, C-terminus; DSC2, desmocollin-2; DSG2, desmoglein-2; DSP, desmoplakin; JUP, plakoglobin; N, N-terminus; PKP2, plakophilin-2.



ARVC (type 10). Of them, around 60% are missense mutations, 20% correspond to small insertions/deletions and other 20% to splice site changes.<sup>35</sup> Despite the fact that most of the identified mutations have a dominant pattern of inheritance, a recessive pattern has been suggested also in ARVC patients,<sup>48</sup> although hitherto, no conclusive relation has been reported.

### Desmoplakin

Desmoplakin is the most abundant protein of the desmosomes, encoded by the *DSP* gene (ENSG00000096696). Desmoplakin has two isoforms produced by alternative splicing: the longest desmoplakin I isoform (ENSP00000369129) and the shorter desmoplakin II (ENSP00000396591). Desmoplakin isoform I has been reported to be a force constituent of desmosomes and the major isoform present in cardiac tissue, although expression of isoform II (DSPII) occurs in different heart compartments<sup>49</sup> (figure 2).

The first report of a pathogenic variation in the *DSP* gene linked to ARVC was published in 2002.<sup>50</sup> Some years later, Yang *et al* performed in vitro and in vivo analyses of the mutant DSP proteins, showing interruption of DSP–DES interaction at intercalated discs and marked ultrastructural changes. Thus, the results suggested that DSP expression in cardiomyocytes is crucial for maintaining cardiac tissue integrity, and that DSP protein abnormalities lead to cardiomyocyte death, changes in lipid metabolism and defects in cardiac development.<sup>51</sup>

Almost 100 pathogenic variations in the *DSP* gene have been linked to ARVC (type 8). In all, 40% are missense variations, 30% nonsense variations, and the rest are small insertions/deletions and splice sites.<sup>35</sup> Heterozygous *DSP* mutations lead to a wide ARVC phenotypic heterogeneity, including forms associated with dermatological phenotypes. The *DSP* gene was also implicated in Carvajal syndrome, an autosomal recessive cardiocutaneous form of ARVC that was described as a variant of Naxos syndrome (see below, Plakoglobin section) in which the cardiomyopathy presents a more pronounced LV involvement, palmoplantar keratoderma and woolly hair.<sup>29 52</sup> Another *DSP* recessive mutation was identified in a large Arab family with a high rate of consanguinity causing ARVC, woolly hair and pemphigus-like skin disorder.<sup>53</sup> A debate still exists about whether the cardiac phenotype in the Arab family appears to be classic ARVC or modified Carvajal syndrome. Finally, since 2010, three studies have reported single heterozygous *DSP* missense mutations in unrelated patients leading to combined phenotypes resembling Naxos–Carvajal syndrome.<sup>54–56</sup> All these mutations are localised in a small DSP region (from 564 to 597 aminoacids), suggesting a key role for this DSP region involved in the interactions with plakophilin or PG leading to combined Naxos–Carvajal phenotypes.

### Plakoglobin

PG is a major component protein of cell adhesion junctions, and the only constituent common to submembranous plaques of both desmosomes and intermediate junctions. PG also forms distinct complexes with cadherins and desmosomal cadherins, being a member of the catenin family since it contains a distinct repeating aminoacid motif (called armadillo repeat). Concretely, PG plays a crucial role in linking the desmosomal cadherins, DSG and DSC, to the cytoskeleton via DP (figure 2). The PG protein (ENSP00000311113) is codified by *JUP* (ENSG00000173801) and implicated in ARVC (type 12).<sup>28</sup>

The first genetic mutation associated with ARVC was a homozygous deletion in the *JUP* gene, with an autosomal recessive pattern of inheritance.<sup>28</sup> The disease was originally identified in

patients on the Greek island of Naxos and hence it is referred to as ‘Naxos disease’.<sup>57</sup> It is characterised by non-epidermolytic palmoplantar keratoderma, woolly hair and right sided cardiomyopathy. Homozygotes for Naxos disease usually have no cardiac symptoms during childhood but penetrance is 100% during adolescence. Patients present with symptomatic arrhythmias, ECG alterations, structural damage of RV in all cases and LV involvement in 35% of cases.<sup>58 59</sup>

To date, less than 20 pathogenic mutations have been identified in *JUP*, and 50% correspond to missense mutations. All other mutations are insertions/deletions and one splice site. Of all mutations, only five correspond to homozygous variations.<sup>35</sup>

### Non-desmosomal genes

While mutations in desmosomal genes are the main cause of ARVC, mutations in six other genes have been also associated with the disease (table 1). These genes encode for intermediate filaments, and regulatory factors, among other proteins. Although there are only a few studies describing the prevalence of pathogenic mutations identified in all these proteins in ARVC, all together they are responsible of approximately 10% of ARVC cases. Despite their low prevalence, we consider that it is important to study them in order to perform a comprehensive genetic testing.

### Desmin

DES is the main intermediate filament protein (ENSP00000363071) in mature skeletal and heart muscle cells, encoded by the *DES* gene (ENSG00000175084). DES gene contains nine exons and spans about 8.4 kb. Intronic sequences contain four AluI repetitive elements, and the promoter region is G rich. It forms a scaffold around the Z-disk and links whole contractile structure with subsarcolemmal cytoskeleton, intercalated disk, nucleus and other components of the cytoplasm. DES-related myopathy is a clinically heterogeneous group of disorders encompassing myopathies, cardiomyopathies, conduction disease and combinations of these disorders.<sup>60</sup>

Even though several mutations related to myopathies have been identified in the *DES* gene, the first study on mutations in ARVC patients was reported in 2009.<sup>61</sup> Later, Otten *et al*<sup>62</sup> reported that a *DES* mutation affects the localisation of DSP and PKP2 proteins at the intercalated disk. So far, only less than 10 pathogenic variants linked to ARVC have been identified in *DES* gene. Overall, 90% of them are missense variations and only a small number of mutations are deletions.<sup>35</sup> Thus, in the absence of skeletal muscle involvement suggestive of a desminopathy, the probability of ARVC-related mutations in the *DES* gene is very low.<sup>63</sup>

### Transforming growth factor $\beta$ -3

TGF $\beta$ 3 (ENSP00000238682) is a member of the TGF family, pleiotropic and multifunctional peptides that exerts diverse effects on different cell types. Three structurally similar isoforms of TGF $\beta$  (TGF $\beta$ 1, 2 and 3), encoded by three distinct genes, have been identified in mammalian species. The *TGF $\beta$ 3* gene (ENSG00000119699) encodes TGF $\beta$ 3 protein, a cytokine that stimulates fibrosis, and modulates cell adhesion and expression of desmosomal genes.<sup>64</sup>

The first association between ARVC (type 1) and this gene was reported in 2005.<sup>65</sup> This study identified two mutations in the untranslated region zone (one in 5' and the other one in 3') in two affected families. Desmosomal genes were not analysed in any patient of this series. Recent findings suggest that the elevated TGF $\beta$  signalling is associated with the pathogenesis of



JUP-related ARVC.<sup>66 67</sup> To date, these are the only two pathogenic variations in *TGFβ3* reported in ARVC patients. Controversy still remains about a clear relation between this gene and ARVC.

#### Transmembrane protein 43

The gene *TMEM43* (ENSG00000170876) contains 12 exons and spans 18.7 kb. It encodes the protein transmembrane protein 43 (TMEM43, ENSP00000303992). The exact function of this gene is still unknown. This gene has been suggested to have a role in an adipogenic pathway because it has a peroxisome proliferator response element (PPAR $\chi$ , an adipogenic transcription factor). Dysregulation of this adipogenic pathway may explain the myocardial fibro-fatty replacement in ARVC patients (type 5).<sup>68</sup>

Only two pathogenic mutations had been described previously, but a recent report has described three additional new pathogenic mutations among 143 UK ARVC probands tested negative for mutations in desmosomal genes.<sup>69</sup> Detailed genotype–phenotype studies of families with *TMEM43* mutations have shown a marked influence of male sex on an earlier and more severe clinical presentation.<sup>20</sup>

#### Lamin A/C

The gene *LMNA* (ENSG00000160789) spans approximately 24 kb and contains 12 exons. Alternative splicing within exon 10 gives rise to two different mRNAs that code for prelamin A and lamin C. Thus, this gene encodes lamin A (ENSP00000357283) and lamin C (ENSP00000357284), by alternative splicing. Both of them belong to the family of type V intermediate filaments that take part in the constitution of the nuclear lamina, a complex of proteins below the inner part of the nuclear membrane.<sup>70</sup> Both lamins A and C contain a 360-residue  $\alpha$ -helical domain with homology to a corresponding  $\alpha$ -helical rod domain that is the structural hallmark of all intermediate filament proteins.

Genetic alterations in *LMNA* are associated with a heterogeneous group of disorders commonly named ‘laminopathies,’ including cardiac disorders,<sup>71</sup> although no relation between the *LMNA* gene and ARVC had been reported until 2012 when Quarta *et al*<sup>72</sup> performed a genetic study in a cohort of 108 ARVC patients, and identified mutations in the *LMNA* gene in the affected individuals. Also in 2012, a study focused on a cohort of SCD samples due to suspected cardiomyopathy has identified a missense mutation in the *LMNA* gene,<sup>73</sup> supporting the addition of this gene to genetic testing in patients with suspected/confirmed ARVC diagnosis.

#### Titin

The cardiac sarcomere contains actin-based thin and myosin-based thick filaments, and the giant protein TTN, ENSP00000343764 (also named connectin). Between its thin and thick filament binding domains TTN contains a large segment that behaves as a molecular spring that extends during sarcomere stretch. TTN protein is encoded by the gene *TTN* (ENSG00000155657) in 363 exons and it is involved in numerous cellular processes including maintaining sarcomere structure and force-dependent signalling.<sup>74</sup>

Despite being the candidate gene responsible for structural cardiac diseases due to its key role in mechanical movement, no reports associated the *TTN* gene with ARVC until 2011, when Taylor *et al*<sup>75</sup> reported for the first time eight missense mutations in the *TTN* gene in ARVC patients. We think that this number will increase progressively if *TTN* is analysed in ARVC

patients as it has occurred in a cohort of patients affected by DCM.<sup>76</sup> To our knowledge, *TTN* genetic testing it is not routinely being performed in ARVC cases due to the technical difficulties to analyse such a long gene.

#### Phospholamban

The gene *PLN* (ENSG00000198523) encodes the protein PLN (ENSP00000350132), a small phosphoprotein closely associated with the cardiac sarcoplasmic reticulum. It is a regulator of the sarcoplasmic reticulum Ca<sup>2+</sup> (SERCA2a) pump in cardiac muscle and therefore important for maintaining Ca<sup>2+</sup> homeostasis.<sup>77</sup> Consequently, the PLN protein is one of the major determinants of cardiac contractility and relaxation.<sup>78</sup>

To date, several *PLN* mutations have been identified in dilated cardiomyopathy (DCM) patients, but recently, a *PLN* pathogenic mutation was published in a Dutch ARVC family, linking for the first time ARVC with mutations in the *PLN* gene.<sup>79</sup> Curiously enough, this study showed that carriers of p.R14del mutation in the *PLN* gene presented with two different phenotypes: DCM or ARVC. This mutation has previously been associated with a low voltage in the ECG.<sup>80</sup> Therefore, the authors suggested that *PLN*\_R14del is responsible for ‘arrhythmogenic cardiomyopathy’ an entity encompassing ARVC, including left-dominant arrhythmogenic cardiomyopathy, and arrhythmogenic forms of DCM. In fact, the genetic overlap between ARVC and DCM has been shown also in other ARVC-related genes identified to date.<sup>72 81 82</sup>

#### $\alpha$ T-catenin

The *CTNNA3* gene (ENSG00000183230) encodes the protein  $\alpha$ T-catenin (catenin cadherin-associated protein  $\alpha$  3 (CTNNA3), ENSP00000389714).  $\alpha$ -Catenins are cytoplasmic molecules thought to be indispensable for dynamic maintenance of tissue morphogenesis by integrating in the cadherin–catenin complex.<sup>83</sup> There are three  $\alpha$ -catenin subtypes in mammals: the ubiquitously expressed  $\alpha$ E-catenin, the neural  $\alpha$ N-catenin and  $\alpha$ T-catenin.<sup>84</sup>  $\alpha$ T-catenin protein is located within the area composite of intercalated disks of cardiomyocytes and in peritubular myoid cells of the testis. The gene is located in a common fragile site epigenetically regulated, transcribed through multiple promoters, and generating a variety of alternate transcripts.<sup>85</sup> It colocalises with CTNNA1 (catenin cadherin-associated protein  $\alpha$  1) and CTNNA2 (catenin cadherin-associated protein  $\alpha$  2) proteins.<sup>86</sup> In addition, it also interacts with the protein CTNNB1 (catenin cadherin-associated protein  $\beta$  1).<sup>87</sup>

In 2003, the gene *CTNNA3* was assessed in order to identify a potential relation to DCM but no DCM-linked *CTNNA3* mutations were identified.<sup>88</sup> Recently, Li *et al*<sup>89</sup> developed an animal model that demonstrates how perturbation in  $\alpha$ T-catenin can affect both the PKP2 and Cx43 proteins and thereby highlights the importance of understanding the crosstalk between the junctional proteins of the intercalar disk and its implications for DCM, suggesting the potential role of this gene in ARVC. Recently, two missense mutations have been identified in two unrelated patients of an ARVC cohort.<sup>90</sup> This report relates for the first time the protein  $\alpha$ T-catenin to ARVC.

## CANDIDATE GENES

### Locus identified

Genetic studies have mapped two different loci for ARVC (10p12–p14 and 14q12–q22) although the gene responsible for the pathology is still unknown. A linkage analysis identified a novel locus (10p12–p14) in ARVC cases but again, no mutation has been identified in known genes. The authors also studied

the possible involvement of the tyrosine phosphatase-like gene (*PTPLA*) that encodes the protein PTPLA but only a polymorphism was identified.<sup>91</sup> The second locus was suggested by a study focusing on three families, which found a genetic linkage in the proximal portion of 14q (14q12–q22). These data indicated that a novel gene causing familial ARVC could be located in the long arm of chromosome 14<sup>92</sup> but it is unknown so far.

### Suspicious genes analysed

Currently, 12 genes have been linked to ARVC but in up to 35%–40% of patients no causal mutation is identified in the candidate genes. New ARVC-related genes are being investigated, and those encoding proteins related to desmosomes, adherent junctions and gap junctions are considered to be possible candidate genes. A genome-wide study focused on a spontaneous canine model of ARVC identified an 8-bp deletion in the 3' untranslated region of the Striatin gene (*STRN*), which encodes the striatin protein (*STRN*), suggesting this gene as a candidate gene for ARVC in humans.<sup>93</sup> However, further studies such as segregation in humans and in vitro analysis should be performed to determine the possible role of this gene in the human pathogenesis of ARVC. Another study on a cohort of ARVC human samples analysed three genes not previously associated with the disease:  $\beta$ -catenin (*CTNNB1*),  $\alpha$ -T-catenin (*CTNNA3*) and PERP (*PERP*), which encode structural proteins of importance for cellular adhesion. No mutations were identified in any of three genes analysed despite their involvement in formation and maintenance of the intercalated disk.<sup>94</sup> However, a recent study on a cohort of ARVC patients have identified two mutations in the *CTNNA3* gene, suggesting a low frequency of mutations in this gene but recommend systematic genetic screening for this gene in ARVC families.<sup>90</sup>

In addition, Friedrich *et al* reported a new gain-of-function variant in the human *ISL1* gene; this study identified the variant in one DCM affected family and in one hypertrophic cardiomyopathy (HCM) patient. It suggests that this gene could potentially lead to greater activation of downstream targets involved in cardiac abnormalities, such as ARVC.<sup>95</sup> Furthermore, the gene *ISL1* is directly regulated by  $\beta$ -catenin (*CTNNB1* gene), another ARVC candidate gene.

Recent studies have shown several limitations in order to perform a classification system for cardiomyopathies mainly due to overlapping clinical features. The distinction of HCM, DCM and ARVC as separate clinical entities has important implications for clinical practice, guiding both diagnostics and treatment.<sup>96–97</sup> Focus on genetics and pathogenic mutations in the same gene can result in phenotypic heterogeneity. For example, so far, mutations in the *DES*, *LMNA*, *TTN* and *PLN* genes, both in DCM and ARVC patients have been identified. Even genetic variations in *TTN* and *PLN* have been identified in HCM cases. All these facts suggest that the term desmosomal disease can no longer be synonymous with ARVC. Recent studies support the concept of 'arrhythmogenic cardiomyopathy' as an entity encompassing arrhythmogenic forms of DCM and ARVC.<sup>23–98</sup> Although it is known that disease-causal gene alterations in desmosomal proteins show definite overlaps between DCM and ARVC, the underlying mechanisms responsible for these differences are not yet understood. Therefore, it has been suggested that environmental factors and interaction between genetic variations as regulatory factors that determine the pathogenic variations lead to ARVC phenotypes.

Our group analysed a cohort of ARVC patients with no mutation in any of disease-related genes. In our cohort, seven genes were assessed -*ACTC1* (actin  $\alpha$  cardiac muscle 1), *CDHN*

(cadherin 2 type 1 or N-cadherin), *CTNNA1* (catenin  $\alpha$  1), *Cx43* or *GJA1* (gap junction protein  $\alpha$  1), *MVCL* (metavinculin), *MYL2* (myosin light chain 2) and *MYL3* (myosin light chain 3)-, all related to desmosome structure but no mutation was identified in any of them.<sup>99</sup> Both studies were performed in a reduced number of ARVC patients suggesting the need to assess all these genes in large cohorts and focus future research into the pathogenesis of ARVC on other components of cardiomyocyte adhesion.

### HIGH-THROUGHPUT TECHNOLOGIES

In the last 10 years, advances in genetics allow us to rapidly sequence human genomes and generate genetic data for a relatively small cost, reducing the issues of possible human error, and opening up numerous opportunities for translation into the clinical area in near future.<sup>100</sup> The new technology named *Next Generation Sequencing* (NGS) allows a massively parallel resequencing in a short period of time, progressively reducing the costs of sequencing itself and the time spent on data analysis and computer storage requirements. Although each identified genetic variant needs to be confirmed by traditional Sanger sequencing mainly due to the poor coverage of gene promoters and repetitive or GC-rich sequences, NGS is being progressively incorporated to clinical practice. It will help to identify genetic defects in families affected by cardiac pathologies in which no pathogenic mutation has been found to date, improving current diagnosis, prevention and treatment, as suggested by Polychronakos and Seng recently.<sup>101</sup> However, the use of NGS to identify gene mutations could face huge challenges, in particular, in small families and sporadic cases, to discern the disease causing variants from those that by chance alone are carried by the affected persons.<sup>63</sup>

Despite all these controversies, some recent studies highlight the high value of integrating genomic approaches in the effective evaluation of patients affected by heterogeneous diseases, rapidly identifying disease-causative variants in known genes, and also revealing novel candidate disease genes.<sup>102–104</sup> Therefore, a recent publication about a family affected by ARVC identified the first pathogenic mutation in the gene *DES* by whole exome sequencing.<sup>105</sup>

Another technology, MassARRAY iPLEX Gold Platform—SEQUENOM—has been used to identify known common pathogenic mutations in patients affected by long QT syndrome,<sup>106</sup> and HCM.<sup>107</sup> Our group has used this technology to identify known pathogenic mutations in ARVC postmortem samples, especially when the DNA quality only enables the identification of limited number of point mutations (Alcalde M *et al*, unpublished data).

### GENETIC RISK STRATIFICATION

Incomplete penetrance and variable expressivity are hallmarks of ARVC, making it difficult for clinicians to evaluate the risk of developing the disease. Several clinical features have been proposed to indicate high risk of ARVC patients, such as SCD family history, syncope, severe RV dysfunction and LV involvement, among others.<sup>108</sup> In recent years, genetics has played a key role in studying phenotype variations although these variations are independent of the gene affected, even among family members sharing the same gene variant, suggesting that additional genetic and/or environmental modifiers can influence the disease phenotype.<sup>27</sup>

As mentioned above, more than 300 pathogenic mutations have been identified in the 12 ARVC-related genes. In addition, more than 250 are classified as of unknown significance, and

more than 900 are considered not to have pathogenic effect.<sup>35</sup> However, a modulator role of these non-pathogenic variations in the phenotype remains not fully understood. All these genetic variations have been identified mainly in desmosomal genes, *PKP2* being the most prevalent gene.<sup>109</sup> The variable expressivity and incomplete penetrance in ARVC patients could be explained by the type of mutation in the causal gene and/or the presence of multiple genetic variants in modifier genes. To elucidate the potential role of a genetic variation in ARVC, in recent years, artificial systems using transfected cell cultures and transgenic mice<sup>110</sup> have been performed. Recently, keratinocyte cultures have been used because of their expression in all cardiac-specific isoforms of desmosomal proteins.<sup>111 112</sup>

A genetic compilation has been published recently by Kapplinger *et al*<sup>39</sup> reporting that most mutations in the *PKP2* gene correspond to 'radical' genetic variations, a term that included inframe, frameshift *indels*, splice junction and nonsense mutations. However, most genetic variations in *DSG2*, *DSC2* and *DSP* genes are missense mutations. While radical mutations are considered highly pathogenic, little is known in ARVC-related genes about the difference between missense mutations and 'background' genetic variations, common genetic variations in population. For example, and as mentioned before, recently a potential pathogenic missense mutation in the *TMEM43* gene has been identified in a study of a limited cohort from UK and Canada. The genetic variation has also been found in control population but at a low frequency. Then the authors have suggested that the missense variation is a 'pathogenic founder mutation'<sup>69</sup> but further studies should be performed to elucidate the role of this novel variation in ARVC patients.

In order to discriminate genetic background and real pathogenic variants is crucial to perform an interpretation in genotype–phenotype correlation, focus in familial cosegregation. The authors suggested that each missense mutation should be assessed with caution, taking into account mutation localisation, gene, evolutionary conservation of the aminoacid residue, and race and ethnicity of patient (to exclude founder mutations). In addition, minor allele frequency (MAF) in population helps to distinguish pathogenic variants from common genetic variations (MAF>1%), and *in silico* databases predict the possible pathogenic role of a new variation.

Referring to causal genes, the *PKP2* gene is the main gene responsible for ARVC, as mentioned above. In addition, it has been also recently published that *PKP2* mutations were most frequently identified to be causal for ARVC,<sup>113</sup> essentially because most of these mutations are 'radical' genetic variations, considered highly pathogenic.<sup>39</sup> The second gene responsible for the pathology is *DSG2*. Mutations in the *DSG2* gene were associated with more frequent LV involvement.<sup>114 115</sup> The other two ARVC-associated genes are *DSP* and *DSC2*. In 2005, mutations in C-terminus of DSP protein have been related to biventricular phenotype.<sup>116</sup>

In recent years, researchers have explored compound heterozygosity in a single gene, double mutations in more than one desmosomal gene (digenic inheritance) and phenotype modification due to polymorphism, hypothesising that ARVC might require multiple genetic hits in the cell adhesion complex to cause the phenotype,<sup>34 117 118</sup> but no clear explanation has been reported for the wide variability and penetrance in families affected by ARVC. In 2009, Bhuiyan *et al*<sup>119</sup> reported that biallelic or digenic *DSC2* and/or *DSG2* gene mutations are frequently identified in ARVC patients, suggesting that a single mutation is less likely to cause the pathology. Accordingly, it has also been reported that carriers of

various genetic mutations exhibit a higher probability to develop ARVC phenotype as compared with patients with a single mutation.<sup>25 118</sup> Fressart *et al*<sup>54</sup> also reported that various mutations identified in the same patient were associated with higher frequency of SCD. Christensen *et al*<sup>40</sup> reported that a substantial proportion of ARVC affected patients carried more than one mutation in any of desmosomal gene, but the phenotype associated with the double mutation carrier status was variable. However, other studies reported a gene dosage effect with patients carrying more than one mutation having a more severe phenotype.<sup>34 120</sup> These studies were performed in small cohorts, which necessitates further investigations to corroborate all these results. Our group also reported possible severe clinical phenotypes in patients carrying more than one mutation, but no greater structural abnormalities were identified in any of them. In the same study, we also reported an apparently more benign phenotype in genetic carriers of polymorphism p.P336L in the *PKP2* gene but the analysis should be interpreted with caution given the small sample size.<sup>121</sup>

Despite all these genetic data, current genotype–phenotype studies are too limited to establish a certain role of genetics in clinical practice. Up to now, the majority of data available on risk stratification and management strategies in patients with ARVC have relied on retrospective analyses in single centres and with rather small patient cohorts. In addition, it is well known that to perform a genetic testing in clinical practice also implies a genetic counselling, helpful adjunct in the diagnosis and management of the pathology.<sup>122</sup> Therefore, because of complex interpretation of genetic results in phenotype, current international recommendations for clinical cardiomyopathies support the involvement and collaboration among cardiologist, geneticists and even psychologists to perform a genetic counselling prior and subsequent to genetic testing.<sup>123 124</sup>

## CONCLUSIONS

In recent years, genetic analysis has been incorporated progressively into ARVC diagnosis. To date, several mutations in 12 genes have been related to the disease. However, around 35%–40% of the ARVC cases remain without a genetic cause identified. Thus, other unknown genes should be responsible for at least almost one part of these cases without genetic diagnosis. In addition to pathogenic point mutations, CNVs in these genes have been also related to the disease. In near future, new genetic technologies will allow faster, and less expensive, testing of more genes (previously related and new genes) and other genetic defects. Genetic testing is especially useful in families with at least one affected member who carries a pathogenic mutation identified because it allows establishing a presymptomatic diagnosis among relatives. However, even though crucial, genetic counselling in current clinical practice, on the basis of current genetic–phenotype knowledge, is speculative to establish an impact of genetics in clinical ARVC risk stratification.

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