Arrhythmogenic Cardiomyopathy

Impact of Genotype, Clinical Course, and Long-Term Outcome

Judith Groeneweg

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Aritmogene Cardiomyopathie
Invloed van het Genotype, Klinisch Beloop en Lange Termijn Uitkomst

(met een samenvatting in het Nederlands)

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“Begin at the beginning,” the King said, very gravely,
“and go on till you come to the end: then stop.”

Alice in Wonderland

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CHAPTER 1

Preface

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a hereditary cardiomyopathy characterized by an increased risk of sudden cardiac death (SCD) and ventricular arrhythmias with primarily right ventricular (RV) involvement.[1-3] However, predominant left ventricular (LV) forms are increasingly recognized.[4] Moreover, on the molecular level, both ventricles are affected in a similar manner.[5] Therefore, the more comprehensive 'Arrhythmogenic Cardiomyopathy' (AC) is the preferred terminology nowadays.[6]

Although familial occurrence was recognized since the first report on AC, only in the last decades has the genetic substrate been identified, mostly in genes encoding desmosomal proteins: plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), and desmocollin-2 (DSC2).[1, 7-11] In 30-70% of index patients a pathogenic AC-related mutation can be identified.[12-15] In The Netherlands, as in other European countries and in North America, mutations are mostly found in the *PKP2* gene.[12-16] However, in a substantial proportion of AC patients no mutation can be identified. Non-desmosomal genes may be involved in these cases. The interpretation of genetic screening results in AC is, as in other cardiomyopathies, puzzling. A pathogenic mutation found in index patients facilitates cascade screening of family members, identifying individuals at risk. However, molecular genetic analysis often results in the finding of genetic variants of uncertain clinical significance. For example, missense variants, resulting in only one amino acid substitution, are found in similar rates in cases and controls and their pathogenicity therefore remains controversial.[17] As stated previously, the distinction of pathogenic mutations from benign variants is important for clinical and risk management purposes. For this reason, the pathogenicity of variants of unknown significance is routinely estimated by *in silico* prediction algorithms. However, these predictions can be conflicting per different programs and the pathogenic potential may be over- or underestimated.[17-19] Functional experimental assessment can unambiguously determine the effect of genetic variants.[20, 21]

Arrhythmogenic Cardiomyopathy predominantly has an autosomal dominant inheritance pattern and is characterized by incomplete penetrance and a highly variable clinical expression.[16] Patients present typically between the second and fourth decade of life with monomorphic sustained ventricular tachycardia (VT). Nonetheless, SCD can occur as early as in adolescence, whereas mutation carriers may also remain without any signs and symptoms into old age. Little is known on the prognostic influence of the genotype on disease course since previous genotype-phenotype studies mostly included overt index patients and were limited by population size.

Arrhythmogenic Cardiomyopathy is diagnosed according to international consensus based Task Force Criteria (TFC), first formulated in 1994.[22] The TFC were revised in 2010, creating a new set of criteria that improved the diagnostic yield.[14, 15, 23, 24] In the classic description, four AC disease stages have been described, which not necessarily proceed from one into the other: 1) concealed stage without or with minimal structural disease, although sudden cardiac death may occur, 2) overt stage with structural alterations of primarily the RV and episodes of monomorphic VT, 3) overt stage with gross structural biventricular involvement, and 4) the end-stage of the disease with heart failure.[2, 3] Information on the long-term follow-up and outcome of AC index patients and, in particular, of at-risk family members is limited.

Diagnosis in the early concealed stage of disease, with increased risk of SCD, is the biggest clinical challenge facing physicians. The substrate for ventricular arrhythmias and SCD in AC is proposed to be intercalated disk remodeling, i.e. down-regulation and/or redistribution of

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desmosomal, gap junction, and sodium channel proteins, and altered tissue architecture due to fibrofatty replacement of cardiomyocytes, both resulting in the hallmark of the disease, activation delay.[3, 25-32] Non-invasive establishment of activation delay, facilitating assessment of diagnosis and risk management, has been shown feasible for the criteria: epsilon wave, prolonged terminal activation duration, and late potentials on signal averaged ECG, all included in the 2010 TFC. However, these parameters predominantly reflect activation delay in the RV outflow tract (RVOT). Non-invasive parameters of activation delay, early disease, and arrhythmogenic substrate when the RVOT is not affected remain to be identified.
In summary, the identification and management of individuals at risk for AC and SCD, both by genetic and clinical assessment, awaits optimization.

Aims

This thesis is based on these issues raised and had the following objectives:

- First, to evaluate the desmosomal and non-desmosomal genetic contribution, facilitate the interpretation of genetic screening results, and to define the impact of genotype on phenotype and disease course in AC.
- And second, to evaluate invasive and non-invasive measurements of arrhythmic substrate and to define the long-term follow-up and outcome in AC index patients and at risk family members.

The ultimate goal of this thesis was to support and improve early diagnosis and patient management in AC.

Outline

This thesis includes genetic and clinical studies in AC index patients and family members. The first part is focused on the impact of genotype in AC. **Chapter 2** provides a general overview of the genetic aspects, diagnosis, and clinical management in AC. The genetic background of AC is detailed in **Chapter 3**. A large family with left-dominant AC is described in **Chapter 4**. Contrary to the supposedly pathogenic *PKP2* variant (c.419C>T, p.Ser140Phe), the non-desmosomal phospholamban (*PLN*) founder mutation c.40_42delAGA (p.Arg14del) was demonstrated to cosegregate with disease. The genetic contribution and phenotypic characteristics of this *PLN* founder mutation in AC is assessed in **Chapter 5**, showing that *PLN* mutation carriers were characterized by low voltages and negative T waves in left precordial leads on the ECG and more often have additional LV structural and functional impairment compared to desmosomal mutation carriers. In **Chapter 6**, the pathogenicity of missense variants that fulfilled the criteria of a newly suggested algorithm, was shown to be similar to radical mutations. The predicted pathogenic potential of 9 AC splice site variants was experimentally validated in **Chapter 7**, demonstrating 6 variants to be pathogenic splice site mutations and 3 variants to be benign. In **Chapter 8**, the impact of genotype on AC disease course is assessed in a large transatlantic cohort of 577 mutation carriers. This study showed that: a clinical presentation with SCD occurs at a significantly younger age than a presentation with a monomorphic VT, differences in mutated genes result in a difference of disease expression, with patients with multiple mutations having a worse outcome, and that male gender negatively influences disease expression.

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The second part of this thesis is focused on the disease course and long-term outcome in AC. In **Chapter 9**, a case study on the end-stage of AC with detailed histopathological, immunohistochemical, and ultrastructural analysis is presented. The invasive evaluation of the arrhythmogenic substrate in AC by electrophysiological studies and voltage mapping is shown in the context of the current hypothesis on AC disease pattern and revised 2010 Task Force Criteria and ultimately related to non-invasive measurements on the ECG in **Chapter 10**. In **Chapter 11**, an editorial comment on the study by Santangeli et al.[33], evaluating the correlation between activation delay detected on signal averaged ECG and histopathological evaluation of the RV outflow tract is provided. The clinical presentation, long-term follow-up, and disease penetrance of AC index patients and at risk family members in a transatlantic cohort of 1001 individuals is described in **Chapter 12**. Long-term outcome was favorable in diagnosed and appropriately treated index patients, one third of family members developed AC and their outcome was related to symptoms at presentation and the presence of pathogenic mutations. Finally, the results and implications of these studies are put into perspective in **Chapter 13**.

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CHAPTER 2

Arrhythmogenic Cardiomyopathy
Diagnosis, Genetic Background, and Risk Management

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is histopathologically characterized by progressive fibrofatty replacement of cardiomyocytes, primarily in the right ventricle (RV).[1-3] However, histopathologically and functionally the left ventricle (LV) is affected in many cases and both ventricles are similarly affected by desmosomal and gap junctional protein redistribution.[4, 5] Because of these observations, at present Arrhythmogenic Cardiomyopathy (AC) is the preferred terminology.[6] AC can be defined as a structural myocardial disease *preceded* by ventricular arrhythmias. Typical ARVD/C with predominant RV abnormalities can be considered as a large and important subcategory of AC. Clinical diagnosis is made according to international consensus-based Task Force Criteria.[7, 8]
The first series of ARVD/C patients was published in 1982.[1] It was described as a developmental disease of the right ventricular musculature, hence the terminology "dysplasia". In the past 25 years, increased insight in the development of the disease as well as the discovery of pathogenic gene mutations involved led to the current understanding that AC is a genetically determined cardiomyopathy. The molecular genetic substrate for the disease is mainly acknowledged in genes encoding desmosomal adhesion proteins in the intercalated disk.[9-14]
This review provides an overview of AC, from phenotypic and genetic features of the disease, to diagnosis, risk stratification and treatment options.

Epidemiology
Estimations of the prevalence of AC in the general population vary from 1:1000 to 1:5000.[15, 16] The real prevalence of AC, however, is unknown and is presumably higher due to many non-diagnosed and misdiagnosed cases. In one study as many as 20% of sudden deaths occurring in people under 35 years of age, features of AC were detected at post mortem evaluation.[17] In nearly half of them, no prior symptoms had been reported. Furthermore in the study by Tabib et al.[18], 26% of forensic autopsy cases following exercise-related sudden cardiac death (SCD) under the age of 30 years, revealed AC.
From the genetic point of view, both men and women should be equally affected. However, men are more frequently diagnosed with AC than women. In a large multicenter study, 57% of affected individuals were male. As many women as men show at least some signs of disease, but women less frequently fulfil criteria to meet the diagnosis.[19] It is speculated that (sports) activity or hormonal factors may play a role in this difference in severity of disease expression.20
Familial disease, with AC diagnosis in at least one other family member besides the index patient, has been demonstrated in more than one third of AC cases.[21, 22]

Presentation
Patients with AC typically present between the second and the fourth decade of life with palpitations, lightheadedness, or syncope due to ventricular ectopy or (monomorphic) ventricular tachycardia (VT) with left bundle branch block (LBBB) morphology, thus originating from the RV (Figure 1). However, SCD may be the first clinical manifestation, often at young age in the concealed stage of disease. In a study by Quarta et al.[22], SCD was the presenting symptom in 50% of index patients, with SCD occurring in 31% at young age, i.e. between 14–20 years.

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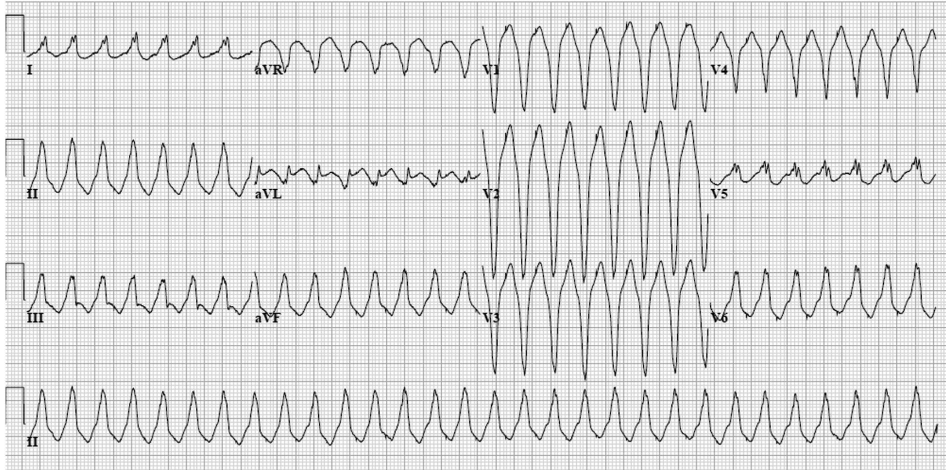

Figure 1. 12-lead ECG of a ventricular tachycardia (VT) in an Arrhythmogenic Cardiomyopathy (AC) index patient with a pathogenic and a most likely pathogenic plakophilin-2 mutation (c.397C>T p.Gln133* and c.2615C>T p.Thr872Ile). The VT has a left bundle branch block morphology, with inferior axis. The QRS complex is predominantly negative in lead aVL and most positive in lead II, suggesting an origin from the right ventricular outflow tract area in the right ventricle.

A presentation with (aborted) SCD occurred less often in other studies.[19, 23-25] In the Dutch AC cohort, 2/149 (1,3%) index patients presented with SCD and 8% (12/149) with aborted SCD.[21] AC can present in four clinical stages which not necessarily proceed from one into the other: 1) concealed stage without or with minimal structural disease, although SCD may occur, 2) overt stage with structural alterations of primarily the RV, and episodes of monomorphic VT, 3) overt stage with obvious structural biventricular involvement, and 4) the end-stage of the disease with heart failure.[1, 26, 27] Recently, LV dominant and primarily biventricular variants have also been described, in analogy with the AC concept.[4, 28]

Diagnosis

Accurate AC diagnosis is critical due to lifelong implications, not only for the index patient but also for family members. Diagnosis has been facilitated by a set of clinically applicable criteria. These criteria, originally formulated for ARVD/C, were defined by an international Task Force based on consensus in 1994 and were revised in 2010.[7, 8] In the revised TFC a minor criterion for (additional) LV involvement, inverted T waves in left precordial leads V4-6, was incorporated. The current Task Force Criteria (TFC) are the essential standard for a correct diagnosis in individuals suspected of AC. In addition, its universal acceptance contributes importantly to unambiguous interpretation of clinical studies and facilitates comparison of results. The TFC include six different categories: 1) global and/or regional dysfunction and structural RV alterations, 2) tissue characterization, 3) depolarization abnormalities, 4) repolarization abnormalities, 5) arrhythmias, and 6) family history and genetics. Within these groups, diagnostic criteria are categorized as major or minor according to their disease specificity. A diagnosis of AC is made

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with the fulfilment of two major, one major and two minor, or with four minor TFC. From each different category, only one criterion can be counted for diagnosis, even when multiple criteria in one group are present. Table 1 provides an overview of the TFC defined in 2010. Figure 2 demonstrates typical repolarization abnormalities in AC patients. Several studies suggested that this new set of criteria improved the diagnostic yield, with equal specificity.[22, 29, 30] Despite these efforts, AC diagnosis in the concealed stage of disease with subsequent risk for SCD still poses a great challenge for physicians.

Specific evaluations are recommended in all patients suspected of AC. Detailed history and family history, physical examination, 12-lead ECG (while off medications), signal averaged ECG, 24-hours Holter monitoring, maximal exercise testing, 2D echocardiography with quantitative wall motion analysis, and more detailed imaging by cardiac magnetic resonance imaging (MRI) with delayed enhancement analysis. Invasive tests are also useful for diagnostic purposes: RV and LV cineangiography, electrophysiological testing, and endomyocardial biopsies for histopathologic and immunohistochemical analysis.[5, 31]

Differential diagnosis

Early and occasionally late stages of AC may show similarities with a few other diseases. In particular, differentiation from idiopathic VT originating from the RV outflow tract (RVOT) can be challenging. However, idiopathic RVOT VT is a benign non-familial condition, in which the ECG shows no depolarization or repolarization abnormalities and no RV structural changes can be detected. Furthermore, VT episodes have a single morphology (LBBB morphology with inferior axis) and are based on abnormal automaticity or triggered activity, whereas reentry is the prevalent arrhythmia mechanism in AC.[32, 32, 33, 33] It is important to differentiate idiopathic RVOT VT from AC with regard to screening of family members, prognosis, and outcome of catheter ablation.

Another disease mimicking AC is cardiac sarcoidosis.[34] Clinical symptoms of cardiac involvement are present in about 5% of all patients with sarcoidosis. In a study by Vasaiwala et al.[35] a remarkably high incidence (15%) of cardiac sarcoidosis was found in patients with suspected AC. Presence of extracardiac sarcoidosis, mediastinal lymphadenopathy, septal conduction abnormalities, and septal scar on cardiac imaging may be indicative of cardiac sarcoidosis rather than AC as disease aetiology.[36] Histopathological findings from endomyocardial biopsies can differentiate between the two entities. Nonetheless, cardiac sarcoidosis may mimic AC even on the molecular level. In a recent study by Asimaki et al.[37] a markedly reduced immunoreactive signal in the intercalated disk of the desmosomal protein plakoglobin was observed in AC as well as in cardiac sarcoidosis patients.

Myocarditis might also be considered in the differential diagnosis. In general, endomyocardial biopsy is required to distinguish myocarditis from AC. In analogy with cardiac sarcoidosis, giant cell myocarditis may be indistinguishable from AC using immunohistochemical analysis. In contrast, viral myocarditis does not result in plakoglobin redistribution from the intercalated disk. Differential profiles of cytokines are implicated to underlie this difference in disruption of desmosomal proteins.[37]

AC may also mimic dilated cardiomyopathy (DCM), especially in the more advanced stages of disease. However, patients with DCM usually present with heart failure rather than arrhythmias.

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Table 1. 2010 Task force criteria for AC diagnosis

I. Global or regional dysfunction and structural alterations

Major:

- By 2D echo

- Regional RV akinesia, dyskinesia, or aneurysm
- And 1 of the following (end diastole): PLAX RVOT ≥32mm (correct for body size [PLAX/BSA] ≥19 mm/m2), PSAX ≥36mm (correct for body size [PSAX/BSA] ≥21 mm/m2, or fractional area change <33%

- By MRI

- Regional RV akinesia or dyskinesia or dyssynchronous RV contraction
- And 1 of the following: ratio of RVEDV to BSA ≥110 mL/m2 (male) or ≥100 mL/m2 (female), or RV ejection fraction ≤40%

- By RV cine-angiography

- Regional RV akinesia, dyskinesia, or aneurysm

Minor:

- By 2D echo

- Regional RV akinesia or dyskinesia
- And 1 of the following (end diastole): PLAX RVOT ≥29mm to <32mm (correct for body size [PLAX/BSA] ≥16 mm/m2 to <19mm/m2), PSAX ≥32mm to <36mm (correct for body size [PSAX/BSA] ≥18mm/m2 to <21mm/m2, 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 RVEDV to BSA ≥100 mL/m2 to <110 mL/m2 (male) or ≥90 mL/m2 to <100 mL/m2 (female), or RV ejection fraction >40% to ≤45%

II. Tissue characterization of wall

Major:

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

Minor:

- Residual myocytes 60-75% by morphometric analysis (or 50-65% 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 (V1, V2, V3) or beyond in individuals >14 years of age

Minor:

- Inverted T waves in leads V1 and V2 in individuals >14 years of age or in V4, V5, V6
- Inverted T waves in leads V1, V2, V3 and V4 in individuals >14 years of age in the presence of complete right bundle- branch block

IV. Depolarization/ conduction abnormalities

Major:

- Epsilon wave (reproducible low- amplitude signals after the end of the QRS complex to onset of the T wave) in right precordial leads (V1, V2, V3)

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Minor:

- Late potentials by SAECG in ≥1 of 3 parameters in the absence of a QRS duration of ≥110ms on the standard ECG
- Filtered QRS duration (fQRS) ≥114ms
- Duration of terminal QRS <40 uV (low-amplitude signal duration) ≥38ms
- Root-mean-square voltage of terminal 40ms ≤20 uV
- Terminal activation duration ≥55ms measured from the nadir of the S wave to the end of all depolarization deflections, including R', in V1, V2 or V3 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:

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

VI. Family History

Major:

- AC confirmed in a first-degree relative who meets current TFC
- AC confirmed pathologically at autopsy or surgery in a first-degree relative
- Indentification of a pathogenic mutation categorized as associated or probably associated with AC in the patient under evaluation

Minor:

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

This table provides an overview of the current Task Force Criteria for Arrhythmogenic Cardiomyopathy (AC) diagnosis. The TFC, originally formulated for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C), include six different categories. Within these groups, diagnostic criteria are categorized as major or minor according to their disease specificity. A diagnosis of AC is made with the fulfilment of two major, one major and two minor, or with four minor TFC. From each different category, only one criterion can be counted for diagnosis, even when multiple criteria in one group are present. BSA= body surface area; First degree family member= parent, sibling or child; PLAX= parasternal long- axis view; PSAX= parasternal short- axis view; RVOT= right ventricular outflow tract; Second degree family member= uncle/aunt, niece/nephew, grandparent.

Thus, patients with sustained VT or SCD as the initial symptom of a supposed DCM, should also be screened for AC.

Finally, there are a number of other rare differential diagnosis to consider: Brugada syndrome with similar electrocardiographic or RV arrhythmias, congenital abnormalities such as Uhl's disease, Ebstein's anomaly, and atrial-septal defects, RV infarction, and possibly pulmonary hypertension.[27]

Molecular genetic background of AC

In 2000, the seminal discovery of mutations in the plakoglobin (*JUP*) gene as the basis of Naxos disease, an autosomal recessive cardio-cutaneous syndrome with AC, directed the search for the genetic substrate to other genes encoding desmosomal proteins.[9] This candidate gene approach identified mutations first in the desmoplakin (*DSP*) gene, and thereafter in the plakophilin-2 (*PKP2*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*) genes.[10, 11, 13, 14]

In The Netherlands, as in most European countries and in North-America, mutations are predominantly found in the *PKP2* gene.[21, 22, 25, 38, 39] *PKP2* mutations are found in 52% of Dutch AC index patients and even in 90% of familial cases.[21] This high yield is partly explained by the occurrence of founder mutations in The Netherlands. Haplotype analysis suggested a founder effect of four different *PKP2* mutations.[38, 40] On the other hand, there are geographical differences in the prevalence of AC-related gene mutations.[13, 41]

Desmosomes are protein complexes located in the intercalated disk and are amongst others important for mechanical integrity of adjacent cardiomyocytes (Figure 3).[42] Desmosomal dysfunction due to a gene mutation may give rise to loss of mechanical cell-cell adhesion, and leads to down-regulation and/or altered distribution of other intercalated disk proteins, i.e. gap junction proteins (Connexin43) and sodium channels (Nav1.5).[43-45] These alterations give rise to electrical cell-cell uncoupling and slow conduction respectively, thereby providing a substrate for early activation delay resulting in ventricular tachyarrhythmia, a hallmark of AC.[5, 12, 46, 47] Presumably, at a later stage myocyte loss and fibrofatty replacement will have a major impact on tissue architecture, giving rise to zig-zag conduction pathways and load mismatch, further contributing to enhanced activation delay.[32, 48]

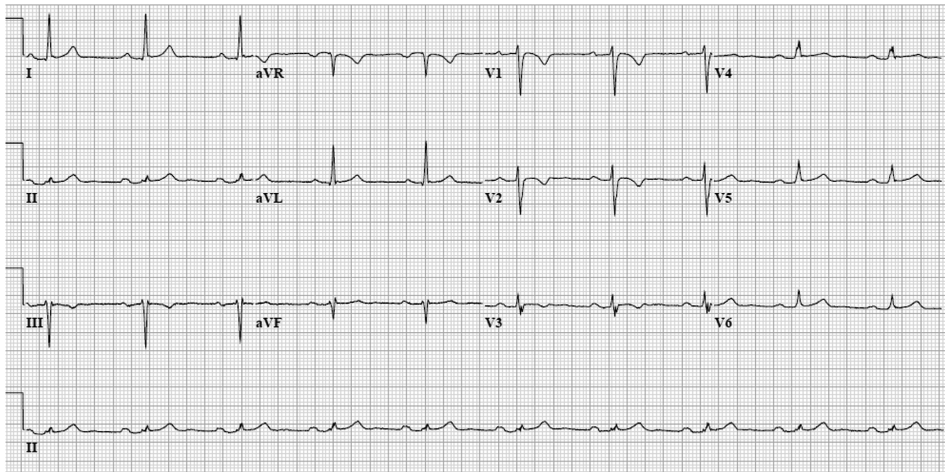

Figure 2. 12-lead ECG (during Atenolol 25mg one time daily) of the same index patient as in Figure 1. The ECG shows sinus rhythm, horizontal axis, and typical negative T waves in right precordial leads V1-3. The terminal activation duration (from the nadir of the S wave to the end of all depolarization deflections) is leads V1-3 in normal (≤55 ms).

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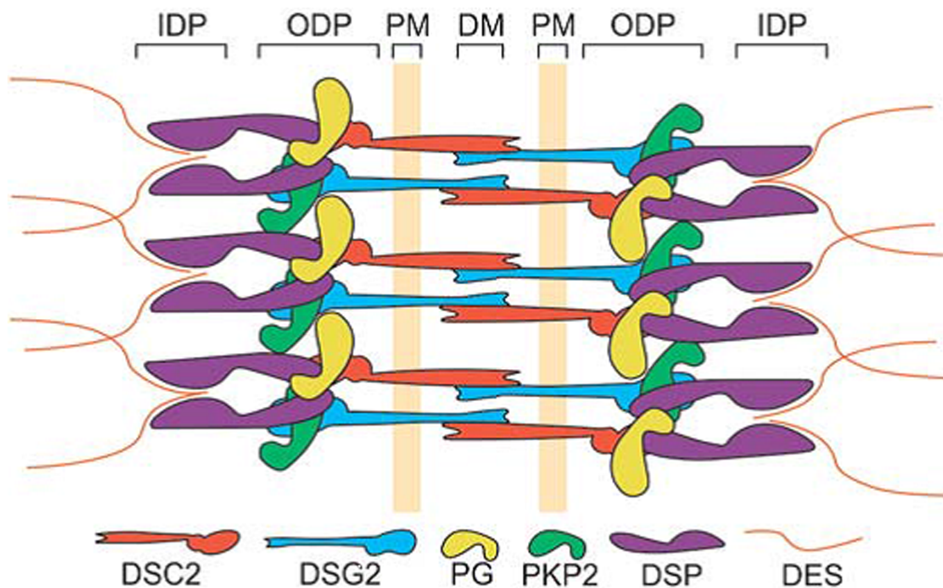

Figure 3. Schematic representation of the molecular organization of cardiac desmosomes. The plasma membrane (PM) spanning proteins desmocollin-2 (DSC2) and desmoglein-2 (DSG2) interact in the extracellular space at the dense midline (DM). At the cytoplasmic side, they interact with plakoglobin (PG) and plakophilin-2 (PKP2) at the outer dense plaque (ODP). The PKP2 and PG also interact with desmoplakin (DSP). At the inner dense plaque (IDP), the C-terminus of DSP anchors the intermediate filament desmin (DES). (Source: Reprint with permission from: Van Tintelen et al. Curr Opin Cardiol. 2007;22:185-92).

Although the function of the desmosome and other components of the intercalated disk becomes increasingly clear, the exact mechanism by which a gene mutation results in the disease remains to be elucidated. Furthermore, not every subject with a mutation and thus a predisposition for AC develops signs and symptoms of the disease. Additional genetic factors, e.g. compound or digenic heterozygosity (carrying more than one mutation in one or more genes), or environmental factors such as exercise, may explain differences in severity of disease evolution within mutation carriers.[49, 50]

In a minority of patients a non-desmosomal gene mutation is associated with the AC phenotype. Mutations in the non-desmosomal transforming growth factor β3 (TGFβ3), transmembrane protein 43 (*TMEM43*), desmin (*DES*), titin (*TTN*), lamin A/C (*LMNA*), αT-catenin (*CTNNA3*), and phospholamban (*PLN*) genes have been related to index patients and/or families with AC.[51-57] Of note, the *PLN* founder mutation c.40_42delAGA has been identified in 13% of AC index patients in The Netherlands. AC patients with the PLN mutation often have low voltage electrocardiograms, negative T waves in left precordial leads V4-6, and additional LV involvement (Figure 4).[58, 59] To facilitate interpretation of genetic data, a large web-based database of genes and mutations underlying AC has been created (www.arvcdatabase.info). This database currently contains information on nearly 900 variants.[60]

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Not all forms of AC are genetically proven. These cases may be explained by mutations in yet unknown genes, by the contribution of genetic variants of unknown significance in the known genes, or epigenetic phenomena. The evaluation of a family history of AC, suggesting a still unknown genetic factor, is crucial in patients without identifiable genetic predisposition. Alternatively, these cases may be due to environmental factors, for example exercise.
Exercise was a trigger and accelerator of the AC phenotype in a mouse model with JUP haploinsufficiency.[61] Very recently, the effect of exercise on AC disease expression was corroborated in humans. Of 87 pathogenic AC mutation carriers, 56 were endurance athletes. Endurance athletes were more likely to have symptoms at young age, fulfil the 2010 TFC, and had lower survival free from VT, ventricular fibrillation (VF), and heart failure. A reversible effect of reduction of sports activities, being a significant decrease in risk for VT or VF, was observed in those individuals that exercised the most (top quartile).[20]

Risk stratification

Risk stratification in AC is imperfect at present. The annual mortality rates reported vary from 0.08-3.6%.[23, 24, 62, 63] Retrospective analysis of clinical and pathologic studies identified several risk factors for sudden death or appropriate ICD therapy, such as previously aborted SCD, syncope, young age, severe RV dysfunction, and LV involvement. Patients without VT had the best prognosis.[23, 63] Presence of sustained and non-sustained VT on Holter monitoring or during exercise testing, and sustained VT/VF during electrophysiologic study were significant predictors of ICD therapy in a multivariate analysis in 84 patients treated with ICDs for primary prevention (appropriate ICD therapy in 48%, of which 19% for ventricular fibrillation/flutter episodes).[64] More recently, Te Riele et al.[65] showed that sustained arrhythmias in AC patients presenting alive seem to coincide with structural abnormalities and that patients with solely electrical abnormalities have a lower arrhythmic risk, implicating a role for RV structural abnormalities in risk stratification.
Molecular genetic analysis is also of importance in risk stratification in AC since cascade screening allows early detection of presymptomatic disease, identification of individuals at risk, and genetic counselling for this sudden death-predisposing disease. However, incorporating genetic results in AC risk stratification is hampered by incomplete penetrance and an extremely variable clinical expression. Mutation carriers may present with SCD but can also remain without signs and symptoms of the disease into old age. Therefore, genetic screening results should be viewed as probabilistic and as part of the overall clinical assessment.
Genotype-phenotype correlation analysis can provide more insight in risk profiles of index patients and family members. In contrast to index patients, mutation-positive family members have a better prognosis, with signs and symptoms of AC present in 50% of relatives in their 5th decades of life.[22] Furthermore, family members with more than one genetic variant had a significant five-fold increase in risk of disease expression, suggesting gene-gene interactions and gene-dose effects. A genotype-phenotype correlation study by our group showed that compared to relatives of index patients without mutations, mutation carrying family members had 1) a six-fold higher risk of AC diagnosis, 2) a markedly enhanced risk for ventricular arrhythmias, and 3) earlier onset of AC signs and symptoms.[21]

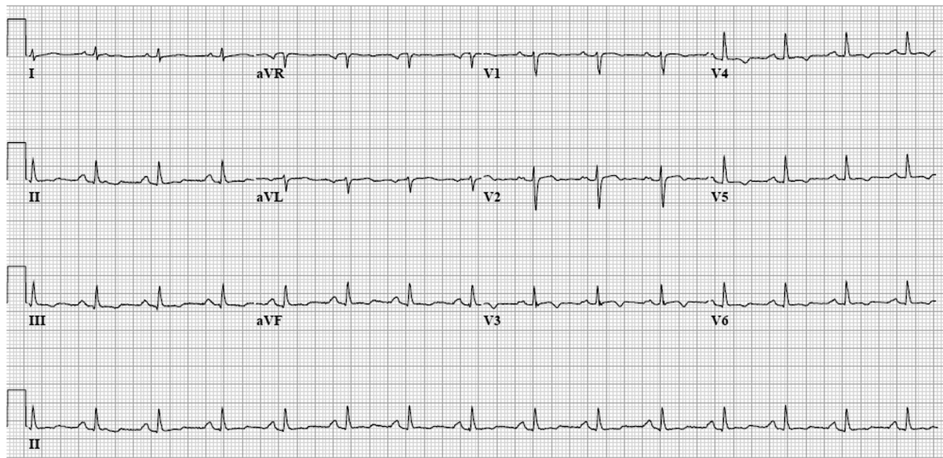

Figure 4. 12-lead ECG (while off medications) of a phospholamban founder mutation carrier (c.40_42delAGA, p.Arg14del). The ECG shows sinus rhythm with intermediate axis, and characteristic negative T waves in left precordial leads from V3-6. The voltages do not meet the criteria for a low voltage ECG (<0.5mV in standard leads). This patient has Arrhythmogenic Cardiomyopathy (AC) based on: a first-degree relative with AC (major criterion), negative T waves in leads V4-6 (minor criterion), and >500 ventricular extrasystoles per 24-hours (minor criterion).

Clinical management

In addition to symptomatic treatment, prevention of SCD is the most important therapeutic goal in AC. As there have been no randomized trials of AC treatment modalities, screening regimens, or medications, most recommendations are based on clinical expertise, results of retrospective registry-based studies, and studies on model-systems.

Evidence suggests that, in the absence of sustained and non-sustained VT and/or high number of ventricular premature complexes (>1000 VPC/24 hours), asymptomatic patients and healthy mutation carriers do not require treatment with an ICD for primary prevention.[64] These individuals should undergo regular cardiac evaluations (every 1-2 years) including 12-lead ECG, 24-hours Holter monitoring, echocardiography, and exercise testing for timely identification of unfavorable signs necessitating ICD implantation.

However, in all patients diagnosed with or having signs or symptoms of AC as well as asymptomatic mutation carriers, specific life style advice is recommended. Sports participation has been shown to increase the risk of SCD five-fold in AC patients.[66] Furthermore, excessive mechanical stress, such as during competitive sports activity and training, may aggravate underlying myocardial abnormalities and accelerate disease progression.[20, 61] Therefore, patients with AC, and in our opinion also all pathogenic mutation carriers, should be advised against practicing competitive and endurance sports.

Therapeutic options in symptomatic patients with AC include antiarrhythmic drugs, catheter ablation, and ICD implantation. However, at present ICD implantation is the only proven lifesaving therapeutic modality for fast VT/VF. In patients who have presented with stable sustained VT but also patients and family members with non-sustained VT or >500 VPC on 24-hours Holter

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monitoring, medication may be considered to reduce arrhythmias and symptoms. This pharmacologic reduction may be critically important in combination with ICD therapy to reduce shock delivery. Since ventricular arrhythmias and cardiac arrest occur frequently during or after physical exercise or may be triggered by catecholamines, non-class III antiadrenergic beta-blockers are recommended. In the absence of adequate anti-arrhythmic response, Sotalol in an appropriate dose is the drug of first choice. Alternatively, Amiodarone and Flecainide have been reported as useful.[67] Efficacy of drug treatment has to be evaluated by serial Holter monitoring and/or exercise testing.

Catheter ablation is an alternative in patients who are refractory to drug treatment and have frequent VT episodes (with a predominantly single morphology).[68] Of note, the role of this therapy in AC patients is to improve quality of life by decreasing the frequency of episodes of sustained VT, symptomatic non-sustained VT, and ventricular ectopy. Accordingly, in a recent study by Philips et al.[69] the overall freedom from VT of 175 ablation procedures in 87 AC patients was 47%, 21%, and 15%, at 1, 5, and 10 years respectively, over a mean follow-up of 88.3±66 months. The outcomes of VT ablation are improved with a combined endocardial and epicardial approach, incorporating a substrate-based strategy.[69, 70]

Although antiarrhythmic drugs and catheter ablation may reduce VT burden, there is no proof from prospective trials that these therapies will also prevent SCD. Implantation of an ICD is indicated in AC patients who are intolerant to antiarrhythmic drug therapy and who are at serious risk for SCD, in patients with aborted cardiac arrest, intolerable fast VT and those with risk factors as mentioned above.

AC in the Netherlands

In The Netherlands, a national collaboration of all in ICIN participating University Medical Centers has resulted in a large dataset with genetic and phenotypic characteristics of AC index patients and family members.[21] Since the prevalence of AC is relatively low, collaboration is essential for adequate studies and thereby improvement of insight in AC. This collaboration has expedited studies on AC related genes, founder mutations, new diagnostic AC parameters, and clinical outcome of index patients and family members. Continuation of inclusion of more individuals, longer follow-up, and implementation of results of next generation DNA sequencing are needed for further risk stratification optimization.

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CHAPTER 3

Genetic Background of Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia

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Introduction

Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC/D) is considered a hereditary disease. Although familial occurrence was recognized since the first report on ARVC/D, only in recent years the genetic substrate has been identified. In 1736, the physician of the Pope, Giovanni Maria Lancisi, reported in his book 'De Motu Cordis et Aneurysmatibus' on a family of four generations with palpitations, heart failure, right ventricular (RV) dilatation and aneurysms, and sudden death. Also in the first systematic study on ARVC/D in 1982 by Marcus et al.[1], the familial occurrence was observed. Nearly two decades later the small Greek island Naxos proved crucial in identification of the genetic basis of the disease. On Naxos, families with a triad of palmoplantar keratoderma, woolly hair and ARVC/D were studied. In 2000 Mckoy et al.[2] identified a mutation in the plakoglobin (*JUP*) gene in affected family members.

Naxos disease is a recessive form of ARVC/D. Affected family members have a homozygous 2 base pair deletion mutation in *JUP* whereas heterozygous carriers show no sign of disease.[2] Another recessive form of ARVC/D, the Carvajal syndrome, is associated with a homozygous mutation in the desmoplakin (*DSP*) gene, and is also characterized by palmoplantar keratoderma, woolly hair, and typically RV and left ventricular (LV) involvement.[3] Following these first publications on rare recessive forms of ARVC/D, other mutations in desmosomal and non-desmosomal genes have been related to the common form of ARVC/D with an autosomal dominant inheritance pattern with solely cardiac involvement.

Autosomal dominant ARVC/D is characterized by a highly variable clinical expression and reduced penetrance of pathogenic gene mutations.[4, 5] Patients with ARVC/D typically present between the second and fourth decade of life with a monomorphic ventricular tachycardia (VT). Nonetheless, sudden cardiac death (SCD) can be the first clinical manifestation as well, often as early as in adolescence, whereas mutation carriers may also remain without signs and symptoms of disease into old age. This variability, as illustrated by the pedigree in Figure 1, may be caused by genetic and environmental factors and poses a great challenge for physicians. Therefore, increasing insight in the genetic background of ARVC/D supports adequate risk stratification and management of ARVC/D patients and their family members.

Desmosomal genes

The molecular genetic era provided new perception of the hypothesis that ARVC/D is a desmosomal disease resulting from defective adhesion between cardiomyocytes. The first genetic ARVC/D locus was detected in 1994 by Rampazzo et al.[6], followed by the detection of ten other loci.[7-11] The seminal discovery of the *JUP* mutation in relation to the ARVC/D phenotype directed the search for the genetic substrate to other genes encoding desmosomal proteins. This candidate gene approach identified mutations first in the desmoplakin (*DSP*) gene, and thereafter in the plakophilin-2 (*PKP2*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*) genes.[12-15] At present, mutations in the five known desmosomal genes (*PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2*) are found in the majority of (familial) ARVC/D cases.[16-18] In The Netherlands, as in most European countries and in North-America, mutations are predominantly found in the *PKP2* gene.[4, 5, 13, 17-19] *PKP2* mutations are found in 52% of Dutch ARVC/D index patients and even in 90% of familial cases.[18] This high yield of *PKP2* mutations is partly explained by the occurrence of founder

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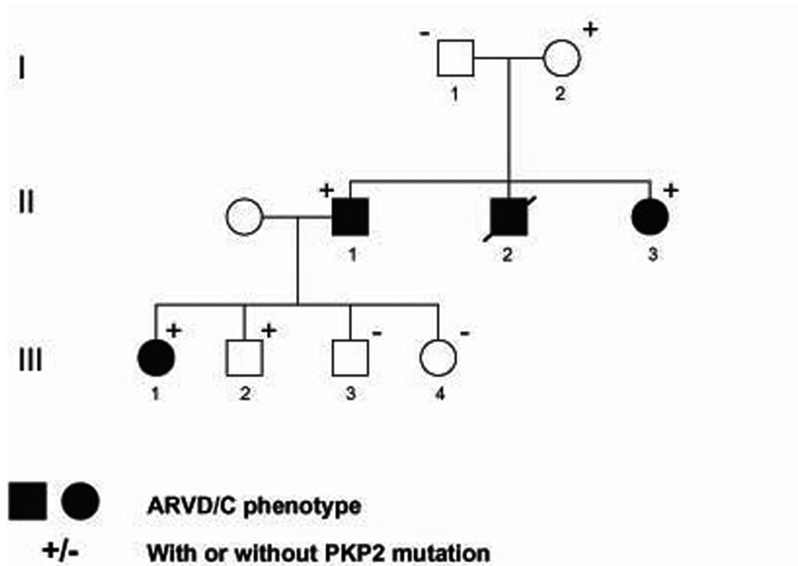

Figure 1. Pedigree of a family with ARVC/D and the PKP2 c.1211dupT mutation, illustrating the variable clinical expression and reduced penetrance which is a characteristic of ARVC/D. The index patient (patient II:1) was analyzed after the sudden cardiac death of his brother at age 17 (patient II:2). The index patient, who was successfully resuscitated after cardiac arrest due to ventricular fibrillation, his sister (patient II:3, with RV aneurysm), and his daughter (patient III:1, with syncope and nonsustained VT) fulfilled 2010 Task Force Criteria for diagnosis, whereas his mother (I:1) and his son (patient III:2) carry the same mutation but do not show any sign or symptom of ARVC/D. Since this mother was over 70 years of age, reduced penetrance is clearly demonstrated in this family.
With kind permission from Springer Science+Business Media: Clinical Cardiogenetics, Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy, 2011, 82, H.F. Baars, P.A.F.M. Doevendans and J.J. van der Smagt (eds.), M.G.P.J. Cox, R.N.W. Hauer (chap. authors), Figure 5.3.

mutations in The Netherlands. Haplotype analysis suggested a founder effect of four different *PKP2* mutations.[5] On the other hand, there are geographical differences in the prevalence of ARVC/D-related gene mutations. In Italy, mutations are most frequently found in the *DSP* gene (16%), followed by the *PKP2* (14%) and *DSG2* (10%) gene, which was confirmed in recent studies.[14, 20]

Desmosomes are protein complexes located in the intercalated disk which are important for mechanical integrity of adjacent cardiomyocytes (Figure 2).[21] Desmosomal dysfunction due to a gene mutation may give rise to loss of mechanical cell-cell adhesion, and leads to down-regulation and/or altered distribution of other intercalated disk proteins, i.e. gap junction proteins (Connexin43) and sodium channels (Nav1.5).[22-24] These alterations give rise to electrical cell-cell uncoupling and slow conduction respectively, thereby providing a substrate for early activation delay resulting in ventricular tachyarrhythmia, a hallmark of ARVC/D.[25-29] Presumably, at a later stage myocyte loss and fibrofatty replacement will have a major impact on tissue architecture, giving rise to zig-zag conduction pathways and load mismatch, further contributing to enhanced activation delay.[30-32]

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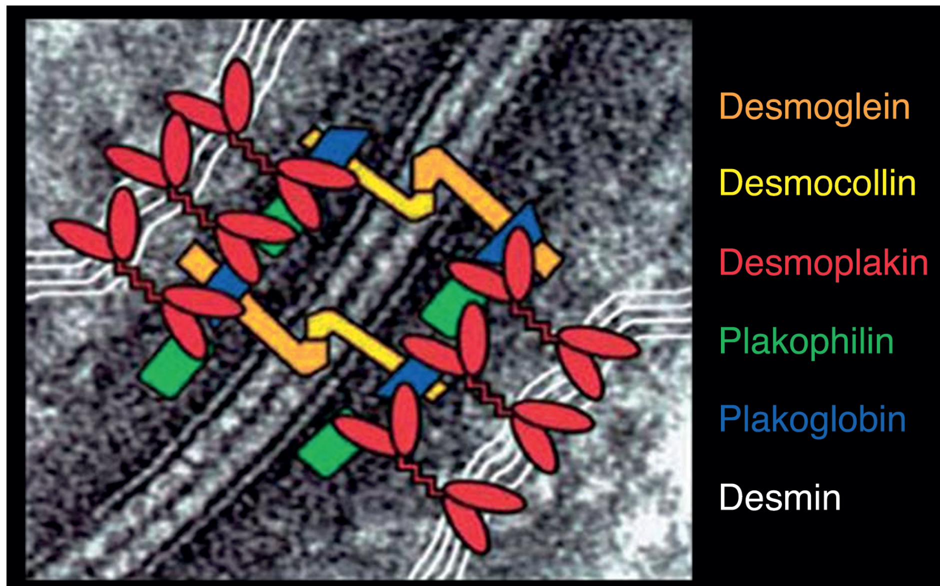

Figure 2. Graphic depiction of the cardiac desmosomal proteins and their localization. Shown are two cardiomyocytes separated by the intercalated disk. The complex of desmosomal proteins functions as 'bridges' between the two adjacent cells, providing mechanical and electrical stability. Desmocollin-2 (yellow), desmoglein-2 (orange), plakophilin-2 (green), plakoglobin (blue), desmoplakin (red), desmin (intermediate filament, white).

Although the function of the desmosome and other components of the intercalated disk seem clear, the exact mechanism by which a gene mutation results in the disease remains to be elucidated. Furthermore, not every subject with a mutation and thus a predisposition for ARVC/D develops signs and symptoms of the disease. Additional genetic factors, e.g. compound or digenic heterozygosity, or environmental factors such as exercise, may explain differences in severity of disease evolution within mutation carriers.[33, 34]

Non-desmosomal genes

In a minority of patients a non-desmosomal gene mutation is associated with the ARVC/D phenotype. This was first described in one Italian family with a mutation in the cardiac ryanodin receptor (*RYR2*) gene, which is responsible for calcium release from the sarcoplasmic reticulum.[35] Affected subjects had exercise-induced polymorphic VT. Generally, *RYR2* mutations lead to catecholaminergic polymorphic VT without structural abnormalities. The *RYR2* mutation associated with ARVC/D has been advocated to act differently from those in familial polymorphic VT without ARVC/D.[36]

Transforming growth factors β (TGFβs) regulate the production of extracellular matrix components and modulates expression of genes encoding desmosomal proteins. The gene (TGFβ3) has been mapped to chromosome 14. A mutation in the '5 UTR promoter region of TGFβ3, with a predicted inhibitory effect, was found in clinically affected members of one large ARVC/D family. In the

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same study, an additional '3 UTR mutation in TGFβ3 was found in an unrelated individual with ARVC/D.[37] These observations implicated that regulatory mutations resulting in overexpression and enhanced activity of TGFβ3 may lead to fibrosis and thereby contribute to the development of ARVC/D.

A missense mutation in the transmembrane protein 43 (*TMEM43*) gene was found in 15 unrelated ARVC/D families from a genetically isolated population in New Foundland and caused a fully penetrant, sex influenced, high-risk form of ARVC/D.[38] The *TMEM43* gene contains the response element for PPARγ, an adipogenic transcription factor. The mutation is thought to cause dysregulation of an adipogenic pathway regulated by PPARγ, which may explain the fibrofatty replacement in these ARVC/D patients.

Mutations in the desmine (*DES*) gene, encoding an intermediate filament protein, have been described to underlie a heterogenous disease spectrum with occasionally an ARVC/D phenotype. In a family with signs and symptoms of ARVC/D but without mutations in the *PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2* genes, the *DES* mutation c.1360C>T was identified. This mutation has been demonstrated to affect the localization of *DSP* and *PKP2* in the intercalated disk, suggesting a link between desmosomal and *DES* associated cardiomyopathies.[39, 40]

The cardiomyopathy gene titin (*TTN*) was evaluated as a candidate gene because of its proximity to an ARVC/D locus at position 2q32 and the connection of *TTN* to the transitional junction at intercalated disks. Mutations in the desmosomal genes *PKP2*, *DSP*, *DSG2*, and *DSC2* were excluded. Of the eight missense *TTN* variants identified in seven families, one (p.Thr2896Ile) showed complete cosegregation with the ARVC/D phenotype in a single large family.[41]

Lamin A/C (*LMNA*) gene mutations were found in 4% of patients (4/108) with borderline or definite ARVC/D diagnosis in a cohort from the United Kingdom, with exclusion of mutations in *PKP2*, *DSP*, *JUP*, *DSG2*, and *DSC2*.[42] These patients mostly had severe structural abnormalities and conduction abnormalities on their ECG. In addition, two of four patients had classic fibrofatty replacement with endomyocardial biopsy.[42]

A founder mutation in the non-desmosomal phospholamban (*PLN*) gene, involved in calcium homeostasis by interaction with the SERCA-pump, was identified in 15% of Dutch patients diagnosed with dilated cardiomyopathy and in 12% of ARVC/D index patients, in the absence of mutations in the five desmosomal genes.[43] Patients with this c.40_42delAGA mutation displayed classic ARVC/D with RV and additional LV involvement and low voltages (voltages <0.5 mV in standard leads) on their ECG (Figure 3). Evidence of pathogenicity was strongly supported by cosegregation analysis in a large family with ARVC/D.[44]

ARVC/D without identified genetic mutations

Not all forms of ARVC/D are proven genetic. Recent preliminary data indicate that in approximately 30% of Dutch index patients no mutation can be found by screening of the desmosomal and non-desmosomal candidate genes. In other cohorts this percentage of proven ARVC/D patients, diagnosed according to internationally consensus based Task Force Criteria (TFC) for diagnosis, without mutations in the known ARVC/D-related genes is even higher.[16, 17, 19] These ARVC/D cases may be explained by mutations in genes unknown yet or by the contribution of genetic variants of unknown significance (VUS) in the known genes. Accordingly, a similar prevalence of familial involvement in probands with and without a mutation in one of the implicated

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desmosomal genes was found in a study by Quarta et al.[17] However, in our study reported by Cox et al.[18] mutation-carrying relatives have a six-fold increased risk of ARVC/D diagnosis compared to relatives of index patients without identified mutation. The evaluation of a family history of ARVC/D, suggesting an unknown genetic factor, is crucial in ARVC/D patients without identifiable genetic predisposition.

Alternatively, these cases may be due to environmental factors, for example exercise. Sports activity, and particularly endurance sports activity, causes volume overload of the RV and mechanical stress on the cardiomyocytes, especially in the thin-walled RV. In a mouse model with *JUP* haploinsufficiency, daily performance of exercise (swimming) was a trigger for ventricular arrhythmias and an accelerator for structural RV abnormalities when compared to mice with the same predisposition by *JUP* haploinsufficiency but with a resting lifestyle.[45, 46] In addition, in a cohort of 47 athletes (subjects performing ≥3 hours per week sports with moderate-intense dynamic component, recreationally or competitive for >5 years) with RV arrhythmias, excluding idiopathic RV outflow tract VT, the prevalence of desmosomal mutations was relatively low.[47] By screening of all five genes *PKP2*, *DSP*, *JUP*, *DSG2*, and *DSC2*, pathogenic desmosomal mutations were identified in 13% of the total cohort. Of the 24 athletes that fulfilled the 1994 TFC for ARVC/D diagnosis [48], only 4 had a pathogenic desmosomal mutation (17%), which is a lower prevalence when viewed from the perspective of previous reports.[16-20, 20, 47] These animal model and athlete studies suggest that exercise is an important contributor to the ARVC/D phenotype, also in the absence of an identifiable genetic predisposition.

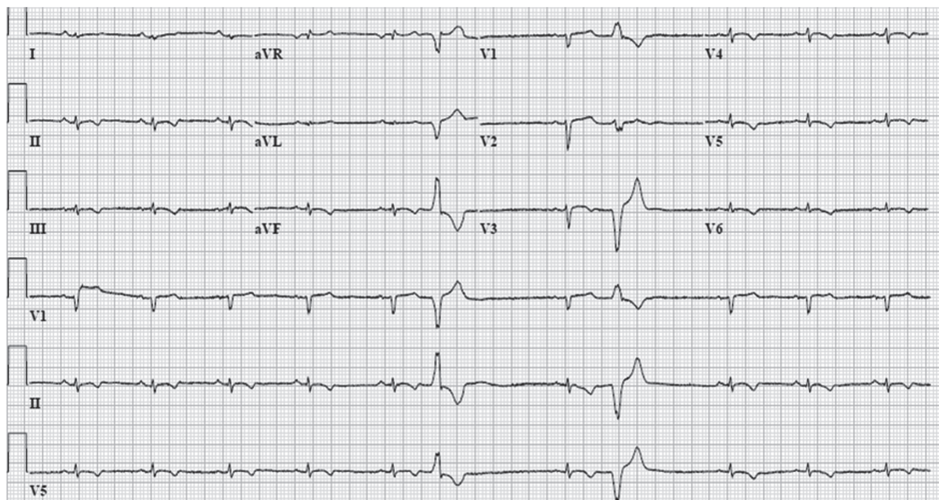

Figure 3. ECG of a phospholamban mutation carrier (while off-drugs). The ECG shows low voltages (voltages <0.5 mV in standard leads) and negative T waves in leads V4-6 (minor criterion in the 2010 Task Force Criteria (TFC) for ARVC/D diagnosis). Two premature ventricular complexes are observed, one with left bundle branch block morphology and one with right bundle branch block morphology. This patient fulfilled the TFC for ARVC/D diagnosis. The phospholamban mutation was not taken into account for the diagnostic criteria.

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Role of genetic screening

It is important to realize that the clinical diagnosis of ARVC/D is based exclusively on fulfilment of the diagnostic 2010 TFC.[49] Mutations underlying the disease show incomplete penetrance and variable clinical expression.[4, 5] Some genetically affected patients may have no signs or symptoms whatsoever, whereas no mutations can be identified in a large minority of clinically diagnosed patients. Therefore, genetic analysis alone can not be of any critical diagnostic value for the index patient who meets the TFC, but can be used to identify family members that are predisposed to disease development.

The current strategy for genetic testing in ARVC/D (in the Netherlands) is as follows. Individuals with a clinical diagnosis of ARVC/D are the first to be tested, i.e. index patients (probands). The detection of a pathogenic mutation contributes as major criterion in the TFC for diagnosis of ARVC/D. However, identification of a pathogenic mutation is not sufficient in the absence of phenotypic criteria. In contrast, if no mutation can be identified in a patient diagnosed with ARVC/D, the clinical diagnosis is still applicable. If a pathogenic mutation is identified in the index patient, parents, siblings and children of this patient can be tested for the mutation via the cascade method. When an (asymptomatic) relative is found to carry a pathogenic mutation, periodic cardiologic screening is required. DNA analysis by direct sequencing of the desmosomal genes *PKP2*, *DSG2*, *DSC2*, *DSP*, and *JUP* is recommended in ARVC/D patients with an appropriate indication for this analysis. When no mutation is found, this analysis can be extended with gene dosage analysis (MLPA) to detect larger deletion or insertion mutations and with the screening of non-desmosomal genes (*RYR2*, TGFβ3, *TMEM43*, *DES*, *TTN*, *LMNA*, and *PLN*). Non-desmosomal genes can be screened according to their geographical prevalence or according to the observed phenotype. For example, in the presence of low voltages on the ECG, *PLN* screening can be performed and conduction disorders could be suggestive of *LMNA* mutations. Genetic analysis in ARVC/D is at present performed with the candidate gene approach. However, the development of new techniques for genetic screening is rapidly progressing. One of those new techniques is analysis by next-generation sequencing (NGS). With this technique the analysis is faster and it permits analysis of larger numbers of genes compared to the candidate gene approach. This might result in the discovery of new ARVC/D-related genes. On the other hand, the findings of associated large numbers of genetic VUS will also result in more uncertainty, both for the patient and the physician.

Interpretation of genetic screening

The interpretation of the outcome of molecular genetic screening in ARVC/D index patients can be challenging. Screening might result in the finding of 1) proven pathogenic mutations, 2) most-likely pathogenic mutations, 3) VUS, 4) polymorphisms, or 5) no abnormalities compared to control populations. An overview of the proven pathogenic and most-likely pathogenic mutations identified in Dutch ARVC/D index patients is shown in Table 1.

The pathogenicity of radical mutations, i.e. truncating or splice site mutations (mutations affecting the invariant AG splice-acceptor and GU splice-donor sites of exons), resulting in truncation or absence of the protein product from the mutated allele, is widely accepted (proven pathogenic). However, the pathogenicity of missense variants (also known as non-synonymous single nucleotide variants), resulting in a single amino acid substitution, is more difficult to delineate.

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Table 1. Mutations identified in Dutch ARVC/D index patients

Gene	Nucleotide change	Amino acid change	Mutation type
Proven pathogenic mutations			
PKP2	deletion exons 1-4	p.(?)	deletion
	deletion exon 8-14	p.(?)	deletion
	deletion exons 1-14	p.(?)	deletion
	deletion exon 8	p.(?)	deletion
	deletion exon 10	p.(?)	deletion
	c.148_151del	p.(Thr50fs)	frameshift
	c.235C>T	p.(Arg79*)	nonsense
	c.258T>G	p.(Tyr86*)	nonsense
	c.397C>T	p.(Gln133*)	nonsense
	c.917_918del	p.(Pro318fs)	frameshift
	c.968_971del	p.(Gln323fs)	frameshift
	c.1211dup	p.(Val406fs)	frameshift
	c.1237C>T	p.(Arg413*)	nonsense
	c.1369_1372del	p.(Gln457*)	nonsense
	c.1378G>A	p.(Val445fs) #	frameshift
	c.1511-2A>G	p.(?)	splice site
	c.1848C>A	p.(Tyr616*)	nonsense
	c.1951C>T	p.(Arg651*)	nonsense
	c.2028G>A	p.(Trp676*)	nonsense
	c.2034G>A	p.(Trp678*)	nonsense
	c.2146-1G>C	p.(Met716fs) #	frameshift
	c.2386T>C	p.(Cys796Arg)	missense
	c.2421C>A	p.(Tyr807*)	nonsense
	c.2489+1G>A	p.(Lys768fs) #	frameshift
	c.2489+4A>C	p.(Lys768fs) #	frameshift
	c.2509del	p.(Ser837fs)	frameshift
	c.2544G>A	p.(Trp848*)	nonsense
	c.2554delG	p.(Glu852fs)	frameshift
DSP	c.3337C>T	p.(Arg1113*)	nonsense
	c.5419C>T	p.(Gln1807*)	nonsense
DSG2	c.378+2T>G	p.(?)	splice site
DSC2	c.942+3A>G	p.(?)	splice site
	c.943-1G>A	p.(?)	splice site
PLN	c.40_42del	p.(Arg14*)	nonsense

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Table 1. Continued

Most-likely pathogenic mutations			
PKP2	c.1844C>T	p.(Ser615Phe)	missense
	c.2062T>C	p.(Ser688Pro)	missense
	c.2615C>T	p.(Thr872Ile)	missense
DSP	c.1982A>T	p.(Asn661Ile)	missense
	c.6881C>G	p.(Ala2294Gly)	missense
DSG2	c.137G>A	p.(Arg46Gln)	missense
	c.614C>T	p.(Pro2015Leu)	missense
	c.874C>T	p.(Arg292Cys)	missense
	c.889G>A	p.(Asp297Asn)	missense
	c.1072G>A	p.(Ala358Thr)	missense
DSC2	c.608G>A	p.(Arg203His)	missense
TMEM43	c.718C>T	p.(Arg240Cys)	missense

Proven pathogenic mutations, i.e. truncating (deletion, frameshift, nonsense) and splice site mutations, and most-likely pathogenic mutations identified in ARVD/C index patients in The Netherlands. Missense mutations were considered most-likely pathogenic mutations when 1) predictive algorithms SIFT and PolyPhen-2 predicted pathogenicity (SIFT<0.02, PolyPhen-2 >0.900) and 2) mutations were rare in the general population (minor allele frequency ≤0.05% in ESP exome dataset). #: RNA experiments by our group showed that this nucleotide substitution leads to the use of a cryptic splice site and results in a frameshift (c.1378G>A r.1333_1378del p.(Val445fs)), (c.2146-1G>C r.2146_2299del p.(Met716fs)), and (c.2489+4A>C r.2300_2489del p.(Lys768fs)), manuscript in preparation.

As with other clinical tests, understanding of the extent and spectrum of genetic variation in the healthy population is necessary for correct interpretation.

In our study by Kapplinger et al.[50], the results of molecular genetic analysis were compared between 175 proven ARVC/D cases and a control population of 427 unrelated ostensibly healthy individuals. Of the ARVC/D cases 58% hosted a genetic variation that met the criteria of a mutation (variation predicted to alter the protein) versus 16% of healthy controls. Radical mutations were significantly more prevalent in the proven ARVC/D cases than in controls (49.9% versus 0.47%, $p<9.8\times10^{-44}$), confirming the accepted assumption of pathogenicity.[50] However, missense variants were found at similar rates in cases and controls. Three associations were identified which might strengthen the pathogenicity of missense variants found: 1) a rare missense variant identified in a Caucasian patient is more likely to be pathogenic than in a non-Caucasian patient, 2) specific amino-terminal regions in *DSP* and *DSG2* contain mutation 'hot-spots', and 3) missense variants that involve a highly conserved residue in *PKP2* and *DSG2* are more likely to be pathogenic.[50] Most likely pathogenicity of specific missense mutations is supported by predictive algorithms.

When properly interpreted, molecular genetic analysis allows ultimately the early detection of presymptomatic disease, identification of individuals at risk, and genetic counselling for this sudden death-predisposing disease. Nonetheless, regardless of the suggestion of pathogenicity of an identified mutation, genetic analysis results should be viewed as probabilistic and as part of the overall clinical assessment.

Genotype-phenotype correlation analysis

The identification of a pathogenic mutation distinguishes individuals with a predisposition for ARVC/D. This is a first step in risk stratification of ARVC/D index patients and family members. Nevertheless, one of the primary therapeutic goals is timely diagnosis of the 'concealed phase', when individuals are at risk for arrhythmias despite absence of symptoms. Genotype-phenotype correlation analysis can provide more insight in risk profiles of index patients and family members. Most genotype-phenotype correlation studies focussed on overt index patients.[4, 5, 16, 19, 20, 51-53] Yet, index patients are by definition affected by the disease. Therefore, the effect of the genotype can be more objectively assessed in family members who provide the opportunity of prospective follow-up from young age.

In a genotype-phenotype study by Quarta et al.[17] including both ARVC/D index patients and family members, it was shown that SCD was the presenting symptom in half of the index patients. SCD occurred in 31% at young age, between 14-20 years old, confirming that adolescence is a vulnerable period for fatal ventricular arrhythmias in ARVC/D. In contrast to index patients, mutation-positive family members had a better prognosis, with signs and symptoms of ARVC/D present in 50% of relatives in their 5th decades of life.[17] The differences between index patients that presented with SCD and relatives with milder disease expression may be explained by the presence of multiple versus single genetic variants. Accordingly, family members with more than one genetic variant had a significant 5-fold increase in risk of disease expression, providing further evidence of gene-gene interactions and gene-dose effects.[17]

A genotype-phenotype follow-up study of ARVC/D index patients and family members by our group showed that a pathogenic mutation in an index patient predicts outcome in family members.[18] Pathogenic desmosomal mutations were identified in 58% of index patients, predominantly truncating *PKP2* mutations (78%). The study included 302 family members, of whom 137 with pathogenic and most likely pathogenic mutations. Compared to relatives of index patients without mutations, mutation carrying family members had 1) a six-fold risk of ARVC/D diagnosis, 2) a markedly enhanced risk for ventricular arrhythmias, and 3) earlier onset of ARVC/D signs and symptoms.[18] In young relatives (below the age of 20 years old), sudden cardiac death and other signs of ARVC/D, occurred in *PKP2* mutation carriers.[18] Diagnosis of ARVC/D was made in 39% of mutation carrying family members with a comparable one third of mutation-positive relatives with a definite ARVC/D diagnosis found in the study by Quarta et al.[17, 18]

Although of great importance for risk stratification in ARVC/D, genotype-phenotype correlation studies are generally hampered by small number of patients and family members. Rare genetic variants are found infrequently. Therefore, large numbers of patients and relatives per specific variant are needed to assess its contribution to the ARVC/D phenotype. Large numbers of family members are also needed to assess variability of disease expression within a family by genetic modifiers or environmental factors. Future studies will have to elaborate further on risk stratification by genotype-phenotype correlation analysis.

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CHAPTER 4

Left-Dominant Arrhythmogenic Cardiomyopathy in a Large Family: Associated Desmosomal or Non-Desmosomal Genotype?

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Abstract

Background

Arrhythmogenic cardiomyopathy (AC) is considered a predominantly right ventricular (RV) desmosomal disease. However, left-dominant forms due to desmosomal gene mutations, including *PKP2* variant c.419C>T have been described. Recently, a non-desmosomal phospholamban (*PLN*) mutation (c.40_42delAGA) has been identified causing dilated cardiomyopathy and arrhythmias.

Objective

By cosegregation analysis of *PKP2* variant c.419C>T versus *PLN* mutation c.40_42delAGA we aimed to gain more insight into pathogenicity.

Methods and results

A Dutch family (13 family members, median age 49, range 34-71 years) with ventricular tachycardia underwent 1) meticulous phenotypic characterization and 2) screening of five desmosomal genes (*PKP2*, *DSC2*, *DSG2*, *DSP*, *JUP*) and *PLN*. Six family members fulfilled 2010 AC Task Force Criteria (TFC). Seven had signs of left ventricular (LV) involvement (inverted T waves in V4-6, LV wall motion abnormalities and late enhancement, reduced LV ejection fraction), including six family members with proven AC. *PKP2* c.419C>T was found as a single variant in three, combined with *PLN* c.40_42delAGA in three others. *PLN* mutation was found in nine family members, including the six with AC and all seven with LV involvement. *PLN* c.40_42delAGA was found as single mutation in six, and combined with *PKP2* c.419C>T in three others. A low voltage ECG was seen in 4/9 *PLN* mutation-positive subjects. None of the family members with the single *PKP2* variant showed any sign of RV or LV involvement.

Conclusion

PLN mutation c.40_42delAGA cosegregates with AC and with electrocardiographic and structural LV abnormalities. In this family there was no evidence of disease causing contribution of the *PKP2* variant c.419C>T.

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Introduction

In the classical description, arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is considered a hereditary cardiac disease, characterized by ventricular arrhythmias of right ventricular origin with fibrofatty replacement of cardiomyocytes, predominantly in the right ventricle (RV).[1-3] More recently, patients and families with ventricular arrhythmia and similar ARVD/C histopathologic changes in the left ventricle (LV) have been recognized and described as left-dominant arrhythmogenic cardiomyopathy (LDAC).[4] Mutations in desmosomal genes are associated with both ARVD/C and LDAC and at the molecular level both ventricles and the interventricular septum are similarly affected.[5-7] These considerations suggested the designation "arrhythmogenic cardiomyopathy" (AC) as preferred terminology.[8]

Desmosomes are protein complexes located in the intercalated disk which are important for mechanical integrity.[9] Desmosomal dysfunction may give rise to loss of mechanical cell-cell adhesion, and leads to down-regulation and/or altered distribution of other intercalated disk proteins, i.e. gap junction proteins (connexin43) and sodium channels (Nav1.5).[6, 10] These alterations give rise to electrical cell-cell uncoupling and slow conduction, thereby providing a substrate for *early* activation delay and thus re-entrant ventricular tachyarrhythmia.[5, 6, 9, 11-17] Presumably, at a *later stage* myocyte loss and fibrofatty replacement will have a major impact on tissue architecture, giving rise to zig-zag conduction pathways and load mismatch, further contributing to enhanced activation delay.[5, 14-16]

Pathogenic mutations in five known desmosomal genes are related to right, left, and biventricular forms of AC: plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*).[18-24] In approximately 60% of Dutch AC patients a pathogenic desmosomal mutation is found, predominantly truncating mutations in the *PKP2* gene.[11, 19]

The *PKP2* variant c.419C>T (p.Ser140Phe) is a missense variant which alters one amino acid. It has been reported to be associated with both classical right-sided AC, and a left-dominant form in individual patients and in families.[4, 20, 25-29] However, the pathogenicity of this variant is not supported by *in silico* prediction algorithms Sorting Intolerant from Tolerant (SIFT) and Polymorphism Phenotyping-2 (PolyPhen-2).[30, 31] No functional tests to assess pathogenicity have been performed thus far.

This report presents a family with monomorphic ventricular tachycardia (VT) with two potentially causative mutations, the desmosomal *PKP2* variant c.419C>T and a recently identified mutation c.40_42delAGA (p.Arg14del) in the non-desmosomal phospholamban (*PLN*) gene.[32] The *PLN* gene is involved in calcium homeostasis and mutation-positive subjects are phenotypically characterized by low voltage electrocardiograms, ventricular arrhythmias, and contractile dysfunction.[32-35]

The aim of the study was to gain insight into the pathogenicity of the *PKP2* variant c.419C>T by cosegregation analysis of the *PKP2* variant c.419C>T versus the *PLN* mutation c.40_42delAGA.

Methods

Study population

A Dutch family of four generations and 30 family members, 13 of whom were available for screening (median age 46, range 34-71 years, 4 men), with VT underwent 1) meticulous phenotypic characterization and 2) mutation analysis.

Clinical evaluation

Standard patient evaluation included detailed assessment of clinical and family history, physical examination, and 12-lead ECG (while off drugs). In the presence of cardiac symptoms and/or an abnormal ECG, analysis was extended with 48-hour ambulatory ECG monitoring (11/13), maximal treadmill exercise testing (7/13), 2D-transthoracic echocardiography (11/13), magnetic resonance imaging (MRI; 9/13) including delayed enhancement (DE; 8/13) analysis, LV/RV cine-angiography (3/13), electrophysiologic study (EPS; 3/13), and endomyocardial biopsy (2/13). Diagnosis of AC was according to the revised 2010 TFC.[36] Involvement of the RV was considered positive in the presence of at least one of the following: negative T waves in right precordial leads V1-3, RV wall akinesia or dyskinesia and/or DE in the RV on imaging studies, and right ventricular ejection fraction (RVEF) ≤ 45%. LV involvement was considered positive in the presence of at least one of the following: negative T waves in left precordial leads V4-6, LV wall akinesia or dyskinesia and/or DE in the LV on imaging studies, and left ventricular ejection fraction (LVEF) < 50%. All patients consented to clinical evaluation according to the TFC.

Histopathological and immunohistochemical analysis

Right ventricular septal tissue was obtained from two patients (III:9 and IV:5 see Figure 1) and stained with hematoxylin/eosin and Masson's trichrome staining. Only from patient IV:5 was tissue available for immunohistochemical analysis. Material from patient IV:5 was flash frozen in liquid nitrogen. Frozen samples were cryo-sectioned at a thickness of 10 μm. Immunohistochemistry was performed as described previously.[37] Primary antibodies against N-Cadherin (mouse, Sigma, 1:800), N-Cadherin (rabbit, Sigma, 1:800), *PKP2* (mouse, Progen, undiluted-1:1000), *JUP* (mouse, Sigma, 1:50.000), Connexin43 (Cx43, rabbit, Invitrogen, 1:250), non-phosphorylated Cx43 (Cx43-NP, mouse, Invitrogen, 1:500) were used. Secondary labeling was performed with appropriate Dylight594 or Alexa Fluor594 (1:250) and FITC (1:250) conjugated whole IgG antibodies (Jackson Laboratories).

Molecular analysis

Genomic DNA was extracted from peripheral blood as described before.[11] Direct sequencing of all coding regions and intron/exon boundaries of the five known desmosomal genes *PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2*, and in addition *PLN* was performed in the index patient. The remaining 12 family members available for screening were analyzed for the *PKP2* variant and the *PLN* mutation found in the index patient. Primer sequences and PCR conditions are available on request.

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Results

Family

At age 51, the index patient (III:8, see pedigree in Figure 1) presented with a monomorphic VT with right bundle branch block (RBBB) morphology, right axis deviation, and cycle length 230 ms. Clinical analysis showed low voltage ECG (<0.5mV in standard leads), and a structurally normal heart on cine-angiography and echocardiography with LVEF 62%. At age 63, she presented again with the same VT, but remarkably longer cycle length (while off drugs) of 350 ms (Figure 2). By then, the LVEF had deteriorated to 32%. The ECG repeatedly showed markedly low voltages in the standard leads (Figure 3). Left and right ventricular cine-angiogram revealed dyskinesia at the LV apex, and akinesia inferiorly of the tricuspid valve with poor RV function. Coronary angiogram (CAG) was unremarkable. The clinical VT was inducible with programmed electrical stimulation (PES) in the RV apex (cycle length 600 ms and two extrastimuli) and additionally, two other RBBB VT morphologies and one non-sustained VT (NSVT) with left bundle branch block (LBBB) morphology and superior axis. All VT episodes were terminated by burst pacing. A cardioverter defibrillator (ICD) was implanted. Heart failure became more prominent at age 64, with a clinical decline in condition when she developed atrial fibrillation.

Family screening was initiated after the index patient's second presentation. There were no sudden cardiac deaths in the family. Family members of the first and second generation reached old age. Findings in the family are summarized in Table 1.

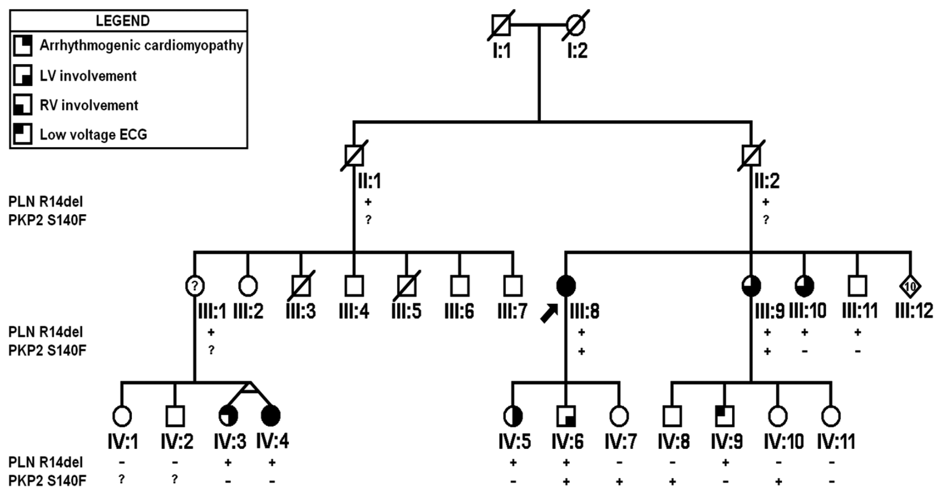

Figure 1. Pedigree with *PKP2* variant c.419C>T (p.Ser140Phe) and *PLN* mutation c.40_42delAGA (p.Arg14del). No other desmosomal mutations identified in the index patient (arrow). In addition, 12 family members screened for *PKP2* variant and *PLN* mutation. Phenotypic characterization with regard to arrhythmogenic cardiomyopathy (AC) diagnosis with left ventricular (LV) and right ventricular (RV) involvement, and presence of low voltages (<0.5mV in standard leads). Square: male, circle: female, +: mutation positive, -: mutation negative, ?: *PKP2* gene not screened, diagonal line: deceased.

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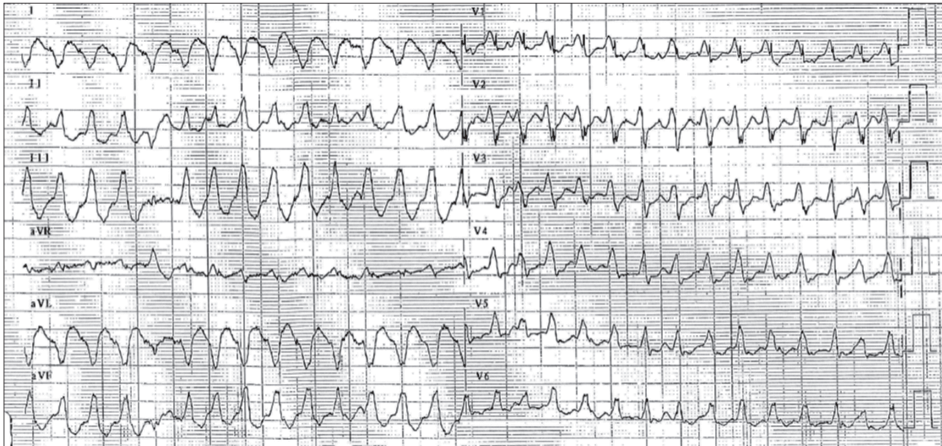

Figure 2. 12-lead ECG recording (while off drugs) of monomorphic ventricular tachycardia with right bundle branch block morphology, right axis deviation and cycle length 350 ms at second presentation of index patient at age 63.

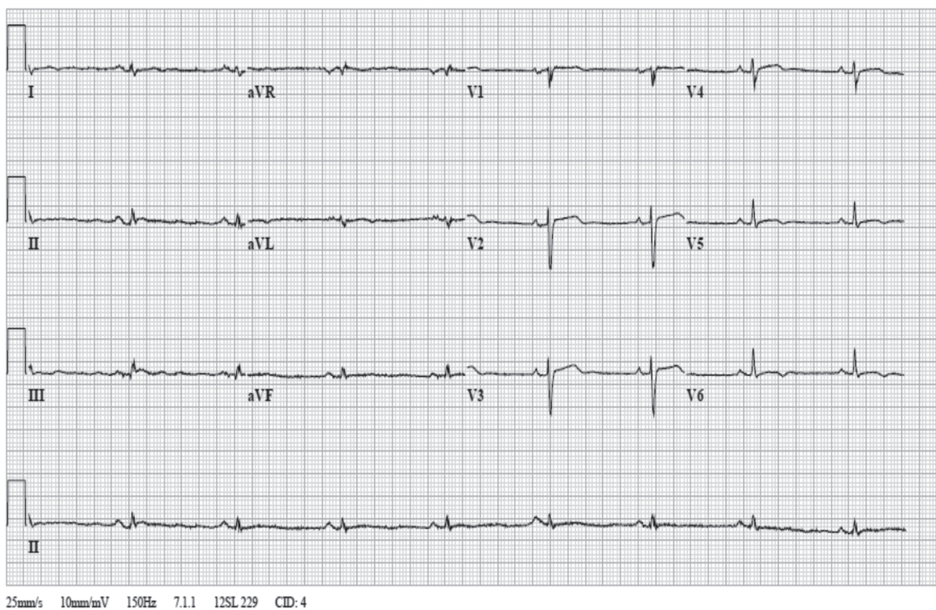

Figure 3. ECG of the index patient (III:8, while off drugs) during normal sinus rhythm. Outstanding are the low voltages (voltages <0.5mV in standard leads) and negative T wave in V5 and V6.

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Table 1.

Family member Age(years)	III:8 71	III:9 66	III:10 64	III:11 62	IV:3 46	IV:4 46	IV:5 46	IV:6 44	IV:7 42	IV:8 42	IV:9 40	IV:10 37	IV:11 34
Molecular genetics													
PLN	+	+	+	+	+	+	+	+	-	-	+	-	-
PKP2	+	+	-	-	-	-	-	+	+	+	-	+	-
ECG													
LowV	+	-	-	-	+	+	-	-	-	-	+	-	-
V1-3	-	+	-	-	-	-	-	-	-	-	-	-	-
V4-6	-	+	+	-	+	+	+	+	-	-	-	-	-
prol TAD	-	-	-	-	-	-	-	-	-	-	-	-	-
Arrhythmias													
LBBB VT	+	+	-	-	-	+	+	-	-	-	-	-	-
RBBB VT	+	-	-	-	-	-	-	-	-	-	-	-	-
>500 VES	+	-	+	-	+	+	+	-	na	-	-	-	na
Imaging													
WMA RV	+	+	-	-	-	+	-	-	-	-	-	-	na
WMA LV	+	-	+	-	-	-	-	-	-	-	-	-	na
DE RV	na	-	-	-	-	+	-	-	na	na	-	na	na
DE LV	na	+	-	-	-	-	+	-	na	na	-	na	na
RVEF (%)	na	47	33	43	47	32	na	56	na	na	54	na	na
LVEF (%)	32	60	50	52	50	51	58	68	na	na	60	na	na
Diagnosis													
AC	+	+	+	-	+	+	+	-	-	-	-	-	-
RV inv.	+	+	+	-	-	+	-	-	-	-	-	-	-
LV inv.	+	+	+	-	+	+	+	+	-	-	-	-	-

Family members in numeric order according to pedigree (Figure 1) with age (years) noted below family number. *PLN*: phospholamban. *PKP2*: plakophilin-2. LowV: ECG, voltages <0.5mV in standard leads. V1-3: negative T waves in leads V1-3. V4-6: negative T waves in leads V4-6. Prol TAD: prolonged terminal activation duration (≥55 ms). LBBB VT: VT with left bundle branch block morphology. RBBB VT: VT with right bundle branch block morphology. 500 VES: fulfilment of Task Force criterion >500 ventricular extrasystoles/24hour. WMA: wall motion abnormalities (akinesia/dyskinesia). DE: delayed enhancement. RV: right ventricle. LV: left ventricle. RVEF: right ventricular ejection fraction. LVEF: left ventricular ejection fraction. AC: arrhythmogenic cardiomyopathy. RV/LV inv.: RV/LV involvement. na: not applicable

Her sister (III:9) had an abnormal ECG with negative T waves in leads V1-6 (Figure 4) and atypical chest pain since age 58 (CAG normal). The 24-Hour ECG showed ventricular ectopy (238/24hour) with left bundle branch block (LBBB) morphology. MRI demonstrated a dyskinetic area in the RV, an enlarged RV end diastolic volume of 107 ml/m^2, and DE in the lateral wall of the LV, with LVEF 60%. At age 65, ventricular ectopy increased and non-sustained VT (NSVT) episodes with

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LBBB morphology were recorded. PES did not induce arrhythmias, late potentials were found in outflow tract, basal-anterior, and inferior-apical region of the RV, and voltage mapping revealed multiple sites with endocardial low voltages (voltages ≤1.5mV in bipolar recordings). Cine-angiography also showed dyskinesia in the outflow tract of the RV. Endomyocardial biopsy showed normal myocardium, with locally some (<10%) subendocardial fibrosis. An ICD was implanted.
Another sister (III:10) had negative T waves in V2-6, frequent ventricular ectopy (3379/24hour), and reduced RV function (RVEF 33%), with LVEF 50%. A brother of the index patient (III:11), aged 62 years, had a normal ECG and structurally normal heart on echocardiography and MRI. The eldest daughter of the index patient (IV:5) had palpitation, negative T waves in V2-6, frequent ventricular ectopy (2431/24hour) and NSVT episodes on Holter recording, and DE in the infero-lateral region of the LV apex. PES did not induce arrhythmias, voltage mapping demonstrated low endocardial voltages in anterior outflow tract, and inferior free-wall region of the RV. Endomyocardial biopsy showed normal myocardium without fibrosis or fatty tissue. However, immunohistochemical analysis of the cardiac tissue showed mild, heterogeneous reduction of the immunoreactive signal of intercalated disk proteins *JUP* (Figure 5a-c) and *PKP2* (Figure 5d-f), fairly normal expression of the gap junction protein Cx43 (Figure 6a-c), and some areas with elevated Cx43-NP signal (Figure 6d-f). An ICD for primary prevention was implanted. The son of the index patient (IV:6) did not have any complaints, his ECG however, showed negative T waves in V3-6, MRI analysis showed a wider RV (47mm) than LV (45mm) but no other signs of AC and no DE. His sister (IV:7) was asymptomatic with a normal ECG and echocardiogram.

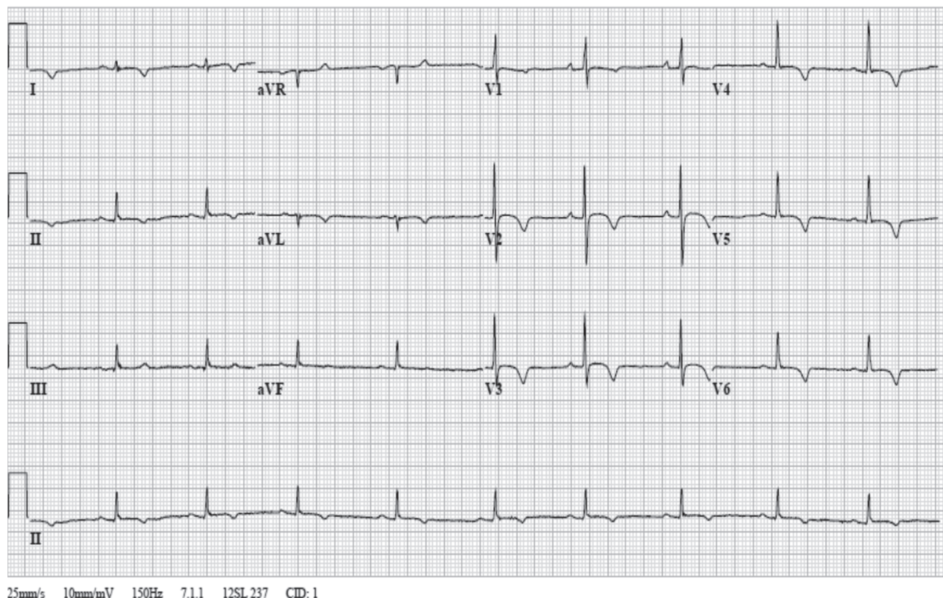

Figure 4. ECG (while off drugs) during normal sinus rhythm of the sister of the index patient (III:9). Remarkable are the negative T waves in leads V1-6, a major criterion according to Task Force Criteria for arrhythmogenic cardiomyopathy.

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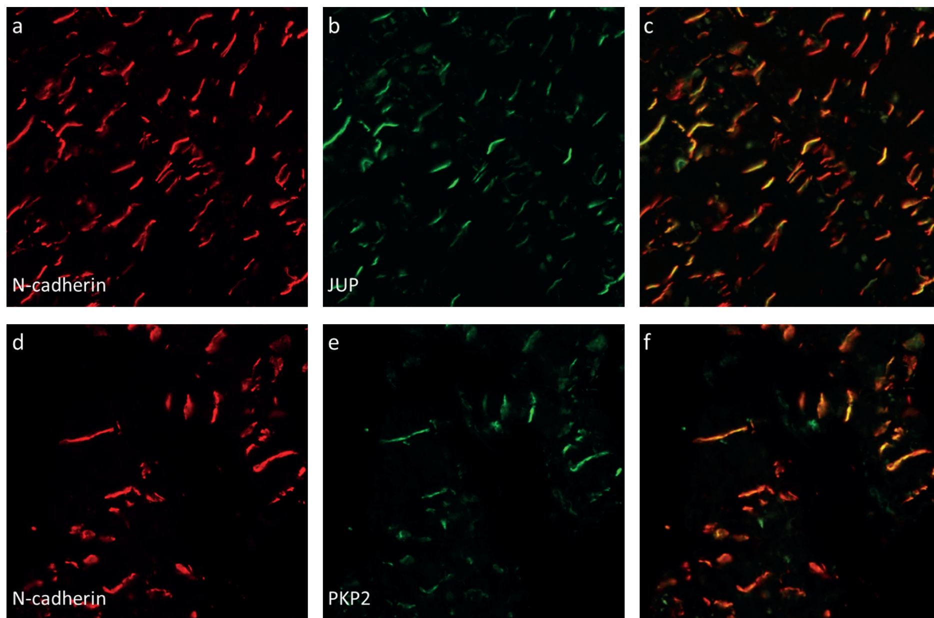

Figure 5. Immunohistochemical analysis of desmosomal proteins plakoglobin (*JUP*) and plakophilin-2 (*PKP2*) in the daughter (IV:5) of the index patient Shown in **a** and **d** is the intercalated disk protein N-cadherin, used as control and reference for presence of intact intercalated disks and appropriate tissue quality, with strong signal. There is a mild, heterogeneous reduction of both JUP and, PKP2 immunoreactive signal as shown in **b** and **e**. **C** and **f** show double-labelings of N-cadherin and *JUP* or *PKP2*.

The eldest son (IV:8) of the sister (III:9) of the index patient was asymptomatic with normal ECG. His brother (IV:9) had an ECG with low voltages but no inverted T waves. Exercise testing and MRI analysis were within normal limits. Their sister (IV:10) and the youngest of these siblings (IV:11) were asymptomatic with normal ECG.

The cousins of the index patient are monozygotic twins (IV:3 and IV:4). At age 43, patient IV:3 showed a low voltage ECG with inverted T waves in V4-6, frequent ventricular ectopy (1042/24hour), and normal ventricular function with RVEF 47% and LVEF 50%. Her sister (IV:4) had low voltages and negative T waves in V4-6, RV wall motion abnormalities and DE, a reduced RV function with RVEF 32%, and LVEF 51%.

Diagnosis of arrhythmogenic cardiomyopathy and LV involvement

According to revised 2010 TFC, AC was diagnosed in six family members (III:8, III:9, III:10, IV:3, IV:4, IV:5, median age 55, range 46-71 years, 0 men), as shown in Table 1 (see also Figure 1). Diagnosis was based on major and minor criteria, specified in Table 1 in the addendum.

LV involvement was identified in seven family members (III:8, III:9, III:10, IV:3, IV:4, IV:5, IV:6, median age 46, range 44-71 years, see Table 1). Those seven included the six family members with AC diagnosis. One family member (IV:6) with LV involvement based on inverted T waves in V3-6 and with prominent ventricular ectopy (407/24 hour) had only one major (family history)

and one minor criterion (negative T waves in left precordial leads), and therefore did not meet the diagnosis of AC. This family member had mild hypertension (ambulatory mean systolic and diastolic blood pressures 147/91 mm Hg) and borderline high electrocardiographic amplitudes (Figure 7) with LV wall thickness of 12 mm on MRI.

DNA analyses

In DNA of the index patient, sequencing of all five desmosomal genes and of the non-desmosomal gene *PLN* (screened because of low QRS voltages), resulted in the identification of the *PKP2* variant c.419C>T and the *PLN* mutation c.40_42delAGA. No other mutation was identified in the index patient. *PKP2* c.419C>T was identified as single variant in three family members (IV:7, IV:8, IV:10, median age 42, range 37-42 years). *PLN* mutation c.40_42delAGA was identified in nine family members, as single mutation in six family members (III:10, III:11, IV:3, IV:4, IV:5, IV:9, median age 46, range 40-64 years), and combined with the *PKP2* variant in three others (III:8, III:9, IV:6, median age 66, range 44-71 years).

Genotype-phenotype correlation

All six family members with AC diagnosis had the *PLN* mutation c.40_42delAGA, four of them as single mutation (III:10, IV:3, IV:4, IV:5, see pedigree in Figure 1). Moreover, all seven patients with LV involvement were *PLN* c.40_42delAGA positive. *PLN* mutation-positive subjects often had a low voltage ECG (voltages <0.5 mV in standard leads, 4/9 subjects, see Table 1), and

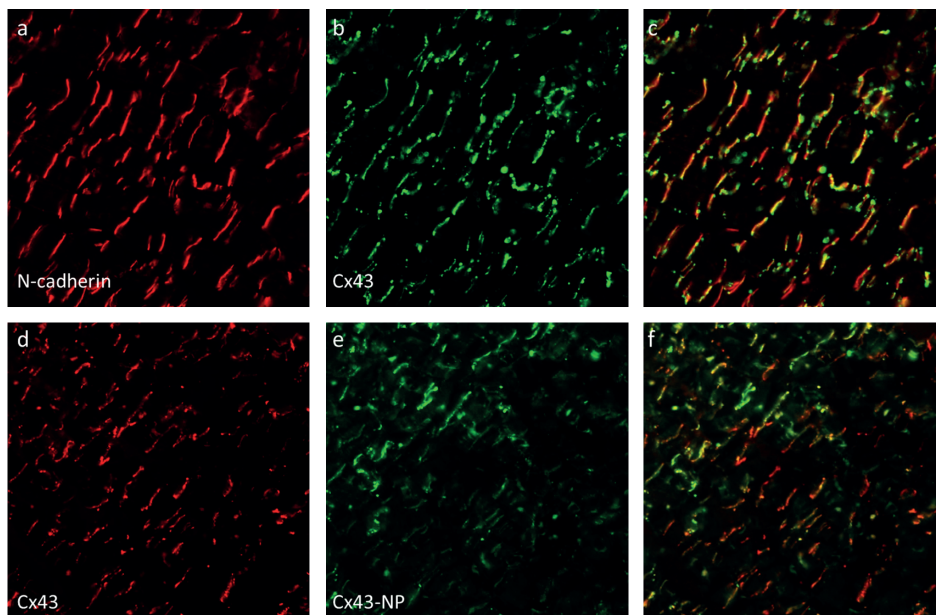

Figure 6. Immunohistochemical analysis of the intercalated disk protein N-cadherin (control) showed strong signal in **a**. Expression of gap junction protein connexin 43 (Cx43) shown in **b**, **c** (double labeling), and **d** is normal, with some expression of non-phosphorylated Cx43 (Cx43-NP) in **e** and in double-labeling in **f**.

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frequently had inverted T waves in V4-6 (6/9 subjects). In contrast, none of the family members with the single *PKP2* variant (IV:7, IV:8, IV:10, aged 42, 42, and 37) showed any sign of RV or LV involvement.

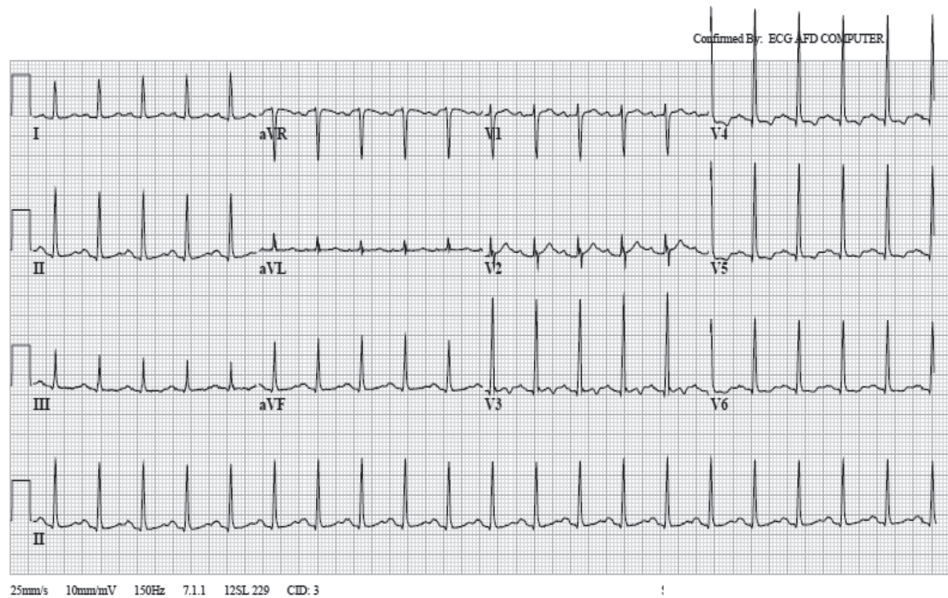

Figure 7. ECG of the son of the index patient (IV:6, while off drugs) during sinus rhythm with remarkably negative T waves in leads V3-6 and rather high voltages, without fulfillment of criteria for left ventricular hypertrophy (R wave in lead aVL 0.4 mV, S wave in lead V1 1.1 mV, R wave in lead V5 2.3 mV).

Discussion

PLN cosegregation and phenotypic characteristics

PLN mutation c.40_42delAGA cosegregated with AC diagnosis and electrocardiographic and structural LV abnormalities. In this family, mutation-positive subjects were often identified by the presence of low voltage ECGs. In addition, *PLN* mutation-positive subjects with AC had RV and LV involvement, negative T waves in V4-6, and wall motion abnormalities and/or DE in the LV.
The c.40_42delAGA (p.Arg14del) mutation results in the deletion of a highly conserved residue and is not or very sporadically (1/473 control samples) found in large control populations.[32, 33, 35] The mutation was not found in the 1000 genomes (http://www.1000genomes.org/) and Exome Sequencing Project datasets ((http://evs.gs.washington.edu/EVS/). *In vitro* studies suggested a dominant negative effect of the mutation on calcium re-uptake in the sarcoplasmatic reticulum.[33] Transgenic mice overexpressing the c.40_42delAGA mutation displayed premature death, massive cardiac fibrosis, and an irreversible superinhibition of calcium re-uptake by the sarcoplasmatic reticulum Ca2+-ATPase (SERCA) pump.[33]

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PLN gene mutations have previously been associated with low voltages on ECG recordings, ventricular tachyarrhythmias, and contractile dysfunction in patients diagnosed with dilated cardiomyopathy (DCM) or AC.[32-34] The attenuated ECG amplitudes were found irrespective of structural abnormalities.[33, 34] Also in our study, patient IV:9 had low voltages without any MRI abnormalities, including DE analysis. Similar patients in whom the electrocardiographic and arrhythmic abnormalities were the earliest disease manifestation have been reported, as have patients in whom arrhythmia and MRI late enhancement preceded ECG or functional imaging abnormalities.[7] Therefore, a mutation specific process leading to fibrosis has been hypothesized. On the other hand, low voltages in the absence of compact (MRI-detectable) fibrosis could indicate either diffuse fibrosis or electrical changes due to chronic increased intracellular calcium levels.[34] The exact mechanism of the low voltage ECG remains to be elucidated.

In accordance with previous studies, this family was also characterized by ventricular arrhythmias.[32-34] Interestingly, the index patient *first* presented with a monomorphic VT with a preserved LVEF and only *later* in life developed progressive heart failure. Correspondingly, in the family described by Posch et al.[34], two *PLN* mutation-positive subjects were reported to be free of heart failure symptoms but displayed sudden cardiac death in their 5th decades as first sign of disease. Furthermore, Haghighi et al.[33] described heterozygous family members that *primarily* had frequent ventricular extrasystoles and VT episodes and only *later* developed heart failure. Finally, Van der Zwaag et al.[32] demonstrate a predominantly arrhythmic presentation (VT or ventricular fibrillation) in a large cohort with the *PLN* mutation. In these studies, including the present study, the same c.40_42delAGA mutation has been identified. Therefore, we hypothesize that this family stands model for the Arrhythmogenic Cardiomyopathy-concept: first arrhythmic manifestations and in a later stage structural abnormalities and heart failure. Confirmation by studying asymptomatic family members from young age is needed.

As in classical forms of AC, modifying factors are important in the clinical presentation of *PLN* mutation-positive subjects. The monozygotic twins IV:3 and IV:4 are clinically different, with markedly reduced RV function in patient IV:4 and preserved RV in patient IV:3. In addition, family member III:11, aged 62 years, did not show any sign of RV or LV disease. The variable clinical expression and reduced penetrance observed in this family is known in AC as well as other cardiomyopathies. At this stage, the role of environmental factors and genetic modifiers is still obscure.

PKP2 cosegregation

Initial molecular genetic screening of the five known desmosomal genes identified the missense variant c.419C>T in the *PKP2* gene. Missense variants in the desmosomal genes are frequently found in AC patients, but also in the normal control population.[38] The literature on the *PKP2* c.419C>T variant (Addendum Table 2), presents conflicting data. The variant is found in healthy controls, in individual AC or DCM patients, and in families with AC. *PKP2* c.419C>T was found in 6/996 alleles in the Genome of the Netherlands dataset (http://www.nlgenome.nl/), in 6/1000 alleles in the 1000 genomes dataset, and in 26/12980 alleles in the Exome Sequencing Project database. On the other hand, identification in controls appears infrequent, and the overrepresentation in AC patients and families has been considered suggestive of a disease-modifying or even pathogenic role of the variant.[20, 25, 27]

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The findings in this study challenge this suggested classification. In our family the *PKP2* variant was the only variant identified after screening of the five desmosomal genes. Nevertheless, the daughter of the index patient had ventricular arrhythmias and structural changes in the LV but did not carry the variant. Furthermore, three family members already aged 37, 42, and 42 years, with *PKP2* c.419C>T as the sole variant did not display any sign of RV or LV involvement. Since the family members with the single *PKP2* variant were all from the youngest generation, a selection bias due to age-related penetrance is possible, but unlikely since these individuals were aged around 40 years. However, in individuals with a *PLN* mutation a modifying effect of the additional *PKP2* variant, suggested by the overrepresentation of the variant in patients in the literature, can not be excluded. Nonetheless, there is no evidence of an individual disease causing contribution of the single *PKP2* variant c.419C>T in this family.
Several arguments support the non-pathogenicity of the single *PKP2* variant. *In silico* prediction algorithms do not support pathogenic classification of the c.419C>T variant.[30, 31] The variant affects a mildly conserved residue[18] and the resulting phenylalanine at position 140 was found at that position in other species.[27] Second, in a study by Christensen et al.[27] this variant was found in 3 out of 53 AC patients but also in 5 out of 650 healthy controls. Moreover, the individual AC patient first described in the study by Dalal et al.[26] carrying the variant, also carried a second, pathogenic mutation in *PKP2* (c.2146-1G>C p.IVS10-1G>C), and an unclassified variant in *DSG2* (c.166G>A p.Val56Met).[28] In addition, the cosegregation of the *PKP2* c.419C>T variant has been studied in five families with AC.[20, 26, 28] Syrris et al.[20] and later Sen-Chowdry et al.[25] describe a family of three generations with sudden cardiac death, VT, and structural alterations in the RV and LV, with affected patients carrying *PKP2* c.419C>T. Nonetheless, a first degree relative had structural abnormalities in the RV and NSVT but did not carry the variant. Similar were the results of a study of Christensen et al.[27] and a study by Xu et al.[28], who described this variant in AC patients but established incomplete cosegregation in three distinct families.

Desmosomal disease in this family?

The non-desmosomal *PLN* mutation c.40_42delAGA cosegregates with AC diagnosis and LV abnormalities whereas the desmosomal *PKP2* variant c.419C>T does not. AC is usually associated with desmosomal gene mutations. There is limited knowledge on the pathogenic mechanism of non-desmosomal *PLN* mutations in AC patients. Redistribution of the desmosomal protein *JUP* has been hypothesized as a final common pathway for the AC phenotype.[5, 12] This hypothesis is supported by the single patient in the present study undergoing immunohistochemical analysis who displayed mild, heterogeneous reduction of the *JUP* signal at the intercalated disks. Reduced immunoreactive signal of *JUP* has also been demonstrated in other AC patients with the *PLN* mutation in the study of Van der Zwaag et al.[32] A reduced immunoreactive signal of the desmosomal protein *PKP2* was additionally observed in the patient from our study, who had the *PLN* c.40_42delAGA mutation and, remarkably, not the *PKP2* c.419C>T variant. The *PLN* mutation may have indirect effects on cardiac desmosomes by altered intracellular calcium handling.[32]

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Limitations

Only one family with 13 family members was available for cosegregation analysis of the *PKP2* c.419C>T variant. This study should be extended in other families. Moreover, functional analysis should be performed to assess the apparent non-pathogenicity of *PKP2* variant c.419C>T. However, we demonstrated cosegregation of the *PLN* mutation with the diagnosis of AC and LV involvement. Available evidence did not reveal other mutations as causal for the familial AC in this family. LV involvement in *PLN* mutation-positive family member IV:6, was diagnosed based on negative T waves in left precordial leads. Since this subject also had hypertension (although very mild) and borderline ECG amplitude values, LV hypertrophy is not excluded with certainty, although detailed MRI findings were within normal limits.

Conclusion

A *PLN* mutation cosegregates with the diagnosis of AC and with electrocardiographic and structural LV abnormalities. Mutation-positive subjects are phenotypically characterized by low voltages and inverted T waves in left precordial leads on the ECG. In this family, there is no evidence of individual disease causing contribution of the single c.419C>T variant in *PKP2*.

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CHAPTER 5

Arrhythmogenic Right Ventricular Dysplasia/ Cardiomyopathy According to Revised 2010 Task Force Criteria with Inclusion of Non-Desmosomal Phospholamban Mutation Carriers

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Abstract

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is frequently associated with desmosomal mutations. However, non-desmosomal mutations may be involved. We assessed the contribution of a phospholamban (*PLN*) gene mutation to ARVD/C diagnosis according to revised 2010 Task Force Criteria (TFC). In 142 Dutch patients (106 males, age 51±13 years) with proven ARVD/C (fulfilment of 2010 TFC for diagnosis), 5 known desmosomal genes: *PKP2*, *DSP*, *DSC2*, *DSG2*, *JUP*, and the non-desmosomal *PLN* gene were screened. After genetic analysis, phenotypic characteristics of desmosomal versus *PLN* mutation carriers were compared. In 59/142 (42%) ARVD/C patients no desmosomal mutation was found. In 19/142 (13%) patients the *PLN* founder mutation c.40_42delAGA (p.Arg14del) was identified. *PLN* mutation carriers more often had low voltage ECG ($p=0.004$), inverted T waves in V4-6 ($p<0.001$), and additional structural ($p=0.007$)/functional ($p=0.017$) LV impairment, whereas desmosomal mutation carriers had more solitary RV abnormalities. Revised TFC included 21/142 proven ARVD/C patients that did not meet the 1994 TFC, including 7 *PLN* mutation carriers. In conclusion, there is a substantial contribution of *PLN* mutation to ARVD/C diagnosis by 2010 TFC. In 32% (19/59) of genetically unexplained proven ARVD/C patients this non-desmosomal mutation was found. *PLN* mutation carriers have ARVD/C characteristics, including important RV involvement, and additionally more often low voltage ECG, inverted T waves in left-precordial leads, and LV involvement.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C), also known as Arrhythmogenic Cardiomyopathy, is a hereditary desmosomal disease with right ventricular (RV) and possibly also left ventricular (LV) involvement.[1-5] ARVD/C is diagnosed according to internationally consensus based Task Force Criteria (TFC) for family history, depolarization and repolarization abnormalities, ventricular arrhythmias, global and/or regional dysfunction and structural abnormalities of the RV, and tissue characterization.[6] The TFC were revised in 2010, creating a new set of criteria that improved the diagnostic yield.[7-9] In the revised 2010 TFC a major criterion was assigned to identification of a pathogenic gene mutation associated with ARVD/C.[6] Pathogenic desmosomal gene mutations have been identified in approximately 60% of Dutch index patients.[10] In a minority of ARVD/C patients a non-desmosomal mutation predisposes to the phenotype.[11-16] The c.40_42delAGA (p.Arg14del) mutation in the non-desmosomal phospholamban (*PLN*) gene was first described in two distinct families with heart failure and ventricular tachyarrhythmias.[17-19] Recently in The Netherlands, we identified the c.40_42delAGA founder mutation in a large series of patients diagnosed with dilated cardiomyopathy or ARVD/C.[16] The aim of this study was 1) assessment of the contribution of the *PLN* mutation to ARVD/C phenotype and diagnosis according to the 2010 TFC and 2) evaluation of the phenotype of ARVD/C patients with desmosomal mutations and those with the non-desmosomal *PLN* mutation.

Methods

A total of 153 Dutch Caucasian ARVD/C index patients from 6 university centers (age 51±14 years, 114 (75%) men) diagnosed according to the 2010 TFC or at autopsy were included.[6] A subset of this cohort has been described in previous studies by Cox et al.[10] and Van der Zwaag et al.[16] An index patient was the first in the family diagnosed with ARVD/C in whom DNA analysis was started. All patients consented to clinical and DNA evaluation.

The diagnostic process included a detailed clinical and family history, physical examination, 12-lead ECG (while off drugs, in 100%), 48-hour ambulant ECG monitoring (in 70%), exercise testing (in 69%), and 2D-transthoracic echocardiography (in 89%). In addition, magnetic resonance imaging (MRI, in 67%) with delayed enhancement analysis (DE, in 30%), electrophysiological study (EPS, in 62%), RV/LV cine-angiography (in 51%), and endomyocardial biopsy (in 47%) were performed when indicated.

Autopsy was performed in 5 patients. The cardiac tissue specimens were stained with hematoxylin and eosin and Masson's trichrome staining. Findings from endomyocardial biopsies were not included in the study due to small numbers of diagnostic biopsies and assessment according to either the 1994 or 2010 TFC.[6, 20]

Genomic DNA was extracted from peripheral blood as described before.[21] Direct sequence analysis of all coding regions and intron/exon boundaries of the desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), desmocollin-2 (*DSC2*), and

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the *PLN* gene was performed. In addition, multiplex ligation-dependent probe amplification (MLPA) analysis was performed to identify large *PKP2* deletions (SALSA MLPA kit P168 ARVC-*PKP2*, MRC Holland, Amsterdam, The Netherlands). Primer sequences and PCR conditions are available on request.

Mutations were considered proven pathogenic when they were nonsense, frameshift, splice site, or exon deletion mutations and not identified as polymorphism. For missense variants predictive algorithms Sorting Tolerant from Intolerant (SIFT) and Polymorphism Phenotyping-2 (PolyPhen-2) were used. Missense variants were considered most likely pathogenic when both algorithms predicted a deleterious effect (SIFT: tolerance index score ≤0.05, PolyPhen-2: probably pathogenic classification) and variants were absent in 200 Dutch Caucasian control individuals.[10] When available, cosegregation analysis data was additionally taken into account.

For phenotype comparison of desmosomal and *PLN* mutation carriers, only those patients with a proven pathogenic or a most likely pathogenic mutation were labeled as pathogenic mutation carriers. Patients carrying unclassified variants in the desmosomal genes were labeled together with those patients in whom no mutation or variant could be identified, as without pathogenic mutation.[10] After clinical and genetic screening, patients were divided into 2 groups: pathogenic 1) desmosomal and 2) *PLN* mutation carriers. The occurrence of 2010 TFC and RV and LV involvement were assessed in both mutation groups. RV involvement was considered positive in the presence of ≥1 of the following: negative T waves in leads V1-3, RV wall motion abnormalities (a/dyskinesia) and/or DE in the RV on imaging studies, and RV ejection fraction (RVEF) ≤45%. LV involvement was considered positive in the presence of ≥1 of the following: negative T waves in leads V4-6, LV wall motion abnormalities (a/dyskinesia) and/or DE in the LV on imaging studies, and LV ejection fraction (LVEF) <50%. Finally, the inclusion of the *PLN* mutation and associated phenotype in ARVD/C diagnosis by the revised TFC was measured.

Continuous variables were compared using Student's t-test. Categorical variables were analyzed using contingency tables and Fisher's Exact Test. Descriptive statistics were reported as mean ± standard deviation. P-values ≤0.05 were considered statistically significant. Results of phenotype comparison were shown in odds ratio estimates and associated confidence intervals. Correction for multiple testing was performed by the Holm-Bonferroni correction. All analyses were performed with PASW Statistics 20.0 software (SPSS, Chicago, IL, USA) and R software (R Development Core Team 2008, http://www.R-project.org).

Results

All 153 patients with proven (definite) ARVD/C underwent screening of desmosomal genes *PKP2*, *DSP*, *JUP*, *DSG2*, and *DSC2*. In 11/153 patients additional screening of *PLN* was impossible due to insufficient DNA or lack of patient permission. Thus, 142/153 patients underwent screening of *both* the desmosomal genes and the non-desmosomal *PLN* gene. A pathogenic desmosomal mutation was identified in 83/142 (58%) patients (Table 1). Of 142 proven AVRD/C patients, 19 (13%) carried the *PLN* c.40_42delAGA (p.Arg14del) mutation. No other mutations besides this founder mutation were identified in the *PLN* gene. More importantly, in all 19 *PLN* mutation carriers this was the only pathogenic mutation found.

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Table 1.

Gene (number of patients with mutation)	**DNA change**	**Protein change**	**Frequency**
PKP2 (n=74)	deletion exons 1-4	p.(?)	1/74
	deletion exons 1-14	p.(?)	1/74
	deletion exon 10	p.(?)	1/74
	c.148_151del	p.(Thr50fs)	1/74
	c.235C>T	p.(Arg79*)	9/74
	c.258T>G	p.(Tyr86*)	1/74
	c.397C>T	p.(Gln133*)	6/74
	c.917_918del	p.(Pro318fs)	3/74
	c.1211dup	p.(Val406fs)	11/74
	c.1369_1372del	p.(Gln457*)	3/74
	c.1378G>A	p.(Val445fs)	1/74
	c.1848C>A	p.(Tyr616*)	4/74
	c.1951C>T	p.(Arg651*)	1/74
	c.2028G>A	p.(Trp676*)	1/74
	c.2034G>A	p.(Trp678*)	1/74
	c.2062T>C	p.(Ser688Pro)	1/74
	c.2146-1G>C	p.(Met716fs)	6/74
	c.2386T>C	p.(Cys796Arg)	10/74
	c.2421C>A	p.(Tyr807*)	1/74
	c.2489+1G>A	p.(Lys768fs)	5/74
	c.2489+4A>C	p.(Lys768fs)	4/74
	c.2509del	p.(Ser837fs)	1/74
	c.2544G>A	p.(Trp848*)	1/74
DSP (n=1)	c.1982A>T	p.(Asn661Ile)	1/1
JUP (n=0)			
DSG2 (n=6)	c.137G>A	p.(Arg46Gln)	2/6
	c.614C>T	p.(Pro205Leu)	1/6
	c.378+2T>G	p.(Ile73_Leu126del)	1/6
	c.874C>T	p.(Arg292Cys)	1/6
	c.1003A>G (homozygous)	p.(Thr335Ala)	1/6
DSC2 (n=3)	c.608G>A (homozygous)	p.(Arg203His)	1/3
	c.942+3A>G	p.(?)	1/3
	c.943-1G>A	p.(?)	1/3

Pathogenic desmosomal gene mutations found in the 5 known desmosomal genes in 83/142 (58%) Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy patients. One patient carried two pathogenic desmosomal mutations: *PKP2* c.2489+4A>C and *DSP* c.1982A>T. *DSC2*: Desmocollin-2, *DSG2*: Desmoglein-2, *DSP*: Desmoplakin, *JUP*: Plakoglobin, *PKP2*: Plakophilin-2.*: Stop codon.

Table 2.

Clinical Parameters	**Desmosomal mutation** n=83	**Phospholamban mutation** n=19	**p-value**	**OR estimate**	**Lower limit**	**Upper limit**	**Adjusted p-value**
Gender	60 men (72%)	12 men (63%)	0.419	1.52	0.45	4.82	1.000
Age mean±SD	50±14	52±11	0.663	-1.28	-7.21	4.65	1.000
Family History							
Family History	1/83 (1%)	0/19 (0%)	1.000	Inf	0.01	Inf	1.000
Autopsy	6/83 (7%)	1/19 (5%)	1.000	1.40	0.15	68.03	1.000
Premature SCD	5/83 (6%)	4/19 (21%)	0.060	0.25	0.05	1.38	0.950
Electrocardiography							
TAD ≥55 ms	44/83 (53%)	11/19 (58%)	0.801	0.82	0.26	2.51	1.000
Epsilon waves	10/83 (12%)	1/19 (5%)	0.685	2.45	0.31	112.82	1.000
Late Potentials	26/61 (43%)	2/16 (13%)	0.039*	5.11	1.03	50.19	0.660
Low voltages	16/73 (22%)	11/19 (58%)	0.004*	0.21	0.06	0.68	0.089
Inverted T V1-3	63/83 (76%)	8/19 (42%)	0.006*	4.26	1.35	14.11	0.127
Inverted T V4-6	6/83 (7%)	10/19 (53%)	<0.001*	0.07	0.02	0.28	<0.001*
Arrhythmias							
>500 VES/24h	30/55 (55%)	14/16 (88%)	0.020*	0.18	0.02	0.88	0.360
LBBB VT	75/83 (90%)	15/19 (79%)	0.229	2.47	0.48	10.78	1.000
LBBB VT sup.	49/83 (59%)	8/19 (42%)	0.207	1.97	0.64	6.29	1.000
RBBB VT	11/72 (15%)	5/19 (26%)	0.311	0.51	0.13	2.17	1.000
Structural							
Structural Major	60/83 (72%)	14/19 (74%)	1.000	0.93	0.24	3.15	1.000
RV WMA	65/73 (89%)	15/19 (79%)	0.262	2.15	0.42	9.39	1.000
RV DE	19/26 (73%)	5/7 (71%)	1.000	1.08	0.08	8.80	1.000
LV WMA	9/71 (13%)	8/19 (42%)	0.007*	0.20	0.06	0.75	0.144
LV DE	10/21 (48%)	3/7 (43%)	1.000	1.20	0.16	10.34	1.000
Functional							
RVEF ≤45%	23/26 (89%)	10/11 (91%)	1.000	0.77	0.01	11.06	1.000
LVEF <50%	12/38 (32%)	11/16 (69%)	0.017*	0.22	0.05	0.86	0.320
Ventricular involvement							
RV-involvement	79/83 (96%)	18/19 (95%)	1.000	1.1	0.02	11.98	1.000
LV-involvement	25/83 (30%)	14/19 (74%)	0.001*	0.16	0.04	0.52	0.026*

Phenotypic characterization of desmosomal versus phospholamban mutation carriers. Ajusted p-value: corrected p-value by Holm-Bonferroni correction. LBBB VT: ventricular tachycardia with left bundle branch block morphology; OR: odds ratio; RBBB VT: VT with right bundle branch block morphology; RV DE: RV delayed enhancement; RV WMA: right ventricular wall motion abnormalities (a/dyskinesia); SCD: sudden cardiac death (<35 years); Structural Major: structural RV abnormalities accounting for a major criterion; sup.: superior axis; TAD: terminal activation duration; VES: ventricular extrasystoles; *: statistical significant result with p-value <0.05.

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Consequently, *PLN* c.40_42delAGA explained nearly one third (19/59, 32%) of the up till then genetically unexplained ARVD/C cases. The non-desmosomal *PLN* gene is at present, after the desmosomal *PKP2* gene, the gene in which second most frequently a mutation is identified in Dutch ARVD/C patients and the c.40_42delAGA mutation is found more frequent than each individual desmosomal mutation.

Baseline characteristics were similar in desmosomal and *PLN* mutation groups. Mean follow-up duration was 12±9 years in the desmosomal mutation carriers and 10±5 years in *PLN* mutation carriers (p=0.258). Findings are summarized in Table 2. Sustained ventricular tachyarrhythmia (VT) with left bundle branch block (LBBB) morphology, i.e. originating from the RV, and RV dysfunction were present in the large majority of ARVD/C patients regardless of the related desmosomal or *PLN* mutation. Desmosomal mutation carriers were more often characterized by negative T waves in right precordial leads V1-3 and beyond (major criterion, Figure 1) in comparison with *PLN* mutation carriers. Nevertheless, this characteristic was also present in nearly half of *PLN* mutation carriers, as demonstrated by Figure 2. *PLN* mutation carriers more often had a low voltage ECG and particularly negative T waves in left precordial leads V4-6 (minor criterion, Figure 3). The minor criterion ">500 ventricular extrasystoles/24 hours by Holter" was furthermore present in the large majority of ARVD/C patients with the *PLN* mutation. Additional LV structural (wall motion abnormalities) and functional (reduced LVEF) abnormalities were considerably more often observed in *PLN* mutation carriers. Accordingly, the combined parameter RV involvement was a common finding in both groups (Figure 4A), whereas additional LV involvement was significantly more frequent a phenotypic expression of the *PLN* mutation (Figure 4B). Assessment of odds ratio estimates of unadjusted p-values and associated confidence intervals seemed to confirm all the above mentioned phenotypical differences between the two mutation groups, except for late potentials.

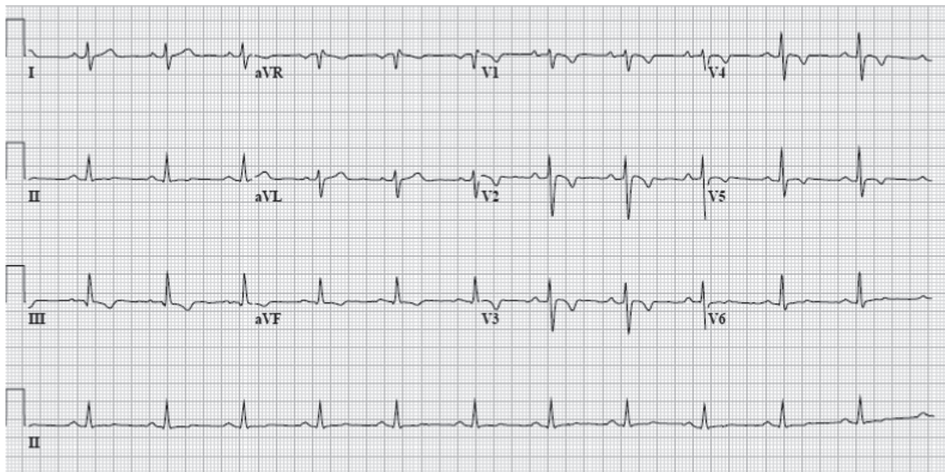

Figure 1. Electrocardiogram of a proven ARVD/C patient with the desmosomal *PKP2* c.1211dupT mutation (while off drugs) showing normal sinus rhythm and right axis deviation with inverted T waves in right precordial leads V1-3 and beyond (V4-5). Moreover, non-specific repolarization abnormalities are present in leads II, III and aVF.

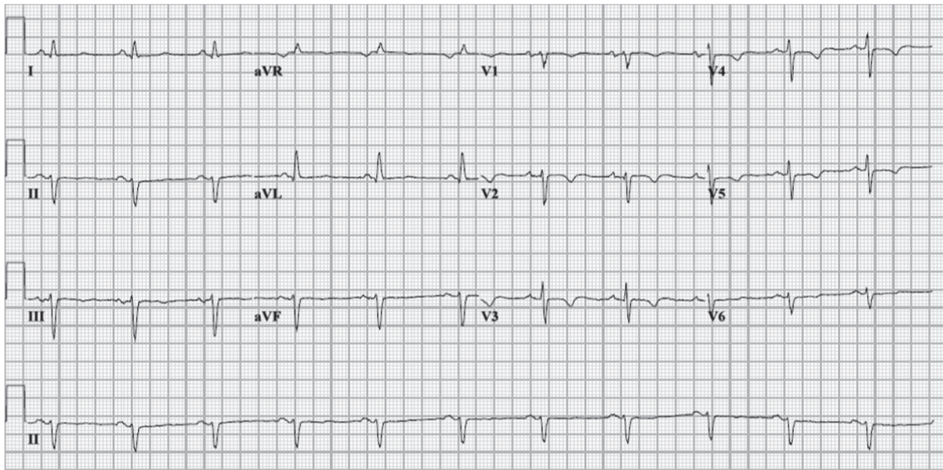

Figure 2. Electrocardiogram of a proven ARVD/C patient with the PLN mutation (while off drugs) showing normal sinus rhythm and left axis deviation with classic negative T waves in right precordial leads and beyond (major criterion in the revised Task Force Criteria). Terminal activation duration is not prolonged, maximum is 50 ms in lead V1.

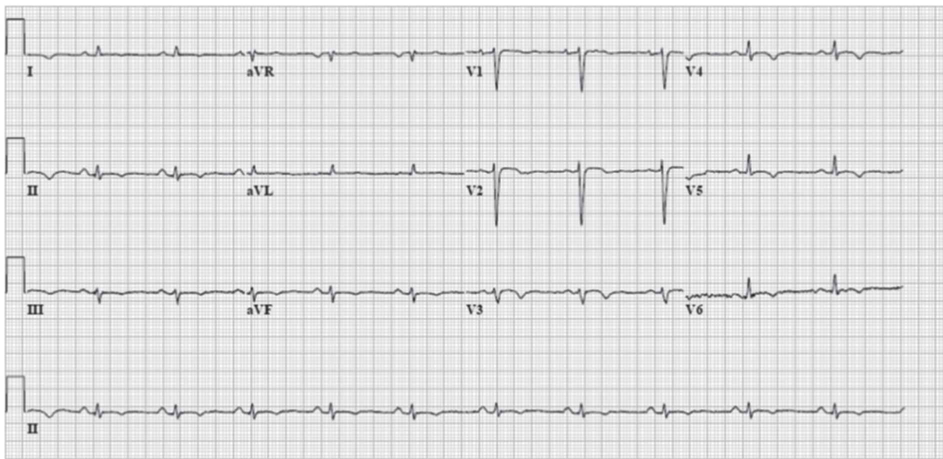

Figure 3. Electrocardiogram of a proven ARVD/C patient with the *PLN* mutation (while off drugs) showing normal sinus rhythm with remarkably low voltages in the standard leads (<0.5mV) and negative T waves in left precordial leads V3-6. In addition negative T waves in II, III, and aVF.

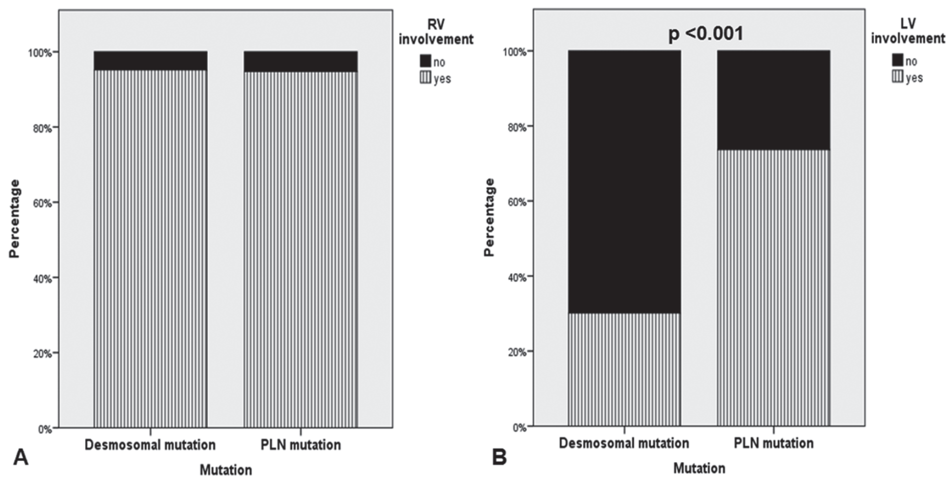

Figure 4A and B. A. RV involvement (considered positive in presence of: inverted T in V1-3, and/or RV wall motion abnormalities (a/dyskinesia), and/or RV delayed enhancement on magnetic resonance imaging, and/or RV ejection fraction ≤45%) is present in large majority of both mutation carrier groups. *PLN*: Phospholamban. B. Additional LV involvement (considered positive in presence of: inverted T in V4-6, and/or LV wall motion abnormalities (a/dyskinesia), and/or LV delayed enhancement on magnetic resonance imaging, and/or LV ejection fraction <50%) on the other hand, is significantly more often a phenotypic expression of the *PLN* mutation.

In addition, even after statistical correction for multiple testing by the conservative Holm-Bonferroni correction, negative T waves in left precordial leads V4-6 and LV involvement stood out as significantly different between desmosomal and *PLN* mutation carriers.

Cardiac explantation material of 2 patients with a desmosomal mutation and 3 with the *PLN* mutation was analyzed. The autopsy of the first desmosomal mutation carrier, dying suddenly at age 27, was diagnostic for ARVD/C with classic fibrofatty replacement of the RV. The second desmosomal mutation carrier, who underwent cardiac transplantation at age 56, had fibrofatty infiltration of virtually the whole RV, severe involvement of the LV and the interventricular septum (Figure 5). The explantation material from the first *PLN* mutation carrier, who died 1 day after cardiac transplantation due to RV failure at age 43, showed fibrofatty replacement of the RV, LV, and interventricular septum. The second *PLN* mutation carrier, who died at age 61 from cardiogenic shock, had transmural fibrofatty degeneration of the RV wall. In addition, the autopsy of the third patient, who died at age 71 from end-stage heart failure, also demonstrated fibrofatty replacement of the RV and to lesser extent of the LV (Figure 6).

The revised criteria for diagnosis additionally included 21/142 proven (2010 TFC) ARVD/C patients that did not meet the 1994 TFC.[20] Among those 21 patients, 7 were *PLN* mutation carriers. Diagnosis in these *PLN* mutation carriers was primarily based on TFC for arrhythmias (7/7), structural and functional RV abnormalities (6/7), prolonged TAD (4/7), right precordial T wave inversion (4/7), and left precordial T wave inversion (3/7).

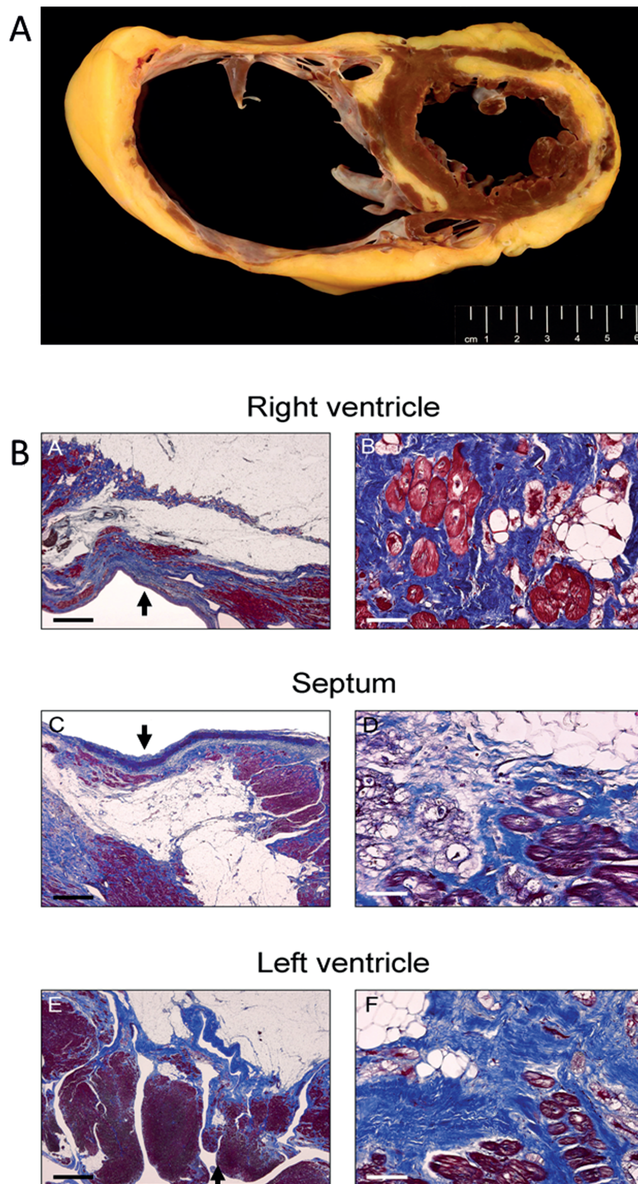

Figure 5. Heart of a 56-year old woman with *PKP2* c.2386T>C mutation, demonstrating the end-stage of desmosomal ARVD/C. **A.** Gross examination of the heart. Cross section through the ventricles of the explanted heart showing severe dilatation of the right ventricle with fibro-fatty replacement of nearly the entire free wall of the right ventricle (RV). In addition, extensive fibro-fatty alteration in the left ventricle (LV) and the septum. **B.** Microscopic examination of the myocardium (Modified AZAN stain). B1 and B2, right ventricle. B1, overview showing fibrofatty replacement of the wall. Cardiomyocytes in red, fibrous tissue in blue and lipocytes. Endocardium is indicated by the arrow. B2, higher magnification of the same area showing large cardiomyocytes (in red) with vacuolar degeneration. B3 and B4, fibrofatty replacement of the myocardium in the septum. Endocardium indicated by the arrow. B5 and B6, fibrofatty replacement of the left ventricular wall. Scale bars in B1, B3, and B5 1mm. Endocardium indicated by the arrow. Scale bars in B2, B4, and B6 100µm.*

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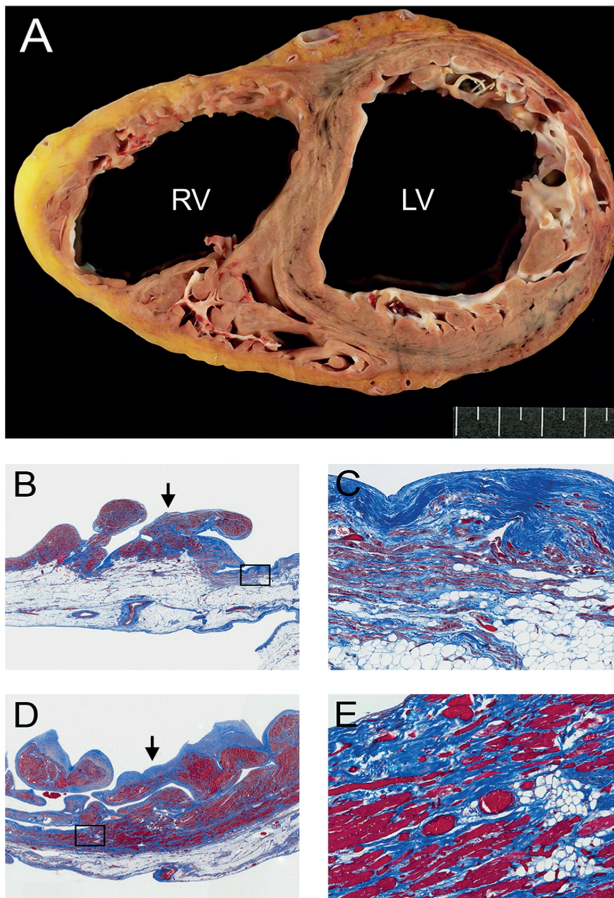

Figure 6. Heart of a 71-year old woman with end-stage ARVD/C with *PLN* c.40_42delAGA mutation. **A.** cross section of the heart showing fibrofatty replacement of the right ventricular wall (right ventricle indicated by RV) and left posterolateral wall (left ventricle indicated by LV). **B.** and **C.** microscopic examination of the right ventricular wall showing severe fibrofatty replacement. Masson's Trichrome stain showing cardiomyocytes in red and fibrosis in blue. Endocardium is indicated by the arrow. **B.** magnification 10x; **C**, magnification 100x of the indicated area B. **D.** and **E.** microscopic examination of the left ventricular wall showing severe fibrosis with areas of fibrofatty alteration. Endocardium is indicated by the arrow. **D.** magnification 10x; **E.** magnification 100x of the indicated area in D.

Discussion

ARVD/C is characterized by electrocardiographic alterations and ventricular arrhythmias in early stages, followed by structural and functional myocardial disease, and frequently sustained monomorphic ventricular tachycardias, in late overt stages. Histopathologically and functionally primarily RV (classical ARVD/C), LV, or biventricular forms exist, although at the level of intercalated disk protein expression and distribution, both ventricles are affected.[4, 5] Because of overlap, discrimination in subforms seems rather artificial. Arrhythmogenic cardiomyopathy, defined as structural heart disease in late stages and preceded by arrhythmias in early or even concealed stage may be considered as preferred terminology by including classical ARVD/C as well as less typical forms.

Criteria for ARVD/C diagnosis were first established in 1994 by an international Task Force and revised in 2010 to improve sensitivity. The 2010 TFC included T wave inversion in left precordial leads V4-6 as a minor criterion and upgraded T wave inversion in V1-3 and beyond to a major criterion.[6, 20] We hypothesized that these changes facilitated fulfilment of ARVD/C criteria in patients with biventricular and primarily LV involvement.

In the present study we compared ARVD/C patients with a desmosomal mutation versus non-desmosomal *PLN* mutation according to the revised 2010 TFC. *PLN* mutation carriers showed more often T wave inversion in the left precordial leads and more ventricular ectopy than desmosomal mutation carriers, who had more often T wave inversion in the right precordial recordings. Nonetheless, both types of mutation carriers showed nearly always RV disease, whereas the *PLN* mutation was more often associated with additional LV involvement and low voltage ECG. With the 2010 TFC the *PLN* c.40_42delAGA founder mutation appeared to be the most prevalent individual mutation associated with ARVD/C in The Netherlands. This mutation was identified in 13% of patients who fulfilled TFC for ARVD/C diagnosis, even without the inclusion of a major criterion for a pathogenic gene mutation. The majority (12/19) of *PLN* mutation carriers already fulfilled the highly specific 1994 TFC for ARVD/C diagnosis. The revised criteria proved more sensitive, including 7 *PLN* mutation carriers, who did not meet old 1994 TFC.

In multiple studies pathogenic desmosomal gene mutations were associated with ARVD/C.22-26 Similar as in our previous study, even 58% of Dutch patients had pathogenic desmosomal mutations.[10] In addition, in 32% of proven TFC positive ARVD/C patients without a pathogenic desmosmal mutation the non-desmosomal *PLN* mutation was identified.

The pathophysiologic mechanism of the *PLN* mutation underlying ARVD/C and specific phenotypic abnormalities is unknown yet. Intra- and extracellular calcium levels are involved in desmosome assembly and disassembly.[27] Hypotheses of *PLN* mediated increased cytosolic calcium levels and thereby desmosome disassembly have been described.[16] In addition, the fibrofatty replacement in cardiac explantation tissue (gold standard for ARVD/C diagnosis) of *PLN* mutation carriers was indistinguishable from desmosomal mutation carriers. In a functional study by Haghighi et al.[18], assessing this specific *PLN* mutation, it was postulated that the mutated *PLN* inhibits adequate response to adrenergic stimulation to facilitate augmentation of cardiac function and thereby causes contractile dysfunction and cardiomyopathy with LV involvement. A worse arrhythmogenic phenotype was reported in ARVD/C patients with the

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PLN mutation compared to patients without the mutation.[16] The *PLN* mutation could trigger arrhythmias by 1) adrenergic stimulation-induced calcium release, 2) cytosolic calcium overload, or 3) tissue architecture alteration by fibrosis.[28-30] Low QRS voltages are previously described to characterize *PLN* mutation carriers.[17, 18] Attenuated voltages were found irrespective of structural abnormalities (WMA, i.e. a/dyskinesia).[17, 18] The mechanism of the attenuated amplitudes remains to be elucidated.

The *PLN* c.40_42delAGA mutation was originally identified in families with dilated cardiomyopathy, ventricular arrhythmias, and premature sudden death.[17-19] ARVD/C was not reported in the functional studies characterizing this mutation. However, since the families with this mutation were initially diagnosed with dilated cardiomyopathy, the focus of these studies was different. Moreover, in the family described by Posch et al.[17], 2 mutation carrying family members were reported to be free of symptoms of heart failure but presented with sudden cardiac death in their 5th decades. Furthermore, Haghighi et al.[18] describe heterozygous carriers that primarily had frequent ventricular extrasystoles and VT episodes and only later developed progressive ventricular dilatation and heart failure. Finally, in a large cohort of *PLN* mutation carriers, Van der Zwaag et al.[16] demonstrate the occurrence of the founder mutation and frequent incidence of revised TFC for ARVD/C and also early VT episodes in patients diagnosed with dilated cardiomyopathy or ARVD/C. These arguments support the ARVD/C or arrhythmogenic cardiomyopathy concept: First ventricular arrhythmias and only later structural alteration and functional impairment.[31] This hypothesis should be examined in prospective studies, following *PLN* mutation carriers from a young age.

This study has several limitations. First, it is a retrospective analysis of ARVD/C patients with a desmosomal or the *PLN* mutation registered since 1997. Not every diagnostic examination was performed in the whole cohort since the clinical evaluations reflect decisions made in daily patient care. In 33% of the patients no MRI analysis was possible because of prior ICD implantation. Subsequently, a selection bias cannot be excluded. Nevertheless, proportions of patients undergoing the examinations were similar in the desmosomal and *PLN* group. Second, the comparison of both mutation carrier groups was hampered by the small numbers in both groups. Third, diagnosis of ARVD/C is dependent on both fulfilment of by consensus obtained TFC and absence of other known aetiologies. One may argue that *PLN* mutation carriers have a form of dilated cardiomyopathy with predominant LV and heart failure features. However, the patients analyzed in this study were already included in the ARVD/C database based on clinical TFC (labelled as without related pathogenic mutation) and the *PLN* mutation was only recently identified. None of the *PLN* mutation carriers presented with heart failure, whereas the majority had arrhythmic events. Finally, the high incidence of the 40_42delAGA mutation is presumably due to a founder effect in The Netherlands.[16] Although most prevalent in The Netherlands, this mutation is also found elsewhere in Europe, Canada and the USA.[16-19] Thus, further studies in other cohorts are needed.

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CHAPTER 6

Prediction of Pathogenicity of Missense Variants in Arrhythmogenic Right Ventricular Dysplasia/ Cardiomyopathy

In preparation

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Abstract

Background

Pathogenicity of truncating and splice site mutations in Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) has been widely accepted. However, pathogenicity of missense variants remains controversial.

Objective: We assessed pathogenicity and associated phenotype of ARVD/C related missense variants.

Methods

A total of 341 family members (age 41±20 years) of proven (fulfilment of 2010 Task Force Criteria for diagnosis) ARVD/C index patients (138 families), with desmosomal (*PKP2*, *DSC2*, *DSG2*, *DSP*, *JUP*) or non-desmosomal (*PLN*, *TMEM43*) mutations were used from US/Dutch cohorts. Family members with >1 mutation were excluded. Missense variants were considered pathogenic and included when 1) both algorithms SIFT and PolyPhen-2 predicted pathogenicity (SIFT score <0.02, PolyPhen-2 score >0.900) and 2) minor allele frequency (MAF) in ESP exome database was low (≤0.05%). Phenotypic characteristics were compared between missense and non-missense (truncating/splice site) mutation carriers.

Results

A pathogenic missense mutation was identified in 48/341 (14%) family members. Frequently occurring missense mutation was *PKP2* c.2386T>C (p.Cys796Arg; 24/48). Phenotypic characteristics, and more importantly, sustained arrhythmia (ventricular tachycardia/fibrillation or appropriate ICD therapy) free survival curves (p=0.803) of family members with missense mutations were indistinguishable from those with non-missense mutations (truncating 215/341, splice site 78/341).

Conclusion

Missense mutations can have a similar pathogenic character as non-missense mutations in family members of proven ARVD/C index patients. Pathogenicity of missense mutations are correctly predicted by combined criteria from SIFT, PolyPhen-2, and MAF in exomes.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C), also known as Arrhythmogenic Cardiomyopathy, is considered a hereditary disease and is phenotypically characterized by ventricular arrhythmias and structural and functional abnormalities with fibrofatty replacement of cardiomyocytes from the right ventricle (RV), left ventricle (LV), or from both ventricles.[1-6] A substantial part of ARVD/C cases is familial with autosomal dominant inheritance pattern, incomplete penetrance, and variable expressivity.[7, 8] Patients present typically between the second and fourth decade of life with episodes of ventricular tachycardia (VT). Nonetheless, sudden cardiac death (SCD) can occur as early as in adolescence, whereas subjects with a pathogenic mutation may also remain without any signs and symptoms into old age. This stresses the importance of timely diagnosis and discrimination of subjects at risk.

The identification of a pathogenic mutation in ARVD/C index patients, allows cascade screening of family members identifying subjects with a predisposition for the disease. Pathogenic mutations in desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmocollin-2 (*DSC2*), and desmoglein-2 (*DSG2*), and non-desmosomal phospholamban (*PLN*) and transmembrane protein-43 (*TMEM43*) genes have been related to ARVD/C.[9-18]

The pathogenicity of radical mutations, i.e. truncating or splice site mutations (mutations affecting the invariant AG splice-acceptor and GU splice-donor sites of exons), resulting in truncation or absence of the protein product from the mutated allele, is widely accepted. However, the pathogenicity of missense variants (also known as non-synonymous single nucleotide variants), resulting in a single amino acid substitution, remains controversial.

Missense variants are found at similar rates in both ARVD/C cases and healthy controls.[19] Although the majority of missense variants is innocuous, the effect of missense variants can be deleterious. *In silico* prediction programmes Sorting Intolerant from Tolerant (SIFT)[20] and Polymorphism Phenotyping-2 (PolyPhen-2)[21] have been designed to predict pathogenicity of missense variants based on sequence conservation, homology, and structure. In addition, conditions to distinguish pathogenic missense variants in ARVD/C, based on race/ethnicity, variant location, and sequence conservation, have been reported.[19] Despite these efforts, decisions on possible pathogenic character of missense variants are still mostly based on expert opinion. In order to enhance objective, transparent classification of missense variants in ARVD/C we suggest an algorithm with synergistic use of SIFT, PolyPhen-2, and the minor allele frequency (MAF) in control populations for the prediction of pathogenicity of missense variants.

Since the establishment of pathogenicity of missense variants has critically important implications for genetic screening and subsequent risk evaluation, the aim of this study was to assess pathogenicity and associated phenotypic characteristics of missense variants in family members of proven ARVD/C index patients. Missense variants were classified according to the newly suggested predictive algorithm.

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Methods

Study population

In the combined cohort of the Interuniversity Cardiology Institute of The Netherlands (ICIN) dataset[7] and the Johns Hopkins University School of Medicine (JHU) ARVD/C registry, only index patients and their family members with a pathogenic mutation (defined as truncating mutation, invariant splice site mutation, or missense variant complying to the algorithm) were included.

We chose to study the pathogenicity of missense variants solely in *family members* of proven ARVD/C patients to minimize ascertainment bias since index patients are by definition affected by the disease. Family members with >1 pathogenic mutation were excluded (n=7). In total, 341 family members (54% ICIN dataset, 46% JHU registry, 138 families, mean age 41±20 years, 160 (47%) men) were included. Virtually the complete cohort of family members was from Caucasian descent (99%). A subset of the Dutch family members has been described previously in the study by Cox et al.[7]

Molecular genetic screening

Genomic DNA was extracted from peripheral blood as described before.[22] Direct sequencing of all coding regions and intron/exon boundaries of the desmosomal genes *PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2*, and non-desmosmal *PLN* and *TMEM43* genes was performed in the index patients. The family members were analyzed for the presence or absence of the mutation identified in the index patients. Primer sequences and PCR conditions are available on request.

Interpretation of missense variants

To predict the pathogenicity of missense variants we employed an algorithm with synergistic use of predictive programmes SIFT (version 4.0.3b)[20] and PolyPhen-2 (version 2.2.2)[21] and the minor allele frequency (MAF) of variants in ethnically matched, ostensibly healthy controls included in the American Exome Sequencing Project (ESP) database, containing data of 6500 exomes (http://evs.gs.washington.edu/EVS/). In addition to evaluation according to the algorithm, the presence of the missense variants was assessed in 1000 Dutch control chromosomes.

Missense variants were included in the study and considered pathogenic upon fulfilment of *all* 3 following criteria of the algorithm: 1) a SIFT score <0.02, 2) a PolyPhen-2 score >0.900, and 3) a MAF ≤0.05 % in the ESP dataset (1 in 2000). In general, SIFT labels a variant with a score <0.05 as not-tolerated. A PolyPhen-2 score >0.920 is corresponding to a 'probably pathogenic' classification. The algorithm was intended to be conservative with low numbers of false positives. Missense variants adhering to the criteria of the algorithm are further referred to as missense mutations.

Phenotype

The diagnostic evaluation of the family members included a detailed clinical and family history, physical examination, 12-lead ECG (while off drugs, 77%), 48-hour ambulant ECG monitoring (63%), exercise testing (48%), 2D-transthoracic echocardiography (62%), and magnetic resonance imaging (MRI, 51%) with delayed enhancement analysis (DE). In addition, electrophysiological study (EPS, 15%), RV/LV cine-angiography (7%), and endomyocardial biopsy (7%) were performed when indicated.

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All index patients in the original combined cohort fulfilled the current TFC for ARVD/C diagnosis (proven ARVD/C).[23] The phenotype of the mutation positive family members of those index patients (the study subjects) varied from possible ARVD/C (1 major criterion, i.e. presence of pathogenic mutation, n=155, 45%), to borderline ARVD/C (1 major and 1 minor criterion, n=53, 16%), and fulfilment of criteria for ARVD/C diagnosis (n=105, 31%). In addition, there were 28 (8%) family members who presented with SCD and that were diagnosed with ARVD/C post-mortem.

Phenotypic characteristics, survival, and arrhythmic outcome were compared between family members with missense mutations and non-missense mutations (i.e. truncating or invariant splice site mutations). Appropriate implantable cardioverter defibrillator (ICD) intervention was defined as antitachycardia pacing or shock for VT or ventricular fibrillation (VF).

Statistics

Continuous variables were compared using Student's t-test. Categorical variables were analyzed using contingency tables and Fisher's Exact Test. Descriptive statistics were reported as mean ± standard deviation (SD). P-values ≤0.05 were considered statistically significant. No log rank test was performed in the Kaplan-Meier analysis of survival and cardiac transplantation since the assumption of proportional hazards was not appropriate (events in the non-missense group versus no events in the missense group). Therefore, the log rank test would be biased. Correction for possible correlation within families by the 'coxph' function of R was applied with the sustained arrhythmia survival analysis. All analyses were performed with PASW Statistics 20.0 software (SPSS, Chicago, IL, USA) and 'rms' package in R software (R Development Core Team 2008, http://www.R-project.org).

Results

Genetic screening

In the total cohort of family members with mutations in the ARVD/C-related desmosomal (*PKP2*, *DSP*, *JUP*, *DSC2*, *DSG2*) and non-desmosomal (*PLN*, *TMEM43*) genes, 48/341 (14%) had a pathogenic missense mutation, fulfilling the 3 criteria of the algorithm. The large majority of family members (215/341, 63%) had truncating mutations. Splice site mutations were found in 78/341 (23%) family members.

Non-missense mutations were mostly found in the *PKP2* gene, 92% of those family members had a *PKP2* mutation (Figure 1A and Supplemental table 1). Mutations in the *PLN*, *DSP*, *DSG2*, and *DSC2* genes were less frequently observed. No single *JUP* or *TMEM43* mutations were identified in the family members. Missense mutations were also predominantly found in the *PKP2* gene, but a substantially larger part of missense mutations was found in the *DSG2* gene when compared to non-missense mutations (Figure 1A and B).

The individual missense mutations that were identified in this cohort and their scores on the 3 criteria of the algorithm are specified in Table 1. The *PKP2* c.2386T>C (p.Arg796Cys) missense mutation was identified in 24/48 missense mutation-positive subjects. Other frequently occurring missense mutations were *DSG2* c.137G>A (p.Arg46Gln, 5/48) and *PKP2* c.1271T>C (p.Phe424Ser, 4/48).

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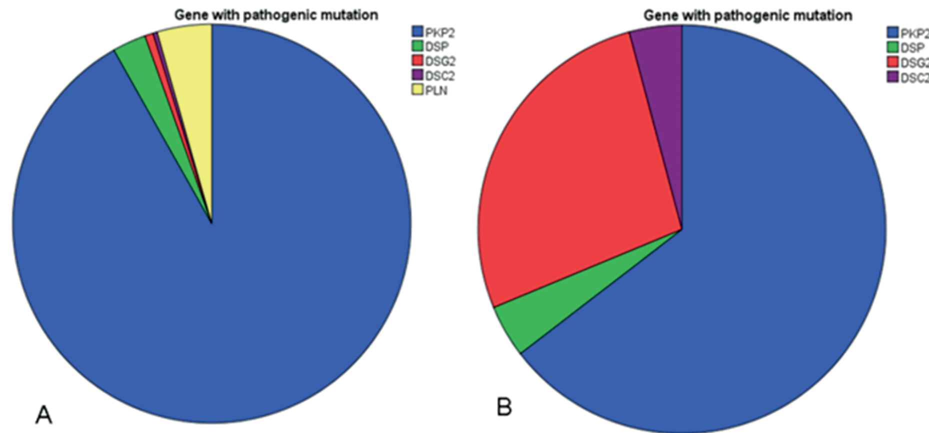

Figure 1A and B. Genes with mutations in family members with non-missense (truncating and splice site mutations) mutations (A) and missense mutations (B). **A.** Non-missense mutations were predominantly found in the plakophilin-2 (*PKP2*) gene (n=269, 91,8%), and less frequently in the phospolamban (*PLN*, n=13, 4,4%), desmoplakin (*DSP*, n=8, 2,7%), desmoglein-2 (*DSG2*, n=2, 0,7%), and desmocollin-2 (*DSC2*, n=1, 0,3%) genes. B. Missense mutations were also predominantly found in the *PKP2* gene (n=31, 65%) but a substantially larger part was found in the *DSG2* gene (n=13, 27%) when compared to non-missense mutations. *DSP* (n=2, 4%) and *DSC2* (n=2, 4%) missense mutations were found at lower rates.

Table 1.

Gene	**Number of family members**	**Nucleotide change**	**Amino acid change**	**SIFT score (<0.02)**	**PolyPhen-2 score (>0.900)**	**MAF ESP % (≤0.05%)**	**Numbers from cohort**
PKP2	4/48	c.1271T>C	p.Phe424Ser	0.00	0.999	0.00	JHU 4
	1/48	c.1844C>T	p.Ser615Phe	0.00	1.000	0.00	ICIN 1
DSP	2/48	c.2062T>C	p.Ser688Pro	0.00	1.000	0.02	ICIN 2
	24/48	c.2386T>C	p.Cys796Arg	0.00	0.988	0.00	ICIN 24
	2/48	c.1264G>A	p.Glu422Lys	0.00	0.992	0.00	JHU 2
DSG2	5/48	c.137G>A	p.Arg46Gln	0.00	1.000	0.00	ICIN 4/ JHU 1
	1/48	c.146G>A	p.Arg49His	0.00	1.000	0.00	JHU 1
	2/48	c.874C>T	p.Arg292Cys	0.00	1.000	0.00	ICIN 2
	2/48	c.1520G>A	p.Cys507Tyr	0.00	1.000	0.00	JHU 2
	2/48	c.2434G>T	p.Gly812Cys	0.00	1.000	0.01	JHU 2
	1/48	c.3140C>G	p.Tyr1047Arg	0.01	0.993	0.00	JHU 1
DSC2	2/48	c.608G>A	p.Arg203His	0.00	1.000	0.00	ICIN 2

Individual pathogenic missense mutations identified in family members of proven ARVD/C index patients. To be considered pathogenic, the missense mutations had to fulfil all 3 criteria of the algorithm: 1) SIFT score <0.02, 2) PolyPhen-2 score >0.900, and 3) MAF in ESP database ≤0.05%. SIFT: Sorting Tolerant from Intolerant, PolyPhen-2: Polymorphism Phenotyping-2, MAF: Minor Allele Frequency, ESP: Exome Sequencing Project, ICIN: Interuniversity Cardiology Institute of The Netherlands. JHU: Johns Hopkins University School of Medicine.

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Phenotypic characteristics of missense and non-missense mutations

Both cohorts of family members with either pathogenic missense mutations or pathogenic non-missense mutations had a similar mean age at last follow-up and a slight female preponderance (Table 2). The follow-up duration in the 2 groups was similar: on average 3.5 years in the missense mutation group and 4.2 years in the non-missense mutation group. Approximately 30% of both groups of family members fulfilled current TFC for proven ARVD/C diagnosis. No obvious differences were observed in occurrence of ventricular arrhythmias, repolarization abnormalities in right or left precordial leads on the ECG, RV structural and functional abnormalities, and ICD implantation rates in the cohorts.

Nonetheless, a prolonged terminal activation duration (TAD) in leads V1, V2, or V3 was observed in 21% of family members in the non-missense group, versus 31% in the missense group. Moreover, 22% of family members with a non-missense mutation presented alive with symptoms, compared to 10% of relatives with a pathogenic missense mutation. Of the 64 subjects that presented with symptoms in the non-missense mutation group, 27 presented with SCD whereas no family members died in the missense mutation group.

Table 2.

Clinical parameter	**Non-missense mutation carriers** n=293	**Missense mutation carriers** n=48
Gender (female)	155/293 (53%)	26/48 (54%)
Mean age at presentation (years)	36 (SD 20,0)	37 (SD 17,5)
Symptomatic presentation		
Alive	64/293 (22%)	5/48 (10%)
Sudden cardiac death	27/293 (9%)	0/48
Mean follow-up duration (years)	4,2 (SD 5,5)	3,5 (SD 3,7)
Mean age at last FU (years)	41 (SD 20,4)	40 (SD 16,6)
TFC proven ARVD/C patients	91/265 (34%)	14/48 (29%)
Negative T waves V1-3	46/232 (20%)	8/42 (19%)
Negative T waves V4-6	14/232 (6%)	2/42 (5%)
Prolonged TAD	49/232 (21%)	13/42 (31%)
>500 VES/24hour	50/233 (22%)	10/42 (24%)
Any NSVT/sust.VT	50/265 (19%)	10/48 (21%)
ICD implantation	52/265 (20%)	7/48 (15%)
Major struct. TFC	36/225 (16%)	15/39 (13%)
Minor struct. TFC	38/225 (17%)	4/39 (10%)
LV dysfunction (LVEF<55%)	21/265 (8%)	3/48 (6%)

Phenotypic characteristics of family members with pathogenic non-missense mutations (truncating and splice site mutations) and pathogenic missense mutations. SD: standard deviation, FU: follow-up, TFC: task force criteria, ARVD/C: arrhythmogenic right ventricular dysplasia/cardiomyopathy, TAD: terminal activation duration (prolonged when ≥55ms), VES: ventricular extrasystole, NSVT: non-sustained ventricular tachycardia, VT: ventricular tachycardia, ICD: implantable cardioverter defibrillator, struct: major or minor diagnostic criterion for structural and functional right ventricular alteration, LV: left ventricular, LVEF: left ventricular ejection fraction.

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These differences are reflected in the survival analysis curves of the 2 groups, with an event defined as death or cardiac transplantation (Supplemental Figure 1). No events were observed in the missense mutation group. In the substantially larger non-missense mutation group, 29 family members had an event (28 deaths and 1 cardiac transplantation).

On the other hand, the sustained arrhythmia free survival interval (event defined as sustained VT, or appropriate ICD intervention, or VF) of family members with a missense mutation was indistinguishable from that of family members with a non-missense mutation (p-value 0.803, Figure 2). There were 4 family members with an event (1 sustained VT, 3 appropriate ICD interventions) among the 48 family members with a missense mutation and 20 (15 sustained VTs, 4 appropriate ICD interventions, and 1 VF) in the larger group of 293 family members with a non-missense mutation. Correction for possible clustering of characteristics in families resulted in similar sustained arrhythmia free survival (p-value 0.759). In those family members that experienced appropriate ICD intervention, the time from ICD implantation to first appropriate intervention was 1.6 years (SD 2,4) in the missense mutation group and 2.9 years (SD 2,5) in the non-missense mutation group.

PKP2 **missense mutation c.2386T>C**

Since *PKP2* c.2386T>C (p.Cys796Arg) was identified in 50% (24/48) of the relatives with pathogenic missense mutations, this mutation has had a large influence on the previously reported phenotypic characteristics of these family members. Subjects with this mutation often had ARVD/C diagnosis (10/14 family members with a pathogenic missense mutation and

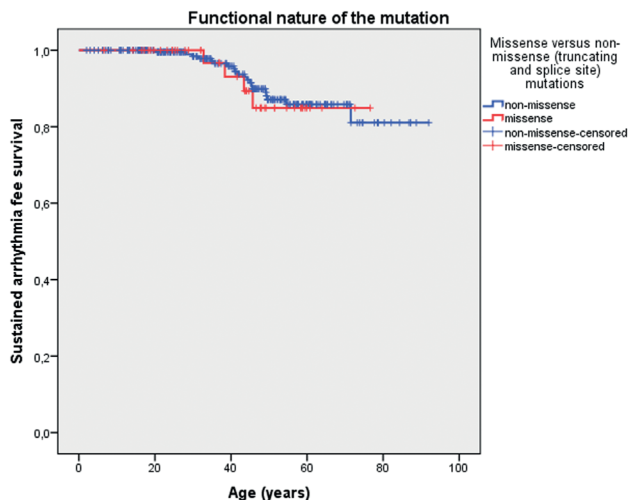

Figure 2. Kaplan-Meier analysis of sustained arrhythmia free survival (event defined as sustained ventricular tachycardia, ventricular fibrillation, or appropriate ICD intervention). The survival curves of family members with missense mutations and non-missense (truncating and splice site) mutations are indistinguishable (p=0.803). In the missense group there were events observed in 4 family members and in the larger non-missense group 20 family members had an event.

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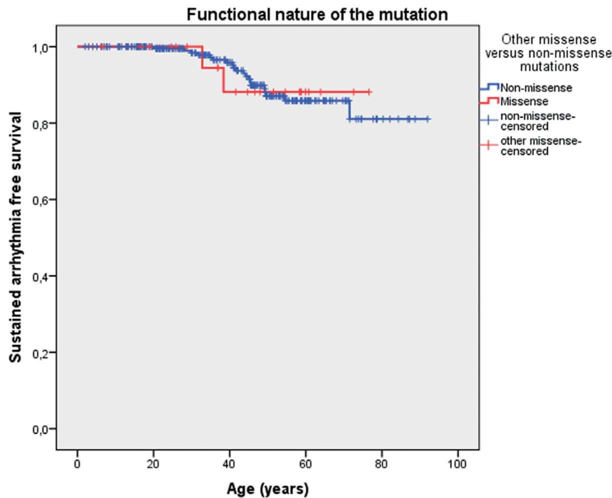

Figure 3. Kaplan-Meier analysis of sustained arrhythmia free survival (event defined as sustained ventricular tachycardia, ventricular fibrillation, or appropriate ICD intervention) of the 24 family members with other missense mutations besides the c.2386T>C mutation. The survival curves of these family members with missense mutations and non-missense (truncating and splice site) mutations are also similar (p=0.936). In the missense group there were events observed in 2 family members and in the larger non-missense group 20 family members had an event.

fulfilment of TFC for ARVD/C diagnosis). Nevertheless, depolarization and repolarization ECG abnormalities, ventricular arrhythmias, and structural and functional RV abnormalities were also observed in family members with the other included missense mutations (Supplemental table 2). In addition, the sustained arrhythmia free survival interval of family members with the other included missense mutations was similar to that of family members with a non-missense mutation (p-value 0.936, Figure 3).

Discussion

ARVD/C is considered a hereditary disease with an autosomal dominant inheritance pattern. Mutations in genes encoding desmosomal and non-desmosomal proteins have been related to the ARVD/C phenotype.[9-18] With a pathogenic mutation identified in an ARVD/C index patient, cascade screening of family members is possible for that mutation. This identifies subjects with a predisposition for disease. Since there is high incidence of sustained VT, VF, and SCD in ARVD/C, careful analysis and follow-up of family members with a pathogenic mutation is critically important.[7,8]

Truncating and invariant splice site mutations have a large impact on form and function of the resulting protein. They are rarely found in healthy control subjects and their pathogenicity is generally undisputed. On the contrary, missense variants, leading to only a single nucleotide

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substitution, are frequently observed in ARVD/C cases and healthy control subjects.[19] Their pathogenicity remains subject of debate and family members of ARVD/C index patients with missense variants are usually not screened for the missense variant but will have to undergo clinical analysis and follow-up. This poses a burden on these families as well as on the healthcare system in general.

Several *in silico* prediction programmes use sequence homology and conservation to predict the pathogenicity of missense variants. In agreement, a recent study by Kapplinger et al.[19] showed that missense variants found in Caucasian patients, within *DSP* and *DSG2* 'hot spots', and in conserved *PKP2* and *DSG2* residues were more likely to be pathogenic. In addition to the importance of sequence homology, conservation, and structure, we hypothesized that pathogenic missense variants are very rare in the normal population. To enhance the objective classification of all ARVD/C missense variants we suggested an algorithm with synergistic use of in silico prediction programmes SIFT and PolyPhen-2 scores and the MAF in a control population in a large exome database. We hypothesized that missense variants can be as pathogenic as non-missense mutations (i.e. truncating and splice site mutations).

In the present study, we assessed the pathogenicity and associated phenotypic characteristics of missense variants, predicted pathogenic by the algorithm, in family members of proven ARVD/C index patients. Of the total cohort of 341 family members, 48 (14%) had a pathogenic missense mutation. Twelve different missense mutations were identified in the desmosmal *PKP2*, *DSP*, *DSG2*, and *DSC2* genes. Family members with a missense mutation had a similar sustained arrhythmia burden compared to family members with a non-missense mutation. Follow-up duration was comparable in both groups, in relatives with a missense mutation even slightly shorter. In addition, in both groups approximately 30% fulfilled 2010 TFC for ARVD/C diagnosis.

The results of this study suggest that pathogenicity of missense mutations can be correctly predicted by the suggested algorithm. This is in correspondence with a study by Guidicessi et al.[24] who demonstrated that the synergistic use of conservation across species, Grantham values, SIFT, and PolyPhen-2, provided the ability to distinguish case-derived from control-derived missense variants in long-QT syndrome. Additional use of the MAF in exome databases may have added value over the exclusive use of *in silico* prediction tools. Due to the conservative nature of the algorithm, only 12 different missense mutations passed its selection. From this observation and the observed phenotypic expression of the selected missense mutations we conclude that the algorithm most-likely selects only those missense mutations that are truly pathogenic. It is possible that missense variants that are classified as VUS for the purpose of this study are in fact pathogenic. Although pathogenic missense mutations might have been missed, these strict criteria presumably result in a low number of false positives. This is more important for genetic screening of family members than false negatives, since the family members of index patients with missense mutations that are incorrectly classified as VUS will all be clinically screened due to the lack of possibility of genetic screening. Optimization of algorithm cut-off values should be the goal of future studies.

The mechanism of pathogenicity might be different in missense and non-missense mutations. Truncating and splice site mutations are believed to inhibit protein formation from the mutated allele by inhibition of RNA transcription, resulting in haploinsufficiency. Missense mutations can

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lead to haploinsufficiency but can also have a dominant negative effect on the formed protein, leading to desmosomal instability by incorporation of the mutated and dysfunctional protein in the desmosomal complex.
The *PKP2* c.2386T>C (p.Cys796Arg) missense mutation was identified in 50% of the relatives with a missense mutation and had therefore large impact on the reported phenotypic characteristics of subjects with a missense mutation. This mutation was already considered pathogenic and a founder effect of the mutation has been established previously.[10] Recently, functional assessment of this specific mutation *in vivo* and *in vitro* demonstrated intrinsic instability and degradation of the formed protein, suggesting haploinsufficiency as the pathogenic mechanism.[25] Accordingly, the algorithm correctly classified this mutation as pathogenic.
Family members with non-missense mutations presented more often with symptoms and moreover, presentation with SCD was solely observed in these family members when compared to relatives with missense mutations. Notably, nearly a third of these SCD cases seemed to cluster in families, there was 1 family with 2 SCD cases and 2 families with 3 SCD cases. Therefore, additional genetic or environmental factors of influence on the occurrence of SCD may be involved and could explain these observed differences. On the other hand, subjects with missense mutations had slightly more often episodes of non-sustained or sustained VT and more appropriate ICD therapy in shorter time period after ICD implantation compared to family members with non-missense mutations, suggesting a similar risk for (fatal) ventricular arrhythmias. Thus, the differences could also be due to the large difference in number of relatives in the 2 groups, possibly SCD will also be observed in subjects with a missense mutation when the group of relatives is larger.

Limitations

This study is a retrospective analysis of a large number of family members with missense and non-missense mutations. Diagnostic examinations performed in the clinical evaluation reflect decisions made in daily practice patient care. Therefore, not every diagnostic examination was performed in the whole cohort. Subsequently, a selection bias cannot be excluded.
The assessment of family members with a missense mutation and the comparison with family members with a non-missense mutation were hampered by the small number of subjects with a missense mutation. Therefore, the difference in the occurrence of SCD in the non-missense mutation group cannot be fully understood. Nevertheless, family members with missense mutations also had a high frequency of ventricular arrhythmias. Although this study showed that missense mutations can have a similar pathogenic character as non-missense mutations, it can not be excluded that in missense mutation carriers there might be a lesser extent of symptoms and better prognosis compared to non-missense mutation carriers.
Results of phenotypic characteristics evaluation largely reflected the associated phenotype of *PKP2* c.2386T>C (pCys796Arg) since this mutation was identified in 50% of relatives with a missense mutation. Large cohorts of family members with other missense mutation are needed.

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Conclusion

Missense mutations have a similar pathogenic character as non-missense mutations (i.e. truncating and splice site mutations) regarding phenotypic characteristics and arrhythmic burden in family members of proven ARVD/C patients. Missense mutations were only classified as pathogenic when they fulfilled all 3 criteria of the algorithm: 1) a SIFT score <0.02, 2) a PolyPhen-2 score >0.900, and 3) a MAF ≤0.05 % in the ESP dataset. The algorithm was able to discriminate pathogenic missense mutations. Pathogenicity of missense mutations can be correctly predicted by the combined criteria from SIFT, PolyPhen-2, and MAF in exome databases. This has important implications for genetic screening, patient management, and subsequent risk evaluation of family members.

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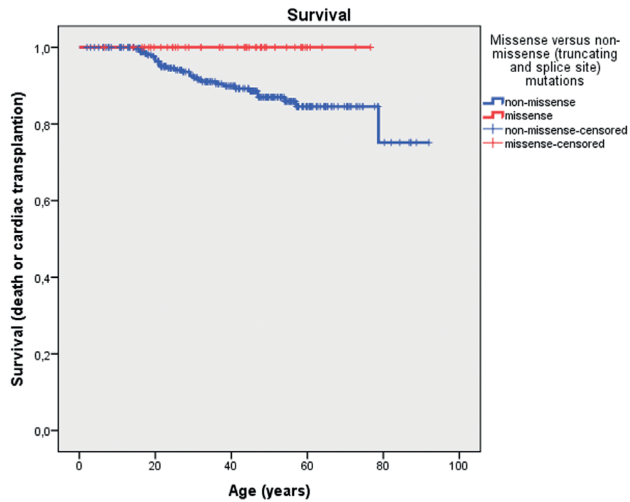
Supplementary material



Supplemental figure 1. Kaplan-Meier survival analysis (event defined as death or cardiac transplantation) of family members with missense mutations versus non-missense (truncating and splice site) mutations. There were no events in the missense mutation carrier group and 29 events (28 deaths and 1 cardiac transplantation) in the much larger non-missense mutation carriers group.

Supplemental table 1.

Gene	Number of family members	Nucleotide change	Amino acid change	Numbers from cohort
PKP2	9/293	deletion exons 1-4	p.(?)	ICIN 9
	7/293	deletion exons 1-14	p.(?)	ICIN 7
	1/293	deletion exon 10	p.(?)	ICIN 1
	7/293	c.148_151del	p.(Thr50fs)	ICIN 3/ JHU 4
	3/293	c.218dup	p.(Asn74fs)	JHU 3
	1/293	c.224-3C>G	p.(?)	JHU 1
	35/293	c.235C>T	p.(Arg79*)	ICIN 25/ JHU 10
	3/293	c.337-2A>T	p.(?)	JHU 3
	15/293	c.397C>T	p.(Gln133*)	ICIN 15
	7/293	c.917_918del	p.(Pro318fs)	ICIN 7
	3/293	c.968_971del	p.(Gln323fs)	ICIN 3
	1/293	c.1171-2A>G	p.(?)	JHU 1
	27/293	c.1211dup	p.(Val406fs)	ICIN 25/ JHU 2
	5/293	c.1237C>T	p.Arg413*	JHU 5
	4/293	c.1369_1372del	p.(Gln457*)	JHU 4
	1/293	c.1378G>A	p.(Val445fs) #	ICIN 1
	5/293	c.1378+1G>C	p.(?)	JHU 5
	9/293	c.1613G>A	p.(Trp538*)	JHU 9
	10/293	c.1643del	p.(Gly548fs)	JHU 10
	2/293	c.1759del	p.(Val587fs)	JHU 2
	1/293	c.1760del	p.(Val587fs)	JHU 1
	1/293	c.1803del	p.(Asp601fs)	JHU 1
	1/293	c.1821dup	p.(Val608fs)	JHU 1
	13/293	c.1848C>A	p.(Tyr616*)	ICIN 13
	2/293	c.1849C>T	p.(Gln617*)	JHU 2
	1/293	c.1951C>T	p.(Arg651*)	ICIN 1
	8/293	c.2013del	p.(Lys672fs)	JHU 8
	3/293	c.2034G>A	p.(Trp678*)	ICIN 3
	26/293	c.2146-1G>C	p.(Met716fs) #	ICIN 3/ JHU 23
	2/293	c.2169_2172dup	p.(Val725fs)	JHU 2
	11/293	c.2197_2202del	p.(His733fs)	JHU 11
	2/293	c.2421C>A	p.(Tyr807*)	ICIN 2
	5/293	c.2484C>T	p.(Gly828Gly) $	JHU 5
	24/293	c.2489+1G>A	p.(Lys768fs) #	ICIN 8/ JHU 16
	9/293	c.2489+4A>C	p.(Lys768fs) #	ICIN 9
	5/293	c.2509del	p.(Ser837fs)	JHU 5
	1/293	c.2544G>A	p.(Trp848*)	ICIN 1

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Supplemental table 1.Continued

Gene	Number of family members	Nucleotide change	Amino acid change	Numbers from cohort
DSP	1/293	deletion exons 1-24	p.(?)	JHU 1
	1/293	c.478C>T	p.(Arg160*)	JHU 1
	2/293	c.3160_3169del	p.(Lys1054fs)	JHU 2
	1/293	c.3337C>T	p.(Arg1113*)	ICIN 1
	2/293	c.6478C>T	p.(Arg2160*)	JHU 2
	1/293	c.6496C>T	p.(Arg2166*)	JHU 1
DSC2	1/293	c.943-1G>A	p.(?)	ICIN 1
DSG2	1/293	c.523+2T>C	p.(?)	JHU 1
PLN	13/293	c.40_42delAGA	p.Arg14del	ICIN 11/ JHU 2

Individual pathogenic non-missense (i.e. truncating or invariant splice site) mutations identified in family members of proven ARVD/C index patients. ICIN: Interuniversity Cardiology Institute of The Netherlands. JHU: Johns Hopkins University School of Medicine. #: RNA experiments by our research group showed that this nucleotide substitution leads to the use of a cryptic splice site and results in a frameshift (c.1378G>A r.1333_1378del p.(Val445fs)), (c.2146-1G>C r.2146_2299del p.(Met716fs)), and (c.2489+4A>C r.2300_2489del p.(Lys768fs)), manuscript in preparation. $: This mutation also generates a cryptic splice site resulting in a frameshift, see Awad et al. Hum Mutat. 2006 Nov;27(11):1157.

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Supplemental table 2.

Clinical parameter	*PKP2* c.2386T>C mutation n=24	Other missense mutations n=24
Gender (female)	12/24 (50%)	10/24 (42%)
Mean age at presentation (years)	34 (SD 14,2)	41 (SD 19,8)
Symptomatic presentation	2/24 (8%)	3/24 (13%)
Mean follow-up duration (years)	3,0 (SD 3,8)	3,9 (SD 3,6)
Mean age at last FU (years)	37 (SD 14,1)	45 (SD 18,1)
TFC proven ARVD/C patients	10/24 (42%)	4/24 (17%)
Negative T waves V1-3	5/23 (22%)	3/19 (16%)
Negative T waves V4-6	1/23 (4%)	1/19 (5%)
Prolonged TAD	10/23 (44%)	3/19 (16%)
>500 VES/24hour	7/21 (33%)	3/21 (14%)
Any NSVT/sust.VT	6/24 (25%)	4/24 (17%)
ICD implantation	4/24 (17%)	3/24 (13%)
Major struct. TFC	2/21 (10%)	3/18 (17%)
Minor struct. TFC	3/21 (14%)	1/18 (6%)
LV dysfunction (LVEF<55%)	3/24 (13%)	0/24

Phenotypic characteristics of family members with the *PKP2* c.2386T>C missense mutation compared to the phenotypic characteristics of family members with another missense mutation adhering to the algorithm. Characteristics of arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) are found in both groups of missense mutation carriers, although fulfilment of Task Force Criteria (TFC) for ARVD/C diagnosis seemed to occur more often in the family members with *PKP2* c.2386T>C. SD: standard deviation, FU: follow-up, TAD: terminal activation duration (prolonged when ≥55ms), VES: ventricular extrasystole, NSVT: non-sustained ventricular tachycardia, VT: ventricular tachycardia, ICD: implantable cardioverter defibrillator, struct: major or minor diagnostic criterion for structural and functional right ventricular alteration, LV: left ventricular, LVEF: left ventricular ejection fraction.

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CHAPTER 7

Functional Assessment of Potential Splice Site Variants in Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

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Abstract

Background

Interpretation of genetic screening results in Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is often difficult. Pathogenicity of variants with uncertain clinical significance may be predicted by software algorithms. However, functional assessment can unambiguously demonstrate the effect of such variants.

Objective

To perform functional analysis of potential splice site variants in ARVD/C patients.

Methods

Nine variants in desmosomal (*PKP2*, *JUP*, *DSG2*, *DSC2*) genes with potential RNA splicing effect were analyzed. The variants were found in patients with fulfilment of 2010 ARVD/C Task Force Criteria (n=7) or suspected ARVD/C (n=2). Total RNA was isolated from fresh blood samples and subjected to reverse transcriptase PCR.

Results

An effect on splicing was predicted by software algorithms for all variants. Of the 9 variants, 5 were intronic and 4 exonic. RNA analysis showed a functional effect on mRNA splicing by exon skipping, the generation of new splice sites, or activation of cryptic sites in 6 variants. All 5 intronic variants tested, severely impaired splicing. Only 1 out of 4 exonic potential splice site variants was shown to have a deleterious effect on splicing. The remaining 3 exonic variants had no detectable effect on splicing and heterozygous presence in mRNA confirmed biallelic expression.

Conclusions

Six variants of uncertain clinical significance in the *PKP2*, *JUP*, and *DSG2* genes showed a deleterious effect on mRNA splicing, indicating these are ARVD/C related pathogenic splice site mutations. These results highlight the importance of functional assessment of potential splice site variants to improve patient care and facilitate cascade screening.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a hereditary cardiomyopathy characterized by ventricular arrhythmias, right ventricular (RV) and possibly left ventricular (LV) dysfunction, with fibrofatty replacement of cardiomyocytes.[1-3] The genetic substrate has been demonstrated in 5 desmosomal genes and, in a minority, in non-desmosomal genes.[4-8] A pathogenic ARVD/C related mutation can be identified in 30-70% of index patients.[9-11] A large web-based dataset of genes and mutations associated with ARVD/C has been created (www.arvcdatabase.info).[12] The identification of a pathogenic mutation in index patients with ARVD/C facilitates cascade screening of family members and subsequent identification of subjects with a disease predisposition. However, not infrequently genetic screening also results in the identification of gene variants of uncertain clinical significance (VUS).[13] The uncertainty of the finding of VUS undermines risk management in ARVD/C and poses a burden on patients and physicians.

The possible pathogenicity of VUS is routinely estimated by *in silico* prediction algorithms. However, the clinical relevance of a VUS may be determined by experimental validation *in vitro*, specifically with potential splice site variants. Not only variants affecting the invariant splice-donor and splice-acceptor splice sites but also more distant intronic and even nonsense, missense, or translationally silent exonic variants can impair gene activity by inducing the splicing machinery to skip the variant-bearing exons and/or activate cryptic splice sites.[14-16] Variants of which the spliceogenic potential is not immediately recognized by prediction algorithms may be incorrectly predicted as innocuous, as demonstrated previously.[16,17] To date in ARVD/C, the spliceogenic character of only a few splice site mutations was experimentally confirmed.[18,19]

In this study, we analyzed 9 distinct ARVD/C VUS for functional impact on splicing by bioinformatic prediction tools and experimental analysis of mRNA. We aimed to confirm or discard pathogenicity of these 9 potential splice site variants for patient management purposes and to improve knowledge on ARVD/C splice site mutations.

Methods

Study population

Nine variants with spliceogenic potential, defined as an anticipated pathogenic effect on splicing according to *in silico* algorithms, that were identified by routine genetic screening as part of the diagnostic evaluation in ARVD/C, were assessed. The potential splice site variants were identified in 9 index patients, 7 with proven (definite) ARVD/C and 2 index patients with suspected ARVD/C. Proven ARVD/C was defined as fulfilment of 2010 Task Force Criteria (TFC) for diagnosis. ARVD/C diagnosis is made with the fulfilment of 2 major TFC, 1 major and 2 minor TFC, or 4 minor TFC in different categories: global and/or regional RV dysfunction and structural alterations, tissue characterization, depolarization abnormalities, repolarization abnormalities, ventricular arrhythmias, and family history/genetics.[20] Suspected ARVD/C was defined as fulfilment of 1 major or ≥1 minor TFC. One patient with suspected ARVD/C and a potential desmoglein-2

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spliceogenic variant, was subsequently shown to have the pathogenic phospholamban (*PLN*) founder mutation c.40_42delAGA (p.Arg14del). None of the other patients had additional pathogenic mutations. All patients consented to clinical and genetic evaluation and in particular to DNA and RNA analysis. The study conformed to the guiding principles of the Declaration of Helsinki and was approved by the Institutional Review Board at our institution.

Molecular genetic analyses

DNA was isolated from peripheral blood lymphocytes according to standard protocols. The coding regions of the desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), desmocollin-2 (*DSC2*) and non-desmosomal genes transmembrane protein 43 (*TMEM43*) and phospholamban (*PLN*) were analyzed using direct Sanger sequence analysis performed with a BigDye Terminator DNA sequencing kit (version 2.0) on a 3730 automated sequencer (Applied Biosystems, Foster City, Calif., USA). All sequences were analyzed using established sequence analysis software.

Total RNA was isolated from fresh blood samples from the ARVD/C index patients using the PAXgene kit (QIAGEN Benelux, Venlo, The Netherlands) and subjected to random hexamer primed reverse transcriptase PCR (RT-PCR). The obtained cDNA products were amplified by PCR with exonic primers specific for the fragment of interest. All cDNA fragments were separated according to size using 2% agarose gel electrophoresis along with a 100-bp DNA ladder (O'GeneRuler; Fermentas, Burlington, Ont., Canada). Both normal and aberrant fragments were subjected to direct sequencing. Primers and PCR details are available upon request.

Prediction of pathogenicity

The pathogenic potential of the splice site variants was assessed using *in silico* prediction analyses of 1) the maximum entropy model (MaxEntScan)[21], 2) the Human Splicing Finder (HSF)[22], 3) NNSPLICE[23], 4) GeneSplicer[24], and 5) SpliceSiteFinder-like[25]. Variant minor allele frequency (MAF) was assessed in large control databases, including Dutch control datasets (the NHLBI 6500 exome dataset (EVS), http://evs.gs.washington.edu/EVS/ and the Genome of the Netherlands dataset (GoNL), http://www.nlgenome.nl/). Variant prevalence was also reviewed in the Dutch ARVD/C cohort, a clinical dataset based on national collaboration of all university medical centers in The Netherlands.

Results

Molecular genetic analyses

Routine diagnostic screening of desmosomal genes *PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2* and non-desmosomal genes *TMEM43* and *PLN*, identified 9 distinct variants with spliceogenic potential (Table 1). In all variants at least 1 *in silico* program predicted an effect on splicing (mRNA processing) and all variants were rare (MAF <0.01). The prediction scores of the 5 bioinformatic splice site programs and the MAF for the 9 variants are specified in supplemental Table 1. Variants were found in the *PKP2* (4), *DSG2* (3), *DSC2* (1), and *JUP* (1) gene. No spliceogenic variants were identified in *DSP*, *TMEM43*, and *PLN*.

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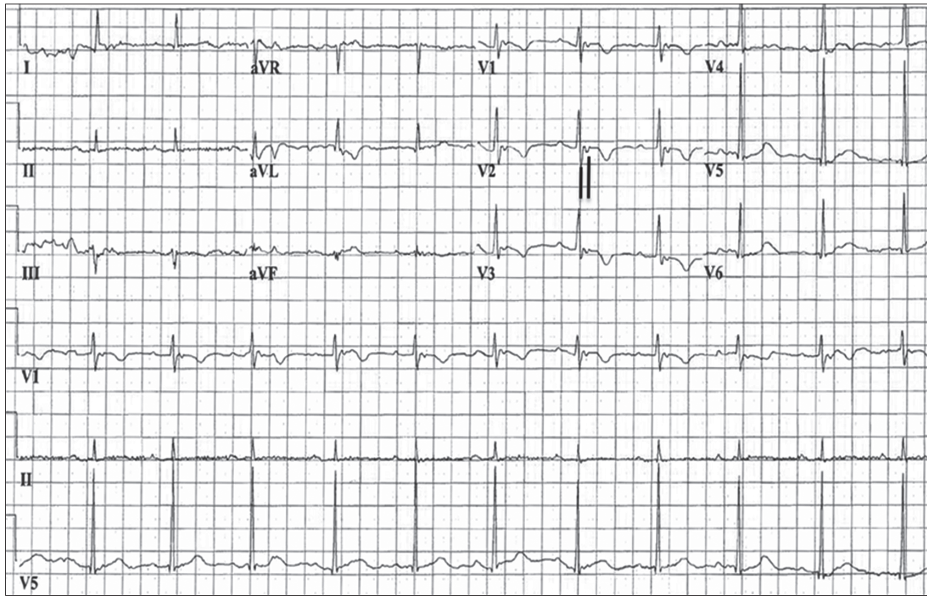

Figure 1. 12-lead ECG (while off drugs) of the index patient with the plakophilin-2 c.1511-2A>G variant. The ECG shows sinus rhythm with a horizontal axis, inverted T waves in leads V1-3 and a prolonged terminal activation duration of 80 ms (vertical lines below lead V2). The terminal activation duration is measured in leads V1-3 from the nadir of the S wave (first line) to the end of depolarization (second line). A prolonged terminal activation duration (≥55 ms) is a minor criterion for ARVD/C diagnosis.

***PKP2* variants**

The c.1378G>A variant, located at the exon/intron border, was the only *exonic PKP2* variant studied. The variant was found in the study patient and 1 other unrelated index patient who both fulfilled the 2010 TFC for ARVD/C diagnosis based on RV structural alterations, inverted T waves in V1-3 and beyond, and ventricular tachycardia (VT) with left bundle branch block (LBBB) morphology. Of both index patients 1 first-degree relative (parent) with the variant also had ARVD/C diagnosis. *In silico* algorithms predicted the disruption of the *PKP2* exon 5 splice donor site, likely resulting in exon 5 skipping in the resulting mRNA. Contrary to the expected exon 5 skipping, RNA analysis showed that this variant inactivates the normal exon 5 splice donor site and leads to activation of a cryptic splice donor site in exon 5, 44 nucleotides upstream of the normal splice donor site (Supplemental Figure 1 and Table 2). The resulting mutant transcript is out of frame.

Of the 3 *intronic PKP2* variants analyzed, c.1511-2A>G and c.2146-1G>C are located within invariant GU donor or AG acceptor dinucleotides. Variant c.1511-2A>G was found in a proven ARVD/C patient with inverted T waves in leads V1-3 and a prolonged terminal activation duration on the ECG (Figure 1). The son of this index patient had the mutation and showed no signs or symptoms of the disease at age 51. Variant c.2146-1G>C was found in the study subject and 6 other seemingly unrelated index patients, with cosegregation of ARVD/C and this variant in 3 families (one family shown in Figure 2A). Both variants were predicted to be damaging according

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Table 1. Potential ARVD/C splice site variants and diagnostic Task Force Criteria

Index patients with splice variants	Gender	Age at evaluation (yrs)	TFC	Struct. abn.	Tissue charact.	Repol. abn.	Depol. abn.	Arrh.
PKP2 c.1378G>A	F	43	+	**Major:** RVEF 23% and WMA of RV free wall	n.a.	**Minor:** inverted T waves in V1-4 with RBBB	-	**Minor:** VT with LBBB morphology
PKP2 c.1511-2A>G *	M	74	+	RV abnor-malities but not quantified	n.a.	**Major:** inver-ted T waves in V1-3	**Minor:** prolonged TAD	VTs of un-known origin
PKP2 c.2146-1G>C	F	34	+	**Major:** RVEF 40% and WMA	-	-	**Minor:** prolonged TAD	**Minor:** VT with LBBB morphology with inferior axis
PKP2 c.2578-3A>G *	M	15	+	RV mildly dilated without WMA and normal RVEF	n.a.	**Major:** inverted T waves in V1-3	-	Frequent ventricular ectopy
DSG2 c.378+2T>G	M	46	+	**Major:** RVEF 20% and WMA	n.a.	**Major:** inverted T waves in V1-5	-	**Minor:** VT with LBBB morphology with inferior axis
DSG2 c.783T>A	M	24	+	**Major:** RV WMA with cine-angiography	-	**Minor:** inverted T waves in V1-2	**Minor:** prolonged TAD	**Major:** VT with LBBB morphology and superior axis
DSG2 c.2759T>G	F	42	-	Apical a/dyskinesia but RV function/volumes not quantified	n.a.	**Minor:** Inverted T waves in V2-6 but during Sotalol treatment	-	Fast VT and VF but morphology unknown
DSC2 c.1350A>G	F	45	-	Fatty infiltration and dyskinesia of RV free wall but RV function/ volumes not quantified	n.a.	**Minor:** inverted T waves in V1-2		**Minor:** 670 VES/24hours

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Table 1. Continued

JUP c.469-8_469-1del *	F	42	+	**Major:** RVEF 41 %, RVEDV 126 ml/m^2, and WMA of RV free wall	n.a.	-	-	-

Clinical characteristics of the 9 index patients with potential Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) splice site variants. *PKP2*: plakophilin-2, *DSG2*: desmoglein-2, *DSC2*: desmocollin-2, *JUP*: plakoglobin. F: female gender, M: male gender. Yrs: age at evaluation in years. TFC: 2010 Task Force Criteria for ARVD/C diagnosis, +: proven ARVD/C, -: suspected ARVD/C, Struct. abn.: global and/or regional RV dysfunction and structural alterations, Tissue charact.: RV tissue characterization, Repol. abn.: repolarization abnormalities, Depol. abn.: depolarization/conduction abnormalities, Arrh.: ventricular arrhythmias. Major: major criterion for ARVD/C diagnosis, minor: minor criterion for ARVD/C diagnosis. RV: right ventricular, RVEF: right ventricular ejection fraction, RVEDV: right ventricular end diastolic volume, WMA: wall motion abnormalities. N.a.: not applicable. RBBB: right bundle branch block. TAD: terminal activation duration measured in V1-3. VT: ventricular tachycardia, LBBB: left bundle branch block. *: These 3 patients fulfilled TFC for ARVD/C diagnosis with the inclusion of a pathogenic (splice site) mutation as major criterion.

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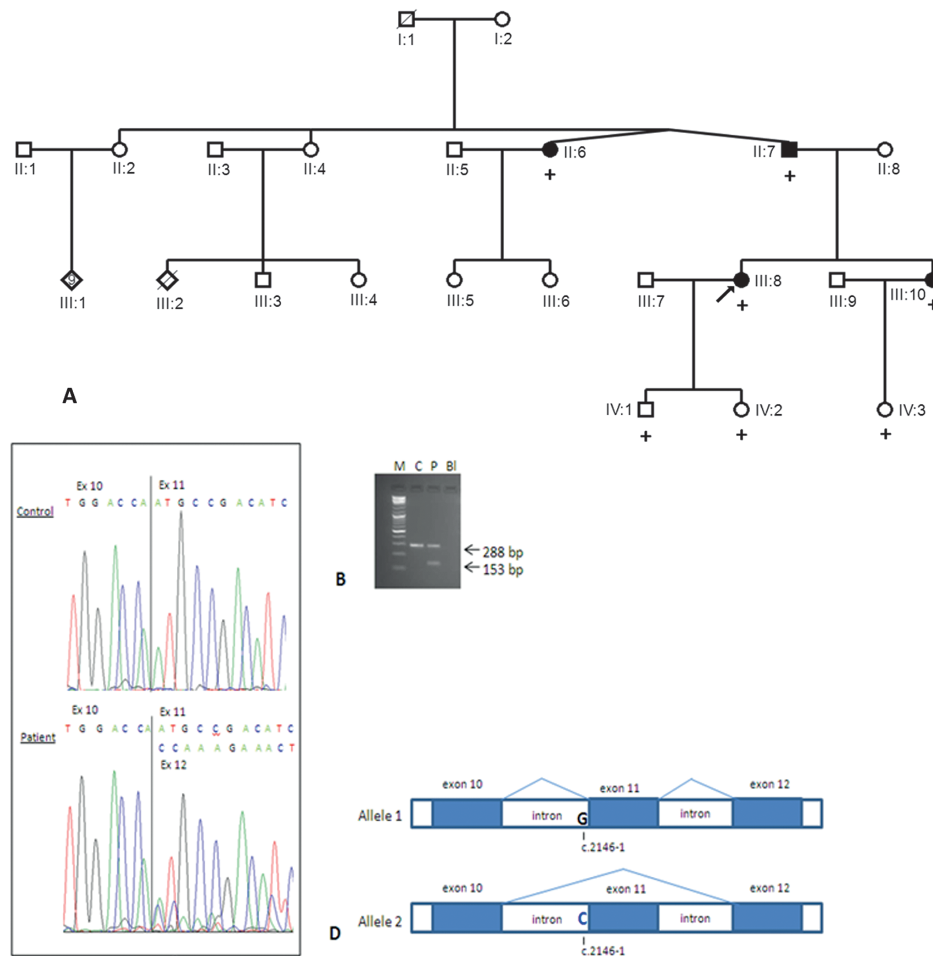

Figure 2A. Pedigree of a family with proven Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy and plakophilin-2 splice site variant c.2146-1G>C. The twin sister of the index patient (II:6), the index patient (II:7, indicated by the arrow), and both his daughters (III:8 and III:10) fulfilled the 2010 Task Force Criteria for diagnosis (black square and circles) with overt disease. The grandson of the index patient (IV:1, age 19) carried the variant and had frequent ventricular ectopy (1903 premature ventricular complexes/24 hours). The other grandchildren of the index patient (IV:2, age 15, and IV:3, age 13) carried the variant and were asymptomatic. **2B.** Agarose gel electrophoresis of RT-PCR amplification products (exons 10 and 12 primers). C (control) showing only 288 base pairs (bp) normally sized PCR products from both *PKP2* alleles containing exons 10, 11 and 12. P (patient) showing, in addition to a 288 base pair RT-PCR product from the normal allele, a smaller PCR fragment from the mutated allele of 153 bp containing exons 10 and 12 only (skipping of exon 11). Bl = water control, M = 100bp size marker. **2C.** Direct sequence analysis of PCR products from Figure 2B. using *PKP2* exon 10 forward primer. Every colored peak represents a nucleotide (G=black, A=green, T=red, C=blue) showing the sequence of exon 10 (Ex 10), 11 (Ex 11), and 12 (Ex 12). The control lane only shows PCR fragments from both wild type PKP2 alleles with normal exon 10 to exon 11 transitions. Patient sample shows a mix (ratio ~1:1) of PCR products from wild type and mutant *PKP2* alleles. PCR products from the normal allele show exon 10 to exon 11 transition. PCR products from the mutated allele show exon 10 to exon 12 transition, with skipping of exon 11. **2D.** A schematic representation of the normal RNA splicing process of the normal allele (Allele1) and splicing of the mutated allele (Allele 2) resulting in exon 11 skipping.

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to the *in silico* programs. RT-PCR analyses in combination with Sanger sequence analysis revealed exclusion of exons 7 and 11 respectively (exon skipping), leading to shifts in the reading frame of the resulting mutant transcripts (Figure 2B-D and Supplemental Figure 2). Intronic variant c.2578-3A>G was found in a patient with proven ARVD/C. He presented with an irregular heartbeat. On examination mild RV abnormalities and patchy LV fibrosis (magnetic resonance imaging), negative T waves in V1-3, and frequent ventricular ectopy were observed. ARVD/C was also confirmed in 1 first-degree relative (parent) with *PKP2* c.2578-3A>G. The variant was predicted to alter mRNA splicing by all *in silico* prediction algorithms. RNA analysis showed that this variant creates a new preferentially used splice acceptor site 2 nucleotides upstream of the normal exon 14 splice acceptor (Supplemental Figure 3). The mutant transcript is out of frame.

***DSG2* variants**

The *DSG2* variant c.378+2T>G is located in the conserved invariant splice donor site of exon 4. It was identified in a patient with proven ARVD/C based on RV structural alterations , inverted T waves in V1-5, and VT originating from the RV outflow tract. The variant was predicted to disrupt normal intron 4 splicing. RNA analysis confirmed the deleterious effect of this variant which was shown to lead to exon 4 skipping of the *DSG2* transcript (Supplemental Figure 4). The resulting transcript is in frame.

The synonymous c.783T>A variant was identified in a proven ARVD/C patient with RV dyskinesia, inverted T waves, and VT with multiple LBBB morphologies. It is rarely found in control populations (MAF 0.00016). Only the SpliceSiteFinder-like analyses predicted an effect of the variant on mRNA maturation. No aberrant transcripts were detectable. Moreover, the c.783T>A variant was seen in heterozygous form in the *DSG2* transcripts indicating biallelic expression (Supplemental Figure 5).

Variant c.2759T>G is a missense variant which was found in a patient with suspected ARVD/C with inverted T waves in leads V2-6, fast ventricular arrhythmias, and local RV a/dyskinesia. Analysis of the *PLN* gene in this patient indentified the pathogenic c.40_42delAGA (p.Arg14del) mutation. The c.2759T>G variant was also found in a family with proven ARVD/C in combination with a pathogenic *PKP2* mutation (c.397C>T, p.Gln133*). The only member of that family with the *DSG2* variant and without the pathogenic *PKP2* mutation had no ARVD/C signs and symptoms. *DSG2* c.2759T>G is found in control populations at low frequency (MAF 0.0039). The variant was predicted to destroy a cryptic splice acceptor site within exon 15. RNA analysis showed no altered *DSG2* transcripts and heterozygous presence of the variant in the transcripts confirmed biallelic expression (Supplemental Figure 6).

***DSC2* variant**

The synonymous variant c.1350A>G in exon 10 of *DSC2* was predicted to create a novel splice donor site, although this variant is found at low frequency in controls (MAF 0.004). The variant was identified in a patient with suspected ARVD/C with inverted T waves in leads V1-2, >500 ventricular extrasystoles/24 hours, and fatty infiltration and RV wall motion abnormalities. RNA analysis showed no detectable aberrant transcripts. Moreover, the variant was seen in heterozygous form in the *DSC2* transcripts indicating normal biallelic expression (Supplemental Figure 7).

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Table 2. RNA analysis and pathogenicity of potential ARVD/C splice site variants

Gene	DNA	mRNA	Protein	Functional effect of variant	Outcome
PKP2	c.1378G>A	r.1333_1378del	p.(Val445fs)	Cryptic splice donor site ctivation in exon 5	Pathogenic
PKP2	c.1511-2A>G	r.1511_1668del	p.(Gly405fs)	Skipping of exon 7	Pathogenic
PKP2	c.2146-1G>C	r.2146_2299del	p.(Met716fs)	Skipping of exon 11	Pathogenic
PKP2	c.2578-3A>G	r.2576_2577dup	p.(Ala860fs)	Splice acceptor site generation in intron 13	Pathogenic
DSG2	c.378+2T>G	r.217_378del	p.(Ile73_Leu126del)	Skipping of exon 4	Pathogenic
DSG2	c.783T>A	r.783u>a	p.(=)	No aberrant splicing detectable (biallelic expression confirmed)	Likely not pathogenic
DSG2	c.2759T>G	r.2759u>g	p.(Val920Gly)	No aberrant splicing detectable (biallelic expression cofirmed)	No effect on splicing
DSC2	c.1350A>G	r.1350a>g	p.(=)	No aberrant splicing detectable (biallelic expression confirmed)	Likely not pathogenic
JUP	c.469-8_469-1del	r.469_483del	p.(Val157_Lys161del)	Cryptic splice acceptor site activation in exon 4	Pathogenic

Experimental mRNA analysis of the 9 rare Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) variants with potential effect on splicing. Six variants demonstrated altered splicing indicative of a pathogenic character. *PKP2*: plakophilin-2, *DSG2*: desmoglein-2, *DSC2*: desmocollin-2, *JUP*: plakoglobin.

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***JUP* variant**

The intronic *JUP* variant c.469-8_469-1del was predicted to lead to disruption of the exon 4 splice acceptor site. The study subject with this variant and proven ARVD/C presented with near-syncope. Paroxysmal atrial fibrillation was observed with rhythm analysis. Magnetic resonance imaging showed a dilated RV, a moderate-severe tricuspid valve regurgitation, and a diminished RV function with regional dyskinesia. RNA analysis confirmed the prediction and demonstrated that this variant activates a cryptic splice acceptor site at positions c.482_483 (Supplemental Figure 8). The resulting *JUP* mutant transcripts remains in frame but lacks 5 amino acids (p.Val157_Lys161del) from the evolutionary conserved and functionally important Armadillo domain.

Discussion

In ARVD/C patients, molecular screening for pathogenic mutations in desmosomal and non-desmosomal genes is common practice.[4,8,11,26-30] However, test results may lead to uncertainty due to the identification of VUS in the concurrent absence of clearly pathogenic mutations. This situation hampers patient management and identification of at-risk family members.

The pathogenic potential of spliceogenic VUS is often assessed using *in silico* prediction programs. With *in silico* predictions, it frequently remains elusive whether spliceogenic variants cause exon skipping, generate new splice sites, and/or activate cryptic splice sites which may be located distant to the wild type sites, and hence are not covered by the prediction algorithms, resulting in erroneous conclusions.[16] Therefore, the functional effects may be demonstrated by experimental mRNA analysis.[14-17]

We analyzed 9 ARVD/C VUS regarding their spliceogenic potential using *in silico* prediction algorithms and *in vitro* mRNA analyses with mRNA isolated from blood from variant carriers. All 5 *intronic* variants studied (*PKP2* c.1511-2A>G, c.2146-1G>C, c.2578-3A>G, *DSG2* c.378+2T>G, *JUP* c.469-8_469-1del) were predicted to be damaging by the *in silico* tools. While the pathogenicity of *PKP2* variants c.1511-2A>G, c.2146-1G>C , and *DSG2* c.378+2T>G might be considered evident since these variants affect invariant splice donor or acceptor sites, the experimental assessment of their functional consequences on pre-mRNA processing was pending. *In vitro* mRNA analyses confirmed the spliceogenic effect of all 3 intronic *PKP2* variants (c.1511-2A>G, c.2146-1G>C, c.2578-3A>G), indicating the pathogenic character of these variants. Formally, *DSG2* variant c.378+2T>G and *JUP* variant c.469-8_469-1del could still result in functional protein products because the resulting mutant transcripts remain in frame. However, since in both cases multiple amino acids in functionally important evolutionary conserved regions are deleted and both variants were not observed in control populations (EVS, GoNL), we considered these variants to be pathogenic mutations.

Of the 4 *exonic* variants (*PKP2* c.1378G>A, *DSG2* c.783T>A, c.2759T>G, *DSC2* c.1350A>G), the *PKP2* c.1378G>A variant was predicted to destroy the normal *PKP2* exon 5 splice-donor site. In general, mutations in splice donor and acceptor sites preferentially lead to exon skipping.[31] RNA analysis however, demonstrated the activation of a cryptic splice donor site within exon 5 leading to a shortened out of frame mutant transcript. The relative low abundance of the mutant

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transcript possibly reflects degradation of this transcript by nonsense mediated decay. Initially, the *PKP2* c.1378G>A variant was regarded as a missense variant (p.Asp460Asn) and its spliceogenic potential was not immediately recognized. The combined results for *DSG2* variant c.783T>A do not support a deleterious effect on *DSG2* expression. We concluded it to most likely be a rare clinically irrelevant polymorphism. Likewise, no deleterious effect on RNA expression could be demonstrated for *DSG2* variant c.2759T>G, although a possible deleterious effect of the resulting single amino acid substitution on DGS2 protein function cannot be excluded. The variant was reported to be pathogenic and related to the ARVD/C phenotype previously.[32] However, in our study this variant was found in combination with the pathogenic *PLN* mutation c.40_42delAGA in one and in combination with the pathogenic *PKP2* c.397C>T nonsense mutation in another ARVD/C index patient. In both patients, the pathogenic *PLN* and *PKP2* mutations are the most likely cause of their phenotype. Moreover, in the GoNL exome dataset, 1 control individual was identified that is homozygous for the c.2759T>G variant. In summary, these results contradict a pathogenic character of this variant and we concluded it to be benign or a modifier of disease expression. No impact on splicing was demonstrated for the synonymous *DSC2* variant c.1350A>G. We concluded this variant to likely be a rare polymorphism. A total of 6 variants, including 1 exonic variant, were identified spliceogenic, either causing/enhancing exon skipping, generating new splice sites, or activating cryptic splice sites. Interestingly, the spliceogenic effect of 5 intronic variants was correctly predicted by all *in silico* prediction programmes, whereas in 3 out of 4 exonic variants the algorithm predictions differed from the experimental results by mRNA analysis. This is in accordance with previous studies, in which possible splice effects of intronic variants were also better predictable compared to exonic variants.[16,17]

In 3 index patients with the *PKP2* c.1511-2A>G, *PKP2* c.2578-3A>G, and *JUP* c.469-8_469-1del variants, the mRNA analysis and subsequent adjudication of pathogenic splice site mutations resulted in the fulfilment of TFC for ARVD/C diagnosis. This diagnostic certainty was beneficial for patient management and family screening purposes. In summary, the present study contributes to the knowledge of ARVD/C splicing mutations and highlights the importance of experimental RNA splicing analysis, particularly for exonic ARVD/C variants. The correct classification of VUS and mutations is of importance for cascade screening and risk estimation in ARVD/C families.

Limitations

In this study we performed *in vitro* analysis of potential splice site variants. However, only *in vivo* analysis can ultimately prove the pathogenicity of the 6 suggested pathogenic splice site variants. No *in vitro* method by itself provides definitive proof of pathogenicity of a variant. Other in vitro methods, like expressing these splice variants in cardiac myocytes or the study of patients iPSCs derived cardiac myocytes, are possible and could potentially add valuable information on the clinical relevance of uncertain variants with a potential effect on splicing. In our study we, focused on functional effects of uncertain variants with a potential effect on mRNA species in peripheral blood mononuclear cells from mutation carriers since we considered this type of study to approach the in vivo situation in the patient.The analyses have been performed with mRNA derived from peripheral blood leukocytes. Since pre-mRNA processing is tissue specific,

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the evaluation of potential splice site variants in peripheral blood leucocytes might not fully reflect the splicing process in cardiac tissue. However, despite extensive genetic screening, no other pathogenic mutations causative of ARVD/C could be demonstrated in the 6 index patients with a suggested pathogenic variant. Two potential splice site variants were identified in patients who had suspected ARVD/C and therefore did not fulfill TFC for diagnosis. It is possible that these patients have another disease aetiology and that these variants are bystanders. Nonetheless, the variants were found in genes commonly associated with ARVD/C.

Conclusion

Nine rare ARVD/C variants with spliceogenic potential were assessed by *in silico* prediction algorithms and additional RNA analysis by mRNA derived from mutation carriers. Six variants (*PKP2* c.1378G>A, c.1511-2A>G, c.2146-1G>C, c.2578-3A>G, *DSG2* c.378+2T>G, and *JUP* c.469-8_469-1del) were considered pathogenic splice site mutations following experimental validation. Three variants (*DSG2* c.783T>A, *DSG2* c.2759T>G, and *DSC2* c.1350A>G) were concluded to be rare polymorphisms, thus family members should not be tested for these variants. *In vitro* splicing analysis is important for ARVD/C diagnosis, and subsequent patient care and risk management, particularly in exonic ARVD/C splice site variants.

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Supplementary material

Supplemental table 1.

Gene	Variant DNA change	In silico prediction algorithm (range)	Wild type	Variant	MAF EA EVS	MAF GoNL
PKP2	c.1378G>A	MaxEntScan (0-12)	6.1	0.0	0.0	0.0
		HSF (0-100)	77.4	66.8		
		NNSPLICE (0-1)	0.9	0.0		
		GeneSplicer (0-15)	0.0	0.0		
		SSF-like (0-100)	72.5	0.0		
PKP2	c.1511-2A>G	MaxEntScan (0-12)	9.7	0.0	0.0	0.0
		HSF (0-100)	88.2	0.0		
		NNSPLICE (0-1)	1.0	0.0		
		GeneSplicer (0-15)	8.1	0.0		
		SSF-like (0-100)	86.6	0.0		
PKP2	c.2146-1G>C	MaxEntScan (0-12)	3.9	0.0	0.0002	0.0
		HSF (0-100)	81.6	0.0		
		NNSPLICE (0-1)	0.0	0.0		
		GeneSplicer (0-15)	0.0	0.0		
		SSF-like (0-100)	0.0	0.0		
PKP2	c.2578-3A>G	MaxEntScan (0-12)	6.8	0.0	0.0	0.0
		HSF (0-100)	82.8	81.9		
		NNSPLICE (0-1)	0.7	0.0		
		GeneSplicer (0-15)	5.2	0.0		
		SSF-like (0-100)	80.0	79.1		
DSG2	c.378+2T>G	MaxEntScan (0-12)	9.4	0.0	0.0	0.0
		HSF (0-100)	92.9	0.0		
		NNSPLICE (0-1)	1.0	0.0		
		GeneSplicer (0-15)	1.6	0.0		
		SSF-like (0-100)	86.2	0.0		
DSG2	c.783T>A	MaxEntScan (0-12)	0.0	0.0	0.0002	0.0
		HSF (0-100)	0.0	0.0		
		NNSPLICE (0-1)	0.0	0.0		
		GeneSplicer (0-15)	0.0	0.0		
		SSF-like (0-100)	61.1	0.0		
DSG2	c.2759T>G	MaxEntScan (0-12)	6.8	0.0	0.005	0.0090
		HSF (0-100)	78.7	0.0		
		NNSPLICE (0-1)	0.9	0.0		
		GeneSplicer (0-15)	2.3	0.0		
		SSF-like (0-100)	76.9	0.0		

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Supplemental table 1. Continued

Gene	Variant DNA change	In silico prediction algorithm (range)	Wild type	Variant	MAF EA EVS	MAF GoNL
DSC2	c.1350A>G	MaxEntScan (0-12)	0.0	7.1	0.0043	0.0070
		HSF (0-100)	0.0	91.8		
		NNSPLICE (0-1)	0.0	0.9		
		GeneSplicer (0-15)	0.0	0.0		
		SSF-like (0-100)	0.0	84.0		
DSC2	c.1350A>G	MaxEntScan (0-12)	0.0	7.1	0.0043	0.0070
		HSF (0-100)	0.0	91.8		
		NNSPLICE (0-1)	0.0	0.9		
		GeneSplicer (0-15)	0.0	0.0		
		SSF-like (0-100)	0.0	84.0		
JUP	c.469-8_469-1del	MaxEntScan (0-12)	12.2	0.0	0.0	0.0
		HSF (0-100)	96.9	0.0		
		NNSPLICE (0-1)	1.0	0.0		
		GeneSplicer (0-15)	16.4	0.0		
		SSF-like (0-100)	90.7	0.0		

Potential splice site variants identified in patients with proven Arrhythmogenic Right Ventricular Dysplasia/ Cardiomyopathy (ARVD/C) or suspected ARVD/C. Scores of *in silico* prediction programmes are noted for the wild type allele and the variant allele. MaxEntScan: the maximum entropy model, HSF: the Human Splicing Finder, SSF-like: SpliceSiteFinder-like, MAF: minor allele frequency, EA: of European American descent, EVS: the NHLBI 6500 exomes dataset, GoNL: the Dutch exomes dataset. *PKP2*: plakophilin-2, *DSG2*: desmoglein-2, *DSC2*: desmocollin-2, *JUP*: plakoglobin.

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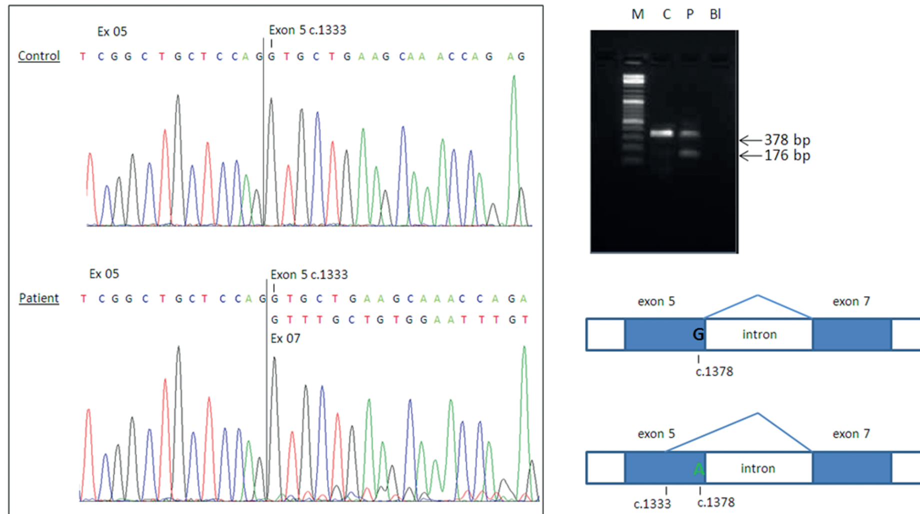
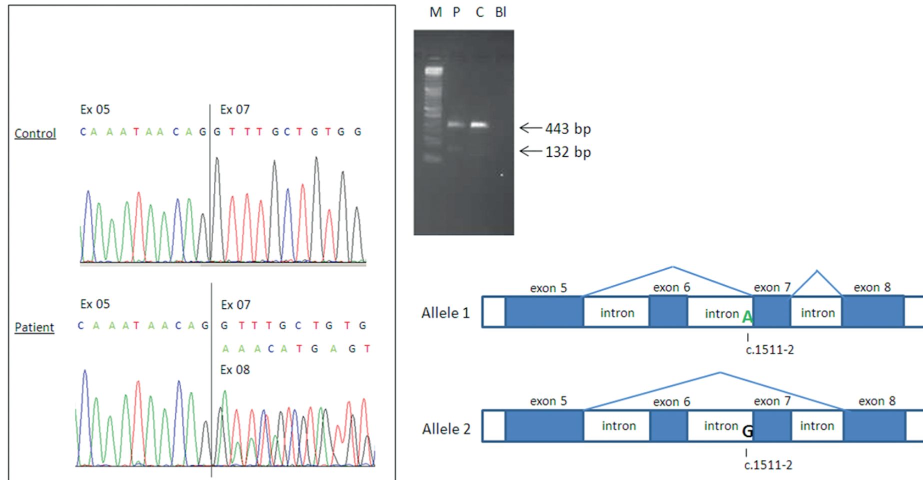
PKP2 c.1378G>A r.1333_1378del p.(Val445fs)



Supplemental figure 1. Results of the experimental mRNA analysis of the plakophilin-2 (*PKP2*) c.1378G>A variant. The variant activates a cryptic splice donor site 44 nucleotides upstream in exon 5 with an out of frame transcript. This results in shorter cDNA fragments when compared to controls as indicated by the gel electrophoresis (top right panel). C (control) showing only 238 base pairs (bp) normally sized PCR products from both *PKP2* alleles. Exon 6 is not expressed in *PKP2* transcripts from blood. P (patient) showing, in addition to a 238 base pair RT-PCR product from the normal allele, a 44 base pairs smaller PCR fragment of 194 base pairs from the mutated allele. Bl = water control, M = size marker. The panel on the left shows the direct sequencing analysis results of the PCR products. Every colored peak represents a single nucleotide showing the sequence of exon 5 (Ex 5) with position of the newly activated cryptic splice site at position c.1333 (Exon 5 c.1333) and exon 7 (Ex 7). The control lane only shows PCR fragments from both wild type *PKP2* alleles with normal exon 5 sequence. Patient sample shows a mix of PCR products from wild type and mutant *PKP2* alleles represented by the double sequence signal at the same position following position c.1333 in exon 5 (also indicated by the difference in letters representing the nucleotides), the larger peaks are from the normal allele and the smaller peaks from the mutated allele. The relative low abundance of the mutant transcript possibly reflects degradation of this transcript by nonsense mediated decay. The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and aberrant splicing of the mutated allele (Allele 2) by the activation of a cryptic splice donor site 44 nucleotides upstream of the normal splice donor site.

PKP2 c.1511-2A>G r.1511_1668del p.(Gly405fs)



Supplemental figure 2. Results of the experimental mRNA analysis of the plakophilin-2 (*PKP2*) c.1511-2A>G variant. The variant results in skipping of exon 7. This results in shorter cDNA fragments when compared to controls as indicated by the gel electrophoresis (top right panel). C (control) showing only 443 base pairs (bp) normally sized PCR products from both *PKP2* alleles containing exons 5, 7, and 8. Exon 6 is not expressed in *PKP2* transcripts from blood. P (patient) showing, in addition to a 443 base pair RT-PCR product from the normal allele, a smaller PCR fragment from the mutated allele of 132 base pairs containing exons 5 and 8 only (skipping of exon 7). Bl = water control, M = size marker. The panel on the left shows the direct sequencing analysis results of the PCR products. Every colored peak represents a nucleotide showing the sequence of exon 5 (Ex 5), exon 7 (Ex 7), and exon 8 (Ex 8). The control lane only shows PCR fragments from both wild type *PKP2* alleles with normal exon 5 to exon 7 transitions. Patient sample shows a mix (ratio ~1:1) of PCR products from wild type and mutant *PKP2* alleles represented by the double sequence signal at the same position following exon 5. PCR products from the normal allele show exon 5 to exon 7 transition (as indicated by the black G and red T sequence and beyond). PCR products from the mutated allele show exon 5 to exon 8 transition (as indicated by the green AA sequence and beyond, with skipping of exon 7). The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and aberrant splicing of the mutated allele (Allele 2) resulting in exon 7 skipping.

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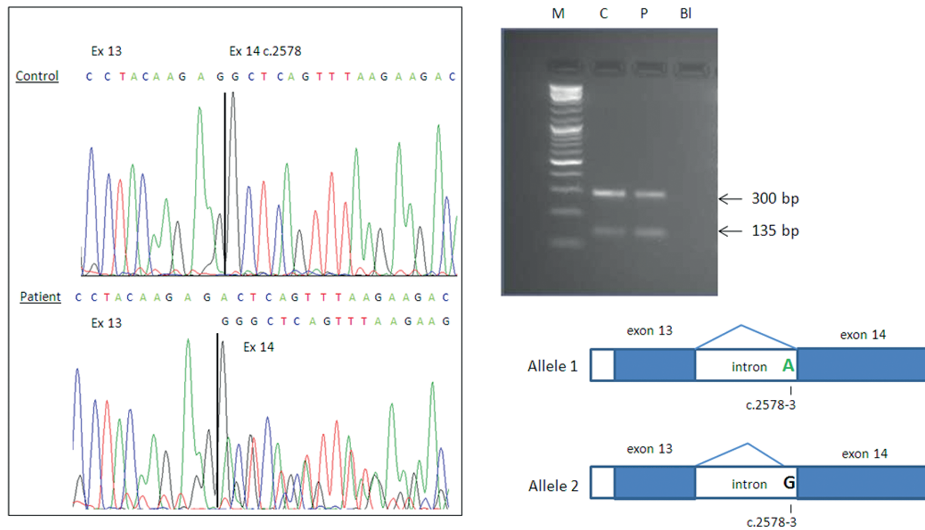
PKP2 c.2578-3A>G r.2576_2577dup p.(Ala860fs)



Supplemental figure 3. Results of the experimental mRNA analysis of the plakophilin-2 (*PKP2*) c.2578-3A>G variant. RNA analysis showed that this variant creates a new preferentially used splice acceptor site 2 nucleotides upstream of the normal exon 14 splice acceptor and an out of frame transcript. This results in longer cDNA fragments when compared to controls. Since the longer transcript contains only 2 additional nucleotides, this difference is not visible on agarose gel electrophoresis (top right panel). C (control) and P (patient) showing 300 base pairs (bp) and 135 base pairs normally sized PCR products from both *PKP2* alleles. The 300 bp fragment was shown to contain retained intron 13 sequence in addition to exon 13 and exon 14 sequence. The 135 bp fragment consisted of correct exon 13-exon 14 sequence without retained intron 13. Subsequently the 135 bp fragments of Patient and Control were gel purified and Sanger sequence analysis of these fragments was performed. Bl = water control, M = size marker. The panel on the left shows the direct sequencing analysis results of the 135 bp gel purified PCR products. Every colored peak represents a nucleotide showing the sequence of exon 13 (Ex 13) and exon 14 (Ex 14) with position of the newly created splice acceptor site at position c.2578-3 (Ex 14 c.2578). The control lane only shows PCR fragments from both wild type *PKP2* alleles with normal exon 13 and 14 sequence. Patient sample shows a mix of PCR products from wild type and mutant *PKP2* alleles represented by double sequence signals at the same position following position c.2578 in exon 14 (also indicated by the difference in letters representing the nucleotides). The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and aberrant splicing of the mutated allele (Allele 2) by the activation of a splice acceptor site 2 nucleotides upstream of the normal splice acceptor site.

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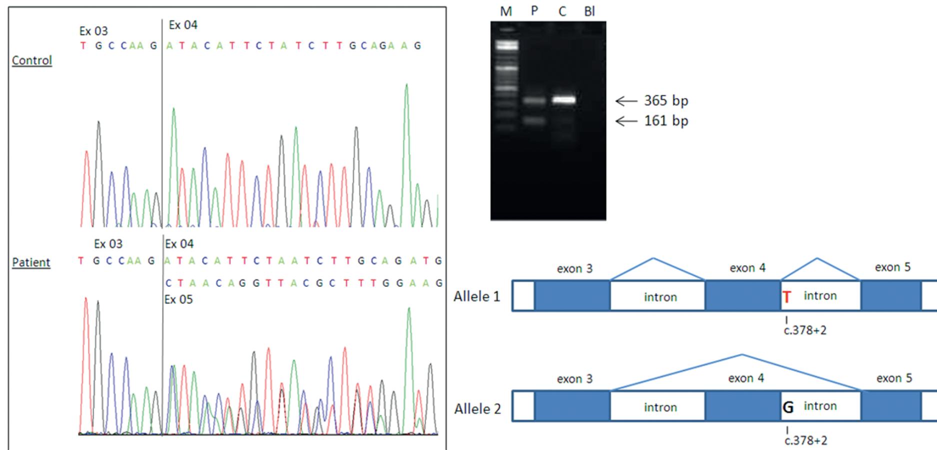
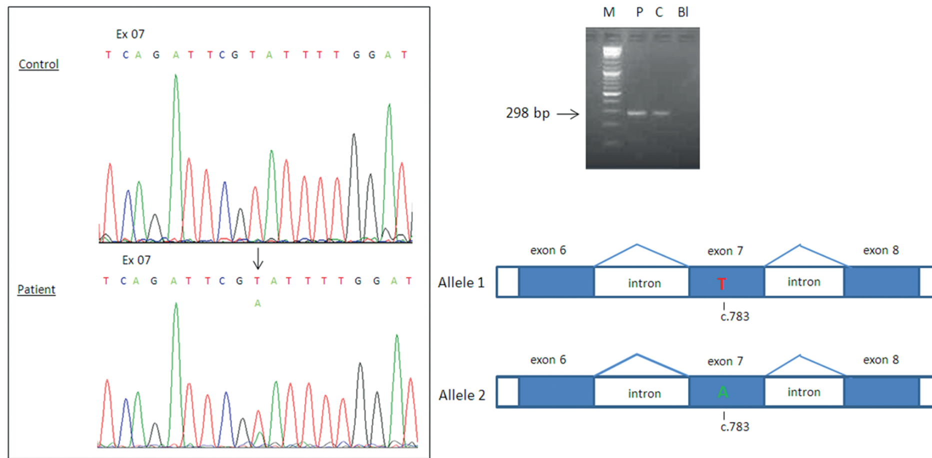
DSG2 c.378+2T>G r.217_378del p.(Ile73_Leu126del)



Supplemental figure 4. Results of the experimental mRNA analysis of the desmoglein-2 (*DSG2*) c.378+2T>G variant. The variant results in skipping of exon 4, which leads to shorter cDNA fragments when compared to controls (161 base pairs versus 365 base pairs) as indicated by the gel electrophoresis (top right panel). C (control) showing only 365 base pairs (bp) normally sized PCR products from both *DSG2* alleles containing exons 3, 4, and 5. P (patient) showing, in addition to a 365 base pair RT-PCR product from the normal allele, a smaller PCR fragment from the mutated allele of 161 base pairs containing exons 3 and 5 only (skipping of exon 4). Bl = water control, M = size marker. The panel on the left shows the direct sequencing analysis results of the PCR products. Every colored peak represents a nucleotide showing the sequence of exon 3 (Ex 3), exon 4 (Ex 4), and exon 5 (Ex 5). The control lane only shows PCR fragments from both wild type *DSG2* alleles with normal exon 3 to exon 4 transitions. Patient sample shows a mix (ratio ~1:1) of PCR products from wild type and mutant *DSG2* alleles represented by the double sequence signal at the same position following exon 3. PCR products from the normal allele show exon 3 to exon 4 transition. PCR products from the mutated allele show exon 3 to exon 5 transition, with skipping of exon 4. The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and aberrant splicing of the mutated allele (Allele 2) resulting in exon 4 skipping.

DSG2 c.783T>A r.783u>a p.(=)



Supplemental figure 5. Results of the experimental mRNA analysis of the desmoglein-2 (*DSG2*) c.783T>A variant. No aberrant transcripts were detectable in peripheral blood as indicated by the gel electrophoresis (top right panel). C (control) and P (patient)showing only 298 base pairs (bp) normally sized PCR products from both *DSG2* alleles containing exons 6, 7, and 8. Bl = water control, M = size marker. The c.783T>A variant is seen in heterozygous form in the *DSG2* transcripts, indicating biallelic expression as is shown by the direct sequencing analysis results of the PCR products (left panel). Every colored peak represents a single nucleotide showing the sequence of exon 7 (Ex 7). The control lane only shows PCR fragments from both wild type *DSG2* alleles with normal sequence. Patient sample shows biallelic expression as demonstrated by the double colored peak at position 783 (red T and green A, indicated by the arrow). The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and splicing of the mutated allele (Allele 2) which does not result in aberrant transcripts.

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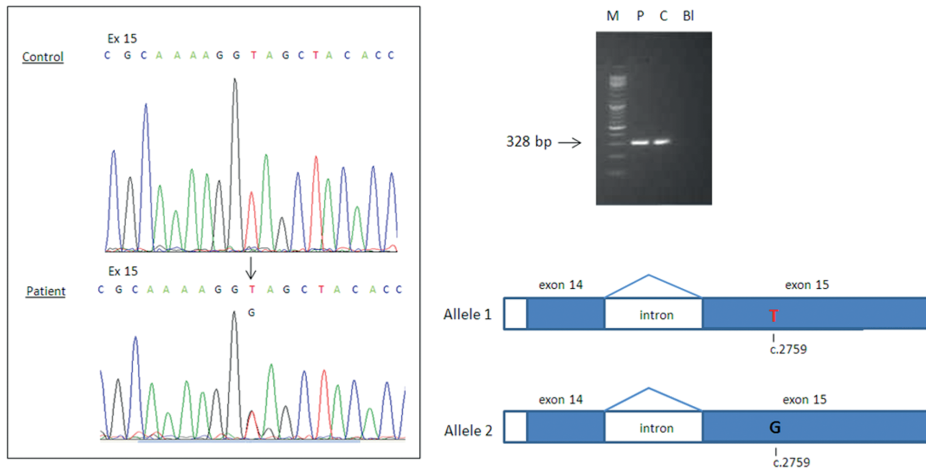
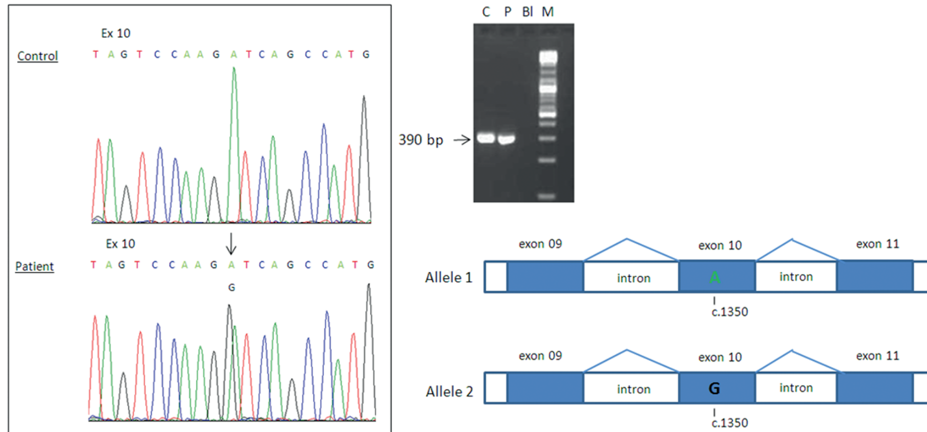
DSG2 c.2759T>G r.2759u>g p.(Val920Gly)



Supplemental figure 6. Results of the experimental mRNA analysis of the desmoglein-2 (*DSG2*) c.2759T>G variant. RNA analysis showed no altered *DSG2* transcripts as indicated by the gel electrophoresis (top right panel). C (control) and P (patient)showing only 328 base pairs (bp) normally sized PCR products from both *DSG2* alleles containing exons 14 and 15. Bl = water control, M = size marker. The c.2759T>G variant is seen in heterozygous form in the *DSG2* transcripts, indicating biallelic expression as is shown by the direct sequencing analysis results of the PCR products (left panel). Every colored peak represents a single nucleotide showing the sequence of exon 7 (Ex 7). The control lane only shows PCR fragments from both wild type *DSG2* alleles with normal sequence. Patient sample shows biallelic expression as demonstrated by the double colored peak at position 2759 (red T and black G, indicated by the arrow). The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and splicing of the mutated allele (Allele 2) which does not result in aberrant transcripts.

DSC2 c.1350A>G r.1350a>g p.(=)



Supplemental figure 7. Results of the experimental mRNA analysis of the desmocollin-2 (*DSC2*) c.1350A>G variant. RNA analysis showed no altered *DSC2* transcripts as indicated by the gel electrophoresis (top right panel). C (control) and P (patient)showing only 390 base pairs (bp) normally sized PCR products from both *DSC2* alleles containing exons 9, 10, and 11. Bl = water control, M = size marker. The c.1350A>G variant is seen in heterozygous form in the *DSC2* transcripts, indicating biallelic expression as is shown by the direct sequencing analysis results of the PCR products (left panel). Every colored peak represents a nucleotide showing the sequence of exon 10 (Ex 10). The control lane only shows PCR fragments from both wild type *DSC2* alleles with normal sequence. Patient sample shows biallelic expression as demonstrated by the double sequence signal at position c.1350 (green A and black G, indicated by the arrow). The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and splicing of the mutated allele (Allele 2) which does not result in aberrant transcripts.

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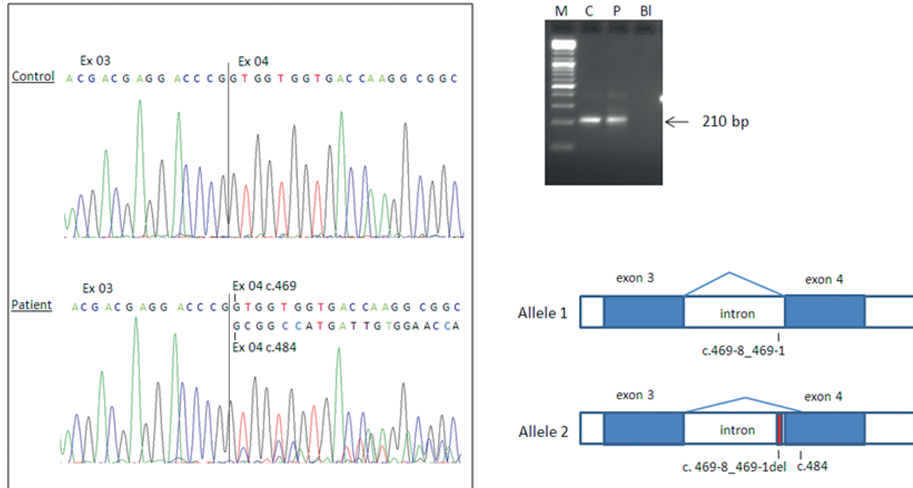
JUP c.469-8_469-1del r.469_483del p.(Val157_Lys161del)



Supplemental figure 8. Results of the experimental mRNA analysis of the plakoglobin (*JUP*) c.469-8_469-1del variant. RNA analysis demonstrated that this variant activates a cryptic splice acceptor site at positions c.482_483. Although this results in shorter cDNA fragments when compared to controls this is not visible by agarose gel electrophoresis since it affects only 15 nucleotides (top right panel). C (control) and P (patient) showing only 210 base pairs (bp) normally sized PCR products from both *JUP* alleles. Bl = water control, M = size marker. The panel on the left shows the direct sequencing analysis results of the PCR products. Every colored peak represents a nucleotide showing the sequence of exon 3 (Ex 3) and exon 4 (Ex 4) with the newly activated cryptic splice site at position c.484 (Exon 4 c.484). The control lane only shows PCR fragments from both wild type *JUP* alleles with normal exon 3 and 4 sequence. Patient sample shows a mix of PCR products from wild type and mutant *PKP2* alleles represented by double sequence signal following position c.469 in exon 4 (also indicated by the difference in letters representing the nucleotides), the larger peaks are from the normal allele and the smaller peaks are from the mutated allele. The bottom panel on the right is a schematic representation of the RNA splicing process of the normal allele (Allele 1) and aberrant splicing of the mutated allele (Allele 2) by the activation of a cryptic splice donor site 15 nucleotides downstream of the normal splice donor site. The resulting *JUP* mutant transcripts stays in frame but lacks 5 amino acids (p.Val157_Lys161del) from the evolutionary conserved and functionally important Armadillo domain.

CHAPTER 8

Impact of Genotype on Clinical Course in Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Associated Mutation Carriers

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Drs. Bhonsale and Groeneweg contributed equally to this article

Abstract

Aims

We sought to determine the influence of genotype on clinical course and arrhythmic outcome among Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) associated mutation carriers.

Methods and results

Pathogenic mutations in desmosomal and non-desmosomal genes were identified in 577 patients (241 families) from U.S. and Dutch ARVD/C cohorts. Patients with sudden cardiac death (SCD)/ ventricular fibrillation (VF) at presentation (n=36) were younger (median 23 vs. 36 years; $p<0.001$) than those presenting with sustained monomorphic ventricular tachycardia (VT). Amongst 541 subjects presenting alive, over a mean follow-up of 6±7 years, 12 (2%) patients died, 162 (30%) had sustained VT/VF, 78 (14%) manifested left ventricular dysfunction (EF<55%), 28 (5%) experienced heart failure (HF) and 10 (2%) required cardiac transplantation. Patients (n=22; 4%) with >1 mutation had significantly earlier occurrence of sustained VT/VF (mean age 28±12 years), lower VT/VF free survival ($p=0.037$), more frequent left ventricular dysfunction (29%), HF (19%) and cardiac transplantation (9%) as compared to those with only one mutation. *DSP* mutation carriers experienced >4 fold occurrence of left ventricular dysfunction (40%) and HF (13%) than *PKP2* carriers. Missense mutation carriers had similar death/transplant free survival and VT/VF penetrance ($p=0.137$) as compared to those with truncating or splice site mutations. Men are more likely to be probands ($p<0.001$), symptomatic ($p<0.001$) and have earlier and more severe arrhythmic expression.

Conclusions

Presentation with SCD/VF occurs at a significantly younger age as compared to sustained monomorphic VT. The genotype of ARVD/C mutation carriers impacts clinical course and disease expression. Male sex negatively modifies phenotypic expression.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is an inherited cardiomyopathy characterized by ventricular arrhythmias, predominantly right ventricular dysfunction, and an increased risk of sudden cardiac death (SCD) [1, 2]. Over the last decade pathogenic mutations have been identified in desmosomal genes: plakoglobin (*JUP*)[3], plakophilin-2 (*PKP2*)[4], desmoplakin (*DSP*)[5], desmoglein-2 (*DSG2*)[6] and desmocollin-2 (*DSC2*)[7]. Mutations in several non-desmosomal genes including *PLN* encoding phospholamban[8] and *TMEM43* encoding transmembrane protein 43[9] have also been reported to cause ARVD/C. While evidence suggests that genotype may influence risk of both life-threatening arrhythmia and heart failure[10], previous genotype–phenotype studies were largely limited by population size. The objective of this study was to 1) define the long-term clinical course among a large cohort of ARVD/C mutation carriers, 2) investigate the prognostic influence of genotype, including impact of multiple mutations, and 3) ascertain the association of sex with phenotypic expression.

Methods

Study population

The study population of 577 subjects was derived from the Johns Hopkins ARVD/C registry (n=259) and the Dutch ARVD/C registry (from the Interuniversity Cardiology Institute of the Netherlands (ICIN); n=318). All patients harbored a pathogenic ARVD/C associated mutation. The study complies with the Declaration of Helsinki and locally appointed ethics committee approved the research protocol and informed consent was obtained from the subjects (or their guardians).

Phenotypic characterization

The medical history of each subject was obtained by review of medical records, clinical evaluation, and patient interviews. Detailed clinical information regarding demographics, presentation, symptoms, noninvasive and invasive studies, and arrhythmia occurrence was obtained (see Supplemental table 1). Patients were prospectively followed and information regarding major clinical events (including syncope, episodes of sustained ventricular tachycardia (VT) or fibrillation (VF), sudden cardiac arrest (SCA), Implantable Cardioverter-Defibrillator (ICD) implantation, appropriate ICD shocks and anti- tachycardia pacing, heart failure, cardiac transplantation, SCD, and other causes of death was obtained (see Supplemental table 2 for definitions). The primary outcome measure was the occurrence of a sustained arrhythmic event (a composite measure of the occurrence of SCA, spontaneous sustained VT/VF or an appropriate ICD intervention for sustained VT/VF). The secondary outcome was the occurrence of death or cardiac transplantation in this cohort (Supplemental Methods A).

Genotype

Comprehensive mutation testing of *PKP2*, *DSP*, *DSG2*, *DSC2*, and *JUP* (dideoxy sequencing or next-generation sequencing) for all probands was performed as reported earlier[11, 12]

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(see Supplemental Methods B). Mutations were identified or confirmed by dideoxy (Sanger) sequencing in all patients. Multiplex ligation dependent probe amplification (MLPA) analysis was performed to identify large deletions in *PKP2* among the Dutch cohort[11, 13]. One U.S family had an incidental finding of a large *DSP* deletion on chromosomal microarray[14]. Non-desmosomal gene analysis included *TMEM43* and *PLN*. Family members were screened only for the pathogenic mutation(s) found in their respective index patient. The identified mutations were characterized by the gene involved and by the nature of the specific mutation (truncating, splice site, missense). Nonsense, frame shift, splice site mutations, and exon deletions, were all considered to be proven pathogenic unless previously identified as polymorphism or non-pathogenic. Missense mutations were reviewed and considered pathogenic for inclusion in the study when these criteria were met: 1) Exome Sequencing Project (ESP) minor allele frequency ≤ 0.05% (the NHLBI 6500 Exome data sets; EVS; http://evs.gs.washington.edu/EVS/) and 2) *in silico* predictive programs (SIFT) and Polymorphism Phenotyping-2 (PolyPhen-2) (see supplemental reference 1 and 2) predicted the genetic variants to affect protein function by a tolerance index score of <0.02 (SIFT) and a PolyPhen-2 score of >0.900. In addition, the presence of the missense mutations was assessed in 1000 Dutch control chromosomes (Genome of The Netherlands, GoNL; www.nlgenome.nl). Subjects who had SCD at presentation and either were obligate carriers or had evidence of ARVD/C on autopsy but did not have genotype studies were assumed to have the same mutation as other genetically affected 1st degree members of their respective family.

Statistical analysis

Continuous variables are summarized as either mean ± SD or median (interquartile range, IQR) and compared across groups using a *t* test, Mann-Whitney *U* test, or Kruskal-Wallis test as appropriate. Categorical variables are reported as frequency (%) and compared between groups by the chi-square or Fisher's exact test. Correlation of relevant clinical phenotypic characteristics including arrhythmic risk with the genotype was performed. The cumulative freedom since birth (i.e. by age) from the arrhythmic outcome was determined by the Kaplan-Meier method, and differences in survival between groups evaluated with the log-rank test. All p-values were corrected for family membership by using robust variance estimates in models clustered by family membership using *logistic*, *stcox*,and *somersd* commands using STATA (version 13; StataCorp, College Station, TX). A p value ≤ 0.050 was considered significant. SPSS (version19; SPSS Inc, Chicago, IL) statistical software was used for all other analyses.

Results

The study population consisted of 577 patients from 241 unrelated families and was derived from the Johns Hopkins ARVD/C registry (259 patients: 102 probands, 157 relatives) and the Dutch ARVD/C cohort (318 patients: 128 probands, 190 relatives). The registries were statistically similar in terms of sex, race and proband/relative ratios. Men constituted 55% of the overall population with the average age at presentation being 35±17 years.

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Genotype

The majority of participants (463, 80%) carried a single copy of a *PKP2* mutation (164 probands, 299 relatives) with significantly fewer heterozygous carriers of mutations in other ARVD/C-associated genes (31 *DSG2*, 31 *PLN*, 19 *DSP*, 8 *DSC2*, 2 *JUP*, and 1 *TMEM43*). Twenty-two individuals (4%) carried two or more pathogenic mutations (compound heterozygote, homozygote, or digenic mutation carriers). Among carriers of single mutations, premature truncating, splice site, and missense mutations were identified in 342 (60%), 130 (23%), and 83 (14%) patients respectively (genotype details in Supplementary Table 3 and 4).

Presentation with sudden cardiac death

Sudden cardiac death, with or without electrocardiographically proven VF, was the presenting symptom in 36 (6%; 29 families) patients. Among patients presenting with SCD, mutations in *DSP* were significantly more represented (11% vs. 3% in alive; p=0.019) (Supplementary Table 5), while missense mutations were less common (3% vs. 15% in alive; p=0.071). The median age (IQR, range) at SCD was 23 (12; 13-57) years, which was significantly younger than those presenting alive with sustained monomorphic VT with a median age of 36 years (20; 14-78) (p<0.001) (Figure 1).

Alive at presentation

Of the 541 patients presenting alive (220 probands; 321 family members), more than half of the population (53%) was asymptomatic at presentation, whereas one quarter presented with sustained VT (54% of probands, 4% of family members) (Table 1). The median age at symptom onset for the large *PKP2* group was 30 years (range 10-84), whereas it was significantly (p=0.027) later for those with *PLN* mutations (40 years; range 16-59) (genotype details in Supplemental table 6). Among probands, no significant difference in sex (p=0.175), occurrence of symptoms

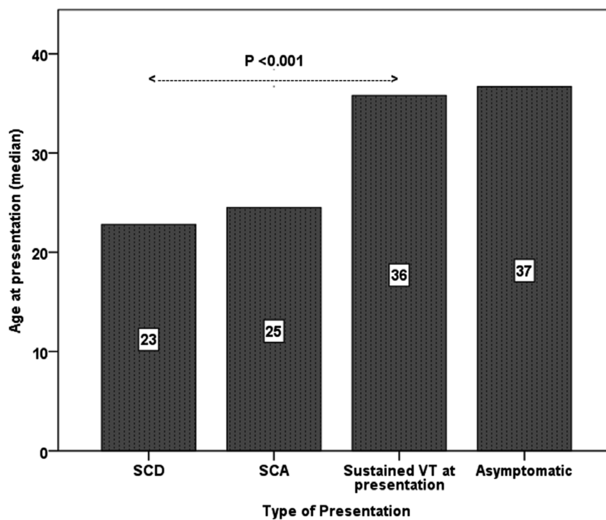

Figure 1. Median age at presentation categorized according to the nature of the presentation. SCD= Sudden cardiac death, SCA= Sudden cardiac arrest, VT= Ventricular tachycardia.

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(p=0.276), or incidence of composite outcome (p=0.719) was seen between major gene groups (Supplemental table 7). The majority of relatives were asymptomatic at presentation (86%) with 24 (8%) experiencing the arrhythmic outcome during follow-up (4± 5years) with no significant differences among gene groups (Supplemental table 8). Data for the single*TMEM43* gene mutation carrier is noted in Supplementary Table 9.

Clinical course

Over a mean duration of follow-up of 6 years, 207 (38%) patients experienced the composite sustained arrhythmic outcome (Table 2). The arrhythmic event free survival for the overall population at age 40 and 60 years was 66% and 42% respectively (Supplemental Figure 1A).

Table 1. Baseline phenotypic characteristics of the study population categorized according to the underlying genotype

Clinical variable	**Study cohort** (n=577)	***PKP2*** (n=463)	***JUP*** (n=2)	***DSG2*** (n=31)	***DSC2*** (n=8)	***DSP*** (n=19)	**>1 mutation** (n=22)‡	***PLN*** (n=31)
Male sex	318(55)	253(55)	2(100)	19(61)	6(75)	10(53)	12(54)	15(48)
Proband	230(40)	164(35)	2(100)	16(52)	5(62)	9(47)	15(68)	18(58)
Age at presentation; m±SD; years	35±17	35±18	41±33	32±17	41±19	33±17	31±13	42±12
SCD as presentation	36(6)	29(6)	0(0)	1(3)	0(0)	4(21)	1(4)	1(3)
Among those presenting alive								
Type of Presentation	(n=541)	(n=434)	(n=2)	(n=30)	(n=8)	(n=15)	(n=21)	(n=30)
Asymptomatic	287(53)	243(56)	0	15(50)	3(37)	8(53)	7(33)	11(37)
SCA	16(3)	13(3)	0(0)	0(0)	1(12)	0(0)	1 (5)	1(3)
VT at presentation	131(24)	99(23)	1(50)	8(27)	2(25)	3(20)	7(33)	11(37)
Cardiac syncope	85(16)	64(15)	0(0)	7(23)	2(25)	3(20)	5(24)	4(13)
Age at symptom onset; m ±SD; years	33± 14	33±14,	41±33	29±12	35±21	33±15	25±10	38±11,
Inducibility at EPS§	146/200 (73)	107/148 (72)	-	12/14 (86)	2/3 (67)	7/9 (78)	10/11 (91)	7/14 50)
Holter PVC count; median	910	694	-	518	3883	1723	2562	3169

* *PKP2*=Plakophilin-2; *JUP*=Plakoglobin; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; *DSP*=Desmoplakin; ‡ digenic, homozygous, and compound heterozygous mutations; *PLN*=Phospholamban; EPS= Electrophysiological study; §among those that had EP study; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; SCD=Sudden cardiac death; SCA= Sudden cardiac arrest; VT= Ventricular tachycardia; PVC= Premature ventricular complex; SD= Standard deviation; m= mean.

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Table 2. Clinical course including data obtained at presentation and during follow-up in ARVD/C mutation carriers presenting alive categorized according to the underlying genotype

Clinical variable	**Study cohort** (n=541)	***PKP2*** (n=434)	***JUP*** (n=2)	***DSG2*** (n=30)	***DSC2*** (n=8)	***DSP*** (n=15)	**>1 mutation‡** (n=21)	***PLN*** (n=30)
Definite ARVD/C	327(60)	251(58)	2(100)	16(53)	5(62)	9(60)	18(86)	25(83)
Follow-up; m±SD; years	6±7	6±7	6±7	5±5	2±3	7±9	10±10	7±5
Composite outcome	207(38)	153(35)	2(100)	13(43)	4(50)	6(40)	12(57)	16(53)
Spontaneous Sustained VT	162(30)	122(28)	1(50)	10(33)	2(25)	5(33)	9(43)	13(43)
Appropriate ICD intervention (VT/VF)	24(4)	14(3)	1(50)	3(10)	1(12)	-	2(9)	2 (7)
Resuscitated SCA/VF	21(4)	17(4)	0(0)	0(0)	1(12)	1(7)	1(5)	1(3)
Age at first sustained VT/VF	35±13	35±13	41±33	32±11	36±23	40±16	28±12	39±11
ICD implantation	241 (44)	179(41)	2(100)	15(50)	4(50)	8(53)	14(67)	18(60)
Appropriate ICD therapy§	117/241	85/179	1/2	7/15	3/4	3/8	9/14	8/18
VT storm	37(7)	28(6)	1(50)	1(3)	1(12)	1(7)	3(14)	1(3)
VT ablation	73(13)	53(12)	1(50)	6(20)	2(25)	3(20)	3(14)	5(17)
LV dysfunction†	78(14)	39(9)	0(0)	4(13)	3(37)	6(40)	6(28)	20(67)
Heart failure	28(5)	15(3)	0(0)	0(0)	0(0)	2(13)	4(19)	7(23)
Cardiac transplant	10(2)	6(1)	0(0)	0(0)	0(0)	0(0)	2(9)	2(7)
Death	12(2)	7(2)	0	0	0	0	1(5)	4(13)

* *PKP2*=Plakophilin-2; *JUP*=Plakoglobin; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; *DSP*=Desmoplakin; ‡ digenic, homozygous, and compound heterozygous mutations; *PLN*=Phospholamban; †EF< 55%; VT= Ventricular tachycardia; VF= Ventricular fibrillation; ICD= Implantable Cardioverter-Defibrillator; §Among those that had ICD implanted; NSVT= Non-sustained VT; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; SCA= Sudden cardiac arrest; SD= Standard deviation; LV=Left ventricular; m= mean;

The median age at the first episode of sustained VT/VF was 35 years in patients with *PKP2* mutations, whereas it was significantly later in *PLN* mutation carriers (42 years) (p=0.031). Ten patients underwent cardiac transplantation (mean age at transplant: 46±15 years, two primarily for intractable arrhythmias, eight primarily as a result of heart failure) and 12 (2%) died during follow-up. The survival free of death/cardiac transplant at age 40 and 60 years for the overall

population was 91% and 85% respectively (Supplemental Figure 1B). SCD in a proband could not be assessed as risk factor in family members since only one relative died suddenly.

Patients with more than one mutation (compound heterozygote, homozygote, or digenic mutation carriers; 4%) had significantly earlier onset of symptoms (median age 23 years [16-46]) as well as earlier occurrence of sustained ventricular arrhythmias (median age 25 years; p=0.041) as compared to those with only one mutation. Overall, these patients had a higher incidence of arrhythmias compared to those with only one mutation (Table 2). The sustained

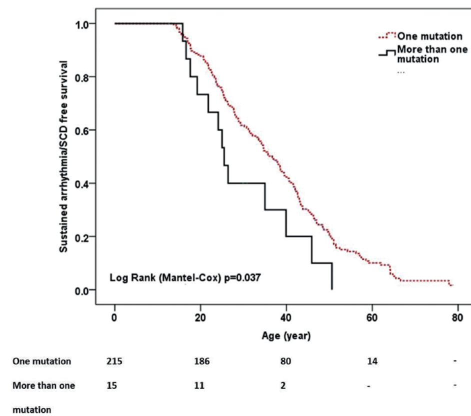

Figure 2. Survival analysis showing significantly lower sustained arrhythmia free survival in index patients with more than one mutation as compared to those with one mutation only.

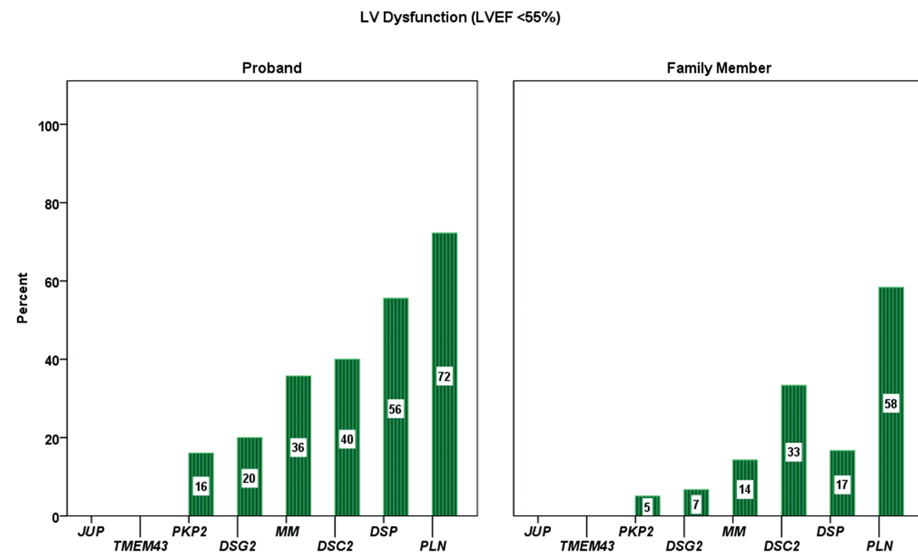

Figure 3. Proportion of patients manifesting left ventricular (LV) dysfunction among probands and family members in each of the major gene groups. *PKP2*=Plakophilin-2; *DSP*=Desmoplakin; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; *PLN*=Phospholamban; *JUP*=Plakoglobin; MM=More than one mutation, including compound heterozygote, homozygote and digenic patients.

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arrhythmia free survival at age 20 and 40 years was significantly lower (71% and 33%, respectively) among probands with more than one mutation than among single heterozygous mutation carriers (Figure 2).
Left ventricular dysfunction (EF< 55%) was seen in 78 (14%) patients while 28 (5%) experienced congestive heart failure during follow-up. *PKP2* carriers were least likely to have left ventricular dysfunction (9%), whereas those with *DSP* mutations had significantly more frequent left ventricular dysfunction (40%) (p=0.001) and heart failure (13%) (p=0.046). Patients with more than one mutation and *PLN* mutation carriers had more than three times the amount of left ventricular dysfunction and heart failure. This relationship, stratified among probands and relatives is shown in Figure 3.

Influence of type of mutation on outcomes

Among those presenting alive, premature truncating, splice site, and missense mutations were identified in 319 (59%), 119 (22%), and 82 (15%) patients respectively (Supplementary Table 6). No significant difference in either the arrhythmic outcome free survival or the cardiac transplant/death free survival was seen in the overall population between patients with premature truncating, splice site, or missense mutations (Figure 4A and B).

Sex

Females constituted nearly half (45%) of the overall cohort. However, men were more likely to be probands (68% of probands; p<0.001), be symptomatic at presentation (50% men vs. 37%; women; p<0.001) and present with SCD (78% men; p=0.004). In the overall population, the composite arrhythmic outcome occurred significantly more in males (53% vs. 29%; p<0.001) (Figure 5). However, occurrence of heart failure (6 vs. 4%; p=0.246) and left ventricular dysfunction (16% vs. 12%; p=0.151) was not significantly higher in males.

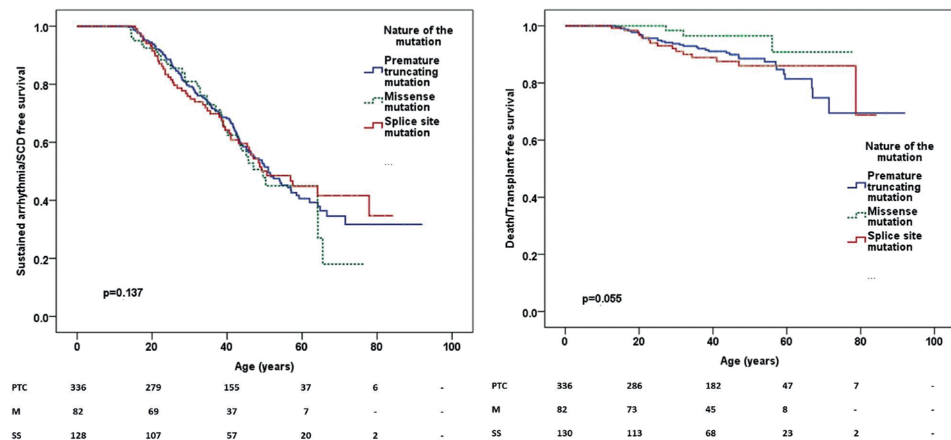

Figure 4A. Sustained ventricular arrhythmia free survival in patients stratified according to the type of underlying mutation. **B.** Death or transplant free survival in patients stratified according to the type of underlying mutation in the study population.

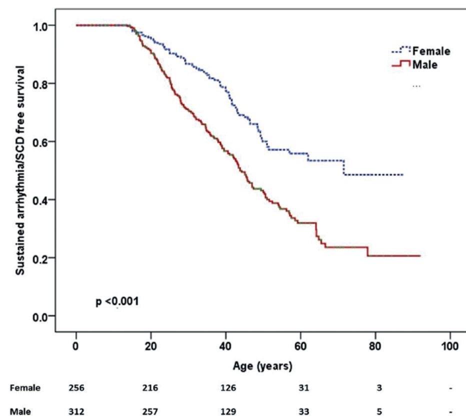

Figure 5. Composite arrhythmic outcome free survival among study patients stratified by sex.

Discussion

Our study of the largest worldwide cohort of 577 patients (230 index patients, 347 relatives) with ARVD/C associated mutations has three important results. First, our study shows that phenotypic first presentation with SCD and/or VF occurs at a significantly younger age (median 23 years) than presentation with sustained monomorphic VT (median 36 years). Secondly, this large cohort demonstrates the association of genotype with clinical course and disease expression. Thirdly, our study provides evidence that male sex negatively modifies the clinical course.

Sudden death as earliest disease manifestation among ARVD/C mutation carriers

Sudden cardiac death is a well-appreciated risk associated with ARVD/C, with up to half of index cases presenting with SCD[10, 15]. Recent studies suggest that histologic changes may be preceded by gap-junction remodeling mediating electrical coupling and altering the amplitude and kinetics of the sodium current[16], giving rise to slow conduction. In cellular and mouse models[17] these changes result in an increased propensity for arrhythmias, also without any evidence of structural heart disease [17]. Our finding that those presenting with SCD/SCA are significantly younger than those presenting with sustained VT supports this mechanism and underscores the importance of molecular, genetic and phenotypic investigation of young family members following the first diagnosis in a family. *DSP* carriers were significantly more likely to present with SCD than other mutation carriers and accounted for 11% of SCD cases (as compared to 3% of the entire population). This observation confirms a prior report from four Italian families[18] and provides evidence for early identification of young at-risk individuals with *DSP* mutations.

Impact of genotype on heart failure and arrhythmic outcomes

In our study, majority of patients presenting alive as well as those presenting with SCD harbored premature truncating *PKP2* mutations. Four percent of our population (6% of probands) carried

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more than one ARVD/C associated mutation. The lower incidence of digenic, homozygous, or compound heterozygous mutations in our population compared to data in some previous reports[10,19] reflects the rigorous approach and stringent criteria we used to define pathogenicity of missense mutations. Prior studies have suggested that individuals with more than one mutation may have worse clinical outcomes[11, 20-24]. Our relatively large sample of individuals with more than one mutation alive at presentation shows that these patients have considerably worse clinical course with significantly earlier onset of symptoms and first sustained arrhythmia, a greater chance of developing sustained VT/VF, and a five-fold increase in the risk of developing left ventricular dysfunction and heart failure than those carrying a single mutation. These results suggest that patients with more than one mutation should be followed carefully for symptoms associated with both arrhythmia and heart failure.

Among carriers of a single mutation presenting alive, our study demonstrates that the risk of left ventricular involvement and development of heart failure is intrinsically related to the mutated gene. Conversely, carriers of single mutations in all the genes had a high risk of developing a life-threatening arrhythmia, with no significant differences in survival from life-threatening VT/VF among the different genes. *PLN* mutation carriers presented at a significantly older age yet had worse long-term prognosis, with more left ventricular dysfunction and heart failure. Also *DSP* mutation carriers were considerably more likely to develop both heart failure and signs of LV involvement, confirming the observation from prior smaller cohorts[25, 26]. In contrast to a prior observation[21], *DSG2* mutation carriers were not disproportionally likely to develop LV involvement in our study.

Pathogenicity of a missense mutation in ARVD/C is difficult to determine and has been questioned [20-22]. We demonstrate that ARVD/C related missense mutations have a similar pathogenic character as non-missense mutations. In contrast to findings of other investigators[21], carriers of missense mutations in our population had no difference in survival from death/transplant or from developing a life-threatening arrhythmia as compared to carriers of premature truncating and splice site mutations. This lack of phenotypic difference or outcome suggests that these highly scrutinized missense alleles are indeed pathogenic. This pathogenicity of ARVD/C associated missense mutations can be correctly predicted by the combined criteria from SIFT, PolyPhen-2, and ESP minor allele frequency.

Sex

Previous reports suggest that the severity of the phenotype is likely influenced by sex[27]. However, the impact of sex upon clinical course among ARVD/C mutations carriers has been unclear. Our study shows that male mutation carriers were more likely to present with sudden death, be symptomatic at presentation, develop VT/VF/SCD, and to have less overall survival free from death/cardiac transplant. However, males were not more likely to have LV involvement or heart failure. The mechanism for lower penetrance among women is uncertain although it is likely that this may reflect different levels of participation in competitive athletics among men and women[28]. There is some evidence from animal models that strenuous exercise is associated with development of ARVD/C[29] and more recently vigorous exercise has been found to be associated with worse clinical outcomes in patients harboring a desmosomal mutation[28].

Limitations

Variations in disease expression between families carrying the same mutation, and among members of the same family, suggest that modifier genes and environmental influences contribute to the overall phenotype in ARVD/C. This study does not evaluate the potential impact of a large number of variants of uncertain significance in modifying the phenotypic expression of a pathogenic mutation. The biophysical function of many missense mutations reported in this study has not been studied in expression systems or models. Also, all *PLN* mutation carriers had the same mutation and results cannot be extrapolated to other mutations in this gene. The presence of founder mutations limits the extent to which the findings may be generalized to other ARVD/C cohorts. Although appropriate ICD therapy constituted a small minority of the composite arrhythmic outcome, overestimation of SCD risk is a possibility. Despite our large cohort, the small number of multiple mutations carriers relative to the overall population demands caution in generalizing these results. Finally, this study focused on patients harboring pathogenic mutations and comparison to those without identifiable genetic abnormality, remains a goal for future studies.

Conclusion

This study of the largest well-defined ARVD/C cohort worldwide, demonstrates that SCD and VF occur at much younger age than monomorphic VT. Genotype-phenotype correlation analysis provides evidence for 1) gene specific differences in the propensity to life-threatening arrhythmias, left ventricular dysfunction, and heart failure, and 2) substantially worse outcome with presence of more than one pathogenic mutation. The study confirms worse outcome with male sex. We also provide evidence that missense variants, defined as pathogenic by predicting algorithms, are associated with a similar prognosis as premature truncating or splice site mutations.

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Supplementary material

Supplemental table 1. Non-invasive and invasive tests available during phenotypic evaluation of the study cohort

	Overall (n=541)	**Probands** (n=220)	**Family members** (n=321)
Electrocardiogram	488(91)	219 (100)	269 (84)
Exercise stress test	323 (60)	155(71)	168(52)
Holter monitoring	369 (68)	148 (68)	221 (69)
Echocardiogram	418 (78)	204 (93)	214 (67)
Magnetic resonance imaging	338 (63)	162 (74)	176 (55)
Electrophysiological study	205 (38)	154(70)	51(16)
RV angiogram	103(19)	79 (36)	24 (7)
Cardiac biopsy	101 (19)	77 (35)	24 (7)

*RV= Right ventricular

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Supplemental table 2. Definitions of clinical and ECG variables employed in this study

Term	Definition
Sudden cardiac death	Death of cardiac origin that occurred unexpectedly within 1 hour of the onset of new symptoms or a death that was unwitnessed and unexpected
Sudden cardiac arrest	An event as described above, that is reversed, usually by cardio-pulmonary resuscitation and/or defibrillation or cardioversion
Sustained ventricular tachycardia	Ventricular tachycardia which lasts 30 seconds or more, or less than 30 s when terminated electrically or pharmacologically
Appropriate ICD Therapy	ICD shock or anti-tachycardia overdrive pacing delivered in response to a ventricular tachyarrhythmia and documented by stored intracardiac ECG data
Electrical storm	Occurrence of ≥ 3 episodes of ventricular tachycardia/ ventricular fibrillation in a 24-h period
Non-sustained ventricular tachycardia	≥ 3 consecutive premature ventricular complexes with a rate >100 /min, lasting <30 s, which was documented during exercise testing, loop monitoring, or 24-h Holter monitoring
Epsilon wave	Distinct waves of small amplitude within the ST segment in the right precordial leads and are distinct from the QRS complex
Terminal activation duration	The longest value in V1–V3, from the nadir of the S wave to the end of all depolarization deflections. Considered abnormal when ≥55 msec
Complete right bundle branch block	QRS duration ≥0.12 s, secondary R wave in right precordial leads, and wide S wave in leads I and V6
Proven or definite ARVD/C (Diagnostic terminology as per 2010 revised Task Force Criteria3)	2 major or 1 major and 2 minor criteria or 4 minor from different categories
Borderline ARVD/C(Diagnostic terminology as per 2010 revised Task Force Criteria3)	1 major and 1 minor or 3 minor criteria from different categories
Heart failure	Stage C heart failure was defined using the American College of Cardiology/American Heart Association heart failure staging system, but required both evidence of structural heart disease including RV abnormalities and symptoms directly attributed to heart failure.
The proband (index patient)	The proband (index patient) was the first affected family member seeking medical attention for ARVD/C in whom the diagnosis was confirmed (i.e. an affected person ascertained independently of family history of ARVD/C and in whom DNA analysis was started)
Family members	Individuals ascertained through family screening.

*ICD= Implantable Cardioverter-Defibrillator; VT= Ventricular tachycardia; VF= Ventricular fibrillation; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

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Supplemental table 3. Genotype of the patients stratified according to the registry

Gene	No. of patient (%)	Premature truncating (%)	Splice site (%)	Missense (%)
JHU registry				
PKP2	210 (81)	125(60)	79(38)	6(3)
DSP	16(6)	12(75)	0(0)	4(25)
DSG2	18(7)	1(6)	1(6)	16(89)
DSC2	3(1)	1(33)	1(33)	1(33)
JUP	2(1)	0	0	2(100)
PLN	2(1)	2(100)	0	0
TMEM43	1(0.4)	0	0	1(100)
More than one mutation	7(3)	-	-	-
Total	259	141	81	30
Dutch registry				
PKP2	253(80)	169(67)	45(18)	39(15)
DSP	3(1)	2(67)	0(0)	1(33)
DSG2	13(4)	1	1	11
DSC2	5(2)	0	3	2
JUP	-	-	-	-
PLN	29(9)	29(100)	0(0)	0(0)
TMEM43	-	-	-	-
More than one mutation	15(5)	-	-	-
Total	318	201	49	53

**PKP2*= Plakophilin-2; *DSP*= Desmoplakin; *DSG2*= Desmoglein-2; *DSC2*= Desmocollin-2; *PLN*= Phospholamban; *TMEM43*= Transmembrane protein 43; *JUP*= Plakoglobin; More than one mutation includes digenic, homozygous, or compound heterozygous mutations.

Supplemental table 4. Mutations represented in the study population

Gene	Nucleotide change	Protein change	No. of probands	No. of family members	Total
PKP2	c. 235C>T	p. Arg79X	15	31	46
	c. 2146-1G>C	Abnormal splice product	20	24	44
	c. 1211-1212insT	p. Val406SerfsX4	15	26	41
	c. 2386T>C	p. Cys796Arg	11	24	35
	c. 2489+1G>A	Abnormal splice product	12	23	35
	c. 397C>T	p. Gln133X	7	15	22
	c. 2197_2202delinsG	p. His733AlafsX8	9	10	19
	c. 1848C>A	p. Tyr616X	4	13	17
	c. 1613G>A	p. Trp538X	6	9	15
	c. 148_151delACAG	p. Thr50SerfsX61	7	7	14
	c. 2489+4A>C	Abnormal splice product	4	9	13
	Deletion exon 1-4		1	9	10
	c. 917-918delCC	p. Pro318GlnfsX29	3	7	10
	c. 2013delC	p. Lys672ArgfsX12	2	8	10
	c. 1643delG	p. Gly548ValfsX15	1	9	10
	c. 1369_1372delCAAA	p. Gln457X	4	4	8
	c. 2509delA	p. Ser837ValfsX94	3	5	8
	Deletion exon 1-14		1	5	6
	c. 1237C>T	p. Arg413X	3	2	5
	c. 1271T>C	p. Phe424Ser	1	4	5
	c. 2484C>T	Abnormal splice product	0	5	5
	c. 337-2A>T	Abnormal splice product	1	3	4
	c. 968_971delAGGC	p. Gln323fs	1	3	4
	c. 2034G>A	p. Trp678X	1	3	4
	c. 1759delG	p. Val587SerfsX69	1	2	3
	c. 2062T>C	p. Ser688Pro	1	2	3
	c. 218dupG	p. Asn74GlnfsX12	0	2	2
	c. 224-3C>G	Abnormal splice product	1	1	2
	c. 1132C>T	p. Gln378X	2	0	2
	c. 1171-2A>G	Abnormal splice product	1	1	2
	c. 1378+1G>C	Abnormal splice product	0	2	2
	c. 1378G>A	p. Asp460Asn	1	1	2
	c. 1803delC	p. Asp601GlufsX55	1	1	2
	c. 1849C>T	p. Gln617X	2	1	3
	c. 1951C>T	p. Arg651X	1	1	2
	c. 2421C>A	p. Tyr807X	1	1	2
	c. 2544G>A	p. Trp848X	1	1	2
	Deletion exon 10		1	1	2
	c. 258T>G	p. Tyr86X	1	0	1
	c. 1307_1315delins8	p. Leu436HisfsX11	1	0	1
	c. 1368delA	p. Lys456AsnfsX3	1	0	1
	c. 1511-2A>G	**Abnormal splice product**	1	0	1
	c. 1760delT	p. Val587fsX69	1	0	1
	c. 1821dupT	p. Val608fs	0	1	1
	c. 1844C>T	p. Ser615Phe	0	1	1
	c. 2028G>A	p. Trp676X	1	0	1

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	c. 2145+1G>C	Abnormal splice product	1	0	1
	c. 2169_2172dupAGTT	p. Val725SerfsX19	0	1	1
	c. 2174T>A	p. Val725Asp	1	0	1
	c. 2554delG	p. Glu852AsnfsX79	1	0	1
	Deletion exons 8-14		1	0	1
DSG2					
	c. 137G>A	p. Arg46Gln	4	5	9
	c. 874C>T	p. Arg292Cys	1	2	3
	c. 1520G>A	p. Cys507Tyr	1	2	3
	c. 146G>A	p. Arg49His	1	1	2
	c. 889G>A	p. Asp297Asn	2	0	2
	c. 2434G>T	p. Gln812Cys	0	2	2
	c. 3140C>G	p. Tyr1047Arg	1	1	2
	c. 136C>T	p. Arg46Trp	1	0	1
	c. 378+2T>G	Abnormal splice product	1	0	1
	c. 464_465insT	**p. Glu156ArgfsX14**	1	0	1
	c. 523+2T>C	Abnormal splice product	0	1	1
	c. 614C>T	p. Pro205Leu	1	0	1
	c. 1038_1040delGAA	p. Lys346del	1	0	1
	c. 1072G>A	p. Ala358Thr	0	1	1
DSC2					
	c. 608G>A	p. Arg203His	0	2	2
	c. 943-1G>A	Abnormal splice product	1	1	2
	c. 154+1G>A	Abnormal splice product	1	0	1
	c. 658G>A	p. Gly220Arg	1	0	1
	c. 942+3A>G	Abnormal splice product	1	0	1
	c. 2582_2585dupGAAG	p. Gly863LysfsX13	1	0	1
DSP					
	c. 1264G>A	p. Glu 422Lys	1	2	3
	c. 478C>T	p. Arg160X	1	1	2
	c. 3337C>T	p. Arg1113X	1	1	2
	c. 151C>T	p. Gln51X	1	0	1
	c. 1333A>G	p. Ile445Val	1	0	1
	c. 3160_3169delAAGAACAA	p. Lys1052fsX26	1	0	1
	c. 4775A>G	p. Lys1592Arg	1	0	1
	c. 5212C>T	**p. Arg1738X**	1	0	1
	c. 6478C>T	p. Arg2160X	0	1	1
	c. 6496C>T	p. Arg2166X	1	0	1
	Deletion 6p24.1p25.1		0	1	1
JUP					
	c. 56C>T	p. Thr19Ile	1	0	1
	c. 475G>T	**p. Val159Leu**	1	0	1
TMEM43					
	c. 1073C>T	p. Ser358Leu	1	0	1
PLN					
	c. 40_42delAGA	p. Arg14del	18	12	30

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More than one mutation					
Digenic					
PKP2,DSP large deletion	*PKP2* c. 2197_2202delinsG, *DSP* deletion 6p2431p25.1	PKP2 p. His733AlafsX8	0	1	1
PLN, DSP	*PLN* c. 40_42delAGA, *DSP* c. 6881C>G	p. Arg14del & p. Ala2294Gly	0	1	1
DSC2,RYR	*DSC2* c. 1276G>A, RYR2 c. 4693C>G	p. Glu426Lys & p. Pro1565Ala	1	0	1
PKP2,DSG2	*PKP2* c. 1237C>T, *DSG2* c. 829-1_835del	p. Arg413X & p. Leu277GlyfsX2	1	0	1
DSG2+ PKP2 exon deletion	*DSG2* c 1072G>A, *PKP2* deletion exon 1-14	p. Ala358Thr	1	0	1
PKP2,DSG2	*PKP2* c. 1211-1212insT, *DSP* 269A>G	p. Val406SerfsX4 & p. Gln90Arg	1	0	1
PKP2,DSG2	*PKP2* c. 2489+4A>C, *DSP* c. 1982A>T	Abnormal splice product & p. Asn661Ile	1	0	1
PKP2, PKP2, DSG2	*PKP2* c. 397C>T , *PKP2* c. 2615C>T, DSG2 c. 1480G>A	p. Gln133X&Thr872Ile & p. Asp494Asn	1	0	1
Compound Heterozygote					
PKP2, PKP2	*PKP2* c. 397C>T, ***PKP2* c. 2615C>T**	p. Gln133X & p. **Thr872Ile**	2	5	7
DSP,DSP	*DSP* c. 6478C>T, *DSP* c. 943C>T	p. Arg2160X & p. Arg315Cys	1	0	1
DSG2,DSG2	*DSG2* c. 523+2T>C, *DSG2* c. 1038_1040delGAA	Abnormal splice product & p. Lys346del	1	0	1
DSG2,DSG2	*DSG2* c. 918G>A, *DSG2* c. 146G>A	p. Trp306X & p. Arg49His	1	0	1
PKP2, PKP2	***PKP2* deletion exon 8, *PKP2* , c. 746G>A**	**deletion& p. Ser249Asn**	1	0	1
Homozygous					
	PKP2 c. 2484C>T homozygosity	Abnormal splice product	1	0	1
	DSC2 c. 608G>A homozygosity	p. Arg203His	1	0	1
Total			14	7	21

* We have noted in bold the novel mutations found in this study that have not been previously reported.

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Supplemental table 5. Genotype of 36 patients presenting with sudden cardiac death

Gene	Nucleotide change	No. of Patients	Age at Death
PKP2	c. 1211-1212insT	1	17
	c. 1237C>T	3	21,38,54
	c. 1369_1372delCAAA	1	14
	c. 1378+1G>C	3	16,20,41
	c. 1643delG	1	36
	c. 1760delT	1	27
	c. 1849C>T	1	32
	c. 2146-1G>C	4	19,23,25,30
	c. 2169_2172dupAGTT	1	27
	c. 218dupG	2	15,16
	c. 2197_2202delinsG	2	15,18
	c. 235C>T	3	21,21,29
	c. 2421C>A	1	18
	c. 2489+1G>A	4	21,23,29,47
	c. deletion exon 1-14	1	20
DSP	c. 3160_3169delAAGAACAA	2	20,47
	c. 6478C>T	1	25
	c. 6496C>T	1	31
DSG2	c. 2434G>T	1	32
PLN	c. 40_42delAGA	1	57
More than one mutation	PLN c. 40_42delAGA, DSP c. 6881C>G	1	25

* *PKP2*= Plakophilin-2; *DSP*= Desmoplakin; *DSG2*= Desmoglein-2; *PLN*= Phospholamban; More than one mutation includes digenic, homozygous, or compound heterozygous mutations.

Supplemental table 6. Genotype of the study population alive at presentation (n=541)

Gene	No. of patients (%)	Premature truncating (%)	Splice site (%)	Missense (%)
PKP2	434(80)	276(64)	113(26)	45(10)
DSP	15(3)	10(67)	0(0)	5(33)
DSG2	30(5.5)	2(7)	2(7)	26(87)
DSC2	8(1.5)	1(12.5)	4(50)	3(37.5)
JUP	2(0.4)	0(0)	0(0)	2(100)
PLN	30(5.5)	30(100)	0(0)	0(0)
TMEM43	1(0.2)	0(0)	0(0)	1(0)
More than one mutation	21(4)	-	-	-
Total	541	319	119	82

* *PKP2*=Plakophilin-2; *DSP*=Desmoplakin; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; *PLN*=Phospholamban; *TMEM43*= Transmembrane protein 43; *JUP*=Plakoglobin; More than one mutation includes digenic, homozygous, or compound heterozygous mutations.

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Supplemental table 7. Clinical characteristics and outcome of the probands (n=220)* presenting alive in the study population

Clinical Variable	***PKP2*** (n=156)	***JUP*** (2)	***DSG2*** (n=15)	***DSC2*** (n=5)	***DSP*** (n=9)	**>1 mutation‡** (n=14)	***PLN*** (n=18)
Male gender	105 (67)	2(100)	12(80)	5(100)	4(44)	8(57)	10(55)
Definite ARVD/C (at last follow-up)	155(99)	2(100)	15(100)	5(100)	8(89)	14(100)	18(100)
Duration of follow-up; ±SD; years	9±9	6±7	6±6	3±4	11±10	14±11	10±5
Symptomatic at presentation	139(89)	2(100)	14(93)	4(80)	7(78)	12(86)	16(89)
Sustained VT at presentation	87(56)	1(50)	8(53)	2(40)	3(33)	7(50)	11(61)
Cardiac syncope	55(35)	0(0)	7(47)	2(40)	3(33)	5(36)	4(22)
VT storm	26(17)	1(50)	1(7)	1(20)	1(11)	3(21)	1(5)
Age at presentation; mean±SD; years	34±13	41±33	31±12	35±21	33±16	25±9	39±12
Age at symptom onset; mean±SD; years	32±13	41±33	30±12	35±21	31±16	24±9	37±11
Inducibility at EPS[II]	95/106(90)	-	11/12(92)	2/3(67)	7/7(100)	10/11(91)	6/12(50)
Antiarrhythmic use	105(67)	2(100)	7(47)	3(60)	5(55)	13(93)	14(78)
Holter PVC count; median	2464	-	4232	3883	8889	3170	6809
ICD implantation	126(81)	2(100)	14(93)	4(80)	8(89)	13(93)	13(72)
Composite outcome	131(84)	2(100)	12(80)	4(80)	6(67)	12(86)	15(83)
Appropriate ICD intervention (VT/VF)(n)	76	1	6	3	3	9	8
Any VT (NSVT or sust VT/VF)	138(88)	2(100)	12(80)	4(80)	7(78)	13(93)	16(89)
Age at first sustained arrhythmic event; (m±SD); years	34±13	41±33	32±12	36±23	40±16	28±12	39±11
VT ablation	39(25)	1(50)	6(40)	2(40)	3(33)	3(22)	5(28)
LV dysfunction (EF< 55%)	25(16)	0	3(20)	2(40)	5(55)	5(36)	13(72)
Heart failure	13(8)	0	0	0	2(22)	4(28)	6(33)
Cardiac transplant/ death	12(8)	0	0	0	0	3(21)	4(22)

PKP2=Plakophilin-2; *JUP*= Plakoglobin; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; ‡Includes compound heterozygote, homozygote, and digenic patients; *PLN*=Phospholamban; *DSP*= Desmoplakin; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; VT= Ventricular tachycardia; NSVT= Non-sustained VT; VF= Ventricular fibrillation; ICD=Implantable cardioverter-defibrillator; LV=Left ventricle; PVC= Premature Ventricular Complex; EPS= Electrophysiological Study; SD= Standard deviation; II Among those undergoing an EP study. *Datafor the one *TMEM43* proband is presented in supplementary table 9; sust=sustained.

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Supplemental table 8. Clinical characteristics and outcome of the family members (n=321) presenting alive in the study population

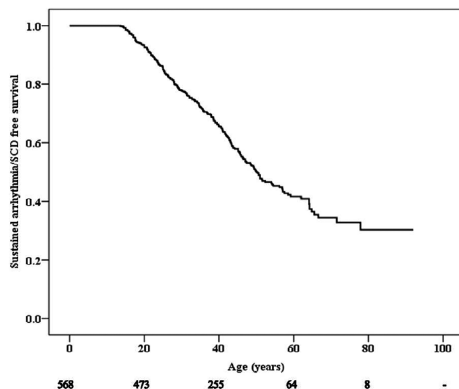
Clinical Variable	*PKP2* (n=278)	*DSG2* (n=15)	*DSC2* (n=3**)**	*DSP* (n=6)	**>1 mutation‡** (n=7)	*PLN***§** (n=12)
Male gender	124(45)	6(40)	1(33)	4(67)	3(43)	5(42)
Definite ARVD/C (at last follow up)	96(34)	1(7)	0(0)	1(17)	4(57)	7(58)
Duration of follow up; m±SD; years	4±5	4±4	0.1±0.1	3±2	2±2	3±2
Symptomatic at presentation	39(14)	1(7)	0	0	1(14)	2(17)
Sustained VT at presentation	13(5)	0	0	0	0	0
Cardiac syncope	9(3)	0	0	0	0	0
VT storm	2(1)	0	0	0	0	0
Age at presentation; mean ±SD; years	37±20	34±22	51±15	35±23	43±12	44±12
Age at symptom onset; mean ±SD; years	36±16	20±1	-	41±13	37	39±15
Inducibility at EPS[II]	12/42(28)	1/2(50)	-	0/2	-	1/3(33)
Antiarrhythmic use	41(15)	0	0	0	1(14)	5(42)
Holter PVC count; median	111	0		9	882	493
ICD implantation	53(19)	1(7)	0	0	1(14)	5(42)
Composite outcome	22(8)	1(7)	0	0	0	1(8)
Appropriate ICD intervention (VT/VF)	9	1	0	0	0	0
Any VT (NSVT or sust VT/VF)	54(19)	1(7)	-	0	2(28)	5(42)
Age at first sustained arrhythmic event; (m±SD); years	42±11	38.5	-	-	-	44
VT ablation	14(5)	0	0	0	0	0
LV dysfunction (EF<55%)	14(5)	1(7)	1(33)	1(17)	1(14)	7(58)
Heart failure	2(1)	0	0	0	0	1(8)
Cardiac transplant/death	2(1)	0	0	0	0	1(8)

PKP2=Plakophilin-2; *DSG2*=Desmoglein-2; *DSC2*=Desmocollin-2; ‡Includes compound heterozygote, homozygote, and digenic patients; *PLN*=Phospholamban; *DSP*= Desmoplakin; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; sust=sustained; VT= Ventricular tachycardia; NSVT= Non-sustained VT; VF= Ventricular fibrillation; ICD=Implantable cardioverter-defibrillator; LV=Left ventricle; PVC= Premature Ventricular Complex; EPS= Electrophysiological Study; SD= Standard deviation; [II] Among those undergoing an EP study.

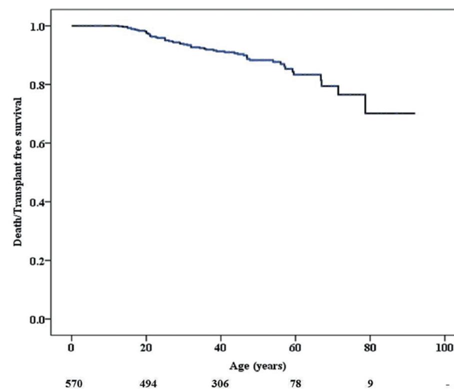
Supplemental table 9. Clinical characteristics and outcome of the *TMEM43* mutation carrier

Clinical Variable	
Male gender	Y
Proband	Y
Definite ARVD/C (at last follow up)	Y
Symptomatic at presentation	Y
Sustained VT at presentation	N
Cardiac syncope	N
VT storm	1
Age at presentation; mean ±SD; years	28.2 years
Inducibility at EPS	Y
Holter PVC count; median	-
ICD implantation	Y
Composite outcome	1(Appropriate ICD intervention)
Age at first sustained arrhythmic event; (m±SD); years	28.7 years
VT ablation	N
LV dysfunction (EF<55%)	N
Heart failure	N
Cardiac transplant/death	N
Duration of follow up; m±SD; years	2.3 years

Y=Yes; N=No; VT= Ventricular tachycardia; VF= Ventricular fibrillation; ICD=Implantable cardioverter-defibrillator; LV=Left ventricle; EPS= Electrophysiological Study; SD= Standard deviation; ARVD/C= Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy



Supplemental Figure 1A. Survival free from sustained ventricular arrhythmia/SCD in the entire study population.



Supplemental Figure 1B. Survival free from death or transplant in the overall study population.

Supplemental references

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Supplemental methods

A. Patients were enrolled at 8 Dutch centers as part of the Interuniversity Cardiology Institute of the Netherlands (ICIN) as well as at the Johns Hopkins ARVD/C registry. All outcome events were predefined prior to initiation of the study and the definition used to classify arrhythmic and heart failure outcomes are detailed in supplementary table 2. Two authors (A.B and J.A.G.) reviewed U.S. and Dutch registry level outcome data respectively to ensure compliance with these definitions. A shared electronic dataset was used to assimilate phenotypic, genotype and outcome data. All genetic data were reviewed by co-authors from both locations, and agreement was reached for each DNA variant, resulting in a single combined opinion. Regularly scheduled teleconferences were used to review data concerns with adjudication by the study senior authors (H.C and R.N.H). There was a shared outcome and phenotypic adjudication protocol that was applied by the ICIN as well the JHU registry for the purpose of this study; however no third party adjudication commitee was involved.

B. In the Dutch part of the dataset, all genetic screening was performed by dideoxy (Sanger) sequencing in diagnostic settings in University Medical Centers (using standard protocols, Bhuiyan et al Circ Cardiovasc Genet. 2009 Oct;2(5):418-27). Next generation sequencing was not performed in these individuals. In the US part of the dataset, genetic testing for some participants was performed by commercial testing in different laboratories according to their respective protocols that in each case included confirmation with dideoxy (Sanger) sequencing. More than one half of the U.S patients had genetic testing in the laboratory of Dr. D.P. Judge (Johns Hopkins University Hospital), this was all performed by dideoxy sequencing.

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CHAPTER 9

The End-Stage of Arrhythmogenic Cardiomyopathy with Severe Involvement of the Interventricular Septum

Heart Rhythm. 2013;10:283-9

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Abstract

Detailed histopathological, immunohistochemical and ultrastructural analysis is described in the heart of a 56-year old woman with end-stage arrhythmogenic cardiomyopathy with a pathogenic plakophilin-2 mutation. The explanted heart revealed severe fibrofatty replacement of nearly the entire right ventricular free wall. Also the left ventricle was severely affected, and most remarkable, there was massive involvement of the interventricular septum. Immunohistochemical and electron microscopy findings of intercalated disks revealed regions with a heterogeneous distribution of Connexin 43 and focal electron microscopic abnormalities amongst these regions. This illustrates again that arrhythmogenic cardiomyopathy is not limited to the right ventricle, but involves the entire myocardium, including the interventricular septum.

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Introduction

Arrhythmogenic Cardiomyopathy (AC), also known as Arrhythmogenic Right Ventricular Dysplasia/ Cardiomyopathy (ARVD/C), can present in 4 clinical stages which not necessarily proceed from one into the other: 1) concealed stage without or with minimal structural disease, although sudden cardiac death may occur, 2) overt stage with structural alterations of primarily the right ventricle (RV), and episodes of monomorphic ventricular tachycardia (VT), 3) overt stage with obvious structural biventricular involvement, and 4) the end-stage of the disease with heart failure.[1-3] Recently, left ventricular dominant variants have been described.[4]

A distinct histopathological feature of AC is fibrofatty replacement of the ventricular myocardium. Cardiomyocytolysis and replacement by fibrous and fatty tissue is a process that progresses from sub-epicardial and mid-myocardial layers to endocardium.[3, 5] These histopathological changes in the RV have been described for the left ventricle (LV) as well.[6] Remarkably, in AC the interventricular septum (IVS) is usually spared from these alterations,[4,5] although it has been reported in up to 20% of AC autopsy patients with biventricular fibrofatty involvement extending to the IVS.[5]

A tentative mechanism for the clinical phenotype and fibrofatty replacement is mechanical and electrical uncoupling of ventricular cardiomyocytes due to desmosomal dysfunction.[7-12] Desmosomes are proteins in the intercalated disk that connect adjacent cardiomyocytes, thereby providing mechanical integrity and electrical stability.[7-12] Alterations in 5 known desmosomal proteins, plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*PKG*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*) have been related to AC.[13-21] Immunohistochemical analysis can be used to visualize the distribution of desmosomal proteins.[13]

Here we present an AC patient with a pathogenic *PKP2* mutation undergoing cardiac transplantation because of progressive heart failure. The explanted heart provided the opportunity to study the end-stage of the AC disease process in great detail. Macroscopically there was extensive damage to the RV, with LV and remarkable severe septal involvement. Histopathological, immunohistochemical, and electron microscopy analyses were performed.

Clinical findings and analysis

A 56-year old woman, since 15 years known with AC, underwent an orthotopic cardiac transplantation because of progressive RV and LV failure. In 1995, at age 41, a first episode of sustained VT was diagnosed. Sotalol treatment was initiated. Coronary angiography showed no abnormalities and LV cine-angiography showed an aneurysm of the LV apex. The following years displayed VT recurrences. Two VT morphologies were recorded: VT 1 had a left bundle branch block (LBBB) morphology, extreme superior axis, and cycle length 320 ms, VT 2 also had LBBB morphology with left superior axis, and cycle length 420 ms (Figure 1A).

She was referred to the Utrecht Cardiac Arrhythmia Unit in 1997 because of suspected AC. Diagnosis of AC was confirmed by the presence of epsilon waves in leads V1-3, negative T waves in all precordial leads (Figure 1B), and LBBB VT episodes with superior axis.

Further analysis showed a dilated RV with poor function and dyskinesia of the RV outflow tract and RV apex on RV cine-angiography. During electrophysiologic study the clinical VT 2 was inducible by programmed electrical stimulation. Body surface mapping, pace mapping, activation

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mapping, and entrainment of the VT confirmed the arrhythmogenic substrate at a right-sided paraseptal infero-basal location. Endocardial radio-frequency ablation was performed and VT 2 was no longer inducible. However, two other VT morphologies were inducible after ablation. Clinical VT 1 was not inducible at all.

Because of recurrent fast VT episodes during sotalol treatment, a cardioverter-defibrillator (ICD) was implanted in 1998. This procedure was complicated by an Addisonian crisis. Chronic treatment of the coincidentally found primary adrenocortical insufficiency significantly improved the patient's general condition.

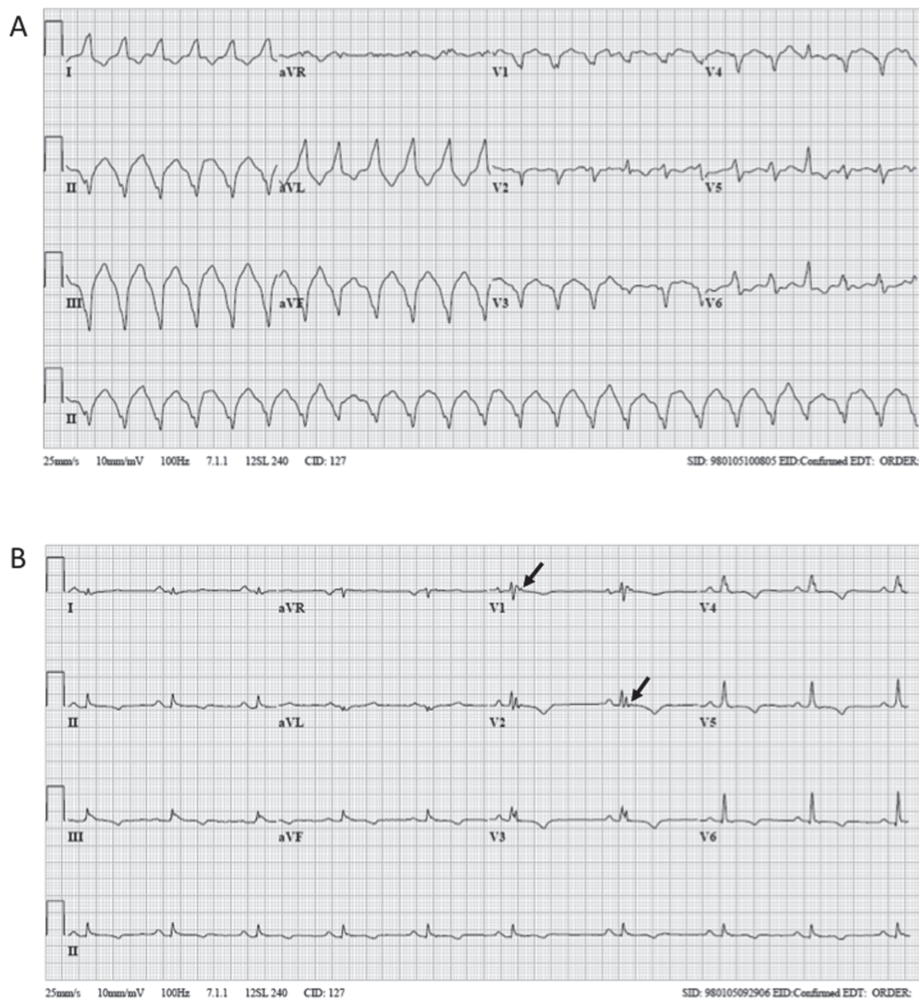

Figure 1A. A 12 lead electrocardiogram of the index patient that shows one of two morphologies of ventricular tachycardia. **B.** Electrocardiogram of the index patient during sinus rhythm, while off drugs, with epsilon waves (indicated by arrows) in leads V1-2 and negative T waves in all precordial leads V1-6.

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Ventricular tachyarrhythmia episodes frequently recurred despite anti-arrhythmic drug treatment. VT recurrences were interrupted by anti-tachycardia pacing and shock therapy from the ICD. In 2005, the heterozygous pathogenic *PKP2* mutation c.2386T>C (p.Cys796Arg) was found by molecular-genetic screening of the desmosomal (*PKP2*, *DSP*, *PKG*, *DSG2*, *DSC2*) and non-desmosomal phospholamban (PLN) and transmembrane protein 43 (*TMEM43*) genes, and family screening was initiated. There were no known arrhythmias or sudden deaths in the family. DNA screening was negative for the *PKP2* mutation in the 3 asymptomatic sons of the patient.
Atrial arrhythmias (atrial fibrillation and flutter) were recorded at age 53. Echocardiography showed an extensively dilated, aneurysmatic RV, significant tricuspid incompetence with volume overload of the RV, LV global and regional wall motion abnormalities with a reduced systolic LV function. An atrial lead was added to the ICD system.
Progression of heart failure and deterioration of the patient's condition started in 2009, at age 55. In 2010 she was admitted in the hospital with backward and forward failure and later with cardiogenic shock. Later on, at age 56, she underwent a successful orthotopic cardiac transplantation. The ICD was explanted. At present, the patient is doing well without any sign of rejection of the cardiac transplant.

Histopathological examination
Gross examination of the explanted heart (695 grams) revealed severe dilatation of the right ventricle with a maximal luminal diameter of 10.5 cm (Figure 2A). Severe fibro-fatty replacement of the free wall of the right ventricle was observed with almost complete absence of viable myocardium. Fibro-fatty replacement was also observed in the wall of the left ventricle, especially in the postero-lateral part. In addition to the free walls of both ventricles, the septum also revealed massive subendocardial fibro-fatty replacement in the anterior and posterior parts. Microscopic examination confirmed the presence of vacuolar degeneration of cardiomyocytes with fibro-fatty replacement in the walls of both ventricles and in the septum (Figure 2B). The areas surrounding those with fibrofatty replacement showed hypertrophic cardiomyocytes with enlarged irregular nuclei and increased number of myofibrils. Very focally a minimal lymphohistiocytic infiltrate around degenerated cardiomyocytes was observed. The coronary arteries revealed concentric intimal fibrosis without significant stenoses.

Immunohistochemical analysis
Formalin-fixed, paraffin-embedded as well as frozen sections were used for immunohistochemical analysis as described previously.[13, 22] N-cadherin, a component of the adherens junction, was used as marker for the intercalated disk. Formalin-fixed, paraffin-embedded material was used for staining of *PKG*, *DSP* and *PKP2* (Figure 3A). As shown in Figure 3A, specific immunoreactive signal for *PKG* was severely reduced both in IVS and LV. Also, signal for *DSP* was markedly reduced. Using an antibody that was able to recognize the C-terminus, the *PKP2* signal was not reduced compared to control (Figure 3A).
Furthermore, again using N-cadherin as the positive control, double labeling on frozen material was performed to visualize localization of the gap junction protein Connexin 43 (Cx43) and to assess potential presence of the non-phosphorylated form of Cx43 (Cx43-NP). In almost the complete LV normal immunoreactive signal for Cx43 was observed at the intercalated disks

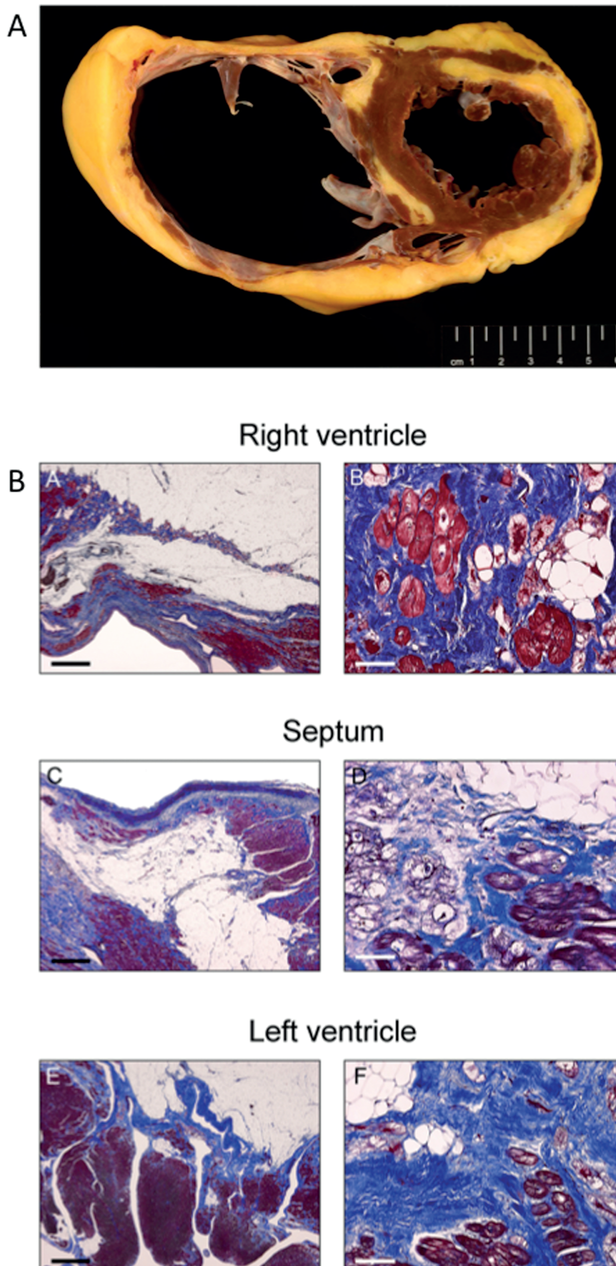

Figure 2A. Gross examination of the heart. Cross section through the ventricles of the explanted heart showing severe dilatation of the right ventricle with fibro-fatty replacement of nearly the entire free wall of the right ventricle (right ventricle indicated by RV). In addition, extensive fibro-fatty alteration in the left ventricle (left ventricle indicated by LV) and the septum. **B.** Microscopic examination of the myocardium (Modified AZAN stain). B1 and B2, right ventricle. B1, overview showing fibro-fatty replacement of the wall. Cardiomyocytes in red, fibrous tissue in blue and lipocytes. B2, higher magnification of the same area showing large cardiomyocytes (in red) with vacuolar degeneration. B3 and B4, fibrofatty replacement of the myocardium in the septum. B5 and B6, fibrofatty replacement of the left ventricular wall. Scale bars in B1, B3, and B5 1mm. Scale bars in B2, B4, and B6 100μm.

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(marked through N-cadherin labelling) with only sporadic some lateralization (Figure 3B, upper panels). In addition, some focal regions within the IVS also displayed normal Cx43 signals, (Figure 3B middle panels). However, heterogeneously diminished Cx43 signal at cell-cell junctions was found in the larger part of the IVS (Figure 3B bottom panels). These observations stress the heterogeneous character of lost Cx43 immunoreactive signal. Double labelling of specimen

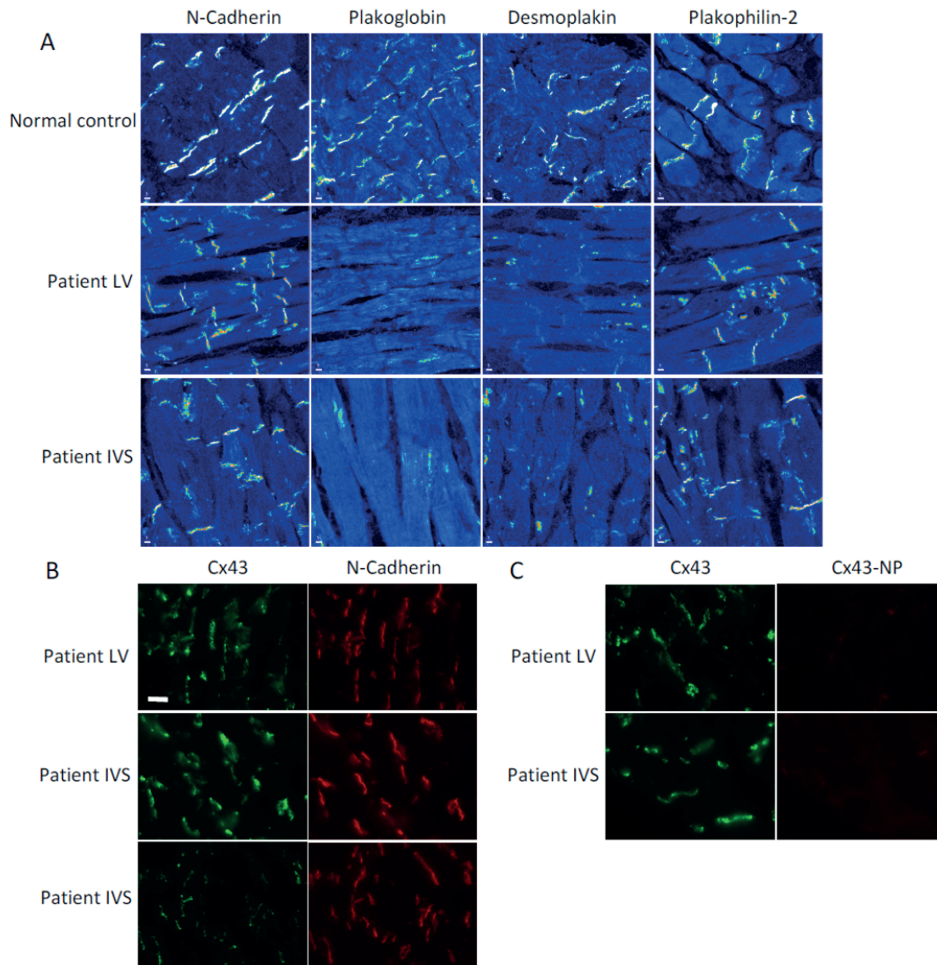

Figure 3A. Immunohistochemical stainings for N-cadherin, plakoglobin, desmoplakin and plakophilin-2 of control, and left ventricle (LV) and interventricular septum (IVS) in the patient. N-cadherin and PKP2 are comparable between normal control, patient-LV and patient-IVS. PKG and DSP are severely reduced in the patient. Scale bars 5μm. **B.** Immunohistochemical analysis of N-Cadherin/Cx43) in specimen derived from the patients' LV and IVS revealed normal signals of Cx43 and a strong overlap with N-cadherin in the LV (upper panels) and in focal regions in the IVS (middle panels) while the larger part of the IVS showed disturbed signals for Cx43 (lower panels). Scale bar 25 μm. **C.** Double labelling with antibodies raised against total Cx43 and the non-phosporylated form of Cx43 (Cx43-NP) revealed that both in the LV sample (upper panels) and the IVS sample (lower panels), dephosphorylated Cx43 (Cx43-NP) is not present.

taken from the LV (Figure 3C, upper panels) and IVS (Figure 3C lower panels) with antibodies against total Cx43 and the nonphosphorylated Cx43 revealed that at sites where Cx43 signals were normal, Cx43-NP was absent (Figure 3C) which suggests a normal intercellular coupling.[23-25] Immunohistochemistry was not performed on the RV, since the wall of the RV showed severe fibrofatty replacement with only a few small areas with intact architecture of the myocardium.

Ultrastructural analysis

Electron microscopic evaluation of the left ventricular and septal myocardium showed focal myofibrillar lysis, T- tubule dilatation and mitochondrial clustering. No alteration in the mitochondrial or nuclear structure was observed. Lipid droplets were rarely present in the myocardial cells. Longitudinal section of cardiomyocytes showed large amounts of dark lipofuscin granules located at the poles of nucleus and among the mitochondria. Abundant collagen fibers interspersed with cardiomyocytes, fibroblasts and inflammatory cells in the interstitial space were noted.

As far as the cell-cell junctions are concerned, highly convoluted intercalated disks and various degrees of intercellular space widening were found in both the LV and the interventricular septum (Figure 4A). Myofibril lysis at their points of attachment to the intercalated disk was also observed. Gap junctions appeared structurally normal in the samples analyzed, without any evidence of lateral localization at the membranes of cardiomyocytes. Proper alignment of sarcomeres, Z-lines, and myofilaments was observed in most cardiomyocytes, although focal accumulation of electron dense material at the Z-lines was observed (Figure 4B).

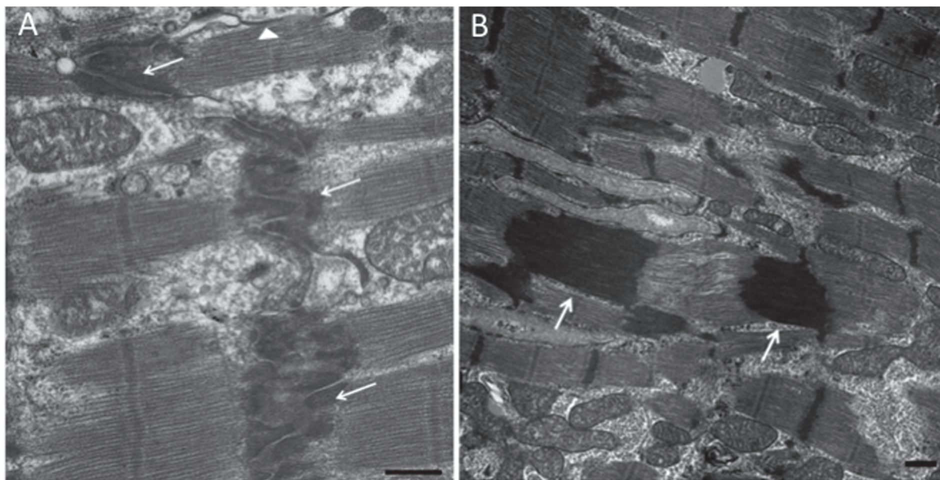

Figure 4. Electron microscopy findings of ventricular cardiomyocytes from the explanted heart
A. Intercalated disks in this sample show focal widening of the intercellular space at the level of the desmosome/fascia adherens (arrows), while the remaining spaces appear normal; gap junctions show a preserved structure (arrow head). Scale bar 500 nm. **B.** Enlarged Z-lines of cardiomyocyte sarcomeres with focal accumulation of electron dense material (arrows) as compared to normal Z lines of other sarcomeres (arrows heads). Scale bar 500 nm.

Discussion

We present a case of severe AC with a pathogenic mutation in the *PKP2* gene, leading to the necessity for a heart transplant. This opened the way to detailed histological, immunohistochemical and ultrastructural analyses on the explanted heart to study the end-stage of AC.

After the concealed phase, the overt stage of the disease developed in which the diagnosis was made based on depolarization and repolarization abnormalities on ECG, left bundle branch block morphology VT, and structural abnormalities of the RV. Gradually, increasing biventricular structural and functional abnormalities were observed, resulting in the end-stage: heart failure and ultimately cardiac transplantation. In addition to ventricular arrhythmias, atrial arrhythmias (atrial fibrillation and flutter) were also recorded in the end-stage, reflecting the atrial stretch and hemodynamic alteration due to reduced RV and LV function, and are often observed in heart failure patients.

Histopathologically, fibrofatty infiltration was found throughout the entire heart. Most severely affected was the RV, with hardly any preservation of viable working myocardium. Furthermore, also the LV free wall showed extensive regions of fibrofatty replacement and with a remarkable severity, regions within the interventricular septum were affected.

Fibrosis or fatty infiltration of the interventricular septum has been previously described in AC, although at the microscopic histopathological level in endomyocardial biopsies or in imaging studies with magnetic resonance imaging with delayed enhancement analysis and never on the extensive macroscopic scale of the patient in the present study. On the contrary, previous studies in large cohorts of AC heart specimens suggested that the septum is usually spared from alterations.[4, 5]

Septal fibrosis is nevertheless frequently observed in cardiac sarcoidosis, and cases of sarcoid myocarditis clinically mimicking AC have been reported.[26-29] However, in this patient there were no clinical signs of systemic sarcoidosis and no sarcoid granulomas were observed with histological analysis of the explanted heart. Moreover, a pathogenic *PKP2* mutation related to AC was identified.

Although this patient specific *PKP2* mutation (c.2386T>C, p.Cys796Arg) is a missense mutation, general consensus is that it is pathogenic. Both *in silico* prediction algorithms SIFT and PolyPhen2 predict a deleterious effect of the amino acid substitution. Furthermore, the mutation has been found in 11 different Dutch families with AC: 11 probands and in addition 11/26 family members carrying solely this mutation fulfilled the revised 2010 Task Force Criteria for AC diagnosis. Even more important, biventricular involvement was identified in 6/37 mutation carriers. A founder effect of the c.2386T>C mutation has been suggested based on haplotype analysis.[15] Finally, in the patient no mutations were identifiable in the other desmosomal (*DSP*, *PKG*, *DSG2*, *DSC2*) and in the non-desmosomal *PLN* and *TMEM43* genes. However, considering the severity of the AC phenotype with necessity for a cardiac transplantation, contribution of other yet unknown/unidentified genetic and/or environmental factors can not be ignored.

In agreement with the previously reported frequent down-regulation of *PKG* in AC[13], severely reduced signal for *PKG* was found with immunohistochemical analysis in regions *without* apparent fibrofatty infiltration, both in LV and IVS. In addition, down-regulation of *DSP* was observed. Normal staining patterns of the adherens junction protein N-cadherin and of the

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desmosomal protein *PKP2* were found, despite the pathogenic *PKP2* mutation. Noteworthy, the staining pattern of the gap junction protein Cx43 was heterogeneous with focal abnormalities, particularly in the IVS.

The remarkable finding of normal LV and heterogeneously down-regulated IVS immunoreactive signal for Cx43 in end-stage is in agreement with the report of Christensen *et al*. who also described severe cases of AC without gap junction remodeling.[30] However, gap junction remodeling has been described in all types of structural heart disease, and in AC even in regions without histopathological alteration.[7, 21] Heterogeneous expression of Cx43 could be highly arrhythmogenic due to dispersion of impulse propagation and conduction velocity. Focal abnormalities, on the molecular level with Cx43 expression but also microscopically with fibrofatty replacement and even macroscopically with wall motion abnormalities, are a hallmark of AC. The exact mechanism for this heterogeneity remains to be elucidated.

The immunostaining of *PKP2* with comparable signal intensities and distribution leads to the important suggestion that apparently both the wildtype protein (from the unaffected allele) and the mutant protein are expressed and localize simultaneously to the cell-cell junctions. This also stands in stark contrast to phenotypes seen in true haploinsufficient patients or in genetically engineered mouse models when there is either heterozygous or homozygous knock out (ie, loss of expression of the wildtype protein). To speculate, this observation suggests that heterozygous expression of the mutant protein is sufficient to cause severe disease and argues strongly for a dominant negative effect, much like that seen in hypertrophic cardiomyopathy.

In the left ventricular free wall and septum we observed focal involvement of myocardium with severe fibrofatty replacement in some regions, whereas other regions were not affected. On the ultrastructural level the intercalated disks of both the LV and septum revealed a heterogeneous appearance with a measured intercellular gap range that was partly close to the normal range and partly revealed extensively widened gaps. In all samples analyzed with electron microscopy, there was evidence for replacement-like fibrosis (i.e. collagen fibers and inflammatory cells in the interstitium).

The mysterious finding of accumulation of electron dense material within the sarcomere reminds of a finding previously reported in boxer dogs suffering from AC.[31] Noteworthy, the presence of electron dense material, corresponding to aggregation of alpha-actinin within sarcomeres, is a hallmark of nemaline myopathy, a disease due to mutations in genes encoding for actin filament proteins and affecting both skeletal and heart muscles.[32, 33] The patient in this report never experienced muscle weakness and creatin phosphokinase levels were within normal range. Future studies are needed to evaluate the origin and meaning of the electron dense material at the Z-lines in AC.

Conclusion

In conclusion, this presentation of the end-stage disease of a *PKP2* mutation associated AC shows that in addition to the right ventricular myocardium and the left ventricular free wall, the septal myocardium can be severely affected as well, not only on the microscopic but moreover on the macroscopic level.

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CHAPTER 10

Beyond the Revised 2010 Task Force Criteria for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy: Electrical and Structural Alteration in the Subtricuspid Area

Submitted

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Abstract

Introduction

ECG criteria for electrical abnormalities in predominantly the right ventricular outflow tract (RVOT) have been included in the revised 2010 Task Force Criteria (TFC) for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C). However, the RVOT is mostly affected in overt ARVD/C, whereas substricuspid involvement starts in the early disease stage.

Methods and results

The findings of electrophysiological studies (EPS, programmed electrical stimulation, endocardial activation/voltage mapping) of 19 ARVD/C patients were related to structural abnormalities (wall motion abnormalities/late gadolinium enhancement) and ventricular tachycardia (VT) morphology. Electrical abnormalities with EPS were defined as local activation duration ≥80 ms, late potentials, or low voltages (≤1.5 mV). Electrical and structural abnormalities were related to the 12-lead ECG during sinus rhythm. All patients had electrical abnormalities and 74% (14/19) had structural abnormalities in the subtricuspid area. This was also the origin of the predominant VT morphology. RVOT electrical abnormalities were found in 18 patients (95%), with structural abnormalities identified in 15 (79%). Electrical abnormalities were found more often but correlated well with structural abnormalities. Three ECG parameters possibly reflecting abnormalities in the subtricuspid area were assessed: 1) late isolated potentials in inferior leads (42%), 2) negative T waves in inferior leads (26%), and 3) left axis deviation (32%).

Conclusion

Abnormal electrical involvement of the subtricuspid area was observed in all 19 ARVD/C patients. ECG criteria reflecting abnormalities in this area combined with the 2010 Task Force Criteria for ARVD/C may increase the diagnostic yield.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C), also referred to as Arrhythmogenic Cardiomyopathy (AC), is a hereditary cardiomyopathy characterized by increased risk of ventricular arrhythmias (VA) and sudden cardiac death (SCD), and right ventricular (RV) and/or left ventricular (LV) structural and functional abnormalities.[1-3] The substrate for VA in ARVD/C is proposed to be remodeling of the intercalated disk, i.e. down-regulation and/or redistribution of desmosomal, gap junction, and sodium channel proteins, followed by altered tissue architecture due to fibrofatty replacement, both resulting in activation delay, the hallmark of the disease.[4-9]

The arrhythmogenic substrate in ARVD/C can be evaluated invasively by electrophysiological study (EPS), including programmed electrical stimulation (PES) and activation and voltage mapping. Activation mapping can identify areas with prolonged RV endocardial activation duration as a measure of local activation delay.[9] Areas of scar (fibrofatty replacement) can be identified with cardiac magnetic resonance imaging (CMR) with late gadolinium enhancement (LGE) analysis, and with more accuracy, with voltage mapping.[10,11, 12] However, the risk for VA and SCD in ARVD/C already exists in the absence of overt structural involvement at a young age in the early asymptomatic stage of disease, and would therefore preferably be identified non-invasively. Non-invasive detection of activation delay in ARVD/C has been shown by electrocardiogram (ECG) recording of epsilon waves and prolonged terminal activation duration (TAD) in right precordial leads and by abnormal features of the signal averaged ECG.[13-15] These ECG markers have been incorporated in the revised 2010 Task Force Criteria (TFC) for ARVD/C diagnosis.[14, 16] Nonetheless, these parameters predominantly reflect activation delay and thus an arrhythmogenic substrate in the RV outflow tract (RVOT). However, these markers obtained during sinus rhythm do not explain the frequent recording of a superior axis during VA. Recently, our group demonstrated early preferential involvement of the subtricuspid region in desmosomal ARVD/C, with RVOT involvement at a later stage.[17] ECG parameters reflecting early electrical abnormalities in the subtricuspid area have not been reported so far.

In this study, we describe the findings of PES and RV endocardial activation and voltage mapping in 19 definite ARVD/C patients and related those to CMR findings and ventricular tachycardia (VT) morphology. Second, we aimed to translate invasive measurements of RV activation delay and electroanatomical scar, particularly in the subtricuspid area, to surface ECG parameters.

Methods

Study population and clinical evaluation

Nineteen definite ARVD/C patients fulfilling the 2010 TFC and invasively analyzed by PES, RV endocardial activation and voltage mapping, and RV and LV cine-angiography as part of the diagnostic process were included in the study (11 index patients and 8 family members). The non-invasive assessments, performed for diagnostic purposes, which were analyzed in this study included a detailed clinical and family history, physical examination, 12-lead ECG (while off medications), 24 hours Holter monitoring, exercise testing, and CMR with wall motion and

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LGE analysis. Comprehensive CMR analysis was performed according to a previously described protocol.[18] The CMR results of 6 patients included in this study were reported in a prior study from our group.[17] Molecular-genetic screening of desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), desmocollin-2 (*DSC2*), and non-desmosomal genes transmembrane protein 43 (*TMEM43*) and phospholamban (*PLN*), was performed in index patients.[19-25] Family members were solely screened for pathogenic mutations identified in their respective index patient. Morphology of all spontaneously occurring or exercise-induced ventricular tachycardia (VT) episodes with 12-lead ECG recording were carefully studied on type of morphology and QRS axis in the vertical plane (see also invasive analysis). All study patients consented to clinical and genetic screening.

Invasive analysis

RV and LV cine-angiography for additional wall motion analysis was performed in 4 projections (2 simultaneously obtained); right (30 degrees) and left (60 degrees) anterior oblique and anterior-posterior and lateral views (Siemens Axiom Artis). Contrast was infused into the RV cavity (40-50 ml per take, flow rate 12-15 ml/sec). To evaluate LV abnormalities, images were recorded until the contrast had passed the LV.

Electrophysiological studies (discontinuation of medications for at least 5 half-lives) were recorded and analyzed with the Prucka system (GE Medical). Up to 3 ventricular premature stimuli after 8 cycles regular pacing at cycle lengths 600ms and 430ms, respectively, were delivered from 2 different sites (RV apex and RVOT). In addition, we used more aggressive stimulation by 1) regular pacing followed by a pause (750ms) and up to 2 extrastimuli , 2) isoproterenol infusion (maximum 4 mcg/min, dependent on the heart rate response, defined as appropriate when a) 60% heart rate increase from baseline, or b) rate >150 per minute) combined with PES with up to 2 extrastimuli, and 3) burst stimulation, if sustained VA were not induced with less aggressive stimulation. Twelve lead ECG recording of all induced VT morphologies was obtained. VT was defined as at least 3 subsequent ectopic ventricular complexes at a rate above 100/minute. A sustained VT episode was defined as an episode with a duration of 30 seconds or longer, or shorter than 30 seconds due to electrical or pharmacological interruption because of hemodynamic instability. VT episodes lasting less than 30 seconds were defined as non-sustained. The morphology of the ventricular complex during VT in the surface ECG (left or right bundle branch block morphology) and the direction of the vector of electrical activation was used to estimate the site of VT origin.

The arrhythmogenic substrate and distribution of scar was assessed during sinus rhythm by RV endocardial activation and voltage mapping using the Localisa mapping system (Medtronic, Minneapolis, Minnesota, USA) and Celsius mapping catheter (Biosense Webster Inc., Diamond Bar, California, USA) (3.5 mm distal tip electrode, 2 mm ring electrode, interelectrode distance 2 mm). Bipolar RV endocardial electrograms (filtered at (Hp) 30- (Lp) 500 Hz) were analyzed with regard to amplitude, local activation duration, and relation to the surface QRS complex. Simultaneously recorded unipolar RV endocardial electrograms (filtered at (Hp) 0.5- (Lp) 500 Hz) were used to support the identification and accurate localization of local endocardial signals. Activation delay was defined as 1) a local activation duration ≥80 ms measured in a bipolar recording from the earliest to the latest activation deflection, and/or 2) local activation signals

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after the QRS complex had ended (late potentials).[10] Areas of abnormal tissue due to at least partly fibrofatty alteration were defined as low endocardial voltages: voltages ≤1.5 mV in at least 3 adjacent sites with similar endocardial activation morphology.[10] Four areas of RV electrical and structural substrate were defined: the subtricuspid area, RVOT, RV septum, and RV apex. The LV was not subdivided and substrate assessment was in the absence of endocardial mapping limited to functional/structural analysis by cine-angiography and CMR. RV and LV wall motion abnormalities (akinesia, dyskinesia) were counted if such an abnormality was detected by either cine-angiography or CMR, or with both techniques.

Statistical analysis

Continuous variables are summarized as either mean±SD or median (interquartile range, range) where appropriate. Categorical variables were presented as numbers (percentages) and compared using the chi-square, Fisher's exact, or chi-square goodness-of-fit test. All analyses were performed with PASW Statistics 20.0 software (SPSS, Chicago, IL, USA).

Results

Summarized in Table 1 and supplemental Table 1 are the baseline characteristics, ARVD/C diagnostic criteria and the genotype of the 19 study patients who underwent invasive and non-invasive diagnostic analysis.

Spontaneous ventricular arrhythmias

In 17 of the 19 patients (89%) spontaneous episodes of ventricular tachycardia (VT) were recorded (sustained VT in 8, non-sustained VT in 9). Of these 17 patients, 8 (47%) had multiple VT morphologies. The predominant VT morphology had left bundle branch block (LBBB) configuration with superior axis (-30 to -90 degrees, in 12/17, 71%). LBBB VT with inferior axis (+60 to +120 degrees) was recorded in 9/17 (53%) patients. VT with right bundle branch block (RBBB) configuration was recorded in 3/17 (18%) patients. The VT morphology and axis could not be determined from a 12-lead recording in 3/17 (18%) patients. Figure 1 shows a typical sustained VT with LBBB morphology and superior axis recorded in a 38-year old male ARVD/C patient (patient 5, see Table 2) with a plakophilin-2 mutation. Of the 2 patients without recorded spontaneous VT episodes, one had a prolonged syncope suspicious for VA and the other had frequent ventricular ectopy with nearly 8000 ventricular extrasystoles per 24 hours on Holter monitoring.

Programmed electrical stimulation

In 15 of the 19 (79%) study patients a VT episode (cycle lengths ranging from 210-310ms) was induced with PES (sustained in 8 and non-sustained in 7). In 5 of the 15 (33%) patients, episodes of VT with multiple morphologies were inducible. Predominant induced VT morphology also had LBBB morphology with superior axis (in 10/15, 67%), see also Figure 2. LBBB VT with inferior axis was induced in 8/15 (53%) patients. VT with RBBB morphology was induced in 1/15 (7%) patients. Polymorphic non-sustained VT was recorded in 2 (13%) patients. Two patients with spontaneous sustained and 2 with non-sustained VT episodes appeared non-inducible with PES.

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Table 1. Baseline characteristics and revised 2010 Task Force Criteria for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy of 19 study patients

Patient characteristics	**Number of patients (%)**
Men	10 (53)
Age at presentation (mean±SD, range)	42±16 (16-70)
Age at EPS (mean±SD, range)	47±17 (16-75)
2010 TFC	
Structural abnormalities	
RV struct./funct. abnormalities major	16 (84)
RV struct./funct. abnormalities minor	1 (5)
Depolarization abnormalities	
Epsilon wave (major)	1 (5)
Prolonged TAD (minor)	11 (58)
Late potentials (minor)	14 (74)
Repolarization abnormalities	
Negative T waves in V1-3 (major)	11 (58)
Negative T waves V1-2 (minor)	3 (16)
Negative T waves V1-4 with RBBB (minor)	1 (5)
Negative T waves in V4-6 (minor)	1 (5)
Arrhythmias	
Total (spontaneous and/or PES induced)	
LBBB VT with superior axis (major)	14 (74)
Total (spontaneous and/or PES induced)	10 (53)
LBBB VT with inferior axis (minor)	12 (63)
>500 VES/24 hours (minor)	
Family history	
Pathogenic mutation (major)	15 (79)
First degree relative with ARVD/C (major)	8 (42)
Autopsy diagnosis in first degree relative (major)	1 (5)
SCD with ARVD/C as probable cause <35 years old (minor)	1 (5)

SD indicates standard deviation, EPS: electrophysiologic study, PES: programmed electrophysiologic stimulation, TFC: Task Force Ciriteria, RV: right ventricular, struct./funct.: structural and functional abnormalities, TAD: terminal activation duration, RBBB: right bundle branch block, LBBB: left bundle branch block, VT: ventricular tachycardia, VES: ventricular extrasystoles, ARVD/C: arrhythmogenic right ventricular dysplasia/cardiomyopathy, SCD: sudden cardiac death.

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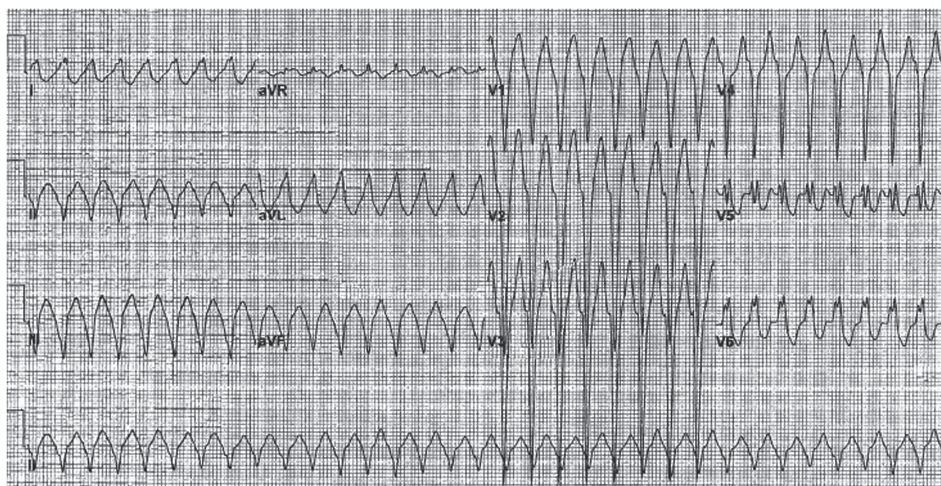

Figure 1. Spontaneous sustained ventricular tachycardia (VT) with left bundle branch block morphology and typical superior axis of -60 degrees recorded in a 38-year old male Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy patient (patient 5, see Table 2). This was the predominant VT morphology observed in our cohort and suggests an origin in the subtricuspid/inferior part of the right ventricle.

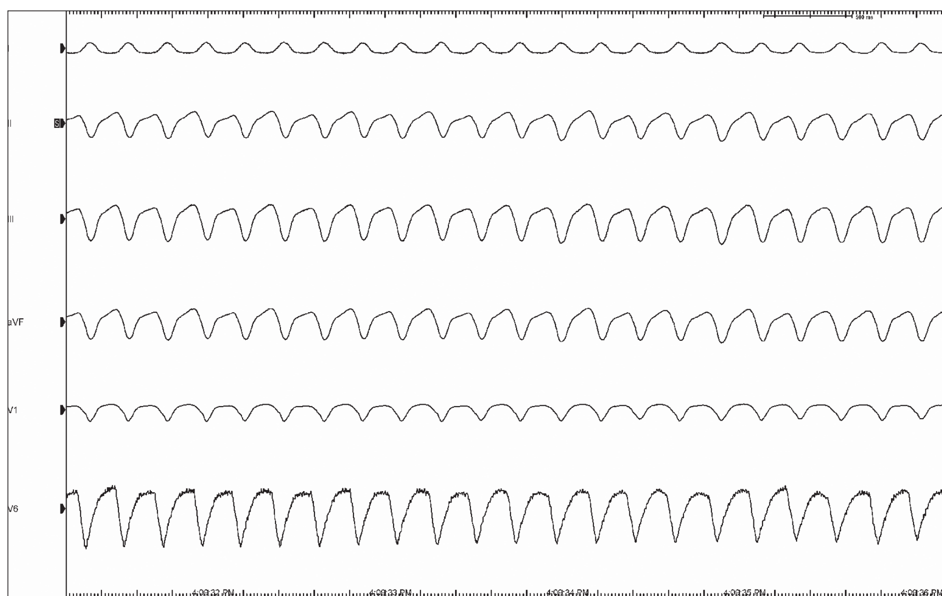

Figure 2. Ventricular tachycardia (shown at 50 mm/sec) with left bundle branch block morphology, superior axis, and a cycle length of 220 ms induced with pacing at cycle length 430 ms and 2 extrastimuli (S1S2 240 ms and S2S3 180ms) at the right ventricular apex in a 62-year old female Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) patient (patient 1, see Table 2) with a plakophilin-2 mutation (c.235C>T, p.Arg79*).

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Table 1. Baseline characteristics and revised 2010 Task Force Criteria for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy of 19 study patients

Region	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12	Pt 13	Pt 14	Pt 15	Pt 16	Pt 17	Pt 18	Pt 19
Subtricuspid area																			
VT sup axis	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-*	-#	+	-#
Mapping																			
Late pot	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	-
Act delay	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+
Low volt	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CMR/Cine-angio																			
WMA	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	-
LGE	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-
ECG																			
Isolated pot	+	-	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-
Neg T II	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-
Neg T III	-	+	-	-	+	+	-	-	-	-	+	+	-	+	+	-	+	-	-
Neg T aVF	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-
Left ax dev	-	+	+	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-
RVOT area																			
VT inf axis	+	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-*	-#	+	-#
Mapping																			
Late pot	+	-	+	+	+	+	-	+	-	+	-	-	+	-	+	+	+	+	-
Act delay	+	-	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	-
Low volt	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
CMR/Cine-angio																			
WMA	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+
LGE	+	-	+	-	+	+	-	+	+	-	+	+	+	-	-	-	-	-	-
ECG																			
Eps wave	-	-	+	RBBB	-	-	-	RBBB	-	-	-	-	-	-	-	-	-	-	-
TAD (ms)	60	80	90	RBBB	80	60	50	RBBB	70	70	50	60	50	60	40	40	40	50	60
Neg T V1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neg T V2	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
Neg T V3	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-
RV septum																			
Mapping																			
Late pot	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
Act delay	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-
Low volt	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
CMR/Cine-angio																			
WMA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LGE	-	-	-	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-

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Table 1. Continued

Region	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12	Pt 13	Pt 14	Pt 15	Pt 16	Pt 17	Pt 18	Pt 19
RV Apex																			
Mapping																			
Late pot	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Act delay	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Low volt	+	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-
CMR/Cine-angio																			
WMA	-	-	-	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-
LGE	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LV																			
RBBB VT	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CMR/Cine-angio																			
WMA	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LGE	+	na	+	na	+	+	+	+	-	+	-	na	na	+	-	+	+	-	-
ECG																			
Neg T V4	-	+	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-
Neg T V5	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	+	-	-
Neg T V6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-

Pt indicates patient, VT sup axis: ventricular tachycardia with superior axis, late pot: late potentials (after the surface QRS has ended), act delay: activation delay (local activation signal ≥80 ms), low volt: low voltages (voltages ≤1.5 mV), CMR: cardiac magnetic resonance imaging, WMA: wall motion abnormalities (a/dyskinesia) detected by cardiac magnetic resonance imaging or cine-angiography, LGE: late gadolinium enhancement detected by cardiac magnetic resonance imaging, isolated pot: isolated potentials, neg T: negative T waves, left ax dev: left axis deviation, VT inf axis: ventricular tachycardia with inferior axis, eps wave: epsilon waves, TAD: terminal activation duration (prolonged when ≥55 ms), RBBB VT: ventricular tachycardia with right bundle branch block configuration, na: not applicable, *: ventricular tachycardia recorded on Holter monitoring therefore the morphology was undetermined, #: polymorphic ventricular tachycardia.

Endocardial mapping and correlation with functional/structural and ECG abnormalities

Summarized in Table 2 (categorized per substrate area) are the individual findings at endocardial mapping during sinus rhythm for the 19 study patients, correlated to VT morphology and structural and ECG abnormalities. With invasive assessment of local activation duration and late potentials as signs of activation delay, abnormalities were found in nearly all 19 patients: 18 (95%) patients had prolongation of local activation signal of ≥80 ms and 15 (79%) had late potentials in one or more regions of the RV. All 19 patients had areas of abnormal tissue and scar as measured by low endocardial voltages (≤1.5 mV). One (5%) patient solely had regional abnormalities in the subtricuspid area, 6 (32%) patients showed involvement of 2 regions, and 12 (63%) patients had abnormalities in 3 or more RV regions.

Subtricuspid area

In all 19 patients, overt electrical abnormalities (presence of activation delay, late potentials, and/or low voltages) were found in the subtricuspid area. Among these 19 patients, 14 had corresponding LBBB VT with superior axis (p=0.039). Two patients had only LBBB VT with

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inferior axis, 2 had polymorphic non-sustained VT, and 1 had non-sustained VT of unknown morphology. In addition, 14 of the 19 (74%) patients with subtricuspid electrical abnormalities also had functional/structural abnormalities (akinesia, dyskinesia, and/or delayed enhancement) in this region (p=0.039). Notably, 4 of the 5 (80%) patients without structural abnormalities in the subtricuspid area had neither spontaneous nor induced *sustained* VT, whereas only 4 of 14 (29%) patients with electrical and structural involvement of this area had no *sustained* arrhythmias (p=0.111). Figure 3 shows activation delay, late potentials, and low voltages in the subtricuspid area in the same patient (patient 1, see Table 2) as shown in Figure 2, with corresponding LBBB VT with superior axis. Moreover, CMR analysis demonstrated wall motion abnormalities (dyskinesia) in the same region (Figure 4).

Right ventricular outflow tract area

The RVOT and adjacent area was second most frequently affected in our cohort, with electrical abnormalities found in 18 (95%) patients. Among these 18 patients, 9 had LBBB VT with inferior axis and 9 did not, including the 2 patients with polymorphic non-sustained VT and the one with non-sustained VT of unknown morphology. In addition, 15 of the 18 (83%) patients with electrical

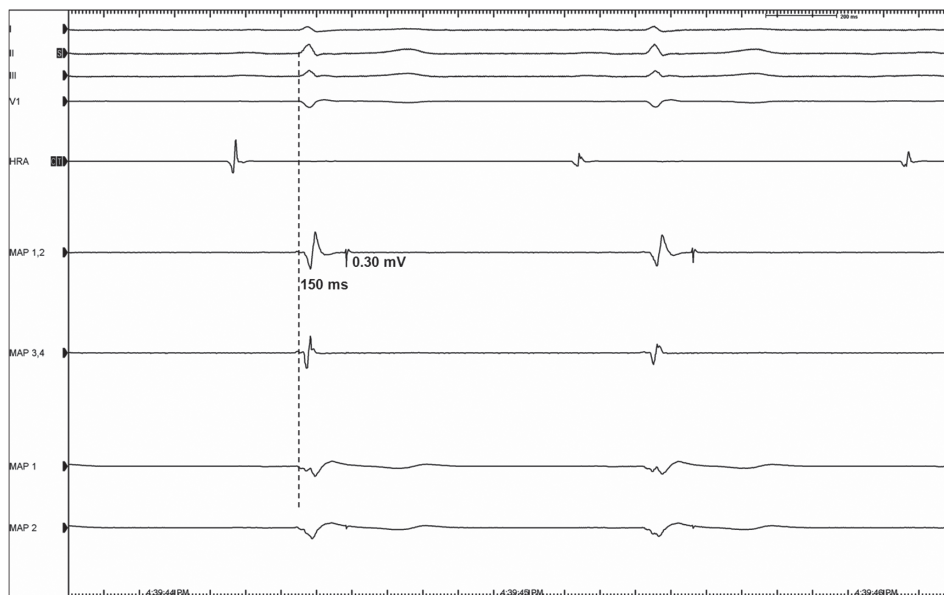

Figure 3. Recordings (paper speed 100 mm/sec) during the electrophyiological study in the same patient as shown in Figure 2 (patient 1, see Table 2). Shown is sinus rhythm with electrocardiographic leads I, II, III, and V1, bipolar recordings HRA (high right atrium), MAP1,2 , and MAP3,4 from the subtricuspid area and corresponding unipolar recordings MAP1 and MAP2 from distal and proximal electrodes, respectively. In MAP1,2 a late, high dV/dt signal with low voltage (0.30 mV) is visible at 150 ms after QRS onset (indicated by the vertical line) and 60 ms after the end of the QRS complex. This signal is also recorded exclusively in MAP2. The local genesis of the low dV/dt signal in MAP1,2 maybe questioned, remote activity cannot be excluded.

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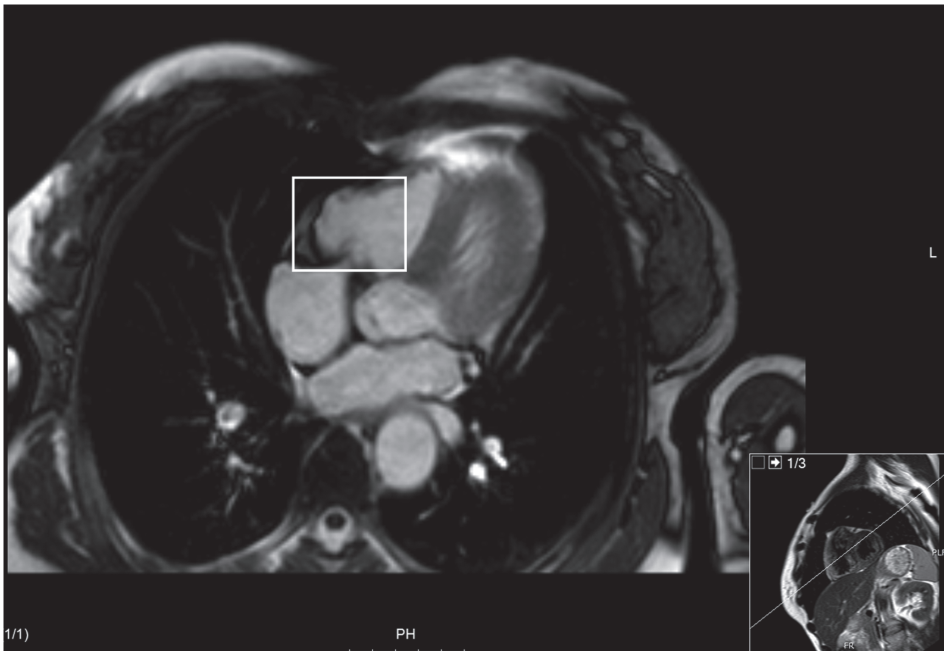


Figure 4. Cardiac magnetic resonance imaging of the patient of whom data are depicted in Figures 2 and 3 (patient 1, see Table 2), showing a dyskinetic wall motion abnormality in the subtricuspid area (indicated by the white frame). These abnormalities correlate well with the induced ventricular tachycardia morphology (Figure 2) and electrical abnormalities found inferior of the tricuspid valve (Figure 3).

abnormalities in the RVOT area also had functional/structural abnormalities in this region and 3 patients did not (p=0.005). These 3 patients did have electrical abnormalities in one or more RV regions, only minimal structural RV involvement (1 patient with subtricuspid structural abnormalities), and none had any spontaneous or induced sustained arrhythmia. Figure 5 displays prolonged activation duration with late potentials and local scar in the RVOT of the same patient as in Figure 1. This patient also had sustained LBBB VT with inferior axis, probably originating from the RVOT, corresponding to the arrhythmogenic substrate identified locally.

Translation to the ECG

The 12-lead ECG at the time of invasive analysis of the 19 study patients was assessed for signs of and correlation with electrical and functional/structural alterations found with invasive analysis (see Table 2). Three parameters that possibly reflect abnormalities in the subtricuspid area were assessed. The first parameter, late isolated potentials prolonging the total depolarization duration, was found in 8 (42%) patients. The most illustrative case is shown in Figure 6, displaying the ECG of the same patient as in Figures 2, 3, and 4 with sustained VT with multiple morphologies, electrical abnormalities in the subtricuspid, RVOT, and apical regions and functional/structural involvement in the first 2 regions. The ECG is remarkably normal compared to the extent of the electrical and structural alteration. Nonetheless, there are late isolated potentials shown in inferior leads II, III, and aVF, indicating the arrhythmogenic substrate. The second parameter, negative

T waves in the inferior leads II, III, and aVF (at least in 2 out of these 3 leads), was found in 5/19 (26%). Shown in Figure 7 is the ECG of the same patient as shown in Figures 1 and 5 with multiple LBBB VT morphologies with both superior and inferior axis and overt electrical and structural abnormalities in the subtricuspid, RVOT, and apical regions. The ECG shows correlation with negative T waves in inferior leads II, III, aVF, right precordial leads V1-4, and a prolonged TAD in V1-2. The third parameter, left axis deviation, was observed in 6/19 (32%) patients. Shown in Figure 8 is the ECG of a 16-year old patient (patient 7, see Table 2) with sustained LBBB VT with superior and inferior axis, electrical abnormalities in the subtricuspid and RVOT area, and functional/structural abnormalities in these regions and the RV apex. These abnormalities correlated to the left axis deviation, remarkable but non-specific fractionation in III and aVF, and negative T waves in V1-4.

Negative T waves in V1-3 on the 12-lead ECG as a sign of predominantly RVOT involvement were identified in 16 (84%) patients, including patient 2 with solely electrical and structural abnormalities in the subtricuspid area with EPS and CMR/cine-angiography. Prolongation of TAD in right precordial leads was observed in 10 of 17 (59%) patients (2 patients had complete RBBB and were, in accordance with the 2010 TFC, excluded from TAD assessment) of whom all but one had electrical and structural abnormalities in the RVOT. Furthermore, one patient with sustained VT with multiple morphologies, electrical and overt structural abnormalities in the subtricuspid and RVOT area, had epsilon waves in V1-3.

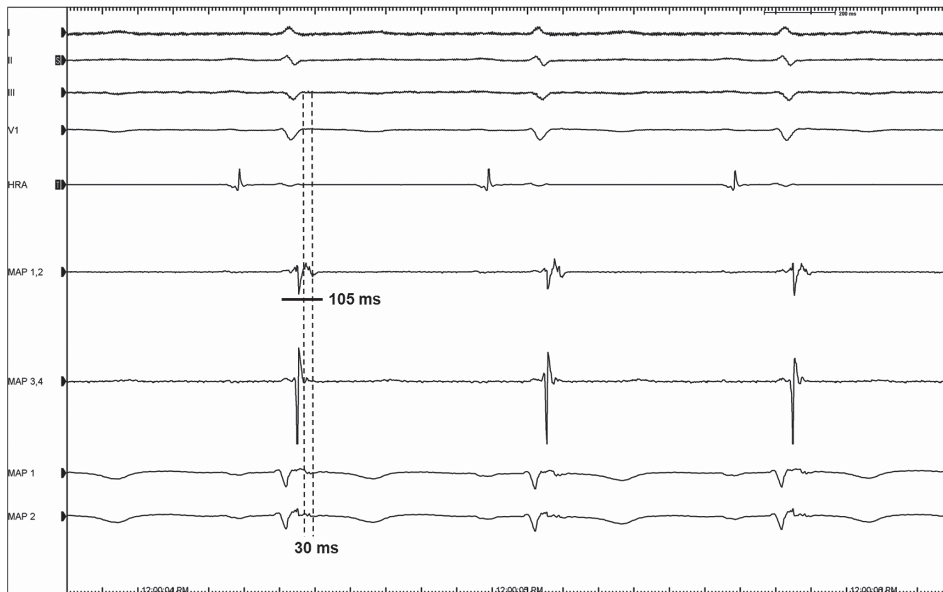
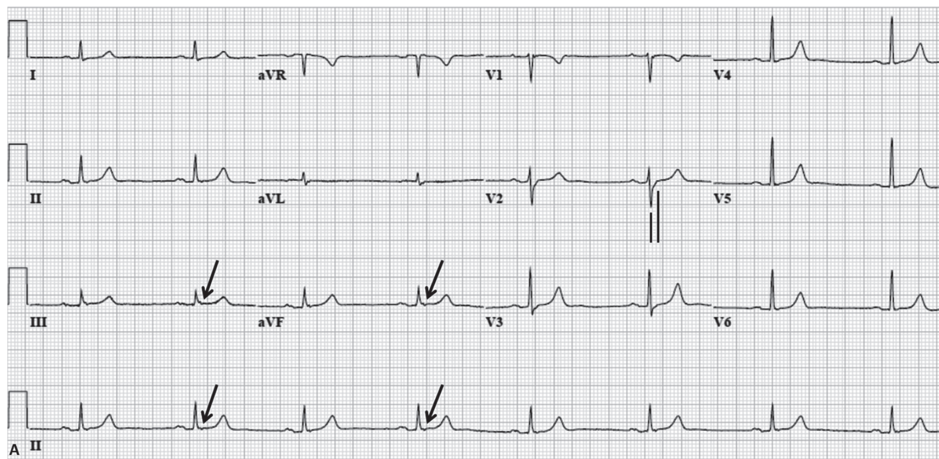


Figure 5. Cardiac magnetic resonance imaging of the patient of whom data are depicted in Figures 2 and 3 (patient 1, see Table 2), showing a dyskinetic wall motion abnormality in the subtricuspid area (indicated by the white frame). These abnormalities correlate well with the induced ventricular tachycardia morphology (Figure 2) and electrical abnormalities found inferior of the tricuspid valve (Figure 3).



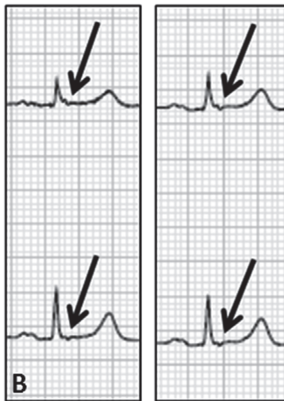


Figure 6A and B. A. ECG of the same patient as in Figures 2, 3, and 4 (patient 1, see Table 2). Although there is extensive arrhythmogenic substrate as demonstrated by the episodes of sustained VT, electrical abnormalities in the subtricuspid, right ventricular outflow tract (RVOT), and apical region, with structural alterations in the first two regions, the ECG is relatively normal. However, in inferior leads II, III, and aVF, late isolated potentials (indicated by the arrows) possibly related to the abnormalities in the subtricupsid area are observed. In addition, there is a prolonged terminal activation duration in leads V1-3 (indicated by black lines), reflecting the substrate in the RVOT. Of note is the absence of any repolarization abnormalities. B. Enlargement of Figure 6A showing in detail the late isolated potentials in leads in inferior leads.

Any of the above mentioned ECG abnormalities in inferior leads II, III, aVF (late isolated potentials, left axis deviation, negative T waves) were found in 13 (68%) patients and any ECG abnormalities in right precordial leads V1-3 (epsilon waves, prolonged TAD, negative T waves) in 17 (89%) patients. Notably, the 2 patients without ECG abnormalities in right precordial leads (patients 4 and 15, see Table 2) did have ECG abnormalities in inferior leads.The depolarization abnormalities late isolated potentials in inferior leads and left axis deviation were present in 11 of 19 (58%) patients, whereas the depolarization abnormalities epsilon waves and prolonged TAD in right precordial leads were present in 10 of 17 (59%) patients. Among the 7 patients without depolarization abnormalities in the right precordial leads, 6 had depolarization abnormalities in inferior leads (3 had late isolated potentials [patients 4, 8, 18] and 3 had left axis deviation [patients 7,11, 13]). The repolarization abnormality negative T waves in inferior leads occurred in 5 of 19 (26%) patients, whereas negative T waves in right precordial leads occurred in 15 of 19 (79%) patients. Nonetheless, in 1 of the 4 patients without repolarization abnormalities in right precordial leads (patient 15), repolarization abnormalities in inferior leads were observed.

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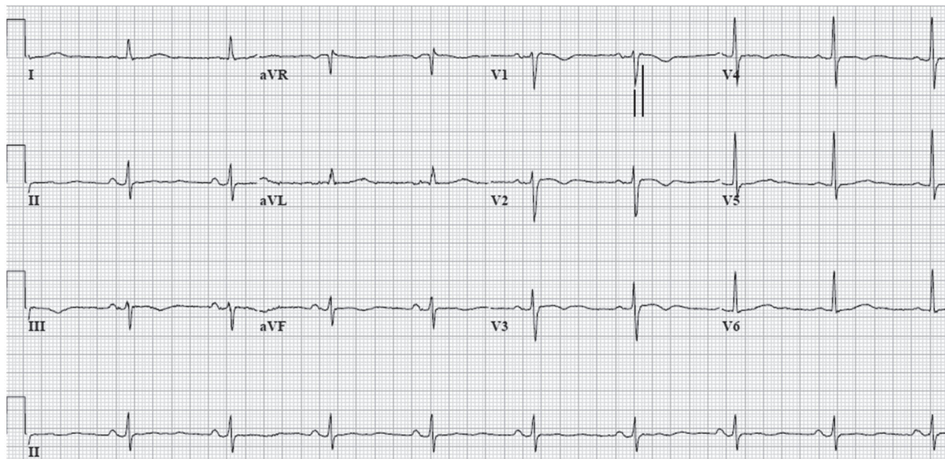

Figure 7. ECG (while of medications) of the same overt ARVD/C patient as in Figures 1 and 5 (patient 5 see Table 2) with multiple LBBB VT morphologies with both superior and inferior axis and electrical and structural abnormalities in the subtricuspid, RVOT, and apical regions. The ECG during sinus rhythm correlates well with the substrate found invasively: negative T waves in inferior leads III and aVF, possibly reflecting abnormalities in the subtricuspid area, and right precordial leads V1-4 and prolonged TAD in V1-3 (indicated by black lines) reflecting abnormalities in the RVOT.

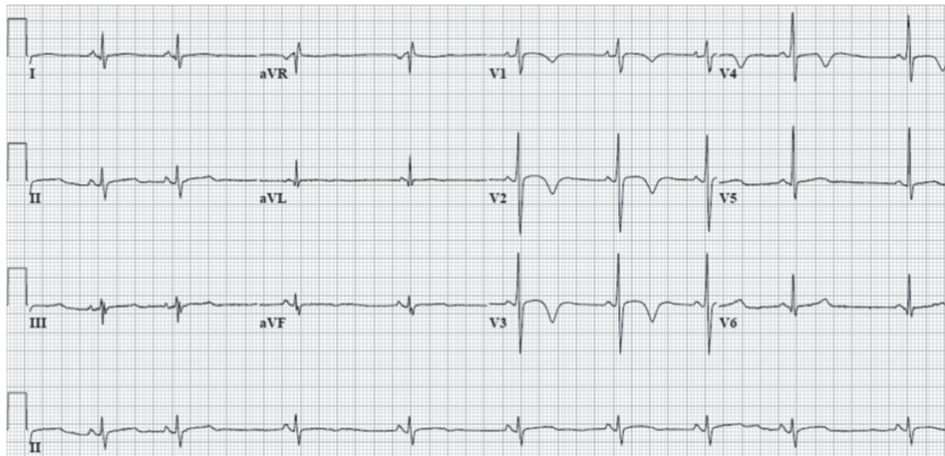

Figure 8. ECG (while off medications) during sinus rhythm of a 16-year old ARVD/C index patient (patient 7, see Table 2) with multiple mutations (plakophilin-2 deletion exon 1-14, desmoglein-2 c.1072G>A p.Ala358Thr, and transmembrane protein 43 c.718C>T p.Arg240Cys). Electrical abnormalities were found in the subtricuspid and right ventricular (RV) outflow tract area with structural alteration in these areas and the RV apex. Subtricuspid abnormalities might be reflected by the left axis deviation and remarkable but non-specific fractionation in leads III and aVF, whereas the RV outflow tract abnormalities can be translated to the negative T waves in right precordial leads V1-4.

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Discussion

The increased risk of SCD and ventricular arrhythmias in ARVD/C is present from the early concealed stage of disease onward.[2, 26] This underscores the need for, preferably non-invasive, parameters for early diagnosis and risk management. In ARVD/C, both intercalated disk remodeling followed by altered tissue architecture due to fibrofatty replacement culminate in activation delay, creating the arrhythmogenic substrate.[4-9] Non-invasive detection of activation delay predominantly in the RVOT has been demonstrated and has therefore been incorporated in the 2010 diagnostic TFC for ARVD/C.[13-16] Diagnostic parameters reflecting the arrhythmogenic substrate when the RVOT is not affected, remain to be elucidated.
In this study, the invasive analysis by PES and activation and voltage mapping, combined with functional/structural analysis by CMR and cine-angiography, in 19 definite ARVD/C patients showed: 1) subtricuspid electrical abnormalities were most prevalent with EPS, with corresponding predominant LBBB VT morphology with superior axis, 2) electrical abnormalities were observed more often but correlated well with regions of functional/structural alteration, and 3) three non-invasive ECG parameters might reflect abnormalities in the subtricucpid area.

Involvement of the subtricuspid area

A new 'triangle of dysplasia' in ARVD/C with early and common involvement of the basal inferior region of the RV, the subtricuspid area, was recently described by our group.[17] Moreover, with CMR analysis, RVOT involvement was identified only in later stages, in patients with moderate or severe disease with at least 3 out of 5 RV regions affected.[17] This implicates that ECG parameters of activation delay predominantly reflecting abnormalities in the RVOT, may not detect abnormalities in the early disease stage.[16] This diagnostic limitation may subsequently affect risk assessment, which is critically important since SCD is frequently the first manifestation of ARVD/C.[26]
In our study, all patients had electrical abnormalities (prolonged local activation duration, late potentials, and low voltages) in the subtricuspid area. Furthermore, the predominant VT morphology observed in our cohort suggests an origin in this area. Nonetheless, the RVOT was usually affected as well in this cohort of definite ARVD/C patients. Therefore, this hypothesis on parameters of early subtricuspid involvement should also be examined in future studies including milder and/or earlier forms of ARVD/C. RV apical abnormalities were solely observed in combination with subtricuspid and RVOT abnormalities. These results are all in accordance with the recently demonstrated ARVD/C disease pattern.[17]
In 17 patients low voltages in the subtricuspid area were observed in combination with prolonged local activation duration and/or late potentials at that area, whereas these combined findings were found in only one patient in the apical region. Since activation delay is important in reentrant arrhythmia mechanisms this observation supports the concept of the subtricuspid site of origin of VT with LBBB morphology and superior axis.

Electrical versus structural abnormalities

In our cohort, electrical abnormalities were observed more often compared to functional and structural alterations. Nonetheless, areas with identified electrical abnormalities correlated well

with areas with structural abnormalities on CMR and cine-angiography analysis. These results are consistent with previous studies indicating that endocardial voltage mapping was sensitive for ARVD/C-related electrical abnormalities and was more accurate in the identification of RV scar areas compared to delayed enhancement on CMR.[10-12] The correlation of electrical and structural abnormalities is in agreement with the studies by Corrado et al.[10] and Santangeli et al.[30], demonstrating a close correlation with histopathological and imaging analysis, respectively. Remarkably, most patients *with* electrical abnormalities but *without* structural involvement in our cohort, had no sustained monomorphic VT. This suggests that both electrical and structural abnormalities are often required as a substrate for maintenance of reentrant circuits resulting in sustained monomorphic VT, as postulated in a prior study.[31]

Translation to the ECG

Activation delay in the RVOT is relatively easily detectable since the RVOT is during normal sinus rhythm, a late activated region of the RV.[27] If the propagation of the electrical signal is delayed on top of the physiologically late activation, the resulting additional activation delay can be observed in the last part of the QRS complex, as prolonged TAD (minor diagnostic TFC), or even after the QRS has ended, as epsilon wave (major diagnostic TFC).[14, 16] However, activation delay in other, physiologically earlier activated parts of the RV, remains frequently hidden in the QRS complex on the 12-lead ECG and identification is therefore challenging.

In our cohort all patients had overt electrical and 74% also had functional and structural abnormalities in the subtricuspid region. These abnormalities may be translated to the 12-lead ECG. Three parameters that possibly reflect the arrhythmogenic substrate in this region, were assessed: 1) late isolated potentials in inferior leads (in 42%), 2) negative T waves in inferior leads (in 26%), and 3) left axis deviation (in 32%). Late isolated potentials might be identified in inferior leads in parallel with epsilon waves in right precordial leads. Negative T waves in inferior leads were observed in a minority in our cohort and did not seem to be a very sensitive marker for disease. This is in accordance with negative T waves in right precordial leads as marker for ARVD/C, which are usually observed in more overt disease.[28] Finally, left axis deviation might reflect loss of electrically excitable myocardium in the lateral and inferior region of the RV and might therefore correlate to abnormalities in the subtricuspid region. In addition, remarkable fractionation of the QRS complex was observed in inferior leads as depicted in Figure 8. Fractionation has been related to altered tissue architecture as a result of myocardial infarction but has also been described as a predictor of adverse outcome in ARVD/C.[4, 29] The potential additional value of ECG abnormalities reflecting subtricuspid involvement in ARVD/C was supported by the observation that 5 patients without or with only a single minor ECG abnormality in right precordial leads did have abnormalities in inferior leads. Overall, non-invasive assessment of electrical abnormalities in the subtricuspid region still awaits further studies and optimization.

Study limitations

This study was limited by the small population size of patients that were comprehensively examined by PES, activation and voltage mapping, CMR with LGE and cine-angiography. However, invasive studies on ARVD/C are typically small in size and the disease pattern observed

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was very consistent with previous descriptions. Since histopathologically the ARVD/C disease process starts from mid- and subepicardial layers and extend towards the subendocardium in later stages, the endocardial mapping approach may be questioned.[3] However, without the intention of an ablation procedure the risks of an epicardial approach were considered inacceptable. The electrical and structural abnormalities found were not confirmed by histopathological analysis since endomyocardial biopsies were taken from the RV septum and were not performed in all patients. Nonetheless, prior studies showed close correlation of electrical abnormalities and areas of fibrofatty replacement and scar.[15] Previous studies in patients with coronary artery disease demonstrated that electrocardiographic estimation of the site of VT origin is hampered by the extension of structural heart disease.[32] Extrapolation of these findings to ARVD/C suggests that precise localization of VT origin is challenging in the presence of extensive structural involvement. Due to the small cohort size, no definite conclusions can be drawn from the suggested ECG parameters reflecting abnormalities in the subtricuspid area. Further studies will have to provide more insight into and validation of these results. The observation of so many ARVD/C patients with VT episodes with LBBB morphology with superior axis (major revised TFC), and frequent occurrence of SCD in the early concealed disease stage strongly support further detailed analysis.

Conclusion

In this study, the findings of PES and RV endocardial activation and voltage mapping in 19 ARVD/C patients showed that the subtricuspid region was electrically affected in all, with structural alteration in 74%. These abnormalities corresponded to the origin of the predominant VT morphology in our cohort. Non-invasive ECG parameters reflecting the abnormalities in this region might be: late isolated potentials in inferior leads, negative T waves in inferior leads, and left axis deviation. At present, non-invasive criteria for abnormalities predominantly derived from the RVOT are included in the 2010 TFC for ARVD/C. Since the subtricuspid area is early and often affected in ARVD/C, criteria reflecting these abnormalities warrant further study.

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Supplemental material

Supplemental table 1. Results of molecular-genetic screening in the 19 study patients

Patient	Gene	DNA change	Protein change
Patient 1	Plakophilin-2	c.235C>T	p.Arg79*
Patient 2	No identified mutation		
Patient 3	Plakophilin-2	c.1848C>A	p.Tyr616*
Patient 4	No identified mutation		
Patient 5	Plakophilin-2	c.1211_1212insT	p.Val406Serfs*
Patient 6	Plakophilin-2	c.2386T>C	p.Cys796Arg
Patient 7	Plakophilin-2	deletion exon 1-14	
	Desmoglein-2	c.1072G>A	p.Ala358Thr
	Transmembrane protein 43	c.718C>T	p.Arg240Cys
Patient 8	No identified mutation		
Patient 9	Plakophilin-2	c.2489+4A>C	splice site mutation
Patient 10	No identified mutation		
Patient 11	Plakophilin-2	c.2146-1G>C	splice site mutation
Patient 12	Plakophilin-2	c.1211_1212insT	p.Val406Serfs*
Patient 13	Plakophilin-2	deletion exon 1-4	
Patient 14	Plakophilin-2	c.2146-1G>C	splice site mutation
Patient 15	Plakophilin-2	c.235C>T	p.Arg79*
Patient 16	Phospholamban	c.40_42delAGA	p.Arg14del
Patient 17	Phospholamban	c.40_42delAGA	p.Arg14del
Patient 18	Plakophilin-2	deletion exon 10	
Patient 19	Plakophilin-2	c. 917-918delCC	p. Pro318Glnfs*

* indicates stopcodon, fs: frame shift

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CHAPTER 11

Non-Invasive Parameters for Evaluation of Activation Delay in Arrhythmogenic Cardiomyopathy

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Judith A. Groeneweg, MD, and Richard N. Hauer, MD, PhD

Editorial

Prolonged electrical activation delay between two sites in the heart is due to slow conduction and/or lengthening of conduction pathways. In the presence of uni-directional block and triggers, enhanced delay promotes reentrant circuits as substrate of arrhythmogenesis in various supraventricular and ventricular arrhythmias. This mechanism is well known in the chronic stage of myocardial infarction and other types of structural heart disease, including arrhythmogenic cardiomyopathy (AC).[1-3]

AC can be defined as an ultimately structural heart disease with signs of heart failure, preceded in the early stages by electrophysiologic alteration resulting in electrocardiographic abnormalities and ventricular arrhythmias. AC includes primarily right ventricular, or primarily left ventricular, or biventricular abnormalities.[4,5] Arrhythmogenic right ventricular dysplasia or cardiomyopathy (ARVD/C) is well known nomenclature for the right-sided variant. However, although histopathologically and functionally predominance of right ventricular abnormalities are found frequently, the left ventricle is affected in most patients as well. Moreover, at the molecular level both ventricles are affected by altered expression and distribution of intercalated disk proteins.[6-8] Because of these considerations AC became favored terminology of this group of frequently difficult to distinguish disorders, recently.

AC is considered to be a genetic disorder, primarily associated with gene mutations encoding desmosomal proteins, although also non-desmosomal genes are involved in a minority, but substantial number, of cases.[9-11] Accumulating evidence suggests that gene mutation-related changes or distribution of desmosomal proteins give rise to altered expression and distribution of other intercalated disk proteins including gap junctions and ion channel proteins, resulting in mechanical and electrical uncoupling and slow conduction and conduction block. These factors favor reentry already in the early concealed stage of the disease. It is very likely that fibrofatty alteration responsible for altered tissue architecture follows in the later overt stage of the disease. This sequence of changes is strongly supported by identification in proven AC patients of altered distribution of the desmosomal protein plakoglobin, in histologically still unaffected left ventricular and septal tissue.[6] In addition, already in the early concealed stage of AC, ventricular fibrillation and sudden death may occur in the absence of identifiable fibrofatty changes.[10] In contrast, in the overt stage the modified tissue architecture characterized by interconnecting vital myocardial bundles embedded in fibrotic tissue, contributes importantly to further aggravation of activation delay by lengthening of conduction pathways and load mismatch at pivotal points.[1,12-14] This highly arrhythmogenic substrate is frequently the cause of monomorphic ventricular tachycardia with left bundle branch block (LBBB) morphology.

During normal sinus rhythm the right ventricular outflow tract (RVOT) is activated late during the ventricular depolarization process. This facilitates detection of prolonged activation delay with non-invasive techniques, i.e. electrocardiography using the right precordial leads V_{1-3} and signal averaged electrocardiography (SAECG).

In the current issue of *Circulation: Arrhythmia and Electrophysiology* Santangeli et al.[15] reported on the correlation between SAECG and histologic evaluation of the myocardial substrate of arrhythmias originating from the RVOT. SAECG was used to detect late potentials due to local

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prolonged activation delay. Late potential recording was fulfilled with at least 2 of the following criteria: 1) fQRSd >114 ms, 2) LAS40 >38ms, and 3) RMS40 < 20µV. Tissue sampling for histologic and immunohistochemical analysis was obtained by endomyocardial biopsies. Sites of biopsies were guided by electroanatomic mapping indicating abnormal myocardium with bipolar signal amplitude < 1.5 mV and scar with amplitude < 0.5 mV. The study was carried out in the limited number of 24 patients, 11 with late potentials and 13 without. All patients with late potential recording showed histologic abnormalities, in 7 patients compatible with AC and in 4 with myocarditis. However, in the absence of late potentials only 5 of 13 patients had normal histologic findings. The other 8 patients showed histologically signs of AC in 4, and myocarditis in the other 4 patients. Although the numbers are low the specificity of late potential recording is high. Thus SAECG positive patients should undergo extensive cardiologic evaluation and molecular-genetic screening, since structural heart disease is very likely. However, the sensitivity of late potential recording with the SAECG technique is very low. The technique is not appropriate to identify patients without structural disease, i.e. those with idiopathic ventricular tachycardia or idiopathic premature ventricular complexes. These arrhythmias from the RVOT are usually not due to reentry and prolonged activation delay is not involved in arrhythmogenicity. Further studies are needed to evaluate if modification of SAECG cut-off values may enhance sensitivity, as also suggested by the authors.

Activation delay in the RVOT can also be studied by routine 12-lead ECG recording, especially derived from V_{1-3}. Cox et al. compared activation delay, while off anti-arrhythmic drugs, in 42 patients with proven AC and 27 controls with idiopathic ventricular tachycardia.[16] Control patients had a normal ECG during sinus rhythm and underwent 2D-transthoracic echocardiography and in equivocal cases magnetic resonance imaging and/or left and right ventricular cine-angiography to exclude structural abnormalities. AC was diagnosed according to the task force criteria from 1994. Activation delay parameters studied were epsilon waves, QRS duration > 110 ms in V_{1-3}, QRS $V_1 + V_2 + V_3 / V_4 + V_5 + V_6 > 1.2$, prolonged S wave upstroke, and prolonged terminal activation duration (TAD). ECG analysis was carried out twice by two physicians independently in randomized sequence and after blinding for all other results. Prolonged TAD (≥ 55 ms) showed the highest sensitivity. This parameter was identified in 30 of 42 proven AC patients, or 71%, and in only 1 of 27 idiopathic ventricular tachycardia patients, or 4% (P < 0.001). Measurements were highly reproducible. The single patient with prolonged TAD and idiopathic ventricular tachycardia was presumably not correctly labelled as such, since in addition to the ventricular tachycardia from the RVOT also a ventricular tachycardia with superior axis has been recorded and catheter ablation was presumably not successful since the patient remained on anti-arrhythmic drug treatment. Because of the superior sensitivity and high specificity of prolonged TAD this criterion was included in the recently revised task force criteria.[17] However, it cannot be excluded with certainty that none of the patients labelled as idiopathic had AC in an early still concealed stage.

In a recent study Cox et al. showed recording of prolonged TAD in 5 of 7 young (age < 20 years) still asymptomatic pathogenic desmosomal mutation carriers, being relatives of symptomatic proven AC index patients.[10] Of these 5 individuals, 4 had prolonged TAD as only clinical abnormality. This finding is in line with the observation that ventricular fibrillation and sudden cardiac death may occur in the early concealed stage of AC. Further studies are needed to

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evaluate prolonged TAD as marker for risk stratification. Since SAECG also detects activation delay, a similar perspective seems reasonable after improvement of methodology.

We have to realize that with both ECG and SAECG detection of prolonged activation delay in the RVOT is relatively easy. However, prolonged activation delay detection in other right ventricular areas and in the left ventricle is hampered by the fact that this delay is frequently hidden within the QRS complex. Solution of this problem is important, since prolonged activation delay is a precursor of arrhythmogenicity due to reentrant mechanisms and is promising for AC diagnosis in the early concealed stage and for risk stratification. Early diagnosis is crucial since sudden death may be the first manifestation of AC.[18]

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CHAPTER 12

Clinical Presentation, Long-Term Follow-up, and Outcomes of 1001 Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Patients and Family Members

Submitted

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Abstract

Background

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a progressive cardiomyopathy. We aimed to define long-term outcome in a transatlantic cohort of 1001 individuals.

Methods and results

Clinical and genetic characteristics and follow-up data of ARVD/C index-patients (n=439, fulfilling of 2010 criteria in all) and family members (n=562) were assessed. Mutations were identified in 276 index-patients (63%). Index-patients presented predominantly with sustained ventricular arrhythmias (VA) (268, 61%). Over a median follow-up of 7 years, 301 of the 416 index-patients presenting alive (72%) experienced sustained VA. Sudden cardiac death (SCD) during follow-up occurred more frequently among index-patients without an ICD (10/63, 16% vs. 2/335, 0.6%). Overall, cardiac mortality and/or the need for cardiac transplantation were low (6% and 4%, respectively). Clinical characteristics and outcome were similar in index-patients with and without mutations, as well as in those with familial and non-familial ARVD/C. ARVD/C was diagnosed in 207 family members (37%). Symptoms at presentation correlated with disease expression. Family members with mutations were more likely to meet Task Force Criteria for ARVD/C (40 vs. 18%), experience a sustained VA (11 vs. 1%), and/or die from cardiac cause (2 vs. 0%) than family members without mutations.

Conclusions

Long-term outcome was favorable in diagnosed and treated ARVD/C index-patients and family members. Outcome in index-patients was modulated by ICD implantation, but not by mutation status and familial background of disease. One third of family members developed ARVD/C. Outcome in family members was determined by symptoms at presentation and mutations.

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Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a hereditary progressive cardiomyopathy characterized by an increased risk of sudden cardiac death (SCD), ventricular arrhythmias (VA), and predominantly right ventricular (RV) dysfunction.[1, 2] ARVD/C related mutations in desmosomal and, less commonly, non-desmosomal genes are identified in approximately 60% of index-patients.[3-11]

Since the first major disease description, considerable progress has been made in understanding of the genetic substrate, pathogenesis, and clinical diagnosis of ARVD/C.[1] However, information on long-term follow-up both in index-patients and family members is limited. Most prior studies predated the availability of genetic testing, were small, focused on overt index-patients, and used the original less sensitive 1994 Task Force Criteria (TFC) for ARVD/C diagnosis.[12-22]

While ARVD/C is considered an inherited cardiomyopathy, it is notable that a significant proportion of index-patients have neither an identifiable mutation nor a family history of disease. Whether the disease course differs in this sizable subset of patients remains unknown. Moreover, whether index-patients with what appears to be isolated disease have yet to be identified mutations or represent a distinct acquired form of ARVD/C, such as 'exercise induced RV cardiomyopathy', remains unknown.[23-25]

In this study we analyzed clinical and genetic characteristics and long-term follow-up data of ARVD/C index-patients and family members in a large transatlantic cohort of more than one-thousand individuals. There were three main goals of this study. First, we sought to define the presenting symptoms, clinical characteristics, and long-term outcome of ARVD/C in a large series of index-patients and family members. Importantly, each index-patient underwent molecular-genetic testing, and the revised 2010 TFC were used for ARVD/C diagnosis. Second, we sought to provide insight into determinants of phenotypic and long-term outcome differences amongst ARVD/C index-patients and family members. And third, we sought to use this analysis as an opportunity to provide insight into the recently proposed hypothesis that isolated ARVD/C may represent 'exercise induced RV cardiomyopathy' with distinct clinical features and more benign outcomes.

Methods

Study population

The study population was comprised of 439 index-patients fulfilling the 2010 TFC for ARVD/C and 562 family members enrolled in the ARVD/C registries from the Johns Hopkins University (n=511) and the Dutch Interuniversity Cardiology Institute of the Netherlands (ICIN, a cardiovascular research institute with collaborative participation of all 8 Dutch University Medical Centers) (n=490). Index-patients were defined as the first affected individuals seeking medical attention for ARVD/C in whom the diagnosis was confirmed (i.e. ascertained independently of ARVD/C family history). Family members were ascertained by family screening. All family members with a mutation predisposing for ARVD/C were included (regardless of degree of relatedness). Only first-degree relatives of individuals with an ARVD/C diagnosis were included

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in families in which mutations were not identified. All subjects or their guardians provided informed consent as per individual institutional protocol. The study was approved by an institutional review committee.

Clinical analysis

The medical history of each individual was obtained by review of medical records, clinical evaluation, and patient interviews. A detailed family history was obtained through patient interview by genetic counselors with special ARVD/C interest for pedigree analysis. Index-patients and family members were prospectively followed and evaluated for TFC, clinical presentation, clinical course, and outcome. Data regarding clinical events including sustained VA episodes (defined as sustained ventricular tachycardia (VT), ventricular fibrillation (VF), cardiac arrest, or appropriate implantable cardioverter-defibrillator (ICD) intervention [shock or anti-tachycardia pacing]), heart failure, cardiac transplantation, SCD, and other causes of death was obtained (see supplemental Table 1 for definitions). ARVD/C diagnosis was based on the presence of major and minor diagnostic criteria according to the 2010 TFC.[26] All family members had 1 major criterion for family history, i.e. either for pathogenic mutations or for first-degree relatives with ARVD/C. Noninvasive and invasive diagnostic studies performed are shown in supplemental Table 2. Clinical characteristics of index-patients and family members were compared between subjects *with* and *without* identified mutations. Additionally, the clinical features of index-patients with *familial* (defined as fulfillment of any 2010 TFC in the category for family history) and *isolated* ARVD/C (defined as absence of family history TFC) were compared.

Molecular-genetic analysis

Mutation analysis of the desmosomal genes encoding plakophilin-2 (*PKP2*), desmoplakin (*DSP*), desmoglein-2 (*DSG2*), desmocollin-2 (*DSC2*), and plakoglobin (*JUP*) was performed in all index-patients as reported previously (see supplemental Table 1 for the definition of a mutation).[18, 27] Non-desmosomal gene analysis included *TMEM43* and *PLN*. Family members were screened for the mutation(s) found in their respective index-patient, if any. Subjects who presented with SCD and were either obligate carriers or had ARVD/C on autopsy but did not have genetic screening were assumed to have the same mutation as their first-degree family members.

Statistical analysis

Continuous variables are summarized as either mean±SD or median (interquartile range (IQR), range) where appropriate and compared across groups using a Student's *t* test. Categorical variables are reported as frequency (%) and compared between groups by the chi-square or Fisher's exact test. The cumulative freedom since birth (i.e. by age) from the clinical outcome was determined by the Kaplan-Meier method, and differences in survival between groups evaluated with the log-rank test. A p-value ≤ 0.05 was considered significant. SPSS statistical software (version 20; SPSS Inc, Chicago, IL) and STATA 13.1 (Stata Corp, College Station, TX) were used for the analyses.

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Results

ARVD/C cohort

Summarized in Tables 1 and 2 are the demographic features and age at presentation of the 1001 subjects (452 unrelated families) including 439 index-patients and 562 family members. Index-patients were followed over a median of 7 years (IQR 12, range 0-37). Of 537 family members presenting alive, 364 (68%) had a median follow-up of 5 years (IQR 8, range 1-38), 173 (32%) were evaluated once.

Genetic screening

Molecular-genetic screening identified mutations in 276 of the 439 index-patients (63%, supplemental Table 3). Mutations were predominantly found in *PKP2* (202, 46%). Less common were mutations in the other desmosomal genes: *DSP* (11, 3%), *JUP* (2, 0.5%), *DSG2* (17, 4%), and *DSC2* (5, 1%). Mutations in non-desmosomal genes *PLN* and *TMEM43* were identified in 21 (5%) and 1 (0.2%) index-patient(s), respectively. Seventeen (4%) had multiple mutations. In 163 (37%) of 439 index-patients no mutation could be identified.

Table 1. Demographic characteristics of 439 index patients with ARVD/C

Demographics	**Index patients** number (%)	**Without identified mutation** number (%)	**With identified mutation** number (%)	**With identified mutation** number (%)				**p-value***
	Total n=439	**Total w/o mutations** n=163	**Total with mutations** n=276	***PKP2*** n=202	***Other desmo-somal*** n=35	***PLN/ TMEM43*** n=22	***CH/DG/ HO*** n=17	
Men	282 (64)	103 (63)	179 (65)	131 (65)	26 (74)	13 (59)	9 (53)	0.725
Race								
Caucasian	432 (98)	162 (99)	270 (98)	200 (99)	33 (94)	22 (100)	15 (88)	
African-American	1 (0.2)	1 (0.6)						
Asian	6 (1)		6 (2)	2 (1)	2 (6)		2 (12)	
Registry								
ICIN	198 (45)	58 (36)	140 (51)	99 (49)	11 (31)	21 (96)	9 (53)	
JHU	241 (55)	105 (64)	136 (49)	103 (51)	24 (69)	1 (4)	8 (47)	
Age at presentation mean ±SD (yrs)	36 ±14	38 ±14	34 ±14	34 ±14	33 ±15	40 ±12	34 ±14	<0.001
Age at last follow-up mean ±SD (yrs)	44 ±15	47 ±14	43 ±15	44 ±16	40 ±14	49 ±12	44 ±16	0.015

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; ICIN: Interuniversity Cardiology Institute of The Netherlands; JHU: Johns Hopkins University; SD: standard deviation; *PKP2*: plakophilin-2; *PLN*: phospholamban; *TMEM43*: transmembrane protein 43; CH: compound heterozygous mutations; DG: digenic mutations; HO: homozygous mutations. *P-value represents comparison between total with mutations (n=276) and without identified mutations (n=163).

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Of 562 family members, 409 (73%) were mutation carriers. Mutations in *PKP2* were found in 342 (61%) family members, *DSP* mutations in 16 (3%), *DSG2* mutations in 13 (2%), *DSC2* mutations in 4 (0.7%), and *PLN* mutations in 26 (5%). No family members with *JUP* or *TMEM43* mutations were identified. Eight (1%) had multiple mutations. In 153 (27%) family members no molecular-genetic screening was performed since no mutation was found in the index-patient.

Presenting clinical characteristics, clinical course, and long-term outcome in index-patients

Figure 1 summarizes the major presenting clinical features, clinical course, and arrhythmia and survival outcomes of each of the 439 index-patients. The mean age at presentation was 36±14 years. Of these, 419 (95%) presented with symptoms. The remaining 20 (5%) were asymptomatic but came to medical attention because of abnormal tests in diverse settings. Forty-eight index-patients (11%) presented with a cardiac arrest among whom 25 were resuscitated and 23 died with the diagnosis established at autopsy (median age at cardiac arrest 25 years, IQR 21, range 13-70). An additional 220 index-patients (50%) presented with a sustained VA.

Table 2. Demographic characteristics of 562 family members of ARVD/C index patients

Demographics	**Family members** number (%)	**Without identified mutation** number (%)	**With identified mutation** number (%)	**With identified mutation** number (%)				**p-value***
	Total n=562	**Total w/o mutations** n=153	**Total with mutations** n=409	***PKP2*** n=342	**Other desmo-somal** n=33	***PLN/ TMEM43*** n=26	***CH/DG/ HO*** n=8	
Men	260 (46)	75 (49)	185 (45)	160 (47)	12 (36)	9 (35)	4 (50)	0.423
Race								
Caucasian	556 (99)	153 (100)	403 (99)	338 (99)	31 (94)	26 (100)	8 (100)	
African-American								
Asian	6 (1)	6 (1)	4 (1)	2 (6)				
Registry								
ICIN	292 (52)	62 (41)	230 (56)	187 (55)	13 (39)	24 (92)	6 (75)	
JHU	270 (48)	91 (59)	179 (44)	155 (45)	20 (61)	2 (2)	2 (25)	
Age at presentation mean ±SD (yrs)	36 ±19	38 ±19	36 ±19	35 ±19	34 ±18	45 ±14	43 ±12	0.283
Age at last follow-up mean ±SD (yrs)	41 ±19	41 ±19	40 ±19	40 ±19	38 ±18	49 ±15	45 ±11	0.789

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; ICIN: Interuniversity Cardiology Institute of The Netherlands; JHU: Johns Hopkins University; SD: standard deviation; *PKP2*: plakophilin-2; *PLN*: phospholamban; *TMEM43*: transmembrane protein 43; CH: compound heterozygous mutations; DG: digenic mutations; HO: homozygous mutations. *P-value represents comparison between total with mutations (n=409) and without identified mutations (n=153).

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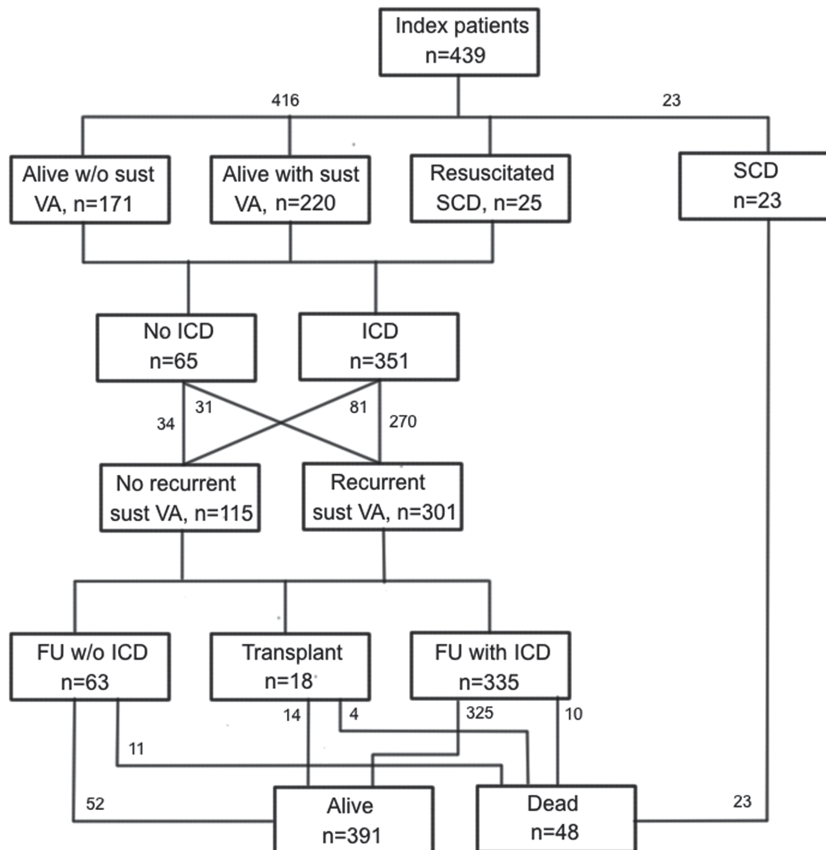

Figure 1. Schematic representation of the presentation, clinical course and outcome in Arrhythmogenic Right ventricular Dysplasia/Cardiomyopathy (ARVD/C) index-patients. The majority presented with sustained ventricular arrhythmias (VA) and received an implantable cardioverter-defibrillator (ICD) during follow-up. Of 63 index-patients without an ICD, 11 patients died, 1 of heart failure and 10 of sudden cardiac death (SCD). In 335 patients with an ICD, 10 died, only 2 of SCD, 3 of heart failure, 2 of heart failure/arrhythmias, and 3 of non-cardiac causes. Overall even in index-patients, mortality was low with 89% alive at last follow-up.

During the clinical disease course, an ICD was implanted in 212 (87%) of the 245 index-patients with a sustained VA or resuscitated SCD and in 139 (81%) of the 171 index-patients presenting without sustained VA. Sixty-five index-patients did not have an ICD implanted during follow-up (33 with and 32 without sustained VA at presentation). Sustained VA during follow-up were observed in more than two-thirds of index-patients (301, 72%). Of the 65 index-patients without an ICD, 31 (48%) experienced a sustained VA during follow-up.

Among index-patients with an ICD, 10 died (3%) (median follow-up 7 years, IQR 11, range 0-37): 2 of SCD, 3 of heart failure, 2 of a combination of heart failure and arrhythmias, and 3 of non-cardiac causes. Among index-patients without an ICD, 11 died (17%)(median follow- up 5 years, IQR 12, range 0-35): 10 of these 11 patients died of SCD and 1 of heart failure. The SCD incidence was remarkably higher in index-patients without an ICD compared to those with an ICD

(16% vs. 0.6%, p<0.001). During long-term follow-up, 54 index-patients (13%) developed symptomatic heart failure and 18 (4%) had a cardiac transplantation. Four patients that underwent transplantation died during follow-up. Overall, 391 of the 416 index-patients (94%) who presented alive were alive at last follow-up.
Shown in supplemental Table 4 are the clinical characteristics of the 416 index-patients presenting alive as reflected in the 2010 TFC. Figure 2A and B show the results of Kaplan-Meier survival analysis by age for these 416 index-patients for the following outcomes: 1) any ARVD/C related symptoms, 2) sustained VA, 3) cardiac mortality, and 4) cardiac transplantation. Of particular note is that all patients presented in their teenage years or later (range 10-78 years, only 4 index-patients presented before the age of 13 years old of whom 1 presented with a sustained VA), the development of symptoms correlated closely with the development of a sustained VA, and cardiac mortality and the need for cardiac transplantation were low.

Impact of mutation status on presenting signs and symptoms and clinical outcome

Index-patients with mutations presented at a significantly younger age compared to index-patients without identified mutations (mean age 34±14 vs. 38±14 years, p<0.001) (Table 1). ARVD/C TFC did not differ between those with and without identified mutations, except for negative T waves in leads V4-6 (4% vs. 0.7%, p 0.037)(supplemental Table 4). Index-patients presenting alive with and without identified mutations had similar proportions of symptoms (99% vs. 97%, p=0.197) and sustained VA (83% vs. 83%, p=0.934) during a median follow-up of 8 versus 6 years (p=0.198). Nonetheless, first symptoms (mean age 33±14 vs. 37±15 years, p=0.002) and sustained VA (mean age 36±14 vs. 40±14 years, p=0.001) occurred at significantly younger age in index-patients with mutations (log-rank test first symptoms p=0.005, log-rank test sustained VA p=0.020). Mutation status of index-patients did not impact cardiac mortality and transplantation outcomes (Figure 2A and B and supplemental Table 5).

Familial versus isolated ARVD/C

ARVD/C TFC for family history were met in 295 of 416 index-patients presenting alive (71%). Of these 295 index-patients with familial ARVD/C, 264 had mutations (89%) and 31 did not (11%). In 121 index-patients (29%), family history TFC (including mutation status) were absent. Phenotype comparison of index-patients with familial and isolated ARVD/C, showed no clinically relevant phenotypic differences (supplemental Table 6). Sustained VA, electrocardiographic abnormalities, structural and functional abnormalities, and heart failure occurred similarly in familial and isolated ARVD/C (median follow-up 8 vs. 6 years, p=0.294). Cardiac mortality during follow-up was higher in familial ARVD/C (7% vs. 0.8%, p=0.013). This difference was mostly due to the 13 index-patients with familial disease that died of SCD during follow-up (median 4 years, IQR 15, range 0-35). Of these 13 patients, 11 did not have an ICD implanted. Five had mutations and 8 were without identified mutations. Although SCD occurred more often in familial ARVD/C, the incidence of VF during follow-up was similar (11% vs. 15%, p=0.303) in index-patients with familial and isolated disease, respectively.

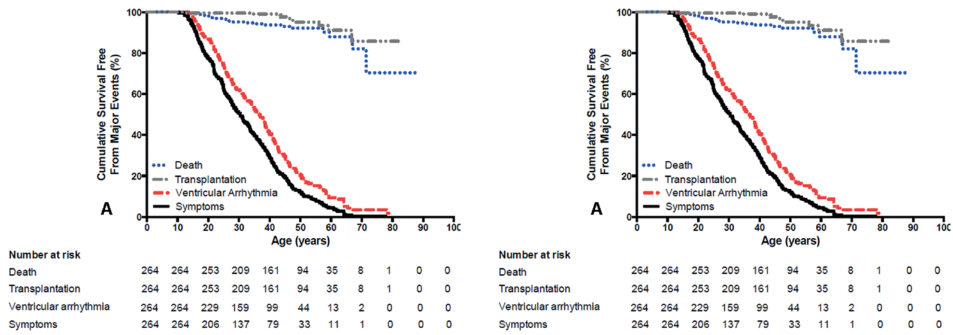

Figure 2A and B. Survival free from any Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) related symptoms, sustained ventricular arrhythmias (VA), cardiac death and cardiac transplantation in ARVD/C index-patients with pathogenic mutations (**A**) and without identified mutations (**B**). Symptoms (p=0.005) and sustained VA (p=0.020) occurred significantly more often at younger age in index-patients with mutations. Survival free from cardiac death (p=0.644) and transplantation (p=0.704) was similar in both groups .

Presenting clinical characteristics, clinical course, and long-term outcome in family members

The 562 family members in the cohort presented at a median age of 35 years (IQR 31, range 1-87). Family members were predominantly asymptomatic (463, 82%), but 27 (5%) presented with a cardiac arrest among whom 2 were resuscitated and 25 died (median age 22 years, IQR 25, range 14-58, 20 autopsy diagnoses, 5 obligate carriers with ARVD/C as probable cause of death). During clinical course, ARVD/C was diagnosed in 207 of 562 family members (37%). Shown in supplemental Table 7 are the clinical characteristics of family members as reflected in the 2010 TFC. Frequent ventricular ectopy and prolonged terminal activation duration (TAD)/ late potentials were the most frequently observed criteria, in 20% and 31%, respectively.

Figure 3 summarizes the clinical presentation and fulfillment of ARVD/C TFC of the 537 family members presenting alive. In 385 family members, the same mutation(s) as in their respective index-patient was identified. The remaining 152 family members were considered as 'without mutations', since no mutation was identified in their respective index-patient. Symptoms at presentation correlated with ARVD/C disease expression. Of the 385 family members with mutations, 61 presented symptomatically (16%) of whom 51 met TFC for diagnosis (84%). Of the 152 family members without mutations, 13 were symptomatic (9%) of whom 10 met TFC for diagnosis (77%). Nevertheless, of 324 asymptomatic family members with mutations and 139 without mutations, 103 (32%) and 18 (13%), respectively, also met TFC for ARVD/C. Overall, family members with mutations had ARVD/C diagnosis twice more often compared to family members without mutations (154/385, 40% versus 28/152, 18%).

Figure 4A and B show the results of Kaplan-Meier survival analysis by age for the outcomes: 1) any ARVD/C related symptoms, 2) sustained VA, 3) cardiac mortality, and 4) cardiac transplantation in the family members presenting alive. Family members had a better clinical event-free survival compared to index-patients. Nonetheless, in those developing symptoms and events, the age

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of onset was only slightly later than that in index-patients. Family members with mutations had worse clinical outcomes than those without. Of 385 family members with mutations, 42 (11%) experienced sustained VA, whereas only 2 of 152 family members without mutations (1%) had sustained VA (Figure 4A and B, $p<0.001$). Eight family members with mutations (2%) died during follow-up: 4 of SCD (all without ICD), 2 of heart failure, and 2 of non-cardiac causes. No deaths during follow-up were observed in family members without mutations. Two family members with (0.5%) and 1 without (0.7%) mutations underwent cardiac transplantation.

An ICD was implanted in 87 of 385 (23%) family members with mutations (appropriate ICD interventions in 18, 21%) compared to 14 of 152 (9%)family members without mutations (appropriate ICD therapy in 2, 14%). However, the percentage of ICDs implanted in family members that fulfilled TFC for ARVD/C diagnosis was not different between those with and without mutations (77/154, 50%, vs. 12/28, 43%, p=0.429).

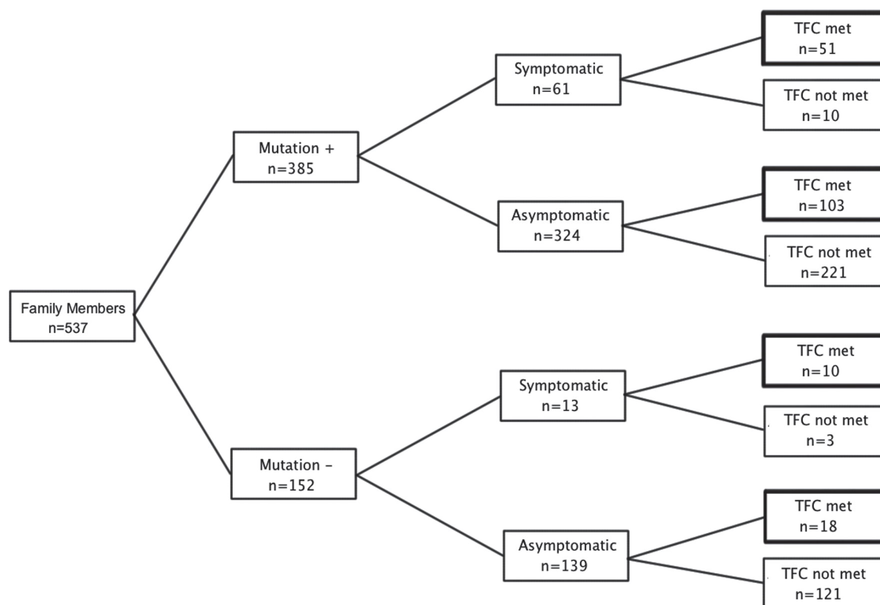

Figure 3. Schematic representation of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) disease penetrance in 537 family members presenting alive. In 385 family members, the same mutation(s) as in their respective index-patients was identified (mutation +). The remaining 152 family members were considered 'without mutations', since no mutation could be identified in their respective index-patient (mutation -). Fulfillment of Task Force Criteria (TFC) for ARVD/C (indicated by the thick black boxes) correlated with symptoms at presentation. Nonetheless, a minority of asymptomatic family members also had ARVD/C diagnosis. This underscores the importance of family screening in ARVD/C.

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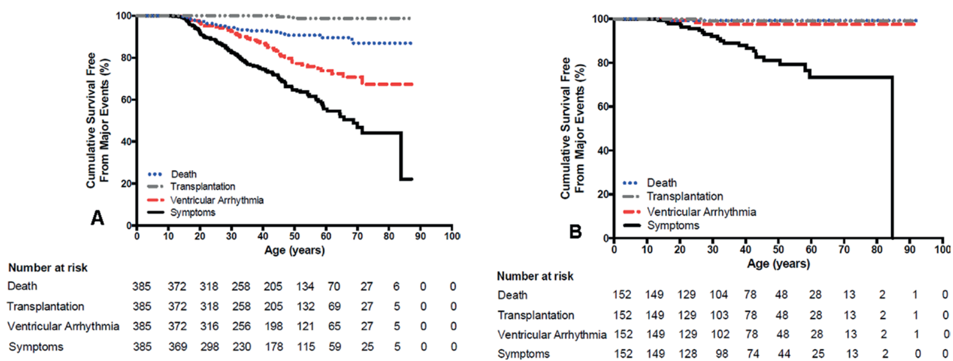

Figure 4A and B. Survival free from any Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) related symptoms, sustained ventricular arrhythmias (VA), cardiac death and cardiac transplantation in family members with pathogenic mutations (**A**) and without mutations (these family members were considered as 'without mutations', since no mutation could be identified in the index-patient)(**B**). Family members without mutations had less symptoms (p=0.004), sustained VA (p<0.001), and cardiac death (no deaths vs. 6 deaths: 4 of sudden cardiac death, 2 of heart failure) compared to family members with mutations. Survival free from cardiac transplantation was similar (p=0.812).

Discussion

Main findings

The results of this study describe in detail the presentation, clinical and genetic characteristics, and long-term outcomes of more than one-thousand ARVD/C index-patients and family members. These results are important and unique for several reasons. First, this is the largest and most comprehensive report detailing the clinical features and outcomes of patients and family members with ARVD/C. Second, this analysis was performed following the advent of widespread molecular-genetic testing for ARVD/C and after implementation of the revised 2010 ARVD/C TFC. And third, this analysis represents the results of a collaborative transatlantic research effort with nearly equal representation of patients from the United States and Europe. There are four main findings of this study. First, long-term outcome was favorable in diagnosed and appropriately treated ARVD/C index-patients and family members. Second, in index-patients phenotype and long-term outcome were modulated by ICD implantation but remarkably not by mutation status and familial background. Third, clinical course and outcome in family members was determined by symptoms at presentation and mutation status. And finally, since index-patients with and without mutations and/or familial and isolated disease had similar clinical features and outcomes, these results challenge the recently proposed concept of an acquired (likely exercise-induced) RV cardiomyopathy with unique clinical features and more benign course.

Prior studies on long-term follow-up in ARVD/C

Since the first major clinical ARVD/C description in 1982, a number of studies reported on the long-term follow-up of patients with ARVD/C.[1, 12-22] While these studies have been invaluable in helping to define the clinical features and outcomes of ARVD/C patients, they also had important

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limitations which limit their applicability to the management of patients with ARVD/C today. First, most studies included small numbers of patients. Second, most of these studies were reports from single centers outside the United States.[12-15, 17, 19-21] Third, more than half of these studies did not include the results of molecular-genetic testing, consistent with the fact that most of these studies included subjects enrolled before the advent of widespread genetic testing. [13, 17, 18, 20, 22] Fourth, nearly all studies relied on the original TFC for ARVD/C diagnosis established in 1994.[12-17, 19] At present, the revised 2010 TFC are used to evaluate patients for ARVD/ C. Finally, almost all of the series focused predominantly on affected patients with ARVD/C. Although family members were included in some of the published series, the number of family members involved was small.[17, 18, 20]

Clinical characteristics, clinical presentation, clinical course and long-term outcome in ARVD/C index-patients

The results of our study provide a number of important insights into the clinical characteristics, presentation, clinical course, and long term outcomes of ARVD/C index-patients. First, it is striking that among the 439 index-patients in this study, only 4 presented before the age of 13 years and none presented before the age of 10 years old. The onset of the disease from teenage years onward might be related to the completion of intercalated disk maturation, or the need for exposure to a certain amount of exercise before ARVD/C becomes manifest.[28-31] During post-natal development, the intercellular junctions in the intercalated disk supposedly undergo drastic reorganization and are eventually localized at the end of cardiomyocytes by early adolescence.[30, 31]

Second, the results of our study demonstrate that diagnosis and subsequent treatment are associated with a good long-term outcome in index-patients. The clinical presentation of index-patients was sustained arrhythmia-related in 61%, of whom 11% presented with cardiac arrest. Sustained VA during follow-up occurred in 72% of index-patients. These findings highlight that arrhythmias are the most important and potentially life-threatening ARVD/C manifestation. Moreover, these results stress the importance of early diagnosis of ARVD/C and family member screening for the prevention of SCD. Progression of ARVD/C resulting in symptomatic heart failure, was observed in a minority of index-patients (13%) and necessity for cardiac transplantation (4%) or heart failure-associated mortality during follow-up (1%) were even more uncommon. Overall, of our large transatlantic cohort of 439 index-patients, 89% were alive at last follow-up.

Determinants of phenotypic and long-term outcome differences in ARVD/C index-patients

Our study assessed the impact of several potential determinants of clinical characteristics and long-term outcome in ARVD/C. First, the results of our study call attention to the important role of ICD implantation in patients with ARVD/C. An ICD was implanted in 85% of index-patients in our cohort. There was a remarkably high incidence of SCD during follow-up in index-patients without an ICD compared to those with an ICD (16% vs. 0.6%). In addition, none of the 4 family members who died of SCD during follow-up had an ICD. At present, ICD implantation is the only proven life-saving therapy in ARVD/C. In the future, other treatment modalities in ARVD/C might improve disease outcome.[32]

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Second, our study demonstrated for the first time that the presence or absence of identifiable mutations did not impact clinical outcome in ARVD/C index-patients, although it did modulate age of disease expression. Phenotypic characteristics and disease course were similar in index-patients with and without identified mutations. Of note, index-patients with mutations had earlier disease onset, with a remarkable 4-year difference in mean age at first symptoms, clinical presentation, and sustained VA compared to index-patients without identified mutations.
Finally, in our study no evidence for impact of familial background on clinical characteristics and long-term outcome index-patients with established disease was observed. This type of analysis has not been performed amongst ARVD/C patients previously. Recently, Heidbüchel and colleagues have proposed a concept of isolated exercise induced RV cardiomyopathy, mimicking ARVD/C. They hypothesized that these patients with isolated disease, although meeting TFC for ARVD/C diagnosis, have distinct clinical characteristics from inherited and familial forms of ARVD/C.[23-25] In our cohort, no evidence supporting this hypothesis was found. The isolated form of ARVD/C was clinically identical to familial ARVD/C. Moreover, our data do not suggest that index-patients with familial or isolated ARVD/C should be treated differently. Although cardiac mortality occurred significantly more often in index-patients with familial ARVD/C, this difference was mostly due to the 13 patients, 11 of whom had no ICD, with familial disease that died of SCD during follow-up. Moreover, the incidence of episodes of sustained VT and VF and structural and functional abnormalities were similar in familial and isolated disease during a comparable follow-up duration. These results suggest the importance of ICD implantation for both groups of index-patients.
Notably, in 11% of index-patients with familial ARVD/C, no mutation could be identified. Nonetheless, in these families an unidentified genetic background for ARVD/C can reasonably be assumed.

Clinical course and long-term outcome in ARVD/C in family members

This study furthermore provides important insights into the disease course and outcomes of family members of index-patients with ARVD/C. Family members presented predominantly asymptomatic. In our cohort, among more than 500 family members, approximately one third (37%) developed ARVD/C. Sustained VA and cardiac mortality during follow-up occurred in a small minority (8% and 2%, respectively). In the family members that developed disease and sustained VA, the age at onset was comparable to that in index-patients.

Determinants of phenotypic and long-term outcome differences in family members

The results of our study expose two important factors influencing disease course and long-term outcome of family members. First, symptoms at presentation corresponded with ARVD/C diagnosis and disease expression. This suggests that particular attention should be paid to symptomatic family members. However, albeit at lower rates, a considerable portion of asymptomatic family members also met 2010 TFC for ARVD/C diagnosis. These results stress the importance of family screening for timely diagnosis and risk management.
Second, in contrast to index-patients, mutation status impacted long-term outcome in family members. A two-fold difference was observed in ARVD/C diagnosis between family members with and without mutations. This is consistent with an autosomal dominant inheritance pattern,

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assuming that all ARVD/C cases are genetically based. However, sustained VA were observed eight times more often in family members with mutations. This divergence could not be explained by the difference in ARVD/C diagnosis or ICD implantations and subsequent arrhythmia detection since ICD implantation rates were comparable in family members with ARVD/C diagnosis between those with and without mutations. Similarly, cardiac death was observed more often in family members with mutations than in those without mutations. It may be that family members of index-patients without identified mutations are at decreased risk of developing disease, sustained VA, and cardiac death or that they have a higher threshold for development of VA.

Overall, our study in the largest cohort reported so far, demonstrates that family members have excellent long-term outcomes. Most family members did not develop ARVD/C and had better sustained arrhythmia-free survival compared to index-patients. Even in family members with mutations, the major clinical event rate was relatively low. Which family members will develop disease and arrhythmias and what their risk factors are, warrants further study.

Limitations

Not all index-patients and family members underwent all diagnostic tests, as indicated in supplemental Table 2. This reflects diagnostic choices made in daily clinical practice in multiple centers. Index-patients were defined as without mutations when no pathogenic mutation was identified. Particularly among the 11% with familial disease, mutations in new ARVD/C-associated genes may be discovered. The contribution of variants of uncertain significance in index-patients with and without identified mutations was not evaluated.

Conclusion

This study of an extensive transatlantic ARVD/C cohort of 1001 individuals, including large numbers of index-patients and family members, provides insights in clinical course and long-term outcome with important clinical implications for current patient management strategies in ARVD/C. Long-term outcome was favorable in diagnosed and appropriately treated index-patients and family members. ICD implantation importantly reduced SCD incidence during follow-up and improved long-term outcome. Index-patients with and without identified mutations had a similar disease course and outcome, although disease onset was earlier in index-patients with mutations. No phenotypic and clinical outcome differences, except for cardiac mortality, were observed between ARVD/C index-patients with familial and isolated disease. Approximately one-third of family members developed ARVD/C during clinical course. Long-term outcome in family members was negatively influenced by symptoms at first presentation and the presence of mutations.

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Supplementary material

Supplementary table 1. Definitions of clinical and ECG variables employed in this study

Term	Definition
Sustained ventricular tachycardia	Ventricular tachycardia which lasts 30 seconds or more, or less than 30 s when terminated electrically or pharmacologically.
Sudden cardiac death	Death of cardiac origin that occurred unexpectedly within 1 hour of the onset of new symptoms or a death that was unwitnessed and unexpected.
Cardiac arrest	An event as described above, that is reversed, usually by cardio-pulmonary resuscitation and/or defibrillation or cardioversion.
Appropriate ICD therapy	ICD shock or antitachycardia overdrive pacing delivered in response to a ventricular tachyarrhythmia and documented by stored intracardiac ECG data.
Heart failure	Stage C heart failure (HF) was defined using the American College of Cardiology/American Heart Association heart failure staging system, but required both evidence of structural heart disease including RV abnormalities and symptoms directly attributed to HF.
ARVD/C diagnosis (as per 2010 revised Task Force Criteria)	2 major or 1 major and 2 minor criteria or 4 minor from different categories.
Index-patient	The index patient (proband) was the first affected family member seeking medical attention for ARVD/C in whom the diagnosis was confirmed (i.e. an affected person ascertained independently of family history of ARVD/C).
Family members	Individuals ascertained through family screening.
Familial ARVD/C	Fulfillment of any 2010 Task Force Criteria in the category for family history (including mutation status).
Isolated ARVD/C	Absence of any 2010 Task Force Criteria in the category for family history (including mutation status).
Mutation	Nonsense, frame shift, splice site mutations and exon deletions were all considered to be proven pathogenic mutations unless previously identified as polymorphism or non-pathogenic. Missense mutations were considered pathogenic when: 1) the minor allele frequency in the Exome Sequencing Project (ESP) dataset was ≤0.05% (NHLBI 6500 Exome data sets; EVS; http://evs.gs.washington.edu/EVS/) and 2) in silico predictive programs (SIFT) and Polymorphism Phenotyping-2 (PolyPhen-2) both predicted the genetic variants to affect protein function by a tolerance index score of <0.02 (SIFT) and >0.900 (PolyPhen-2), respectively.
DNA variants of uncertain significance	Genetic variants that did not fulfill the criteria for mutations. These variants were not included in this study.
Epsilon wave	Distinct waves of small amplitude within the ST segment in the right precordial leads and are distinct from the QRS complex.
Terminal activation duration	The longest value in V1–V3, from the nadir of the S wave to the end of all depolarization deflections. Considered abnormal when ≥55 msec.
Right bundle branch block	QRSd ≥0.12 s, secondary R wave in right precordial leads, and wide S wave in leads I and V6.

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

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Supplementary table 2. Non-invasive and invasive tests available during phenotypic evaluation of the study cohort presenting alive

	Index-patients (n=416)	**Family members** (n=537)
Electrocardiogram	413/416 (99%)	517/537 (96%)
Exercise stress test	337/416 (81%)	346/537 (64%)
Holter monitoring	309/416 (74%)	416/537 (78%)
Echocardiogram	403/416 (97%)	459/537 (86%)
Magnetic resonance imaging	319/416 (77%)	309/537 (58%)
Electrophysiological study	304/416 (73%)	78/537 (15%)
RV angiogram	153/416 (37%)	37/537 (7%)
LV angiogram	117/416 (28%)	16/537 (3%)

RV indicates right ventricular; LV: left ventricular.

Supplementary table 3. Pathogenic mutations identified in ARVD/C index-patients and family members

Gene	**Nucleotide change**	**Protein change**	**No. of index-patients**	**No. of family members**	**Total**
PKP2	c.235C>T	p.(Arg79*)	22	47	69
	c.2146-1G>C	Abnormal splice product	26	30	56
	c.1211-1212insT	p.(Val406fs)	15	29	44
	c.2386T>C	p.(Cys796Arg)	12	29	41
	c.2489+1G>A	Abnormal splice product	17	30	47
	c.397C>T	p.(Gln133*)	7	12	19
	c.2197_2202delinsG	p.(His733fs)	11	14	25
	c.1848C>A	p.(Tyr616*)	5	11	16
	c.1613G>A	p.(Trp538*)	6	9	15
	c.148_151delACAG	p.(Thr50fs)	12	6	18
	c.2489+4A>C	Abnormal splice product	4	16	20
	Deletion exon 1-4		1	5	6
	c.917-918delCC	p.(Pro318fs)	3	7	10
	c.2013delC	p.(Lys672fs)	2	7	9
	c.1643delG	p.(Gly548fs)	1	10	11
	c.1369_1372delCAAA	p.(Gln457*)	4	5	9
	c.2509delA	p.(Ser837fs)	4	4	8
	Deletion exon 1-14		1	9	10
	c.1237C>T	p.(Arg413*)	4	3	7
	c.1271T>C	p.(Phe424Ser)	1	2	3
	c.2484C>T	Abnormal splice product	0	5	5
	c.337-2A>T	Abnormal splice product	1	3	4
	c.968_971delAGGC	p.(Gln323fs)	1	3	4
	c.2034G>A	p.(Trp678*)	1	2	3
	c.1759delG	p.(Val587fs)	1	2	3
	c.2062T>C	p.(Ser688Pro)	1	2	3
	c.218dupG	p.(Asn74fs)	1	3	4

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	c.224-3C>G	Abnormal splice product	1	1	2
	c.1132C>T	p.(Gln378*)	3	2	5
	c.1171-2A>G	Abnormal splice product	1	1	2
	c.1378+1G>C	Abnormal splice product	0	5	5
	c.1378G>A	Abnormal splice product	2	3	5
	c.1803delC	p.(Asp601fs)	1	0	1
	c.1849C>T	p.(Gln617*)	2	2	4
	c.1951C>T	p.(Arg651*)	2	1	3
	c.2421C>A	p.(Tyr807*)	1	2	3
	c.2544G>A	p.(Trp848*)	1	1	2
	Deletion exon 10		1	2	3
	c.258T>G	p.(Tyr86*)	1	0	1
	c.1307_1315delins8	p.(Leu436fs)	1	0	1
	c.1368delA	p.(Lys456fs)	1	0	1
	c.1511-2A>G	Abnormal splice product	1	2	3
	c.1760delT	p.(Val587fs)	1	2	3
	c.1821dupT	p.(Val608fs)	1	2	3
	c.1844C>T	p.(Ser615Phe)	0	1	1
	c.2028G>A	p.(Trp676*)	1	0	1
	c.2145+1G>C	Abnormal splice product	1	0	1
	c.2169_2172dupAGTT	p.(Val725fs)	0	2	2
	c.2174T>A	p.(Val725Asp)	1	0	1
	c.2554delG	p.(Glu852fs)	1	0	1
	c.2197_2202delCACACCinsG	p.(His733fs)	3	0	3
	c.517C>T	p.(Gln173*)	1	1	2
	c.663C>A	p.(Tyr221*)	2	1	3
	c.253_256delGAGT	p.(Glu85fs)	1	2	3
	c.14delG	p.(Gly5fs)	1	0	1
	c.1828A>G	p.(Gln617*)	1	1	2
	c.451delT	p.(Ser151fs)	1	0	1
	c.841delA	p.(Thr281fs)	1	2	3
	Deletion exons 8-14		1	1	2
DSG2					
	c.137G>A	p.(Arg46Gln)	4	6	10
	c.874C>T	p.(Arg292Cys)	2	2	4
	c.1520G>A	p.(Cys507Tyr)	1	1	2
	c.146G>A	p.(Arg49His)	2	1	3
	c.889G>A	p.(Asp297Asn)	1	0	1
	c.2434G>T	p.(Gln812Cys)	1	1	2
	c.3140C>G	p.(Tyr1047Arg)	1	1	2
	c.136C>T	p.(Arg46Trp)	1	0	1
	c.378+2T>G	Abnormal splice product	1	0	1
	c.464_465insT	p.(Glu156fs)	1	0	1
	c.523+2T>C	Abnormal splice product	0	1	1
	c.614C>T	p.(Pro205Leu)	1	0	1
	c.1038_1040delGAA	p.(Lys346del)	1	0	1
DSC2					
	c.608G>A	p.(Arg203His)	0	2	2
	c.943-1G>A	Abnormal splice product	1	1	2

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	c.154+1G>A	Abnormal splice product	1	0	1
	c.658G>A	p.(Gly220Arg)	1	0	1
	c.942+3A>G	Abnormal splice product	1	0	1
	c.1276G>A	p.(Glu426Lys)	0	1	1
	c.2582_2585dupGAAG	p.(Gly863fs)	1	0	1
DSP					
	c.1264G>A	p.(Glu422Lys)	1	2	3
	c.478C>T	p.(Arg160*)	1	0	1
	c.3337C>T	p.(Arg1113*)	1	1	2
	c.151C>T	p.(Gln51*)	1	0	1
	c.1333A>G	p.(Ile445Val)	1	0	1
	c.3160_3169delAAGAACAA	p.(Lys1052fs)	1	3	4
	c.5212C>T	p.(Arg1738*)	1	3	4
	c.6478C>T	p.(Arg2160*)	1	3	4
	c.6496C>T	p.(Arg2166*)	1	1	2
	c.4395T>G	p.(Tyr1465*)	1	1	2
	c.5419C>T	p.(Gln1807*)	1	1	2
	Deletion 6p24.1p25.1		0	1	1
JUP					
	c.56C>T	p.(Thr19Ile)	1	0	1
	c.475G>T	p.(Val159Leu)	1	0	1
TMEM43					
	c.1073C>T	p.(Ser358Leu)	1	0	1
PLN					
	c.40_42delAGA	p.(Arg14del)	22	26	48
Multiple mutations					
Digenic and trigenic					
PKP2,DSP large deletion	*PKP2* c.2197_2202delinsG, *DSP* deletion 6p2431p25.1	p.(His733fs) & deletion	0	1	1
PLN, DSP	*PLN* c.40_42delAGA, *DSP* c.6881C>G	p.(Arg14del) & p.(Ala2294Gly)	0	1	1
DSC2,RYR	*DSC2* c.1276G>A, *RYR2* c.4693C>G	p.(Glu426Lys) & p.(Pro1565Ala)	1	0	1
PKP2,DSG2	*PKP2* c.1237C>T, *DSG2* c.829-1_835del	p.(Arg413*) & p.(Leu277fs)	1	0	1
DSG2, PKP2, TMEM43	*DSG2* c.1072G>A, *PKP2* deletion exon 1-14, *TMEM43* c.718C>T	p.(Ala358Thr), exon deletion 1-14, p.(Arg240Cys)	1	0	1
PKP2,DSG2	*PKP2* c.1211-1212insT, *DSP* c.269A>G	p.(Val406fs) & p.(Gln90Arg)	1	0	1
PKP2,DSG2	*PKP2* c.2489+4A>C, *DSP* c.1982A>T	Abnormal splice product & p.(Asn661Ile)	1	0	1
PKP2, PKP2, DSG2	*PKP2* c.397C>T , *PKP2* c.2615C>T, DSG2 c.1480G>A	p.(Gln133*) & p.(Thr872Ile) & p.(Asp494Asn)	1	0	1
PKP2,	*PKP2* c.235C>T, c.2487delT	p.(Arg79*) & p.(Phe829fs)	1	1	2
Compound Heterozygote					
PKP2, PKP2	*PKP2* c.[397C>T;2615C>T]	p.[(Gln133*);(Thr872Ile)]	3	5	8
DSP,DSP	*DSP* c.6478C>T, *DSP* c.943C>T	p.(Arg2160*) & p.(Arg315Cys)	1	0	1
DSG2,DSG2	*DSG2* c.523+2T>C, *DSG2* c.1038_1040delGAA	Abnormal splice product & p.(Lys346del)	1	0	1
DSG2,DSG2	*DSG2* c.918G>A, *DSG2* c.146G>A	p.(Trp306*) & p.(Arg49His)	1	0	1

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PKP2, PKP2	*PKP2* deletion exon 8, *PKP2* c.746G>A	deletion & p.(Ser249Asn)	1	0	1
DSG2, DSG2	*DSG2* c.1038_1040delGAA, *DSG2* c.1072G>A	p.(Lys346del) & p.(Ala358Thr)	1	0	1
Homozygous					
	PKP2 c.2484C>T homozygosity	Abnormal splice product	1	0	1
	DSC2 c.608G>A homozygosity	p.(Arg203His)	1	0	1
Total			276	409	685

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; *PKP2*: plakophilin-2; *DSG2*: desmoglein-2; *DSC2*: desmocollin-2; *DSP*: desmoplakin; *JUP*: plakoglobin; *TMEM43*: transmembrane protein 43; *PLN*: phospholamban; no.: number.

Supplementary table 4. Revised 2010 Task Force Criteria for ARVD/C in 416 index-patients presenting alive

2010 TFC	**Index-patients** number (%)	**Without identified mutation** number (%)	**With mutation** number (%)	**With mutation** number (%)				**p-value***
	Total n=416	**Total w/o mutations** n=152	**Total with mutations** n=264	***PKP2*** n=192	**Other desmo-somal** n=33	***PLN/ TMEM43*** n=22	***CH/DG/ HO*** n=17	
Structural RV abnormalities								
Major	264 (63)	93 (61)	171 (65)	119 (62)	23 (70)	15 (68)	14 (82)	0.539
Minor	50 (12)	20 (13)	30 (11)	25 (13)	2 (6)	1 (4)	2 (12)	0.564
Depolarization abnormalities								
Epsilon wave (major)	63 (15)	22 (14)	41 (15)	32 (17)	2 (6)	3 (14)	4 (23)	0.833
prol TAD/ Late potentials (minor)#	277 (67)	105 (69)	172 (65)	125 (65)	20 (61)	17 (77)	10 (59)	0.316
Repolarization abnormalities								
Negative T waves V1-3 (major)	308 (74)	106 (70)	202 (76)	151 (79)	26 (79)	9 (41)	16 (94)	148
Negative T waves V1-2 (minor)	29 (7)	12 (8)	17 (6)	16 (8)	0	1 (4)	0	0.564
Negative T waves V1-4 in case of RBBB (minor)	30 (7)	16 (10)	14 (5)	10 (5)	3 (9)	0	1 (6)	0.045
Negative T waves V4-6 (minor)	13 (3)	1 (0.7	12 (4)	2 (1)	4 (12)	6 (27)	0	0.037

Supplementary table 4. Continued

Ventricular arrhythmias								
LBBB VT with superior axis (major)	181 (44)	69 (45)	112 (42)	83 (43)	11 (33)	10 (45)	8 (47)	0.909
LBBB VT with inferior or unknown axis (minor)	268 (64)	95 (63)	173 (66)	126 (66)	18 (55)	17 (77)	12 (71)	0.176
>500 VES/24 hours	205 (49)	79 (52)	126 (48)	82 (43)	19 (58)	15 (68)	10 (60)	0.425
Family history								
1st degree relative fulfilling 2010 TFC (major)	27 (6)	6 (4)	21 (8)	18 (9)	3 (9)	0	0	
Pathogenic mutation (major)	242 (58)	0	242 (92)	192 (100)	33 (100)	0	17 (100)	
Histopathological ARVD/C diagnosis in 1st degree relative (major)	17 (4)	5 (3)	12 (4)	11 (6)	1 (3)	0	0	
SCD <35 years suspected due to ARVD/C in 1st degree relative (minor)	25 (6)	4 (3)	21 (8)	13 (7)	3 (9)	4 (18)	1 (6)	
History of ARVD/C not possible or practical to determine (minor)	11 (3)	6 (4)	5 (2)	4 (2)	0	0	1 (6)	
2nd degree relative fulfilling 2010 TFC (minor)	5 (1)	3 (2)	5 (2)	3 (2)	2 (6)	0	0	

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; Revised 2010 TFC: Task Force Criteria for diagnosis; RV: right ventricular; prol TAD: prolonged terminal activation duration; RBBB: right bundle branch block; LBBB: left bundle branch block; VT: ventricular tachycardia; VES: ventricular extrasystoles; SCD: sudden cardiac death; *PKP2*: plakophilin-2; *PLN*: phospholamban; *TMEM43*: transmembrane protein 43; CH: compound heterozygous mutations; DG: digenic mutations; HO: homozygous mutations. *P-value represents comparison between total with mutations (n=264) and without identified mutations (n=152), #: minor criteria prolonged TAD and late potentials were grouped together since signal averaged ECG was not performed routinely in the Dutch individuals and it was not possible to evaluate prolonged TAD in all U.S. based individuals. Criteria of the same TFC category can only be counted once.

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Supplementary table 5. Clinical presentation and outcomes of 416 index-patients presenting alive stratified by genotype

Clinical characteristics	With mutations n=264 (63%)	Without identified mutations n=152 (37%)	p-value
Clinical presentation			
Asymptomatic	14 (5%)	6 (4%)	0.534
Symptomatic without			
sustained VA	93 (35%)	58 (38%)	0.618
Sustained VA	137 (52%)	83 (55%)	0.951
Resuscitated SCD	20 (8%)	5 (3%)	0.077
Clinical Outcome			
FU (yrs, median, IQR)	8 (12)	6 (10)	0.198
Sustained VA during FU	194 (73%)	107 (70%)	0.565
ICD implantation	225 (85%)	126 (83%)	0.528
Primary prevention	56 (25%)	40 (32%)	0.160
Secondary prevention	169 (75%)	86 (68%)	
Appropriate ICD intervention	134 (51%)	75 (49%)	0.935
Primary prevention	22 (16%)	22 (29%)	0.026
Secondary prevention	112 (84%)	53 (71%)	
Symptomatic heart failure	33 (13%)	21 (14%)	0.701
Cardiac transplantation	10 (4%)	8 (5%)	0.476
Cardiac death	12 (5%)	9 (6%)	0.628
SCD	5 (2%)	8 (5%)	
Heart failure	5 (2%)	1 (1%)	
Heart failure and arrhythmias	2 (1%)	-	

VA indicates ventricular arrhythmias; SCD: sudden cardiac death; FU: follow-up duration; yrs: years; IQR: interquartile range; ICD: implantable cardioverter-defibrillator.

Supplementary table 6. Phenotype comparison of index-patients presenting alive with familial and isolated ARVD/C

Clinical characteristics	**Index-patients with familial ARVD/C** n= 295	**Index-patients with isolated ARVD/C and without mutations** n= 121	**p-value**
FU (yrs, median, IQR)	8 (12)	6 (10)	0.294
Negative T waves V1-3	221/291 (76%)	87/121 (72%)	0.389
Negative T waves V1-2	19/291 (7%)	10/121 (8%)	0.531
Negative T waves V1-4 with RBBB	16/291 (6%)	14/121 (12%)	0.031
Negative T waves V4-6	12/291 (4%)	1/121 (0.8%)	0.120
Epsilon wave V1-3	43/291 (15%)	20/119 (17%)	0.605
Prolonged TAD/ Late potentials	192/291 (66%)	85/120 (71%)	0.340
Sustained VA	244/295 (83%)	100/121 (83%)	0.987
VT with LBBB morph. and superior axis	126/273 (46%)	55/121 (46%)	0.898
VT with LBBB morph. and inferior axis	187/274 (68%)	81/121 (67%)	0.798
>500 VES/24 hours	137/253 (54%)	68/121 (56%)	0.710
Structural major abn.	186/292 (64%)	78/121 (65%)	0.883
Structural minor abn.	33/292 (11%)	17/121 (14%)	0.436
RVEF (%) (mean±SD)	37±10 (87/295)	38±11 (64/121)	0.596
LVEF (%) (median, IQR)	55 (14) (136/295)	55 (11) (100/121)	0.783
Symptomatic heart failure	41/295 (14%)	13/121 (11%)	0.385
ICD implantation	248/295 (84%)	103/121 (85%)	0.788
Cardiac transplantation	12/295 (4%)	6/121 (5%)	0.685
Cardiac death	20/295 (7%)	1/121 (0.8%)	0.013
SCD	13/20 (65%)	-	
Heart failure	5/20 (25%)	1/1	
Heart failure and arrhythmias	2/20 (10%)	-	

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; FU: follow-up duration; yrs: years; IQR: interquartile range; RBBB: right bundle branch block; TAD: terminal activation duration; VA: ventricular arrhythmias; VT: ventricular tachycardia; LBBB: left bundle branch block; VES: ventricular extrasystoles; abn: abnormalities; RVEF: right ventricular ejection fraction; SD: standard deviation; LVEF: left ventricular ejection fraction; ICD: implantable cardioverter-defibrillator; SCD: sudden cardiac death.

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Supplementary table 7. Revised 2010 Task Force Criteria for ARVD/C in 537 family members presenting alive

2010 TFC	Family members	Without identified mutation	With mutation	With mutation				p-value*
	number (%)	number (%)	number (%)	number (%)				
	Total	**Total w/o mutations**	**Total with mutations**	***PKP2***	**Other desmo-somal**	***PLN/ TMEM43***	***CH/DG/ HO***	
	n=537	n=152	n=385	n=325	n=28	n=25	n=7	
Structural RV abnormalities								
Major	49 (9)	3 (2)	46 (12)	41 (13)	1 (4)	3 (12)	1 (14)	<0.001
Minor	44 (8)	8 (5)	36 (9)	32 (10)	1 (4)	3 (12)	0	0.094
Depolarization abnormalities								
Epsilon wave (major)	12 (2)	1 (0.7)	12 (3)	10 (3)	0	0	2 (29)	0.023
prol TAD/								
Late potentials (minor)#	164 (31)	48 (32)	116 (30)	102 (31)	7 (25)	3 (12)	4 (57)	0.834
Repolarization abnormalities								
Negative T waves V1-3 (major)	89 (17)	13 (9)	76 (19)	71 (22)	3 (11)	1 (4)	1 (14)	0.001
Negative T waves V1-2 (minor)	42 (8)	7 (5)	35 (9)	32 (10)	1 (4)	1 (4)	1 (14)	0.071
Negative T waves V1-4 in case of RBBB (minor)	5 (0.9)	1 (0.7)	4 (1)	4 (1)	0	0	0	1.000
Negative T waves V4-6 (minor)	15 (3)	2 (1)	13 (3)	4 (1)	0	8 (32)	1 (14)	0.251
Ventricular arrhythmias								
LBBB VT with superior axis (major)	20 (4)	1 (0.7)	20 (5)	19 (6)	0	1 (4)	1 (14)	0.003
LBBB VT with inferior or unknown axis (minor)	47 (9)	7 (5)	40 (10)	35 (11)	1 (4)	4 (16)	1 (14)	0.022
>500 VES/24 hours	107 (20)	16 (10)	91 (24)	77 (24)	1 (4)	8 (32)	5 (71)	<0.001

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Supplementary table 7. Continued

Family history							
1st degree relative fulfilling 2010 TFC (major)	430 (80)	141 (93)	313 (81)	261 (80)	23 (82)	23 (92)	6 (86)
Pathogenic mutation (major)	360 (67)	0	360 (94)	325 (100)	28 (100)	0	7 (100)
Histopathological ARVD/C diagnosis in 1st degree relative (major)	95 (18)	44 (29)	51 (13)	47 (14)	3 (11)	0	1 (14)
SCD <35 years suspected due to ARVD/C in 1st degree relative (minor)	48 (9)	7 (5)	41 (11)	37 (11)	1 (4)	2 (8)	1 (14)
History of ARVD/C not possible or practical to determine (minor)	10 (2)	0	10 (3)	10 (3)	0	0	0
2nd degree relative fulfilling 2010 TFC (minor)	70 (13)	20 (13)	50 (13)	46 (14)	3 (11)	1 (4)	0

ARVD/C indicates Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy; Revised 2010 TFC: Task Force Criteria for diagnosis; RV: right ventricular; prol TAD: prolonged terminal activation duration; RBBB: right bundle branch block; LBBB: left bundle branch block; VT: ventricular tachycardia; VES: ventricular extrasystoles; SCD: sudden cardiac death; *PKP2*: plakophilin-2; *PLN*, phospholamban; *TMEM43*: transmembrane protein 43; CH: compound heterozygous mutations; DG: digenic mutations; HO: homozygous mutations. *P-value represents comparison between total with mutations (n=385) and without identified mutations (n=152), #: minor criteria prolonged TAD and late potentials were grouped together since signal averaged ECG was not performed routinely in the Dutch individuals and it was not possible to evaluate prolonged TAD in all U.S. based individuals. Criteria of the same TFC category can only be counted once.

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CHAPTER 13

General Discussion and Future Perspectives

The studies in this thesis evaluated genetic and clinical aspects of Arrhythmogenic Cardiomyopathy (AC). The aims in the first part of this thesis were to evaluate the desmosomal and non-desmosomal genetic contribution, to facilitate the interpretation of genetic screening results, and to define the impact of genotype on phenotype and disease course in AC. The second part addresses the aim to evaluate invasive and non-invasive measurements of arrhythmic substrate, and to define the long-term follow-up and outcome in AC index patients and at risk family members. The ultimate goal of this thesis was to support and improve early diagnosis and patient management.

Impact of genotype in Arrhythmogenic Cardiomyopathy

Arrhythmogenic Cardiomyopathy is usually related to desmosomal gene mutations, as described in Chapter 3. An important genetic contribution (13%) of the non-desmosomal phospholamban (*PLN*) founder mutation c.40_42delAGA to AC to the Dutch population was observed in Chapter 5. This mutation was the most frequently identified single AC related mutation in The Netherlands. In addition, the *PLN* gene is the second most frequently mutated gene in AC in The Netherlands at present, following the plakophilin-2 (*PKP2*) gene. Notably, the phenotype of most AC patients with the *PLN* mutation, implied the underlying genotype. The AC patients with the *PLN* mutation had a classic phenotype with predominant right ventricular involvement and arrhythmias, fulfilling the Task Force Criteria for diagnosis, disproving the argument that these patients have in fact dilated cardiomyopathy. Moreover, dilated cardiomyopathy patients with the *PLN* mutation had significantly more ventricular arrhythmias compared to dilated cardiomyopathy patients without the mutation and often fulfilled diagnostic AC criteria, supporting the AC concept: first ventricular arrhythmias and only later structural involvement (Chapter 4).[1] Although one could argue that the *PLN* mutation is a Dutch affair, the mutation is also found in Germany, Greece, Spain, and North America albeit at lower rates.[1-4] In the national AC dataset, a pathogenic mutation, including the *PLN* mutation, is now found in 72% of index patients.

The results of the study on the *PLN* mutation and the study on the long-term outcomes in AC showed that still in a substantial proportion of index patients (28% and 37%, respectively) no mutation could be identified with screening of the desmosomal genes *PKP2*, desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*) and non-desmosomal genes transmembrane protein 43 (TMEM43) and *PLN* (Chapters 5 and 12).[5-9] In addition, a family history of AC, i.e. suggesting a genetic background for the disease, was present in 21% of index patients without identified mutations. The percentage of patients without identified mutations was even larger in other studies.[10-12] It can be questioned whether these patients are truly 'mutation negative'. With the assessment of more cardiomyopathy-related genes in addition to the candidate genes, pathogenic mutations are found in genes usually associated with other forms of cardiomyopathy (unpublished data). This suggests that pathogenic mutations can result in important phenotypic heterogeneity, not only in disease severity, but even within disease entities. This overlap is also observed in other cardiomyopathies. Meticulous genetic and clinical assessment of family members, cosegregation analysis, and collaborative cataloging of mutations are crucial in understanding and unraveling of this conundrum.

The most important limitation of genetic screening currently, is the misbalance between the amount of new findings and increase in knowledge on the interpretation of these findings.

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Therefore, genetic testing frequently results in the finding of genetic variants of uncertain clinical significance. Missense variants are especially controversial since they were found in similar rates in AC cases and controls.[13] Conditions to distinguish pathogenic AC-related missense mutations, including race/ethnicity, variant location, and sequence conservation, have been reported.[13] In order to enhance objective, transparent classification of missense variants in AC an algorithm with synergistic use of *in silico* algorithms SIFT, PolyPhen-2, and the minor allele frequency (MAF) in control populations for the prediction of pathogenicity of missense variants was deployed in Chapter 6. The algorithm was able to discriminate pathogenic missense variants. These results are consistent with a study by Guidicessi et al.[14] that demonstrated that the combined use of conservation across species, Grantham values, SIFT, and PolyPhen-2, provided the ability to distinguish case-derived from control-derived missense variants in long-QT syndrome. Additional use of the MAF in control populations, as we did in our algorithm, may have added value over the use of exclusively conservation information and i*n silico* prediction tools. The relevance of this algorithm in other genetic cardiomyopathies will have to confirm this hypothesis. Similarly, prediction algorithms for the potential deleterious effect of genetic variants on splicing are suboptimal presently, with over or underestimation of the spliceogenic potential as previously demonstrated.[15, 16] The pathogenic potential of 9 AC related splice site variants with a predicted deleterious effect, was experimentally validated in Chapter 7 by mRNA analysis. The pathogenic potential was accurately predicted in 6 variants, but for 3 variants no deleterious effect could be demonstrated. The correct classification of genetic variants is important for patient management and family screening purposes. Summarizing these data, at present i*n silico* predictions assist in the assessment of pathogenicity of variants but only functional analysis can ultimately prove pathogenicity. Information on the genetic variation in large numbers of control individuals is essential in the interpretation and classification of genetic testing results.

The extent of the influence of genotype on the disease course and outcome was largely unknown. Previous genotype-phenotype studies, although limited by population size and predominant inclusion of index patients, suggested that genotype affects arrhythmia and heart failure propensity in AC.[12, 17-21] In Chapter 8, the prognostic impact of genotype on AC disease course was assessed in a large transatlantic US/Dutch cohort of mutation carriers. The study showed that individuals with *DSP* mutations presented significantly more often with sudden cardiac death and had more left ventricular dysfunction and heart failure. In addition, individuals with multiple pathogenic mutations had earlier onset of symptoms and more sustained ventricular arrhythmias and heart failure compared to patients with single mutations. Patients with the *PLN* mutation presented at later age and had more left ventricular dysfunction in comparison with desmosomal mutation carriers. Remarkably, the type of mutation did not influence outcomes. These results imply that genetic screening and identification of pathogenic mutations may assist in gene-specific, 'tailored' management of AC patients.

A limitation of this and most other studies, is that the impact of genetic variants of unknown clinical significance as genetic modifiers of disease course was not assessed. A gene dose effect was demonstrated in the study by Quarta et al.[12]: family members with more genetic variants had more penetrant disease. The AC phenotype may also be modified by environmental factors. Exercise was previously demonstrated in a mouse model to be a trigger and accelerator of the AC phenotype in a mouse model with *JUP* haploinsufficiency.[22] Recently, the effect of

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exercise on AC disease expression was corroborated in humans. Endurance athletes were more likely to have symptoms at young age, fulfil the 2010 Task Force Criteria, and had lower survival free from ventricular tachycardia (VT), ventricular fibrillation (VF), and heart failure.[23]

Clinical course and long-term outcome in Arrhythmogenic Cardiomyopathy

In the study in Chapter 8, it was shown that a presentation with sudden cardiac death (SCD) occurred at a significantly younger age than a presentation with sustained monomorphic VT. Quarta et al.[12], did a similar observation with the ages at SCD of 51 index patients. Of these index patients, 23 (45%) died before the age of 30 years old and the incidence of SCD decreased with age. For some reason AC patients present with SCD mostly in the early concealed stages of disease, whereas sustained monomorphic VT is the presenting symptom in later overt disease stages. It may be that this phenomenon is related to the current hypothesis on the pathogenesis in AC. Desmosomal dysfunction with loss of mechanical cell-cell adhesion, leads to down-regulation and/or redistribution of other intercalated disk proteins, i.e. gap junction proteins (connexin43) and sodium channels (Nav1.5). These alterations give rise to electrical cell-cell uncoupling and slow conduction respectively, thereby providing a substrate for *early* activation delay. Presumably, at a *later stage* myocyte loss and fibrofatty replacement will have a major impact on tissue architecture, giving rise to zig-zag conduction pathways and load mismatch, further contributing to enhanced activation delay. This hypothesis on the difference in arrhythmogenic substrate in early and later disease stages has been studied in animal models. In a *PKP2* haploinsufficient mouse model, Cerrone at al.[24] demonstrated sodium channel dysfunction and increased susceptibility of ventricular arrhythmias and death upon pharmacological challenge with flecainide. Moreover, ventricular arrhythmias and SCD occurred in the absence of structural heart disease, although ultrastructural changes were present. Similarly, slowed conduction and increased arrhythmia propensity as a consequence of intercellular space widening and sodium channel dysfunction was observed in transgenic mice overexpressing a *DSG2* mutation.[25] In addition, in *DSP* haploinsufficient mice, delayed conduction and inducible ventricular arrhythmias were observed prior to overt structural changes. Mislocalization of connexin43 seemed to associate with the activation delay and arrhythmias.[26] Diagnosis and adequate risk management of AC patients in early concealed disease stages with increased risk of VT/VF and SCD is at present the biggest challenge facing physicians.

As activation delay plays a pivotal role in increased arrhythmia susceptibility also in early stages of the disease, parameters reflecting activation delay may improve early AC diagnosis and thereby risk management. Activation delay as parameter of the arrhythmogenic substrate can be evaluated invasively by electrophysiological studies.[27] Non-invasive assessment of activation delay has been shown feasible in previous studies by the recording of epsilon waves and terminal activation duration (TAD) in right precordial leads on the ECG and by the signal averaged ECG, which is also described in Chapter 11.[28-30] The recording of epsilon waves, prolonged TAD, and late potentials on signal averaged ECG have been incorporated in the revised 2010 Task Force Criteria (TFC) for ARVD/C diagnosis.[31] However, these parameters predominantly reflect activation delay and possible substrate for arrhythmias in the RVOT. Recently, our group demonstrated early involvement of the subtricuspid area in ARVD/C followed by RVOT involvement in later stages.[32] Non-invasive parameters reflecting early electrical and structural abnormalities in the

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subtricuspid area have not been reported. The study described in Chapter 10, showed electrical abnormalities in the subtricuspid area in all 19 patients included. Moreover, predominant VT morphology in this cohort suggested a VT origin in this area. Three ECG parameters were considered: 1) late isolated potentials in inferior leads II, III, aVF (observed in 37%), 2) negative T waves in II, III, aVF (observed in 26%), and 3) left axis deviation (observed in 37%). ECG criteria reflecting abnormalities in the subtricuspid area combined with the Task Force Criteria for ARVD/C, may increase the diagnostic yield. However, identification of these new ECG parameters is challenging and awaits optimization.

Arrhythmogenic Cardiomyopathy is considered a progressive disease, as illustrated by Chapter 9. Nevertheless, information on the long-term follow-up and outcome of AC index patients and particularly of family members is limited. Previous studies mostly predated genetic testing availability, were largely limited by population size, and had little universal relevance since small national datasets were assessed.[33-39] Moreover, the conclusions regarding the long-term outcome varied importantly among the studies. Assessment of the long-term outcome in an extensive transatlantic cohort of 1001 individuals (Chapter 12) show that, despite the fact that the clinical presentation and ARVD/C disease course is characterized by sustained VA, diagnosed and appropriately treated index patients have a favorable outcome. Index patients with mutations have an earlier ARVD/C onset, whereas the disease course and burden is similar between those with and without identified mutations. Furthermore, one-third of family members develop ARVD/C and their long-term outcome is related to symptoms at first presentation and the presence of mutations. This study confirmed and extended insights in the long-term follow-up in ARVD/C and provided important clinical implications for current patient management strategies.

One of those implications for patient management strategies is the use of an implantable cardioverter-defibrillator. At present, ICD implantation, contrary to anti-arrhythmic medication, is the only proven lifesaving strategy to prevent SCD. A remarkably high number of index patients without ICDs included in the study described in Chapter 12 died of SCD during follow-up in comparison with index patients that had an ICD implanted (16% vs. 06%). Nonetheless, increasing knowledge on the epicardial substrate of arrhythmias and improved techniques have led to better outcomes of catheter ablation as treatment option in AC.[40, 41] However, anti-arrhythmic medication, ICD implantation, and catheter ablation are all symptomatic treatment modalities. Future therapeutic treatment modalities preferable include' upstream therapy'.

Future perspectives

Overall, collaboration is essential for adequate studies to improve insight into relatively rare diseases such as AC. In The Netherlands, a national collaboration of all in ICIN participating University Medical Centers has resulted in a large dataset with genetic and phenotypic characteristics of AC index patients and family members.[21] This collaboration has expedited studies on AC related genes, founder mutations, new diagnostic AC parameters, and clinical outcome of index patients and family members. In addition, since 2011 a transatlantic collaboration has laid the foundation for studies with large numbers of index patients and family members with presumably global applicability (Chapters 8 and 12). Continuation of inclusion of more individuals, longer follow-up, and implementation of results of genetic and clinical studies are needed for further risk stratification optimization.

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Genetical perspectives

The availability of new genetic techniques such as mutation detection arrays, next generation sequencing, including whole exome or even whole genome sequencing, may improve insight into the genetic background of AC. However, more questions will be raised with these new techniques, such as the genetic overlap between cardiomyopathy entities, which will need further exploration. Whether all forms of AC will be genetically explained by these techniques or an isolated environmentally induced phenotype exists, remains to be elucidated.

More information on the genetic background of the disease may also result in increased mechanistic insights of the disease. In particular, functional analysis of mutations and genetic variants by high throughput systems (zebra fish), exploration of potential new proteins involved in remodeling of the intercalated disk (proteomics) and application of patient specific induced pluripotent stem cells may improve pathophysiological knowledge in AC.

The modifying role of genetic variants of unknown clinical significance in AC should be further addressed. A gene dose effect was previously shown in a relatively small subset of family members.[12] The cumulative effect of genetic variants was unexpectedly high in a genome wide association study in Brugada syndrome patients.[42] A similar contribution of genetic variance can be expected in AC. Finally, epigenetic phenomena may also play a role in the phenotypic heterogeneity characterizing AC.

Clinical perspectives

Insight into the early concealed stage of AC is elusive presently. Further studies with meticulous histopathological, immunohistochemical, and ultrastructural evaluation of autopsy cases and extensive clinical evaluation, with amongst others electrophysiological studies with possible pharmacological challenges, of patients with aborted SCD are warranted. Exploration of early subtricuspid involvement, by electrophysiological, imaging, and ECG studies, will have to be subject of future studies. Electrical abnormalities in the subtricuspid area are found in the large majority of AC patients (Chapter 10).[32] Structural involvement of the subtricuspid area is early and frequently found with echocardiographic deformation imaging and MRI analysis.[32, 43] Preferably, these findings are translated to risk assessment in AC.

In addition to optimization of risk stratification in AC, optimization of therapeutic options warrants future studies. A new compound, SB216763, a drug previously studied for bipolar disorder, has been shown to be able to reverse the AC phenotype and seems very promising as future ‘upstream’ therapeutic option.[44]

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APPENDIX

Summary
Nederlandse Samenvatting
Acknowledgements/Dankwoord
Curriculum Vitae
List of Publications

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Summary

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a heart muscle disease with an estimated prevalence of 1:1000-1:5000. The disease is characterized by ventricular arrhythmias, increased risk for sudden cardiac death (SCD), and structural and functional abnormalities, predominantly in the right ventricle (RV). Nonetheless, abnormalities in both the RV and left ventricle (LV), or even predominantly in the LV have also been described. Therefore, Arrhythmogenic Cardiomyopathy (AC) is the preferred terminology nowadays.

In approximately 60% of AC patients a genetic mutation that is responsible for the disease can be identified. Most mutations are found in genes encoding desmosomal proteins. Desmosomes are complexes of proteins that are involved in the adhesion between cardiac cells. Gene mutations can give rise to dysfunction of desmosomal proteins. Desmosomal dysfunctions causes a less stable mechanical connection but also negatively influences the electrical coupling between the cells. At this early disease stage, ventricular arrhythmias and SCD can already occur. The mechanical and electrical uncoupling of cardiac cells most likely result in cell death and additional slowing of the electrical conduction in later disease stages. The cardiac cells that have died are replaced by scar and fat tissue. This gives rise to structural and functional alteration in the ventricles and provides the substrate for ventricular arrhythmias.

Since the first major description of AC, tremendous progress has been made in the understanding of the genetic substrate for AC. When a disease causing mutation is found in index patients, cascade screening of family members for that mutation and the disease can be initiated. However, genetic testing frequently results in the finding of genetic variants of uncertain significance. The analysis of such variants is suboptimal presently. Moreover, in a substantial portion of AC patients, no disease causing mutation can be identified.

The diagnosis of AC is made by international, consensus based criteria. These diagnostic criteria (Task Force Criteria) were first formulated in 1994. In 2010 the diagnostic criteria were revised, including amongst others criteria for left ventricular involvement and gene mutations. This revised set of criteria improved the diagnostic yield. Despite the increased sensitivity of these diagnostic criteria, diagnosis in the early concealed stage of AC remains the biggest challenge for physicians.

This thesis

This thesis includes genetic and clinical studies in AC index patients and family members. The aims were two-fold. First, the aim was to evaluate the contribution of desmosomal and non-desmosomal gene mutations to AC, to facilitate the interpretation of genetic testing results, and to study the impact of the genotype on clinical course in AC. Second, the aim was to assess invasive and non-invasive measurements of arrhythmogenic substrate and to provide insight into the clinical course and long-term follow-up of AC index patients and family members. The ultimate goal of this thesis was to support and improve early diagnosis and patient management in AC. The background, aims, and outline of this thesis are detailed in **Chapter 1**. A general introduction into and extensive literature overview of AC is provided in **Chapter 2**. The epidemiology, presenting signs and symptoms, criteria for diagnosis, differential diagnosis, molecular genetic background, risk stratification, clinical management, and AC research in The Netherlands are described.

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Impact of genotype in Arrhythmogenic Cardiomyopathy

In **Chapter 3**, the genetic background of AC is detailed. The familial occurrence of AC has been recognized since the first descriptions of the disease. The majority of mutations are found in the desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmocollin-2 (*DSC2*), desmoglein-2 (*DSG2*). In a minority of cases non-desmosomal gene mutations have been associated with AC. The inheritance pattern is usually autosomal dominant but autosomal recessive forms are also known. Mutations underlying AC show incomplete penetrance and variable clinical expression. Therefore, genetic testing results should be viewed as probabilistic and part of the overall clinical assessment. Genotype-phenotype correlation analysis in AC may assist in the risk stratification of patients and family members.

Chapter 4 describes the cosegregation analysis in a large family with left-dominant AC. The *PKP2* variant c.419C>T, previously reported as disease causing, and the recently identified non-desmosomal founder mutation in phospholamban (*PLN*, c.40_42delAGA) were identified in this family. Six family members had AC and seven (including the six with AC) had LV abnormalities (inverted T waves in V4-6, LV wall motion abnormalities and late enhancement, reduced LV ejection fraction). The *PLN* mutation was found in nine family members, including the six with AC and all seven with LV abnormalities. Low voltages on the ECG (<0.5mV in standard leads) were seen in four of the nine *PLN* mutation-positive subjects. None of the three family members with the single *PKP2* variant showed any sign of RV or LV involvement. Thus, the *PLN* mutation cosegregated with AC and electrocardiographic and structural LV abnormalities. In this family there was no evidence for the disease causing contribution of the *PKP2* variant.

The study of the non-desmosomal founder mutation in *PLN* (c.40_42delAGA) is continued in **Chapter 5**. In this chapter, the contribution of desmosomal mutations and the non-desmosomal *PLN* mutation to AC diagnosis according to the revised 2010 diagnostic criteria was evaluated in 142 Dutch AC index patients. Of these, 83 (58%) had disease causing desmosomal mutations, 19 (13%) tested positive for the *PLN* mutation, and in 40 (28%) no mutation could be identified. Phenotypic comparison of desmosomal and non-desmosomal *PLN* mutation carriers, showed that *PLN* mutation carriers more often had a low voltage ECG, inverted T waves in V4-6, and additional structural and functional LV abnormalities, whereas desmosomal mutation carriers had more solitary RV abnormalities. The revised 2010 diagnostic criteria, including criteria for LV involvement, included 7 *PLN* mutation carriers that did not fulfil the original 1994 diagnostic criteria. This study showed that there is a substantial contribution of the non-desmosomal *PLN* founder mutation to AC in The Netherlands and that *PLN* mutation carriers have AC with a distinct phenotype.

As stated previously, genetic testing in AC frequently results in the finding of genetic variants of uncertain significance. The analysis and classification of these variants is challenging. The disease causing potential of genetic variants is routinely estimated by in bioinformatic prediction algorithms. Optimization of the categorization of genetic variants of uncertain significance in AC was the objective of the studies described in **Chapter 6** and **7**. The prediction of the disease causing potential of AC missense variants was evaluated in **Chapter 6**. The study included 341 family members with desmosomal or non-desmosomal mutations from US and Dutch AC cohorts. Missense variants were considered disease causing when they fulfilled the following criteria: 1) both bioinformatic algorithms SIFT and PolyPhen-2 predicted pathogenicity (SIFT

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score <0.02, PolyPhen-2 score >0.900) and 2) minor allele frequency (MAF) in ESP exome database was low (≤0.05%). Disease causing missense variants were identified in 48 (14%) family members. Phenotypic characteristics and ventricular arrhythmia free survival curves were similar in family members with missense and non-missense (truncating/splice site) variants. Thus, missense variants can have a similar disease causing potential as non-missense variants in family members of AC index patients. The disease causing potential was correctly predicted by the combined criteria from SIFT, PolyPhen-2, and MAF in exomes. In **Chapter 7**, the disease causing potential of AC splice site variants is studied. Using RNA derived from variant carriers, the effect of the variants on splicing was evaluated *in vitro*. For all nine variants in *PKP2*, *JUP*, *DSG2*, and *DSC2*, an effect on splicing was predicted by software algorithms. Six of the nine variants had a deleterious effect on splicing indicating the disease causing potential, whereas of three variants no effect on splicing was observed. In three patients the RNA analysis and subsequent adjudication of disease causing gene mutations resulted in the fulfilment of the diagnostic criteria for AC. These results highlight the importance of functional assessment of potential splice site variants to improve patient care and facilitate cascade screening.

In **Chapter 8**, the impact of genotype in AC is assessed. In a large transatlantic cohort of 577 index patients and family members enrolled in the Johns Hopkins University (JHU) and Interuniversity Cardiology Institute of the Netherlands (ICIN) AC cohorts, all with disease causing gene mutations, the influence of the genotype on clinical course and arrhythmic outcome was studied. Individuals that presented with SCD or ventricular fibrillation were significantly younger than those presenting with sustained monomorphic ventricular tachycardia. Individuals with multiple disease causing mutations had significantly earlier occurrence of ventricular arrhythmias, a lower ventricular arrhythmia free survival, and more frequent left ventricular dysfunction, heart failure, and cardiac transplantations as compared to those with only one disease causing mutation. Left ventricular dysfunction and heart failure were more often observed in *DSP* and *PLN* mutation carriers. Men were more likely to be index patient, symptomatic, and had earlier and more severe arrhythmic expression.

Clinical course and long-term outcome in Arrhythmogenic Cardiomyopathy

The end-stage of AC is studied in detail in **Chapter 9**. The clinical, histological, immunohistological, and ultrastructural features of a patient that underwent a cardiac transplantation is described. The explanted heart revealed severe fibrofatty replacement of nearly the entire RV, the LV, and most remarkable, of the interventricular septum. Immunohistochemical and electron microscopy findings of intercalated disks revealed regions with a heterogeneous distribution of connexin 43 and focal electron microscopic abnormalities amongst these regions. These results underscore that AC is not limited to the RV, but involves the entire myocardium, including the interventricular septum.

In **Chapter 10**, the correlation of the invasive evaluation by electrophysiological studies to non-invasive studies in 19 AC index patients is described. Subtricuspid abnormalities occur presumably in an early stage of AC, whereas abnormalities in the RV outflow tract occur mostly in later stages. However, in the 2010 diagnostic criteria, only ECG criteria for predominantly RV outflow tract abnormalities are represented. Therefore, ECG criteria for subtricuspid involvement may improve early diagnosis and risk stratification in AC. With activation and voltage mapping,

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all 19 patients had abnormalities in the subtricuspid region and 18 had abnormalities in the RV outflow tract. Abnormalities found with activation and voltage mapping correlated well with structural abnormalities found with imaging studies. Three ECG parameters possibly reflecting subtricuspid abnormalities were studied: 1) late isolated potentials in inferior leads leads II, III, and aVF (in 42%), 2) negative T waves in II, III, and aVF (in 26%), and 3) left axis deviation (in 32%). The identification of these new ECG parameters is challenging and awaits optimization. The non-invasive detection of activation delay in AC is discussed to further extent in **Chapter 11** as an editorial comment on an article by Santangeli et al.

The clinical presentation, clinical course, and long-term outcomes of AC in a cohort of 1001 individuals are described in **Chapter 12**. The aim of the study was to define the long-term follow-up and determinants of clinical outcome in the largest AC cohort worldwide, comprising of 439 index patients and 562 family members from JHU and ICIN AC registries. In index patients, clinical presentation and clinical course were characterized by the occurrence of ventricular arrhythmias. Sudden cardiac death during follow-up (median 7 years) occurred more frequently among index patients without an ICD. Overall, cardiac mortality and/or the need for cardiac transplantation were low. AC was diagnosed in 207 family members (37%). Symptoms at presentation correlated with disease expression. Family members with mutations were more likely to meet criteria for AC diagnosis, experience ventricular arrhythmias, and/or die from cardiac cause than family members without mutations. The study showed that the long-term outcome was favorable in diagnosed and appropriately treated AC index patients and family members. In index patients, phenotype and long-term outcome were modulated by ICD implantation but remarkably not by mutation status and familial background. Clinical course and outcome in family members was determined by symptoms at presentation and mutation status. These results have important clinical implications for current patient management strategies in AC.

Finally, in **Chapter 13** all preceding chapters are put into perspective in the general discussion.

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Nederlandse Samenvatting

Aritmogene Rechter Ventrikel Dysplasie/Cardiomyopathie (ARVD/C) is een hartspierziekte die bij ongeveer 1:1000-1:5000 mensen voorkomt. De ziekte wordt gekenmerkt door ventriculaire ritmestoornissen (kamerritmestoornissen), een verhoogde kans op een plotse dood en structurele en functionele afwijkingen voornamelijk in de rechter ventrikel (RV). Echter, het komt ook voor dat er afwijkingen aanwezig zijn in zowel de RV als de linker ventrikel (LV) of zelfs met name in de LV. Om deze reden spreken we tegenwoordig van Aritmogene Cardiomyopathie (AC).
In ongeveer 60% van AC patiënten kan een genetische mutatie die verantwoordelijk is voor het ontstaan van de ziekte gevonden. De meeste mutaties worden gevonden in genen die coderen voor desmosomale eiwitten. Desmosomen zijn complexen van eiwitten die betrokken zijn in de verbinding tussen hartspiercellen. Gen mutaties kunnen aanleiding geven tot dysfunctie van de desmosomale eiwitten. Dysfunctie van de desmosomen zorgt voor een minder stabiele verbinding en heeft een negatieve invloed op de elektrische koppelingen tussen de hartspiercellen. In dit vroege stadium van de ziekte kunnen ventriculaire ritmestoornissen en plotse dood al optreden. De mechanische en elektrische koppeling van de cellen leidt vermoedelijk tot celdood en extra vertraging van de elektrische geleiding in latere ziektestadia. The hartspiercellen die zijn afgestorven worden vervangen door vet- en bindweefsel. Dit geeft aanleiding tot structurele en functionele veranderingen in de ventrikels en creëert de voorwaarden voor het ontstaan van ventriculaire ritmestoornissen.
Sinds de eerste beschrijving van AC is er enorm veel bekend geworden over het genetische substraat voor AC. Wanneer er een ziekte veroorzakende mutatie wordt gevonden in een index patiënt is cascade screening van familieleden op de mutatie en de ziekte mogelijk. Helaas resulteert genetische screening ook vaak in het vinden van genetische varianten van onzekere betekenis. De analyse van deze varianten kan nog verbeterd worden op dit moment. Bovendien kan in een substantieel gedeelte van de patiënten geen ziekte veroorzakende mutatie worden gevonden.
Aritmogene Cardiomyopathie wordt gediagnosticeerd op basis van internationale op consensus gebaseerde criteria. Deze diagnostische criteria zijn voor het eerst opgesteld in 1994. In 2010 zijn de diagnostische criteria herzien. Criteria voor LV betrokkenheid en gen mutaties zijn geïncludeerd in deze versie. Met deze herziene diagnostische criteria werden meer patiënten gediagnosticeerd. Echter, ondanks de verhoogde sensitiviteit van de criteria blijft de diagnose in het vroege stadium, als de ziekte nog grotendeels verborgen is, de grootste uitdaging voor artsen.

Dit proefschrift

Dit proefschrift omvat genetische en klinische studies van AC index patiënten en familieleden. De doelen van het proefschrift waren tweeledig. Ten eerste was het doel om de bijdrage van desmosomale en non-desmosomale gen mutaties te evalueren, de interpretatie van de resultaten van genetische screening te vergemakkelijken en om de invloed van het genotype op het klinisch beloop in AC te bestuderen. Ten tweede was het doel om de invasieve en non-invasieve parameters van substraat voor ritmestoornissen te bekijken en om meer inzicht te krijgen in het klinisch beloop en de lange termijn uitkomsten van AC index patiënten en familieleden. Het ultieme doel van dit proefschrift was om de vroege diagnose en behandeling van AC index

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patiënten en familieleden te ondersteunen en verbeteren. De achtergrond, doelen en inhoud van dit proefschrift zijn in detail beschreven in **Hoofdstuk 1**. Een algemene inleiding in en uitgebreid literatuur overzicht van AC wordt gegeven in **Hoofdstuk 2**. De epidemiologie, klinische presentatie, criteria voor diagnose, differentiaal diagnose, moleculair genetische achtergrond, risicostratificatie, klinisch management, en AC onderzoek in Nederland worden beschreven.

Invloed van het genotype in Aritmogene Cardiomyopathie

In **Hoodstuk 3** wordt de genetische achtergrond van AC uitgelegd. Het familiair voorkomen van de ziekte is al bekend sinds de eerste beschrijvingen van de ziekte. Het merendeel van de gen mutaties wordt gevonden in de desmosomale genen plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmocollin-2 (*DSC2*), desmoglein-2 (*DSG2*). Non-desmosomale gen mutaties zijn geassocieerd met AC in een minderheid van de patiënten. Aritmogene Cardiomyopathie erft meestal in autosomaal dominante vorm over maar autosomaal recessieve vormen zijn ook bekend. Mutaties verantwoordelijk voor AC hebben een incomplete penetrantie en variabele klinische expressie. Om deze reden moet genetische screening worden beschouwd als richtinggevend maar onderdeel van de algemene klinische evaluatie. Genotype-fenotype correlatie analyse zou een rol kunnen spelen in de risicostratificatie van patiënten en familieleden.
Hoofdstuk 4 beschrijft de cosegregatieanalyse in een grote familie met links-dominante AC. De *PKP2* variant c.419C>T, in het verleden bestempeld als ziekte veroorzakend, en de recent ontdekte non-desmosomale founder mutatie in phospholamban (*PLN*, c.40_42delAGA) werden in deze familie gevonden met genetische screening. Zes familieleden hadden AC en zeven LV afwijkingen (negatieve T toppen in afleiding V4-6, LV wandbewegingsstoornissen en contrast aankleuring of een verminderde LV ejectiefractie). De *PLN* mutatie werd gevonden in negen familieleden, waaronder de zes met AC en zeven met LV afwijkingen. Lage voltages op het ECG (<0.5mV in extremiteitsafleidingen) werden gezien in vier van de negen *PLN* mutatie positieve familieleden. Geen van de drie familieleden met alleen de *PKP2* variant hadden RV of LV afwijkingen. Concluderend cosegregeerde de *PLN* mutatie met AC en ECG en structurele LV afwijkingen. In deze familie werden geen aanwijzingen gevonden voor een ziekte veroorzakende bijdrage van de *PKP2* variant.
De bestudering van de non-desmosomale founder mutatie in *PLN* (c.40_42delAGA) wordt vervolgd in **Hoofdstuk 5**. In dit hoofdstuk wordt de bijdrage van desmosomale mutaties en de non-desmosomale *PLN* mutatie aan AC volgens de in 2010 herziene diagnostische criteria bekeken in 142 Nederlandse AC index patiënten. Van deze patiënten had 83 (58%) een ziekte veroorzakende desmosomale mutatie, 19 (13%) bleken de *PLN* mutatie te dragen en in 40 (28%) kon er geen ziekte veroorzakende mutatie worden gevonden. Vergelijking van het fenotype van desmosomale en non-desmosomale *PLN* mutatie dragers liet zien dat *PLN* mutatie dragers vaker een laag gevolteerd ECG, negatieve T toppen in V4-6 en additionele structurele en functionele LV afwijkingen hadden, waar desmosomale mutatiedragers meer solitaire RV afwijkingen hadden. De herziene 2010 diagnostische criteria zorgden ervoor dat 7 patiënten met de *PLN* mutatie de diagnose kregen die ze met de originele criteria uit 1994 niet hadden gekregen. Deze studie liet dus zien dat er een grote bijdrage van de non-desmosomale *PLN* founder mutatie aan AC in Nederland is en dat *PLN* mutatiedragers AC hebben met een kenmerkend fenotype.

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Zoals al eerder genoemd, resulteert genetische screening in AC vaak in het vinden van genetische varianten van onzekere betekenis. De analyse en classificatie van deze varianten is niet altijd makkelijk. De kans dat zo'n variant ziekte veroorzakend is, wordt gewoonlijk ingeschat met behulp van bioinformatische voorspellende algoritmen. Optimalisering van de categorisatie van genetische varianten van onzekere betekenis was het onderwerp van de studies beschreven in **Hoofdstuk 6** en **7**. De voorspelling van de ziekte veroorzakende potentieel van missense varianten wordt geëvalueerd in **Hoofdstuk 6**. Deze studie includeerde 341 familieleden met desmosomale of non-desmosomale gen mutaties vanuit het Amerikaanse en Nederlandse AC cohort. Missense varianten werden beschouwd als ziekte veroorzakend als er aan de volgende voorwaarden werd voldaan: 1) beide bioinformatische algoritmen SIFT en PolyPhen-2 voorspelden pathogeniciteit (SIFT score <0.02 en PolyPhen-2 score <0.900) en 2) de 'minor allele frequency' (MAF) in de ESP exome dataset was klein (≤0.05%). Ziekte veroorzakende missense varianten werden gevonden in 48 (14%) familieleden. Fenotypische kenmerken en ventriculaire ritmestoornis vrije overleving waren vergelijkbaar tussen familieleden met missense en non-missense (truncerende of splice site) varianten. Concluderend, missense varianten kunnen net zo ziekte veroorzakend zijn als non-missense varianten in familieleden van AC index patiënten. De kans dat een missense variant ziekte veroorzakend kan zijn, werd goed voorspeld met de gecombineerde criteria van SIFT, PolyPhen-2 en de MAF in exomen. In **Hoofdstuk 7** wordt het ziekte veroorzakende potentieel van AC splice site varianten beschreven. Het effect van de varianten op splicing wordt *in vitro* bestudeerd met behulp van RNA afkomstig van variant dragers. Een negatief effect op splicing was voorspeld door bioinformatische algoritmen voor alle negen varianten in *PKP2*, *JUP*, *DSG2*, en *DSC2*. Zes van de negen varianten hadden ook daadwerkelijk een negatief effect op splicing, duidend op het ziekte veroorzakend potentieel van deze varianten, waar in drie van de negen varianten geen effect op splicing werd gezien. In drie patiënten gaf de RNA analyse en toekenning van het predicaat 'ziekte veroorzakende mutatie' de doorslag voor het toekennen van de diagnose AC. Deze resultaten benadrukken het belang van goede analyse van genetische varianten van onzekere betekenis in het algemeen en splice site varianten in het bijzonder. Daarnaast draagt goede analyse van deze varianten bij aan patiëntenzorg en familiescreening.

De invloed van het genotype is onderzocht in **Hoofdstuk 8**. In een groot transatlantisch cohort van 577 index patiënten en familieleden uit de databases van de Johns Hopkins Universiteit (JHU) en het Interuniversitair Cardiologisch Instituut Nederland (ICIN), allemaal met ziekte veroorzakende mutaties, werd de invloed van het genotype op het klinisch beloop en het voorkomen van ritmestoornissen bestudeerd. Patiënten die zich presenteerden met plotse dood of ventrikel fibrilleren waren significant jonger dan patiënten die zich presenteerden met een monomorfe ventrikel tachycardie. Patiënten met meerdere ziekte veroorzakende mutaties hadden significant eerder en vaker ventriculaire ritmestoornissen, meer LV dysfunctie, hartfalen en harttransplantaties dan patiënten met maar een ziekte veroorzakende mutatie. Linker ventrikel dysfunctie en hartfalen werden vaker gezien in patiënten met *DSP* en *PLN* gen mutaties. Mannen waren vaker index patiënt, symptomatisch en hadden eerder en meer ventriculaire ritmestoornissen in vergelijking met vrouwen.

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Klinisch beloop en lange termijn uitkomst in Aritmogene Cardiomyopathie

Het eindstadium van AC is beschreven in **Hoofdstuk 9**. De klinische, histologische, immunohistologische en ultrastructurele kenmerken van een patiënt die een harttransplantatie heeft ondergaan worden gerapporteerd. Het uitgenomen hart liet zeer uitgebreide vervanging van het normale hartspierweefsel door vet- en bindweefsel zien in de RV, LV en zelfs van het interventriculaire septum. Met immunohistologische en electronen microscopische analyse van de intercalairschijven in het hartspierweefsel werden regio's met heterogene distributie van connexin43 en focale afwijkingen en verwijdingen van de intercalairschijven in deze regio's geobserveerd. Deze resultaten onderstrepen dat AC niet beperkt is tot de RV maar het gehele ventriculaire hartspierweefsel omvat, inclusief het interventriculaire septum.

In **Hoofdstuk 10** is de correlatie van invasief onderzoek door middel van electrofysiologisch onderzoek met non-invasieve onderzoeken in 19 AC index patiënten beschreven. Afwijkingen in het subtricuspidale gebied in de RV komen waarschijnlijk al in een vroeg stadium van de ziekte voor, terwijl afwijkingen in de RV uitstroombaan meestal later optreden. Echter, in de in 2010 herziene diagnostische criteria, zijn alleen ECG criteria voor voornamelijk RV uitstroombaan afwijkingen vertegenwoordigd. Het zou kunnen dat ECG criteria voor subtricuspidale afwijkingen de vroegdiagnostiek en risicostratificatie in AC kunnen verbeteren. Met activatie en voltage mapping hadden alle 19 index patiënten elektrische afwijkingen in het subtricuspidale gebied en 18 hadden afwijkingen in de RV uitstroombaan. Elektrische afwijkingen gevonden met activatie en voltage mapping kwamen goed overeen met structurele afwijkingen gevonden met beeldvormende onderzoeken. Drie ECG parameters die mogelijk afwijkingen in het subtricuspidale gebied weergeven werden bekeken: 1) late geïsoleerde potentialen in de onderwandsafleidingen II, III, aVF (in 42%), 2) negatieve T toppen in afleiding II, III en aVF (in 26%) en 3) linker as draaiing (in 32%). De indetificatie van dit soort nieuwe ECG parameters is lastig en moet nog worden geoptimaliseerd. Het non-invasief vaststellen van vertraging van de elektrische activatie wordt verder bediscussieerd in **Hoofdstuk 11**, als een 'editorial comment' op een artikel door Santangeli et al.

De klinische presentatie, het klinisch beloop en de lange termijn uitkomsten van AC in een cohort van 1001 personen zijn bestudeerd in **Hoofdstuk 12**. Het doel van de studie was om de lange termijn follow-up en determinanten van de klinische uitkomst te definiëren in het grootste AC cohort wereldwijd, bestaande uit 439 index patiënten en 562 familieleden vanuit de JHU en ICIN AC datasets. De klinische presentatie en het klinisch beloop van index patiënten werd voornamelijk gekarakteriseerd door het voorkomen van ventriculaire ritmestoornissen. Plotse dood tijdens follow-up (mediane duur 7 jaar) kwam vaker voor bij index patiënten zonder een ICD. De cardiale sterfte en noodzaak tot harttransplantatie waren over het algemeen laag. Aritmogene Cardiomyopathie werd in 207 (37%) familielieden vastgesteld. Klachten bij presentatie correleerde met uitingen van de ziekte. Familieleden met een ziekte veroorzakende mutatie hadden vaker de diagnose, ventriculaire ritmestoornissen en overleden vaker als gevolg van cardiale oorzaken dan familieleden zonder mutaties. De studie liet zien dat de lange termijn uitkomsten van AC gunstig waren in gediagnosticeerde en goed behandelde AC index patiënten als familieleden. De lange termijn uitkomsten van index patiënten werden bepaald door ICD implantatie maar opvallende genoeg niet door de aanwezigheid van een mutatie of een familiaire achtergrond van de ziekte. Het klinisch beloop en de uitkomst in familieleden werd wel bepaald

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door de aanwezigheid van mutaties en door de aanwezigheid van klachten bij presentatie. Deze resultaten hebben belangrijke klinische implicaties voor de huidige patiëntenzorg strategieën in AC.
Tenslotte worden in **Hoofdstuk 13** alle voorgaande hoofdstukken in perspectief geplaatst in de algemene discussie.

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By the end of 2011, an international transatlantic collaboration with the ARVD team of the Johns Hopkins University School of Medicine in Baltimore, Maryland, under supervison of **Hugh Calkins** was created. In this collaborative effort, I was the cat who got the canary (hah Anneline, at least one Dutchie that got the American sayings right, ☺), since I got to write two beautiful papers with this fantastic team. Dear **Hugh**, **Cindy**, **Anneline**, **Crystal**, **Hari**, **Brittney**, **Aditya**, **Abhishek**, and **Dan**, thank you so much for your hospitality, guidance, and the educational and fun period that I spent in Baltimore. **Cindy**, as said it is an honor to have you at my thesis defense and I am looking forward to showing you Utrecht and The Netherlands.

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Het aritmogene cardiomyopathie project is, zoals al eerder aangestipt, goed ingebed in de wetenschappelijke structuur van het UMC. Bij cardiologie overstijgende vragen waren er altijd mensen van andere afdelingen die ik om raad kon vragen. Van de afdeling Medische Fysiologie, **Prof. Marc Vos**, **Prof. Jacques de Bakker**, **Toon van Veen**, **Prof. Harold van Rijen**, **Tonny van Woudenberg**, **Sanne de Jong** en **Maartje van Kempen-Noorman**, dank voor het inzicht in de basale kant van aritmogene cardiomyopathie, de discussies, goede adviezen en gezelligheid. **Maartje**, mijn AC-maa(r)tje, we waren onverslaanbaar als team bij de gezamenlijke projecten! We hebben samen overlegd, gewerkt, geschreven en nogal wat doorzettingsvermogen nodig gehad maar het resultaat mag er zijn. Je staat me met raad en daad bij, zelfs nu je allang een nieuwe baan hebt, waarvoor ongelooflijk veel dank! Gelukkig krijg ik die goede raad vaak bij een koffie of lunch waarvan ik hoop dat we ze erin houden, ook als ik een nieuwe baan heb! Van de afdeling Pathologie, **Aryan Vink**, bedankt voor het enthousiasme, de mooie figuren en nauwgezette analyses. Van de afdeling Medische Genetica, **Jasper van der Smagt**, **Angela Schoemaker** en **Jan Post**, bedankt voor de patiëntenzorg en betrokkenheid in het onderzoek.

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Curriculum Vitae

Judith Groeneweg was born on April 9th 1983 in Leiden, The Netherlands. She attended the Minkema College in Woerden and later the STEBO college in Utrecht where she graduated in 2001. Thereafter, she moved to Leiden to study History at the University of Leiden. In 2003, she started Medical School at the Leiden University Medical Center.

During her clinical Internal Medicine rotation, including a two-week Cardiology rotation, Judith became fascinated by the wonders of the ECG and heart rhythms. After graduation from Medical School in 2009, she started working as a resident (ANIOS) at the Cardiology department of the HagaZiekenhuis in The Hague under supervision of dr. Delemarre. Here, she became convinced of her interest in general Cardiology and electrophysiology in particular.

In 2010, she started working as a PhD student on the Arrhythmogenic Cardiomyopathy research project at the Cardiology department of the University Medical Center Utrecht under supervision of Prof. dr. R.N. Hauer, Prof. dr. P.A. Doevendans, dr. J.P. van Tintelen, and dr. A.A.B van Veen. The Arrhythmogenic Cardiomyopathy research project is based on national and international collaboration. This provided Judith the opportunity to share ideas and work with many talented researchers both in The Netherlands and internationally, present her research results at different national and international conferences, and travel for scientific purposes. Her research proposal on genotype-phenotype correlation analysis in Arrhythmogenic Cardiomyopathy was awarded with a dr. E. Dekker personal grant from the Netherlands Heart Foundation. In January 2014 she had the opportunity to work with the ARVD research program team of the Johns Hopkins School of Medicine in Baltimore, Maryland, which resulted in a manuscript included in this thesis (Chapter 12).

In September 2014, Judith started with her clinical work, which will be followed by her formal Cardiology training at the Cardiology department of the University Medical Center Utrecht under supervision of dr. J.H. Kirkels starting December 1st 2014.

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Geographical distribution of plakophilin-2 mutation prevalence in patients with arrhythmogenic cardiomyopathy. Jacob KA, Noorman M, Cox MG, **Groeneweg JA**, Hauer RN, van der Heyden MA. *Neth Heart J* 2012;20:234-9.

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Impact of new electrocardiographic criteria in arrhythmogenic cardiomyopathy. Hauer RN, Cox MG, **Groeneweg JA**. *Front Physiol* 2012;3:352.

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